

BMJ Open

BMJ Open is committed to open peer review. As part of this commitment we make the peer review history of every article we publish publicly available.

When an article is published we post the peer reviewers' comments and the authors' responses online. We also post the versions of the paper that were used during peer review. These are the versions that the peer review comments apply to.

The versions of the paper that follow are the versions that were submitted during the peer review process. They are not the versions of record or the final published versions. They should not be cited or distributed as the published version of this manuscript.

BMJ Open is an open access journal and the full, final, typeset and author-corrected version of record of the manuscript is available on our site with no access controls, subscription charges or pay-per-view fees (<http://bmjopen.bmj.com>).

If you have any questions on BMJ Open's open peer review process please email info.bmjopen@bmj.com

BMJ Open

Cohort profile: An early life observational cohort in China: Bone And MicroBiOme Onset (BAMBOO) study

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2023-075417
Article Type:	Cohort profile
Date Submitted by the Author:	07-May-2023
Complete List of Authors:	<p>Wang, Jing; Tianjin Women's and Children's Health Center Jiang, Chang; Tianjin Women's and Children's Health Center Wang, Shuo; Tianjin Women's and Children's Health Center Feng, Lingyan; Tianjin Women's and Children's Health Center Zhang, Yu; Tianjin Women's and Children's Health Center Guo, Yuanyuan; Tianjin Women's and Children's Health Center Liu, Gongshu; Tianjin Women's and Children's Health Center Li, Xi; BGI; Shenzhen Engineering Laboratory for Birth Defects Screening Zhang, Guohong; BGI; Shenzhen Engineering Laboratory for Birth Defects Screening Zhu, Xiaowei; BGI Ren, Fangyi; BGI; China National GeneBank Guan, Lingyao; BGI; China National GeneBank Chen, Jiayu; BGI; China National GeneBank Gao, Ya; BGI; Shenzhen Engineering Laboratory for Birth Defects Screening Chen, Mo; Nestlé Institute of Health Sciences Darwish, Noura; Clinical Research Unit Mottaz, Sara Colombo; Clinical Research Unit Horcajada, Marie Noelle; Nestlé Institute of Health Sciences Bonnet, Nicolas; Nestlé Institute of Health Sciences Dogra, Shaillay Kumar; Nestlé Institute of Health Sciences Wang, Dantong; Nestlé Institute of Health Sciences</p>
Keywords:	Paediatric gastroenterology < GASTROENTEROLOGY, EPIDEMIOLOGIC STUDIES, NUTRITION & DIETETICS, Bone diseases < ORTHOPAEDIC & TRAUMA SURGERY

SCHOLARONE™
Manuscripts



I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our [licence](#).

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which [Creative Commons](#) licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

Cohort profile: An early life observational cohort in China: Bone And MicroBiOme Onset (BAMBOO) study

Jing Wang¹, Chang Jiang¹, Shuo Wang¹, Lingyan Feng¹, Yu Zhang¹, Yuanyuan Guo¹, Gongshu Liu¹, Xi Li^{2,3}, Guohong Zhang^{2,3}, Xiaowei Zhu², Fangyi Ren^{2,4}, Lingyao Guan^{2,4}, Jiayu Chen^{2,4}, Ya Gao^{2,3}, Mo Chen⁵, Noura Darwish⁶, Sara Colombo Mottaz⁶, Marie Noelle Horcajada⁵, Nicolas Bonnet⁵, Shailay Kumar Dogra⁵, Dantong Wang^{5*}

¹Tianjin Women's and Children's Health Center, Tianjin, China; ²BGI-Shenzhen, Shenzhen, 518083, China; ³Shenzhen Engineering Laboratory for Birth Defects Screening, Shenzhen, 518083, China; ⁴China National Gene Bank, BGI-Shenzhen, Shenzhen, 518120, China; ⁵Nestlé Institute of Health Sciences, Nestlé Research, Lausanne, Switzerland; ⁶Clinical Research Unit, Nestlé Research, Lausanne, Switzerland

*Corresponding author

Jing Wang: wangjing8162012@163.com

Chang Jiang: jackiechristina@163.com

Shuo Wang: wangshuotjfe@163.com

Lingyan Feng: haiyangfly_2000@163.com

Yu Zhang: zhangyuwork2@163.com

Yuanyuan Guo: zhangchen71@163.com

Gongshu Liu: liugongshu727@163.com

Xi Li: lixi2@genomics.cn

Guohong Zhang: zhangguohong@genomics.cn

Xiaowei Zhu: zhuxiaowei@genomics.cn

Fangyi Ren: renfangyi@genomics.cn

Lingyao Guan: guanlingyao@genomics.cn

Jiayu Chen: chenjiayu@genomics.cn

Ya Gao: gaoya@genomics.cn

Mo Chen: Mo.chen@rd.nestle.com

Noura Darwish: Noura.Darwish@rd.nestle.com

Sara Colombo Mottaz: sara.colombomottaz@rd.nestle.com

Marie Noelle Horcajada: MarieNoelle.Horcajada@rdls.nestle.com

Nicolas Bonnet: Nicolas.Bonnet@rd.nestle.com

Shailay Kumar Dogra: ShailayKuma.Dogra@rd.nestle.com

Dantong Wang: Dantong.Wang@rdls.nestle.com

WORD COUNT: 4007

ABSTRACT

Purpose: The Bone And MicroBiOme Onset (BAMBOO) study is an ongoing prospective observational cohort study conducted in Tianjin, China, aiming to determine age-appropriate trajectories for microbiome maturation and bone development, and to identify the influence of dietary factors in the process.

Participants: The recruitment started in September 2021 and was completed in February 2023. In total 1380 subjects were recruited, 690 at birth (group 1) and 690 at 6 months of age (group 2). Group 1 and 2 will be followed up to 12 months and 36 months, respectively.

Findings to date: The age of the mothers was 31.1 ± 3.7 (mean \pm SD), and the birth weight of infants was 3.3 ± 0.5 kg with an incidence of Caesarean section 50.4%. Food diary information of the first 100 subjects showed that 64 food items were introduced by 6 months. A pilot microbiome analysis revealed that at the species level, bacterial communities were composed of mostly *Bacteroides dorei*, *Bacteroides vulgatus* and *Escherichia coli*, that were consistent with previous reports. A cross validation of breast milk vitamin D and HMOs measurement was also conducted. The early data assessment showed a high reliability of the data generated from this study.

Future plans: The data collection will be completed in August 2025. Four stage-statistical analyses will be performed as the cohort reaches certain age thresholds before the final report. Analysis of BAMBOO data will be used to develop age-appropriate trajectories for microbiome maturation and bone development for children 0-3 years and investigate the contribution of dietary factors in the process.

Registration: Trial registration No.: ChiCTR2100049972 (August 16th, 2021)

Strengths and limitations

- Bone And MicroBiOme Onset (BAMBOO) study is a large prospective longitudinal cohort study with 3 years follow up period, measuring multiple aspects of health consistently at multiple timepoints. To our knowledge, this is the first study with such comprehensive coverage in infant and toddlers in China, making it possible to investigate the interplay of dietary factors, microbiome maturation and bone development, as well as growth and health status.
- The findings will provide insights that may help to develop scientific hypotheses and identify potentially relevant timing and interventions to support optimal microbiome or bone development and contribute new evidence to inform nutrition policy setting.
- As an observational study, a limitation is that only associations between early life nutrition, microbiome maturation and bone development can be investigated, their causal relationships could not be determined.
- The study is conducted in a major metropolitan area of northern China, we could anticipate differences in infant feeding and dietary habits from other regions across China. Future studies could focus on such regional differences.

Keywords: Microbiome; bone health; early life nutrition; early life growth

INTRODUCTION

Early childhood is a critical period for growth and development benefitting long term health. The role of the gut microbiome in health has been investigated in many studies. Differences in gut microbiome composition and function have been associated with various chronic diseases such as metabolic conditions, neurological disorders, and cardiovascular illnesses^{1,2}. Microbiome maturation in infants parallels human neurodevelopmental processes and growth. It plays a role in gut-brain signaling³, and is associated with cognitive development⁴. An essential aspect of microbiome function is its impact on immune system. The establishment of host-microbial symbiosis relies on the mutualistic co-development of the host microbiota and immune system⁵. Microbiome diversity has been associated with respiratory tract infections⁶ and its composition may influence mucosal IgA response to vaccine in infants⁷.

A critical window for microbiome maturation is from birth to 4 years of age⁵. Stewart and colleagues reported three distinct phases of microbiome composition evolution in early life: a developmental phase (months 3-14), a transitional phase (months 15-30), and a stable phase (months 31-46)⁸. Considering that after these phases the microbiome composition remains fairly stable across life stages, it is important to define the optimal age-appropriate microbiome acquisition, selection, and maturation from birth up to toddlerhood in relation to health benefits. Moreover, further understanding on the influence of weaning and nutrition during early childhood on an age-appropriate microbiome maturation is needed. While multiple factors influence the establishment and development of infant gut microbiome, such as delivery mode and day care attendance^{9,10}, there is also growing evidence of the influence of dietary factors on microbiome maturation, both breastfeeding and complementary foods. Breastfeeding has a critical role, especially during the first 6 months of age^{8,9,11}. After 6 months of age when weaning foods are gradually being introduced to the diet, microbiota composition and environment changed rapidly¹². An intervention with microbiota-directed complementary foods (i.e. the combination of chickpea flour, peanut flour, soy flour, and raw banana) modified the microbiota and increased plasma biomarkers and mediators of growth, bone formation, neurodevelopment, and immune function in a randomized, double-blind controlled feeding study in malnourished infants in Bangladesh¹³. Understanding the impact and mechanisms underlying these maturation changes requires information on two aspects: establishment of normal age-appropriate microbiome maturation trajectory and investigation of the relationship between specific dietary factors and microbiome⁵. Although some studies have been carried out in Bangladesh¹⁴, US and Europe^{8,12,15}, no longitudinal data have been reported for Chinese infants.

Healthy skeleton development is an important aspect of child growth, particularly around 2-4 years where fast growth occurs with some consequence on fracture risk elevated in child with high height at age 3 years¹⁶. Bone is a dynamic and highly specialized connective tissue. Major functions of bone include provision of a mechanical support for muscular activity, physical protection to the tissues and internal organs, and to act as a repository for systemic mineral homeostasis¹⁷. Bone mass is related to fracture risk. Studies have shown that, for every standard deviation decrease in size-adjusted bone mass, there is an 89% increase in fracture risk in childhood¹⁸. By the early 20s, a 10–15% higher peak bone mass has been estimated to decrease the risk of fracture by 25–50% later in life¹⁹. Therefore, one strategy to reduce later life fracture risk is to build peak bone mass. There are two windows of opportunity to maximize peak bone mass, infancy and adolescence²⁰. Bone mass is assessed by both geometry measures (length and width, which are relatively easier to measure in clinical studies) and mineral deposition. Bone mineralization provides rigidity and resistance to the skeleton while maintaining a certain degree of elasticity¹⁷. Chronological bone physiology highlights the importance of bone mineralization in infants and toddlers to achieve developmental milestones, such as climbing, walking, and running. However, the knowledge on infant bone mineral deposition is limited due to the difficulties in measurement. Moreover, in this rapid growing phase, the balance between rigidity and elasticity at different ages also requires further investigation²¹. Breastmilk was found to have a positive influence on peak bone microarchitecture²², and might be protective for long term bone health²³. Non-digestible human milk oligosaccharides (HMOs), which have a significant influence on infant microbiome²⁴, were found to improve bone mass and structure in different mouse models suggesting a potential HMO-microbiome- bone interaction²⁵⁻²⁷. A prospective randomized clinical trial showed that calcium supplementation increased the

1
2
3 acquisition of bone mass in children, adolescence, and early adulthood²⁸. Notwithstanding such studies, age-
4 appropriate bone development, and the influence of feeding types and complementary food introduction on bone
5 development, in infants and toddlers is still less investigated.
6

7 To fill the knowledge gaps on both optimal age-appropriate microbiome maturation and bone development, the
8 interaction of these two and influence of dietary factors, the Tianjin Women and Children's Health Center
9 (TJWCH), Beijing Genomic Institute (BGI) and Nestlé Research (NR) jointly designed and initiated the BAMBOO
10 study. The results of this unique Chinese project will help researchers to establish age-appropriate trajectories for
11 microbiome maturation and bone development from birth to pre-school age toddlers. The insights gathered may
12 support local nutrition - healthcare programs for infants and toddlers to benefit long term health status.
13
14

15 **COHORT DESCRIPTION**

16 - **Aim of BAMBOO**

17
18 BAMBOO is a prospective cohort observational study aiming to characterize normal age-appropriate
19 microbiome maturation and bone development, and to assess how early life nutrition influences these
20 development processes.
21

22 - **Setting of BAMBOO**

23
24 The BAMBOO study is conducted in Tianjin, the fourth largest municipality in China, covering an area of
25 12,000 square kilometers with a resident population of 13.8 million. It is comprised of 16 districts, including 6
26 central urban districts, 4 surrounding urban districts and 6 suburb districts. All 0–6-year-old children in Tianjin
27 are registered in the Maternal and Child Healthcare system administered by TJWCH. Routine health care
28 records from the pregnancy until children's 6 years are kept in the system. Participants in this cohort are mainly
29 from the 6 central urban districts (Hedong district, Nankai district, Hebei district, Hexi district and Hongqiao
30 district) and 4 surrounding urban districts (Beichen, Jinnan, Xiqing and Dongli district).
31

32 The cohort is composed of children between 0 to 3 years of age. In an accelerated cohort design, two parallel
33 groups covering different age ranges are recruited with an overlap age from 6 to 12 months. Group 1 subjects
34 will be followed from birth to 12 months of age with infant/toddler development information and samples
35 collected at birth and then 1, 3, 4, 6, 9, 12 months of age. Group 2 subjects will be followed from 6 to 36
36 months of age with infant/toddler development information and samples collected at 6, 9, 12, 18, 24, 30 and 36
37 months of age. Detailed visit timepoints and sample collection schedule are shown in figure 1.
38

39 This international scientific collaboration was approved by China Human Genetic Resource Admission
40 Committee (HGRAC) in August 2021. The study is conducted according to the principles of the Declaration of
41 Helsinki and in accordance with the guidelines and regulations of HGRAC. Ethics approval of research was
42 obtained from Tianjin Women's and Children's Health Center Medical Ethical Committee and the Institutional
43 Review Board of BGI-Shenzhen (BGI-IRB 21056). For this study the informed consent will be obtained from
44 parents or legal guardians of the participants. Informed consent will also be obtained from all the mothers
45 involved in the study. The study is registered in the clinical trial registration and the trial number is
46 ChiCTR2100049972.
47
48

49 - **Recruitment**

50
51 Children who meet the following requirements are invited to participate in the study: 1) full-term gestational
52 birth (≥ 37 and ≤ 42 weeks); 2) singleton; and 3) signed informed consent by infant's parents (or his/her legally
53 accepted representative) and agree to fulfill the requirements of the study protocol.
54
55
56
57
58
59
60

The exclusion criteria are: 1) birth after a complicated pregnancy, such as pre-eclampsia, gestational diabetes, or bowel disease, determined by medical interview/ medical record; 2) infant's parents/LAR not willing and/or not able to comply with scheduled visits and the requirements of the study protocol; and 3) currently participating or having participated in another clinical trial within 4 weeks prior to the start of this cohort.

For group 1, infants begin their journey in the study within 10 days after birth. Participant recruitment is ongoing at 7 local delivery hospitals. Investigators introduce the study to the parents and ask their consent, with screening based on the inclusion/exclusion criteria at 3-5 days after giving birth. For group 2, infants join the cohort at 6 months of age. Investigators introduce the study to the parents based on the inclusion/exclusion criteria and ask for their consent when the child is around 5.5 months.

Sample size was calculated based on bone development outcomes. For the bone trajectory analysis, Limanovitz et al found that a sample size of 60 infants per group would be required to demonstrate a significant difference of 70m/s in SoS between breastfed and formula-fed infants with a standard deviation of 133 m/s, assuming an overall type I error of 5% and 80% statistical power²⁹. Based on a previous study in Tianjin, 10% of women performed exclusively formula feeding, 45% performed exclusively breastfeeding and 45% performed mixed feeding. Therefore, given that at least 60 infants are required in each feeding group, a total of 600 completed infants are needed. Considering a drop-out rate of 13%, the total number of infants to be enrolled is 690 per group. For the microbiome trajectory development, based on literature in which a small number of subjects with frequent time points of fecal sampling were used to derive a trajectory¹⁴, we believe we will have sufficient reference subset of infants selected based on our criteria, starting from 690 subjects per group. A total of 1380 healthy normal developing Chinese infants and toddlers are planned to be recruited in this study. Recruitment of participants started in September 2021 and completed in February 2023.

- **Data collection**

Data collection includes questionnaires, medical history (MH), concomitant medication (CM), adverse events/serious adverse events (AE/SAE) record, food diary, biological samples collection and anthropometric measurements (length/height, weight and bone measurements).

- Mother and infant questionnaires. A mother's questionnaire is filled at enrolment (group 1 at around birth; group 2 when infants are 6 months old), including basic information (mother's height, weight, education level and history of gestation), tobacco and alcohol use, work and physical activities after 28 weeks of gestation, vitamin, mineral and probiotic supplements, health and medication during pregnancy, and the last B-ultrasound examination result during pregnancy (only for Group 1).

The infant's questionnaires are gathered at each visit from main caregivers in a face-to-face interview or phone-call interview if the person cannot attend the visit. The information on baby feeding practice, sleep, vitamin, mineral and probiotic supplements, health status, growth and development and antibiotic use is collected from the infant's questionnaires. Infant Gastrointestinal Symptom Questionnaire (IGSQ) (up to 12 months of age) and Toddler Gut Comfort Questionnaire (TGCQ)(18m to 3 years of age) are also administered³⁰. MH, AEs/SAEs as well as concomitant medications and non-pharmacological treatments are recorded from the beginning till the final visit.

- Definition of feeding types. In this study, exclusive breastfeeding (BF) is defined as a subject reported as exclusively breast fed at 1 and 3 months. For exclusive infant formula feeding (IF), a subject is either exclusively infant formula fed at 1 and 3 months or with a mixed feeding and an IF ratio higher than 80% at 1 and 3 months. Feeding practice is classified as mixed feeding if the subject has an estimated IF ratio lower than

80% of total intake at 1 and 3 months. We applied the principle of flexible feeding type description²⁹, IF ratio = total daily infant formula intake in ml/780ml.

- Food diary. Starting from 4 months of age, a food diary is filled by the main caregiver at home, assisted by photos and tools, for 3 consecutive days, starting 4 days prior to each visit. The content is reviewed during the visit by a dietitian. A nutrition evaluation report is shared with the parents by the dietitian at 6 months and 1 year of age. Each food diary is checked by the principal investigator to ensure quality. Nutrient intakes are calculated using Chinese Food Composition database³¹, complemented by other sources such as pack labels and literature review in case of lack of information in the current database.
- Biological samples collection. All biological samples (child fecal and urine samples, and human milk samples) are temporarily stored at -80° freezer at TJWCH, and regularly shipped on dry ice to different labs for analysis. The samples flow chart is shown in Figure 2. Detailed methods for sample handling and analysis are described in Supplementary material.
- Height, Weight measurement. Children's length/height and weight are measured by trained investigators using calibrated electronic scale and measuring bed (Suhong RCS-20), with the nearest accuracy of 0.1cm and 5g, respectively.
- Bone length and mass index measurement. Bone mass index measurement is conducted using a non-invasive and radiation-free ultra-sound sonometer (Sunlight® Omnisense Mini) measuring bone transmission time and speed of sound at the tibia site, together with measurements of both tibia and radius length. In the study, two ultrasound devices are used to measure the bone transmission time and speed of sound at the radius and tibia site. To calibrate the two devices, a phantom is scanned to detect deviation of the ultrasound source daily. In order to maximize the reproducibility, the research staff who had past experience with similar devices were trained and tested in children with the reproducibility of the two devices CV<6%.
- **Data management and Statistical analysis**

Data are entered from the source document into an electronic Case Report Form (eCRF - web database named ClinFlash) within 15 days after the subject's visit. Data quality review meetings are held quarterly, and the statistical results on sample enrolment and descriptive statistics on key measurements are included into a data quality report and submitted to the research committee for review. Protocol deviations are predefined and divided into major and minor categories, which trigger corresponding corrective action and preventive action in time. All protocol deviations in listing format will be reported to the TJWCH Ethics Committee.

Data analyses are planned to be conducted in R (R Core Team, 2014) and figures will be produced using the package ggplot2 (Wickham, 2009). Microbiome data will be further explored using Microbiome-toolbox (version 1.0)³⁹. Data will be initially checked for normality of distribution (using qq-plot and residuals vs. fitted values plot). Feeding groups, complementary food and nutrient intakes will be summarized. Dietary patterns will be identified using principal component analysis (PCA) and/or cluster analysis. Microbiome trajectories were derived using a machine learning model to approximate Microbiome Maturation Index (MMI). Microbiota-for-age z-scores will be calculated and associations between normal/abnormal microbiota-for-age z-scores and various health and dietary factors will be investigated using chi-square test of independence. Bone (tibia and radius length and SoS) trajectories over time will be summarized. Associations between different measures will also be investigated using appropriate method, such as linear mixed-effect model adjusting for confounding factors. The model will include the computed propensity scores as weights.

FINDINGS TO DATE

1
2
3 The Bamboo study was started in September 2021 and recruitment was completed by the end of February 2023. A
4 total of 1380 mother-child pairs were recruited in this study (690 in group 1 and 690 in group 2). Table 1 shows the
5 basic characteristics of the participants, including mother's age, delivery weeks, birth mode, infants' gender, nation,
6 birth weight or birth length. In total, 239 subjects dropped out from the study. Figure 3 shows the reasons for drop
7 out, where the top 3 reasons are "inconvenient to follow up", "not interested in the study" and "leaving Tianjin". We
8 compared the basic characteristics between participants still in the group and those who dropped out, and found no
9 difference except that the dropped-out mothers were more likely to be younger than the mothers remaining in the
10 study (Table 1). The population characteristics of this study are comparable to those in another large cohort study,
11 the Tianjin Birth Cohort (TJBC), conducted in the same city with a large number of mother and infant dyads [40]. In
12 both studies, the average mothers' age was around 30-31 years, children's average body length and weight at birth
13 were around 50cm and 3.3kg, respectively. We observed a slightly lower proportion of girls enrolled in this study
14 (45%) as compared to TJBC (48%) but the difference is not statistically significant (detailed data of TJBC have not
15 yet been published)

16
17
18 A review of dietary intake data was conducted using food diary information from the first 100 subjects at 6 months
19 of age in group 2 since most children have been exposed to the complementary foods at this age. In total 64 food
20 items were reported (Table 2). Nutrient intake estimation is ongoing based on the food intake using Chinese Food
21 Composition database³¹, complemented by labels on the pack.

22
23 A data assessment was conducted using early available samples, which is a small proportion of the cohort data. The
24 data that support the findings of this study have been deposited into CNGB Sequence Archive (CNSA)^{40,41}, with
25 accession number CNP0003576. A total of 20 stool samples were sequenced and subjected to subsequent
26 metagenomic analysis. All DNA concentrations of the 20 samples are greater than 3 ng/ μ L, which is the minimum
27 amount required for library construction. Adaptor contaminated, low-quality and host reads were removed from the
28 raw sequencing read sets. An average of 33.6 GB (9.52 to 48.36 GB) data of high-quality clean reads per sample
29 was generated, equivalent to 96.1% of raw reads on average. Comparison of the compositional features of the gut
30 microbiota alongside the age spectrum revealed several characteristic patterns. At the species level, bacterial
31 communities were composed of mostly *Bacteroides dorei*, *Bacteroides vulgatus* and *Escherichia coli* (Figure 4).
32 Progressive changes in microbial diversity and abundance of the infant gut microbiome are likely to re-shape the
33 metabolic functions of the hosts over time. The results of the pilot assessment were consistent with the discoveries
34 reported previous^{8,42}. The urine creatine assay has been tested and validated previously in infants and toddlers with
35 similar age to the BAMBOO cohort, with results in the correct range^{43,44}. A cross validation of breast milk vitamin
36 D and HMOs measurement has been conducted with internal data previously published and also validated through
37 certified reference values^{45,46}. In summary the early data assessment shows that high reliability of the data generated
38 from this study.

39
40
41 The next step will focus on completing recruitment and minimizing the drop out, especially in group 1. Additionally,
42 an interim statistical analysis will be performed to provide an initial overview of different aspects of the study,
43 including microbiome maturation, bone development and dietary intake of children at different ages. Finally, 4-
44 stage-statistical analyses before final data analysis will be performed as the complete cohort reaches certain age
45 thresholds.

46 47 **FURHER DETAILS**

48 49 **Strengths and limitations**

50
51 There are several limitations in this study. First, as an observational study, only associations between early life
52 nutrition, microbiome maturation and bone development can be investigated, but their causal relationships could not
53 be determined. However, these findings will provide insights that may help to develop scientific hypotheses and
54 identify potentially relevant timing and interventions to support optimal microbiome or bone development and
55 contribute new evidence to inform nutrition policy setting. Second, as the study is conducted in a major metropolitan
56

1
2
3 area of northern China, we could anticipate differences in feeding and dietary habits from other regions across
4 China. Future studies could focus on such regional differences.
5

6 The strength of the study is its large sample size with longitudinal follow up, measuring multiple aspects of health
7 consistently at multiple timepoints. To our knowledge, this is the first study with such comprehensive coverage in
8 infant and toddlers in China, making it possible to investigate the interplay of dietary factors, microbiome
9 maturation and bone development, as well as growth and health status.
10

11 **Collaboration and Data availability statement**

12 Microbiota data that support the findings of this study have been deposited into CNGB Sequence Archive (CNSA)
13 and made publicly available upon publication of the results in scientific articles. Ethical considerations related to
14 personal data, informed consent and human research act restrict human data sharing in public repositories. Fully
15 anonymized data may be shared upon request to the corresponding author accompanied by a proposed research plan
16 for evaluation and approval by the Bamboo scientific council, followed by relevant ethics committee approval.
17
18

19 **ETHICS STATEMENTS**

20 **Ethics approval**

21 This international scientific collaboration was approved by China Human Genetic Resource Admission Committee
22 (HGRAC) in August 2021. The study is conducted according to the principles of the Declaration of Helsinki and in
23 accordance with the guidelines and regulations of HGRAC. Ethics approval of research was obtained from Tianjin
24 Women's and Children's Health Center Medical Ethical Committee and the Institutional Review Board of BGI-
25 Shenzhen (BGI-IRB 21056). The study is registered in the clinical trial registration and the trial number is
26 ChiCTR2100049972.
27
28

29 **Consent to participate**

30 For this study the informed consent was obtained from parents or legal guardians of the infants. Informed consents
31 were also obtained from all the mothers involved in the study.
32

33 **Patient consent for publication**

34 Not applicable.
35

36 **Funding**

37 The study is funded by Société des Produits Nestlé SA and BGI-Research.
38

39 **Competing interests**

40 Xi Li, Guohong Zhang, Xiaowei Zhu, Fangyi Ren, Lingyao Guan, Jiayu Chen, and Ya Gao are employed by BGI-
41 Research. Mo Chen, Noura Darwish, Sara Colombo Mottaz, Marie Noelle Horcajada, Nicolas Bonnet, Shaillay
42 Kumar Dogra, and Dantong Wang are employed by Nestle Research. The authors declared that they have no
43 competing interests.
44

45 **Authors' contributions**

46 All authors contributed to the study design, interpretation of the data and writing the manuscript. Data and sample
47 collection were performance by Jing Wang, Chang Jiang, Shuo Wang, Lingyan Feng, Yu Zhang, Yuanyuan Guo,
48 and Gongshu Liu. Sample measurement and analyses were performed by Xi Li, Guohong Zhang, Xiaowei Zhu,
49 Fangyi Ren, Lingyao Guan, Jiayu Chen, and Ya Gao. Data management and statistical protocol were set up by Mo
50 Chen, Noura Darwish, Marie Noelle Horcajada, Nicolas Bonnet, Shaillay Kumar Dogra, and Dantong Wang. The
51
52
53
54
55
56
57
58
59
60

1
2
3 first draft of the manuscript was written by Jing Wang, Sara Colombo Mottaz, Marie Noelle Horcajada, Nicolas
4 Bonnet, Shaillay Kumar Dogra and Dantong Wang. All authors read and approved the manuscript.
5

6 **Acknowledgement**

7
8 We thank Dr. Norbert Sprenger for his contribution in questionnaire design and guidance in microbiome data
9 analysis, Dr. Giles Major for his critical review of this manuscript. We thank Qiaoji Li, Dr. Alice Pannérec, Dr.
10 Marie Boutant Lys and Dr. Marie Bachelet for setting-up the collaboration and their professional management in
11 planning and executing the project. We thank all the investigators from Tianjin Women and Children's Health
12 Center for conducting the field investigation and sample collection, research staff from BGI-Shenzhen for preparing
13 sample collecting kits and performing laboratory testing. This work was supported by China National GeneBank
14 (CNGB). We thank all participants in this study.
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

REFERENCES

1. Vijay A, Valdes AM. Role of the gut microbiome in chronic diseases: a narrative review. *Eur J Clin Nutr* 2022;76(4):489-501. doi: 10.1038/s41430-021-00991-6 [published Online First: 2021/09/30]
2. Parekh PJ, Balart LA, Johnson DA. The Influence of the Gut Microbiome on Obesity, Metabolic Syndrome and Gastrointestinal Disease. *Clin Transl Gastroenterol* 2015;6:e91. doi: 10.1038/ctg.2015.16 [published Online First: 2015/06/19]
3. Cong X, Xu W, Romisher R, et al. Gut Microbiome and Infant Health: Brain-Gut-Microbiota Axis and Host Genetic Factors. *Yale J Biol Med* 2016;89(3):299-308. [published Online First: 2016/10/05]
4. Carlson AL, Xia K, Azcarate-Peril MA, et al. Infant Gut Microbiome Associated With Cognitive Development. *Biol Psychiatry* 2018;83(2):148-59. doi: 10.1016/j.biopsych.2017.06.021 [published Online First: 2017/08/11]
5. Dogra SK, Kwong Chung C, Wang D, et al. Nurturing the Early Life Gut Microbiome and Immune Maturation for Long Term Health. *Microorganisms* 2021;9(10) doi: 10.3390/microorganisms9102110 [published Online First: 2021/10/24]
6. Woodall CA, McGeoch LJ, Hay AD, et al. Respiratory tract infections and gut microbiome modifications: A systematic review. *PLoS One* 2022;17(1):e0262057. doi: 10.1371/journal.pone.0262057 [published Online First: 2022/01/14]
7. Zhao T, Li J, Fu Y, et al. Influence of gut microbiota on mucosal IgA antibody response to the polio vaccine. *NPJ Vaccines* 2020;5(1):47. doi: 10.1038/s41541-020-0194-5 [published Online First: 2020/06/23]
8. Stewart CJ, Ajami NJ, O'Brien JL, et al. Temporal development of the gut microbiome in early childhood from the TEDDY study. *Nature* 2018;562(7728):583-88. doi: 10.1038/s41586-018-0617-x
9. Ho NT, Li F, Lee-Sarwar KA, et al. Meta-analysis of effects of exclusive breastfeeding on infant gut microbiota across populations. *Nat Commun* 2018;9(1):4169. doi: 10.1038/s41467-018-06473-x
10. Amir A, Erez-Granat O, Braun T, et al. Gut microbiome development in early childhood is affected by day care attendance. *NPJ Biofilms Microbiomes* 2022;8(1):2. doi: 10.1038/s41522-021-00265-w [published Online First: 2022/01/13]
11. Baumann-Dudenhoefter AM, D'Souza AW, Tarr PI, et al. Infant diet and maternal gestational weight gain predict early metabolic maturation of gut microbiomes. *Nat Med* 2018;24(12):1822-29. doi: 10.1038/s41591-018-0216-2
12. Backhed F, Roswall J, Peng Y, et al. Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. *Cell Host Microbe* 2015;17(5):690-703. doi: 10.1016/j.chom.2015.04.004 [published Online First: 2015/05/15]
13. Gehrig JL, Venkatesh S, Chang HW, et al. Effects of microbiota-directed foods in gnotobiotic animals and undernourished children. *Science* 2019;365(6449) doi: 10.1126/science.aau4732 [published Online First: 2019/07/13]
14. Subramanian S, Huq S, Yatsunenko T, et al. Persistent gut microbiota immaturity in malnourished Bangladeshi children. *Nature* 2014;510(7505):417-21. doi: 10.1038/nature13421 [published Online First: 2014/06/05]
15. Depner M, Taft DH, Kirjavainen PV, et al. Maturation of the gut microbiome during the first year of life contributes to the protective farm effect on childhood asthma. *Nat Med* 2020;26(11):1766-75. doi: 10.1038/s41591-020-1095-x [published Online First: 2020/11/04]
16. Jones IE, Williams SM, Goulding A. Associations of birth weight and length, childhood size, and smoking with bone fractures during growth: evidence from a birth cohort study. *Am J Epidemiol* 2004;159(4):343-50. doi: 10.1093/aje/kwh052 [published Online First: 2004/02/11]
17. Ma NS, Gordon CM. Pediatric osteoporosis: where are we now? *J Pediatr* 2012;161(6):983-90. doi: 10.1016/j.jpeds.2012.07.057 [published Online First: 2012/09/15]
18. Clark EM, Ness AR, Bishop NJ, et al. Association between bone mass and fractures in children: a prospective cohort study. *J Bone Miner Res* 2006;21(9):1489-95. doi: 10.1359/jbmr.060601 [published Online First: 2006/08/31]
19. Weaver CM, Gordon CM, Janz KF, et al. The National Osteoporosis Foundation's position statement on peak bone mass development and lifestyle factors: a systematic review and implementation recommendations. *Osteoporos Int* 2016;27(4):1281-386. doi: 10.1007/s00198-015-3440-3 [published Online First: 2016/02/10]

20. Lu J, Shin Y, Yen MS, et al. Peak Bone Mass and Patterns of Change in Total Bone Mineral Density and Bone Mineral Contents From Childhood Into Young Adulthood. *J Clin Densitom* 2016;19(2):180-91. doi: 10.1016/j.jocd.2014.08.001 [published Online First: 2014/12/03]
21. Ambrose CG, Soto Martinez M, Bi X, et al. Mechanical properties of infant bone. *Bone* 2018;113:151-60. doi: 10.1016/j.bone.2018.05.015 [published Online First: 2018/05/26]
22. Yang Y, Wu F, Dwyer T, et al. Associations of Breastfeeding, Maternal Smoking, and Birth Weight With Bone Density and Microarchitecture in Young Adulthood: a 25-Year Birth-Cohort Study. *J Bone Miner Res* 2020;35(9):1652-59. doi: 10.1002/jbmr.4044 [published Online First: 2020/07/09]
23. Carter SA, Parsons CM, Robinson SM, et al. Infant milk feeding and bone health in later life: findings from the Hertfordshire cohort study. *Osteoporos Int* 2020;31(4):709-14. doi: 10.1007/s00198-020-05296-1 [published Online First: 2020/02/18]
24. Berger B, Porta N, Foata F, et al. Linking Human Milk Oligosaccharides, Infant Fecal Community Types, and Later Risk To Require Antibiotics. *mBio* 2020;11(2) doi: 10.1128/mBio.03196-19 [published Online First: 2020/03/19]
25. Charbonneau MR, O'Donnell D, Blanton LV, et al. Sialylated Milk Oligosaccharides Promote Microbiota-Dependent Growth in Models of Infant Undernutrition. *Cell* 2016;164(5):859-71. doi: 10.1016/j.cell.2016.01.024 [published Online First: 2016/02/24]
26. Blanton LV, Barratt MJ, Charbonneau MR, et al. Childhood undernutrition, the gut microbiota, and microbiota-directed therapeutics. *Science* 2016;352(6293):1533. doi: 10.1126/science.aad9359 [published Online First: 2016/06/25]
27. Cowardin CA, Ahern PP, Kung VL, et al. Mechanisms by which sialylated milk oligosaccharides impact bone biology in a gnotobiotic mouse model of infant undernutrition. *Proc Natl Acad Sci U S A* 2019;116(24):11988-96. doi: 10.1073/pnas.1821770116 [published Online First: 2019/05/30]
28. Dibba B, Prentice A, Ceesay M, et al. Effect of calcium supplementation on bone mineral accretion in gambian children accustomed to a low-calcium diet. *Am J Clin Nutr* 2000;71(2):544-9. doi: 10.1093/ajcn/71.2.544 [published Online First: 2000/01/29]
29. Litmanovitz I, Davidson K, Eliakim A, et al. High Beta-palmitate formula and bone strength in term infants: a randomized, double-blind, controlled trial. *Calcif Tissue Int* 2013;92(1):35-41. doi: 10.1007/s00223-012-9664-8 [published Online First: 2012/11/28]
30. Riley AW, Trabulsi J, Yao M, et al. Validation of a Parent Report Questionnaire: The Infant Gastrointestinal Symptom Questionnaire. *Clin Pediatr (Phila)* 2015;54(12):1167-74. doi: 10.1177/0009922815574075 [published Online First: 2015/03/12]
31. Society CN. China Food Composition Table. Beijing: Peking University Medical Press 2019.
32. Fang C, Zhong H, Lin Y, et al. Assessment of the cPAS-based BGISEQ-500 platform for metagenomic sequencing. *Gigascience* 2018;7(3):1-8. doi: 10.1093/gigascience/gix133
33. Xu Y, Lin Z, Tang C, et al. A new massively parallel nanoball sequencing platform for whole exome research. *BMC Bioinformatics* 2019;20(1):153. doi: 10.1186/s12859-019-2751-3
34. Drmanac R, Sparks AB, Callow MJ, et al. Human genome sequencing using unchained base reads on self-assembling DNA nanoarrays. *Science* 2010;327(5961):78-81. doi: 10.1126/science.1181498
35. Huang J, Liang X, Xuan Y, et al. A reference human genome dataset of the BGISEQ-500 sequencer. *Gigascience* 2017;6(5):1-9. doi: 10.1093/gigascience/gix024
36. Zhang Y, Gu Y, Ren H, et al. Gut microbiome-related effects of berberine and probiotics on type 2 diabetes (the PREMOTe study). *Nat Commun* 2020;11(1):5015. doi: 10.1038/s41467-020-18414-8
37. Jie Z, Yu X, Liu Y, et al. The Baseline Gut Microbiota Directs Dieting-Induced Weight Loss Trajectories. *Gastroenterology* 2021;160(6):2029-42 e16. doi: 10.1053/j.gastro.2021.01.029
38. Beghini F, McIver LJ, Blanco-Miguez A, et al. Integrating taxonomic, functional, and strain-level profiling of diverse microbial communities with bioBakery 3. *Elife* 2021;10 doi: 10.7554/eLife.65088
39. Banjac J, Sprenger N, Dogra SK. Microbiome Toolbox: Methodological approaches to derive and visualize microbiome trajectories. *bioRxiv* 2022:2022.02.14.479826. doi: 10.1101/2022.02.14.479826
40. Guo X, Chen F, Gao F, et al. CNSA: a data repository for archiving omics data. *Database (Oxford)* 2020;2020 doi: 10.1093/database/baaa055 [published Online First: 2020/07/25]
41. Chen FZ, You LJ, Yang F, et al. CNGBdb: China National GeneBank DataBase. *Yi Chuan* 2020;42(8):799-809. doi: 10.16288/j.yczs.20-080 [published Online First: 2020/09/22]
42. Niu J, Xu L, Qian Y, et al. Evolution of the Gut Microbiome in Early Childhood: A Cross-Sectional Study of Chinese Children. *Front Microbiol* 2020;11:439. doi: 10.3389/fmicb.2020.00439

- 1
2
3 43. Wang W, Du C, Lin L, et al. Anthropometry-based 24-h urinary creatinine excretion reference for Chinese
4 children. *PLoS One* 2018;13(5):e0197672. doi: 10.1371/journal.pone.0197672 [published Online First:
5 2018/05/24]
6 44. Kwak BO, Lee ST, Chung S, et al. Microalbuminuria in normal Korean children. *Yonsei Med J* 2011;52(3):476-
7 81. doi: 10.3349/ymj.2011.52.3.476 [published Online First: 2011/04/14]
8 45. Oberson JM, Benet S, Redeuil K, et al. Quantitative analysis of vitamin D and its main metabolites in human
9 milk by supercritical fluid chromatography coupled to tandem mass spectrometry. *Anal Bioanal Chem*
10 2020;412(2):365-75. doi: 10.1007/s00216-019-02248-5 [published Online First: 2019/12/14]
11 46. Austin S, De Castro CA, Benet T, et al. Temporal Change of the Content of 10 Oligosaccharides in the Milk of
12 Chinese Urban Mothers. *Nutrients* 2016;8(6) doi: 10.3390/nu8060346 [published Online First: 2016/06/25]
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

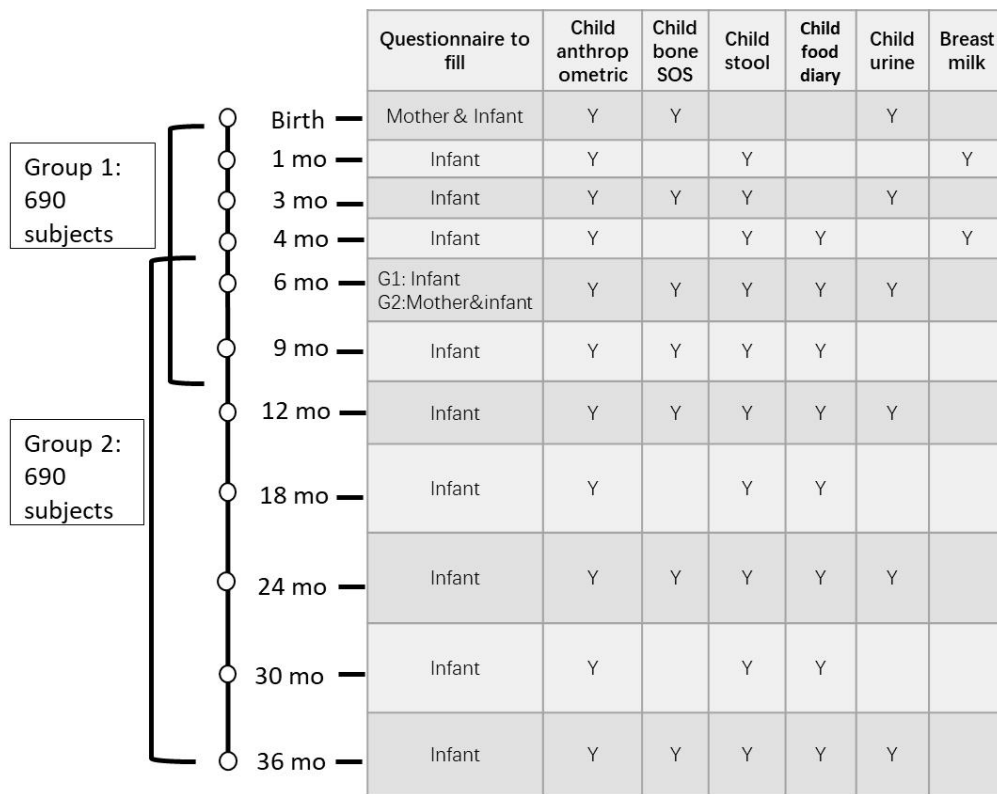
Table 1. Characteristics of enrolled participants (February 2023)

Characteristics	Group 1	Group 2	Total	Active	Drop out	Statistics	P-value
N	690	690	1380	1141	239		
Mother's age	30.83±3.80	31.44±3.59	31.14±3.71	31.28±3.73	30.44±3.54	t=3.205	0.001
Delivery weeks	39.05±0.937	39.19±1.072	39.12±1.01	39.14±1.01	39.03±1.02	t=1.605	0.109
Birth mode	Natural childbirth	381 (55.2%)	695 (49.6%)	586 (51.4%)	109 (45.6%)	$\chi^2=2.615$	0.106
	Caesarean section	314 (45.5%)	309 (44.8%)	685 (50.4%)	555 (48.6%)		
Infant's gender	Male	396 (57.4%)	369 (53.5%)	765 (55.4%)	631 (55.3%)	$\chi^2=0.047$	0.829
	Female	294 (42.6%)	321 (46.5%)	615 (44.6%)	510 (44.7%)		
Nation	Han	619 (89.7%)	639 (92.6%)	1258 (91.2%)	1041 (91.2%)	$\chi^2=0.048$	0.827
	Other	71 (10.3%)	51 (7.4%)	122 (8.8%)	100 (8.8%)		
Birth weight (kg)	3.33±0.372	3.33±0.389	3.33±0.380	3.34±0.375	3.29±0.401	t=1.733	0.083
Birth length (cm)	49.63±1.36	49.97±1.29	49.80±1.33	49.84±1.32	49.65±1.39	t=1.925	0.054

Table 2 Foods recorded in the food diary*

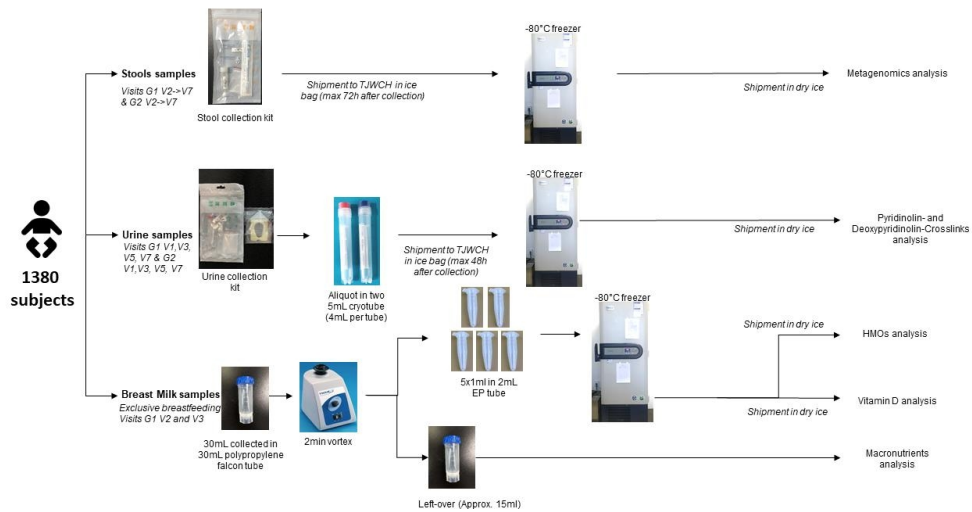
Food name	Count	Percent, %	Food name	Count	Percent, %
Breastmilk	1552	54.0	Orange	8	0.3
Formula	510	17.8	Tangerine	6	0.2
Ground rice	179	6.2	Avocado	1	0.1
Ground rice with vegetable puree	1	0.0	Grape	2	0.1
Ground rice with zucchini puree	2	0.1	Pomegranate	1	0.0
Ground rice with potato puree	1	0.0	Red date	2	0.1
Formula with ground rice	1	0.0	Fruit puree	6	0.2
Rice cracker	8	0.3	Banana	7	0.2
Rice water	2	0.1	Yolk	14	0.5
Rice porridge	1	0.0	Liver	3	0.1
Noodle	5	0.2	Shrimp	3	0.1
Small steamed buns	3	0.1	Dried meat	1	0.0
Millet puree	1	0.0	Cookie	1	0.1
Millet porridge	3	0.1	Puff	1	0.0
Millet sweet potato porridge	1	0.0	Mousse	1	0.0
Mixed porridge	1	0.0	Oil	23	0.8
Purple rice porridge	2	0.1	Linseed oil	2	0.1
Sweet potato	4	0.1	Sesame butter	3	0.1
Potato	3	0.1	Soy sauce	3	0.1
Purple sweet potato	1	0.0	Water	34	1.2
Yum	7	0.2	Vitamin AD	188	6.5
Spanish	4	0.1	Vitamin D3	61	2.1
Bok choy	1	0.0	Vitamin supplement	1	0.0
Broccoli	5	0.2	Mineral supplement	7	0.2
Carrot	14	0.5	Calcium	47	1.6
Cucumber	5	0.2	Zinc	2	0.1
Pumpkin/Squash	21	0.7	Probiotics	37	1.3
Apple	29	1.0	Fish oil	3	0.1
Pitaya	1	0.0	DHA	25	0.9
Blueberry	2	0.1	B12	2	0.1
Pear, blueberry	1	0.0	Albumen powder	2	0.1
Pear	2	0.1	Protein iron succinate oral liquid	2	0.1

*Data was based on the first 100 subjects at 6 months in group 2.

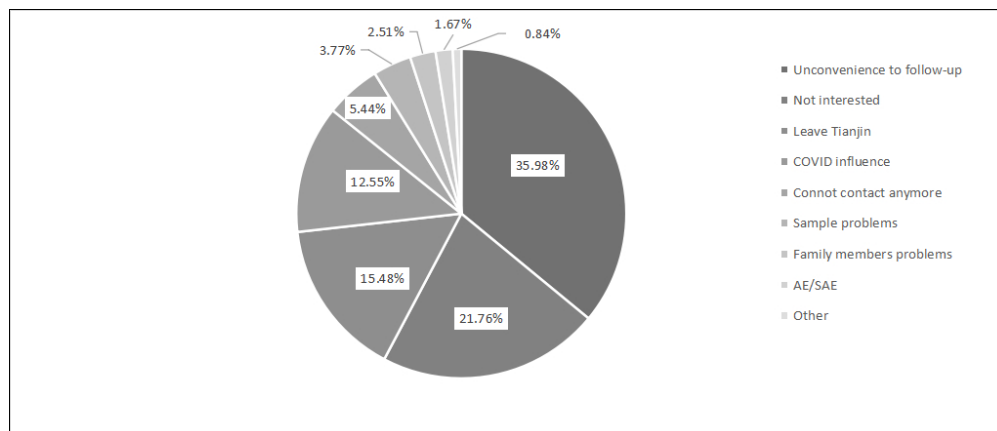


180x142mm (150 x 150 DPI)

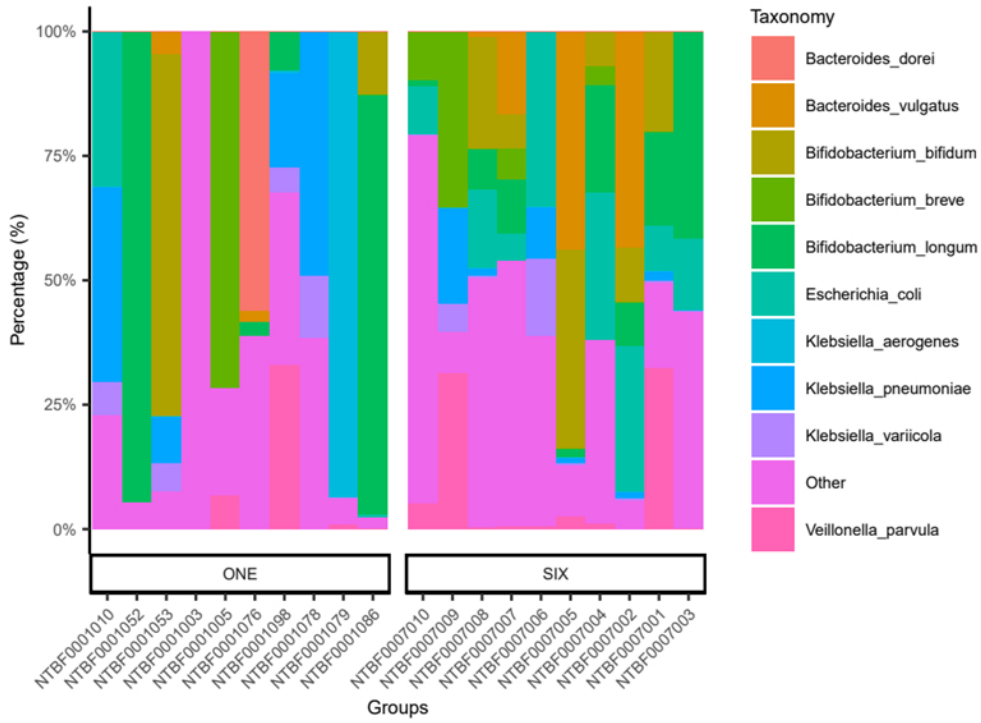
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



338x190mm (96 x 96 DPI)



553x236mm (47 x 47 DPI)



337x244mm (59 x 59 DPI)

Supplementary material: Biological samples collection and measurement

All biological samples are temporarily stored at -80° freezer at Tianjin Women and Children's Health Center (TJWCH), and regularly shipped on dry ice to different labs for analysis.

- Fecal samples:

- Fecal samples are collected a maximum of 72 hours before the planned visit, stored at 2-8°C and bring to the hospital using a cooling bag. For subjects aged 6 months and under ($\leq 6m$), 1 flat spoon of stool sample is collected. For subjects over 6 months of age ($> 6m$), 2 swabs of stool samples is collected. The collected samples are put in a pre-prepared tube and the stabilizer should merge the sample. A sampling guideline is included in the stool sample kits.
- The methods of library construction and sequencing of microbiota in fecal samples are adopted from the protocol described previously¹. Genomic DNA is firstly isolated from stool samples using Magpure Stool DNA KF Kit B (Magen, China) following the manufacturer's instruction. The isolated DNA is subjected to random fragmentation with Covaris E220 (Covaris, Brighton, UK). After ligation, a MGIEasy™ DNA Library Prep Kit (MGI, Shenzhen, China) is applied and the resulting ssDNA circles are used to generate DNA nanoballs (DNBs) by rolling circle amplification (RCA)^{2,3}. After RCA and the formation of DNBs, the final products are measured by Qubit using the ssDNA HS Assay kit (Invitrogen), and loaded on a DIPSEQ platform (MGI, Shenzhen, China) for sequencing using paired-end 100 bp mode following the manufacturer's instructions⁴. Metagenome analysis by DNA shotgun sequencing using DIPSEQ platforms has been previously established to study human gut microbiome^{5,6}.
- For bioinformatics analysis, the high-quality cleaned reads are used for the annotation and profile acquisition of taxons using MetaPhlan3 (version 3.0.14, code: `metaphlan.py input.fastq -input_type fastq -nproc 10 > profiled_metagenome.txt`)⁷. For functional abundance calculation, the HumanN3 pipeline (version 3.0.1, code: `humann --input input.fastq --threads 10 --output output`)⁷ is used to map the sequences against the UniRef90 database with default parameters to obtain functional profiles. Taxonomic profiles include the stratified relative abundances from phylum to species levels. The stratified relative abundances are extracted according to the taxonomic levels of interest. The Chao1 and Shannon indexes are calculated to estimate microbial alpha-diversity.

- Breast milk samples:

- Breast milk samples are collected in group 1 at 1-month and 4-month timepoints from mothers who provide exclusive breastfeeding to their infants. The volume of a breast milk sample is 30ml. A breast milk pump is available at the site and milk is collected in the morning from 9 to 11am. For mothers who cannot complete such collection during the visit, the study team will arrange another time (within 48h). A delay of additional 48h is accepted if the mother is sick.
- Macronutrient analyses, including protein, lactose, energy, fat, and calcium, are performed on HLIFE MR-1011 automatic breast milk analyzer in the lab in TJWCH.
- Quantitative analysis of Vitamin D in human milk. Vitamin D₃ and 25-OH D₃ are target analytes for quantitative analysis. Analysis is performed on Agilent 1290

Infinity liquid chromatography coupled to Agilent 6495 tandem mass spectrometry (Agilent). The analysis is conducted by SMQ Group Medical Laboratory (Shen Zhen, China). Phenomenex Kinetex PFP (2.1×150 mm, 1.7 μm) has been chosen as the analytical column. The separation gradient is shown in Supplementary Table S1. Mass spectrometric detection is carried out on Atmospheric Pressure Chemical Ionization operating in positive mode at unit resolution (APCI⁺). Compound parameters are shown in Supplementary Table S2. The detection limit and quantitation limit of Vitamin D₃ are 0.02 ng/mL and 0.07 ng/mL, respectively. For 25-OH D₃, the detection limit is 0.05 ng/ml and the quantitation limit is 0.16 ng/ml.

- Quantification of HMOs in human milk by UHPLC-FLD. The method has been developed for the quantification of the major human milk oligosaccharides (HMOs) in human milk using UHPLC with fluorometric detection (Thermo U3000).
- Child urine sample
 - Urine samples are collected using urine collection sterile bags and then transferred into the two 5ml cryotubes. The samples are collected a maximum of 48hr before the planned visit, stored at 2-8°C. If samples are not collected prior the visit, the study team will propose to collect the sample during the visit or to collect the samples at home in the following 48 hours, stored at 2-8°C.
 - Analysis for urine creatinine and pyridinoline (PYD) is performed using commercial ELISA kits (MicroVue Creatinine 8009 and MicroVue PYD 8010, respectively), according to the manufacturer's instructions. The analysis is conducted by SMQ Group Medical Laboratory (Shen Zhen, China).

Reference

1. Fang C, Zhong H, Lin Y, et al. Assessment of the cPAS-based BGISEQ-500 platform for metagenomic sequencing. *Gigascience* 2018;7(3):1-8. doi: 10.1093/gigascience/gix133
2. Xu Y, Lin Z, Tang C, et al. A new massively parallel nanoball sequencing platform for whole exome research. *BMC Bioinformatics* 2019;20(1):153. doi: 10.1186/s12859-019-2751-3
3. Drmanac R, Sparks AB, Callow MJ, et al. Human genome sequencing using unchained base reads on self-assembling DNA nanoarrays. *Science* 2010;327(5961):78-81. doi: 10.1126/science.1181498
4. Huang J, Liang X, Xuan Y, et al. A reference human genome dataset of the BGISEQ-500 sequencer. *Gigascience* 2017;6(5):1-9. doi: 10.1093/gigascience/gix024
5. Zhang Y, Gu Y, Ren H, et al. Gut microbiome-related effects of berberine and probiotics on type 2 diabetes (the PREMOTÉ study). *Nat Commun* 2020;11(1):5015. doi: 10.1038/s41467-020-18414-8
6. Jie Z, Yu X, Liu Y, et al. The Baseline Gut Microbiota Directs Dieting-Induced Weight Loss Trajectories. *Gastroenterology* 2021;160(6):2029-42 e16. doi: 10.1053/j.gastro.2021.01.029
7. Beghini F, McIver LJ, Blanco-Miguez A, et al. Integrating taxonomic, functional, and strain-level profiling of diverse microbial communities with bioBakery 3. *Elife* 2021;10 doi: 10.7554/eLife.65088

An early life observational cohort in China: Bone And MicroBiOme Onset (BAMBOO) study

Supplementary Table S1 Separation gradient of chromatographic separation

Time (min)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0	0.300	74.0	26.0
0.50	0.300	74.0	26.0
5.00	0.300	76.0	24.0
6.00	0.300	80.0	20.0
13.00	0.300	82.0	18.0
13.01	0.300	74.0	26.0

Supplementary Table S2 Compound parameters of mass spectrometric detection

Analyte	Transition Reactions (m/z) used for Quantification
D3-PTAD	560.31 →298.1
25-OHD3-PTAD	558.31 →298.1
D3- d3-PTAD	563.31 →301.1
25-OHD3-d6-PTAD	564.41 →298.0

BMJ Open

Cohort profile: An early life observational cohort in China: Bone And MicroBiOme Onset (BAMBOO) study

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2023-075417.R1
Article Type:	Cohort profile
Date Submitted by the Author:	16-Feb-2024
Complete List of Authors:	<p>Wang, Jing; Tianjin Women's and Children's Health Center Jiang, Chang; Tianjin Women's and Children's Health Center Wang, Shuo; Tianjin Women's and Children's Health Center Feng, Lingyan; Tianjin Women's and Children's Health Center Zhang, Yu; Tianjin Women's and Children's Health Center Guo, Yuanyuan; Tianjin Women's and Children's Health Center Liu, Gongshu; Tianjin Women's and Children's Health Center Li, Xi; BGI-Shenzhen Zhang, Guohong; BGI; Shenzhen Engineering Laboratory for Birth Defects Screening Zhu, Xiaowei; BGI Ren, Fangyi; BGI; China National GeneBank Guan, Lingyao; BGI; China National GeneBank Chen, Jiayu; BGI; China National GeneBank Gao, Ya; BGI; Shenzhen Engineering Laboratory for Birth Defects Screening Chen, Mo; Nestlé Institute of Health Sciences Darwish, Noura; Clinical Research Unit Mottaz, Sara Colombo; Clinical Research Unit Horcajada, Marie Noelle; Nestlé Institute of Health Sciences Bonnet, Nicolas; Nestlé Institute of Health Sciences Dogra, Shaillay Kumar; Nestlé Institute of Health Sciences Wang, Dantong; Nestle Institute of Health Sciences SA</p>
Primary Subject Heading:	Epidemiology
Secondary Subject Heading:	Paediatrics, Gastroenterology and hepatology, Epidemiology, Nutrition and metabolism, Research methods
Keywords:	Paediatric gastroenterology < GASTROENTEROLOGY, NUTRITION & DIETETICS, Bone diseases < ORTHOPAEDIC & TRAUMA SURGERY, EPIDEMIOLOGIC STUDIES

SCHOLARONE™
Manuscripts

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our [licence](#).

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which [Creative Commons](#) licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

Cohort profile: An early life observational cohort in China: Bone And MicroBiOme Onset (BAMBOO) study

Jing Wang¹, Chang Jiang¹, Shuo Wang¹, Lingyan Feng¹, Yu Zhang¹, Yuanyuan Guo¹, Gongshu Liu¹, Xi Li^{2,3,4}, Guohong Zhang^{2,3}, Xiaowei Zhu², Fangyi Ren^{2,5}, Lingyao Guan^{2,5}, Jiayu Chen^{2,5}, Ya Gao^{2,3}, Mo Chen⁵, Noura Darwish⁷, Sara Colombo Mottaz⁷, Marie Noelle Horcajada⁶, Nicolas Bonnet⁶, Shaillay Kumar Dogra⁶, Dantong Wang^{6*}

¹Tianjin Women's and Children's Health Center, Tianjin, China; ²BGI-Shenzhen, Shenzhen, 518083, China; ³Shenzhen Engineering Laboratory for Birth Defects Screening, Shenzhen, 518083, China; ⁴BGI Research, Wuhan 430074, China; ⁵China National Gene Bank, BGI-Shenzhen, Shenzhen, 518120, China; ⁶Nestlé Institute of Health Sciences, Nestlé Research, Lausanne, Switzerland; ⁷Clinical Research Unit, Nestlé Research, Lausanne, Switzerland

*Corresponding author

Jing Wang: wangjing8162012@163.com

Chang Jiang: jackiechristina@163.com

Shuo Wang: wangshuotjfe@163.com

Lingyan Feng: haiyangfly_2000@163.com

Yu Zhang: zhangyuwork2@163.com

Yuanyuan Guo: zhangchen71@163.com

Gongshu Liu: liugongshu727@163.com

Xi Li: lixi1@genomics.cn

Guohong Zhang: zhangguohong@genomics.cn

Xiaowei Zhu: zhuxiaowei@genomics.cn

Fangyi Ren: renfangyi@genomics.cn

Lingyao Guan: guanlingyao@genomics.cn

Jiayu Chen: chenjiayu@genomics.cn

Ya Gao: gaoya@genomics.cn

Mo Chen: Mo.chen@rd.nestle.com

Noura Darwish: Noura.Darwish@rd.nestle.com

Sara Colombo Mottaz: sara.colombomottaz@rd.nestle.com

Marie Noelle Horcajada: MarieNoelle.Horcajada@rdls.nestle.com

Nicolas Bonnet: Nicolas.Bonnet@rd.nestle.com

Shaillay Kumar Dogra: ShaillayKuma.Dogra@rd.nestle.com

Dantong Wang: Dantong.Wang@rdls.nestle.com

WORD COUNT: 3880

ABSTRACT

Purpose: The Bone And MicroBiOme Onset (BAMBOO) study is an ongoing prospective observational cohort study conducted in Tianjin, China, aiming to determine age-appropriate trajectories for microbiome maturation and bone development, and to identify the influence of dietary factors in the process.

Participants: The recruitment started in September 2021 and was completed in February 2023. In total 1380 subjects were recruited, 690 at birth (group 1) and 690 at 6 months of age (group 2). Group 1 and 2 will be followed up to 12 months and 36 months, respectively.

Findings to date: The age of the mothers was 31.1 ± 3.7 (mean \pm SD), and the birth weight of infants was 3.3 ± 0.5 kg with an incidence of Caesarean section 50.4%. Food diary information of the first 100 subjects showed that 64 food items were introduced by 6 months. A pilot microbiome analysis revealed that at the species level, bacterial communities were composed of mostly *Bacteroides dorei*, *Bacteroides vulgatus* and *Escherichia coli*, that were consistent with previous reports. Feasibility assessments of breast milk vitamin D and HMOs were validated through certified reference measurements. The early data assessment showed a high reliability of the data generated from this study.

Future plans: The data collection will be completed in August 2025. Four stage-statistical analyses will be performed as the cohort reaches certain age thresholds before the final report. Analysis of BAMBOO data will be used to develop age-appropriate trajectories for microbiome maturation and bone development for children 0-3 years and investigate the contribution of dietary factors in the process.

Registration: Trial registration No.: ChiCTR2100049972 (August 16th, 2021)

Strengths and limitations

- This is a large prospective longitudinal cohort study covering the first 3 years of life, measuring multiple aspects of health at multiple timepoints.
- The study investigates the interplay of dietary factors, microbiome maturation and bone development, as well as growth and health status.
- As an observational study, a limitation is that only associations between early life nutrition, microbiome maturation and bone development can be investigated, the causal relationships could not be determined.
- The study is being conducted in a major metropolitan area of northern China, the infant feeding and dietary habit could differ in other regions across China.

Keywords: Microbiome; bone health; early life nutrition; early life growth

INTRODUCTION

Early childhood is a critical period for growth and development benefitting long term health. The role of the gut microbiome in health has been investigated in many studies. Differences in gut microbiome composition and function have been associated with various chronic diseases such as metabolic conditions, neurological disorders, and cardiovascular illnesses [1-2]. Microbiome maturation in infants parallels human neurodevelopmental processes and growth. It plays a role in gut-brain signaling [3], and is associated with cognitive development [4]. An essential

1
2
3 aspect of microbiome function is its impact on immune system. The establishment of host-microbial symbiosis relies
4 on the mutualistic co-development of the host microbiota and immune system [5]. Microbiome diversity has been
5 associated with respiratory tract infections [6] and its composition may influence mucosal IgA response to vaccine
6 in infants [7].
7

8 A critical window for microbiome maturation is from birth to 4 years of age[5]. Stewart and colleagues reported
9 three distinct phases of microbiome composition evolution in early life: a developmental phase (months 3-14), a
10 transitional phase (months 15-30), and a stable phase (months 31-46) [8]. Considering that after these phases the
11 microbiome composition remains fairly stable across life stages, it is important to define the optimal age-appropriate
12 microbiome acquisition, selection, and maturation from birth up to toddlerhood in relation to health benefits.
13 Moreover, further understanding on the influence of weaning and nutrition during early childhood on an
14 ageappropriate microbiome maturation is needed. While multiple factors influence the establishment and
15 development of infant gut microbiome, such as delivery mode and day care attendance[9-10], there is also growing
16 evidence of the influence of dietary factors on microbiome maturation, both breastfeeding and complementary
17 foods. Breastfeeding has a critical role, especially during the first 6 months of age [8-9, 11]. After 6 months of age
18 when weaning foods are gradually being introduced to the diet, microbiota composition and environment changed
19 rapidly[12]. An intervention with microbiota-directed complementary foods (i.e. the combination of chickpea flour,
20 peanut flour, soy flour, and raw banana) modified the microbiota and increased plasma biomarkers and mediators of
21 growth, bone formation, neurodevelopment, and immune function in a randomized, double-blind controlled feeding
22 study in malnourished infants in Bangladesh [13]. Understanding the impact and mechanisms underlying these
23 maturation changes requires information on two aspects: establishment of normal age-appropriate microbiome
24 maturation trajectory and investigation of the relationship between specific dietary factors and microbiome[5].
25 Although some studies have been carried out in Bangladesh[14], US and Europe[8, 12, 15], no longitudinal data
26 have been reported for Chinese infants.
27
28

29 Healthy skeleton development is an important aspect of child growth, particularly around 2-4years where fast growth
30 occurs with some consequence on fracture risk elevated in child with high height at age 3 years [16]. Bone is a
31 dynamic and highly specialized connective tissue. Major functions of bone include provision of a mechanical
32 support for muscular activity, physical protection to the tissues and internal organs, and to act as a repository for
33 systemic mineral homeostasis [17]. Bone mass is related to fracture risk. Studies have shown that, for every standard
34 deviation decrease in size-adjusted bone mass, there is an 89% increase in fracture risk in childhood [18]. By the
35 early 20s, a 10–15% higher peak bone mass has been estimated to decrease the risk of fracture by 25–50% later in
36 life [19]. Therefore, one strategy to reduce later life fracture risk is to build peak bone mass. There are two windows
37 of opportunity to maximize peak bone mass, infancy and adolescence[20]. Bone mass is assessed by both geometry
38 measures (length and width, which are relatively easier to measure in clinical studies) and mineral deposition. Bone
39 mineralization provides rigidity and resistance to the skeleton while maintaining a certain degree of elasticity[17].
40 Chronological bone physiology highlights the importance of bone mineralization in infants and toddlers to achieve
41 developmental milestones, such as climbing, walking, and running. However, the knowledge on infant bone mineral
42 deposition is limited due to the difficulties in measurement. Moreover, in this rapid growing phase, the balance
43 between rigidity and elasticity at different ages also requires further investigation[21]. Breastmilk was found to have
44 a positive influence on peak bone microarchitecture[22], and might be protective for long term bone health[23].
45 Non-digestible human milk oligosaccharides (HMOs), which have a significant influence on infant microbiome[24],
46 were found to improve bone mass and structure in different mouse models suggesting a potential HMO-microbiome-
47 bone interaction [25-27]. A prospective randomized clinical trial showed that calcium supplementation increased the
48 acquisition of bone mass in children, adolescence, and early adulthood[28]. Notwithstanding such studies,
49 ageappropriate bone development, and the influence of feeding types and complementary food introduction on bone
50 development, in infants and toddlers is still less investigated.
51
52
53

54 To fill the knowledge gaps on both optimal age-appropriate microbiome maturation and bone development, the
55 interaction of these two and influence of dietary factors, the Tianjin Women and Children's Health Center
56 (TJWCH), Beijing Genomic Institute (BGI) and Nestlé Research (NR) jointly designed and initiated the BAMBOO
57
58
59

1
2
3 study. The results of this unique Chinese project will help researchers to establish age-appropriate trajectories for
4 microbiome maturation and bone development from birth to pre-school age toddlers. The insights gathered may
5 support local nutrition - healthcare programs for infants and toddlers to benefit long term health status. The focus of
6 this article is to report the study protocol used in this longitudinal cohort study.
7

9 **COHORT DESCRIPTION - Aim of BAMBOO**

11 BAMBOO is a prospective cohort observational study aiming to characterize normal age-appropriate
12 microbiome maturation and bone development, and to assess how early life nutrition influences these
13 development processes.
14

15 - **Setting of BAMBOO**

16
17 The BAMBOO study is conducted in Tianjin, the fourth largest municipality in China, covering an area of
18 12,000 square kilometers with a resident population of 13.8 million. It is comprised of 16 districts, including 6
19 central urban districts, 4 surrounding urban districts and 6 suburb districts. All 0–6-year-old children in Tianjin
20 are registered in the Maternal and Child Healthcare system administered by TJWCH. Routine health care
21 records from the pregnancy until children's 6 years are kept in the system. Participants in this cohort are mainly
22 from the 6 central urban districts (Hedong district, Nankai district, Hebei district, Hexi district and Hongqiao
23 district) and 4 surrounding urban districts (Beichen, Jinnan, Xiqing and Dongli district).
24

25 The cohort is composed of children between 0 to 3 years of age. In an accelerated cohort design, two parallel
26 groups covering different age ranges are recruited with an overlap age from 6 to 12 months. Group 1 subjects
27 will be followed from birth to 12 months of age with infant/toddler development information and samples
28 collected at birth and then 1, 3, 4, 6, 9, 12 months of age. Group 2 subjects will be followed from 6 to 36
29 months of age with infant/toddler development information and samples collected at 6, 9, 12, 18, 24, 30 and 36
30 months of age. Detailed visit timepoints and sample collection schedule are shown in figure 1.
31

32 This international scientific collaboration was approved by China Human Genetic Resource Admission
33 Committee (HGRAC) in August 2021. The study is conducted according to the principles of the Declaration of
34 Helsinki and in accordance with the guidelines and regulations of HGRAC. Ethics approval of research was
35 obtained from Tianjin Women's and Children's Health Center Medical Ethical Committee and the Institutional
36 Review Board of BGI-Shenzhen (BGI-IRB 21056). For this study the informed consent will be obtained from
37 parents or legal guardians of the participants. Informed consent will also be obtained from all the mothers
38 involved in the study. The study is registered in the clinical trial registration and the trial number is
39 ChiCTR2100049972.
40

41 - **Recruitment**

42
43 Children who meet the following requirements are invited to participate in the study: 1) full-term gestational
44 birth (≥ 37 and ≤ 42 weeks); 2) singleton; and 3) signed informed consent by infant's parents (or his/her legally
45 accepted representative) and agree to fulfill the requirements of the study protocol.
46
47

48 The exclusion criteria are: 1) birth after a complicated pregnancy, such as pre-eclampsia, gestational diabetes, or
49 bowel disease, determined by medical interview/ medical record; 2) infant's parents/LAR not willing and/or not
50 able to comply with scheduled visits and the requirements of the study protocol; and 3) currently participating
51 or having participated in another clinical trial within 4 weeks prior to the start of this cohort.
52

53 For group 1, infants begin their journey in the study within 10 days after birth. Participant recruitment is
54 ongoing at 7 local delivery hospitals. Investigators introduce the study to the parents and ask their consent, with
55 screening based on the inclusion/exclusion criteria at 3-5 days after giving birth. For group 2, infants join the
56
57
58
59

cohort at 6 months of age. Investigators introduce the study to the parents based on the inclusion/exclusion criteria and ask for their consent when the child is around 5.5 months.

Sample size was calculated based on bone development outcomes. For the bone trajectory analysis, Limanovitz et al found that a sample size of 60 infants per group would be required to demonstrate a significant difference of 70m/s in SoS between breastfed and formula-fed infants with a standard deviation of 133 m/s, assuming an overall type I error of 5% and 80% statistical power[29]. Based on a previous study in Tianjin, 10% of women performed exclusively formula feeding, 45% performed exclusively breastfeeding and 45% performed mixed feeding. Therefore, given that at least 60 infants are required in each feeding group, a total of 600 completed infants are needed. Considering a drop-out rate of 13%, the total number of infants to be enrolled is 690 per group. For the microbiome trajectory development, based on literature in which a small number of subjects with frequent time points of fecal sampling were used to derive a trajectory[14], we believe we will have sufficient reference subset of infants selected based on our criteria, starting from 690 subjects per group. A total of 1380 healthy normal developing Chinese infants and toddlers are planned to be recruited in this study. Recruitment of participants started in September 2021 and completed in February 2023.

- **Data collection**

Data collection includes questionnaires, medical history (MH), concomitant medication (CM), adverse events/serious adverse events (AE/SAE) record, food diary, biological samples collection and anthropometric measurements (length/height, weight and bone measurements).

- Mother and infant questionnaires. A mother's questionnaire is filled at enrolment (group 1 at around birth; group 2 when infants are 6 months old), including basic information (mother's height, weight, education level and history of gestation), tobacco and alcohol use, work and physical activities after 28 weeks of gestation, vitamin, mineral and probiotic supplements, health and medication during pregnancy, and the last B-ultrasound examination result during pregnancy (only for Group 1).

The infant's questionnaires are gathered at each visit from main caregivers in a face-to-face interview or phonecall interview if the person cannot attend the visit. The information on baby feeding practice, sleep, vitamin, mineral and probiotic supplements, health status, growth and development and antibiotic use is collected from the infant's questionnaires. Infant Gastrointestinal Symptom Questionnaire (IGSQ) (up to 12 months of age) and Toddler Gut Comfort Questionnaire (TGCQ)(18m to 3 years of age) are also administered [30]. MH, AEs/SAEs as well as concomitant medications and non-pharmacological treatments are recorded from the beginning till the final visit.

- Definition of feeding types. In this study, exclusive breastfeeding (BF) is defined as a subject reported as exclusively breast fed at 1 and 3 months. For exclusive infant formula feeding (IF), a subject is either exclusively infant formula fed at 1 and 3 months or with a mixed feeding and an IF ratio higher than 80% at 1 and 3 months. Feeding practice is classified as mixed feeding if the subject has an estimated IF ratio lower than 80% of total intake at 1 and 3 months. We applied the principle of flexible feeding type description [29], IF ratio = total daily infant formula intake in ml/780ml.

- Food diary. Starting from 4 months of age, a food diary is filled by the main caregiver at home, assisted by photos and tools, for 3 consecutive days, starting 4 days prior to each visit. The content is reviewed during the visit by a dietitian. A nutrition evaluation report is shared with the parents by the dietitian at 6 months and 1 year of age. Each food diary is checked by the principal investigator to ensure quality. Nutrient intakes are calculated using Chinese Food Composition database [31], complemented by other sources such as pack labels and literature review in case of lack of information in the current database.

- 1
2
3 - Biological samples collection. All biological samples (child fecal and urine samples, and human milk samples)
4 are temporarily stored at -80° freezer at TJWCH, and regularly shipped on dry ice to different labs for analysis.
5 The samples flow chart is shown in Figure 2. Detailed methods for sample handling and analysis are described
6 in Supplemental material.
7
8
9 - Height, Weight measurement. Children's length/height and weight are measured by trained investigators using
10 calibrated electronic scale and measuring bed (Suhong RCS-20), with the nearest accuracy of 0.1cm and 5g,
11 respectively.
12
13 - Bone length and mass index measurement. Bone mass index measurement is conducted using a non-invasive
14 and radiation-free ultra-sound sonometer (Sunlight® Omnisense Mini) measuring bone transmission time and
15 speed of sound at the tibia site, together with measurements of both tibia and radius length. In the study, two
16 ultrasound devices are used to measure the bone transmission time and speed of sound at the radius and tibia
17 site. To calibrate the two devices, a phantom is scanned to detect deviation of the ultrasound source daily. In
18 order to maximize the reproducibility, the research staff who had past experience with similar devices were
19 trained and tested in children with the reproducibility of the two devices CV<6%.
20

21
22 - **Data management and Statistical analysis**

23 Data are entered from the source document into an electronic Case Report Form (eCRF - web database named
24 ClinFlash) within 15 days after the subject's visit. Data quality review meetings are held quarterly, and the
25 statistical results on sample enrolment and descriptive statistics on key measurements are included into a data
26 quality report and submitted to the research committee for review. Protocol deviations are predefined and
27 divided into major and minor categories, which trigger corresponding corrective action and preventive action in
28 time. All protocol deviations in listing format will be reported to the TJWCH Ethics Committee.
29
30

31 Data analyses are planned to be conducted in R (R Core Team, 2014) and figures will be produced using the
32 package ggplot2 (Wickham, 2009). Microbiome data will be further explored using Microbiome-toolbox
33 (version 1.0) [32]. Data will be initially checked for normality of distribution (using qq-plot and residuals vs.
34 fitted values plot). Feeding groups, complementary food and nutrient intakes will be summarized. Dietary
35 patterns will be identified using principal component analysis (PCA) and/or cluster analysis. Microbiome
36 trajectories were derived using a machine learning model to approximate Microbiome Maturation Index (MMI).
37 Microbiota-for-age z-scores will be calculated and associations between normal/abnormal microbiota-for-age
38 zscores and various health and dietary factors will be investigated using chi-square test of independence. Bone
39 (tibia and radius length and SoS) trajectories over time will be summarized. Associations between different
40 measures will also be investigated using appropriate method, such as linear mixed-effect model adjusting for
41 confounding factors. The model will include the computed propensity scores as weights.
42
43

44 - **Patient and public involvement**

45 Patients and/or the public were not involved in the development of study design and dissemination of this
46 research.
47
48
49

50 **FINDINGS TO DATE**

51
52 The Bamboo study was started in September 2021 and recruitment was completed by the end of February 2023. A
53 total of 1380 mother-child pairs were recruited in this study (690 in group 1 and 690 in group 2). Table 1 shows the
54 basic characteristics of the participants, including mother's age, delivery weeks, birth mode, infants' gender, nation,
55 birth weight or birth length. In total, 239 subjects dropped out from the study. Figure 3 shows the reasons for drop
56
57
58
59

out, where the top 3 reasons are “inconvenient to follow up”, “not interested in the study” and “leaving Tianjin”. We compared the basic characteristics between participants still in the group and those who dropped out, and found no difference except that the dropped-out mothers were more likely to be younger than the mothers remaining in the study (Table 1). The population characteristics of this study are comparable to those in another large cohort study, the Tianjin Birth Cohort (TJBC), conducted in the same city with a large number of mother and infant dyads [33]. In both studies, the average mothers’ age was around 30-31 years, children’s average body length and weight at birth were around 50cm and 3.3kg, respectively. We observed a slightly lower proportion of girls enrolled in this study (45%) as compared to TJBC (48%) but the difference is not statistically significant (detailed data of TJBC have not yet been published).

Table 1. Characteristics of enrolled participants (February 2023)

Characteristics	Group 1	Group 2	Total	Active	Drop out	Statistics	P-value	
Number of subjects	690	690	1380	1141	239			
Mother’s age	30.83±3.80	31.44±3.59	31.14±3.71	31.28±3.73	30.44±3.54	t=3.205	0.001	
Delivery weeks	39.05±0.937	39.19±1.072	39.12±1.01	39.14±1.01	39.03±1.02	t=1.605	0.109	
Birth mode	Natural childbirth	314 (45.5%)	381 (55.2%)	695 (49.6%)	586 (51.4%)	109 (45.6%)	$\chi^2=2.615$	0.106
	Caesarean section	376 (54.5%)	309 (44.8%)	685 (50.4%)	555 (48.6%)	130 (54.4%)		
Infant’s gender	Male	396 (57.4%)	369 (53.5%)	765 (55.4%)	631 (55.3%)	134 (56.1%)	$\chi^2=0.047$	0.829
	Female	294 (42.6%)	321 (46.5%)	615 (44.6%)	510 (44.7%)	105 (43.9%)		
Nation	Han	619 (89.7%)	639 (92.6%)	1258 (91.2%)	1041 (91.2%)	217 (90.8%)	$\chi^2=0.048$	0.827
	Other	71 (10.3%)	51 (7.4%)	122 (8.8%)	100 (8.8%)	22 (9.2%)		
Birth weight (kg)	3.33±0.372	3.33±0.389	3.33±0.380	3.34±0.375	3.29±0.401	t=1.733	0.083	
Birth length (cm)	49.63±1.36	49.97±1.29	49.80±1.33	49.84±1.32	49.65±1.39	t=1.925	0.054	

A review of dietary intake data was conducted using food diary information from the first 100 subjects at 6 months of age in group 2 since most children have been exposed to the complementary foods at this age. In total 64 food items were reported (Table 2). Nutrient intake estimation is ongoing based on the food intake using Chinese Food Composition database [31], complemented by labels on the pack.

Table 2 Foods recorded in the food diary*

Food name	Count	Percent, %	Food name	Count	Percent, %
Breastmilk	1552	54.0	Orange	8	0.3
Formula	510	17.8	Tangerine	6	0.2
Ground rice	179	6.2	Avocado	1	0.1
Ground rice with vegetable puree	1	0.0	Grape	2	0.1
Ground rice with zucchini puree	2	0.1	Pomegranate	1	0.0
Ground rice with potato puree	1	0.0	Red date	2	0.1

Formula with ground rice	1	0.0	Fruit puree	6	0.2
Rice cracker	8	0.3	Banana	7	0.2
Rice water	2	0.1	Yolk	14	0.5
Rice porridge	1	0.0	Liver	3	0.1
Noodle	5	0.2	Shrimp	3	0.1
Steamed buns	3	0.1	Dried meat	1	0.0
Millet puree	1	0.0	Cookie	1	0.1
Millet porridge	3	0.1	Puff	1	0.0
Millet sweet potato porridge	1	0.0	Mousse	1	0.0
Mixed porridge	1	0.0	Oil	23	0.8
Purple rice porridge	2	0.1	Linseed oil	2	0.1
Sweet potato	4	0.1	Sesame butter	3	0.1
Potato	3	0.1	Soy sauce	3	0.1
Purple sweet potato	1	0.0	Water	34	1.2
Yum	7	0.2	Vitamin AD	188	6.5
Spanish	4	0.1	Vitamin D3	61	2.1
Bok choy	1	0.0	Vitamin supplement	1	0.0
Broccoli	5	0.2	Mineral supplement	7	0.2
Carrot	14	0.5	Calcium	47	1.6
Cucumber	5	0.2	Zinc	2	0.1
Pumpkin/Squash	21	0.7	Probiotics	37	1.3
Apple	29	1.0	Fish oil	3	0.1
Pitaya	1	0.0	DHA	25	0.9
Blueberry	2	0.1	B12	2	0.1
Pear, blueberry	1	0.0	Albumen powder	2	0.1
Pear	2	0.1	Iron protein succinylate oral liquid	2	0.1

*Data was based on the first 100 subjects at 6 months in group 2.

A data assessment was conducted using early available samples, which is a small proportion of the cohort data. The data that support the findings of this study have been deposited into CNGB Sequence Archive (CNSA)[34-35], with accession number CNP0003576. A total of 20 stool samples were sequenced and subjected to subsequent metagenomic analysis. All DNA concentrations of the 20 samples are greater than 3 ng/ μ L, which is the minimum amount required for library construction. Adaptor contaminated, low-quality and host reads were removed from the raw sequencing read sets. An average of 33.6 GB (9.52 to 48.36 GB) data of high-quality clean reads per sample was generated, equivalent to 96.1% of raw reads on average. Comparison of the compositional features of the gut microbiota alongside the age spectrum revealed several characteristic patterns. At the species level, bacterial communities were composed of mostly *Bacteroides dorei*, *Bacteroides vulgatus* and *Escherichia coli* (Supplemental Figure S1). Microbial abundances were showed in the Supplemental materials. Progressive changes in microbial diversity and abundance of the infant gut microbiome are likely to re-shape the metabolic functions of the hosts over time. The results of the pilot assessment were consistent with the discoveries reported previous [8, 36]. The urine creatine assay has been tested and validated previously in infants and toddlers with similar age to the BAMBOO cohort, with results in the correct range [37-38]. A cross validation of breast milk vitamin D and HMOs measurement has been conducted with internal data previously published [39-40] and also validated through certified reference values (Supplemental Tables S1, S2 and S3). In summary the early data assessment shows that high reliability of the data generated from this study.

1
2
3 The next step will focus on completing recruitment and minimizing the drop out, especially in group 1. Additionally,
4 an interim statistical analysis will be performed to provide an initial overview of different aspects of the study,
5 including microbiome maturation, bone development and dietary intake of children at different ages. Finally, 4stage-
6 statistical analyses before final data analysis will be performed as the complete cohort reaches certain age
7 thresholds.
8

9 **FURHER DETAILS Strengths and limitations**

10
11 There are several limitations in this study. First, as an observational study, only associations between early life
12 nutrition, microbiome maturation and bone development can be investigated, but their causal relationships could not
13 be determined. However, these findings will provide insights that may help to develop scientific hypotheses and
14 identify potentially relevant timing and interventions to support optimal microbiome or bone development and
15 contribute new evidence to inform nutrition policy setting. Second, as the study is conducted in a major metropolitan
16 area of northern China, we could anticipate differences in feeding and dietary habits from other regions across
17 China. Future studies could focus on such regional differences.
18

19 The strength of the study is its large sample size with longitudinal follow up, measuring multiple aspects of health
20 consistently at multiple timepoints. To our knowledge, this is the first study with such comprehensive coverage in
21 infant and toddlers in China, making it possible to investigate the interplay of dietary factors, microbiome
22 maturation and bone development, as well as growth and health status.
23

24 **Collaboration and Data availability statement**

25
26 Microbiota data that support the findings of this study have been deposited into CNGB Sequence Archive (CNSA)
27 and made publicly available upon publication of the results in scientific articles. Ethical considerations related to
28 personal data, informed consent and human research act restrict human data sharing in public repositories. Fully
29 anonymized data may be shared upon request to the corresponding author accompanied by a proposed research plan
30 for evaluation and approval by the Bamboo scientific council, followed by relevant ethics committee approval.
31
32
33

34 **ETHICS STATEMENTS Ethics approval**

35 This international scientific collaboration was approved by China Human Genetic Resource Admission Committee
36 (HGRAC) in August 2021. The study is conducted according to the principles of the Declaration of Helsinki and in
37 accordance with the guidelines and regulations of HGRAC. Ethics approval of research was obtained from Tianjin
38 Women's and Children's Health Center Medical Ethical Committee and the Institutional Review Board of
39 BGIShenzhen (BGI-IRB 21056). The study is registered in the clinical trial registration and the trial number is
40 ChiCTR2100049972.
41
42

43 **Consent to participate**

44 For this study the informed consent was obtained from parents or legal guardians of the infants. Informed consents
45 were also obtained from all the mothers involved in the study.

46 **Patient consent for publication** Not

47 applicable.
48
49

50 **Funding**

51 The study is funded by Société des Produits Nestlé SA and BGI-Research.
52
53

54 **Competing interests**

55 Xi Li, Guohong Zhang, Xiaowei Zhu, Fangyi Ren, Lingyao Guan, Jiayu Chen, and Ya Gao are employed by BGI-
56
57
58
59
60

1
2
3 Research. Mo Chen, Noura Darwish, Sara Colombo Mottaz, Marie Noelle Horcajada, Nicolas Bonnet, Shaillay
4 Kumar Dogra, and Dantong Wang are employed by Nestle Research. The authors declared that they have no
5 competing interests.
6

7 **Authors' contributions**

8 All authors contributed to the study design, interpretation of the data and writing the manuscript. Data and sample
9 collection were performance by Jing Wang, Chang Jiang, Shuo Wang, Lingyan Feng, Yu Zhang, Yuanyuan Guo,
10 and Gongshu Liu. Sample measurement and analyses were performed by Xi Li, Guohong Zhang, Xiaowei Zhu,
11 Fangyi Ren, Lingyao Guan, Jiayu Chen, and Ya Gao. Data management and statistical protocol were set up by Mo
12 Chen, Noura Darwish, Marie Noelle Horcajada, Nicolas Bonnet, Shaillay Kumar Dogra, and Dantong Wang. The
13 first draft of the manuscript was written by Jing Wang, Sara Colombo Mottaz, Marie Noelle Horcajada, Nicolas
14 Bonnet, Shaillay Kumar Dogra and Dantong Wang. All authors read and approved the manuscript.
15
16

17 **Acknowledgement**

18 We thank Dr. Norbert Sprenger for his contribution in questionnaire design and guidance in microbiome data
19 analysis, Dr. Giles Major for his critical review of this manuscript. We thank Qiaoji Li, Dr. Alice Pannérec, Dr.
20 Marie Boutant Lys and Dr. Marie Bachelet for setting-up the collaboration and their professional management in
21 planning and executing the project. We thank all the investigators from Tianjin Women and Children's Health
22 Center for conducting the field investigation and sample collection, research staff from BGI-Shenzhen for preparing
23 sample collecting kits and performing laboratory testing. This work was supported by China National GeneBank
24 (CNGB). We thank all participants in this study.
25
26
27
28

29 **Figure caption**

30 Figure 1. Data and sample collection timepoints Figure
31
32 2. Biological sample collection and storage Figure3
33
34 Drop-out reasons breakdown.
35
36
37
38
39

40 **REFERENCES**

- 41
42 1. Vijay A, Valdes AM. Role of the gut microbiome in chronic diseases: a narrative review. *Eur J Clin Nutr*
43 2022;76(4):489-501. doi: 10.1038/s41430-021-00991-6 [published Online First: 2021/09/30]
44 2. Parekh PJ, Balart LA, Johnson DA. The Influence of the Gut Microbiome on Obesity, Metabolic Syndrome and
45 Gastrointestinal Disease. *Clin Transl Gastroenterol* 2015;6:e91. doi: 10.1038/ctg.2015.16 [published Online
46 First: 2015/06/19]
47 3. Cong X, Xu W, Romisher R, et al. Gut Microbiome and Infant Health: Brain-Gut-Microbiota Axis and Host
48 Genetic Factors. *Yale J Biol Med* 2016;89(3):299-308. [published Online First: 2016/10/05]
49 4. Carlson AL, Xia K, Azcarate-Peril MA, et al. Infant Gut Microbiome Associated With Cognitive Development.
50 *Biol Psychiatry* 2018;83(2):148-59. doi: 10.1016/j.biopsych.2017.06.021 [published Online First: 2017/08/11]
51 5. Dogra SK, Kwong Chung C, Wang D, et al. Nurturing the Early Life Gut Microbiome and Immune Maturation
52 for Long Term Health. *Microorganisms* 2021;9(10) doi: 10.3390/microorganisms9102110 [published Online
53 First: 2021/10/24]
54
55
56
57
58
59

6. Woodall CA, McGeoch LJ, Hay AD, et al. Respiratory tract infections and gut microbiome modifications: A systematic review. *PLoS One* 2022;17(1):e0262057. doi: 10.1371/journal.pone.0262057 [published Online First: 2022/01/14]
7. Zhao T, Li J, Fu Y, et al. Influence of gut microbiota on mucosal IgA antibody response to the polio vaccine. *NPJ Vaccines* 2020;5(1):47. doi: 10.1038/s41541-020-0194-5 [published Online First: 2020/06/23]
8. Stewart CJ, Ajami NJ, O'Brien JL, et al. Temporal development of the gut microbiome in early childhood from the TEDDY study. *Nature* 2018;562(7728):583-88. doi: 10.1038/s41586-018-0617-x
9. Ho NT, Li F, Lee-Sarwar KA, et al. Meta-analysis of effects of exclusive breastfeeding on infant gut microbiota across populations. *Nat Commun* 2018;9(1):4169. doi: 10.1038/s41467-018-06473-x
10. Amir A, Erez-Granat O, Braun T, et al. Gut microbiome development in early childhood is affected by day care attendance. *NPJ Biofilms Microbiomes* 2022;8(1):2. doi: 10.1038/s41522-021-00265-w [published Online First: 2022/01/13]
11. Baumann-Dudenhoefter AM, D'Souza AW, Tarr PI, et al. Infant diet and maternal gestational weight gain predict early metabolic maturation of gut microbiomes. *Nat Med* 2018;24(12):1822-29. doi: 10.1038/s41591-018-0216-2
12. Backhed F, Roswall J, Peng Y, et al. Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. *Cell Host Microbe* 2015;17(5):690-703. doi: 10.1016/j.chom.2015.04.004 [published Online First: 2015/05/15]
13. Gehrig JL, Venkatesh S, Chang HW, et al. Effects of microbiota-directed foods in gnotobiotic animals and undernourished children. *Science* 2019;365(6449) doi: 10.1126/science.aau4732 [published Online First: 2019/07/13]
14. Subramanian S, Huq S, Yatsunenko T, et al. Persistent gut microbiota immaturity in malnourished Bangladeshi children. *Nature* 2014;510(7505):417-21. doi: 10.1038/nature13421 [published Online First: 2014/06/05]
15. Depner M, Taft DH, Kirjavainen PV, et al. Maturation of the gut microbiome during the first year of life contributes to the protective farm effect on childhood asthma. *Nat Med* 2020;26(11):1766-75. doi: 10.1038/s41591-020-1095-x [published Online First: 2020/11/04]
16. Jones IE, Williams SM, Goulding A. Associations of birth weight and length, childhood size, and smoking with bone fractures during growth: evidence from a birth cohort study. *Am J Epidemiol* 2004;159(4):343-50. doi: 10.1093/aje/kwh052 [published Online First: 2004/02/11]
17. Ma NS, Gordon CM. Pediatric osteoporosis: where are we now? *J Pediatr* 2012;161(6):983-90. doi: 10.1016/j.jpeds.2012.07.057 [published Online First: 2012/09/15]
18. Clark EM, Ness AR, Bishop NJ, et al. Association between bone mass and fractures in children: a prospective cohort study. *J Bone Miner Res* 2006;21(9):1489-95. doi: 10.1359/jbmr.060601 [published Online First: 2006/08/31]
19. Weaver CM, Gordon CM, Janz KF, et al. The National Osteoporosis Foundation's position statement on peak bone mass development and lifestyle factors: a systematic review and implementation recommendations. *Osteoporos Int* 2016;27(4):1281-386. doi: 10.1007/s00198-015-3440-3 [published Online First: 2016/02/10]
20. Lu J, Shin Y, Yen MS, et al. Peak Bone Mass and Patterns of Change in Total Bone Mineral Density and Bone Mineral Contents From Childhood Into Young Adulthood. *J Clin Densitom* 2016;19(2):180-91. doi: 10.1016/j.jocd.2014.08.001 [published Online First: 2014/12/03]
21. Ambrose CG, Soto Martinez M, Bi X, et al. Mechanical properties of infant bone. *Bone* 2018;113:151-60. doi: 10.1016/j.bone.2018.05.015 [published Online First: 2018/05/26]
22. Yang Y, Wu F, Dwyer T, et al. Associations of Breastfeeding, Maternal Smoking, and Birth Weight With Bone Density and Microarchitecture in Young Adulthood: a 25-Year Birth-Cohort Study. *J Bone Miner Res* 2020;35(9):1652-59. doi: 10.1002/jbmr.4044 [published Online First: 2020/07/09]
23. Carter SA, Parsons CM, Robinson SM, et al. Infant milk feeding and bone health in later life: findings from the Hertfordshire cohort study. *Osteoporos Int* 2020;31(4):709-14. doi: 10.1007/s00198-020-05296-1 [published Online First: 2020/02/18]

24. Berger B, Porta N, Foata F, et al. Linking Human Milk Oligosaccharides, Infant Fecal Community Types, and Later Risk To Require Antibiotics. *mBio* 2020;11(2) doi: 10.1128/mBio.03196-19 [published Online First: 2020/03/19]
25. Charbonneau MR, O'Donnell D, Blanton LV, et al. Sialylated Milk Oligosaccharides Promote Microbiota-Dependent Growth in Models of Infant Undernutrition. *Cell* 2016;164(5):859-71. doi: 10.1016/j.cell.2016.01.024 [published Online First: 2016/02/24]
26. Blanton LV, Barratt MJ, Charbonneau MR, et al. Childhood undernutrition, the gut microbiota, and microbiota-directed therapeutics. *Science* 2016;352(6293):1533. doi: 10.1126/science.aad9359 [published Online First: 2016/06/25]
27. Cowardin CA, Ahern PP, Kung VL, et al. Mechanisms by which sialylated milk oligosaccharides impact bone biology in a gnotobiotic mouse model of infant undernutrition. *Proc Natl Acad Sci U S A* 2019;116(24):11988-96. doi: 10.1073/pnas.1821770116 [published Online First: 2019/05/30]
28. Dibba B, Prentice A, Ceesay M, et al. Effect of calcium supplementation on bone mineral accretion in Gambian children accustomed to a low-calcium diet. *Am J Clin Nutr* 2000;71(2):544-9. doi: 10.1093/ajcn/71.2.544 [published Online First: 2000/01/29]
29. Litmanovitz I, Davidson K, Eliakim A, et al. High Beta-palmitate formula and bone strength in term infants: a randomized, double-blind, controlled trial. *Calcif Tissue Int* 2013;92(1):35-41. doi: 10.1007/s00223-0129664-8 [published Online First: 2012/11/28]
30. Riley AW, Trabulsi J, Yao M, et al. Validation of a Parent Report Questionnaire: The Infant Gastrointestinal Symptom Questionnaire. *Clin Pediatr (Phila)* 2015;54(12):1167-74. doi: 10.1177/0009922815574075 [published Online First: 2015/03/12]
31. China Nutrition Society. China Food Composition Table. Beijing: Peking University Medical Press 2019.
32. Banjac J, Sprenger N, Dogra SK. Microbiome Toolbox: Methodological approaches to derive and visualize microbiome trajectories. *bioRxiv* 2022:2022.02.14.479826. doi: 10.1101/2022.02.14.479826
33. Wang S, Zhang G, Wang J, et al. Study Design and Baseline Profiles of Participants in the Tianjin Birth Cohort (TJBC) in China. *J Epidemiol* 2022;32(1):44-52. doi: 10.2188/jea.JE20200238 [published Online First: 2020/10/06]
34. Guo X, Chen F, Gao F, et al. CNSA: a data repository for archiving omics data. *Database (Oxford)* 2020;2020 doi: 10.1093/database/baaa055 [published Online First: 2020/07/25]
35. Chen FZ, You LJ, Yang F, et al. CNGBdb: China National GeneBank DataBase. *Yi Chuan* 2020;42(8):799-809. doi: 10.16288/j.ycz.20-080 [published Online First: 2020/09/22]
36. Niu J, Xu L, Qian Y, et al. Evolution of the Gut Microbiome in Early Childhood: A Cross-Sectional Study of Chinese Children. *Front Microbiol* 2020;11:439. doi: 10.3389/fmicb.2020.00439
37. Wang W, Du C, Lin L, et al. Anthropometry-based 24-h urinary creatinine excretion reference for Chinese children. *PLoS One* 2018;13(5):e0197672. doi: 10.1371/journal.pone.0197672 [published Online First: 2018/05/24]
38. Kwak BO, Lee ST, Chung S, et al. Microalbuminuria in normal Korean children. *Yonsei Med J* 2011;52(3):476-81. doi: 10.3349/ymj.2011.52.3.476 [published Online First: 2011/04/14]
39. Oberson JM, Benet S, Redeuil K, et al. Quantitative analysis of vitamin D and its main metabolites in human milk by supercritical fluid chromatography coupled to tandem mass spectrometry. *Anal Bioanal Chem* 2020;412(2):365-75. doi: 10.1007/s00216-019-02248-5 [published Online First: 2019/12/14]
40. Austin S, De Castro CA, Benet T, et al. Temporal Change of the Content of 10 Oligosaccharides in the Milk of Chinese Urban Mothers. *Nutrients* 2016;8(6) doi: 10.3390/nu8060346 [published Online First: 2016/06/25]

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

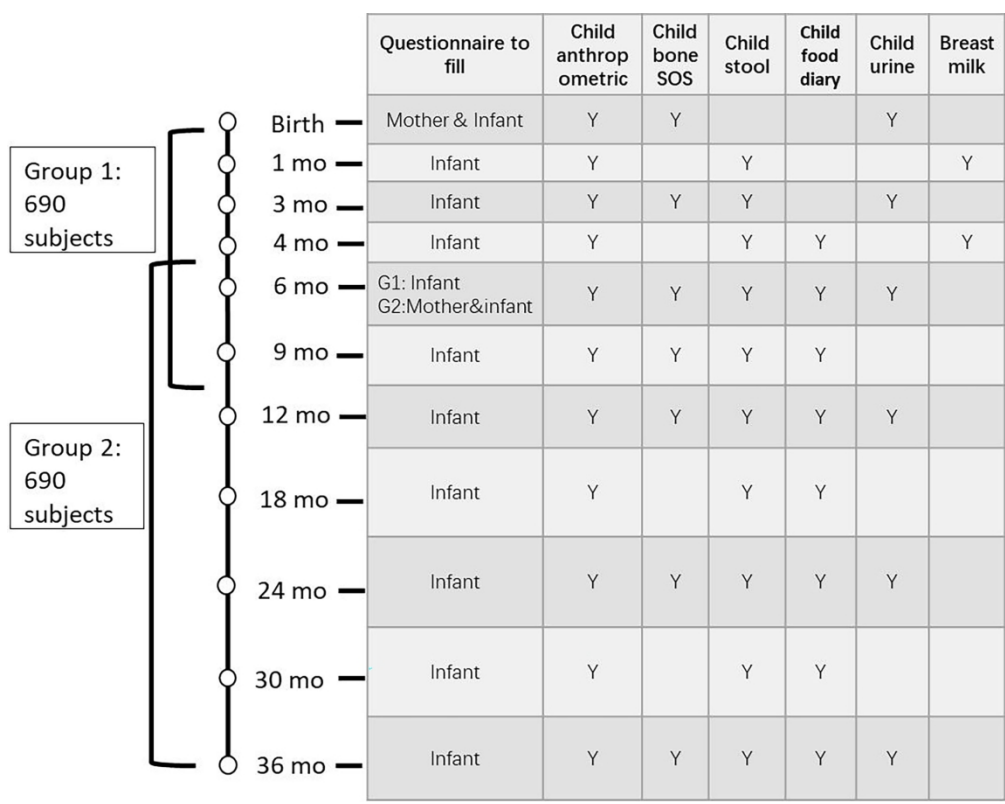


Figure 1. Data and sample collection timepoints

203x160mm (300 x 300 DPI)

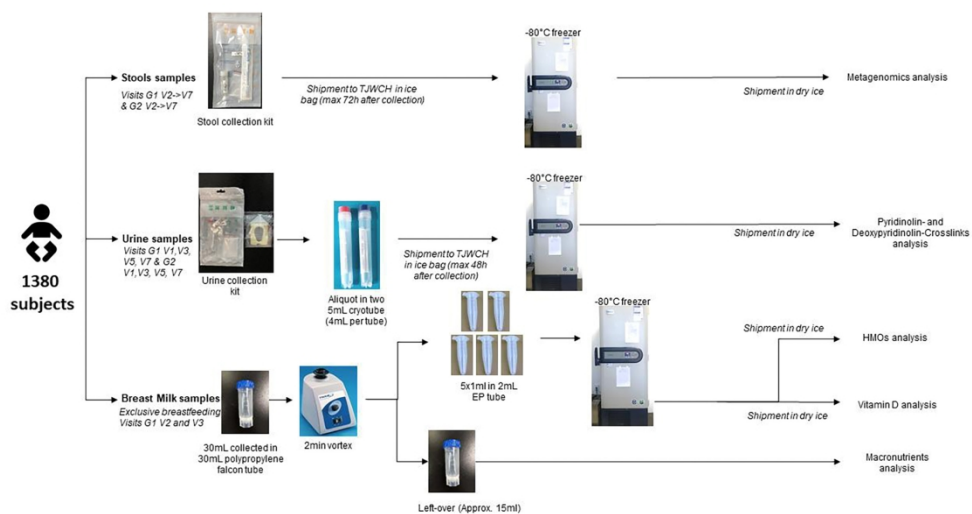


Figure 2. Biological sample collection and storage

203x114mm (300 x 300 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

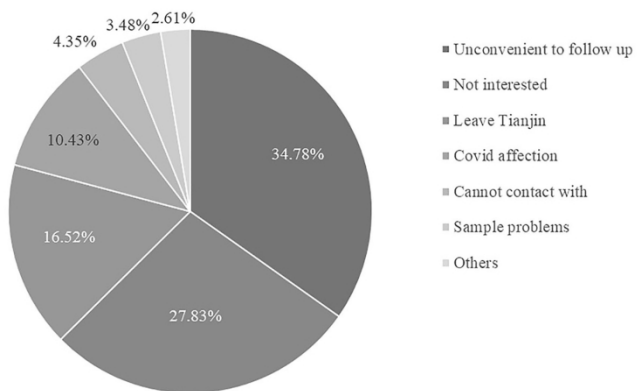


Figure3 Drop-out reasons breakdown
203x114mm (300 x 300 DPI)

Supplemental material: Biological samples collection and measurement

All biological samples are temporarily stored at -80° freezer at Tianjin Women and Children's Health Center (TJWCH), and regularly shipped on dry ice to different labs for analysis.

- Fecal samples:

- Fecal samples are collected a maximum of 72 hours before the planned visit, stored at 2-8°C and bring to the hospital using a cooling bag. For subjects aged 6 months and under ($\leq 6m$), 1 flat spoon of stool sample is collected. For subjects over 6 months of age ($> 6m$), 2 swabs of stool samples is collected. The collected samples are put in a pre-prepared tube and the stabilizer should merge the sample. A sampling guideline is included in the stool sample kits.
- The methods of library construction and sequencing of microbiota in fecal samples are adopted from the protocol described previously¹. Genomic DNA is firstly isolated from stool samples using Magpure Stool DNA KF Kit B (Magen, China) following the manufacturer's instruction. The isolated DNA is subjected to random fragmentation with Covaris E220 (Covaris, Brighton, UK). After ligation, a MGIEasy™ DNA Library Prep Kit (MGI, Shenzhen, China) is applied and the resulting ssDNA circles are used to generate DNA nanoballs (DNBs) by rolling circle amplification (RCA)^{2,3}. After RCA and the formation of DNBs, the final products are measured by Qubit using the ssDNA HS Assay kit (Invitrogen), and loaded on a DIPSEQ platform (MGI, Shenzhen, China) for sequencing using paired-end 100 bp mode following the manufacturer's instructions⁴. Metagenome analysis by DNA shotgun sequencing using DIPSEQ platforms has been previously established to study human gut microbiome^{5,6}.
- For bioinformatics analysis, the high-quality cleaned reads are used for the annotation and profile acquisition of taxons using MetaPhlan3 (version 3.0.14, code: `metaphlan.py input.fastq -input_type fastq -nproc 10 > profiled_metagenome.txt`)⁷. For functional abundance calculation, the HumanN3 pipeline (version 3.0.1, code: `humann --input input.fastq --threads 10 --output output`)⁷ is used to map the sequences against the UniRef90 database with default parameters to obtain functional profiles. Taxonomic profiles include the stratified relative abundances from phylum to species levels. The stratified relative abundances are extracted according to the taxonomic levels of interest. The Chao1 and Shannon indexes are calculated to estimate microbial alpha-diversity.

- Breast milk samples:

- Breast milk samples are collected in group 1 at 1-month and 4-month timepoints from mothers who provide exclusive breastfeeding to their infants. The volume of a breast milk sample is 30ml. A breast milk pump is available at the site and milk is collected in the morning from 9 to 11am. For mothers who cannot complete such collection during the visit, the study team will arrange another time (within 48h). A delay of additional 48h is accepted if the mother is sick.
- Macronutrient analyses, including protein, lactose, energy, fat, and calcium, are performed on HLIFE MR-1011 automatic breast milk analyzer in the lab in TJWCH.
- Quantitative analysis of Vitamin D in human milk. Vitamin D₃ and 25-OH D₃ are target analytes for quantitative analysis. Analysis is performed on Agilent 1290

Infinity liquid chromatography coupled to Agilent 6495 tandem mass spectrometry (Agilent). The analysis is conducted by SMQ Group Medical Laboratory (Shen Zhen, China). Phenomenex Kinetex PFP (2.1×150 mm, 1.7 μm) has been chosen as the analytical column. The separation gradient is shown in Supplementary Table S1. Mass spectrometric detection is carried out on Atmospheric Pressure Chemical Ionization operating in positive mode at unit resolution (APCI⁺). Compound parameters are shown in Supplementary Table S2. The detection limit and quantitation limit of Vitamin D₃ are 0.02 ng/mL and 0.07 ng/mL, respectively. For 25-OH D₃, the detection limit is 0.05 ng/ml and the quantitation limit is 0.16 ng/ml.

- Quantification of HMOs in human milk by UHPLC-FLD. The method has been developed for the quantification of the major human milk oligosaccharides (HMOs) in human milk using UHPLC with fluorometric detection (Thermo U3000). External standard used for the quantification of identified HMOs is listed in Supplemental Table S3.
- Child urine sample
 - Urine samples are collected using urine collection sterile bags and then transferred into the two 5ml cryotubes. The samples are collected a maximum of 48hr before the planned visit, stored at 2-8°C. If samples are not collected prior the visit, the study team will propose to collect the sample during the visit or to collect the samples at home in the following 48 hours, stored at 2-8°C.
 - Analysis for urine creatinine and pyridinoline (PYD) is performed using commercial ELISA kits (MicroVue Creatinine 8009 and MicroVue PYD 8010, respectively), according to the manufacturer's instructions. The analysis is conducted by SMQ Group Medical Laboratory (Shen Zhen, China).

Reference

1. Fang C, Zhong H, Lin Y, et al. Assessment of the cPAS-based BGISEQ-500 platform for metagenomic sequencing. *Gigascience* 2018;7(3):1-8. doi: 10.1093/gigascience/gix133
2. Xu Y, Lin Z, Tang C, et al. A new massively parallel nanoball sequencing platform for whole exome research. *BMC Bioinformatics* 2019;20(1):153. doi: 10.1186/s12859-019-2751-3
3. Drmanac R, Sparks AB, Callow MJ, et al. Human genome sequencing using unchained base reads on self-assembling DNA nanoarrays. *Science* 2010;327(5961):78-81. doi: 10.1126/science.1181498
4. Huang J, Liang X, Xuan Y, et al. A reference human genome dataset of the BGISEQ-500 sequencer. *Gigascience* 2017;6(5):1-9. doi: 10.1093/gigascience/gix024
5. Zhang Y, Gu Y, Ren H, et al. Gut microbiome-related effects of berberine and probiotics on type 2 diabetes (the PREMOTÉ study). *Nat Commun* 2020;11(1):5015. doi: 10.1038/s41467-020-18414-8
6. Jie Z, Yu X, Liu Y, et al. The Baseline Gut Microbiota Directs Dieting-Induced Weight Loss Trajectories. *Gastroenterology* 2021;160(6):2029-42 e16. doi: 10.1053/j.gastro.2021.01.029
7. Beghini F, McIver LJ, Blanco-Miguez A, et al. Integrating taxonomic, functional, and strain-level profiling of diverse microbial communities with bioBakery 3. *Elife* 2021;10 doi: 10.7554/eLife.65088

Supplemental Table S1 Separation gradient of chromatographic separation

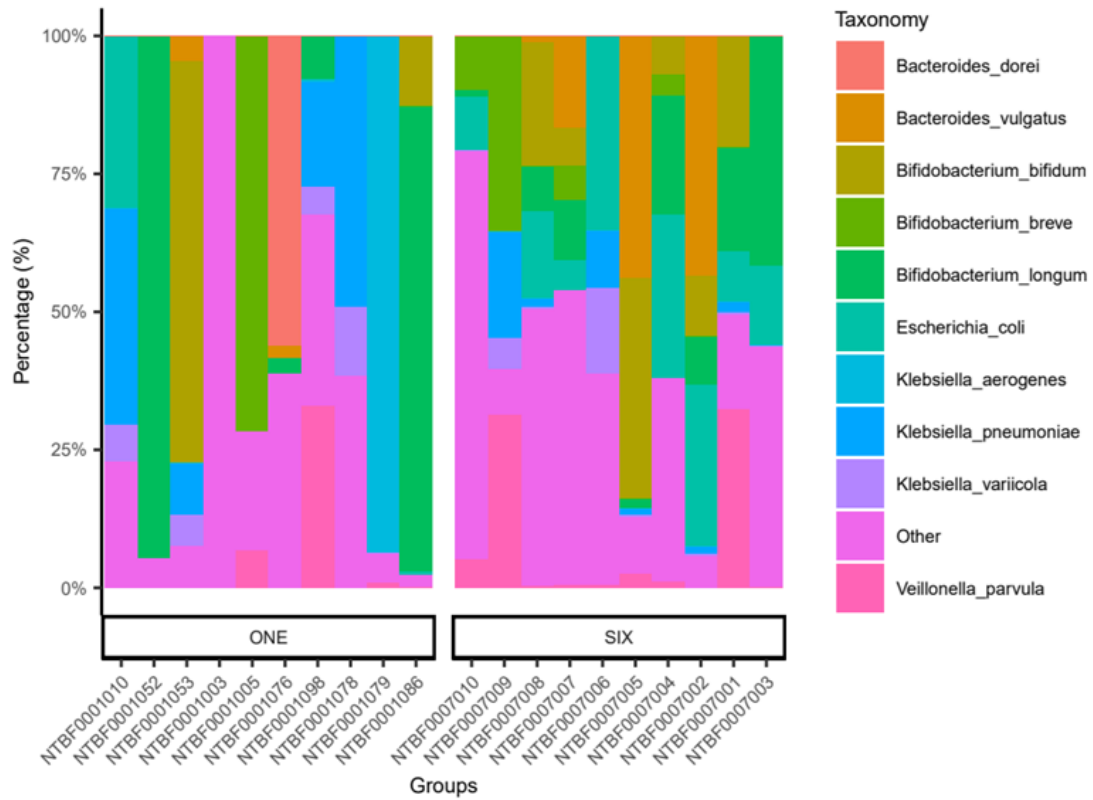
Time (min)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0	0.300	74.0	26.0
0.50	0.300	74.0	26.0
5.00	0.300	76.0	24.0
6.00	0.300	80.0	20.0
13.00	0.300	82.0	18.0
13.01	0.300	74.0	26.0

Supplemental Table S2. Compound parameters of mass spectrometric detection for vitamin D

Analyte	Transition Reactions (m/z) used for Quantification
D3-PTAD	560.31 →298.1
25-OHD3-PTAD	558.31 →298.1
D3- d3-PTAD	563.31 →301.1
25-OHD3-d6-PTAD	564.41 →298.0

Supplemental Table S3 External standard used for the quantification of identified HMOs

Analyte	Unit	Concentration range	
		Min	Max
2'FL	mg/L	19.4	5324.2
3FL	mg/L	17.8	4890.3
LDFT	mg/L	16.9	1011.3
3'SL	mg/L	16.0	799.3
LNT	mg/L	18.3	1643.8
LNnT	mg/L	18.1	723.7
6'SL	mg/L	17.7	1237.1
LNFP-I	mg/L	29.2	2923.5
Maltotriose	mg/L	30.1	6867.2



Supplemental Figure S1. Relative abundances of top 10 Species in the two groups.

SampleID	NTBF1	NTBF2	NTBF3	NTBF4	NTBF5	NTBF6	NTBF7	NTBF8	NTBF9	NTBF10	NTBF11	NTBF12	NTBF13	NTBF14	NTBF15	NTBF16	NTBF17	NTBF18	NTBF19	NTBF20
ABS_Faecalicatena_oroitica	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.1509	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
ABS_Staphylococcus_epidermidis	1.3977	0.4174	0.0003	0.1842	0.0685	0.0000	0.0030	0.1413	0.0044	0.0000	0.0000	0.0024	0.0050	0.0015	0.0007	0.0016	0.0000	0.0025	0.0000	0.0000
ABS_Klebsiella_quasipneumoniae	0.0000	0.0000	5.6669	0.0006	1.8392	0.0000	6.0017	0.0000	0.0012	2.1501	0.1293	0.1078	0.0215	0.0137	0.1814	1.9353	0.0000	1.6888	2.3907	0.0096
ABS_Streptococcus_sp_HMSC071D03	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0002	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
ABS_Streptococcus_sp_SK643	0.0000	0.0000	0.0002	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0002	0.0000	0.0000	0.0000	0.0000	0.0000
ABS_Coprobacillus_cateniformis	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.2939
ABS_Bacteroides_faecis	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.4202	3.2738	0.0000	0.0000
ABS_Actinomyces_turicensis	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0030	0.0000	0.0000	0.0027
ABS_Clostridium_bolteae	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.6623	0.0000	0.0000	0.0000	0.0000
ABS_Solobacterium_moorei	0.0023	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0012	0.0000	0.0000
ABS_Escherichia_coli	0.0036	0.0017	31.2641	0.0041	0.0007	0.0000	0.0061	0.0025	0.6622	0.0015	9.0171	29.2320	14.4045	29.9202	0.0030	35.2999	5.3620	15.6868	0.0006	9.6427
ABS_Atopobium_deltae	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0025	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0003	0.0000	0.0000
ABS_Sutterella_parvirubra	0.0000	0.0000	0.0000	0.0000	0.0000	0.0005	0.0000	0.0000	0.0000	0.0000	0.0017	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
ABS_Ruminococcus_torques	0.0000	0.0000	0.0000	0.0000	0.0000	1.1498	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
ABS_Chryseobacterium_gleum	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0008
ABS_Klebsiella_oxytoca	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.1162	0.0000	0.0003	0.8083	0.0000	0.0000	0.0012	0.0005	0.1648	0.0999
ABS_Porphyromonas_sp_HMSC065F10	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0738	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
ABS_Veillonella_dispar	0.4179	0.0000	0.0664	0.0084	0.0000	0.0000	0.0000	0.0233	0.0000	0.0002	0.1344	0.0000	0.0000	0.0879	0.0027	0.0000	0.0000	0.0000	0.0042	0.2820
ABS_Staphylococcus_haemolyticus	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0015
ABS_Acidaminococcus_intestini	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	3.2004	0.0000	0.0000	0.0000
ABS_Enterococcus_casseliflavus	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.7789	0.0000	0.0000	0.0000	2.3237	0.0217	0.0191	0.0069	1.0522	0.0000	0.0076	0.0000	0.0000	0.0000
ABS_Haemophilus_parainfluenzae	0.6164	0.0000	0.9920	0.0000	0.0000	0.0000	0.0000	0.0075	0.0000	0.0000	0.0028	0.1211	0.0133	0.0030	0.0014	0.0023	0.0000	0.0000	0.0001	0.4024
ABS_Granulicatella_elegans	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0002	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0004	0.0014
ABS_Bifidobacterium_animalis	0.0000	0.0031	0.0473	0.0000	0.0165	0.0000	0.0000	0.2920	0.0000	0.0000	0.0000	0.0000	0.9509	0.0091	0.0000	0.0000	0.0000	0.0000	0.0000	0.7734
ABS_Pandoraea_thiooxydans	0.0008	0.0000	0.0000	0.0083	0.0004	0.0000	0.0000	0.0003	0.0000	0.0003	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
ABS_Delftia_tsuruhatensis	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0020
ABS_Acinetobacter_ursingii	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0763
ABS_Streptococcus_macedonicus	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0004	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
ABS_Prevotella_buccalis	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0005	0.0000	0.0000	0.0000	0.0027	0.0459	0.0000	0.0000
ABS_Actinomyces_sp_HP40247	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0041	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
ABS_Serratia_nematodiphila	1.2363	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
ABS_Streptococcus_sp_M334	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
ABS_Lactobacillus_plantarum	0.0000	0.0000	0.0000	0.0000	0.0000	0.0146	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
ABS_Prevotella_sp_oral_taxon_473	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0003	0.0000	0.0000
ABS_Holdemanela_biformis	0.0000	0.0000	0.0000	0.0000	0.0000	0.0196	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
ABS_Rothia_mucilaginoso	0.0000	0.0000	0.0000	0.0000	0.0027	0.0000	0.0000	0.0000	0.0005	0.0003	0.0554	0.0034	0.0000	0.0000	0.0003	0.0000	0.0000	0.0003	0.0003	0.0111
ABS_Acinetobacter_guillouiae	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
ABS_Prevotella_histicola	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0084	0.0000	0.0006	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
ABS_Enterococcus_aviium	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0011	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0496	0.0000	0.0000	0.0000