Long-term Diet Quality and Gut Microbiome Functionality: A Prospective, Shotgun Metagenomic Study among Urban Chinese Adults

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ABSTRACT

Background: Diet is known to affect human gut microbiome composition; yet, how diet affects gut microbiome functionality remains unclear. Objective: We compared the diversity and abundance/presence of fecal microbiome metabolic pathways among individuals according to their long-term diet quality.

Methods: In 2 longitudinal cohorts, we assessed participants’ usual diets via repeated surveys during 1996–2011 and collected a stool sample in 2015–2018. Participants who maintained a healthy or unhealthy diet (i.e., stayed in the highest or lowest quintile of a healthy diet score throughout follow-up) were selected. Participants were excluded if they reported a history of cancer, cardiovascular disease, diabetes, or hypertension; had diarrhea or constipation in the last 7 d; or used antibiotics in the last 6 mo before stool collection. Functional profiling of shotgun metagenomics was performed using HUMAnN2. Associations of dietary variables and 420 microbial metabolic pathways were evaluated via multivariable-adjusted linear or logistic regression models.

Results: We included 144 adults (mean age = 64 y; 55% female); 66 had an unhealthy diet and 78 maintained a healthy diet. The healthy diet group had higher Shannon α-diversity indexes of microbial gene families and metabolic pathways (both P < 0.02), whereas β-diversity, as evaluated by Bray-Curtis distance, did not differ between groups (both P > 0.50). At P < 0.01 [false discovery rate (FDR) <0.15], the healthy diet group showed enriched pathways for vitamin and carrier biosynthesis (e.g., tetrahydrofolate, acetyl-CoA, and l-methionine) and tricarboxylic acid (TCA) cycle, and increased degradation (or reduced biosynthesis) of certain sugars [e.g., cytidine monophosphate (CMP)-legionaminate, deoxyxymethidine diphosphate (dTDP)-l-rhamnose, and sucrose], nucleotides, 4-aminobutanoate, methylglyoxal, sulfate, and aromatic compounds (e.g., catechol and toluene). Meanwhile, several food groups were associated with the CMP-legionaminate biosynthesis pathway at FDR <0.05.

Conclusions: In a small longitudinal study of generally healthy, older Chinese adults, we found long-term healthy eating was associated with increased α-diversity of microbial gene families and metabolic pathways and altered symbiotic functions relevant to human nutrition and health.

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Keywords: gut microbiota, shotgun metagenomics, nutrition, diet quality, dietary pattern, prospective cohort study, epidemiology

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Supplemental Figures 1 and 2 and Supplemental Tables 1–3 are available from the “Supplementary data” link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/cdn/.

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Abbreviations used: CMP, cytidine monophosphate; dTDP, deoxyxymethidine diphosphate; FDR, false discovery rate; GABA, 4-aminobutanoate; GLM, general linear model; HDS, healthy diet score; PERMANOVA, permutational multivariate ANOVA; SMHS, Shanghai Men’s Health Study; SWHS, Shanghai Women’s Health Study; TCA, tricarboxylic acid.

Introduction

Diet is one of the most important, modifiable determinants of human health. Emerging research in recent years has indicated that diet may affect human health, in part through modifying the gut microbiota (1, 2). The commensal gut microbes are usually harmless or beneficial to humans; they protect the host against pathogens, regulate immune responses, synthesize essential nutrients, and play a major role in metabolizing xenobiotics, including various dietary components, environmental chemicals, and pharmaceuticals (3, 4). On the other hand, a disrupted gut microbiota, characterized by loss of diversity, loss of commensals, gain of pathogens, and change in functionality, has been linked to multiple human diseases, from gastrointestinal diseases to cancer, cardiometabolic diseases, and neurobehavioral disorders (2, 5). An improved understanding of how diet influences the composition and functionality of the human gut microbiota will advance nutrition science and
inform potential diet- or microbiota-based interventions to improve human health.

An increasing number of population-based studies have collected fecal samples to examine habitual diets’ influence on the gut microbiota (6–12). However, due to the high cost of shotgun metagenomics, most of the studies, including our prior research, conducted 16S rRNA sequencing and can only evaluate associations of dietary variables with microbial taxonomic composition and diversity. Meanwhile, although a few population studies conducted shotgun metagenomics to assess microbial functional diversity and pathways, few had comprehensive long-term dietary data to evaluate habitual food intakes and overall dietary patterns (10, 11, 13, 14). Given the functional redundancy of gut microbiota in which taxonomically unrelated species may perform similar functions, it is important to investigate microbial functionality beyond taxonomy (5, 15). Understanding how habitual diets affect microbial pathways may reveal novel biological mechanisms underlying the diet–gut microbiota–host interactions (11).

In the present study, we leveraged resources of 2 population-based, prospective cohorts—the Shanghai Women’s Health Study (SWHS) and Shanghai Men’s Health Study (SMHS)—including longitudinally collected dietary data and recently collected fecal samples. We compared the diversity, abundance, and presence of microbial metabolic pathways among generally healthy participants who had a long-term healthy or unhealthy diet, determined by a healthy diet score (HDS). The HDS incorporates 8 major food groups (fruits, vegetables, dairy, fish and seafood, nuts and legumes, refined grains, red meat, and processed meat) and is significantly associated with a reduced risk of type 2 diabetes and increased species-level α-diversity and abundance of multiple fiber-fermenting bacteria in the SWHS and SMHS (12, 16).

Methods

Study population

The SWHS and SMHS are population-based prospective cohort studies conducted in Shanghai, China, which enrolled 74,940 women during 1996–2000 (response rate = 93%) and 61,491 men during 2002–2006 (response rate = 74%), respectively (17, 18). In-person interviews were conducted at baseline and follow-up visits every 2–4 y (follow-up rates = 93%, respectively). In-person interviews were conducted at baseline and follow-up visits every 2–4 y (follow-up rates >92% in both cohorts) to collect and update participants’ sociodemographic, disease history, diet, lifestyle, and anthropometric information. Biospecimens were also collected, including blood, urine, and oral rinse samples at baseline and stool samples at the latest follow-up (see details in the section below). Participants’ death and disease outcomes were also updated via linkages to Shanghai Vital Statistics and Cancer Registries (completion rate >99% in both cohorts). The SWHS and SMHS were approved by Institutional Review Boards of the Shanghai Cancer Institute and Vanderbilt University Medical Center; informed consent was obtained from each participant.

Stool sample collection

During the cohort follow-up in 2015–2018, we collected a stool sample using the 95% ethanol method from ~10,000 living and willing cohort participants, as previously described (12). Briefly, participants were provided with stool sample collection kits, each of which included disposable collection tools, a tube containing 5-mL 95% ethanol and glass beads, an instruction sheet, and a sample collection form. They were asked to collect a peanut-sized stool into the tube and shake the tube until the sample was well mixed with ethanol. The collection form asked the date and time of stool collection, use of antibiotics and other medications in the last 7 d and 6 mo, bowel movement frequency, and any diet or body weight changes in the last 7 d before stool collection. The samples were stored at room temperature and transported to a research laboratory within 24 h, and then placed into aliquots and stored at −80°C.

Assessment of long-term diet

Semi-quantitative FFQs were used to estimate participants’ usual food intake over the past 12 mo. The FFQs used in the SWHS and SMHS were very similar, and contained 77 and 81 common food items, respectively. Both FFQs were validated against 24-h dietary recalls that were administered monthly over 12 mo; correlation coefficients for major food groups were 0.41–0.66 in SWHS and 0.42–0.72 in SMHS, suggesting good validity of the FFQs (19, 20). The FFQ was administered 3 times in the SWHS, capturing food intakes during 1996–2011, and twice in the SMHS, capturing intakes during 2002–2011. We generated an HDS to evaluate overall diet quality, incorporating 8 food groups with equal weights: fruits, vegetables (excluding potatoes), dairy, fish and seafood, nuts and legumes, refined grains, red meat, and processed meat (16). Based on energy-adjusted, sex-specific intake quintiles, the first 5 groups were assigned by ascending values (1 to 5) and the last 3 groups were assigned by descending values (5 to 1). Their sum represented the HDS, ranging from 8 to 40; a higher score reflects a higher diet quality. We calculated the HDS for each participant at each FFQ and generated a cumulative average HDS reflecting long-term diet quality. Participants who stayed in the highest quintile of HDS at each FFQ were defined as having a long-term healthy diet, whereas participants who stayed in the lowest quintile of HDS at each FFQ were defined as having a long-term unhealthy diet. We have previously reported in SWHS and SMHS that a high HDS, especially if maintained for a long term, was associated with a 15–25% reduced risk of type 2 diabetes (16). Furthermore, in a recent 16S rRNA metagenomics study, we reported that a high HDS was associated with an increased species-level Shannon index and abundances of fiber-fermenting bacteria (e.g., genera Clostridium, Faecalibacterium, and Bifidobacterium) (12).

Participant selection

Participants were selected based on their availability of fecal microbial DNA as well as their disease history, recent use of medications, recent bowel movements, and long-term diet quality. Among 3194 participants whose fecal DNA was extracted in the aforementioned 16S study, we excluded 692 participants who did not have long-term dietary data (those who only had 1 dietary survey or extreme calorie intake, i.e., <500 or >3500 kcal/d for women and <800 or >4200 kcal/d for men). We also excluded participants who reported a history of cancer, cardiovascular diseases, diabetes, or hypertension (n = 1026); who were aged >80 y (n = 141); who had antibiotic use in the last 6 mo (n = 68); who had constipation or diarrhea (n = 139; i.e., ≤2 or ≥21 bowel movements in the last 7 d); or who had use of antidiabetic, anti-hypertensive, or antidiabetic medications or substantial changes in body weight or diet in the last 7 d (n = 125) at the time of stool collection. After those exclusions, we selected participants who had a healthy or unhealthy long-term diet using repeated HDS data, which yielded
TABLE 1  Participant characteristics by long-term diet quality

| Characteristics                     | Unhealthy diet group (n = 66) | Healthy diet group (n = 78) | P       |
|-------------------------------------|------------------------------|----------------------------|---------|
| Healthy diet score                  | 17.0 ± 1.8                   | 32.3 ± 2.0                 | <0.0001 |
| Age at stool collection, y          | 63.8 ± 6.3                   | 64.5 ± 6.9                 | 0.53    |
| Female, %                          | 56.1                         | 55.1                       | 0.91    |
| High income, %                     | 3.0                          | 18.0                       | 0.0003  |
| Current smoking cigarette, %       | 37.8                         | 23.1                       | 0.04    |
| Current alcohol drinking, %        | 21.2                         | 21.8                       | 0.93    |
| Leisure-time exercise, %           | 40.9                         | 53.9                       | 0.02    |
| BMI, kg/m²                         | 24.1 ± 3.2                   | 23.7 ± 3.2                 | 0.52    |
| Dietary intakes                    |                              |                            |         |
| Total energy, kcal/d               | 1844 ± 412                   | 1786 ± 388                 | 0.38    |
| Fruit, g/d                         | 76.8 ± 57.6                  | 294 ± 151                  | <0.0001 |
| Vegetables, g/d                    | 221 ± 84.3                   | 478 ± 166                  | <0.0001 |
| Dairy products, g/d                | 39.3 ± 72.7                  | 201 ± 236                  | <0.0001 |
| Fish and seafood, g/d              | 22.6 ± 13.1                  | 85.6 ± 53.4                | <0.0001 |
| Legumes and nuts, g/d              | 15.7 ± 9.3                   | 31.5 ± 12.2                | <0.0001 |
| Refined grains, g/d                | 372 ± 59.9                   | 277 ± 63.2                 | <0.0001 |
| Red meat, g/d                      | 63.3 ± 28.9                  | 42.5 ± 22.4                | <0.0001 |
| Processed meat, time/wk            | 0.62 ± 0.79                  | 0.25 ± 0.44                | <0.0001 |
| Shannon index of gene families      | 5.44 ± 0.09                  | 5.48 ± 0.13                | 0.03    |
| Shannon index of metabolic pathways | 5.10 ± 0.21                  | 5.18 ± 0.23                | 0.03    |

Values are means ± SDs unless otherwise indicated. t tests for continuous variables and chi-square tests for categorical variables were used. All characteristics were updated during cohort follow-up, and the latest available data are presented, except for dietary variables, which were the cumulative averages using data from repeated FFQs during follow-ups.

49 men and 51 women in the healthy diet group and 36 men and 39 women in the unhealthy diet group. Among those 166 samples sent for shotgun metagenomics, 21 were further excluded due to low microbial DNA amount and 1 failed to pass sequencing quality control. Finally, 144 participants were included in our analysis. A participant selection flowchart is provided in Supplemental Figure 1.

Shotgun metagenomics
DNA was extracted from stool samples using Qiagen’s DNeasy PowerSoil kit. DNA libraries were constructed using the Illumina Nextera XT kit. Sequencing was performed on the Illumina HiSeq X TEN platform using a 150 paired-end strategy, generating a median of 27.3 million raw reads per sample (range: 20.1 to 47.0 million reads). Trimmomatic (v0.39) was utilized to trim low-quality bases in raw reads, then removal of human reads, on average, 27.1 million reads were retained for each sample (range: 20.1 to 46.9 million reads). These reads were then used for functional profiling using HUMAnN2 (v2.8.1) to estimate the relative abundance of microbial gene families and MetaCyC pathways within each sample. For both gene families and metabolic pathways, α-diversity was evaluated by Shannon index and β-diversity was evaluated by Bray–Curtis distance (23). Statistical analyses were performed for α- and β-diversity indexes of gene families and metabolic pathways and relative abundance of microbial metabolic pathways.

Statistical analysis
Participant characteristics between healthy and unhealthy diet groups were compared by using t tests for continuous variables and chi-square tests for categorical variables. The associations between diet groups and α-diversity indexes were evaluated using general linear models (GLMs), adjusting for sequencing depth, sociodemographics (age at stool sample collection, sex, and income), lifestyle (cigarette smoking, alcohol drinking, and leisure-time exercise), BMI, and total calorie intake. We used the latest available information on income, lifestyle, and BMI, and average calorie intake from repeated FFQs. The associations between diet groups and Bray-Curtis distance were evaluated using permutational multivariate ANOVA (PERMANOVA) with 999 permutations, adjusting for sequencing depth and the same covariates. A pathway was considered present if its relative abundance was ≥1 × 10⁻⁵ (0.001%). We defined pathways as common if present in >50% of samples and rare if present in 10–50% of samples; pathways present in <10% of samples were excluded from analysis as the sample size was too small. For common pathways, centered log-ratio transformation was applied to normalize relative abundance with zeros imputed by adding 1 pseudo count to the total reads. Then GLM was used to evaluate their associations with dietary variables, controlling for the covariates listed above. For rare pathways, logistic regression models were used to compare their presence between diet groups with adjustment for the covariates. Based on crude P values of both common and rare pathways, Benjamini and Hochberg false discovery rates (FDRs) were calculated; an FDR <0.10 was considered significant given the small sample size of the current study. We assessed potential effect modifications of participants’ age, sex, lifestyle, and obesity status on diet–pathway associations by adding an interaction term of these covariates with diet to the regression model. Statistical analyses were performed using SAS (version 9.4; SAS Institute) and R (version 3.6.3; R Foundation for Statistical Computing).

Results
Table 1 shows the characteristics of study participants with a long-term unhealthy or healthy diet. The HDS and intakes of all 8 food groups were
FIGURE 1  Fecal microbial gene family and metabolic pathway α-diversity by long-term diet quality. Violin plots of Shannon diversity indexes of microbial gene families (A) and metabolic pathways (B); P values were obtained from general linear models adjusting for sequencing depth, age at stool sample collection, sex, income, cigarette smoking, alcohol drinking, leisure-time exercise, BMI, and total calorie intake.

substantially different between groups, but total calorie intake was not. Two groups had similar distributions of age (mean: ~64 y), sex (~55% female), and BMI (kg/m²; mean: ~24). However, the healthy diet group had a significantly higher income and a higher rate of leisure-time exercise and a lower rate of cigarette smoking than the unhealthy diet group.

Compared with the unhealthy diet group, the healthy diet group had small, but significant increases in Shannon indexes of both microbial gene families and metabolic pathways \((P = 0.02\) and 0.01, respectively; Figure 1). However, there were no significant differences between diet groups in Bray-Curtis distances of gene families or metabolic pathways (Supplemental Figure 2). The diet group explained 0.84% of variance in microbial gene families and 0.75% of variance in metabolic pathways (adjusted PERMANOVA, \(P = 0.30\) and 0.29, respectively).

When examined individually, among a total of 366 common and 54 rare pathways, 20 common and 4 rare pathways were associated with diet group at \(P < 0.01\) (Table 2), and an additional 44 common pathways and 1 rare pathway were associated with diet group at \(0.01 < P < 0.05\) (Supplemental Table 1); all of these associations had an FDR between 0.10 to 0.15. The most significant results included associations of healthy diet with reduced pathway abundances for biosyntheses of nucleotide sugars (e.g., CMP-legionaminate and dTDP-l-rhamnose), nucleotides (e.g., guanosine and adenosine), and fatty acids, and increased pathway abundances for biosyntheses of tetrahydrofolate, acetyl-CoA, l-methionine, and dTDP-N-acetylthomosamine and for degradation of sucrose, 4-aminobutanoate (GABA), methylglyoxal, and sulfate. Meanwhile, the healthy diet group had a higher prevalence of pathways for degrading aromatic compounds (e.g., catechol and toluene). The adjusted mean relative abundances (95% CIs) of 20 common pathways and the prevalence of 4 rare pathways with \(P < 0.01\) are shown in Figures 2 and 3, respectively. The MetaCyC classes of those pathways are summarized in Supplemental Table 2. We did not find significant effect modifications by participants’ age, sex, lifestyle (smoking, drinking, and exercise), or obesity status on the diet–microbial pathway associations.

We then evaluated the associations of 8 major food groups with microbial metabolic pathways. At \(P < 0.01\), many food groups were associated with the CMP-legionaminate biosynthesis pathway, including inverse associations for fruits, vegetables, fish/seafood, and nuts/legumes, and a positive association for refined grains (Supplemental Table 3). Of note, associations of refined grains, fruits, and fish/seafood with CMP-legionaminate biosynthesis reached an FDR <0.05. Meanwhile, dairy intake was associated with reduced tetrapyrrole biosynthesis at FDR = 0.03.

Discussion

Among generally healthy, older, urban Chinese adults, we found that a long-term healthy diet is associated with increased α-diversity of fecal microbial gene families and metabolic pathways and enhanced pathways related to cofactor, carrier, and vitamin biosynthesis (e.g., folate, acetyl-CoA, and l-methionine) and tricarboxylic acid (TCA) cycle, but increased degradation (or reduced biosynthesis) of certain sugars, nucleotides, amines, methylglyoxal, sulfate, and aromatic compounds. These findings support the influence of habitual diets on gut microbial functionality and reveal potential microbial pathways through which long-term healthy or unhealthy diets may subsequently affect human health.

Diet quality and gut microbial functional diversity

We observed an increased microbial functional diversity in the healthy diet group. Diversity is generally considered a feature of healthy gut microbiota, as higher diversity usually means a more resilient and efficient ecosystem (5, 15, 24). In line with our finding, the LifeLines DEEP cohort, a population-based study of 1135 Dutch adults, reported that more frequent intakes of fruit and tea and lower intakes of bread, snacks, and soda were associated with higher microbial gene richness (10), although they did not evaluate an overall diet quality. Meanwhile, like our previ-
Diet quality and gut microbial metabolic pathways

Among specific pathways, the most significant findings were related to decreased biosyntheses of CMP-legionamine and dTDP-l-rhamnose, 2 nucleotide sugars, in the healthy diet group. A reduced CMP-legionaminase biosynthesis pathway was also related to multiple food groups, including higher intakes of fruits, vegetables, fish/seafood, and nuts/legumes and a lower intake of refined grains. Legionaminase and l-rhamnose are building blocks of the glycan component (e.g., O-antigens) of lipopolysaccharide (LPS) of gram-negative bacteria. Many pathogenic bacteria (e.g., *Legionella pneumophila*, *Campylobacter coli*, and *Salmonella enterica*) display O-antigens on their surface to help them invade host cells (27, 28). Bacteria can also release LPS into the environment, which then triggers host inflammatory cascades (29). Our findings may suggest a mechanism underlying the anti-inflammatory effect of healthy diets via reducing gut bacteria-derived, proinflammatory nucleotide sugars. However, we did not observe a significant change in the overall LPS biosynthesis super-pathway ($P = 0.30$), which may be because we also observed counteractively increased biosyntheses of other LPS-contributing molecules such as dTDP-N-acetylthiomyosamine and lipid IV A (tetra-acylated lipid A) ($P = 0.008$ and 0.03, respectively). The evidence regarding the gut microbiome LPS biosynthesis pathway and human health remains inconsistent. Studies have reported higher abundances of LPS pathway in the fecal microbiome of morbidly obese individuals than in lean individuals (BMI: 37 vs. 20) and among colorectal adenoma/carcinoma patients than healthy controls (30, 31), but also higher abundances among healthy controls than rheumatoid arthritis patients and after metformin treatment among diabetic patients with no concurrent change in circulating C-reactive protein (32, 33). It is unclear to what extent the metagenomic abundance of this pathway affects secreted LPS concentration and, more importantly, the host’s immune response to LPS exposure. Unfortunately, we did not collect blood samples at the time of stool collection for participants involved in this study. Future studies incorporating fecal metagenomics with blood concentrations of LPS or LPS-binding protein and inflammatory markers are needed.

Next, we observed pathways related to cofactor, carrier, and vitamin biosynthesis enriched in the healthy diet group, involving tetrahy-
drofolate, acetyl-CoA, l-methionine, and also NAD, S-adenosyl-l-methionine, heme, and pantothenate (vitamin B-5) (see Supplemental Table 1). These molecules are involved in biochemical reactions as acceptors or donors of electrons, hydrons, and methyl or acyl groups, and hence may affect energy metabolism, glycolysis, glucogenesis, oxidative stress, and epigenetic regulation. Previous case-control studies of type 2 diabetes, liver cirrhosis, and rheumatoid arthritis among Chinese and European adults have reported higher abundances of these pathways among healthy controls than in patients (32, 34, 35), suggesting a potential role of gut microbial cofactor/carrier/vitamin biosynthesis against host metabolic and inflammatory diseases. However, it is unclear to what extent gut microbial biosynthesis contributes to human needs, given that most of those molecules can be synthesized by the human body or ingested from foods. Nevertheless, vitamin deficiencies,
including folate deficiency, have been observed after antibiotic use, implying that commensal microbial vitamin production can be significant (36, 37).

Finally, we observed a series of pathway changes in the healthy diet group regarding microbial degradation and fermentation of various substrates as sources of energy and nutrients or means of detoxification, including increased degradation of sugars (e.g., sucrose, fructose, and D-glucarate), amines (e.g., GABA, ornithine, and allantoin), methylglyoxal, sulfate, and aromatic compounds (e.g., catechol and tolune), which can generate energy, pyruvate, lactate, SCFAs (acetate, propionate, and butyrate), and other metabolites (e.g., succinate and ethanol). Supporting our observational findings, intervention studies have found that a 5-d plant-based diet or an 8-wk Mediterranean diet increased abundances of microbial genes for carbohydrate and amino acid degradation, particularly glutamate-family amino acids: glutamate, arginine, and GABA (38, 39). Metformin treatment may also increase microbial pathways for carbohydrate and amino acid degradation (33).

Meanwhile, in a unique study comparing the gut microbiome of centenarians (99–109 y) with young and middle-aged Italians (40), centenarians had significantly enriched microbial genes for aromatic xenobiotics degradation (including tolune, an industrial chemical used in paint and glue). Furthermore, research has increasingly focused on gut microbiota–related metabolites, which can mediate gut microbiota–host cross-talks, enter the host circulatory system, and affect multiple aspects of human health (41). For example, SCFAs are known for their potential health benefits to reduce obesity, inflammation, and insulin resistance (42, 43), whereas methylglyoxal is a toxic byproduct of glycolysis, which can cause endothelial cell loss and inflammation (44).

Our current findings regarding increased fermentation and generation of lactate and SCFAs are in line with our previous results from a 16S study that higher diet quality was associated with higher abundances of fiber-fermenting, SCFA-/lactate-generating bacteria (e.g., *Faecalibacterium* and *Bifidobacterium*) (12). Together, previous studies and our current study have revealed certain microbial functions that may mediate the effects of habitual diets on human health and aging. Prospective studies are needed to evaluate potential causal relations of those gut microbial functions with health outcomes.

**Strengths and limitations**

To our knowledge, this is one of the few longitudinal, population-based studies on the influence of habitual diets on the gut microbiome and one of the first reports on overall diet quality and microbial functionality among Asian populations. Our strengths include the use of validated, repeated dietary surveys to assess participants’ long-term diets and comprehensive metadata (e.g., other lifestyle factors, disease history, and medications) to minimize and control for potential confounding. Our limitations include a small sample size, and thus studies with larger sample sizes are needed to examine further the associations of habitual diets with microbial functionality. Second, although we used repeated dietary surveys and adjusted for potential confounders, we cannot rule out measurement errors and residual confounding. We also acknowledge a lack of dietary assessment proximal to stool collection as a limitation. We aimed to evaluate the effect of long-term, overall diet and thus selected participants who had maintained a stable diet quality during follow-up and excluded those who reported substantial recent diet changes; therefore, potential misclassification in the diet group should be minimized. Third, we could not further evaluate the potential health implications of those observed diet–microbial pathway associations due to excluding participants with major diseases and lack of disease risk biomarkers in the current study. Also, given that our current study participants were highly selected, our current findings may not be directly applicable to other populations (e.g., younger or rural Chinese adults or those with metabolic diseases). Finally, although shotgun metagenomics can imply microbial composition and functional potential, it is ideal to incorporate other data (e.g., fecal and/or blood metabolomics) and evaluate microbial functional activities or circulating microbial metabolites that may directly affect host physiology. The relations between diet, gut microbiota, and disease risk are yet to be evaluated in our future studies after enough incident cases have accrued.

**Conclusions**

In summary, among 2 groups of generally healthy, older, urban Chinese adults who had a healthy or an unhealthy long-term diet, we found the healthy diet to be associated with increased α-diversity of fecal microbial gene families and metabolic pathways and enhanced pathways related to cofactor, carrier, and vitamin biosynthesis and TCA cycle, and increased degradation (or reduced biosynthesis) of certain sugars, sugar nucleotides, amines, and aromatic compounds. Future large-scale prospective studies are needed to validate our findings and evaluate those gut microbial metabolic pathways for their associations with human disease risk.

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**Data Availability**

Data described in the manuscript will be made available upon research study application and approval by the cohort committees.

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