Gut microbiota and fecal short-chain fatty acids are not associated with bone mass in healthy Chinese children: a cross-sectional study

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Research

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Abstract

Background

The link between the gut microbiota, short-chain fatty acids (SCFAs), and bone loss has already been observed in animal models and a few human studies in adults, but no such study has been conducted in children. We aimed to investigate whether the gut microbiota and fecal SCFAs are associated with bone mass in healthy Chinese children aged 6–9 years.

Methods

In this study, 236 healthy children including 145 boys and 91 girls were enrolled. Fecal samples from children were collected, and DNA was extracted. 16S rRNA gene sequencing was used to characterize the composition of their gut microbiota. Total and 10 subtypes of SCFAs in the fecal samples were determined by high-performance liquid chromatography. Dual X-ray absorptiometry was used to measure the bone mineral density and bone mineral content (BMC) for total body and total body less head (TBLH). Size-adjusted BMC for TBLH was calculated.

Result

The boys showed less gut microbial diversity than the girls, as indicated by the Chao1 index (364.02 (63.07) vs. 375.12 (43.50), $P = 0.018$) and abundance-based coverage estimator (362.65 (54.48) vs. 381.07 (40.19), $P = 0.007$). No significant sex difference was found in the relative abundance of the gut microbiota at any level. Multiple regression analysis after adjustment for covariates and multiple test correction showed that neither gut microbial richness (β: −0.15–0.16, *P*: 0.376–0.984) nor fecal subtypes of or total SCFAs (β: −0.06–0.17, *P*: 0.699–0.979) were correlated with bone mass measures in total samples. Similar results were observed in sex-specific analysis.

Conclusions

Our results did not support the hypothesis that the gut microbiota and fecal SCFA concentrations are associated with bone mass in children.

Introduction

Osteoporosis is a systemic skeletal disease characterized by low bone mineral density (BMD) and alterations in the bone microstructure, which eventually increase bone fragility and susceptibility to fracture [1]. The prevalence of osteoporosis in China in people aged over 65 years reached 32.0% in 2018; specifically, the prevalence was 10.7% in men and 51.6% in women [2]. It is speculated that the population with osteoporosis will increase sharply from 83.9 million in 1997 to 212 million by 2050 [3].

The two main factors related to the risk of osteoporosis are the peak bone mass reached during the bone maturity period and the rate of bone loss after adulthood. Bone tissue accumulated during childhood accounts for approximately half of the bone mass in adulthood [4]. An increase of 10% in the peak bone mass during childhood would delay the onset of osteoporosis by 13 years and reduce the risk of osteoporotic fractures by 50% [5, 6]. Therefore, maximizing bone health during growth may represent an important strategy in the prevention of osteoporosis and fractures during aging.

The human gut microbiota is composed of more than 1000 distinct microbial species. Gut colonization by microbes occurs rapidly after birth [7]. The composition and activity of the gut microbiota change with age, development of the immune system [8], and changes in geographical location and dietary structure [9]. Short-chain fatty acids (SCFAs), which are key regulatory factors in human metabolism, are generated from the interplay between the diet and gut microbiota in the gut luminal environment. Evidence from animal studies suggests that through their metabolites, such as SCFAs, intestinal microbes can disrupt the balance between bone formation and resorption by regulating insulin-like growth factors or altering the bone immune status, and can also alter the metabolism of serotonin, cortisol, and sex hormones, thereby affecting bone mass [10]. Alterations of the gut microbiota by intestinal microflora transplantation, antibiotic treatments, probiotic supplements, or dietary interventions can also increase or decrease bone density [11]. Therapeutic supplementation of SCFAs or diets that increase the endogenous production of SCFAs have been shown to significantly enhance bone mass and prevent postmenopausal and inflammation-induced bone loss [12]. Currently, some human studies on the associations of the gut microbiota and SCFAs with bone health are still underway, but three previous studies in adults that compared microbiota composition between people with normal and low bone mass have not shown consensus on their observations of individual bacterial taxa [13–15]. For example, the phylum *Firmicutes* was reported to be significantly higher in the osteoporosis group than in the control group in the study by Wang et al. [13], but was lower in the study by Li et al. [15]. Another study on U.K. adults revealed a weak correlation between the gut microbiota and BMD of the pelvis but not of other skeletal regions [16]. Notably, no such epidemiologic evidence is available for the role of the gut microbiota in bone mass accumulation during childhood.

Therefore, the aim of this study was to determine the association of the gut microbiota composition and SCFAs with bone health in Chinese children aged 6–9 years.

Results

Children’s characteristics

A total of 236 children (91 girls and 145 boys) were included in this study. The demographic characteristics of the study group are presented in Table 1. The median (interquartile range) ages of the girls and boys were 8.17 (1.17) and 8.25 (1.17) years, respectively. Compared with the girls, the boys had higher weight, body mass index, physical activity, and daily energy intake. In addition, the TB BMC, TB

BMD, SA-TB BMC, and SA-TBLH BMC were also higher in the boys than the girls ($P < 0.05$). No significant differences were found in the other characteristics between the sexes.

Table 1
Characteristics of 236 children in the cross-sectional study

Variables	Total (N = 236)	Girls (N = 91)	Boys (N = 145)	*P*
Age (years)	8.25 (1.17)	8.17 (1.17)	8.25 (1.17)	0.307
Height (cm)	129.45 (10.83)	128.60 (10.50)	130.90 (10.90)	0.187
Weight (kg)	25.64 (8.71)	24.86 (8.33)	26.39 (9.09)	**0.038**
BMI (kg/m^2)	15.48 (3.94)	15.06 (3.68)	15.58 (4.32)	**0.033**
Physical activity (MET × h/d)	39.44 (5.22)	38.67 (5.22)	40.02 (5.04)	**0.007**
Daily energy intake (kcal/d)	1390 (576)	1303 (628)	1425 (578)	**0.005**
Daily fiber intake (g/d)	6.88 (3.87)	7.06 (3.90)	6.71 (3.89)	0.820
Delivery mode, *N* (%)				0.164
Natural	111 (47.0)	48 (52.7)	63 (43.4)	
Cesarean	125 (53.0)	43 (47.3)	82 (56.6)	
Maternal educational level, *N* (%)				0.072
Primary or less	40 (16.9)	11 (12.1)	29 (20.0)	
Secondary	38 (16.1)	20 (22.0)	18 (12.4)	
Graduate or above	158 (66.9)	60 (65.9)	98 (67.6)	
Paternal educational level, *N* (%)				0.283
Primary or less	22 (9.3)	5 (5.5)	17 (11.7)	
Secondary	66 (28.0)	30 (33.0)	36 (24.8)	
Graduate or above	146 (61.9)	55 (60.4)	91 (62.8)	
Use of calcium supplement, *N* (%)				0.396
No	145 (61.4)	59 (64.8)	86 (59.3)	
Yes	91 (38.6)	32 (35.2)	59 (40.7)	
Use of multivitamin supplement, *N* (%)				0.404
No	199 (84.3)	79 (86.8)	120 (82.8)	

SCFAs, short-chain fatty acids; TSCFAs, total short-chain fatty acids; TB BMC: total body bone mineral content; TB BMD: total body bone mineral density; SA- TB BMC: Size-adjusted total body bone mineral content; TBLH BMC: total body less head bone mineral content; TBLH BMD: total body less head bone mineral density; SA-TBLH BMC: Size-adjusted total body less head bone mineral content; BMI: body mass index. Bold indicates $P < 0.05$.

Variables	Total (N = 236)	Girls (N = 91)	Boys (N = 145)	*P*
Yes	37 (15.7)	12 (13.2)	25 (17.2)	
Household income, Yuan × month^{-1}, *N* (%)				0.994
≤ 150,00	109 (46.2)	42 (46.2)	67 (46.2)	
>150,00	127 (53.8)	49 (53.8)	78 (53.8)	
Acetic acid (μmol/g)	54.52 (39.06)	54.93 (41.58)	54.28 (36.25)	0.506
Propionic acid (μmol/g)	25.94 (16.05)	25.08 (16.12)	26.44 (15.09)	0.633
Crotonic acid (μmol/g)	0.13 (0.27)	0.09 (0.28)	0.13 (0.27)	0.341
Isobutyric acid (μmol/g)	1.99 (1.51)	2.02 (1.33)	1.97 (1.63)	0.704
n-Butyric acid (μmol/g)	19.07 (14.46)	18.37 (16.93)	19.20 (13.11)	0.734
2-Methylbutyric acid (μmol/g)	1.03 (1.01)	1.06 (0.92)	1.00 (0.95)	0.748
Iso-Valeric acid (μmol/g)	1.77 (1.87)	1.82 (1.73)	1.74 (1.84)	0.380
Valeric acid (μmol/g)	2.30 (2.67)	2.29 (1.96)	2.42 (2.87)	0.857
4-Methylvaleric acid (μmol/g)	0.04 (0.09)	0.05 (0.09)	0.03 (0.07)	0.365
Hexanoic acid (μmol/g)	0.10 (0.20)	0.10 (0.10)	0.12 (0.29)	0.122
TSCFAs (μmol/g)	110.18 (68.24)	108.81 (69.73)	111.11 (66.92)	0.678
TB BMC (g)	934.52(177.77)	920.29 (180.50)	948.35 (177.22)	**0.048**
TB BMD (g/cm^2)	0.79 (0.08)	0.77 (0.09)	0.80 (0.08)	**0.001**
SA-TB BMC (g)	951.25 (72.40)	927.98 (62.47)	970.60 (63.38)	**< 0.001**
TBLH BMC (g)	589.44 (132.16)	584.07 (129.65)	590.00 (132.54)	0.842
TBLH BMD (g/cm^2)	0.62 (0.08)	0.61 (0.08)	0.62 (0.08)	0.109
SA-TBLH BMC (g)	603.57 (32.51)	597.21 (35.71)	608.73 (31.58)	**0.008**

SCFAs, short-chain fatty acids; TSCFAs, total short-chain fatty acids; TB BMC: total body bone mineral content; TB BMD: total body bone mineral density; SA- TB BMC: Size-adjusted total body bone mineral content; TBLH BMC: total body less head bone mineral content; TBLH BMD: total body less head bone mineral density; SA-TBLH BMC: Size-adjusted total body less head bone mineral content; BMI: body mass index. Bold indicates $P < 0.05$.

Differences in gut microbial diversity between healthy girls and boys

After paired-end read merging and error correction of the 16S rRNA sequencing data, a total of 15,925,947 high-quality sequences were obtained. The amplicons were clustered into 87,987 OTUs based on ≥ 97% sequence identity. As presented in Fig. 1, the boys exhibited a lower gut microbial biodiversity than the girls, as indicated by the Chao1 index (364.02 (63.07) vs. 375.12 (43.50), $P = 0.018$) and ACE (362.65 (54.48) vs. 381.07 (40.19), $P = 0.007$).

The sex differences in β-diversity based on the PCoA of the Bray-Curtis dissimilarity, weighted UniFrac distances, and unweighted UniFrac distances. The PCoA calculated on the weighted UniFrac distances revealed that the gut microbiota of the boys subjects clustered apart from that of girls subjects ($P = 0.003$, Figs. 2). No difference in other β-diversity indexes was detected between boys and girls (P: 0.052–0.172).

Differences in gut microbiota composition and SCFAs between healthy boys and girls

The gut microbiota in both boys and girls was found to be dominated by four main bacterial phyla, namely *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Proteobacteria* (Supplemental Table S1). The relative abundance of Order *Coriobacteriia* and Class *Coriobacteriia* were higher in boys compared with the girls ($P = 0.043$, $P_{adjusted} = 0.020$). No significant sex difference was found in the microbial composition at other level with ($P_{adjusted}$: 0.050–0.957) or without (P: 0.052–0.983) covariates and FDR correction (P_{FDR}: 0.616–0.986) (Fig. 3 and Supplemental Tables S1). Similar results were observed for the ratio of *Firmicutes/Bacteroidetes* (1.63 vs. 1.58, $P_{FDR} = 0.819$). Similarly, with regard to SCFAs, no difference was found in total and subtypes of SCFAs between boys and girls (P_{FDR}: 0.677–0.973)

Associations Between Gut Microbiota, Fecal Scfas, And Bone Measurements

The correlations between the 10 most abundant taxa in the gut microbiota and bone measures are shown in Figs. 4. No significant association was observed between the gut microbiota and the BMC/BMD for TB and TBLH using Spearman correlation analysis (r: 0.14–0.16, P: 0.731–0.996; Supplemental Figure S1). In the covariate-adjusted multiple regression model, the BMC for TBLH was significantly positively associated with *Prevotellaceae* at the family level (β = 0.10, $P = 0.007$) and *Prevotella-9* at the genus level (β = 0.09, $P = 0.009$); however, these relationships were nullified after FDR correction (Fig. 4). The genus *Pseudobutyrivibrio* was negatively associated with the BMC for TBLH after adjusting for the covariates (β = −0.08, $P = 0.034$). Similar significant associations were observed between the family *Prevotellaceae*, the genus *Pseudobutyrivibrio* and *Prevotella-9* and the TB BMC, TB BMD, and TBLH BMD; however, these associations were nullified after FDR correction in boys, girls, and total children (Fig. 4 and Supplemental Figure S3).

Spearman correlation analysis was used to identify the association between fecal SCFAs and bone mass measurements. The results showed no significant association between them (r: −0.22–0.11, P: 0.057–

0.992; Supplemental Figure S2). Multiple regression analysis showed that the BMC/BMD for TB and TBLH and SA-BMC for TBLH had no significant association with SCFAs after adjusting for covariates and FDR correction in boys (β: −0.17–0.17, *P*: 0.708–0.956), girls (β: −0.15–0.36, *P*: 0.115–0.990), or total children (β: −0.06–0.17, *P*: 0.699–0.979) (Fig. 5 and Supplemental Figure S4).

Discussion

This cross-sectional study was the first to examine the relationship between the gut microbiota, SCFAs, and bone mass measurements in healthy children. After controlling for potential covariates and multiple comparison correction, the findings showed no significant associations between the fecal gut microbiota, SCFAs, and bone measurements in children aged 6–9 years.

Gut microbiota and bone mass

Recent investigations in germ-free mice linking the presence and contents of the gut microbiota with the accumulation of bone mass during growth have reported conflicting results. Some studies showed that young germ-free mice had greater bone mass and skeletal strength measures than conventionally raised mice [17, 18]. Germ-free mice colonized with conventional microbiotas exhibited lower trabecular bone mass and increased bone resorption marker [17]. In contrast, another study reported that 8-week-old germ-free mice had reduced femoral length, cortical thickness, and BMD compared with conventionally raised mice [18]. Experimental data from mice in another study demonstrated that the modulation of the gut microbiota in germ-free mice using probiotics can increase bone mass [19]. To date, limited studies on human adults have directly evaluated the correlation between the gut microbiota and bone health. A study using the U.K. Biobank resource suggested a weak correlation of the gut microbiota with pelvic BMD in healthy humans aged 37–76 years ($P = 0.044$), but no significant relationships were observed between the gut microbiota and BMD at other sites (e.g. femur neck and spine) [16]. In 181 individuals aged 55–75 years, Mrinmoy et al. did not identify any taxa that differed significantly in abundance between individuals with osteopenia and those with normal BMD, but the patients with osteoporosis had higher abundance of the genera *Actinomyces*, *Eggerthella*, *Clostridium* cluster XIVa, and *Lactobacillus* than the individuals with normal BMD [14]. Wang et al. reported that the proportions of the genera *Blautia* and *Parabacteroides*, the family *Ruminococcaceae*, and the phyla *Firmicutes*, *Gemmatimonadetes*, and *Chloroflexi* were significantly higher in a group of elderly osteoporosis patients than in a group of elderly people with normal bone density in China [13]. In another study on 102 elderly people aged ≥60 years in China, individuals with low BMD were shown to have fewer OTUs and less bacterial diversity at the phylum, family, and genus levels, such as *Firmicutes*, *Actinobacteria*, *Bifidobacteriaceae*, and *Bifidobacterium* [20]. Alterations in the gut microbiota have also been observed in other clinical conditions (e.g. inflammatory bowel disease) in which osteopenia develops [21]. Although current evidence from studies on adults supports the link between the gut microbiota and bone loss, our findings revealed no connection between the gut microbiota and bone mass in children.

Although the gut microbiota displays a robust response to external stimuli such as antibiotics [22] and short-term diet shifts [23], it rapidly returns to its original steady state after the disturbing factor is eliminated. This makes it difficult to sustain controlled changes in the microbiota over extended time periods required to detect changes in skeletal phenotype [24]. Additionally, the activities of osteoblasts and osteoclasts change with advancing age; particularly, the activity of osteoclasts in older people begins to outweigh the activity of osteoblasts [25]. The gut microbiota may regulate these two types of cells differently through its metabolites. Lucas et al. showed that therapeutic supplementation of SCFAs or diets that increase the endogenous production of SCFAs could inhibit osteoclast differentiation and bone resorption in vitro and in vivo, without affecting bone formation [12]. This provides another possible explanation for the differing results between adults and children.

SCFAs and bone mass

Gut microbes ferment dietary or prebiotic fibers to SCFAs. SCFAs have the capacity to diffuse to distant organs and induce potent bone regulatory effects, which indicates that SCFAs may function as potent regulators of osteoclast metabolism and bone homeostasis. For example, propionate and butyrate have been shown to inhibit osteocalcin differentiation in a dose- and time-dependent manner in vitro [10]. A study on a mouse model revealed that treatment of mice with SCFAs in addition to feeding a high-fiber diet significantly increased the bone mass and prevented postmenopausal and inflammation-induced bone loss [12]. In a weanling rat, intervention for SCFA production resulted in a significant increase in bone mass [26]. To date, no human study has directly investigated the association between SCFAs and the bone mineral status. The results from a randomized controlled trial indicated that daily consumption of a combination of prebiotic short- and long-chain inulin-type fructans elicited significant bone mineralization enhancement during pubertal growth [27]. However, in our study, no significant relationship was detected between total and subtypes of SCFAs in feces and bone mass measures in children. More human studies are needed to verify this finding.

Strengths and Limitations

This study is the first to explore the associations of the gut microbiota and SCFAs with bone mass in a relatively large sample size of children. However, several limitations also merit consideration. First, the cross-sectional nature of our data did not permit us to make causal inferences. Second, DXA examination was conducted for TB, but not for the spine and hip, where osteoporosis fractures most frequently occur. However, data derived from our previous study suggested a strong correlation between the BMC for TB, lumbar spine, and femur neck (r: 0.730–0.894, $P < 0.0001$) [28]. Third, the fecal microbiota and SCFA data were not tracked as they changed over time within each individual. Future studies should collect fecal samples of the study population over time to evaluate longitudinal changes. Fourth, we did not measure SCFA concentrations in blood, which may better represent SCFA absorption. Fifth, the gut microbiota composition was assessed by 16S rRNA sequencing, which may be biased by primer choice and PCR-related issues and does not provide information about bacterial genes. Sixth, children were recruited from an urban area and covered a narrow age range, which may limit the generalizability of the findings. Last,

although a range of dietary and lifestyle confounders were applied for adjustment, residual or unmeasured confounding is still inevitable.

Conclusions

In conclusion, the findings of this first study in healthy Chinese children aged 6–9 years do not support the hypothesis that the gut microbiota and fecal SCFAs are correlated with bone mass in children. Future longitudinal studies with repeated assessment of other metabolic markers of the microbiota and SCFAs (cecum, blood, genetic partial metabolizer, etc.) are required to verify our findings.

Subjects And Methods

Subjects

Healthy children aged 6–9 years were recruited from a kindergarten and primary school in Guangzhou between 2015 and 2017 as reported previously [29, 30]. Two recruitment methods were used: recruitment leaflets distributed in five primary schools in different areas of Guangzhou, and recruitment through parental mutual acquaintances and WeChat public accounts. The following exclusion criteria were adopted: (1) use of antibiotics within 3 months before fecal sample collections; (2) twins and preterm births; (3) incomplete general data or incomplete collection of feces or bone density testing; or (4) history of serious disease. Eventually, 236 participants (145 boys and 91 girls) were included in this study.

This study was approved by the Ethics Committee of the School of Public Health at Sun Yat-sen University (No. 201549). The parents or legal guardians of all participants provided written informed consent prior to enrollment.

Dual X-ray absorptiometry (DXA) measurements for bone mineral content (BMC) and BMD

The weight of all participants was measured to the nearest 0.1 kg using a Tanita MC-780A scale (Tanita Corporation, Tokyo, Japan), with the participants wearing light clothes and no shoes. Height was measured to the nearest 0.1 cm using a portable fixed stadiometer, with the participants in an upright position with no shoes. Whole body DXA scans were performed using the Hologic Discovery W System (Discovery W; Hologic Inc., Waltham, MA, USA) in accordance with the manufacturer's instructions. The BMC and bone area (BA) values for total body (TB) and total body less head (TBLH) were determined. The BMDs for the TB and TBLH were calculated by dividing the BMC by the BA. The coefficients of variation between two consecutive measurements of BMC (BMD) for TB and TBLH, with repositioning among 33 children on the same day, were 1.09% (1.58%) and 1.37% (2.04%), respectively.

Measurement of gut microbiota

About 90% of the children provided their stool specimens during the process of physical examinations. The specimens were frozen at −80°C within 10 minutes and stored until DNA extraction. The parents or

legal guardians of the remaining 10% of the children were asked to collect stool samples at home and place them in a sterile plastic container. The samples were refrigerated at home and transported to the research facility within 12 hours in coolers with ice packs.

The gut microbiota was measured as described previously [29]. Briefly, fecal microbial DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). The bacterial genomic DNA was then used as the template for amplifying the V3–V4 hypervariable region of the 16S rRNA gene with the primer pair 341F (Illumina adapter sequence 1 + CCTAYGGGRBGCASCAG) and 806R (Illumina adapter sequence 2 + GGACTACHVGGGTWTCTAAT) by Phusion® High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs). The reaction volume (30 µl) comprised Phusion High-Fidelity PCR Master Mix (New England Biolabs) (15 µl), forward primer (0.2 pmol /µl), 5 µM reverse primer (0.2 pmol /µl), and template DNA (10 ng). Cycling proceeded as follows: 1 min at 98 °C thirty cycles (10 sat 98 °C, 30 sat 50 °C, 60 sat 72 °C); 5 min at 72 °C. The PCR products were purified by electrophoresis on 2% agarose gel in 1× TAE buffer. The purity and concentration of the sample DNA were determined using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA).

An Illumina TruSeq DNA PCR-free kit was used to construct the library. After quantification and library testing using Qubit@ 2.0 Fluorometer (Thermo Scientific), the Illumina HiSeq2000 platform was used for high-throughput sequencing. Operational taxonomic units (OTUs) were assigned by clustering the sequences with a threshold of 97% pairwise identity, and chimeras were removed using UPARSE. OTUs were taxonomically assigned at a confidence threshold of 80% based on the Ribosomal Database Project database by the Mothur classifier [31]. All samples were sequenced together and at the same laboratory. Bioinformatics analysis was performed by Novogene Bioinformatics Technology Co., Ltd. (Tianjin, China).

Measurement of SCFAs

Fecal SCFA concentrations were measured as described previously [30] using high-performance liquid chromatography (HPLC) [32]. Approximately 0.3 g of feces were added to 5.0 mL of 70% ethanol and centrifuged at 20°C, 2500 rpm for 10 min. The supernatants were filtered through a 0.45 µm syringe filter. A mixture of 300 µL of the supernatant and 50 µL of 2-ethylbutyric acid (800 mM), an internal standard, was pre-labeled with 2-nitrophenylhydrazide using a Short- and Long-Chain Fatty Acid Analysis Kit (YMC Co., Ltd., Kyoto, Japan). The SCFA derivatives were extracted with *n*-hexane and diethyl ether and subsequently evaporated to dryness. The obtained fatty acid hydrazide was dissolved in 200 µL of methanol, and 30 µL of the resulting solution was injected into an HPLC system (Agilent 1200, CA, USA) with a YMC-Pack FA column (250 × 6.0 mm; YMC Co., Ltd.). HPLC was performed under the following conditions. The column oven temperature was 50°C; the mobile phase consisted of acetonitrile-methanol-water [30:16:54 v/v/v, pH 4.5 adjusted using 0.1% trifluoroacetic acid (Wako Pure Chemical industries, Japan)]; the flow rate was 1.1 mL/min; and the eluate absorbance was monitored online at a wavelength of 400 nm. A sample of pooled fecal supernatants was analyzed with certain batches of study samples to monitor the analytic precision, and the resulting coefficients of variation were calculated as 2.4%–9.8%

and 3.9%–11.4% for intra-day and inter-day reproducibility, respectively, with recovery rates of 80–120% for the SCFAs. A total of 90 samples were tested in triplicate and the intra-class correlation coefficient of fecal SCFAs was from 0.797 to 0.967.

Covariates

Data on household income, parental educational level, and delivery method were collected by trained interviewers through face-to-face interviews. The parental education level was classified into three categories: primary or lower, secondary, and graduate or above. Household income per month was classified into two categories: ≤15,000 yuan and >15,000 yuan. The delivery mode was defined as a binary variable: cesarean or vaginal. Dietary intake for the past year was assessed by a quantitative food frequency questionnaire, and the dietary consumption of energy and fiber was calculated using the 2009 China Food Composition Table [33]. Information on physical activity was obtained using a three-day physical activity questionnaire, and physical activity was calculated by combining the metabolic equivalent score for each type of physical activity after multiplying it by its duration (hours) per day [34].

Bioinformatics analysis

Alpha- and beta-diversity estimates, which indicate within-sample richness and between-sample dissimilarity, respectively, were computed using the R software. The indicators of alpha-diversity included the abundance-based coverage estimator (ACE), Chao1 index, and Shannon index. Principal coordinate analysis (PCoA) was used to depict the beta-diversity at the OTU level and the phylogenetic tree. The Adonis statistical analysis method in permutational multivariate analysis of variance was used to test the significance of the difference in the gut microbial colony structure between the boys and girls.

Statistical analysis

Continuous variables are expressed as means ± standard deviations if normally distributed, or as medians with interquartile ranges if not normally distributed. The Box-Cox method was used to normalize the data. Categorical variables are presented as percentages. Differences in baseline characteristics and the relative abundances of taxa between boys and girls were assessed using Student's *t*-test for continuous variables and using the χ^2 test for categorical variables.

The BMC is affected by bone size, so we used the residual method to calculate the BMC after size adjustment [35]. Size-adjusted BMC (SA-BMC) for TB and TBLH were derived using the following models [28]:

$$BMC = \beta_1 \times Weight + \beta_2 \times Height + \beta_3 \times BA + Constant + Residual$$

The β_i, constant, and residual were derived from the linear regression model:

$$SA\text{-}BMC = \beta_1 \times Weight_{mean} + \beta_2 \times Height_{mean} + \beta_3 \times BA_{mean} + Constant + Residual$$

Spearman correlation analysis was conducted to evaluate the associations of microbial abundance and SCFAs with DXA-derived bone mass measurements. Multiple linear regression models using the enter method were applied to examine whether the correlations of the gut microbiota abundance and SCFA concentrations with bone mass were independent after adjusting for other potential covariates. In the regression models, we adjusted for weight, age, sex, delivery mode, household income, parental education, physical activity, use of calcium and multivitamin supplements, dietary energy intake, and dietary fiber intake. The false discovery rate (FDR) was used for the *P*-value correction upon multiple comparisons using the Benjamini-Hochberg method [36]. All analyses were conducted using R version 4.0.0. The significance level was conventionally set at 0.05.

Abbreviations

SCFA: total short chain fatty acids; TB BMC:total body bone mineral content; TB BMD:total body bone mineral density; SA- TB BMC:Size-adjusted total body bone mineral content; TBLH BMC:total body less head bone mineral content; TBLH BMD:total body less head bone mineral density; SA-TBLH BMC:Size-adjusted total body less head bone mineral content; FDR:false discovery rate; PCoA:Principal coordinate analysis.

Declarations

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Availability of data and materials:

The sequencing data that support the findings of this study has been made publicly available at the NIH National Center for Biotechnology Information Sequence Read Archive (SRA) with BioProject ID SUB7613496.

Author contributions:

Conception and design of the study: Z.Q.Z and Z.C. Collection of the data: F.Y.C, Y.H.W, D.F.X, J.W, W.K.A and L.J.P. Analysis: F.Y.C and Y.H.W., Q.Z.W provided critical comments during the manuscript revision. Drafting of the paper: F.Y.C and Z.Q.Z. All authors read, revised, and approved the final draft.

Ethics approval and consent to participate

All procedures in this work were carried out following the principles expressed in the Declaration of Helsinki and have been approved by the Ethics Committee of the School of Public Health at Sun Yat-sen

University (No. 201549). The parents or legal guardians of all participants provided written informed consent prior to enrollment.

Conflict of Interest:

The authors declare no conflict of interest.

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Figures

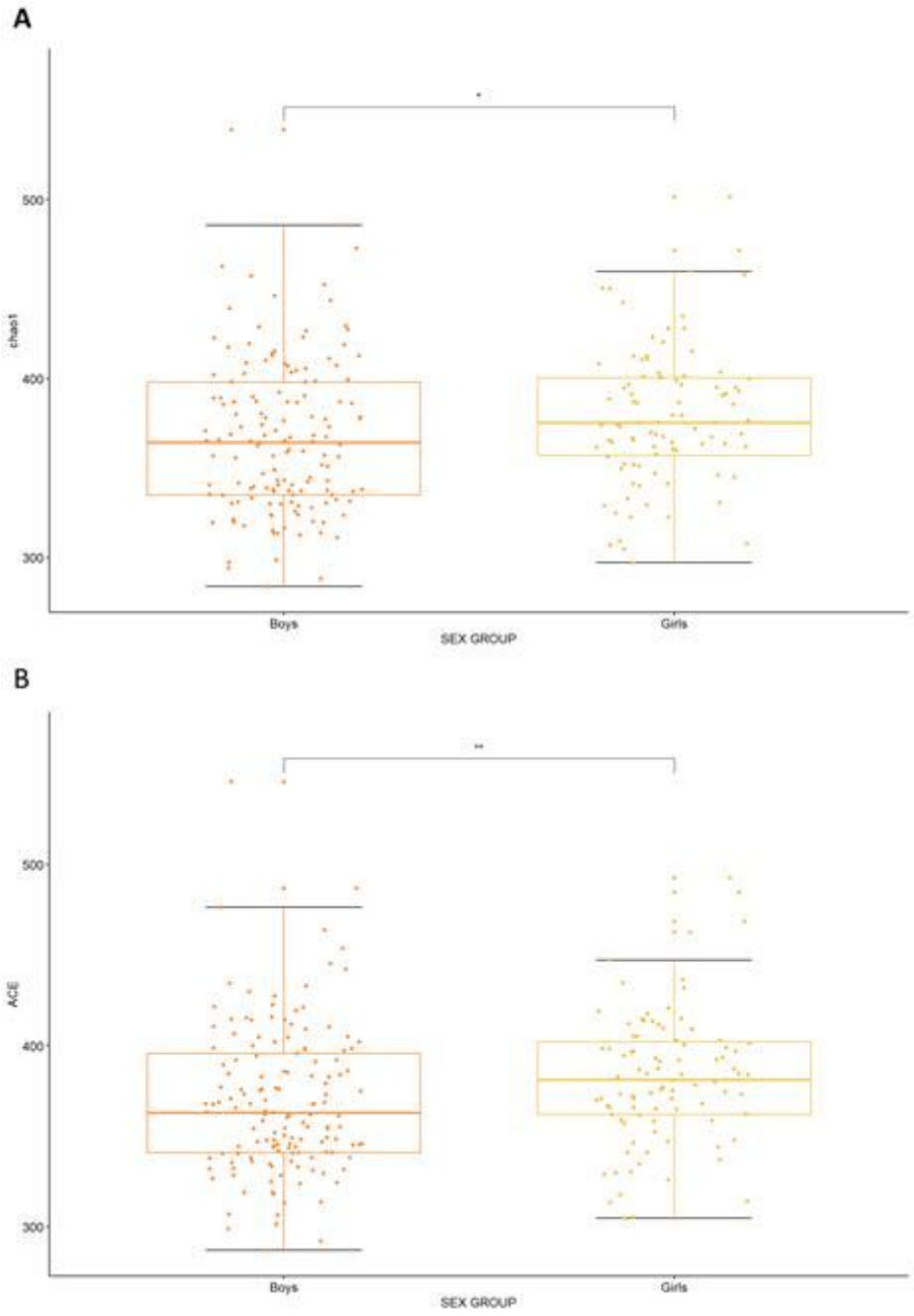

Figure 1

Alpha diversity of gut microbiota in boys and girls based on the chao1 (A) and ACE indexes (B). *P < 0.05.

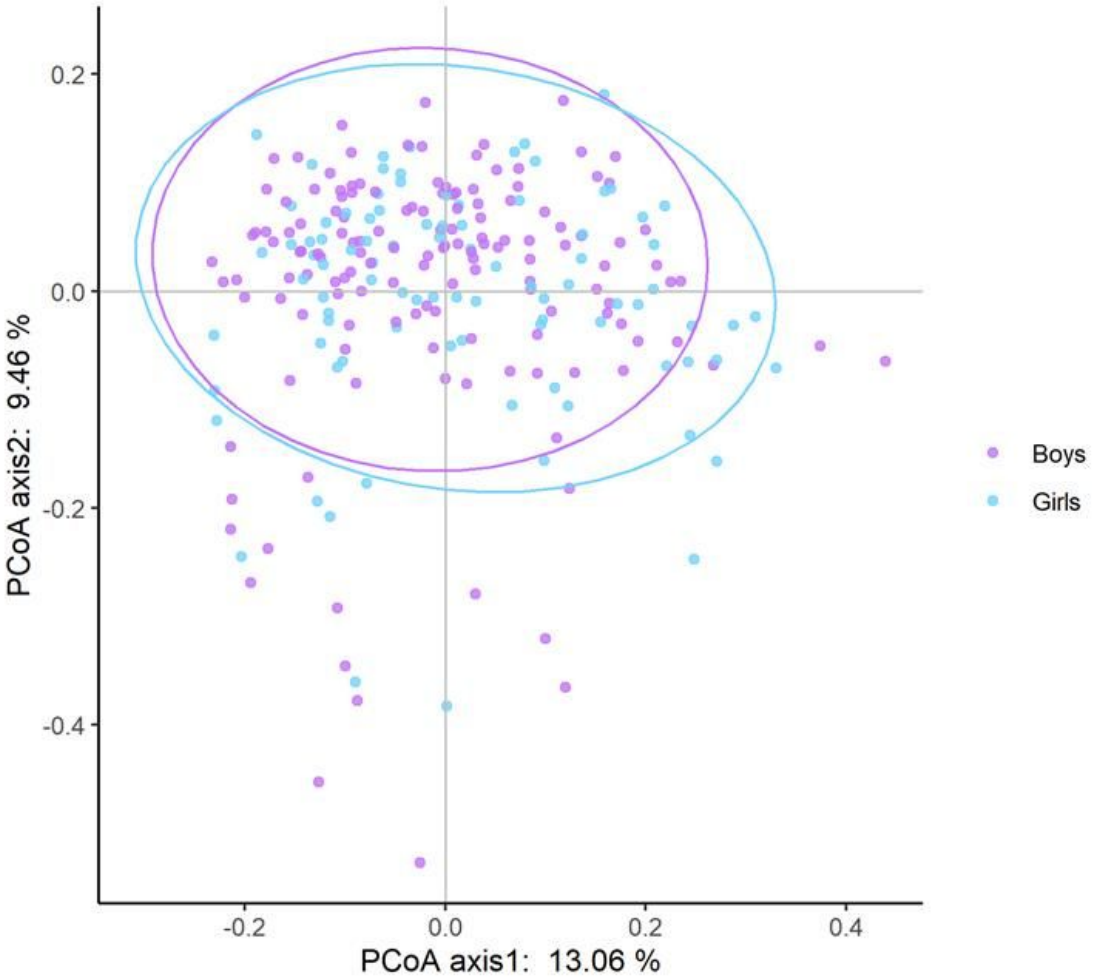

Figure 2

Beta diversity of gut microbiota in boys and girls based on weighted UniFrac distances. PCoA: Principal coordinate analysis.

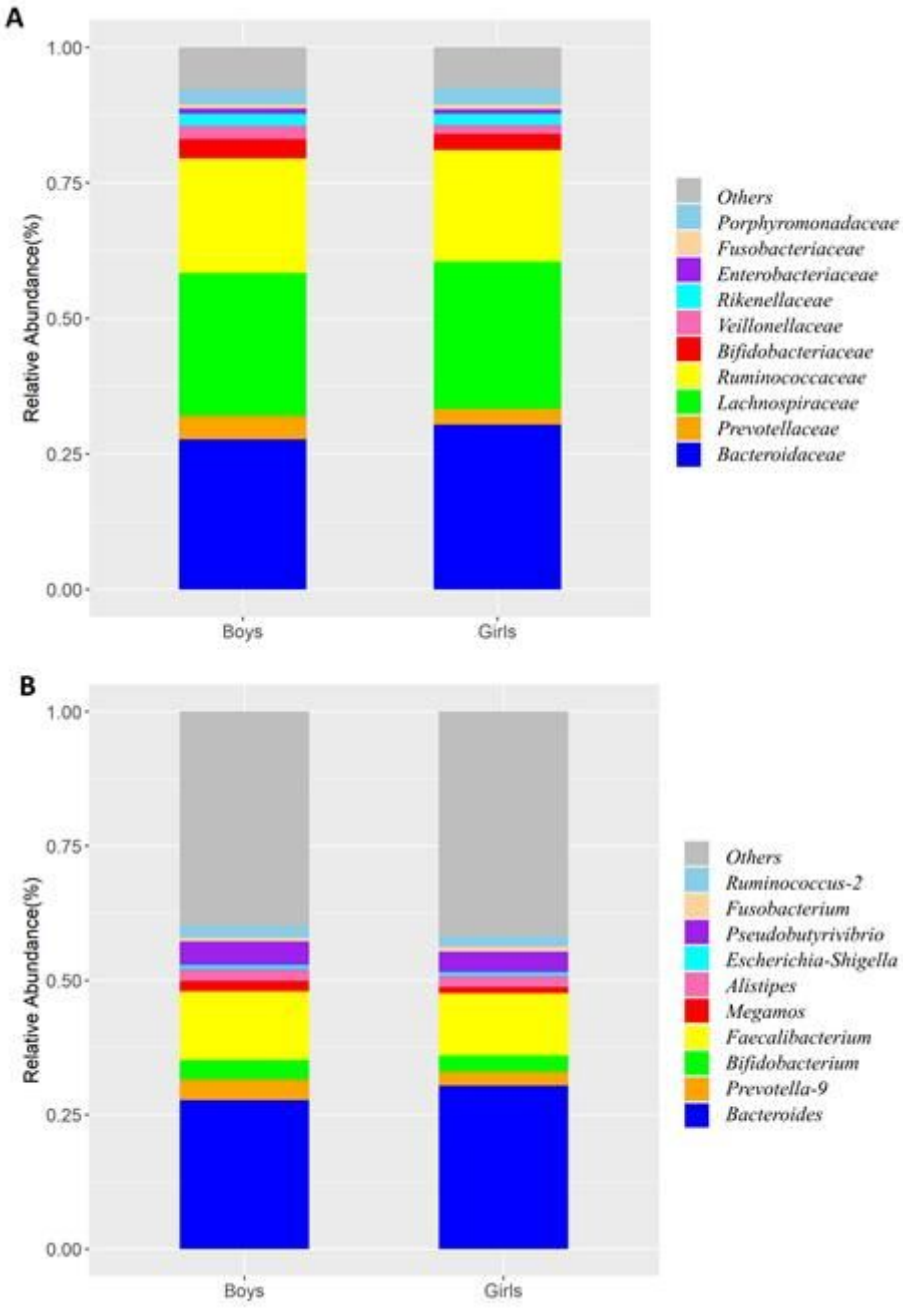

Figure 3

Top 10 abundant of gut microbiota in boys and girls at the level of (A) families and (B) genera.

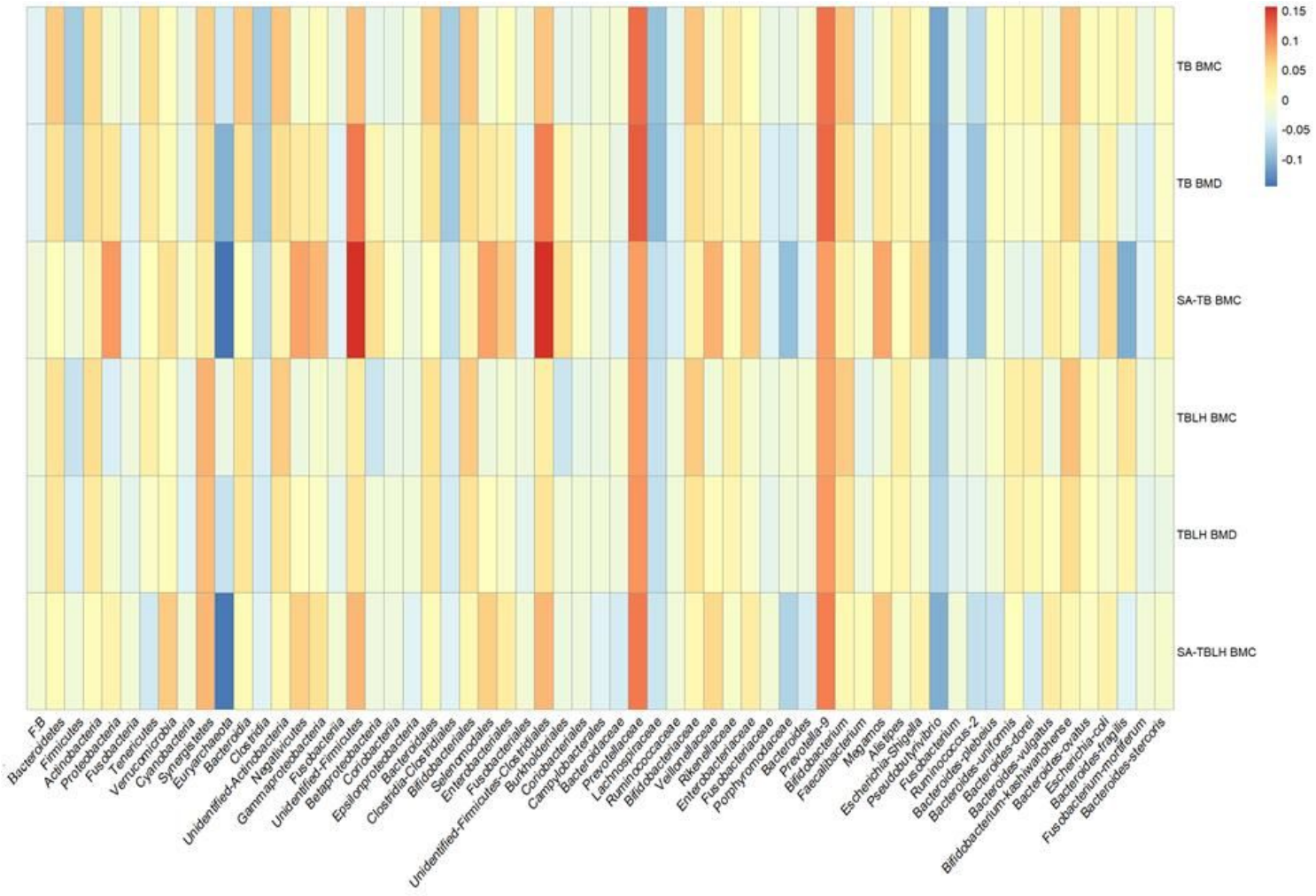

Figure 4

The multiple linear regression analysis of the associations between gut microbiota richness and bone mass. Results were adjusted for weight, age, sex, delivery mode, household income, parental education, physical activity, use of calcium and multivitamin supplements, dietary energy intake, and dietary fiber intake and multiple comparisons correction. TB BMC: total body bone mineral content; TB BMD: total body bone mineral density; SA- TB BMC: Size-adjusted total body bone mineral content; TBLH BMC: total body less head bone mineral content; TBLH BMD: total body less head bone mineral density; SA-TBLH BMC: Size-adjusted total body less head bone mineral content

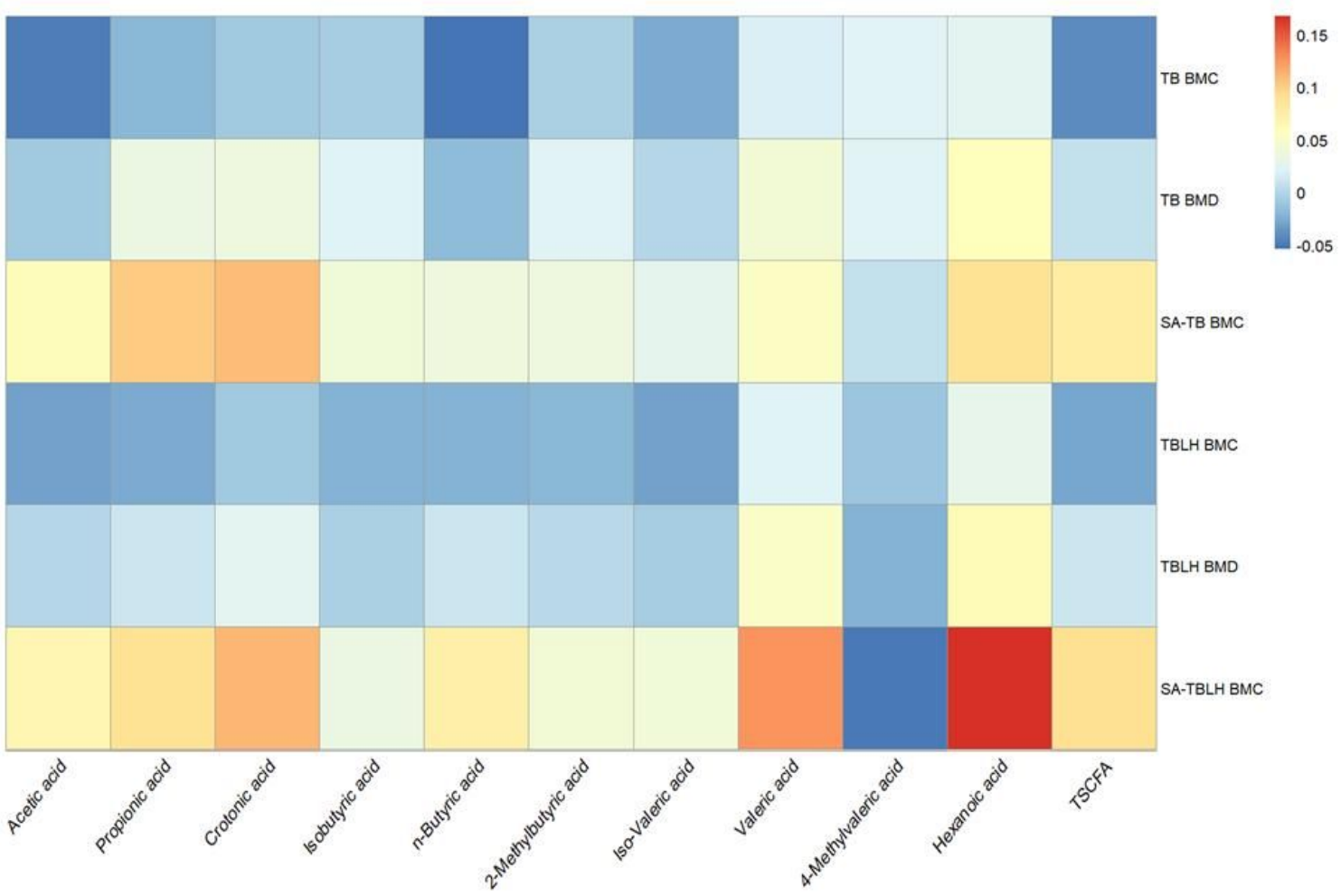

Figure 5

The multiple linear regression analysis of the associations between fecal SCFAs and bone mass. Results were adjusted for weight, age, sex, delivery mode, household income, parental education, physical activity, use of calcium and multivitamin supplements, dietary energy intake, and dietary fiber intake and multiple comparisons correction. TSCFA: total short chain fatty acids; TB BMC: total body bone mineral content; TB BMD: total body bone mineral density; SA- TB BMC: Size-adjusted total body bone mineral content; TBLH BMC: total body less head bone mineral content; TBLH BMD: total body less head bone mineral density; SA-TBLH BMC: Size-adjusted total body less head bone mineral content.

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