



# MFGM components promote gut *Bifidobacterium* growth in infant and in vitro

Junying Zhao<sup>1,2</sup> · Wei Yi<sup>3</sup> · Bin Liu<sup>1,2</sup> · Yaohua Dai<sup>4</sup> · Tiemin Jiang<sup>1,5</sup> · Shuxing Chen<sup>6</sup> · Jianwu Wang<sup>7</sup> · Baowen Feng<sup>8</sup> · Weicang Qiao<sup>1,2</sup> · Yanpin Liu<sup>1,2</sup> · Huihuang Zhou<sup>9</sup> · Jianying He<sup>10</sup> · Juncai Hou<sup>11</sup> · Lijun Chen<sup>1,2</sup>

Received: 4 March 2021 / Accepted: 5 July 2021 / Published online: 29 July 2021  
© Springer-Verlag GmbH Germany, part of Springer Nature 2021

## Abstract

**Purpose** Infant gut microbiota which plays an important role in long-term health is mainly shaped by early life nutrition. However, the effect of nutrients on infants gut microbiota is less researched. Here, we present a study aiming to investigate in vitro a modified formula that is supplemented with milk fat globule membrane (MFGM) that were missing in common formulas when compared with human milk and to assess the impact of feeding scheme on microbiota and metabolism.

**Methods** A total of 44 infants including 16 from breast milk feeding, 13 from common formula feeding and 15 from modified formula feeding were analyzed, and A cross-sectional sampling of fecal and urine was done at 1 month-of-age. Stool microbiota composition was characterized using high-throughput DNA sequencing, and urinary metabolome was profiled by nuclear magnetic resonance (NMR). In vitro growth experiment of *Bifidobacterium* with key components from MFGM was performed and analyzed by both DNA and RNA.

**Results** Stool samples from the infants who were breastfed had a higher relative abundance of *Bifidobacterium* and a lower relative abundance of *Escherichia* than the formula-fed infants. The stool microbiome shifts were associated with urine metabolites changes. Three substances including lactadherin, sialic acid and phospholipid, key components of MFGM were significantly positively correlated to *Bifidobacterium* of stool samples from infants, and stimulated the growth rate of *Bifidobacterium* significantly by provided energy in vitro growth experiment with RNA analysis.

**Conclusions** These findings suggest that the key components from MFGM could improve infants' health by modulating the gut microbiome, and possibly supporting the growth of *Bifidobacterium*.

**Registration** Clinicaltrials.gov NCT02658500 (registered on January 20, 2016).

**Keywords** Microbiome · *Bifidobacterium* · MFGM · Metabolome

✉ Lijun Chen  
chenlijun@sanyuan.com.cn

<sup>1</sup> National Engineering Center of Dairy for Maternal and Child Health, Beijing Sanyuan Foods Co. Ltd., No. 8, Yingchang Street, Yinghai Town, Daxing District, Beijing 100163, China

<sup>2</sup> Beijing Engineering Research Center of Dairy, Beijing Technical Innovation Center of Human Milk Research, Beijing Sanyuan Foods Co. Ltd., No. 8, Yingchang Street, Yinghai Town, Daxing District, Beijing 100163, China

<sup>3</sup> Beijing Ditan Hospital Capital Medical University, Beijing 100015, China

<sup>4</sup> Capital Institute of Pediatrics, Beijing 100020, China

<sup>5</sup> South Asia Branch of National Engineering Center of Dairy for Maternal and Child Health, Guilin University of Technology, Guilin 541006, China

<sup>6</sup> Henan University of Science and Technology, Luoyang 471000, Henan Province, China

<sup>7</sup> XiangYa School of Public Health, Central South University, Changsha 410078, Hunan Province, China

<sup>8</sup> Tongzhou Maternal and Child Health Hospital of Beijing, Beijing 101101, China

<sup>9</sup> Maternal and Child Health Hospital of Liuyang of Hunan Province, Changsha 410300, Hunan Province, China

<sup>10</sup> Luoyang Northern Enterprises Group Hospital, Luoyang 471000, Henan Province, China

<sup>11</sup> Food Science College of Northeast Agricultural University, Harbin 150030, China

## Introduction

Gut microbiome's major role in human health is being increasingly understood and emphasized in recent years. Various gut microbes are essential for the normal function of host immune system [1]. In adults, many disease-oriented studies illustrated associations between nutrition and gut microbiota or metabolites of the gut microbiota [2–4]. In contrast, more studies are needed to understand the structuring forces of the infant gut microbiome [5, 6].

Infants acquire gut microbiome at birth, and studies of infant meconium suggest that bacteria are present in the foetal gut prior to birth [7]. The infant's gastrointestinal microbiome gradually matures over time after birth. This period is also recognized as a key window of immune system development, during which immune tolerance is built for common microbes. It has been reported that a dysbiosis in the gut microbiome may underlie the development of type I diabetes and allergies before 4 years old [8]. Understanding the structuring factors and the possible modulation of the gut microbiome development in infants would thus be vitally important for improving infant health and preventing certain diseases, even into adulthood.

Patterns of infant feeding would impact the development of gut microbiome [9–11]. Human milk contains essential nutrients including protein, fat, carbohydrates and vitamins primarily for the infants, and some of them would be utilized by microbes. Milk fat globule membrane (MFGM) is a unique three-layered biological membrane surrounding the milk fat globule. There are approx 76% protein, 7% phospholipids and 1.9% sialic acid in MFGM with bioactivity of anti-adhesion of bacteria and viruses, which are beneficial for the infant gut microbiome [12, 13]. In contrast, common formulas may lack those key components with essential functions in modulating gut microbiome, and could be accounted for the observed differences between breast-fed and formula-fed infant's gut microbiome such as *Bifidobacterium* [14–16]. More importantly, the health consequences associated with feeding pattern and gut microbiome differences call for a mechanistic investigation and potential interventions in infants, as it has been demonstrated that formula-fed infants have a higher tendency in developing asthma, type I diabetes compared to those breastfed [17, 18]. However, the effect of MFGM on *Bifidobacterium* which is beneficial for infant health such as anti-allergy and antidiabetic activity has not been proven [19, 20].

Here, we studied a modified formula that is supplemented with specific proteins and fat that were from MFGM but missing in common formulas when compared to human milk. We hypothesize that MFGM components could promote gut *Bifidobacterium* growth in infant. The

effect of modified formula on stool microbiome was analyzed. The influence on infant metabolism was investigated by relation analysis between stool microbiome and urine metabolites. The mechanism was researched by in vitro growth experiment (Supplemental Fig. 1).

## Methods

### Study design

We have administrated three different feeding strategies to infants from birth to 1 month of age, including breastfeeding (BF), common formula feeding (FF1) and modified formula feeding (FF2), the average nutrient compositions of which are listed in Table 1. MFGM, rich in lipids, is applied in the modified formula, leading to higher content of phospholipid and sphingolipid compared to breast milk. Formula-fed infants were randomized to FF1 or FF2. Formula taken records and the empty formula box were collected to ensure the fidelity of the intervention.

### Infant study population

The Ethics Committee of Beijing Ditan Hospital affiliated with Capital Medical University approved all aspects of the study (#2015-027-01), and informed consent was obtained from all of the subjects. The procedures were in accordance with the ethical standards. Study subjects were recruited from Tongzhou Maternal and Child Health Hospital (Tongzhou, Beijing, China) and Maternal and Child Health Hospital of Liuyang (Liuyang, Hunan, China).

The primary outcome measure was the relative abundance of gut *Bifidobacterium* in stool samples between human milk-fed infant and formula-fed infant at 1 month. To demonstrate

**Table 1** Composition of nutrients in breastfeeding (BF), common formula feeding (FF1) and modified formula feeding (FF2)

Nutrients	BF g/L	FF1 g/L	FF2 g/L
Total MFGM protein	0.3	Trace	0.36
Phospholipid	0.25–0.37	0.219	0.431
Sphingomyelin	0.07–0.150	Trace	0.0986
Sialic acid	0.61	0.122	0.245
Total lactoferrin	1–2	Trace	0.0493
IgG	0.7–2	Trace	0.246
Lactadherin	0.093	Trace	0.0616
Ganglioside	0.009	Trace	0.0123
Mucin1	0.73	Trace	0.0863

BF breastfeeding, FF1 common formula feeding, FF2 modified formula feeding

a clinically significant difference in the relative abundance of *Bifidobacterium* by 76% between two groups (i.e. from 54 to 13%) with 90% power and an alpha value of 0.05, we estimated a sample size of 13 infants in each group for a total of 39 infants [14].

This report, which is a cross-sectional analysis of a single time point, was comprised of 44 participants (16 from BF, 13 from FF1 and 15 from FF2 group) whose data passed all quality control criteria (See details below). All analyses involving stool microbiome and urine metabolites data were restricted to 44 individuals (Supplemental Fig. 1 and Supplemental Table 1).

## Criteria

Newborns were eligible to be included if they were: healthy term newborns (gestational age ranges from 37 to 41 weeks) with a birth weight range from 2500 to 4000 g appropriate for gestational age and Apgar scores larger than 7. Infants were excluded from the entire study if any the following exclusion criteria were met at any time of the study: diagnosis of a significant chronic medical condition including: HIV infection, cancer, bone marrow or organ transplantation, blood product administration within the last 3 months, bleeding disorder, known congenital malformation or genetic disorder; if the parent or legal guardian was unable to read and/or comprehend Chinese; if the family moved outside of the city they lived during the study period; if infant take antibiotics during the research period. Feeding practices were assessed by careful chart review and classified as exclusive formula or breast-fed within the first month of life.

## Sampling and growth data collecting

Parents were trained for fecal and urine collection when they agreed to participate in the research. At the age of 1 month ( $\pm 2$  days), fecal samples were collected by parents at home from diapers after defaecation, immediately put into the stool collection tubes with stool DNA stabilizer (Stratec, Germany) and stored at  $-20\text{ }^{\circ}\text{C}$  until they were transported (in dry ice, within 24 h) to hospital. Urine was collected by parents at home with urine collection bag which was stuck on the uriding area and transferred into sterile tubes and stored at  $-20\text{ }^{\circ}\text{C}$  until they were transported to the hospital (in dry ice, within 24 h). Then the samples were stored at  $-80\text{ }^{\circ}\text{C}$  until analyses. Growth data including height, weight, and disease frequency were also collected at the same time.

## High-throughput sequencing of the 16S rRNA gene of the stool microbiome

Genomic DNA was extracted from 100 mg of homogenized fecal samples using the QIAamp Fast DNA Stool Mini Kit

(Qiagen, GmbH, Hilden, Germany) following the manufacturer's protocol. PCR amplification of the V3-V4 region of 16S rRNA genes (primers 341F: CCTACGGGNGGCWGC AG and 805R: GACTACHVGGGTATCTAATCC) was performed with 10 ng DNA as a template, using 15  $\mu\text{L}$  of Phusion High-Fidelity PCR Master Mix (New England Biolabs), 0.2  $\mu\text{M}$  of forward and reverse primers in a 30  $\mu\text{L}$  total reaction volume. The PCR program included 3 min at  $95\text{ }^{\circ}\text{C}$ , 25 cycles of 30 s at  $95\text{ }^{\circ}\text{C}$  and 30 s at  $55\text{ }^{\circ}\text{C}$ , and then 30 s at  $72\text{ }^{\circ}\text{C}$ . Sequencing libraries were generated using NEB Next Ultra DNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's recommendations. Amplicons were sequenced using  $2\times 250$  bp paired-end protocol by Illumina HiSeq 2500 according to the manufacturer's instructions.

## Infant urine NMR metabolites

Samples were centrifuged at 15,870 rcf for 2 min at  $4\text{ }^{\circ}\text{C}$ , the 540  $\mu\text{L}$  aqueous layer was transferred to a clean 2 mL centrifuge tube. 60  $\mu\text{L}$  3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS) standard solution (Anachro, Canada) was added. Samples were mixed well before transfer to 5 mm NMR tube (Norwell, USA). Spectra were collected using a nuclear magnetic resonance (NMR) spectroscopy (Bruker AV III 600 MHz). The first increment of a 2D- $^1\text{H}$ ,  $^1\text{H}$ -NOESY pulse sequence was utilized for the acquisition of  $^1\text{H}$  NMR data and for suppressing the solvent signal. Experiments used a 100 ms mixing time along with a 990 ms pre-saturation ( $\sim 80$  Hz  $\gamma_{\text{B}_1}$ ). Spectra were collected at  $25\text{ }^{\circ}\text{C}$ , with a total of 128 scans over a period of 15 min. The collected Free Induction Decay (FID) signal was automatically zero filled and Fourier transform in Processing module in Chenomx NMR Suite 8.1 (Chenomx Inc., Edmonton, Canada). The data was then carefully phased and baseline corrected in Chenomx Processor. All the spectra were referenced to the internal standard, DSS and analyzed against Chenomx Compound Library. All metabolites' concentration was normalized by weight across all parallel samples before used in the later on multivariable analysis.

A total of 77 metabolites concentrations and derived measures were obtained from the NMR spectroscopy platform. These metabolites cover several types of metabolite classes, including vitamin/cofactors, organic acids, amino acids, amino acid derivatives, ketones, nucleic acid components, ammoniums compounds, amines, sugars, alcohols, and amides.

## In vitro growth experiment

Bacteria strain *B. longum* subsp. *infantis* DSMZ20090, provided by China General Microbiological Culture Collection Center (CGMCC) (<http://www.cgmcc.net/>) was used for the

in vitro growth experiment. *B. infantis* were first cultured in TPY (Trypticase Soy-Yeast) liquid broth under anaerobic conditions with Anaerocult A system (Merck, Darmstadt, Germany) for 48 h, and then diluted to  $10^5$  cells/mL in each media. Key MFGM components (lactadherin, L; sialic acid, S; phospholipid, P) were provided by Arla Foods (Denmark), and added to TPY medium alone (L, S, P), or combining two out of three (L + S, L + P, S + P), or all three components (L + S + P), with concentration identical to that of BF provided in Table 1. TPY medium without additives was used as a control. All groups were grown in triplicate under anaerobic conditions in a modular atmosphere-controlled system (Davidson and Hardy, Belfast, Ireland) at 37 °C for 18 h to reach mid-exponential phase, and the optical density at 600 nm (OD600) was determined, using a Shimadzu UV-1280 UV-399 VIS Spectrophotometer (Shimadzu Corp., Kyoto, Japan) directly after inoculation as well as after 18 h; bacterial growth was measured as changes in OD600. After growth, tubes were centrifuged at  $10,000\times g$  for 5 min, supernatant was removed, and the pellet was resuspended in 1 mL of Trizol, and the RNA was extracted and purified using RNeasy Mini Kit (Qiagen, Hilden, Germany, 74104) following the instructions. RNA libraries were prepared using a TruSeq Stranded Total Library Preparation Kit (Illumina, USA) following the instructions. Samples were sequenced at the Institute of Microbiology, Chinese Academy of Sciences, using 150-bp pair-end reads chemistry on a HiSeq 2500 instrument.

### Bioinformatic analysis

USEARCH [21] and VSEARCH [22] were used to quality filter, cluster, and removing chimera of demultiplexed 16S rRNA raw sequencing data of stool sample. The clustered sequences at 97% similarity level were utilized to construct operational taxonomy unit (OTU) tables and representative sequences were classified into the respective taxonomical level from phylum to species based on the RDP 16S rRNA gene database [23].

All statistical analyses were performed in the statistical computing language R [24]. For 16S rRNA data, total sum normalization (TSS) was firstly used to normalize count data by dividing feature read counts by the total number of reads in each sample. Rarefaction analysis, principal coordinates analysis (PCoA) using Bray–Curtis distance of stool microbiome data were performed using Phyloseq [25] with vegan (<https://cran.r-project.org/web/packages/vegan/index.html>) and ade4 [26] packages. Boxplots with significance level were plotted using “ggpubr” package version 0.1.6 (<https://cran.r-project.org/web/packages/ggpubr/index.html>). Correlation of microbes and metabolites were performed using Spearman correlation in the R package “neuropsychology” package 0.5.0 [27], 16S rRNA abundance data were TSS

and square root transformed. Urinary metabolites data were log<sub>2</sub>-transformed, and the *p* value was corrected by false discovery rate (FDR) ( $< 0.01$ ). RNA-Seq raw reads were quality control using FastQC [28], then trimmed into clean reads using Trim-Galore ([https://www.bioinformatics.babraham.ac.uk/projects/trim\\_galore/](https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/)). Next, clean reads were aligned to the reference genome (NCBI Accession ID: GCF\_000020425.1) with HISAT2 [29], then.sam files were converted to.bam files using SamTools [30], and an expression table was generated using featureCounts of Subread [31]. Differential expression analysis was performed using DESeq2 [32]. KEGG pathway annotation was performed using clusterProfiler [33] and DOSE [34] packages in R.

## Results

### Feeding strategies lead to different microbiome in infants

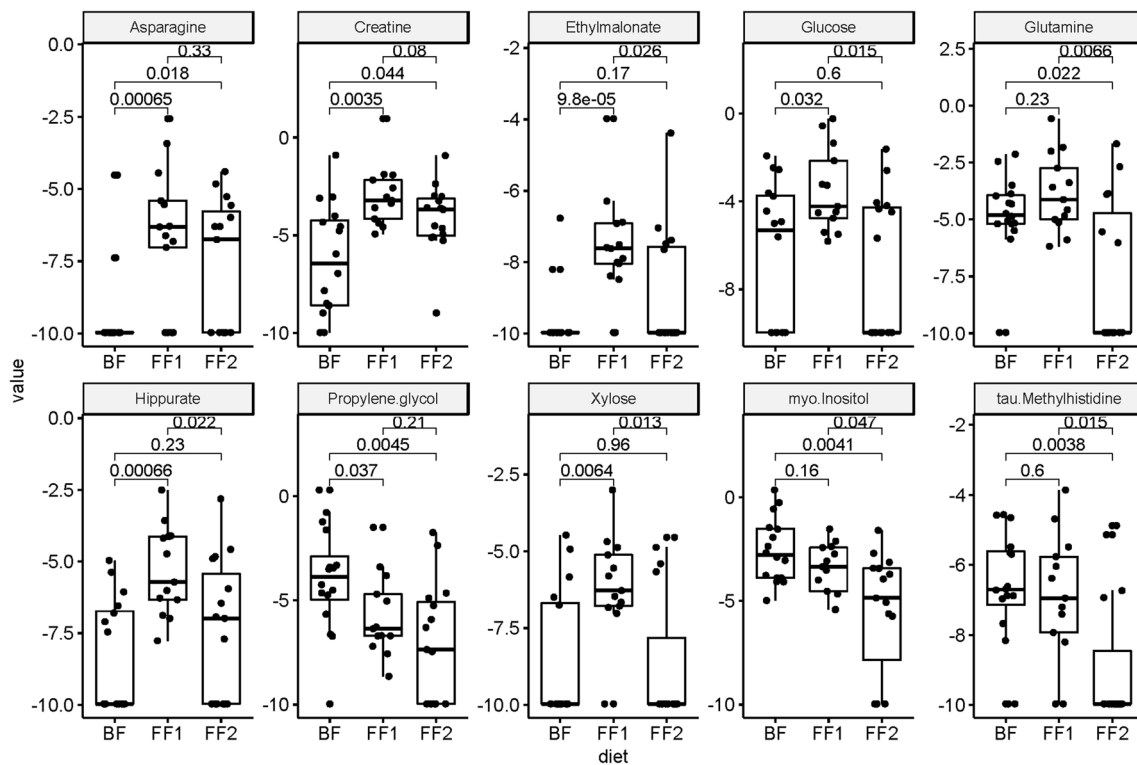
We first analyzed the alpha-diversity of each group, both Simpson index and Shannon evenness were higher in the BF group than FF1 and FF2 (Supplemental Fig. 2), while Chao 1 index was higher in FF1 group than BF and FF2. Further beta-diversity analysis revealed that stool microbiota of BF group was also distinct from that of FF1 and FF2 (Supplemental Fig. 3). Overall, 40.28% of the total microbiota variations could be explained by the feeding scheme.

We further examined the urine metabolites, anthropometric measures as well as clinical parameters among the three groups. In urine metabolites, 10 out of 77 tested metabolites showed significant differences among the three groups (FDR  $< 10\%$ ) (Fig. 1). Hippurate was significantly lower in FF2 group than that in FF1 group but was not different significantly from that in BF group. Creatine was significantly lower in BF group than that in both FF2 group and FF1 group.

Among the infants’ anthropometric measures as well as clinical parameters of infant, FF2 group and FF1 group were significantly lower in height and weight compared to BF both at birth and 1 month, while the growth of height and weight and other anthropometric measures, clinical parameters at 1 month were not significantly different from BF (Supplemental Table 2).

### *Bifidobacterium* as key modulator of stool microbiome

We then have performed further compositional analysis of the stool microbiota from different groups, especially at the genus level. Among all major genera (average relative abundance  $> 1\%$ ), *Bifidobacterium* was found to be the main source of differences in infants fed different strategies.



**Fig. 1** Differential urinary metabolites of three feeding schemes. The analysis was performed using Kruskal–Wallis test with posthoc analysis by Wilcoxon-test (0.05 level). Black dot show the result of each

sample. *BF* breastfeeding, *FF1* common formula feeding, *FF2* modified formula feeding

The average relative abundance of *Bifidobacterium* for three groups reaches 12% in our study, and 29.89%, 2.66% and 2.27% in BF, FF1 and FF2 groups, respectively (Supplemental Fig. 4). Negatively correlated with *Bifidobacterium*, *Veillonella* was among the major genera that are different among the three groups, accounting for 0.26%, 6.80% and 5.06% in BF, FF1 and FF2 groups, respectively; and *Escherichia/Shigella* accounting for 0.03%, 8.48% and 8.03% in BF, FF1 and FF2 groups, respectively.

We further performed an association analysis between the different nutrients measured within BF, FF1 and FF2 formula and the relative abundances of the major genera, especially *Bifidobacterium* (Fig. 2). Three nutrient components, lactadherin, sialic acid and phospholipid showed significant positive correlations to the relative abundance of *Bifidobacterium* and consequently negative correlation to that of *Veillonella* and *Escherichia/Shigella* (Figs. 2 and 3); they are the major active components within MFGM, a complex structure surrounding milk fat globules secreted in human and other mammals' milk. Other components including mucin, lactoferrin, IgG and sphingomyelins demonstrated a negative correlation with *Veillonella* and *Escherichia/Shigella* as well, yet they do not correlate with *Bifidobacterium*. Mucin and lactoferrin are negatively correlated with *Bacteroides* as well as *Clostridium*, and despite

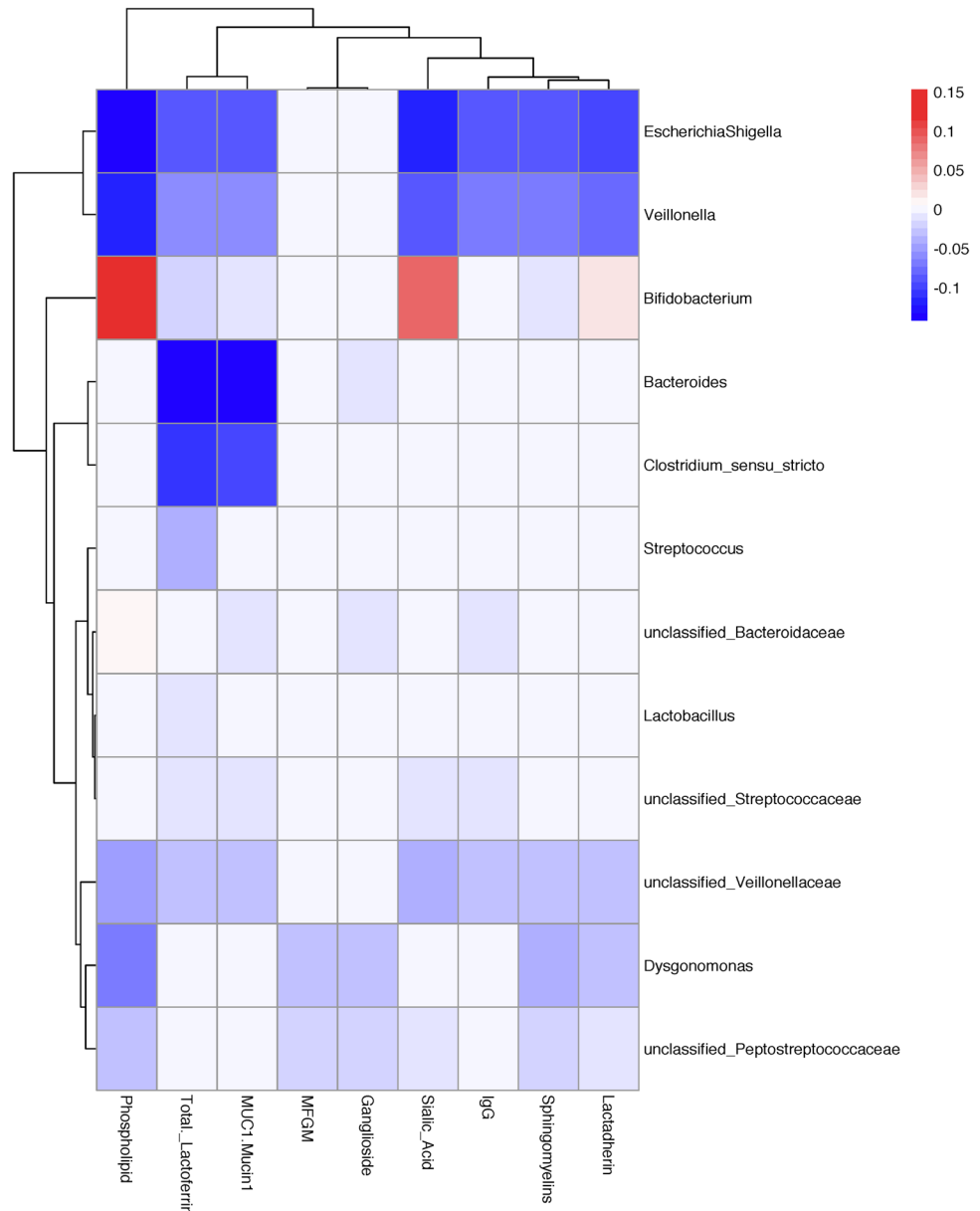
they are usually key members of adult gut microbiota, in infants their abundances are relatively low (9% and 16% on average, respectively, Supplemental Fig. 4).

Lastly, we investigated the potential sources of differential metabolites within the infants' urine by performing an association analysis of stool microbiota members to that of metabolites (Supplemental Table 3). In total, 40 correlations, including 24 positive correlations and 16 negative correlations were found with a FDR of 10%. Of all metabolites with significant differences among the three groups, creatine and benzoate showed a positive correlation with *Veillonella*. None of any metabolites were found to significantly correlate with both *Bifidobacterium* and *Escherichia/Shigella*. Benzoate was positively correlated with *Veillonella*, as well as other short-chain fatty acids (e.g., propionate).

### MFGM components stimulates *Bifidobacterium* growth in vitro

Since the concentrations of lactadherin, sialic acid and phospholipid are correlated within human milk as well as in modified formula, it is yet difficult to elucidate the effects of the three components simply from infants' stool microbiota data. Moreover, the effect of three components in modified formula on infant stool microbiota is not yet

**Fig. 2** Association analysis and heatmap visualization of nutrients measured within formula and the relative abundances of the gut microbial genera. The red and blue color demonstrated a significant positive and negative association between the nutrients and the microbiome, respectively

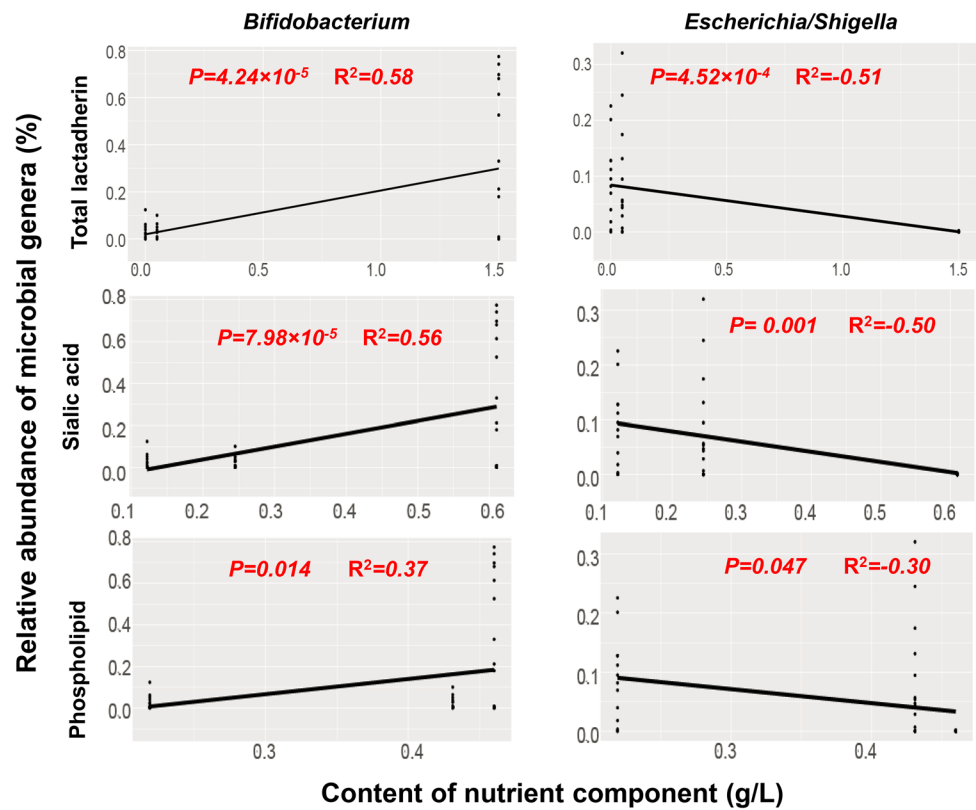


clear. To investigate whether the *Bifidobacterium* enrichment effect comes from single components or rather a combination and the effect of three components in FF2 formula, we have used one *Bifidobacterium longum* subsp. *infantis* strain isolated from infant fecal samples, and tested its growth on simple TPY (Trypticase Soy-Yeast extract) liquid medium versus addition of one, two or all three components, of which the concentrations reflect those in FF2 formula, each with three technical replicates. After 18 h of anaerobic growth under the same temperature (37 °C), bacterial growth was measured with OD600 absorption (with blank medium as background). Interestingly, the only medium with all three added showed significantly higher bacterial cell density compared to the

control medium while adding one or two components did not lead to a significant increase in cell density (Fig. 4).

Finally, to investigate the potential mechanism underlying the *Bifidobacterium* enrichment under MFGM components, we isolated total RNA from strains grown in a simple medium of triplicate as well as medium with all three components added, and carried out transcriptome analysis with RNA-Seq on Illumina HiSeq. Data were analyzed with Hisat2 using *B. infantis* reference genome sequences, and differential genes were defined as adjusted  $p < 0.05$  in paired  $t$  tests. This analysis revealed 74 genes that were differentially expressed between the two culture conditions, and top enriched genes with all three components included 2-hydroxyacid dehydrogenase (gene2390),

**Fig. 3** Regression of three nutrient components, lactadherin, sialic acid and phospholipid with the relative abundances of *Bifidobacterium* and *Escherichia/Shigella*. Black dot show the result of each sample. X axis stands for the content of nutrient component, Y axis stands for the relative abundance of microbial genera



4-hydroxy-tetrahydrodipicolinate synthase (gene2170), and ABC transporter ATP-binding protein (gene2468) (Fig. 5). A gene enrichment analysis revealed that glyoxylate and dicarboxylate metabolism pathway ( $p=0.0001$ ) is among the top enriched pathways in *Bifidobacterium* with MFGM components (Supplemental Fig. 5).

## Discussion

Diet is known to be a potent and persistent modifier of the microbiota in both adults and children [35, 36] and breastfeeding is one of the main factors shaping infants' gut microbiota [37]. Previous studies showed that breastfeeding versus non-breastfeeding significantly influenced the gut microbiome [38, 39]. Digestion and metabolism of nutrients are likely influenced by the intestinal microbiome [37], and there is a well-established connection between breast feeding and lower risk for childhood and adult-onset obesity possibly mediated in part by the microbiome in early life [40]. Compared with formula-fed infants, breastfed infants showed an impact on several gut microbes, such as *Escherichia coli*, *Veillonella dispar* and *Ruminococcus gnavus* as well as *Klebsiella*, known to be pro-inflammatory or opportunistic pathogens, and *Bifidobacterium longum*, who plays an important role in maintaining a healthy gut environment especially in infants, as reported by previous studies [11,

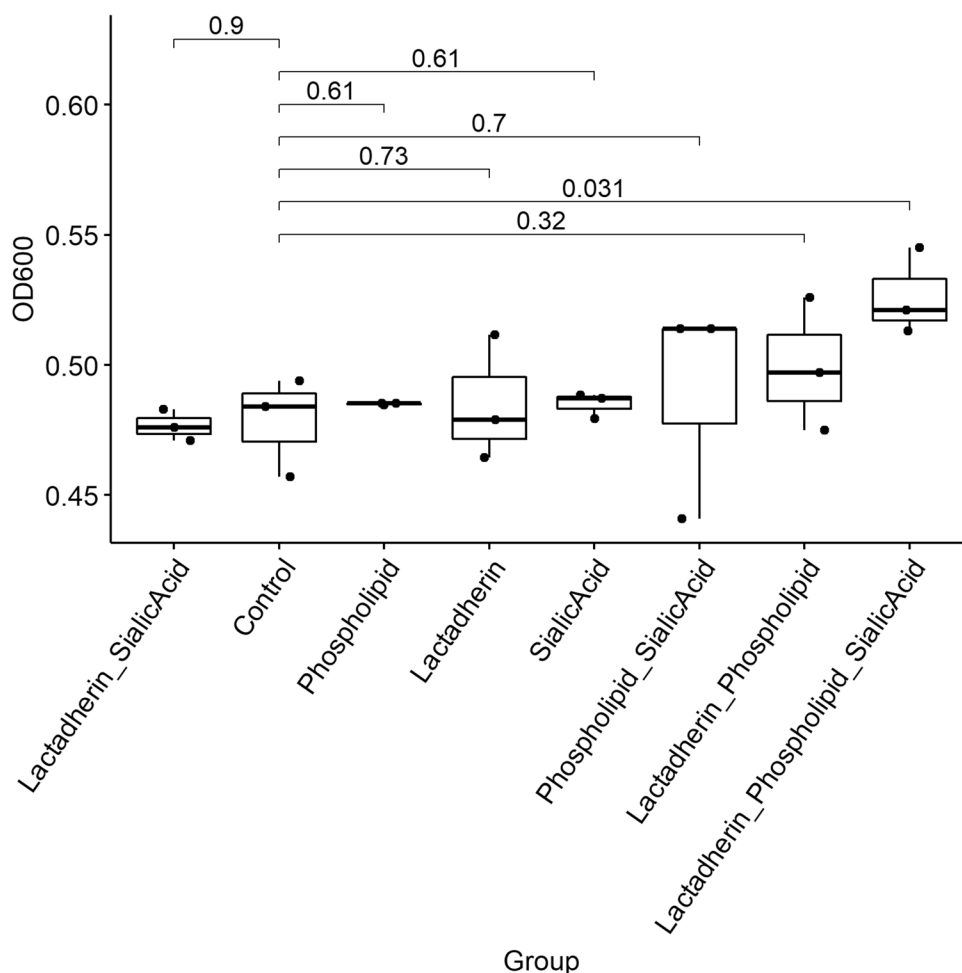
35]. In most of the cases, no significant alpha-diversity was found between breast- and formula-fed infants [10]. Some cases found that the alpha-diversity of gut microbiota of breast-fed infant was higher than that of formula-fed infants [10]. This might be due to the components of infant formula.

In this study, we studied a modified formula that is partially replenished with specific protein and fat components that were missing in common formulas when compared with human milk. Three nutrient components added in the modified formula were positively related to *Bifidobacterium* and really enriched *Bifidobacterium* in vitro experiment.

### Adding three nutrient components, lactadherin, sialic acid and phospholipid shifts stool microbiome of three feeding strategies

Assessment of association between major genera of stool microbiome and nutrition components revealed that those three nutrient components, lactadherin, sialic acid and phospholipid were significantly and positively correlated with *Bifidobacterium* and negatively correlated with *Veillonella* and *Escherichia/Shigella*. MFGM components were reported to resist several pathogens in vitro, such as *Campylobacter jejuni*, *Salmonella enteritidis*, and *Listeria monocytogenes* [41], and also protect against pathogens in vivo, such as *L. monocytogenes*. Addition of MFGM in formula also showed many beneficial effects, such as leading to a microbiome

**Fig. 4** OD600 nm absorption of bacterial growth under different combinations of nutrient components. Control stand for control TPY medium. Black dots show three replicates of each group. MFGM components (lactadherin, sialic acid, phospholipid). The analysis was performed using *t* test (0.05 level), which compared each group with control separately



more similar to that of mother's milk-fed rat, protecting against *C. difficile* toxins [13]. MFGM also modulates microbiota in mouse studies, but no effect on *Bifidobacterium* is mentioned [13].

In this study, it was shown that *Bifidobacterium* was stimulated in vitro by the combination of lactadherin, sialic acid and phospholipid. Thus, the combination of lactadherin, sialic acid and phospholipid is required for the enrichment of *Bifidobacterium*. Three components may also enrich *Bifidobacterium* of infant. In-depth RNA-Seq sequencing showed that 2-hydroxyacid dehydrogenase, 4-hydroxy-tetrahydrodipicolinate synthase, ABC transporter ATP-binding protein genes were differentially expressed in the existence of these three components. These differentially expressed genes were significantly enriched in glyoxylate and dicarboxylate metabolism pathway. This pathway belongs to energy and carbohydrate metabolism, both of which are critical for the tricarboxylic acid cycle (TCA cycle), and therefore, it could be a potential mechanism for stimulating the growth of *Bifidobacterium* [42]. It might be those components that provide energy specifically towards *Bifidobacterium*. However, the abundance of *Bifidobacterium* in FF2 group was not

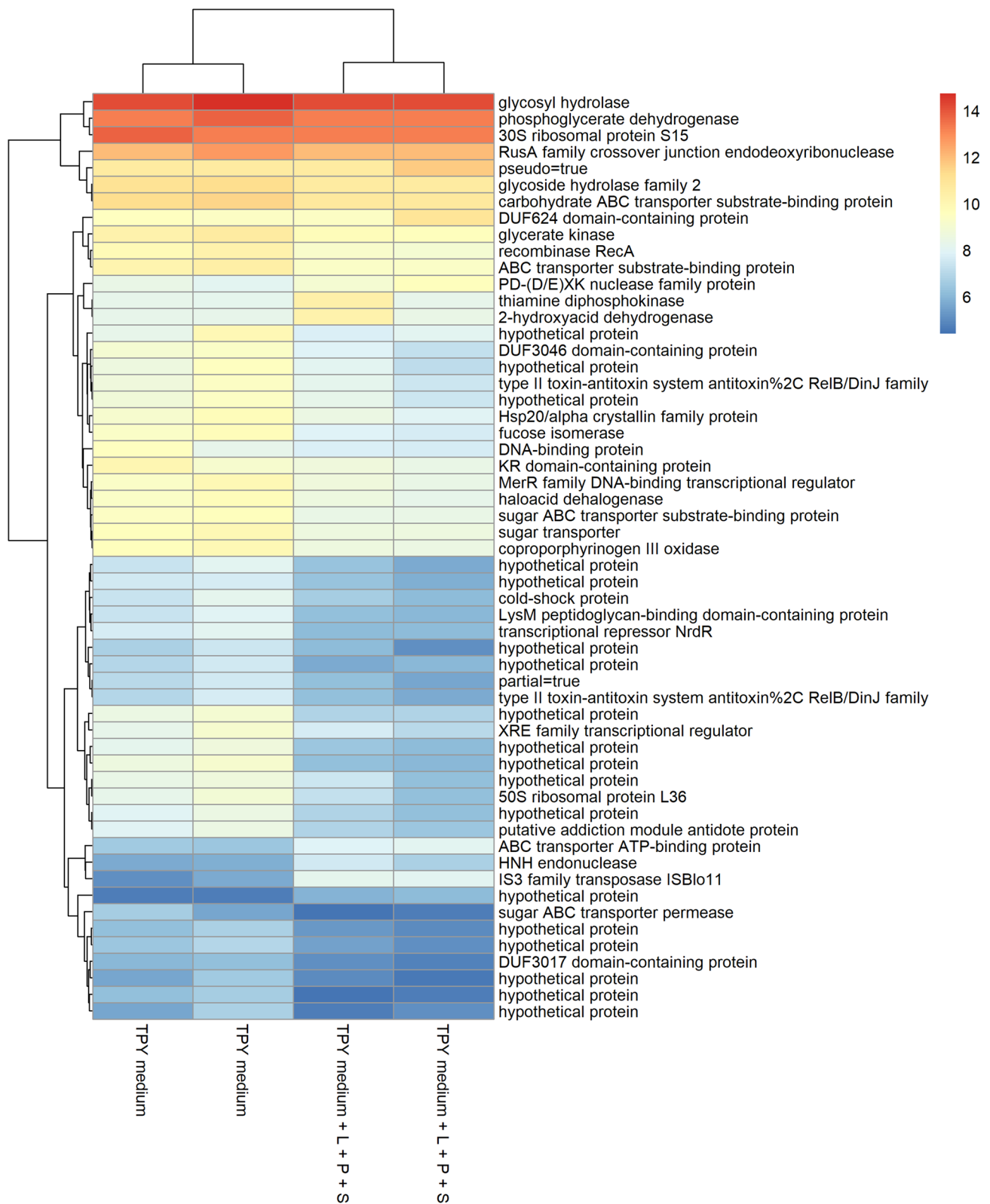
higher than that of FF1 group. A possible reason could be interactions between MFGM and other components of FF2.

### Change of microbiota correlated with metabolites and health

Urine contains metabolic breakdown products from various sources, such as foods, drinks, and microbial by-products [43]. Metabolites, which were affected by gut microbiota, are directly associated with health conditions and disease phenotypes [44].

Hippurate content in three group increased as  $BF < FF2 < FF1$ . The difference was significant between BF and FF1, FF2 and FF1 but not between FF2 and BF (Fig. 1). Hippurate is synthesized by the liver and kidney with glycine and benzoic acid. Benzoic acid is a metabolite produced by intestinal bacteria from aromatic compounds of diet [45]. Therefore, hippurate, together with Trimethylamine *N*-oxide, acetate and formate, were generally accepted as metabolites sourced from the gut microbiome [46]. It could be deduced that FF2 alters the microbiome regarding hippurate in a way that it becomes more similar to that of breastfed infants.





**Fig. 5** Heatmap visualization of FPKM (Fragments Per Kilobase per Million mapped reads) abundances of differentially expressed genes in *Bifidobacterium* grown in medium with or without all three com-

ponents (lactadherin-L, sialic acid-S, phospholipid-S) added. “1” and “2” stand for technical replicates of RNA sequencing of the two groups

Creatine content in three groups increased as BF < FF2 < FF1. The difference was not significant between FF2 and FF1 but significant between FF2 and BF as well as FF1 and BF. Creatine is an amino acid derivative participated in cellular energy production, which is known as an anti-inflammation agent [47] and can improve maintenance of ATP content and reduce oxidative stress [48]. Creatine was positively associated with *Veillonella* (the correlation coefficient was 0.48) in this study. *Veillonella*, which was 0.26%, 6.80% and 5.06% in BF, FF1, FF2, respectively, participated in the modulation of linked pathways including short-chain fatty acids production [49]. It is resident of oral cavity, genito-urinary, respiratory, and intestinal tracts of humans, and can cause severe human infections such as endocarditis, osteomyelitis, and prosthetic joint infection [50]. Lower content of creatine and *Veillonella* demonstrate better health status in BF and FF2 group.

Taken together, this study suggests that the key components from MFGM could improve infants' health by modulating the gut microbiome, and possibly supporting the growth of *Bifidobacterium*. These results offer a first step in changing formula for the good of children. Our follow-on studies will expand the number of recruits from more areas, potentially follow the infants for a longer time, and employ metagenomic sequencing to further profile the metabolic functions.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00394-021-02638-5>.

**Acknowledgements** We thank the infants and their parents participating in this study for their trust. We also thank the study team for their involvement in this work, especially nurses and coordinators. We thank Dr Jufang Li and Dr Xueyan Dong for helping collecting samples, and we also thank Dr Jun Wang and Dr Yue Ma for bioinformatic analysis and revision.

**Author contributions** JZ: investigation, data curation, writing original draft preparation; WY: methodology, investigation, data curation; BL: data curation, writing original draft preparation; YD: methodology, investigation; TJ: methodology, investigation; SC: investigation; JW: investigation; BF: investigation; WQ: investigation; YL: investigation; HZ: investigation; JH: investigation; JH: methodology; LC: conceptualization, methodology, infant formula design, funding acquisition, resources.

**Funding** This work was supported by National Natural Science Foundation of China No. 32072191, Beijing Science and Technology Plan No. Z201100008020005, Daxing District Major Scientific and Technological Achievements Transformation Project No. 2020006, National Key Research and Development Program No. 2019YFF0216702, Beijing Science and Technology Plan No. Z201100002620005.

**Data availability** The datasets generated and/or analyzed during the current study are not publicly available due to ethical and privacy considerations but are available from the corresponding author on reasonable request.

**Code availability** Not applicable.

## Declarations

**Conflict of interest** There was no conflicts of interest/competing interests for this article.

**Ethical approval** The Ethics Committee of Beijing Ditan Hospital affiliated to Capital Medical University approved all aspects of the study (#2015-027-01).

**Consent to participate** Informed consent was obtained from all of the parents.

**Consent for publication** All authors were consent for publication.

## References

- Human Microbiome Project Consortium T (2012) Structure, function and diversity of the healthy human microbiome. *Nature* 486:207–214. <https://doi.org/10.1038/nature11234>
- Qin JJ, Li YR, Cai ZM et al (2012) A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* 490:55–60. <https://doi.org/10.1038/nature11450>
- Schulz MD, Atay Ç, Heringer J et al (2014) High-fat-diet-mediated dysbiosis promotes intestinal carcinogenesis independently of obesity. *Nature* 514:508–512. <https://doi.org/10.1038/nature13398>
- Ridaura VK, Faith JJ, Rey FE et al (2013) Gut microbiota from twins discordant for obesity modulate metabolism in XZmice. *Science* 341:1241214. <https://doi.org/10.1126/science.1241214>
- Zimmermann P, Curtis N (2020) Breast milk microbiota: a review of the factors that influence composition. *J Infect* 81:17–47. <https://doi.org/10.1016/j.jinf.2020.01.023>
- Sindi AS, Geddes DT, Wlodek ME et al (2021) Can we modulate the breastfed infant gut microbiota through maternal diet? *FEMS Microbiol Rev*. <https://doi.org/10.1093/femsre/fuab011>
- Walker RW, Clemente JC, Peter I, Loos RJF (2017) The prenatal gut microbiome: are we colonized with bacteria in utero? *Pediatr Obes* 12:3–17. <https://doi.org/10.1111/ijpo.12217>
- Kostic ADD, Gevers D, Siljander H et al (2015) The dynamics of the human infant gut microbiome in development and in progression toward type 1 diabetes. *Cell Host Microbe* 17:260–273. <https://doi.org/10.1016/j.chom.2015.01.001>
- Le Huërrou-Luron I, Blat S, Boudry G (2010) Breast- v. formula-feeding: impacts on the digestive tract and immediate and long-term health effects. *Nutr Res Rev* 23:23–36. <https://doi.org/10.1017/S0954422410000065>
- Guaraldi F, Salvatori G (2012) Effect of breast and formula feeding on gut microbiota shaping in newborns. *Front Cell Infect Microbiol* 2:1–4. <https://doi.org/10.3389/fcimb.2012.00094>
- Bezirtzoglou E, Tsiotsias A, Welling GW (2011) Microbiota profile in feces of breast- and formula-fed newborns by using fluorescence in situ hybridization (FISH). *Anaerobe* 17:478–482. <https://doi.org/10.1016/j.anaerobe.2011.03.009>
- Zhao LL, Du M, Gao J et al (2019) Label-free quantitative proteomic analysis of milk fat globule membrane proteins of yak and cow and identification of proteins associated with glucose and lipid metabolism. *Food Chem* 275:59–68. <https://doi.org/10.1016/j.foodchem.2018.09.04413>
- Bhinder G, Allaire JM, Garcia C et al (2017) Milk fat globule membrane supplementation in formula modulates the neonatal

- gut microbiome and normalizes intestinal development. *Sci Rep* 7:45274. <https://doi.org/10.1038/srep45274>
14. Aimee MBD, Alaric WDS, Phillip IT, Barbara BW, Gautam D (2018) Infant diet and maternal gestational weight gain predict early metabolic maturation of gut microbiomes. *Nat med* 24:1822–1829. <https://doi.org/10.1038/s41591-018-0216-2>
  15. Martin CR, Ling PR, Blackburn GL (2016) Review of infant feeding: key features of breast milk and infant formula. *Nutrients* 8:279. <https://doi.org/10.3390/nu8050279>
  16. Brink LR, Katelin M, Piccolo BD et al (2019) Neonatal diet impacts bioregional microbiota composition in piglets fed human breast milk or infant formula. *J Nutr* 149:2236–2246. <https://doi.org/10.1093/jn/nxz170>
  17. Johnson CC, Ownby DR (2017) The infant gut bacterial microbiota and risk of pediatric asthma and allergic diseases. *Transl Res* 179:60–70. <https://doi.org/10.1016/j.trsl.2016.06.010>
  18. Stewart CJ, Ajami NJ, O'Brien JL et al (2018) Temporal development of the gut microbiome in early childhood from the TEDDY study. *Nature* 7728:583–588. <https://doi.org/10.1038/s41586-018-0617-x>
  19. Cukrowska B, Biera JB, Zakrzewska M et al (2020) The Relationship between the infant gut microbiota and allergy. The role of *Bifidobacterium breve* and prebiotic oligosaccharides in the activation of anti-allergic mechanisms in early life. *Nutrients* 12:946. <https://doi.org/10.3390/nu12040946>
  20. Li T, Yang JJ, Zhang HX et al (2020) Bifidobacterium from breastfed infant faeces prevent high-fat-diet-induced glucose tolerance impairment, mediated by the modulation of glucose intake and the incretin hormone secretion axis. *J Sci Food Agric* 100:3308–3318. <https://doi.org/10.1002/jsfa.10360>
  21. Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26:2460–2461. <https://doi.org/10.1093/bioinformatics/btq461>
  22. Rognes T, Flouri T, Nichols B, Quince C, Mahé F (2016) VSEARCH: a versatile open source tool for metagenomics. *PeerJ* 4:e2409v1. <https://doi.org/10.7717/peerj.2584>
  23. Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 73:5261–5267. <https://doi.org/10.1128/AEM.00062-0>
  24. R Core Team (2015) R: a language and environment for statistical computing. R Foundation for Statistical Computing. Available from <http://www.r-project.org/>
  25. McMurdie PJ, Holmes S (2013) phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* 8:e61217. <https://doi.org/10.1371/journal.pone.0061217>
  26. Dray S, Dufour A-BB (2007) The ade4 package: implementing the duality diagram for ecologists. *J Stat Softw* 22:1–20. <https://doi.org/10.18637/jss.v022.i04>
  27. Makowski D (2016) Package “neuropsychology”: an R toolbox for psychologists, neuropsychologists and neuroscientists. Available from: <https://github.com/neuropsychology/neuropsychology.R> Accessed 30 June 2019
  28. Patel RK, Jain M (2012) NGS QC toolkit: a toolkit for quality control of next generation sequencing data. *PLoS ONE* 7:e30619. <https://doi.org/10.1371/journal.pone.0030619>
  29. Perteua M, Kim D, Perteua GM, Leek JT, Salzberg SL (2016) Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. *Nat Protoc* 11:1650–1667. <https://doi.org/10.1038/nprot.2016.095>
  30. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R (2009) The sequence alignment/map format and SAMtools. *Bioinformatics* 25:2078–2079. <https://doi.org/10.1093/bioinformatics/btp352>
  31. Liao Y, Smyth GK, Shi W (2013) The Subread aligner: fast, accurate and scalable read mapping by seed-and-vote. *Nucleic Acids Res* 41:e108–e108. <https://doi.org/10.1093/nar/gkt214>
  32. Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15:1–34. <https://doi.org/10.1186/s13059-014-0550-8>
  33. Yu G, Wang LG, Han Y, He QY (2012) ClusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS* 16:284–287. <https://doi.org/10.1089/omi.2011.0118>
  34. Yu G, Wang LG, Yan GR, He QY (2015) DOSE: an R/Bioconductor package for disease ontology semantic and enrichment analysis. *Bioinformatics* 31:608–609. <https://doi.org/10.1093/bioinformatics/btu684>
  35. Cani PD, Hul MV (2020) Mediterranean diet, gut microbiota and health: when age and calories do not add up. *Gut* 69:1167–1168. <https://doi.org/10.1136/gutjnl-2020-320781>
  36. Forbes JD, Azad MB, Lorena V et al (2018) Association of exposure to formula in the hospital and subsequent infant feeding practices with gut microbiota and risk of overweight in the first year of life. *JAMA Pediatr* 172:e181161. <https://doi.org/10.1001/jamapediatrics.2018.1161>
  37. Newburg DS, Morelli L (2015) Human milk and infant intestinal mucosal glycans guide succession of the neonatal intestinal microbiota. *Pediatr Res* 77:115–120. <https://doi.org/10.1038/pr.2014.178>
  38. Stearns JC, Zulyniak MA, de Souza RJ et al (2017) Ethnic and diet-related differences in the healthy infant microbiome. *Genome Med* 9:32. <https://doi.org/10.1186/s13073-017-0421-5>
  39. Li N, Yan F, Wang N et al (2020) Distinct gut microbiota and metabolite profiles induced by different feeding methods in healthy Chinese infants. *Front Microbiol* 11:714. <https://doi.org/10.3389/fmicb.2020.00714>
  40. Thompson AL (2012) Developmental origins of obesity: early feeding environments, infant growth, and the intestinal microbiome. *Am J Hum Biol* 24:350–360. <https://doi.org/10.1002/ajhb.22254>
  41. Doueellou T, Montel MC, Sergentet DT (2017) Anti-adhesive properties of bovine oligosaccharides and bovine milk fat globule membrane-associated glycoconjugates against bacterial food enteropathogens. *J Dairy Sci* 100:3348–3359. <https://doi.org/10.3168/jds.2016-11611>
  42. Underwood MA, German JB, Lebrilla CB, Mills DA (2015) *Bifidobacterium longum* subspecies *infantis*: champion colonizer of the infant gut. *Pediatr Res* 77:229. <https://doi.org/10.1038/pr.2014.156>
  43. Bouatra S, Aziat F, Mandal R et al (2013) The human urine metabolome. *PLoS ONE* 8:e73076. <https://doi.org/10.1371/journal.pone.0073076>
  44. Bowling FG, Thomas M (2014) Analyzing the metabolome. *Methods Mol Biol* 1168:31–45. [https://doi.org/10.1007/978-1-4939-0847-9\\_3](https://doi.org/10.1007/978-1-4939-0847-9_3)
  45. Gonthier MP, Cheynier V, Donovan JL, Manach C, Morand C, Mila I, Lapiere C, Révész C, Scalbert A (2003) Microbial aromatic acid metabolites formed in the gut account for a major fraction of the polyphenols excreted in urine of rats fed red wine polyphenols. *J Nutr* 133(461–467):42. <https://doi.org/10.1046/j.1365-277X.2003.00411.x>
  46. O'Sullivan A, He X, McNiven EMS, Hinde K, Haggarty NW, Lönnerdal B, Slupsky CM (2013) Metabolomic phenotyping validates the infant rhesus monkey as a model of human infant metabolism. *J Pediatr Gastroenterol Nutr* 56:355–363. <https://doi.org/10.1097/MPG.0b013e31827e1f07>
  47. Riesberg LA, Weed SA, McDonald TL, Eckerson JM, Drescher KM (2016) Beyond muscles: the untapped potential of creatine. *Int Immunopharmacol* 37:31–42. <https://doi.org/10.1016/j.intimp.2015.12.034>

48. Cunha MP, Martin-De-Saavedra MD, Romero A, Javier E, Fabiana KL, Carla IT, Marcelo F, Ana LSR, Manuela GL (2014) Both creatine and its product phosphocreatine reduce oxidative stress and afford neuroprotection in an in vitro Parkinson's model. *ASN Neuro* 6:1–6. <https://doi.org/10.1177/1759091414554945>
49. Muccioli GG, Naslain D, Bäckhed F, Reigstad CS, Lambert DM, Delzenne NM, Cani PD (2010) The endocannabinoid system links gut microbiota to adipogenesis. *Mol Syst Biol* 6:392. <https://doi.org/10.1038/msb.2010.46>
50. Carlier JP (2015) Veillonella. In: David H (ed) *Bergey's manual of systematics of archaea and bacteria*, 2nd edn. John Wiley & Sons, Hoboken, pp 1–11