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# Population-based metagenomics analysis reveals altered gut microbiome in sarcopenia: data from the Xiangya Sarcopenia Study

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## Abstract

**Background** Several studies have examined gut microbiota and sarcopenia using 16S ribosomal RNA amplicon sequencing; however, this technique may not be able to identify altered specific species and functional capacities of the microbes. We performed shotgun metagenomic sequencing to compare the gut microbiome composition and function between individuals with and without sarcopenia.

**Methods** Participants were from a community-based observational study conducted among the residents of rural areas in China. Appendicular skeletal muscle mass was assessed using direct segmental multi-frequency bioelectrical impedance and grip strength using a Jamar Hydraulic Hand dynamometer. Physical performance was evaluated using the Short Physical Performance Battery, 5-time chair stand test and gait speed with the 6 m walk test. Sarcopenia and its severity were diagnosed according to the Asian Working Group for Sarcopenia 2019 algorithm. The gut microbiome was profiled by shotgun metagenomic sequencing to determine the microbial composition and function. A gut microbiota-based model for classification of sarcopenia was constructed using the random forest model, and its performance was assessed using the area under receiver-operating characteristic curve (AUC).

**Results** The study sample included 1417 participants (women: 58.9%; mean age: 63.3 years; sarcopenia prevalence: 10.0%).  $\beta$ -diversity indicated by Bray–Curtis distance (genetic level:  $P = 0.004$ ; taxonomic level of species:  $P = 0.020$ ), but not  $\alpha$ -diversity indicated by Shannon index (genetic level:  $P = 0.962$ ; taxonomic level of species:  $P = 0.922$ ), was significantly associated with prevalent sarcopenia. After adjusting for potential confounders, participants with sarcopenia had higher relative abundance of *Desulfovibrio piger* ( $P = 0.003$ ,  $Q = 0.090$ ), *Clostridium symbiosum* ( $P < 0.001$ ,  $Q = 0.035$ ), *Hungatella effluvii* ( $P = 0.003$ ,  $Q = 0.090$ ), *Bacteroides fluxus* ( $P = 0.002$ ,  $Q = 0.089$ ), *Absiella innocuum* ( $P = 0.002$ ,  $Q = 0.072$ ), *Coprobacter secundus* ( $P = 0.002$ ,  $Q = 0.085$ ) and *Clostridium citroniae* ( $P = 0.001$ ,  $Q = 0.060$ ) than those without sarcopenia. The relative abundance of six species (*Desulfovibrio piger*, *Clostridium symbiosum*, *Hungatella effluvii*, *Bacteroides fluxus*, *Absiella innocuum*, and *Clostridium citroniae*) was also positively associated with sarcopenia severity. A differential species-based model was constructed to separate participants with sarcopenia from controls. The value of the AUC was 0.852, suggesting that model has a decent discriminative performance. *Desulfovibrio piger* ranked the highest in this model. Functional annotation analysis revealed that the phenylalanine, tyrosine, and tryptophan biosynthesis were depleted ( $P = 0.006$ ,  $Q = 0.071$ ), while alpha-Linolenic acid metabolism ( $P = 0.008$ ,  $Q = 0.094$ ), furfural degradation ( $P = 0.001$ ,  $Q = 0.029$ ) and staurosporine biosynthesis ( $P = 0.006$ ,  $Q = 0.072$ ) were enriched in participants with sarcopenia. *Desulfovibrio piger* was significantly associated with staurosporine biosynthesis ( $P < 0.001$ ).

**Conclusions** This large population-based observational study provided empirical evidence that alterations in the gut microbiome composition and function were observed among individuals with sarcopenia.

**Keywords** Sarcopenia; Gut microbiome; Metagenomics; Population-based study

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## Introduction

Sarcopenia, characterized by the age-associated degenerative loss of skeletal muscle strength and mass, has recently been recognized as a disease by the World Health Organization.<sup>1</sup> Approximately 10–27% of people aged  $\geq 60$  years have sarcopenia,<sup>2</sup> and this condition is associated with poor health outcomes, including frailty, disability, and mortality.<sup>3</sup> There is a paucity of data on the pathophysiologic mechanisms underlying sarcopenia; thus, no specific treatment regimen, including pharmacological treatment, has been developed for it.<sup>4</sup>

The human gut microbiome is a reservoir of 10–100 trillion microorganisms that play a vital role in human health through their involvement in metabolic interactions (e.g. food decomposition and nutrient intake) and the host's immune responses.<sup>5</sup> More importantly, the composition and function of the gut microbiome can be modified through lifestyle interventions, including diet, physical activity, and probiotics, by enhancing the abundance of beneficial microbes and reducing that of harmful microbes.<sup>6</sup> Aberrant microbiome compositional profiles can alter critical biological processes, such as host metabolism and inflammatory milieu.<sup>7</sup> Additionally, microbes may have a detrimental effect on muscle mass and function by reducing nutrient bioavailability, promoting insulin resistance, and increasing oxidative stress.<sup>8</sup> In animal studies on sarcopenia, a 'gut-muscle axis' has been observed.<sup>9–15</sup> Several human studies have also reported an association between alterations in the gut microbiota and the prevalence of sarcopenia.<sup>16–19</sup> However, these studies used the 16S rRNA method to examine the gut microbiota profile. Although 16S rRNA sequencing is technically robust, it has a limited ability to differentiate between closely related species and cannot provide direct evidence on the microbiome community's functional capabilities.<sup>20</sup> Further elucidation of the association between gut microbiome and the risk of sarcopenia using more advanced sequencing technologies, that is, shotgun metagenomic sequencing, is needed to elucidate the role of the gut microbiome in the development of sarcopenia and to contribute to potential translational opportunities for the prevention and treatment of this common disease.

Although shotgun metagenomic sequencing can provide detailed functional annotations of microbial communities

with species-level resolution<sup>21</sup>; to date, few studies have examined the association between gut microbiome and sarcopenia using this technology. Therefore, we performed shotgun metagenomic sequencing to compare the gut microbiome composition and function among individuals with and without sarcopenia.

## Methods

### Study participants

The Xiangya Sarcopenia Study is piggybacked on the Xiangya Osteoarthritis Study, a large community-based longitudinal study of the natural history and risk factors of osteoarthritis in a rural area in China (NCT04033757).<sup>51</sup> The Xiangya Osteoarthritis Study comprises three subcohorts, that is, subcohort I ( $n = 1469$ ), II ( $n = 1271$ ), and III ( $n = 1340$ ), which were initiated in 2015, 2018, and 2019, respectively. Participants in the Xiangya Osteoarthritis Study were a randomly selected sample of residents aged  $\geq 50$  years from rural mountainous villages of Longshan County in Hunan Province. In brief, we adopted a probability proportionate to size sampling method to select 14 communities. All villages in the selected communities were listed in a random order. Village-to-village recruitment began from the first village in the first community until the number of participants in that community met the pre-determined proportion in the age stratum (50–60, 60–70, and  $\geq 70$  years) and sex according to the Sixth National Census Data of Longshan County (2010). Overall, 25 rural mountain villages of Longshan County were included in the Xiangya Osteoarthritis Study. This study was approved by the Research Ethical Committee of Xiangya Hospital, Central South University (201510506), and all participants provided informed written consent before participating in the studies.

The Xiangya Sarcopenia Study consisted of participants in subcohort II ( $n = 1222$ ), subcohort III ( $n = 1251$ ) and those who participated in the fifth year follow-up visit of subcohort I ( $n = 993$ ). We evaluated the participants of these subcohorts for the presence of sarcopenia. The aim of the Xiangya Sarcopenia Study was to describe the natural history of sarcopenia and investigate the risk factors of this disease. Trained health

professionals conducted a face-to-face interview of participants at their homes or site of identification. A standardized questionnaire was administered to obtain demographic characteristics, health-related habits, muscle symptoms, and potential risk factors for sarcopenia. The current analysis only included participants from the subcohort II and subcohort III because data on shotgun metagenomic sequencing of gut microbiome were not collected from the participants in subcohort I. In addition, participants were excluded if they either did not provide stool samples or did not have a sufficient amount of DNA extracted from their stool samples for shotgun metagenomic sequencing, or reported antibiotic use 1 month prior to stool sample collection, or had a history of inflammatory bowel disease, gastrointestinal tract surgery, or cancer.

### *Sarcopenia assessment*

The grip strength of the dominant hand of the participants was measured using a calibrated Jamar dynamometer with the participants in the sitting position (Patterson Medical, Ltd. Nottinghamshire, UK). Three grip strength measurements were taken at 10 s intervals and the maximum value of the three measurements was used as the participant's final grip strength.<sup>52</sup> The participant had their appendicular skeletal muscle mass measured using bioimpedance analyses (InBody 770 analyser, InBody Japan, Tokyo, Japan)<sup>53</sup> if their grip strength was low (<28 kg for men and <18 kg for women). In addition, we evaluated each participant's physical performance using a series of tests, including the Short Physical Performance Battery (SPPB), 5-time chair stand test, and gait speed with the 6 m walk test.<sup>54</sup> Low physical performance was defined as an SPPB score of  $\leq 9$ , 5-time chair stand test of  $\geq 12$  s, or gait speed of <1.0 m/s. If a participant had both low appendicular skeletal muscle mass (<7.0 kg/m<sup>2</sup> in men and <5.7 kg/m<sup>2</sup> in women) and low grip strength, s/he was considered to have sarcopenia, according to the Asian Working Group of Sarcopenia 2019 algorithm.<sup>22</sup> If a participant had low grip strength, low muscle mass, and low physical performance, s/he was considered to have severe sarcopenia.<sup>22</sup>

### *Assessment of covariates*

Demographic characteristics were collected from face-to-face interview using standard questionnaires. We defined participants as 'ever smokers' if they reported smoking at least seven cigarettes or seven pipes of tobacco per week for at least 1 year.<sup>55</sup> If participants did not smoke this amount, they were classified as 'non-smokers'. Participants were classified as 'current smokers' if they smoked cigarettes on a regular basis and still smoke at the time of interview. Those who have

given up smoking were classified as 'past smokers'. Any alcohol consumption was defined as the intake of alcohol more than once in each month during the past year.<sup>56</sup> Participants who did not consume this amount were classified as 'non-drinkers'. Participants who have given up drinking were classified as 'past drinkers', while those who kept drinking were classified as 'current drinkers'. Self-reported histories of any fractures (yes vs. no) from the participants were obtained from the interview.<sup>57,8</sup>

Physical activity level was assessed using the International Physical Activity Questionnaire Short Form (IPAQ-SF).<sup>59–11</sup> This questionnaire comprises nine items across four generic sections covering several types and domains of physical activity and sitting behaviours, and quantifies the physical activity as the metabolic equivalent of task (MET)-min/week. According to the guidelines for the data processing and analysis of the IPAQ-SF,<sup>512</sup> high physical activity was defined as any one of the following criteria: (i) vigorous-intensity activity on at least 3 days and accumulating at least 1500 MET-minutes per week and (ii)  $\geq 7$  days of any combination of walking, moderate- or vigorous-intensity activities accumulating at least 3000 MET-minutes per week. Moderate physical activity was defined as one of the following three criteria: (i)  $\geq 3$  days of vigorous activity of at least 20 min per day; (ii)  $\geq 5$  days of moderate-intensity activity and/or walking of at least 30 min per day; and (iii)  $\geq 5$  days of any combination of walking, moderate-intensity or vigorous intensity activities achieving a minimum of at least 600 MET-minutes/week. Low physical activity was defined as either no physical activity is reported or no physical activity meets moderate or high levels.

We used a semi-quantitative food frequency questionnaire (SFFQ) to obtain dietary information. This questionnaire was specifically developed for the population living in Hunan Province which was validated and used in previous studies.<sup>51,13</sup> The SFFQ contains 63 food items that are commonly consumed in the Hunan Province in China. Participants were queried about frequency (i.e. never, once per month, two to three times per month, one to three times per week, four to five times per week, once per day, twice per day, or three times and more per day) of each food item they consumed during the past year and the average amount of food consumption for each time (i.e. <100 g, 100–200 g, 201–300 g, 301–400 g, 401–500 g, or more than 500 g). Colour pictures showing food samples with labelled weights were given to participants as a reference.

Height was measured to the nearest 0.5 cm using a portable stadiometer without shoes and weight was measured to the nearest 0.1 kg using the InBody 770 analyser after removing shoes and heavy outer clothing. Body mass index (BMI) was calculated as weight (kg) divided by the square of height (m<sup>2</sup>). All covariates were collected on the same day when the participants underwent sarcopenia assessment.

## Stool sample collection and DNA extraction

Stool samples from the participants were collected at the recruitment site. Collected stool samples were frozen immediately, transported on dry ice within 20 min, and stored at  $-80^{\circ}\text{C}$  until DNA extraction. In accordance with the manufacturer's protocol, 200 mg of stool was used for DNA extraction using the Magen HiPure Soil DNA Kit (Magen, Guangzhou, China). First, the stool samples were homogenized and treated with lysis buffers. Subsequently, a bead-beating process was performed using TissueLyser-24L (Jingxin, Shanghai, China) (25 Hz for 5 min) to completely lyse the cells, followed by a 10 min incubation at  $65^{\circ}\text{C}$ . The cellular debris was then removed, and the sample was purified using a HiPure DNA Mini Column II (Magen, Guangzhou, China) by centrifuging and washing steps according to the manufacturer's protocol. After the rounds of washing, DNA was eluted using elution buffers. The eluted DNA was quantified with a Qubit Fluorometer by using Qubit dsDNA BR Assay kit (Invitrogen, Oregon, USA) and the quality was checked by running aliquot on 1% agarose gel. Extracted DNA samples were stored at  $-80^{\circ}\text{C}$  until shotgun metagenomic sequencing.

## DNA library construction and shotgun metagenomic sequencing

Extracted DNA samples were randomly fragmented by Covaris. Magnetic beads were used to select the fragmented DNA at an average size of 200 to 400 base pairs. The selected fragments went through end repair, 3' adenylation, adapters ligation, PCR amplification and magnetic-bead purification. The double-stranded PCR products were heat-denatured and circularized by the splint oligo sequence. The single-strand circular DNA was formatted as the final library and qualified. The qualified libraries were sequenced on the MGISEQ-2000 platform (BGI, Shenzhen, China), and paired-end reads of 150-base pair nucleotides were generated.<sup>514</sup>

To obtain clean data, the original sequencing reads were processed as follows: (i) excluding reads containing 10% uncertain bases (N bases); (ii) excluding reads containing adapter sequences (15 bases or more extended sequence aligned to the adapter sequence); (iii) excluding reads containing low-quality bases of 20% (bases of  $Q < 20$ ); (iv) a filtering step to remove the sequence of the host genome (SOAP2,<sup>515</sup>  $> 90\%$  similarity, a host reference sequence is required). After quality control, high-quality reads were assembled using MEGAHIT.<sup>516</sup> DNA reads were assessed for taxonomic using Kraken2 based on UHGG for human stool samples.<sup>517</sup> The alignment results were mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.<sup>518</sup>

## Statistical analyses

The richness and evenness of the gut microbiome composition were assessed using  $\alpha$ -diversity, indicated by the Shannon index. The similarities of gut microbiome composition were evaluated using  $\beta$ -diversity, measured by the Bray–Curtis distance.<sup>519</sup> We compared the genetic and taxonomic differences (from phylum to species) in  $\alpha$ -diversity using a generalized linear regression and in  $\beta$ -diversity using a permutation multivariate analysis of variance (PERMANOVA) test between participants with and those without sarcopenia.<sup>520</sup> Age, sex, body mass index (BMI), smoking history, alcohol intake, fracture history, physical activity, and frequency of dietary intake of meat/eggs, dairy products and vegetables were entered as separate variables in the multivariate-adjusted model.<sup>521</sup> In addition, we performed multivariate association with linear models (MaAsLin)<sup>522</sup> to examine the association between microbial taxa and sarcopenia on the phylum, class, order, family, genus and species levels, respectively. The regression coefficient in MaAsLin represents the mean difference in the log-transformed relative abundances of taxa between participants with and without sarcopenia. We also examined the association between microbial species and sarcopenia severity using MaAsLin. Specifically, the relative abundance of microbial species was analysed as a continuous dependent variable, and the regression coefficient and Q for the trend were calculated by entering sarcopenia status (i.e. 0: non-sarcopenia, 1: sarcopenia, 2: severe sarcopenia) as an ordinal independent variable into the multivariate model. In these analyses, we removed microbial taxa present in  $< 10\%$  of samples. Furthermore, we performed the random forest model using species identified in MaAsLin to classify sarcopenia status. We evaluated the model's discriminative performance using the area under receiver-operating characteristic curve (AUC). As the outcome variables showed a significant imbalance, we first used the Synthetic Minority Over-sampling Technique to balance the categorical data.<sup>523–26</sup> Then we randomly split (7:3) the synthetic data into a training set and a test set, and constructed a random forest classifier with the relative abundance of the differential species.<sup>527</sup> We plotted the receiver-operating characteristic curve and calculated the AUC to evaluate the performance of the random forest classifier in the test set. The variable importance of each selected species was measured based on mean decrease accuracy and mean decrease Gini index. In addition, we performed the MaAsLin to assess differences in KEGG level 3 pathways or modules (present in  $> 10\%$  of samples) between participants with and without sarcopenia, adjusting for the aforementioned confounders. Finally, we assessed the correlation between microbial species and altered KEGG pathways and modules using Spearman's correlation test with adjustment for potential confounders.<sup>528</sup>

*P*-values were corrected for multiple testing with the Benjamin & Hochberg False discovery rate method and a corrected *P*-value (*Q* value) < 0.1 was considered as statistically significant.

## Results

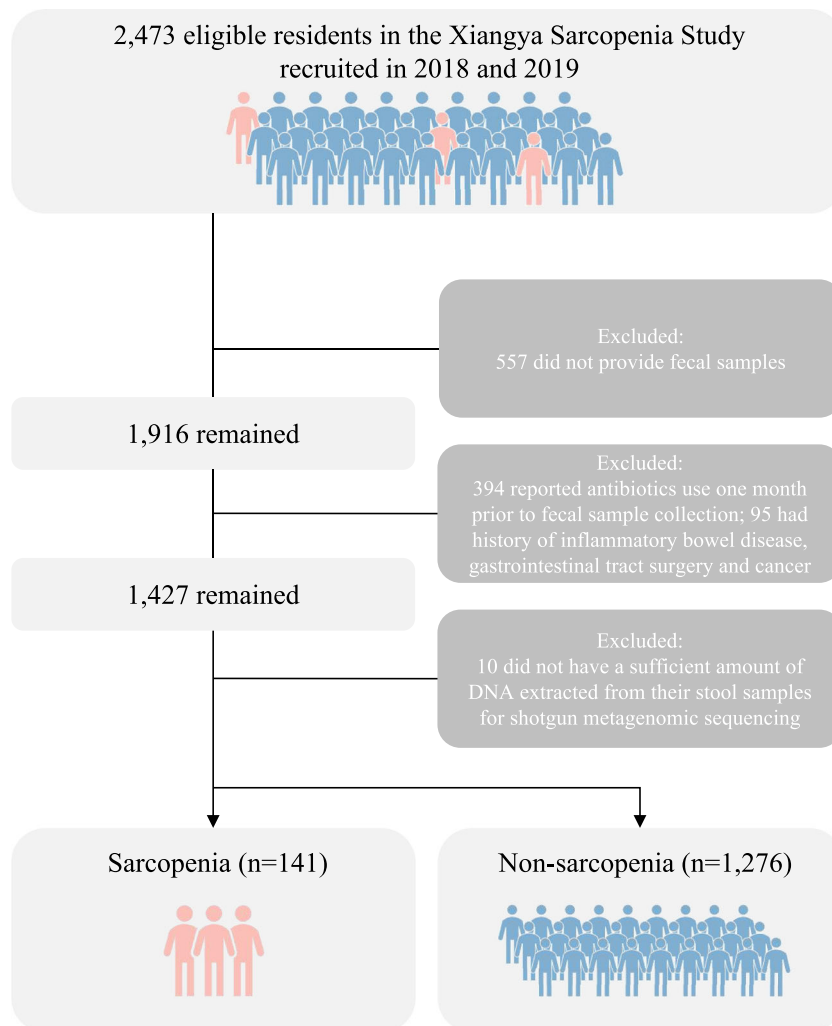
The flowchart depicting the selection process of participants is shown in *Figure 1*. Of 2473 eligible residents, we excluded 557 who did not provide stool samples, 394 who reported antibiotic use 1 month prior to stool sample collection, 95 who had a history of inflammatory bowel disease, gastrointestinal tract surgery or cancer, and 10 who did not have a sufficient amount of DNA extracted from their stool samples for shotgun metagenomic sequencing. The remaining 1417 participants were included in the final analysis.

### Basic characteristics of the included participants

Among 1417 participants, 141 (10.0%) had sarcopenia according to the Asian Working Group of Sarcopenia 2019 algorithm.<sup>22</sup> Participants with sarcopenia (*n* = 141) were older (72.2 vs. 62.3 years), had lower mean BMI (21.4 vs. 24.2 kg/m<sup>2</sup>) and had a higher proportion of men (51.8% vs. 39.9%) than those without sarcopenia (*n* = 1276). Basic characteristics of the included participants are shown in *Table 1*.

### Sequencing characteristics

All stool samples were sequenced on MGISEQ-2000 platform. A mean of 12.2G raw base were generated, after quality control and read filter steps, a mean of 11.7G clean base of each sample were obtained.



**Figure 1** Selection process of the included subjects in the study.

**Table 1** Basic characteristics of the included participants

	Sarcopenia	Non-sarcopenia	P
N	141	1276	-
Age, mean (SD), years	72.2 (8.5)	62.3 (8.5)	<0.001
50–59 years (%)	9.2	41.6	
60–69 years (%)	28.4	36.5	
≥70 years (%)	62.4	21.9	
Sex (%)			0.007
Male	51.8	39.9	
Female	48.2	60.1	
BMI, mean (SD), kg/m <sup>2</sup>	21.4 (2.5)	24.2 (3.4)	<0.001
Smoking status (%)			0.002
None	51.8	64.9	
Past	11.0	5.3	
Current	37.2	29.8	
Alcohol intake (%)			0.014
None	50.4	48.2	
Past	18.2	10.9	
Current	31.4	40.9	
Fracture history (%)	17.0	12.9	0.166
Physical activity level (%)			0.608
Low and moderate	16.3	14.7	
High	83.7	85.3	
Dietary intake of meat/eggs, mean (SD), times/week	3.1 (4.0)	3.3 (3.4)	0.101
Dietary intake of dairy products, mean (SD), times/week	0.4 (1.5)	0.2 (0.9)	0.008
Dietary intake of vegetables, mean (SD), times/week	10.3 (5.9)	10.8 (6.6)	0.659

N, number; SD, standard deviation; BMI, body mass index.

### Comparison of gut microbial diversity between participants with and those without sarcopenia

There was no significant difference in genetic and taxonomic  $\alpha$ -diversities (from phylum to species) indicated by the Shannon index between participants with and those without sarcopenia (Figure 2A,B). The structure and composition (i.e.  $\beta$ -diversity) indicated by Bray-Curtis distance for gut microbial genes (PERMANOVA test  $P = 0.004$ ) and taxonomic level of genus and species (PERMANOVA test  $P = 0.042$  and  $0.020$ , respectively) differed significantly between the two groups (Figure 2C,D).

### Specific taxa differences between participants with and those without sarcopenia

Compared with those without sarcopenia, participants with sarcopenia had higher adjusted relative abundance of *Clostridium* ( $P = 0.002$ ,  $Q = 0.067$ ) and *Lawsonibacter* ( $P = 0.003$ ,  $Q = 0.095$ ) at the genus level (Figure 3A, Supporting Information, Table S1), as well as *Desulfovibrio piger* ( $P = 0.003$ ,  $Q = 0.090$ ), *Clostridium symbiosum* ( $P < 0.001$ ,  $Q = 0.035$ ), *Hungatella effluvii* ( $P = 0.003$ ,  $Q = 0.090$ ), *Bacteroides fluxus* ( $P = 0.002$ ,  $Q = 0.089$ ), *Absiella innocuum* ( $P = 0.002$ ,  $Q = 0.072$ ), *Coprobacter secundus* ( $P = 0.002$ ,  $Q = 0.085$ ) and *Clostridium citroniae* ( $P = 0.001$ ,  $Q = 0.060$ ) at species level (Figure 3B, Table S2), with  $\beta$  coefficients of seven species ranging from 0.51 (*C. citroniae*) to 1.34 (*D. piger*). The relative abundance of these species, except for *Coprobacter secundus*, was also positively associated with sar-

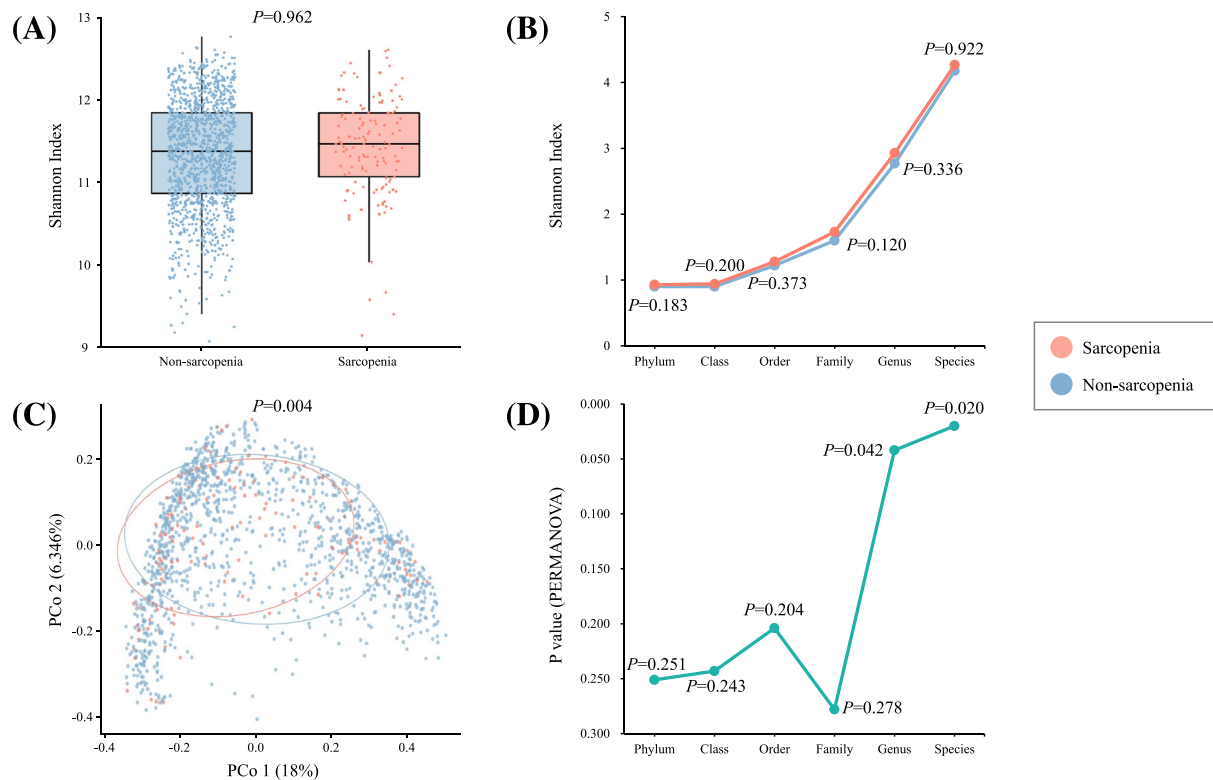
copenia severity (Figure 3C, Table S3). There was no apparent difference in microbiome taxa at the phylum, class, order, family levels between participants with and without sarcopenia.

### Microbiota-based model for classification of sarcopenia

A microbiota-based classifier was constructed by random forest model to differentiate participants with sarcopenia from controls. Microbiota contained in this model were bacterial species identified in former analysis with significantly different relative abundance between participants with and without sarcopenia. The value of the AUC was 0.852, suggesting a microbiota-based classifier had a decent discriminative ability (Figure 4A). The variable importance of these differentially abundant species is shown in Figure 4B,C and Table S4. Of them, *D. piger* was considered as the most important one, with a remarkably higher mean decrease accuracy and mean decrease Gini index.

### Associations of microbial functions with sarcopenia

Compared with those without sarcopenia, the gut microbiome of participants with sarcopenia showed significant differences in the relative abundance of KEGG pathways and modules (Figure 5). KEGG pathways related to phenylalanine, tyrosine, and tryptophan biosynthesis ( $P = 0.006$ ,  $Q = 0.071$ ) were depleted, while alpha-Linolenic acid metabolism ( $P = 0.008$ ,  $Q = 0.094$ ), furfural degradation



**Figure 2** Comparison of gut microbial diversity and composition of participants with and without sarcopenia. Box plot comparing the participants with and without sarcopenia of  $\alpha$ -diversity measured by the Shannon index at the genetic level (A). The horizontal bar within each box represents the median. The bottom and top of each box represent the 25th and 75th percentiles, respectively. Line plot comparing participants with and without sarcopenia of  $\alpha$ -diversity indicated by the Shannon index at the taxonomic levels from phylum to species (B). Each plot represents the mean of the Shannon index. PCoA plot comparing participants with and without sarcopenia of  $\beta$ -diversity measured by the Bray-Curtis distance at the genetic level (C). Line plot comparing participants with and without sarcopenia of  $\beta$ -diversity indicated by the Bray-Curtis distance at the taxonomic levels from phylum to species (D). Each plot represents the mean of the Shannon index. PCoA, principal coordinates analysis; PERMANOVA, permutation multivariate analysis of variance.

( $P = 0.001$ ,  $Q = 0.029$ ) and staurosporine biosynthesis ( $P = 0.006$ ,  $Q = 0.072$ ) were enriched in the gut microbiome among participants with sarcopenia (Figure 5A, Table S5). In addition, eight KEGG modules, including staurosporine biosynthesis (M00805,  $P = 0.001$ ,  $Q = 0.028$ ), were more abundant in the gut microbiome among participants with sarcopenia than those without sarcopenia (Figure 5A, Table S6). In particular, *D. piger* was significantly associated with staurosporine biosynthesis (KEGG pathways:  $P < 0.001$ ; KEGG modules:  $P < 0.001$ , Figure 5B, Tables S7 and S8).

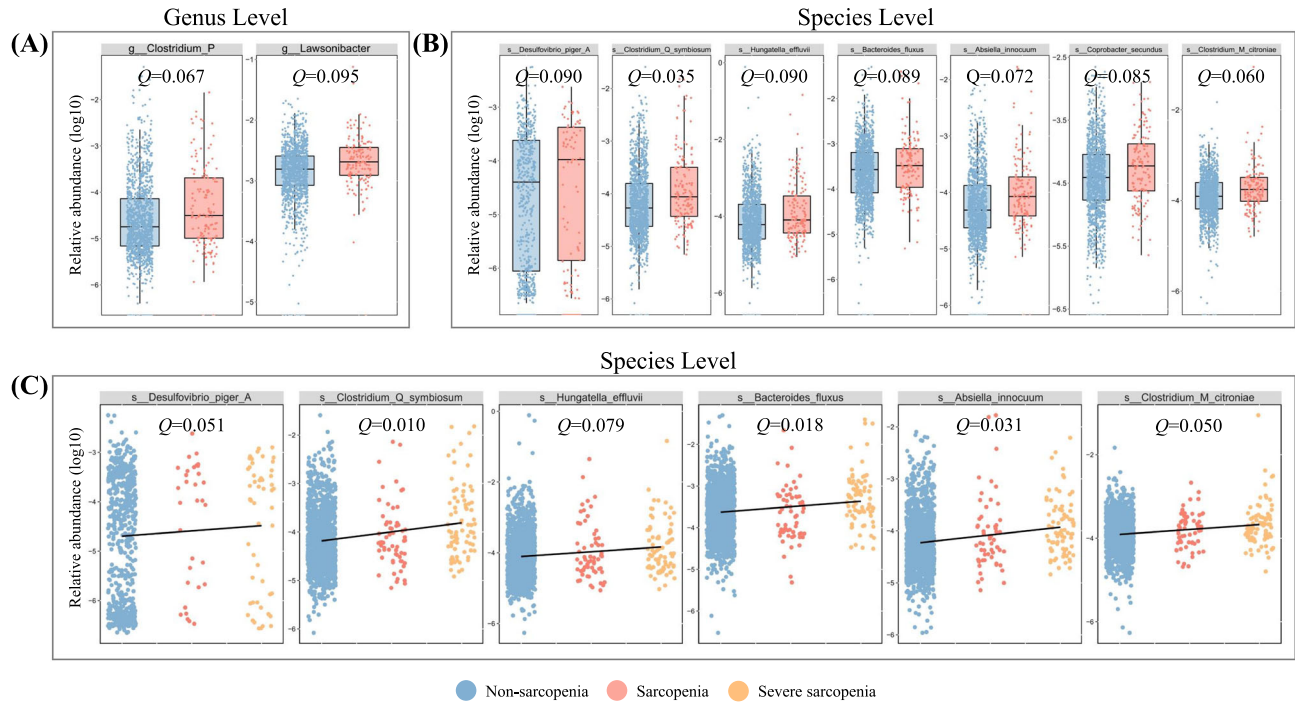
## Discussion

Using a large population-based observational study, we demonstrated microbiome alterations in participants with sarcopenia. Our study identified seven bacterial species enriched in participants with sarcopenia, and of them, six species (i.e. *D. piger*, *C. symbiosum*, *Hungatella effluvii*, *B.*

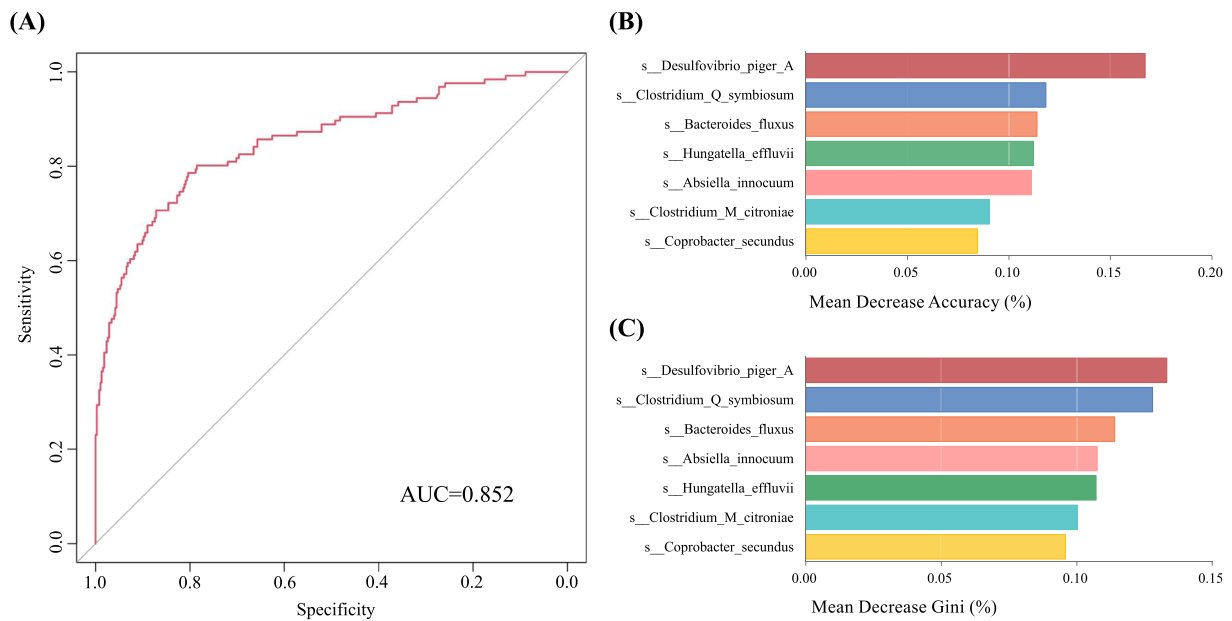
*fluxus*, *Absiella innocuum*, and *C. citroniae*) were positively associated with the severity of sarcopenia. In addition, we found 12 KEGG pathways and modules, including phenylalanine tyrosine and tryptophan biosynthesis, staurosporine biosynthesis and alpha linolenic acid metabolism, were differentiated between participants with sarcopenia and those without it.

Previous studies have suggested that the gut microbiome may affect the skeletal muscle mass and function; however, most of these findings were based on animal models.<sup>9–15</sup> Although animal studies have shown promise, there are complexities and limitations inherent in translating reductionist animal models to complex human diseases. Until now, only a few studies have examined the association between gut microbiota and sarcopenia in human beings.<sup>16–19,23</sup> Four studies have shown that several bacterial genera differed between sarcopenia cases and controls<sup>16–19</sup>; however, all these studies used 16S rRNA amplicon sequencing, which limited their ability to identify closely related species. Only one study found significant differences in the composition and functionality of the

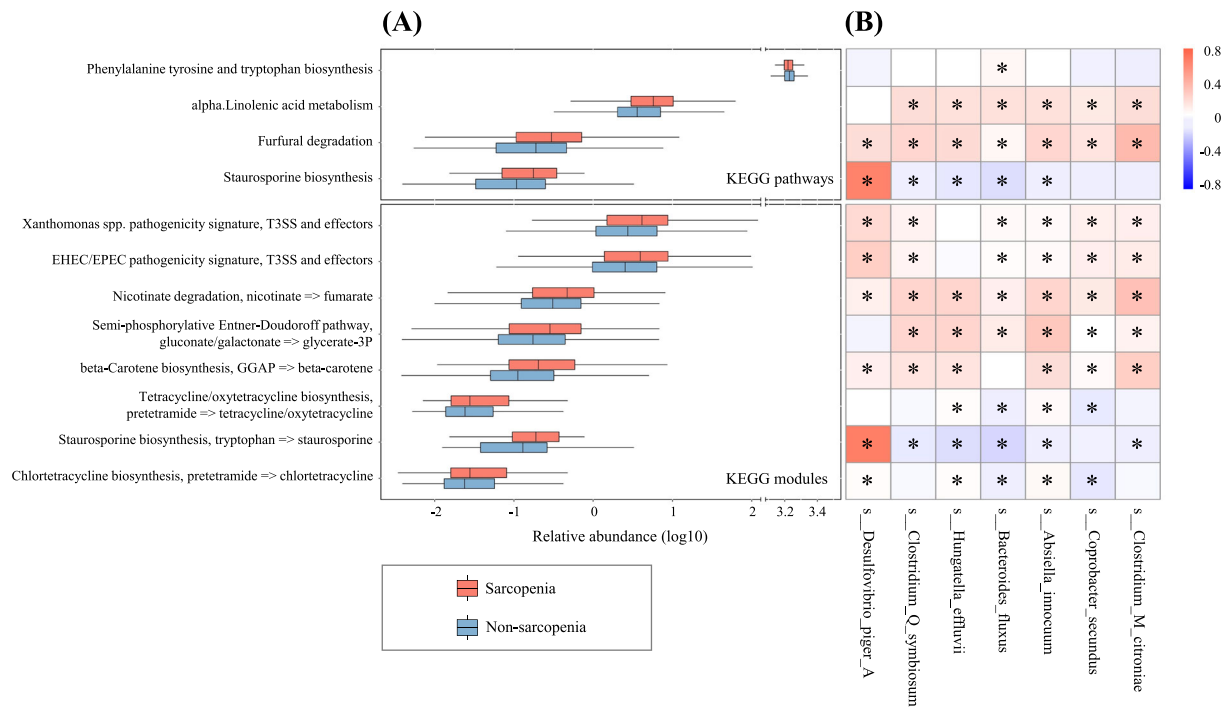




**Figure 3** Microbiota genus and species alterations in sarcopenia. Relative abundance of the differential gut microbiota between participants with and without sarcopenia at the genus (A) and species (B) levels. The horizontal bar within each box represents the median. The bottom and top of each box represent the 25th and 75th percentiles, respectively. Linear associations between the relative abundance of bacterial species and sarcopenia severity (C). The trend line is fitted using a linear model.



**Figure 4** Random forest model based on differentially abundant species classifying participants with sarcopenia and controls. Receiver-operating characteristic curve of the test set. The diagonal line in the graph represents an AUC of 0.5 (A). Species are ranked in descending order of importance to the model's accuracy based on mean decrease accuracy (B) and Gini index (C). AUC, area under receiver-operating characteristic curve.



**Figure 5** Functional characterization alterations of gut microbiota in sarcopenia. Difference in the relative abundances of predicted functions based on KEGG pathways and modules between individuals with and without sarcopenia (A). The vertical bar within each box represents the median. The bottom and top of each box represent the 25th and 75th percentiles, respectively. The association between bacterial species and KEGG pathways and modules (B). Red indicates a positive association; blue indicates a negative association. KEGG, Kyoto Encyclopaedia of Genes and Genomes.

gut microbiome between sarcopenic patients and non-sarcopenic controls using shotgun metagenomic sequencing.<sup>23</sup> However, this study used only muscle mass to diagnose sarcopenia. According to the latest algorithm for defining sarcopenia, sarcopenia is now considered a muscle disease, with low muscle strength overtaking the role of low muscle mass as a principal determinant.<sup>3</sup> Moreover, the sample size of this study was relatively small (5 cases vs. 12 controls). Population-based studies with large sample sizes are crucial to minimize the effect of the inter-individual variability of microbiome profiles.<sup>24</sup> Using shotgun metagenomic sequencing in a sizable population-based sample and a standardized definition to diagnose sarcopenia, we identified seven specific bacterial species and 12 KEGG pathways and modules that may be involved in the pathogenesis of sarcopenia.

Our study found that *D. piger* was enriched in participants with sarcopenia. Desulfovibrio species are Gram-negative bacteria characterized by their ability to reduce sulphate to hydrogen sulfide during the anaerobic respiration of organic matter.<sup>25</sup> *D. piger* is one of the most common and abundant Desulfovibrio species in the human digestive tract.<sup>26</sup> This sulphate-reducing bacteria can reduce both sulfites and sulfates from the diet and sulfated mucopolysaccharides in mucin, leading to the generation of hydrogen sulfide (a cytotoxic compound).<sup>27</sup> Hydrogen sulfide can inhibit fatty acid oxida-

tion, a process of energy production, resulting in reduced muscle endurance.<sup>28</sup> Hydrogen sulfide also damages the intestinal epithelium, causing systemic and chronic inflammation, which appears to have an important role in the pathogenesis of sarcopenia.<sup>29</sup> Moreover, *D. piger* was correlated with altered KEGG pathways and modules, including the biosynthesis of staurosporine, a broad specificity kinase inhibitor, and a commonly used apoptotic stimulus.<sup>30</sup> A recent study demonstrated that muscle cells were sensitive to staurosporine-induced apoptosis<sup>31</sup> and this type of cell death is involved in skeletal muscle degeneration.<sup>32</sup> In addition, staurosporine can cause a rapid loss in phosphorylase activity and impair the ability of muscle cells to generate adenosine triphosphate,<sup>33</sup> thereby leading to muscular damage and dysfunction. In addition, six other species were enriched in participants with sarcopenia. Most of them are strictly anaerobic bacteria (e.g. *C. symbiosum*, *Hungatella effluvii*, and *B. fluxus*) and have potentially pathogenic effects, including bacteraemia<sup>34</sup>; however, their roles in the pathogenesis of sarcopenia warrants further study.

Our results from the functional annotation of the metagenomic sequences suggest that the gut microbiome of participants with sarcopenia had decreased phenylalanine tyrosine and tryptophan biosynthesis as well as increased alpha linolenic acid metabolism. Previous studies have re-

ported that phenylalanine and tyrosine can stimulate muscle protein anabolism, increase muscle strength, or enhance physical performance.<sup>35,36</sup> Additionally, tryptophan, a necessary amino acid, is thought to regulate skeletal muscle mass.<sup>37</sup> Alpha linolenic acid is a plant-derived n-3 fatty acid and contributes to the anabolic pathway for muscle protein synthesis.<sup>38</sup> Dietary intake of alpha linolenic acid has been positively associated with muscle strength and function among older women.<sup>39</sup> In addition, a randomized controlled trial reported that supplementation with alpha linolenic acid decreased the plasma inflammatory cytokine levels (tumour necrosis factor alpha and interleukin-6) in 35 participants with a mean age of 60.6 years.<sup>40</sup> Taken together, these functional results indicate that aberrant microbiome profiles may induce significant shifts in the anabolic-catabolic balance and inflammation, resulting in reduced activity of muscle cells.

Several characteristics of our study are worth noting. First, this was a population-based observational study with a relatively large sample size (total: 1417 participants; sarcopenia: 141 participants); thus, we could minimize the effect of the inter-individual variability of microbiome profiles. Second, we used a standard definition, that is, the Asian Working Group of Sarcopenia 2019 algorithm,<sup>22</sup> to define sarcopenia. This algorithm focuses on assessing muscle mass and integrates muscle strength as part of a more comprehensive definition of sarcopenia.<sup>3</sup> The prevalence of sarcopenia in our study (10.0%) was similar to previous reports, making our findings more generalizable. Third, we used deep sequencing (i.e. metagenomic shotgun sequencing) on the stool samples to profile gut microbiome and the latest definition to define sarcopenia (i.e. muscle mass and grip strength); thus, our study could provide an in-depth understanding of gut microbiome in sarcopenia. Finally, our findings were independent of several potential confounding variables, suggesting that the observed associations were robust.

Some limitations in our study should be acknowledged. First, the current study was cross-sectional; thus, we could not establish the temporal relationship between the gut microbiome and the occurrence of sarcopenia. Longitudinal studies, animal model experiments, and randomized controlled trials are required to verify the potential causality. Second, participants were residents in a rural area of China; thus, we should be cautious when generalizing the findings to other populations with different characteristics. Third, our results have not been verified in other cohorts. Changes in specific bacterial species may not be replicable in other populations given the heterogeneity of the gut microbiome in different geographical locations and culture differences; therefore, further studies are therefore needed to confirm these findings.

Sarcopenia affects various daily life activities, especially among the elderly. The current prevention and treatment plans for sarcopenia remain unsatisfactory.<sup>4</sup> Our study showed that microbiome alterations and several specific bacterial species were associated with the presence of sarcopenia and its

severity. If confirmed by longitudinal observational studies, animal model experiments, and randomized controlled trials, these findings may open new paths in the development of novel treatment strategies for sarcopenia based on microbiome manipulation. This could be accomplished by developing highly selective antibiotics<sup>529</sup> and bacteriophages<sup>530</sup> targeting key aggressive microbial species (e.g. *D. piger*), inhibiting microbial attachment,<sup>531</sup> blocking microbial receptors,<sup>532</sup> and normalizing microbial function properties (e.g. inhibiting the biosynthesis of staurosporine).<sup>533,34</sup> Although increased physical activity has been recommended as the primary treatment of sarcopenia,<sup>41</sup> some frail elderly people may not be able to engage in physical activity; therefore, targeting the gut microbiome to influence skeletal muscle among those individuals is an attractive approach.<sup>42</sup> Finally, our study identified the compositional and functional patterns of the gut microenvironment in individuals with sarcopenia. These findings shed light on our understanding of physiological changes linked to sarcopenia and indicate a new direction for future studies that aim to understand the host-gut microbiome interplay in the pathogenesis of sarcopenia.

This large population-based observational study utilizing metagenomic shotgun sequencing provided empirical evidence that alterations in the gut microbiome composition and function were observed among individuals with sarcopenia. Future studies are warranted to validate our findings to better understand the underlying mechanisms of the gut microbiome in sarcopenia.

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## Conflict of interest

The authors declare no conflict of interest.

## Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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