Original Article

Association between gut microbiota and obesity combined with high carotid intima-media thickness among Chinese children

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Background and Objectives: Obesity and related target organ damage such as high carotid intima-media thickness (cIMT) in children is associated with cardiovascular disease (CVD) later in life. However, the association between gut microbiota and obesity combined with high cIMT among children remains unclear. Therefore, we compared differences in composition, community diversity, and richness of gut microbiota among normal children and obesity combined with or without high cIMT to identify differential microbiota biomarkers. **Methods and Study Design:** A total of 24 children with obesity combined with high cIMT (OB+high-cIMT), 24 with obesity but normal cIMT (OB+non-high cIMT), and 24 with normal weight and normal cIMT aged 10-11 years matched by age and sex from the "Huantai Childhood Cardiovascular Health Cohort Study" were included. All included fecal samples were tested using 16S rRNA gene sequencing. **Results:** The community richness and diversity of gut microbiota in OB+high-cIMT children were decreased compared with OB+non-high cIMT children and normal children. At the genus level, the relative abundances of *Christensenellaceae_R-7_group*, *UBA1819*, *Family_XIII_AD3011_group*, and *unclassified_o_Bacteroidales* were associated with reduced odds of OB+high-cIMT among children. Receiver operating characteristic (ROC) analysis showed that combined *Christensenellaceae_R-7_group*, *UBA1819*, *Family_XIII_AD3011_group*, and *unclassified_o_Bacteroidales* performed a high ability in identifying OB+high-cIMT. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) showed that several pathways such as biosynthesis of amino acids and aminoacyl-tRNA pathways were lower in the OB+high-cIMT group compared with the normal group. **Conclusions:** We found that the alteration of gut microbiota was associated with OB+high-cIMT among children, which indicates that the gut microbiota may be a marker for obesity and related cardiovascular damage among children.

Key Words: gut microbiota, cardiovascular disease, obesity, carotid intima-media thickness, children

INTRODUCTION

Cardiovascular disease (CVD) is a major public health issue worldwide, which can result in a decline in life expectancy and an increase in health and economic burden. CVD in adulthood can be tracked from cardiovascular risk factors in childhood, such as obesity, hypertension, and other related metabolic disorders.[1] Therefore, prevention of CVD risk factors in childhood can originally reduce the huge health burden later in life.

Based on data from the China Health and Nutrition Survey of 14,888 children and adolescents aged 6-17 years, the prevalence of obesity defined by the World Health Organization standard increased from 1.86% in 1991 to 10.75% in 2015.[2] It has been demonstrated that obesity, even for metabolically healthy obesity, was associated with high carotid intima-media thickness (cIMT) in children and adolescents,[3] which is an important predictor for cardiovascular events later in life.[4] Previous evidence

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showed that a reduction in the degree of CVD can be predicted by the intervention on the progress of obesity and high cIMT.[5,6] Therefore, early detection of obesity and high cIMT among children is of great significance to track cardiovascular health among children, which can provide guidance for early prevention of CVD later in life.

The gut microbiota has been considered to be a very promising target for the detection, prevention, and treatment of CVD risk factors.[7,8] Changes in the ability of gut microbiota to transport, synthesize, and compete for compounds and substrates had direct and indirect effects on human health (such as metabolic diseases, inflammatory diseases, CVD, and atherosclerotic).[9-12] A large number of clinical and experimental studies have shown that gut microbiota plays a key role in the occurrence and development of obesity mainly by regulating host energy metabolism, substrate metabolism, and inflammatory pathophysiological mechanisms in adults and children.[13,14] Previous studies mainly based on adults have shown that the disturbance in gut microbiota such as genera *Christensenellaceae_R-7_group* and *Family_XIII_AD3011_group* were significantly associated with obesity.[15,16] However, whether these gut microbiota biomarkers in adults also play an important role in the development of obesity among children remains unclear. In addition, little is known about the specific gut microbiota associated with obesity and related cardiovascular damage in children, such as high cIMT, which can be considered as a progress status of obesity.

Therefore, in this study, we aimed to compare the composition, relative abundance, and community diversity of the gut microbiota among children with obesity combined with high cIMT (OB+high-cIMT), obesity but normal cIMT (OB+non-high cIMT), and children with normal weight and normal cIMT, and screen for predominant biomarkers using 16S rRNA sequencing.

METHODS

Study population

Data were collected from baseline survey of the "Huantai Childhood Cardiovascular Health Cohort Study" conducted in a public elementary school in Huantai County, Zibo, China, between November 2017 and January 2018. In the present study, a total of 24 children aged 10-11 years with OB+high-cIMT, 24 children with OB+non-high cIMT, and 24 children with normal weight and normal cIMT matched by age and sex were included. Based on the Shannon index between obesity and normal weight reported by previous studies among children ($\alpha = 0.05$, Power = 0.90), 17 the sample size was estimated to be a total of 26 (13 per group) using a paired t-test. The total sample size in this study was 72 (24 /group * 3 groups), which met the requirement. This study was approved by the Ethics Committee of Shandong University, and written informed consent was obtained from all investigated children and their parents or guardians.

Anthropometric measurements and variables

The ultrasonic stadiometer (Shengyuan Co. Ltd, HGM-300) was used to measure the height (0.1 cm precision) and weight (0.1 kg precision). Body mass index (BMI, kg/m2) was calculated by the following equation: weight/height^2 (kg/m^2). Obesity was defined as BMI ≥ the sex- and age-specific BMI cutoffs in Chinese children and adolescents aged 2-18 years.[18] Waist circumference (WC, cm) was measured twice at the end of expiration using an inelastic measuring tape. The average of two records was used for analysis. Blood pressure (BP) was measured thrice using a clinically verified electronic sphygmomanometer Omron HEM-7012 by trained staff. The average of the last two records was used for analysis. A portable ultrasound instrument (Royal Philips, L12-4, CX30) was used to measure the cIMT of the left and right anterior and posterior walls at the proximal end of the common carotid sinus. The mean values were used for analysis. High cIMT was defined as cIMT ≥ the age- and sex-specific 90th percentile for children aged 6 to 11.[19]

Blood biochemical indicators, including glucose (GLU), triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C), were measured using an automatic analyzer (Beckman Coulter, AU480). The metabolic syndrome (MetS) of children was defined as at least three of the following components including elevated BP, elevated GLU, high TG, low HDL-C, and abdominal obesity.[20] Demographic information (such as age and gender) and lifestyle factors (such as intake of soft drinks during the past 30 days [≥3 times/week vs. <3 times/week], intake of fruit and vegetable during past 30 days [≥3 times/day vs. <3 times/day], parental education level [≥high school vs. <high school], parental smoking status [yes vs. no], and physical activity [≥1 hour/day vs. <1 hour/day]) were collected through a self-reported questionnaire.

Fecal collection and gut microbiome profiling

Fecal samples of all included children who had not received antibiotics and any other medications within the past three months were collected between 8:00 am and 10:00 am within three days, and then frozen immediately at -80°C. After extraction of gDNA and detection by 1% agarose gel electrophoresis, TransStart FastPfu DNA Polymerase (TransGen AP221-02) was used to amplify the V3–V4 region of 16S rDNA from gDNA. The primer sequences of the V3–V4 region were as follows: 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). After quality control, detection, and quantification, TruSeqTM DNA Sample Prep Kit was used to construct the Miseq library, fix the generated single-stranded DNA fragments, and perform PCR synthesis and amplification. MiSeq Reagent Kit v3-600 cycles (Illumina, California, USA) on the Illumina MiSeq platform was then used to sequence the library.

Bases with a quality value lower than 20 in the tail of Paired-end (PE) reads were filtered out to obtain the final optimized sequence. After removing single sequences without repetitions, non-repetitive sequences were extracted (http://drive5.com/usearch/manual/dereplication.html) and compared using the Silva database (Release138 http://www.arb-silva.de). Operational taxonomic units (OTU) clustering was performed according to 97% similar non-repetitive sequences. After preprocessing,

data were imported into Quantitative Insights into Microbial Ecology (QIIME version 1.9.1) for further analysis. The ribosomal database project classifier Bayesian algorithm was used to perform taxonomic analysis of OTU representative sequences on the 97% similar level, to obtain the species classification information corresponding to each OTU. The raw sequence reads have been stored in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (PRJNA811365).

Statistical analysis

Continuous variables with non-normal distribution were presented as Median (P25, P75), and categorical variables were presented as proportion and 95% confidence interval (CI). Non-parametric Kruskal-Wallis H test was used to compare the differences of continuous variables, and Chi-square test was used to compare categorical variables, across the normal group, OB+non-high cIMT group, and OB+high-cIMT group. Two-sided *p* values <0.05 indicate significant differences.

Venn diagram was used to visualize the number of overlapping and unique OTUs in the three groups. A bar chart was used to show the percent of community abundance of gut microbiota. Alpha diversity indexes including Sobs, Shannon, Simpson, Ace, and Chao indicators were used to estimate the community richness and diversity of gut microbiota, and the Wilcoxon rank-sum test was used to compare differences between each two of the three groups. Benjamini-Hochberg was used to calculate *p* values with the adjustment of the false discovery rate (FDR). The rarefaction curve was used to determine whether the sequencing data are sufficient or not.

The principal coordinate analysis (PCoA) and nonmetric multidimensional scaling (NMDS) analysis based on the Bray-Curtis distance algorithm with ANOSIM analysis were used to compare the differences in the community composition among the three groups. The linear discriminant analysis effect size (LEfSe) was conducted to screen for predominate microbial biomarkers, and then linear discriminant analysis (LDA) was performed to evaluate their effect size (LDA ≥2.5). Given good anti-noise ability and robust models, the random forest model analysis was used to evaluate the extent of contribution of important biomarkers. After adjusting for intake of soft drink intake, fruit and vegetable, parental education level, parental smoking status, physical activity, and MetS, logistic regression analyses were used to examine the associations of gut microbiota with OB+non-high cIMT and OB+high cIMT. The receiver operating characteristic (ROC) was conducted to evaluate the performance of gut microbiota in identifying the OB+high-cIMT group from normal children. All analyses were performed using R version 3.3.1 and SPSS version 23.0.

Based on the Greengene ID, the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis was used to standardize the OTU abundance table. The pathway information was obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) database, and the abundance of each pathway was performed according to the OTU abundance. The predicted functional profiling of gut microbiota was obtained by Welch's t-test for every two groups using the extended error bar plot in the STAMP version 2.1.3.

RESULTS

Study population

A total of 72 children with a median age of 10.8 years (boys 66.7%) were included in this study (OB+high-cIMT: n=24; OB+non-high cIMT: n=24; normal weight with normal cIMT: n=24; matched by age and sex). The demographic characteristics were presented in Table 1. The BMI (16.6 vs. 23.8 vs. 26.2 kg/m2), WC (60.6 vs. 81.9 vs. 85.9 cm), cIMT (0.54 vs. 0.57 vs. 0.64 mm), systolic BP (SBP, 104.3 vs. 113.3 vs. 116.8 mmHg), and TG (0.8 vs. 1.2 vs. 1.2 mmol/L) were highest in the OB+high-cIMT group, followed by the OB+non-high cIMT group and normal group. The HDL-C (1.8 vs. 1.4 vs. 1.4 mmol/L) was highest in the normal group, followed by the OB+non-high cIMT group and OB+high-cIMT group. No significant difference was observed in age, sex, TC, LDL-C, GLU, and lifestyle factors (e.g., intake of soft drinks, fruit and vegetable, and physical activity) across the three groups.

Gut microbial composition

A total of 3,909,618 optimization sequence numbers, 1,611,082,076 bases with an average length of 412.2 (min length: 208; max length: 529) was generated. Venn diagram showed that a total of 642 OTUs were overlapped by three groups, with 95 unique OTUs in the normal group, 68 unique OTUs in the OB+non-high cIMT group, and 45 unique OTUs in the OB+high-cIMT group, respectively (Figure 1A).

At the phylum level, the proportions of Proteobacteria were significantly different in the OB+high-cIMT group (3.90%), OB+non-high cIMT group (2.35%), and the normal group (0.79%) (p=0.001, Figure 1B), while no significant difference was observed in the *Firmicutes/Bacteroidetes* (F/B) ratio among the three groups (p = 0.773, Figure 1B). The proportions of gut microbiota at the genus level among the three groups were shown in Figure 1C.

Gut microbial community diversity

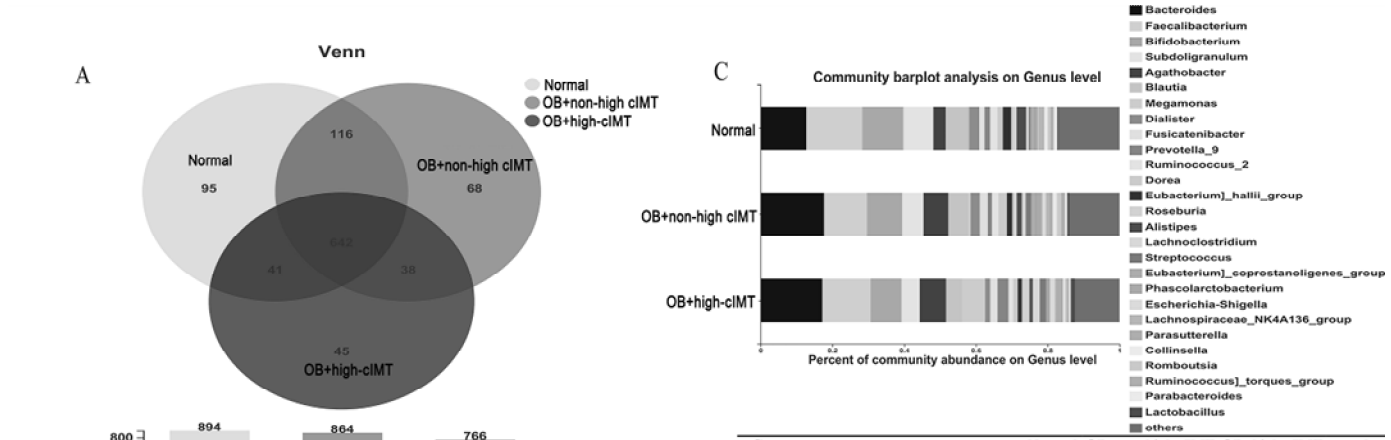
Alpha diversity showed that the richness (Sobs, Ace, and Chao) in the OB+high-cIMT group was significantly lower than the OB+non-high cIMT group and normal group (p<0.05, Figure 2A-C), as well as the community diversity (Shannon and Simpson, p<0.05, Figure 2D-E).

The flattened rarefaction curves indicated that the sequencing depth was enough to represent the most microbial species (Sobs, Ace, Chao, Shannon, Simpson; Supplementary Figure 1A-E). Given the intra- and inter-group differences, PCoA and NMDS analyses showed almost no significant separation of the microbial composition at the OTU level between the OB+non-high cIMT group and the normal group (r=0.076, p=0.015, Figure 3A and 3D) and between the OB+non-high cIMT group and the OB+high-cIMT group (r=0.055, p=0.013, Figure 3C and 3F), but a marginal separation between the OB+high-cIMT group and the normal group (r=0.120, p=0.001, Figure 3B and 3E).

Table 1. Characteristics of study participants

	Overall (N=72)	Normal (N=24)	OB+non-high cIMT (N=24)	OB+high-cIMT (N=24)	*p* value
Boys, %	66.7 (55.5, 77.8)	66.7 (44.7, 84.4)	66.7 (44.7, 84.4)	66.7 (44.7, 84.4)	1.000
Age, years	10.8 (10.5, 11.1)	11.0 (10.6,11.2)	10.7 (10.5, 11.0)	10.7 (10.4, 11.0)	0.248
BMI, kg/m^2	23.8 (17.5, 25.8)	16.6 (15.5, 17.5)	23.8 (23.0, 24.8)	26.2 (25.0, 28.9)	<0.001
WC, cm	80.0 (62.3, 84.9)	60.6 (57.5, 62.7)	81.9 (75.4, 84.8)	85.9 (83.0, 91.9)	<0.001
cIMT, mm	0.57 (0.54, 0.63)	0.54 (0.51, 0.56)	0.57 (0.53, 0.59)	0.64 (0.61, 0.65)	<0.001
SBP, mmHg	112 (105, 119)	104 (101, 110)	113 (109, 120)	117 (112, 124)	<0.001
TG, mmol/L	1.0 (0.8, 1.3)	0.8 (0.6, 0.9)	1.2 (0.8, 1.5)	1.2 (1.0, 1.6)	<0.001
TC, mmol/L	4.4 (3.9, 5.0)	4.2 (3.8, 4.8)	4.4 (4.0, 5.0)	4.5 (4.0, 5.3)	0.558
LDL-C, mmol/L	2.4 (2.0, 3.0)	2.2 (2.0, 2.7)	2.6 (2.0, 3.0)	2.8 (2.2, 3.1)	0.113
HDL-C, mmol/L	1.5 (1.3, 1.8)	1.8 (1.5, 2.0)	1.4 (1.2, 1.6)	1.4 (1.2, 1.6)	<0.001
GLU, mmol/L	5.0 (4.7, 5.4)	4.9 (4.5, 5.4)	5.0 (4.8, 5.4)	4.9 (4.6, 5.4)	0.387
Soft drinks intake, %					0.686
< 3 times/week	90.3 (83.3, 97.3)	95.8 (78.9, 99.9)	87.5 (67.6, 97.3)	87.5 (67.6, 97.3)	
≥ 3 times/week	9.7 (2.7, 16.7)	4.2 (0.1, 21.1)	12.5 (2.7, 32.4)	12.5 (2.7, 32.4)	
Fruit and vegetable intake, %					0.481
< 3 times/day	55.6 (43.8, 67.3)	45.8 (25.6, 67.2)	62.5 (40.6, 81.2)	58.3 (36.6, 77.9)	
≥ 3 times/day	44.4 (32.7, 56.2)	54.2 (32.8, 74.4)	37.5 (18.8, 59.4)	41.7 (22.1, 63.4)	
Parental education level, %					0.411
< high school	25.0% (14.8%, 35.2)	25.0 (9.8, 46.7)	16.7 (4.7, 37.4)	33.3 (15.6, 55.3)	
≥ high school	75.0 (64.8, 85.2)	75.0 (53.3, 90.2)	83.3 (62.6, 95.3)	66.7 (44.7, 84.4)	
Parental smoking, %					0.087
no	33.3 (22.2, 44.5)	37.5 (18.8, 59.4)	16.7 (4.7, 37.4)	45.8 (25.6, 67.2)	
yes	66.7 (55.5, 77.8)	62.5 (40.6, 81.2)	83.3 (62.6, 95.3)	54.2 (32.8, 74.4)	
Children's physical activity, %					0.167
< 1 hour/day	59.7 (48.1, 71.3)	50.0 (29.1, 70.9)	54.2 (32.8, 74.4)	75.0 (53.3, 90.2)	
≥ 1 hour/day	40.3 (28.7, 51.9)	50.0 (29.1, 70.9)	45.8 (25.6, 67.2)	25.0 (9.8, 46.7)	

cIMT: carotid intima-media thickness; OB+non-high cIMT: obesity with normal cIMT; OB+high-cIMT: obesity with high cIMT; BMI: body mass index; WC: waist circumference; SBP: systolic blood pressure; TG: triglyceride; TC: total cholesterol; LDL-C: low-density lipoprotein cholesterol; GLU: glucose; HDL-C: high-density lipoprotein cholesterol The median (P25, P75) and the prevalence (95% confidence interval) were used to represent continuous and category variables.



Phylum	Normal	OB+non-high cIMT	OB+high-cIMT	*p* value
Firmicutes	66.03%	63.56%	63.89%	0.381
Bacteroidetes	19.43%	21.63%	22.79%	0.846
Actinobacteria	12.86%	11.42%	9.25%	0.285
Proteobacteria	0.79%	2.35%	3.90%	0.001
others	0.89%	1.04%	0.16%	0.144
Firmicutes/Bacteroidetes ratio	5.12	5.04	12.06	0.773

Genus	Normal	OB+non-high cIMT	OB+high-cIMT	*p* value
Bacteroides	12.72%	17.65%	17.17%	0.583
Faecalibacterium	15.64%	11.99%	13.53%	0.540
Bifidobacterium	11.44%	9.80%	8.58%	0.386
Subdoligranulum	8.33%	5.94%	5.06%	0.024
Agathobacter	3.40%	6.86%	7.29%	0.041
Blautia	6.49%	5.53%	4.47%	0.360
Megamonas	0.10%	0.75%	6.47%	0.131
Dialister	2.76%	2.50%	1.37%	0.790
Fusicatenibacter	1.52%	2.29%	2.09%	0.213
Prevotella_9	1.53%	1.12%	3.01%	0.686
Ruminococcus_2	2.10%	1.89%	1.39%	0.621
Dorea	1.47%	2.30%	1.21%	0.258
Eubacterium]_hallii_group	2.32%	1.49%	1.16%	0.172
Roseburia	1.50%	1.27%	2.14%	0.430
Alistipes	2.64%	1.39%	0.88%	0.001
Lachnoclostridium	0.75%	1.12%	2.01%	0.001
Streptococcus	0.41%	1.45%	1.74%	0.297
Eubacterium]_coprostanoligenes_group	1.31%	1.37%	0.55%	0.003
Phascolarctobacterium	0.77%	1.71%	0.50%	0.094
Escherichia-Shigella	0.24%	1.16%	1.46%	0.017
Lachnospiraceae_NK4A136_group	1.30%	1.03%	0.51%	0.072
Parasutterella	0.21%	0.75%	1.67%	0.006
Collinsella	0.96%	1.17%	0.48%	0.163
Romboutsia	0.81%	1.19%	0.55%	0.031
Ruminococcus]_torques_group	0.89%	1.03%	0.62%	0.123
Parabacteroides	1.06%	0.71%	0.54%	0.017
Lactobacillus	0.28%	0.45%	1.07%	0.958
others	17.03%	14.09%	12.49%	0.016

Figure 1. Visualization of the taxonomic composition of gut microbiota in the normal, OB+non-high cIMT, and OB+high-cIMT groups. (A) The Venn diagram at OTUs levels in these three groups; (B) The Bar diagram of the proportions of gut microbial at the phylum level in these three groups; (C) The Bar diagram of the proportions of gut microbial at genus level in these three groups. The Kruskal-Wallis H test was used to compare the composition between groups.

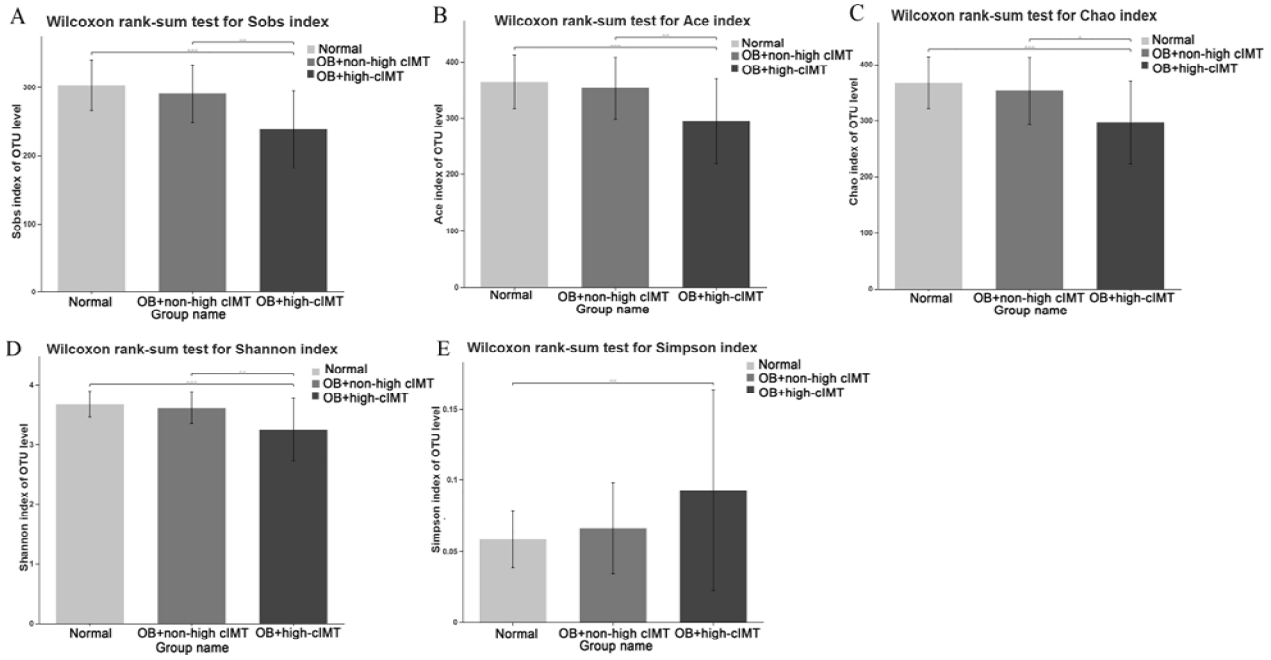


Figure 2. Differences in α-diversity indices between each two of the three groups based on the Wilcoxon rank sum test. (A) Sobs; (B) Ace; (C) Chao; (D) Shannon; (E) Simpson indexes. ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$)

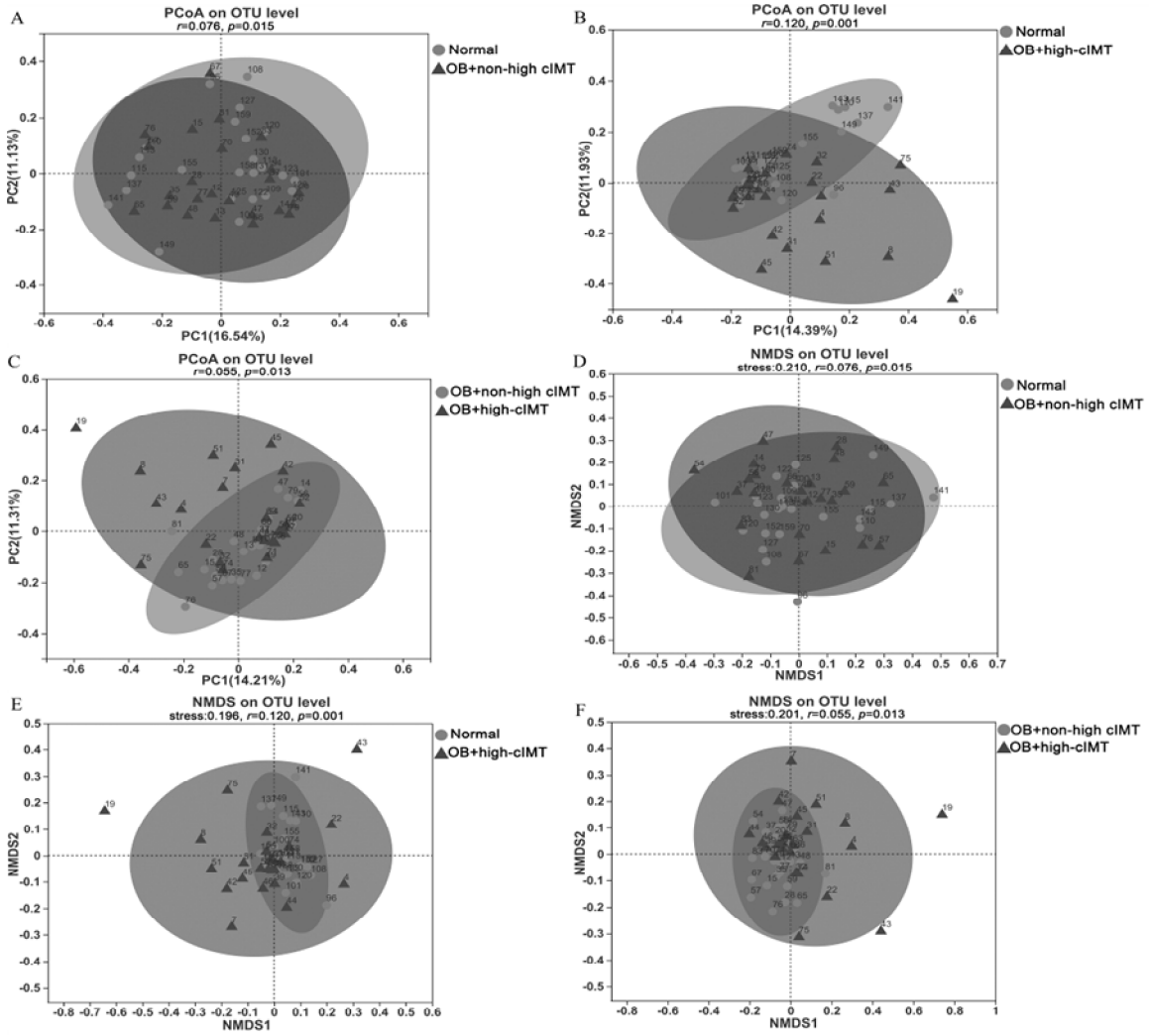


Figure 3. Principal coordinate analysis (PCoA) based on the Bray-Curtis distance algorithm to show the β-diversity among these three groups. (A) OB+non-high cIMT group vs. normal group; (B) OB+high-cIMT group vs. normal group; (C) OB+non-high cIMT group vs. OB+high-cIMT group. Nonmetric multidimensional scaling (NMDS) based on the Bray-Curtis distance algorithm to show the β-diversity among the three groups. (D) OB+non-high cIMT group vs. normal group; (E) OB+high-cIMT group vs. normal group; (F) OB+non-high cIMT group vs. OB+high-cIMT group. The differences between groups were detected according to the ANOSIM analysis.

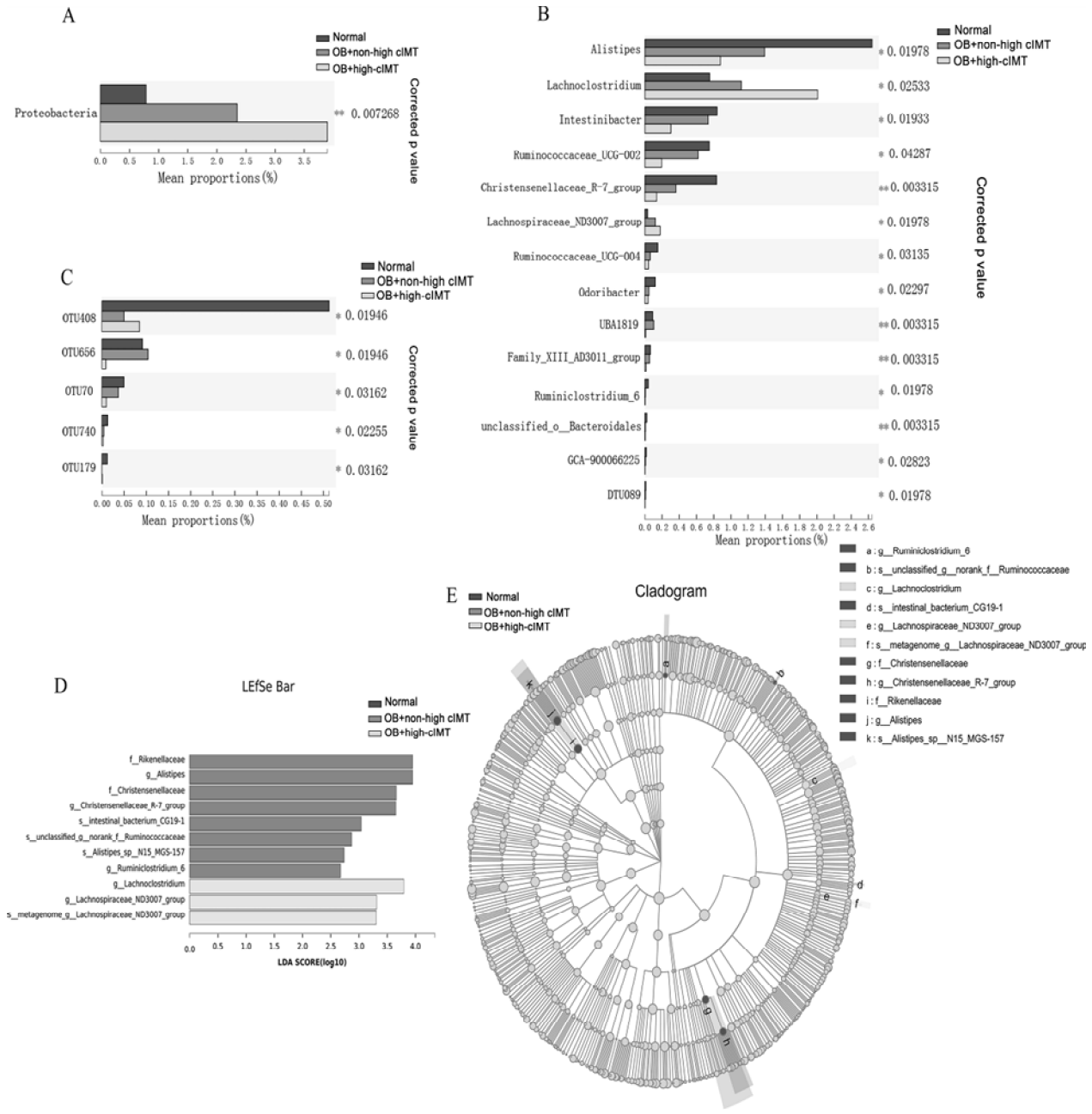


Figure 4. Difference in gut microbiota among these three groups based on the non-parametric Kruskal-Wallis H test. (A) at species levels; (B) at genus levels; (C) at OTU levels. (D) Linear discriminant analysis (≥2.5) score of each microbial biomarker; (E) Cladogram of the main bacterial biomarkers from phylum to species. The larger the diameter of each circle, the greater the relative abundance of the taxa. (p: phylum; c: class; o: order; f: family; g: genus; s: species; Unclassified as a mark without classification information; $^{*}p<0.05$, $^{**}p<0.01$)

Gut microbial biomarkers and potential value in risk assessment

At the phylum level, *Proteobacteria* was significantly enriched in the OB+high-cIMT group, followed by OB+non-high cIMT and normal groups (Figure 4A, $p_{FDR}<0.05$). At the genus level, 14 significant genera such as *Alistipes*, *Christensenellaceae_R-7_group* (top 4 significant genera), and *unclassified_o__Bacteroidale* (top 4 significant genera) were enriched in the normal group followed by OB+non-high cIMT and OB+high-cIMT groups; *Intestinibacter*, *Ruminococcaceae_UCG-002*, *Family_XIII_AD3011_group* (top 4 significant genera), and *UBA1819* (top 4 significant genera) enriched in both normal and OB+non-high cIMT groups, followed by OB+high-cIMT group; and *Lachnoclostridium and Lachnospiraceae_ND3007_group* enriched in the OB+high-cIMT group, followed by OB+non-high cIMT and normal groups (Figure 4B, $p_{FDR}<0.05$). OTU 408 in genus *Christensenellaceae_R-7_group*, OTU656 in genus *UBA1819*, OTU740 in genus *unclassified_o_Bacteroidales*, and OTU70 in genus *Family_XIII_AD3011_group* were significantly enriched in normal and OB+non-high cIMT groups compared with OB+high-cIMT group (Figure 4C, $p_{FDR}<0.05$). Based on the LDA threshold of 2.5, LEfSe analysis showed that at the genus level, *Alistipes* and *Christensenellaceae_R-7_group* were significantly enriched in the normal group, while *Lachnoclostridium* was significantly enriched in the OB+high-cIMT group (Figure 4D and 4E).

Random forest analysis further showed that 2 of the top 4 significant genera (*Christensenellaceae_R-7_group* and *UBA1819*) ranked as the two most important biomarkers

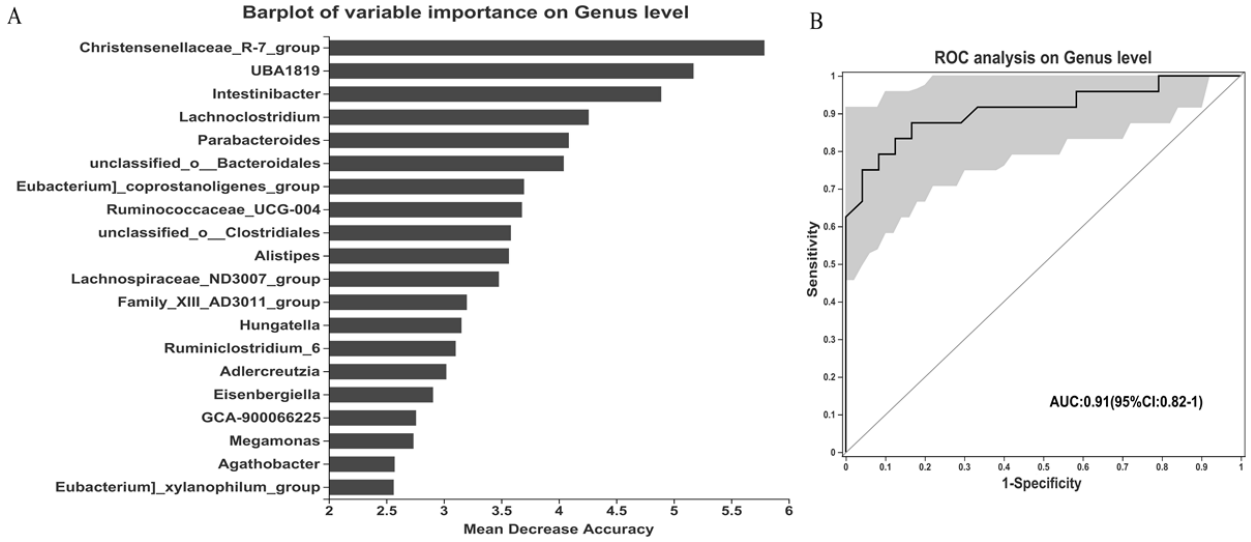


Figure 5. Further screening of significant gut microbiota and evaluation of ability in identifying OB+high-cIMT from normal children. (A) Random forest model showed the relative abundance ranking of genera; (B) ROC analysis assessed the ability of the biomarker genera to identify OB+high-cIMT vs. normal.

(Figure 5A). The other 2 of the top 4 significant genera (*unclassified_o__Bacteroidale* and *Family_XIII_AD3011_group*) were identified as the top 6 and 12 important biomarkers, respectively. These four gut microbiota biomarkers were significantly associated with OB+high-cIMT after adjusted for soft drink intake, fruit and vegetable intake, parental education level, parental smoking status, physical activity, and MetS (p<0.05, Supplemental Table 1), while the association between biomarkers and OB+non-high cIMT did not reach statistical significance. The ROC analysis showed that these four significant biomarkers had a high ability in discriminating the OB+high-cIMT group from the normal group (area under the curve [AUC]: 0.91, 95% CI: 0.82–1.00, Figure 5B).

KEGG pathways contributing to the OB+high-cIMT children

We observed 5 significant KEGG pathways, including two-component system, flagellar assembly, biosynthesis of secondary metabolites, ribosome, and biosynthesis of amino acids between OB+non-high cIMT group and normal group (p<0.05, Figure 6A). Compared with OB+high-cIMT group, the metabolism of thiamine and methane was higher in OB+non-high cIMT group (p<0.05, Figure 6B). In addition to 5 shared pathways between OB+high-cIMT group and normal group, we additionally found other several significant pathways such as biosynthesis of amino acids and aminoacyl-tRNA between OB+high-cIMT group and normal group (p<0.05, Figure 6C).

DISCUSSION

In this cross-sectional study, we identified dysbiosis of gut microbiota associated with OB+high cIMT among children. The richness and diversity of gut microbiota in OB+high-cIMT children and OB+non-high cIMT children were lower compared with the normal group. Genera *Christensenellaceae_R-7_group*, *UBA1819*, *Family_XIII_AD3011_group*, and *unclassified_o_Bacteroidales* had high ability in discriminating children with obesity combined with high cIMT from normal ones.

Emerging evidence among adults has shown that the dysbiosis of gut microbiota was associated with obesity, diabetes, and CVD.[21,22] Turnbaugh et al reported that reduced microbiota diversity was associated with obesity among female monozygotic twin pairs aged 25-32 years.[23] Kashtanova et al found that higher IMT was associated with reduced microbiota diversity among Moscow participants aged 25-76 years.[22] In addition, it has previously been shown that children with obesity with related factors, such as elevated BP, have a lower community diversity of gut microbiota, compared with normal controls.[24] However, to the best of our knowledge, few studies have been reported on the associations between gut microbiota and obesity combined with subclinical CVD (e.g., high cIMT) among children. In this study, we innovatively found that the dysbiosis of gut microbiota was associated with an increased risk of OB+high-cIMT among children. Our findings suggest that the high diversity of gut microbiota might be a protective factor for obesity and related cardiovascular damage.

We found no significant difference in the F/B ratio among the three groups. Although the increased F/B ratio has been considered as a possible marker of obesity in adults,[25] evidence on the association of the F/B ratio with childhood obesity remains inconsistent.[26] Goffredo et al reported that the F/B ratio was positively associated with obesity among US youth aged 9-18 years.[27] However, Mbakwa et al. supported our findings that no significant difference in the F/B ratio was found between children with obesity and normal ones.[28] This discrepancy may be explained by differences in methods, study design, sample size, and ethnicity. Well-designed studies are warranted to confirm these associations. We identified that the relative abundance of phylum *Proteobacteria* was the highest among OB+high-cIMT children followed by those with OB+non-high cIMT and normal ones. Studies based on adults and children similarly reported that the

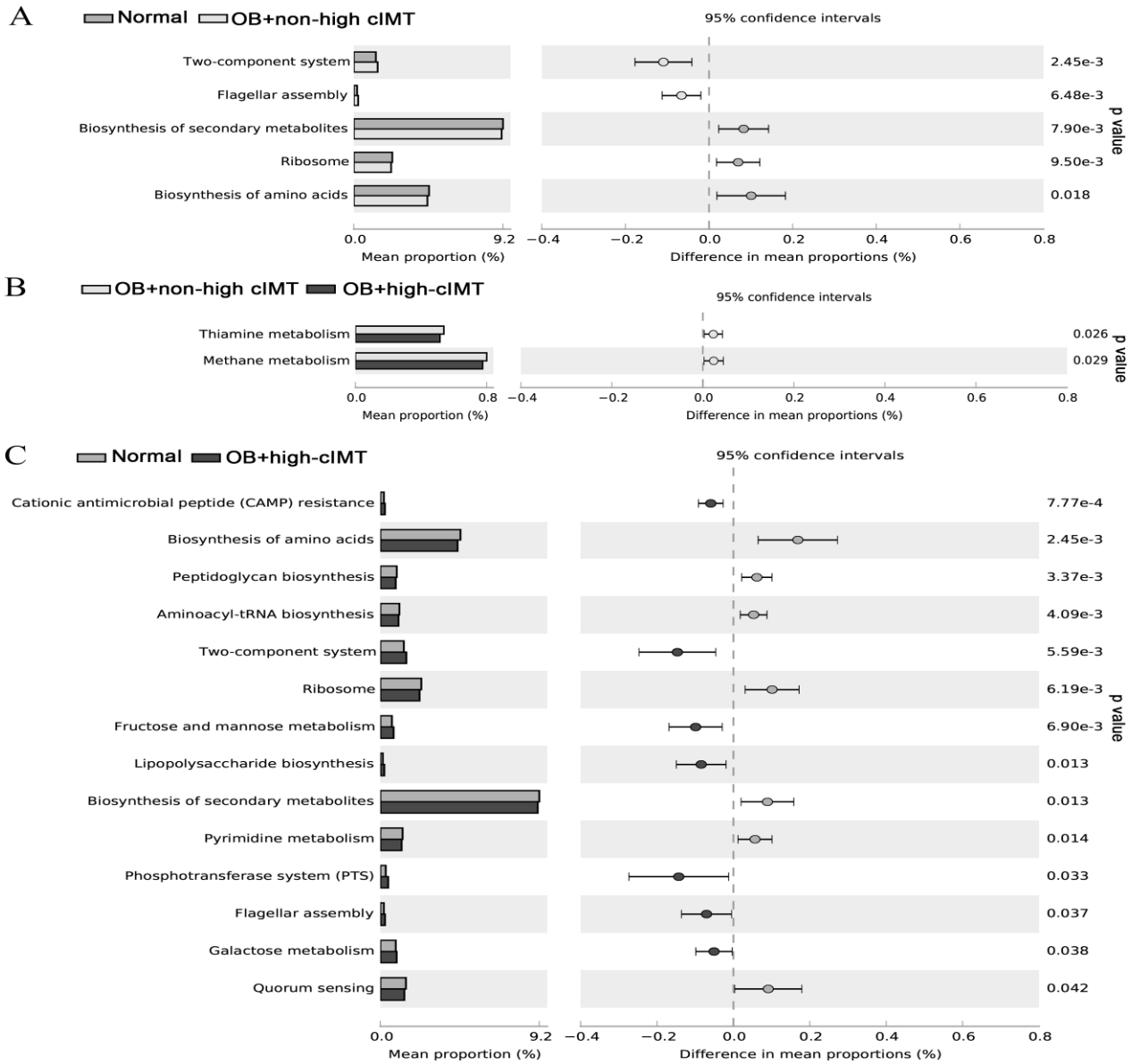


Figure 6. Extended error bars plot of the KEGG functional pathways predicted by PICRUSt in each two of the three groups. (A) normal group vs. OB+non-high cIMT group; (B) OB+non-high cIMT group vs. OB+high-cIMT group; (C) normal group vs. OB+high-cIMT group

relative abundance of *Proteobacteria* was positively associated with obesity and its related metabolic disorder.[29-32] A recent systematic review showed that the phylum *Proteobacteria* was associated with obesity in both adults and children.[29] *Proteobacteria* (e.g., *Proteus mirabilis* and *Escherichia coli*) were potential drivers of gastrointestinal inflammation,[31] which might be associated with the development of metabolic disorders (such as obesity, atherosclerosis, insulin resistance, and diabetes).[32]

Moreover, the genus *Lachnoclostridium* was the highest among OB+high-cIMT children followed by those with OB+non-high cIMT and normal ones, while genus *Alistipes* showed an opposite trend. A large population-based cohort based on the TwinsUK registered adult twins showed that Lachnoclostridium was positively associated with visceral fat and increased the risk of cardiometabolic diseases.[33] However, no studies have reported the association between *Lachnoclostridium* and obesity or related metabolic diseases in children. We found that the relative abundance of *Lachnoclostridium* was higher in OB+high-cIMT and OB+non-high cIMT children. Consistent with our results among children, *Alistipes* can be considered as an important protective biomarker for CVD and MetS among Chinese adults.[34,35] In contrast, a study of 54 American adults suggested that *Alistipes* was associated with an increased BP.[36] In addition, *Alistipes* was also found to be positively associated with serum adipokines (adropin and angiopoietin-like 4) among 65 Chinese children with obesity.[37] The discrepancy may be due to differences in sample size, sex distribution, ethnic groups, and different dietary patterns.[38] In summary, our findings add to the existing evidence and suggest that increased phylum *Proteobacteria* and genus *Lachnoclostridium* and decreased genus *Alistipes* might contribute to the development of obesity and related cardiovascular damage among children.

We found that the relative abundance of genera *Christensenellaceae_R-7_group*, *UBA1819*, *Fami-*

ly_XIII_AD3011_group, and *unclassified_o_Bacteroidales* were associated with reduced odds of OB+high-cIMT and these four gut microbiotas had high ability in identifying OB+high-cIMT from normal groups, which suggest that a decreased abundance of these four biomarkers might play a vital role in the development of obesity combined with damage of carotid intima-media. It has been demonstrated that the potential mechanisms might be due to adipose tissue inflammation and glucolipid metabolism disorder.[15,39,40] Several human and experimental studies supported the protective effects of these biomarkers on CVD risk factors. For example, a study based on hypertensive adults aged 18-65 showed a protective effect of increased relative abundance of *Christensenellaceae_R-7_group* on BP.[15] A recent experimental study by Shi et al. showed that overexpressed *UBA1819* was associated with reduced body weight of high-fat-fed rats by reducing adipose tissue inflammation and glucolipid metabolism disorder.[41] Lüll et al reported that the *Family_XIII_AD3011_group* was decreased in females with obesity among polycystic ovary syndrome women in Finland.[16] One experimental study showed that increased abundance of *unclassified_o_Bacteroidales* was associated with improved glycolipid metabolism in mice with type 2 diabetes,[39] whereas the other study showed that it was positively associated with pro-inflammatory cytokines in the colitis mice model.[40] The discrepancy may be due to differences in animal models and dietary patterns.[38] In addition, in this present study, the association between the four gut microbiota biomarkers at genera level and OB+non-high cIMT did not reach significance after adjusting for lifestyle factors, suggesting that the altering of the gut microbiota might start to appear when obesity progressed to high cIMT. These biomarkers may be used as one of the non-invasive diagnoses of obesity combined with damage to carotid intima-media among children.

We found that several compounds and substrates pathways may contribute to differentiating obesity and related cardiovascular damage from normal children, possibly due to chronic inflammation caused by intestinal permeability and microbiota density.[42] A review showed that the biosynthesis of amino acids could significantly affect the macrophage atherogenicity through the regulation of cellular triglyceride metabolism, thereby leading to CVD.[42,43] Aminoacyl-tRNA synthetases catalyzed amino acids to provide raw materials for protein translocation, which have been reported to be closely associated with obesity and CVD.[44,45] We additionally found that thiamine metabolism and methane metabolism might have potential role in discriminating OB+high-cIMT and OB+high cIMT. It may be explained by the essential effect of thiamine on the synthesis and secretion of insulin and the regulation of leptin concentration, which were markers of diabetes and obesity.[46] In addition, human arterial smooth muscle cells mediated by insulin and glucose play a key role in the development of atherosclerotic plaques.[47] Methane, which can be produced by Methanomassiliicoccales, was involved in the progress of adult CVD and plays a protective effect by anti-inflammation, anti-oxidation, and anti-apoptosis.[48,49] Our findings suggest that the association of obesity and related cardiovascular damage with gut microbiota might be mediated by compounds and substrates pathways.

To the best of our knowledge, this is the first study to explore the association between gut microbiota and obesity and related cardiovascular damage in children, and we have found several biomarkers which have high ability in identifying OB+high-cIMT children from normal children. However, several limitations should be noted. First, 16S rRNA sequencing generally cannot provide a level of species resolution, and more comprehensive technologies, such as *in vivo* experiments in mice and metagenomic sequencing, are needed to reveal the underlying mechanisms of the gut microbiota in depth. Second, our case-control study cannot be used for causal inference. Third, our sample size was relatively small compared with studies in adults. However, our sample size met the requirement which can provide statistical confidence to the results and can easily measure outcomes with significant changes in the microbiota.[50] The flattened rarefaction curves also indicated that the sequencing data of the samples were sufficient and reasonable, and larger sample size will only produce a few new features. Fourth, our study was based on a single center, which needs to be validated in multiple centers and other ethnic groups. Finally, all children with high cIMT screened from 1,515 children at the baseline of the "Huantai Childhood Cardiovascular Health Cohort Study" were all accompanied by obesity and we were unable to include children with normal weight and high cIMT. Future studies with a larger population are needed to address this issue.

In conclusion, we found that dysbiosis of gut microbiota was associated with obesity combined with carotid intima-media damage in children. Genera *Christensenellaceae_R-7_group*, *UBA1819*, *Family_XIII_AD3011_group*, and *unclassified_o_Bacteroidales* had a high ability in identifying obesity and related cardiovascular damage. Our study provides effective and targeted guidance for interventions for children with obesity and related cardiovascular damage.

DATA AVAILABILITY

The raw sequence reads can be found under NCBI accession number PRJNA811365 and are also available from the corresponding author Bo Xi (Email: xibo2007@126.com) upon request.

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AUTHOR DISCLOSURES

No competing interests are reported.

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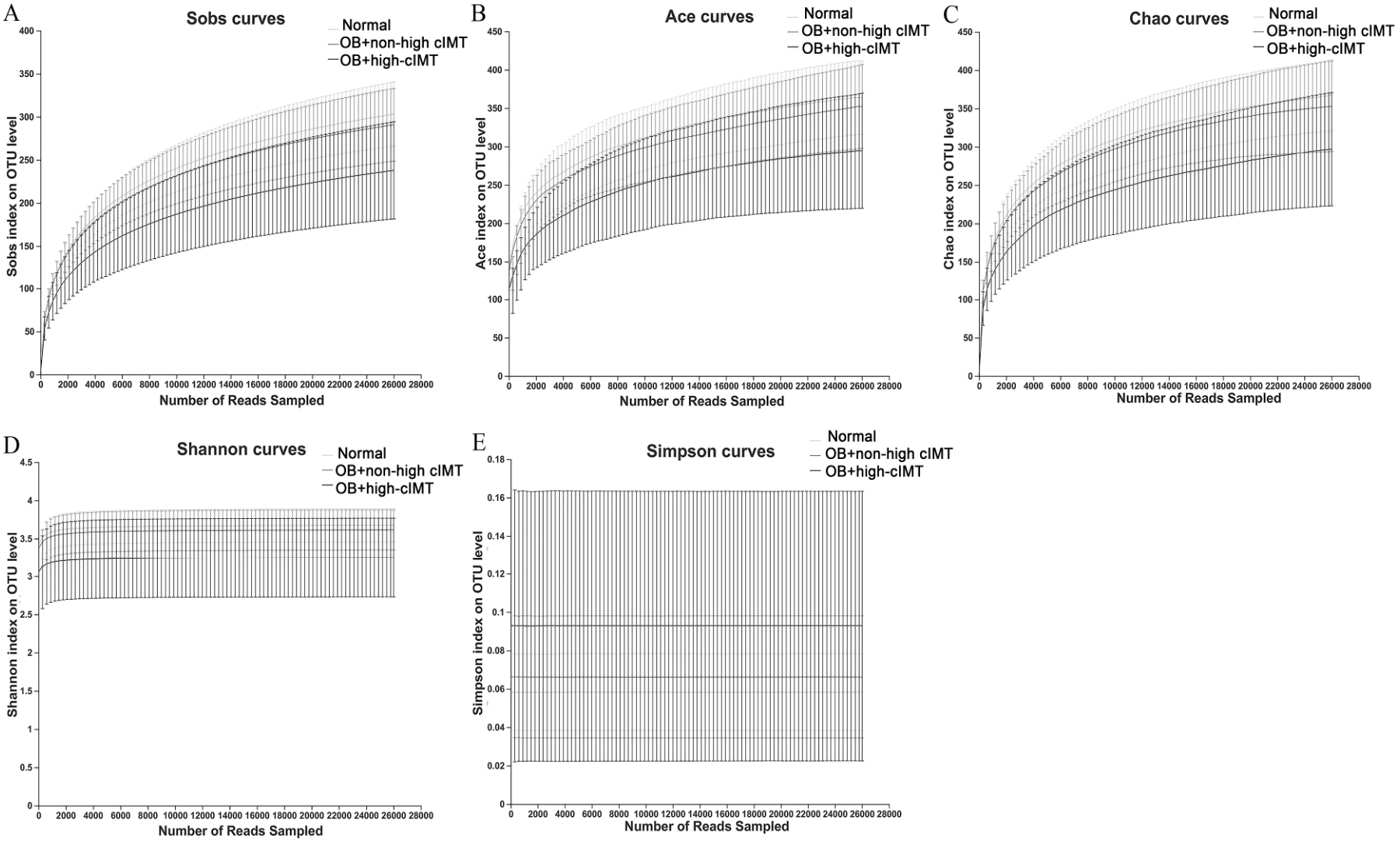
Supplementary table 1. Associations between gut biomarkers and obesity with and without high cIMT

	OR (95% CI)			
	Model 1	*p* value	Model 2	*p* value
OB+non-cIMT vs. Normal				
Christensenellaceae_R-7_group	0.96(0.88,1.04)	0.326	0.96(0.89,1.04)	0.340
UBA1819	1.01 (0.99,1.03)	0.412	1.01(0.99,1.03)	0.342
Family_XIII_AD3011_group	1.05(0.84,1.31)	0.666	1.07(0.86,1.34)	0.542
unclassified_o__Bacteroidales	0.35(0.11,1.15)	0.084	0.23(0.05,1.15)	0.073
OB+high-cIMT vs. Normal				
Christensenellaceae_R-7_group	0.74(0.60,0.91)	< 0.01	0.74(0.58,0.94)	< 0.05
UBA1819	0.68(0.52,0.89)	< 0.01	0.44(0.22,0.87)	< 0.05
Family_XIII_AD3011_group	0.21(0.08,0.54)	< 0.01	0.10(0.02,0.51)	< 0.01
unclassified_o__Bacteroidales	0.04(0.00,0.41)	< 0.01	0.05(0.00,0.60)	< 0.05

OR: odds ratio; CI: confidence interval.
Model 1: Adjusted for soft drink intake, fruits and vegetables intake, parental education level, parental smoking status, and children's physical activity.
Model 2: Model 1 plus metabolic syndrome which was defined as at least three of following components including elevated BP, elevated GLU, high TG, low HDL-C, and abdominal obesity.



Supplementary figure 1. The rarefaction curves of each sample at the OTU level. (A) Sobs; (B) Ace; (C) Chao; (D) Shannon; (E) Simpson.