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Xinhua Xiao *et al.*

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Gut microbiota might be a crucial factor in deciphering the metabolic benefits of perinatal genistein consumption in dams and adult female offspring†

Liyuan Zhou,  Xinhua Xiao, * Qian Zhang, Jia Zheng, Ming Li, Xiaojing Wang, Mingqun Deng, Xiao Zhai and Jiaying Liu

Adverse early-life exposures program an increased risk of chronic metabolic diseases in adulthood. However, the effects of genistein consumption in early life on metabolic health are unclear. Our objective was to investigate whether perinatal genistein intake could mitigate the deleterious effects of a high-fat diet (HF) on metabolism in dams and female offspring and to explore the role of the gut microbiota in mediating the transgenerational effects. C57BL/6 female mice were fed a HF, HF with genistein (0.6 g kg⁻¹ diet) or normal control diet for 3 weeks before mating and throughout pregnancy and lactation. The offspring had free access to normal diet from weaning to 24 weeks of age. A glucose tolerance test was performed and the levels of serum insulin and lipid were measured. The cecal contents were collected for 16s rDNA sequencing. The results showed that perinatal genistein intake could not only significantly reduce blood glucose levels, insulin and free fatty acids (FFA) in dams, but also improve glucose tolerance, insulin sensitivity and serum lipid profiles in adult female offspring. Significant enrichment of short-chain fatty acid (mainly butyrate)-producing bacteria might play crucial roles in deciphering the metabolic benefits of perinatal genistein intake in dams. The obvious decrease in harmful microorganisms and increase in *Erysipelotrichaceae_incertae_sedis* were associated with the protective effects of maternal genistein intake on female offspring. In addition, *Bifidobacterium* might be an important factor for deciphering the metabolic improvement in both dams and female offspring by dietary genistein. Overall, perinatal genistein intake attenuated the harmful effects of HF on metabolism in both dams and female offspring, and the protective effects were associated with the alterations in the gut microbiota, which provides new evidence and targets for mitigating the poor effects of adverse early-life exposures on metabolic health in later life.

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1. Introduction

The global prevalence of metabolic disturbances, including glucose intolerance, insulin resistance and obesity, has brought unprecedented challenges. It is traditionally accepted that chronic metabolic diseases arise at the interface of genetics and environmental factors, including physical inactivity, smoking and high-calorie diet. In recent years, a large number of observational studies and experimental animal models have consistently highlighted the importance of the early-life environment in determining disease trajectories in later life.^{1,2} More specifically,

adverse intrauterine or postnatal exposures, including maternal obesity and gestational diabetes mellitus (GDM), programmed elevated risks of developing chronic metabolic diseases in adult life, such as obesity and glucose intolerance.³ Thus, the hypothesis of developmental origins of health and disease (DOHaD) has been put forward, which proposes that environmental exposures during critical windows of development program permanent changes in later life. Although epigenetics, microbiome and metabolome were all considered the potential mechanisms, the specific mechanism of the DOHaD still remains unclear.⁴ Substantial amounts of research and our previous studies both showed that maternal high-fat diet during pregnancy and lactation not only resulted in metabolic disorders *per se*, but significantly increased susceptibility to glucose intolerance, insulin resistance and lipid disorders in adult offspring.^{5–7} Therefore, the perinatal period might be a critical window for preventing the transgenerational transmission of metabolic diseases. Effective measures should be taken to reset the trajectories of chronic metabolic disease.

Key Laboratory of Endocrinology, Translational Medicine Center, Ministry of Health, Department of Endocrinology, Peking Union Medical College Hospital, Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing, China.
E-mail: xiaoxh2014@vip.163.com

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Over the last few decades, increasing research has provided evidence to support the beneficial role of bioactive dietary compounds in the prevention and treatment of metabolic disorders.⁸ Genistein, as one of the major components in soy isoflavone, has a similar structure to 17 β -estradiol and is also known as a phytoestrogen. The protective effects of genistein on cancer,⁹ bone metabolic disease¹⁰ and cardiovascular disease¹¹ have been extensively researched. The safety of genistein consumption has been confirmed in both animals and humans.¹² In terms of metabolic diseases, several large epidemiological studies preliminarily showed that the dietary intake of soy isoflavone was negatively correlated with the risk of type 2 diabetes mellitus (T2DM), especially in obese population.^{13,14} Subsequently, emerging clinical trials and animal experiments verified the benefits of genistein on glucose intolerance, insulin sensitivity and lipid profile disorders.^{15,16} It has been indicated that the potential mechanisms deciphering the protective effects of genistein against metabolic disorders included directly enhancing the proliferation of β -cells, inhibiting apoptosis, promoting insulin secretion, epigenetic modifications, regulating the cAMP/PKA signaling pathway, and modulating the gut microbiota.^{12,17} However, studies exploring the effects and mechanism of perinatal genistein consumption on transgenerational metabolic health are lacking.

The gut microbiota, as an environmental factor, plays crucial roles in the metabolic health. Limited studies have found that genistein intake could significantly modify the imbalance of the gut microbiota induced by a high-fat diet or in type 1 diabetic mice.^{17,18} Thus, the role of the intestinal microbial community in the effects of genistein intake on metabolism still needs further exploration. In addition, recent research evidence challenged the traditional view of the intrauterine sterile environment, since the microbiota was detected in amniotic fluid, cord blood and placenta during normal pregnancy.¹⁹ Poor perinatal exposures could impair the balance of the gut microbiota in both mothers and offspring.²⁰ Therefore, increasing studies have suggested that the gut microbiota might play a key role in the transgenerational effects of adverse early-life exposures on metabolism in later life. However, whether the genistein intake experienced by mothers could regulate the gut microbiota in both dams and offspring is unclear.

Therefore, we aimed to investigate the effects of perinatal genistein intake for 3 weeks prior to mating, and throughout pregnancy and lactation on glucose and lipid metabolism in both dams and female offspring. In addition, we further explored whether the gut microbiota plays vital roles in the transgenerational effects of genistein consumption on metabolic health.

2. Materials and methods

2.1 Animals and study design

C57BL/6 mice used in this experiment were obtained from the National Institutes for Food and Drug Control (Beijing, China, SYXK-2018-0019). The mice were kept in a controlled environ-

ment of room temperature at 22 ± 2 °C with a 12 h light/dark cycle. All mice had *ad libitum* access to food and sterile water during the study. After one week of acclimation, five-week-old female mice were randomly divided into three groups: the control group (MC, $n = 8$), which received a standard rodent diet (AIN-93G) (15.8% of the calories as fat); high-fat group (MHF, $n = 8$), which received a high-fat diet (60% of the calories as fat); and high-fat with genistein group (MHFG, $n = 7$), which received a high-fat diet with genistein (CAS: 466-72-0, G0272, TCI Development Co., Ltd) (0.6 g kg⁻¹ diet). The ingredients are shown in ESI Table S1.† Females were fed these diets for 3 weeks prior to mating and throughout pregnancy and lactation.

After the first three weeks of intervention, the female mice were mated with normal control C57BL/6 males. At birth, the litters were adjusted to 5 pups for each dam to ensure that there was no nutritional bias between litters. The offspring were weaned at 3 weeks of age. At weaning, all dams were sacrificed, and female offspring from the three groups (FC, FHF and FHFG) ($n = 7-8$ per group) were given *ad libitum* access to a standard chow diet until 24 weeks of age. At the end of the experiment, one female offspring from different litters ($n = 7-8$ per group) was sacrificed. Blood samples were collected from the intraorbital retrobulbar plexus after 10 h of fasting from anesthetized mice, and the inguinal subcutaneous adipose tissue (SAT) and perirenal visceral adipose tissue (VAT) were removed and weighed; the cecal contents were quickly removed, snap frozen in liquid nitrogen, and then stored at -80 °C for further analysis in both dams and female offspring. All of the procedures were approved by the animal care and use committee of the Peking Union Medical College Hospital (Beijing, China, SYXC-2014-0029). All of the animal operations were conducted in compliance with the National Institutes of Health guide for the care and use of laboratory animals.

2.2 Body weight, gestational weight gain (GWG) and birth weight

The body weights of dams and female offspring were measured per week. The gestational weight gain was calculated from the body weight before delivery (6th week of intervention) minus that before mating (3rd week of intervention). At birth, the litters were weighed and the values were recorded as birth weights.

2.3 Fasting blood glucose (FBG) levels at the first week of pregnancy

To detect the effects of genistein intake on maternal blood glucose levels during pregnancy, we only measured the FBG levels at the first week of pregnancy based on concerns over safety. The dams were fasted for 6 h. The FBG levels were measured from tail vein blood using a Contour TS glucometer (ACCU-CHEK Mobile, Beijing, China).

2.4 Oral glucose tolerance tests (OGTT)

The OGTT were performed in dams at weaning and female offspring at 4, 12 and 24 weeks of ages. The mice were fasted for 6 h and given a glucose load (2 g per kg of body weight) by



gavage. Blood glucose (BG) levels were measured at 0, 30, 60, and 120 min after the gavage also from the tail vein blood. The area under the curve (AUC) of the OGTT was calculated as previously described.²¹

2.5 Serum biochemical parameter measurement

The blood samples collected from dams at weaning and female offspring at 24 weeks of age were centrifuged at 3000g for 10 min at 4 °C, and the serum was stored in aliquots at -80 °C. The serum insulin concentrations, total cholesterol (TC), triacylglycerol (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and free fatty acids (FFA) were measured as previously described.²² Insulin sensitivity was assessed using the homeostasis model assessment of insulin resistance (HOMA-IR). The HOMA-IR was calculated as previously described.²¹

2.6 Gut microbiota analysis

To determine the effects of genistein consumption on the maternal and female offspring gut microbiota, we performed the 16s rDNA sequencing. Microbial DNA was extracted from the cecal contents of dams at weaning and female offspring at 24 weeks of age using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's protocols. The V3-V4 regions of the 16S rRNA genes were amplified using the primers 341F 5'-CCTACGGGSGCAGCAG-3' and 806R, 5'-GGACTACVGGGTATCTAATC-3'. Amplicons were purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) and quantified using Qubit @2.0 (Invitrogen, U.S.). The tags were sequenced on the Illumina HiSeq platform (Illumina, Inc., CA, USA).

After merging paired-end reads, reads were performed by quality filtering. High quality reads were clustered into operational taxonomic units (OTUs) with a 97% similarity using UPARSE software (version 7.0.1001),²³ and representative sequences for each OTU were screened using QIIME software (version 1.7.0, Quantitative Insights into Microbial Ecology).²⁴ Then, the GreenGene Database²⁵ was used to annotate taxonomic information based on the RDP classifier version 2.2 algorithm.²⁶ Alpha and beta diversity analyses were performed using QIIME software (Version 1.7.0) and R software (Version 2.15.3). For alpha diversity, Chao1, observed_species and PD_whole_tree were analyzed. For beta diversity, principal coordinates analysis (PCoA), Adonis, and analysis of similarities (ANOSIM) were performed using unweighted UniFrac. In addition, linear discriminant analysis (LDA) of the effect size (LEfSe) was used to determine differences among the groups.

2.7 Statistical analysis

The data were expressed as mean ± standard error of the mean (S.E.M). The statistics were analyzed by one-way ANOVA and two-way ANOVA, with Tukey *post hoc* analyses. The differences in the relative abundances of the gut microbiota were analyzed by the Kruskal-Wallis test, with Benjamini and Hochberg *post hoc* analyses. Correlation analyses between the relative abundances of bacterial taxa at genus levels and metabolic para-

eters were performed by the Spearman correlation coefficient test. A *p* value < 0.05 was considered statistically significant. Prism version 7.0 (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analysis.

3. Results

3.1 The effects of high-fat diet and genistein consumption on body weight as well as glucose and lipid metabolism in dams

As shown in Fig. 1a, there was no difference in the body weights among the three groups at the baseline and before the 6th week of intervention. Subsequently, the body weights of the dams in the MHF group were significantly higher than those in the MC group from the time of delivery (6th week of intervention) (*p* < 0.05) to weaning (9th week of intervention) (*p* < 0.0001). However, the GWG among the three groups were not significantly different. To determine the effects of high-fat diet and genistein consumption on glucose metabolism during pregnancy, we detected the FBG levels at the first week of pregnancy. After 4 weeks of intervention, HF fed dams had dramatically elevated FBG than that in the MC group (*p* < 0.0001), whereas genistein intake significantly reduced the FBG levels compared with those in the MHF group (*p* < 0.0001). At weaning, 9 weeks of HF led to significantly elevated BG levels at 30 min (*p* < 0.01), 60 min (*p* < 0.01) and 120 min (*p* < 0.05) during the OGTT and larger AUC values (*p* < 0.01) compared to those fed the control diet. However, perinatal genistein consumption did not alter the BG levels induced by HF in dams at weaning (Fig. 1d and e). In order to explore the effects of diet intervention on insulin sensitivity in dams, we measured the fasting serum insulin levels and calculated the HOMA-IR index. The results showed that perinatal 9 weeks of HF significantly elevated the serum insulin levels (*p* < 0.001) and the HOMA-IR index (*p* < 0.05) in the dams of the MHF group compared with those in the MC group, whereas genistein intake tended to reduce the fasting insulin levels induced by HF in dams at weaning (*p* = 0.05) (Fig. 1f and g).

In addition to glucose metabolism, we further analyzed the effects of 9 weeks of diet intervention on lipid metabolism and fat mass in dams at weaning. As shown in Fig. 1h-l, HF fed dams had higher serum levels of TC (*p* < 0.001), TG (*p* < 0.01), LDL-C (*p* < 0.001) and FFA (*p* < 0.05) than those in the MC group. Perinatal genistein consumption tended to decrease the FFA levels elevated by HF in the MHFG fed dams (*p* = 0.09). Furthermore, in comparison with the dams of the MC group, perinatal HF exposure also dramatically increased the relative SAT (*p* < 0.01), VAT (*p* < 0.001) and WAT (SAT + VAT) (*p* < 0.0001) mass in the dams of the MHF group. Nevertheless, perinatal 9 weeks of genistein intake did not significantly change the relative fat mass in dams at weaning.

3.2 The changes of the gut microbiota in dams at weaning

To investigate the effects of perinatal diet intervention on the gut microbiota in dams at weaning, we performed the 16s



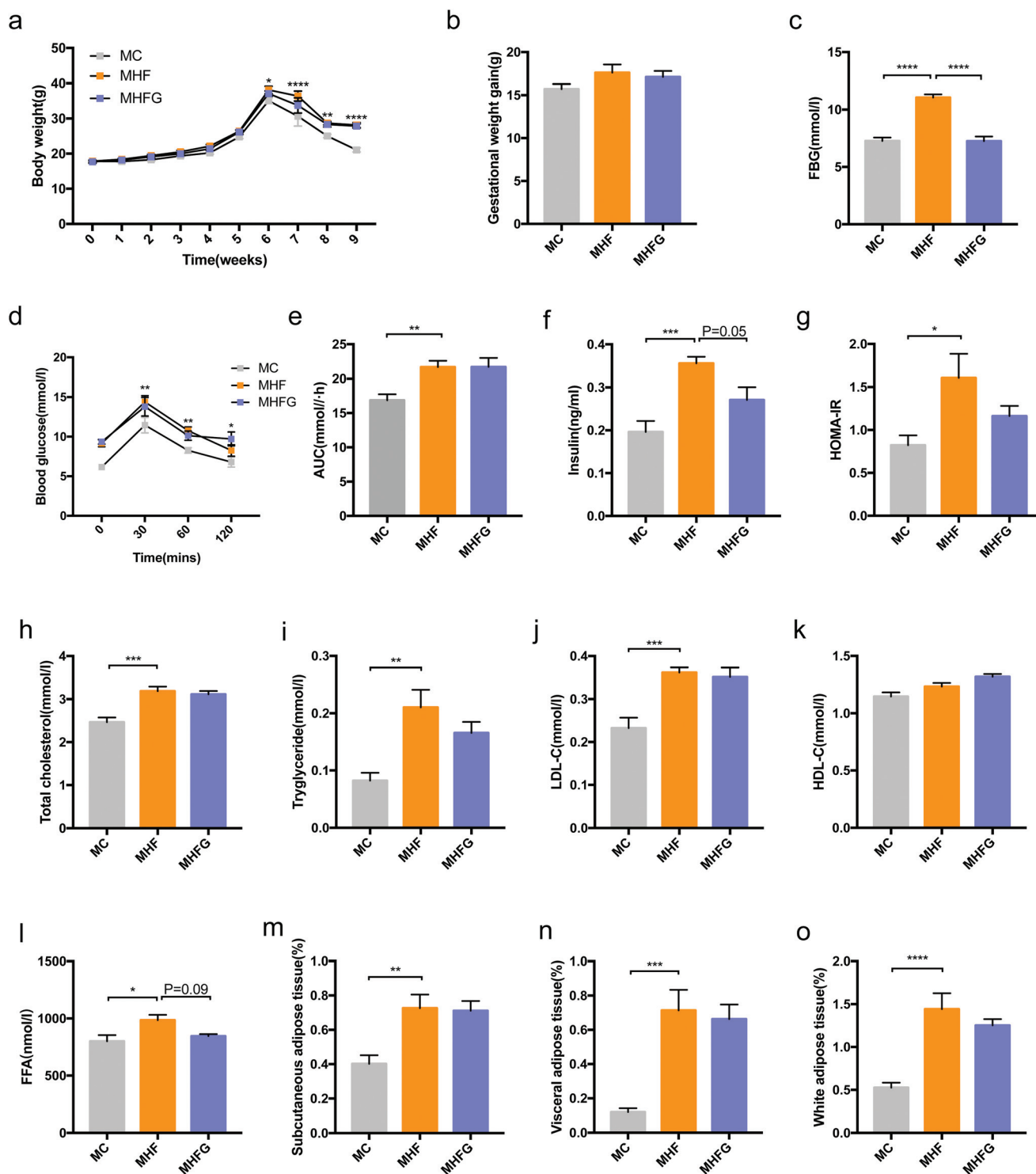


Fig. 1 Metabolic parameters in dams. (a) Body weight changes between interventions; (b) GWG; (c) FBG at the first week of pregnancy; (d) OGTT; (e) AUC; (f) serum insulin levels; (g) HOMA-IR; (h) TC; (i) TG; (j) LDL-C; (k) HDL-C; (l) FFA; (m) relative content of subcutaneous adipose tissue; (n) relative content of visceral adipose tissue; and (o) relative content of white adipose tissue. MC, normal control diet in dams; MHF, high-fat diet in dams; MHFG, high-fat diet with genistein in dams; GWG, gestational weight gain; FBG, fasting glucose level; OGTT, oral glucose tolerance test; AUC, area under the curve; HOMA-IR, homeostasis model assessment of insulin resistance; TC, total cholesterol; TG, triacylglycerol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; and FFA, free fatty acid. Data are expressed as means \pm S.E.M. ($n = 7-8$ per group). Mean values were significantly different between the MC group and the MHF group in body weight and during the OGTT: *, mean values were significantly different between the groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.



rDNA sequencing for the cecal contents. The sequencing data have been submitted to the Sequence Read Archive (SRA) database (PRJNA543285). Firstly, we assessed the OTUs among the three groups to identify the shared and unique species. A Venn diagram showed that there were 275 shared OTUs, 56 unique OTUs in the dams of the MC group, 2 unique OTUs in HF fed dams and 8 unique OTUs in the dams of the MHFG group

(Fig. 2a). Then we evaluated the composition of the gut microbial community in dams. At the phylum level, Firmicutes, Bacteroidetes, Verrucomicrobia and Proteobacteria were the most abundant, whereas the relative abundances of the four phyla were not significantly different among the three groups (Fig. 2b). The abundance of phylum Defferribacteres was dramatically elevated in the HF fed dams than that in the

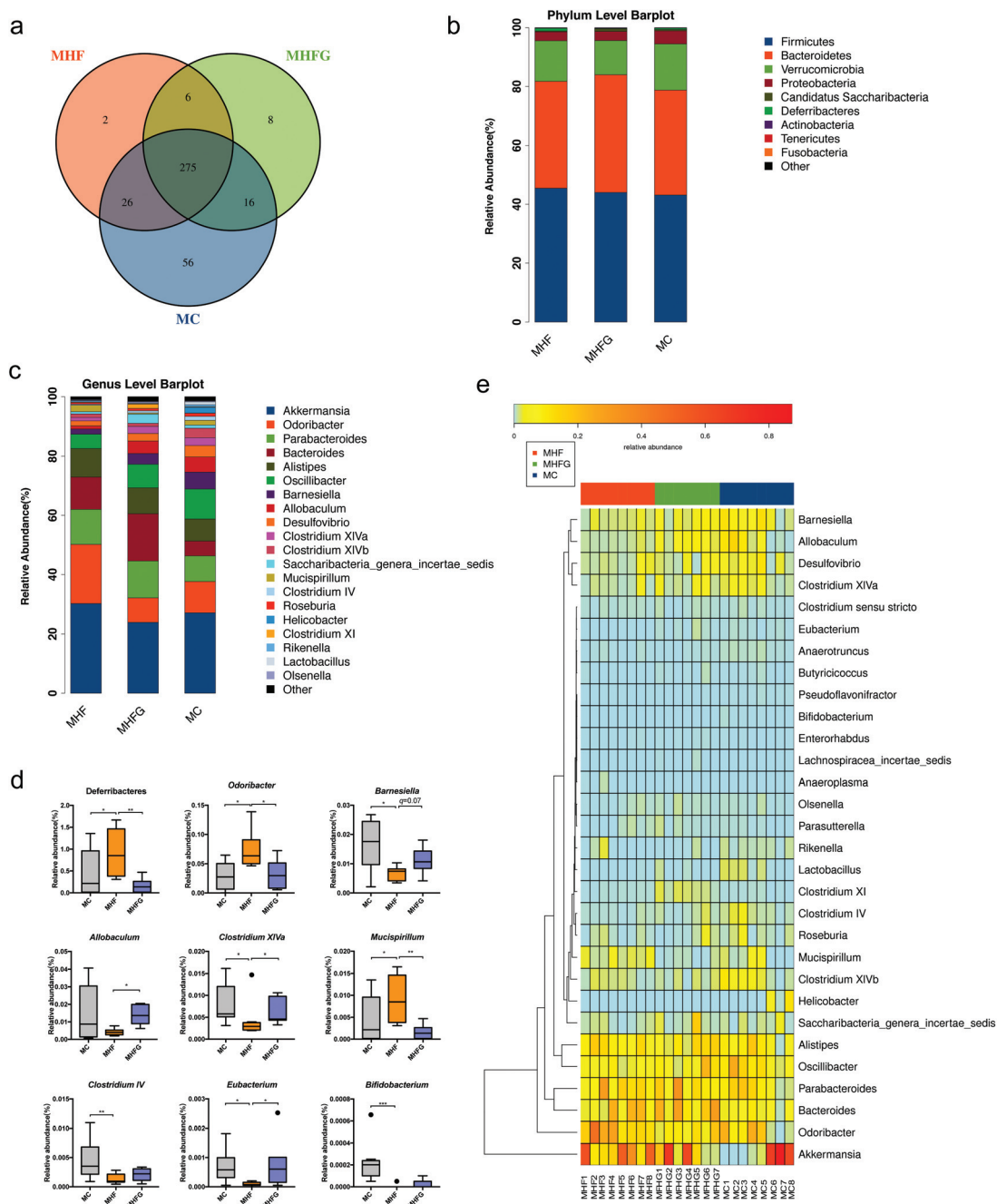


Fig. 2 The changes of the gut microbiota in dams at weaning. (a) Venn diagram of the OUTs; (b) relative abundances of the top ten phyla; (c) relative abundances of the top twenty genera; (d) significantly different germs at the phylum and genus levels; and (e) heat map analysis of the different genera among the three groups. MC, normal control diet in dams; MHF, high-fat diet in dams; and MHFG, high-fat diet with genistein in dams. Data are expressed as means \pm S.E.M. ($n = 7-8$ per group). Mean values were significantly different between the groups: * $q < 0.05$, ** $q < 0.01$, *** $q < 0.001$.



MC group ($q < 0.05$), and perinatal genistein intake could significantly reduce this phylum ($q < 0.01$). Fig. 2c shows the relative abundances of the top 20 species at the genus level among the three groups. The abundances of *Odoribacter* ($q < 0.05$) and *Mucispirillum* ($q < 0.05$) were significantly higher in HF fed dams compared with those in the MC group, and were dramatically decreased ($q < 0.05$, $q < 0.01$) in dams fed genistein (MHFG group). In contrast, 9 weeks of HF resulted in lower abundances of *Barnesiella* ($q < 0.05$), *Clostridium XIVa* ($q < 0.05$), *Clostridium IV* ($q < 0.01$), *Eubacterium* ($q < 0.05$) and *Bifidobacterium* ($q < 0.001$) in the dams of the MHF group compared to those of the MC group. Perinatal addition of genistein to the HF could dramatically increase the abundance of *Allobaculum* ($q < 0.05$), *Barnesiella* ($q = 0.07$), *Clostridium XIVa* ($q < 0.05$) and *Eubacterium* ($q < 0.05$) (Fig. 2d). Significantly different species at the genus level among the three groups were summarized in a heatmap (Fig. 2e).

Then we evaluated the structure of the gut microbiota in dams among the three groups. Alpha diversity analysis showed that HF fed dams had significantly decreased community richness and diversity compared with the mice in the MC group (Fig. 3a–c). No significant differences in alpha diversity were observed between the mice in the MHFG group and the MHF group. PCoA based on unweighted UniFrac distances showed that the gut microbial community was significantly separated in dams among the three groups (Fig. 3d). ANOSIM analysis demonstrated that the differences among the groups were significant ($R = 0.686$, $p = 0.001$) (Fig. 3e).

3.3 The effects of maternal high-fat diet and genistein intake on energy metabolic parameters in female offspring

In addition to the influences of perinatal genistein consumption on dams, we further detected the effects of maternal HF and genistein intake on body weight as well as glucose and lipid metabolism in female offspring. As shown in Fig. 4a,

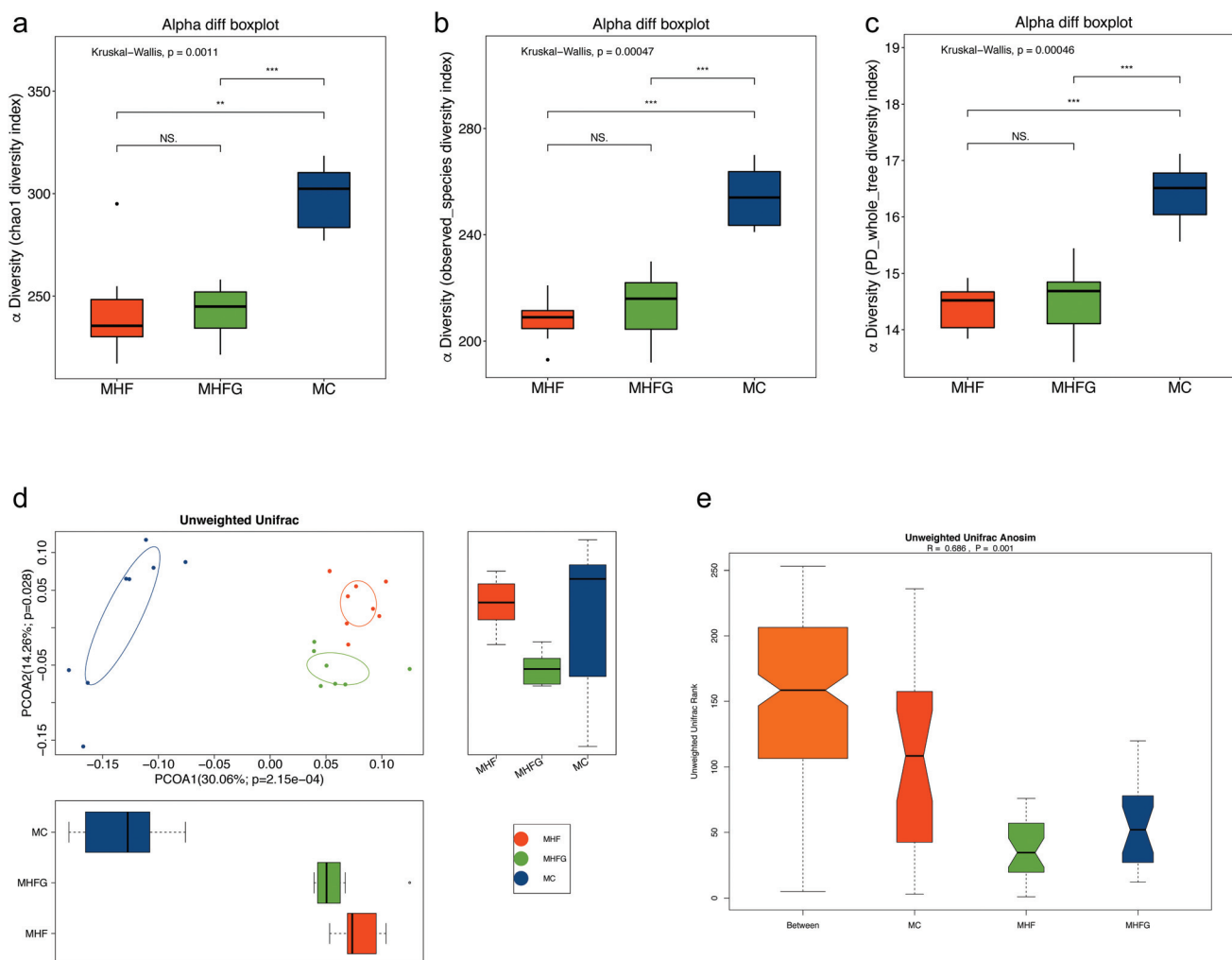


Fig. 3 Alpha-diversity and beta-diversity analyses of the gut microbiota in dams at weaning. (a) Chao1 index; (b) observed_species index; (c) PD_whole_tree index; (d) PCoA plots of gut communities; and (e) unweighted UniFrac ANOSIM analysis between the three groups ($n = 7-8$ per group). MC, normal control diet in dams; MHF, high-fat diet in dams; and MHFG, high-fat diet with genistein in dams. Data are expressed as means \pm S.E.M. ($n = 7-8$ per group). Mean values were significantly different between the groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



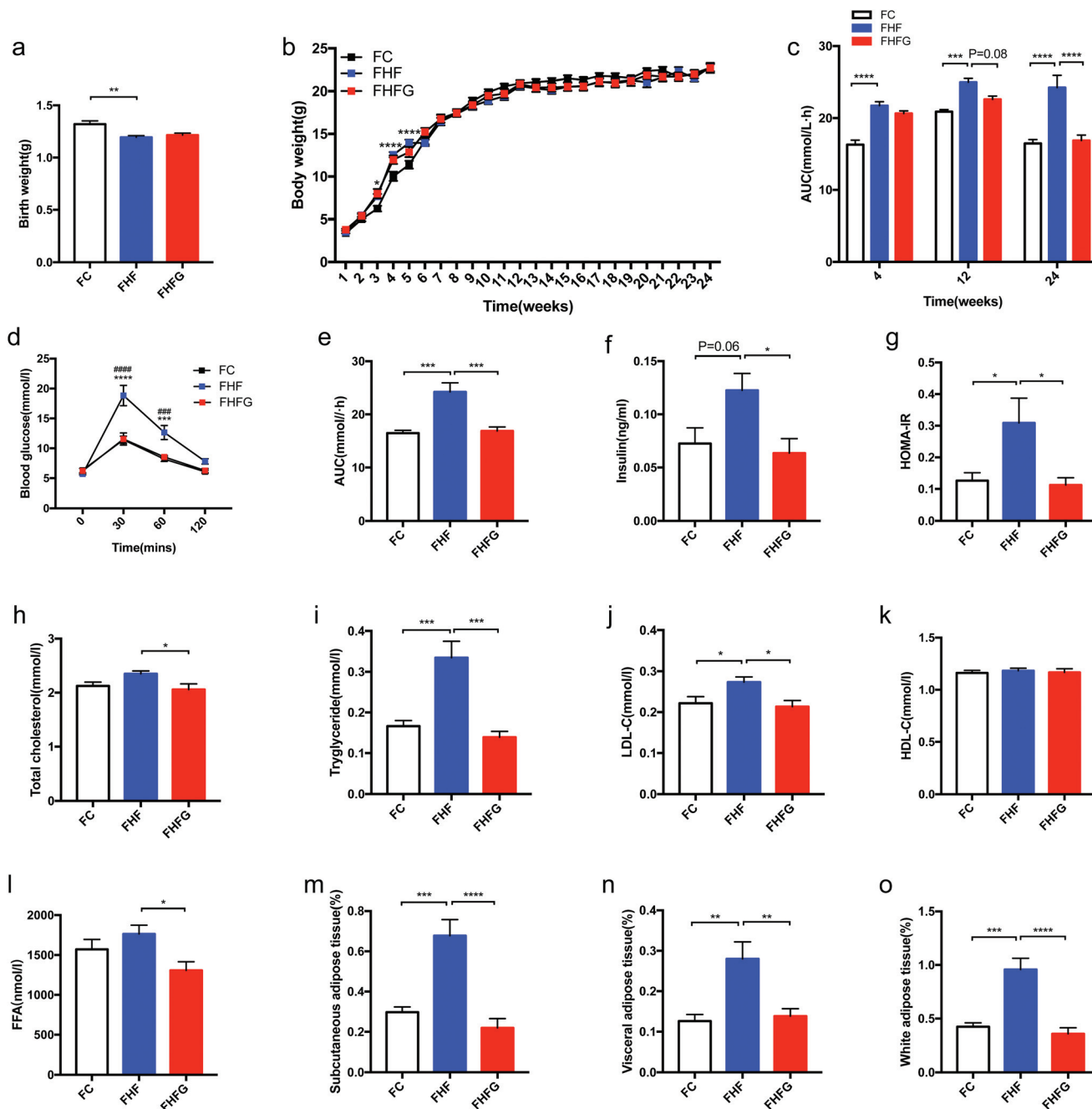


Fig. 4 Metabolic parameters in female offspring. (a) Birth weight; (b) body weight from birth to 24 weeks of age; (c) AUC at 4, 12 and 24 weeks of ages; (d) OGTT; (e) AUC; (f) serum insulin levels; (g) HOMA-IR; (h) TC; (i) TG; (j) LDL-C; (k) HDL-C; (l) FFA; (m) relative content of subcutaneous adipose tissue; (n) relative content of visceral adipose tissue; and (o) relative content of white adipose tissue. FC, female offspring of normal control diet fed dams; FHF, female offspring of high-fat diet fed dams; FHFG, female offspring of high-fat diet with genistein fed dams; OGTT, oral glucose tolerance test; AUC, area under the curve; HOMA-IR, homeostasis model assessment of insulin resistance; TC, total cholesterol; TG, triacylglycerol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; and FFA, free fatty acid. Data are expressed as means \pm S.E. M. ($n = 7-8$ per group). Mean values were significantly different between the FC group and the FHF group in body weight and during the OGTT: *, mean values were significantly different between the FHFG group and the FHF group during the OGTT: #. Mean values were significantly different between the groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

maternal HF led to significantly lower birth weight in offspring compared with that of the control diet fed dams ($p < 0.01$). However, the body weight was dramatically higher in female offspring at the end of lactation (3rd week) in the

FHF group than that in the FC group ($p < 0.0001$). Thus, a catch-up growth existed in the offspring of the HF fed dams (Fig. 4b). Subsequently, there were no differences in body weights between female offspring in the FHF group and the



FC group. Nevertheless, no effects of maternal genistein intake on the birth weight or body weight induced by HF were found in female offspring.

Maternal HF during 3 weeks before mating and throughout pregnancy and lactation resulted in significantly larger AUC of the OGTT in female offspring from early life (4 weeks of age) ($p < 0.0001$) to adult life (12 and 24 weeks of age) than that of the C fed dams ($p < 0.001$, $p < 0.0001$). Although there were no effects of perinatal genistein intake on glucose tolerance in early life of female offspring, the AUC tended to decrease at 12 weeks of age ($p = 0.08$) and was significantly reduced when the mice were 24 weeks of age compared with those in the FHF group ($p < 0.0001$). In the meantime, perinatal genistein consumption reduced the elevated BG levels at 30 min ($p < 0.0001$) and 60 min ($p < 0.001$) induced by maternal HF in female offspring. Furthermore, we analyzed the transgenerational effects of diet intervention on insulin sensitivity in female offspring at 24 weeks of age. The results showed that serum insulin levels ($p = 0.06$) and HOMA-IR ($p < 0.05$) of offspring in the FHF group were significantly higher than those from the FC group. Maternal dietary genistein prevented the deleterious effects of maternal HF on insulin sensitivity, with significantly lower insulin levels ($p < 0.05$) and HOMA-IR ($p < 0.05$) (Fig. 4f and g).

Moreover, the transgenerational effects on lipid metabolism were also assessed. The fasting serum TG ($p < 0.001$) and LDL-C ($p < 0.05$) were significantly elevated in female offspring at 24 weeks of age by maternal HF compared with that of the control diet fed dams. Maternal genistein consumption could dramatically reduce the TC ($p < 0.05$), TG ($p < 0.001$), LDL-C ($p < 0.05$) and FFA ($p < 0.05$) levels in the dams of the FHF group compared to those of the FHF group (Fig. 4h, i, j and l). No significant differences were observed in serum HDL-C levels among the three groups (Fig. 4k). Although no differences were found in body weights in female offspring at 24 weeks of age, we further detected whether fat mass was influenced. Interestingly, maternal HF significantly increased the relative mass of SAT ($p < 0.001$), VAT ($p < 0.01$) and WAT ($p < 0.001$) in offspring, which were all dramatically reduced by perinatal genistein intake ($p < 0.0001$, $p < 0.01$, and $p < 0.0001$, respectively) (Fig. 4m, n and o).

3.4 The alterations of the gut microbiota in female offspring at 24 weeks of age

Next, we analyzed how maternal genistein intake modulated the gut microbiota in female offspring at 24 weeks of age. A Venn diagram showed that there were 301 shared OTUs among the three groups, 11 unique OTUs in the female offspring of the control diet fed dams, 21 OTUs in the offspring of the FHF group and 7 unique OTUs in the offspring of the genistein fed dams (Fig. 5a). Fig. 5b shows the top 10 phyla among the three groups. The relative abundances of Bacteroidetes ($q < 0.01$) and Proteobacteria ($q < 0.05$) were significantly increased in the female offspring of the HF fed dams compared with those of control diet fed dams, and were dramatically reduced by maternal genistein supplementation ($q < 0.05$, $q < 0.001$). The

top 20 species at the genus level are shown in Fig. 5c. Maternal HF dramatically elevated the abundance of *Alloprevotella* ($q < 0.05$) while reducing the abundance of *Bifidobacterium* ($q < 0.01$) in female offspring compared with that in the FC group. Perinatal genistein intake significantly decreased the *Alloprevotella* in female offspring ($q < 0.05$) (Fig. 5d). The different germs at the genus level in female offspring at 24 weeks of age among the three groups are summarized in Fig. 5e.

The structure of the gut microbiota in female offspring was also assessed. Alpha diversity analysis showed that there were parallel community richness and diversity among the three groups. PCoA based on unweighted Unifrac distances demonstrated that the microbiota community in female offspring among the groups was dramatically separated, which was supported by ANOSIM analysis ($R = 0.42$, $p = 0.001$) (Fig. 6a and b).

3.5 The significantly enriched microbiota at different levels in dams and female offspring

We next identified individual microbial species which differed between groups from the phylum level to the genus level in dams and offspring, respectively. At the genus level, maternal high-fat diet significantly enriched *Odoribacter* from the *Odoribacteraceae* family and *Mucispirillum* from the *Deferribacteraceae* family, while *Allobaculum* from *Erysipelotrichaceae*, *Eisenbergiella* from *Lachnospiraceae*, and *Clostridium IV* from *Ruminococcaceae*, *Lactobacillus*, and *Rikenella* were abundant in control diet fed dams. Perinatal genistein consumption enriched the genus *Eubacterium* and some other germs in dams at weaning (Fig. 7a). As for female adult offspring, *Alloprevotella* from the *Prevotellaceae* family was significantly increased in the FHF group. Control diet fed dams resulted in significant enrichment of *Parvibacter*, *Bifidobacterium* and *Rikenella* in female offspring. The genus *Erysipelotrichaceae_incertae_sedis* from the *Erysipelotrichaceae* family was significantly enriched in the female offspring of genistein fed dams (Fig. 7b).

3.6 Correlation analysis of the differential bacteria at the genus level and metabolic parameters in dams and female offspring

In order to analyze the relationship between the gut microbiota and metabolism, we performed a correlation analysis between the abundance of significantly altered bacteria at the genus level and metabolic parameters in both dams and female offspring. Fig. 8 shows the relationship between the parameters and gut microbiota in dams. The significantly enriched genus *Eubacterium* in the MHFG group was negatively correlated with the FBG during pregnancy, BG60 during the OGTT and LDL-C in dams. The relative abundances of *Clostridium XIVa*, *Clostridium IV* and *Barnesiella*, which were elevated by 9 weeks of genistein intake, were all negatively correlated with the FBG during pregnancy, HOMA-IR, and TG and LDL-C levels in dams. In contrast, the significantly reduced *Odoribacter* and *Mucispirillum* in the dams of the MHFG group



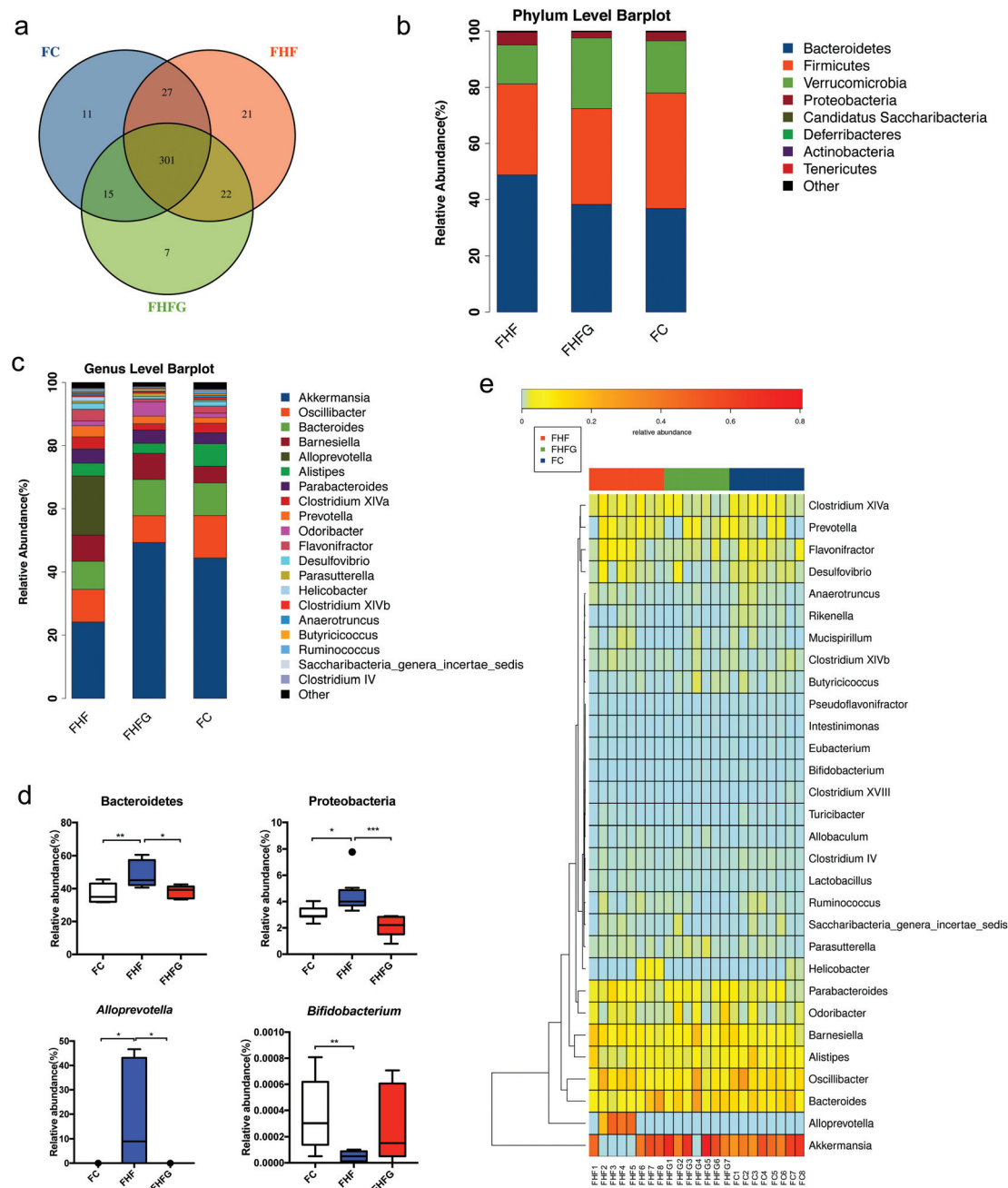


Fig. 5 The changes of the gut microbiota in adult offspring. (a) Venn diagram of the OTUs; (b) relative abundances of the top ten phyla; (c) relative abundances of the top twenty genera; (d) significantly different germs at the phylum and genus levels; and (e) heat map analysis of the different genera among the three groups. FC, female offspring of normal control diet fed dams; FHF, female offspring of high-fat diet fed dams; and FHFG, female offspring of high-fat diet with genistein fed dams. Data are expressed as means \pm S.E.M. ($n = 7-8$ per group). Mean values were significantly different between the groups: * $q < 0.05$, ** $q < 0.01$, *** $q < 0.001$.

were positively related to the FBG levels during pregnancy and BG60 during the OGTT. As for female offspring, elevated *Erysipelotrichaceae_incertae_sedis* by maternal genistein intake in adult female offspring was negatively correlated with BG0 during the OGTT, serum insulin levels, and SAT and WAT mass. However, the relative abundance of *Alloprevotella*, which was dramatically reduced in the female offspring of the genistein fed dams compared with that in the FHF group, was posi-

tively correlated with the serum TC levels, and SAT, VAT and WAT mass (Table 1). Interestingly, the relative abundance of *Bifidobacterium* was significantly decreased in both the dams of the MHF group and the female offspring of the FHF group compared with that in the control diet fed dams. It was negatively correlated with not only the BG levels during the OGTT, insulin levels, HOMA-IR, serum lipid profiles (TG, TC and LDL-C) and fat mass in dams, but also the insulin levels,



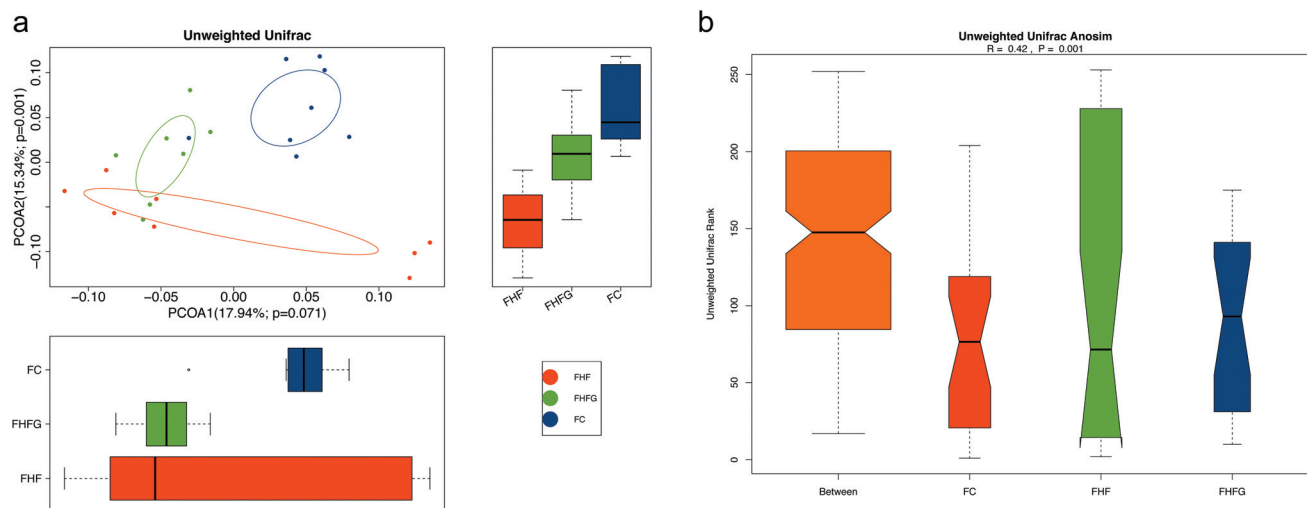


Fig. 6 Beta-diversity analysis of the gut microbiota in the adult female offspring ($n = 7-8$ per group). (a) PCoA plots of gut communities; and (b) unweighted Unifrac ANOSIM analysis between the three groups. FC, female offspring of normal control diet fed dams; FHF, female offspring of high-fat diet fed dams; and FHFG, female offspring of high-fat diet with genistein fed dams.

HOMA-IR, serum LDL-C levels, FFA and fat mass in adult female offspring.

4. Discussion

The global prevalence of chronic metabolic diseases has imposed enormous financial burden. Therefore, it is urgent to clarify the pathogenesis and develop new strategies to fight against the development of metabolic disturbances and obesity. In recent years, it has been widely reported that adverse early-life exposures could significantly increase the risks of metabolic disorders in later life.^{2,27} Consistent with the previous studies, our research also showed that perinatal 9 weeks of a high-fat diet led to significantly higher body weight, increased fat mass, glucose intolerance, insulin resistance and disorders of serum lipid profiles in not only dams at weaning but also adult female offspring. Maternal high-fat diet intervention during 3 weeks prior to mating, and throughout pregnancy and lactation led to an overnutritional early-life environment, which programmed abnormal metabolism in offspring. Thus, the perinatal period might be a critical window for preventing the occurrence and development of metabolic disorders.

The metabolic benefits of bioactive components have been extensively studied. Genistein is one of the major components of soy isoflavone. Both clinical trials and experimental animal models showed the protective effects of genistein supplementation against glucose and lipid metabolic disorders.^{15,17} However, it is still unclear whether early-life genistein intake could inhibit the transgenerational effects of perinatal overnutrition exposures on metabolism. Based on the proved safety of genistein intake in both animals and humans,¹² to the best of our knowledge, this is the first study to investigate whether

perinatal addition of genistein could counteract the deleterious effects of maternal high-fat diet on metabolism in both dams and female offspring. Our study showed that perinatal genistein consumption significantly reduced the FBG levels at the first week of pregnancy and decreased the serum insulin and FFA levels induced by high-fat diet in dams at weaning. In addition, there were larger metabolic benefits in adult female offspring. Perinatal addition of genistein to high-fat diet significantly programmed improved glucose tolerance, insulin sensitivity and serum lipid profiles, as well as lower fat mass in female offspring at 24 weeks of age, which were independent of the body weight. Therefore, this study confirmed the transgenerational metabolic benefits of perinatal genistein consumption.

Given the vital role of the gut microbiota in host health, we hypothesized that the intestinal microbial community might play crucial roles in deciphering the transgenerational metabolic benefits of genistein consumption. Indeed, our results showed that perinatal high-fat diet significantly increased the relative abundances of *Odoribacter* and *Mucispirillum* in dams at weaning. The genus *Odoribacter* has been reported to be associated with opportunistic infections, particularly intra-abdominal and systemic infections.²⁸ Walker *et al.*²⁹ found an unusual class of bacterial sulfonolipids in mice fed the high-fat diet and showed that *Odoribacter* was responsible for their production. The positive correlation between the abundance of *Odoribacter* and the adiposity index and visceral fat has been demonstrated in a human study.³⁰ In addition, Granado-Serrano *et al.*³¹ showed that men with hypercholesterolemia were characterized by the higher relative abundance of *Odoribacter*, which was also significantly correlated with the lipid parameters. In contrast, Garcia-Mazcorro *et al.*³² showed reduced abundance of *Odoribacter* in obese diabetic db/db mice supplemented with raspberry. Oligosaccharide sup-



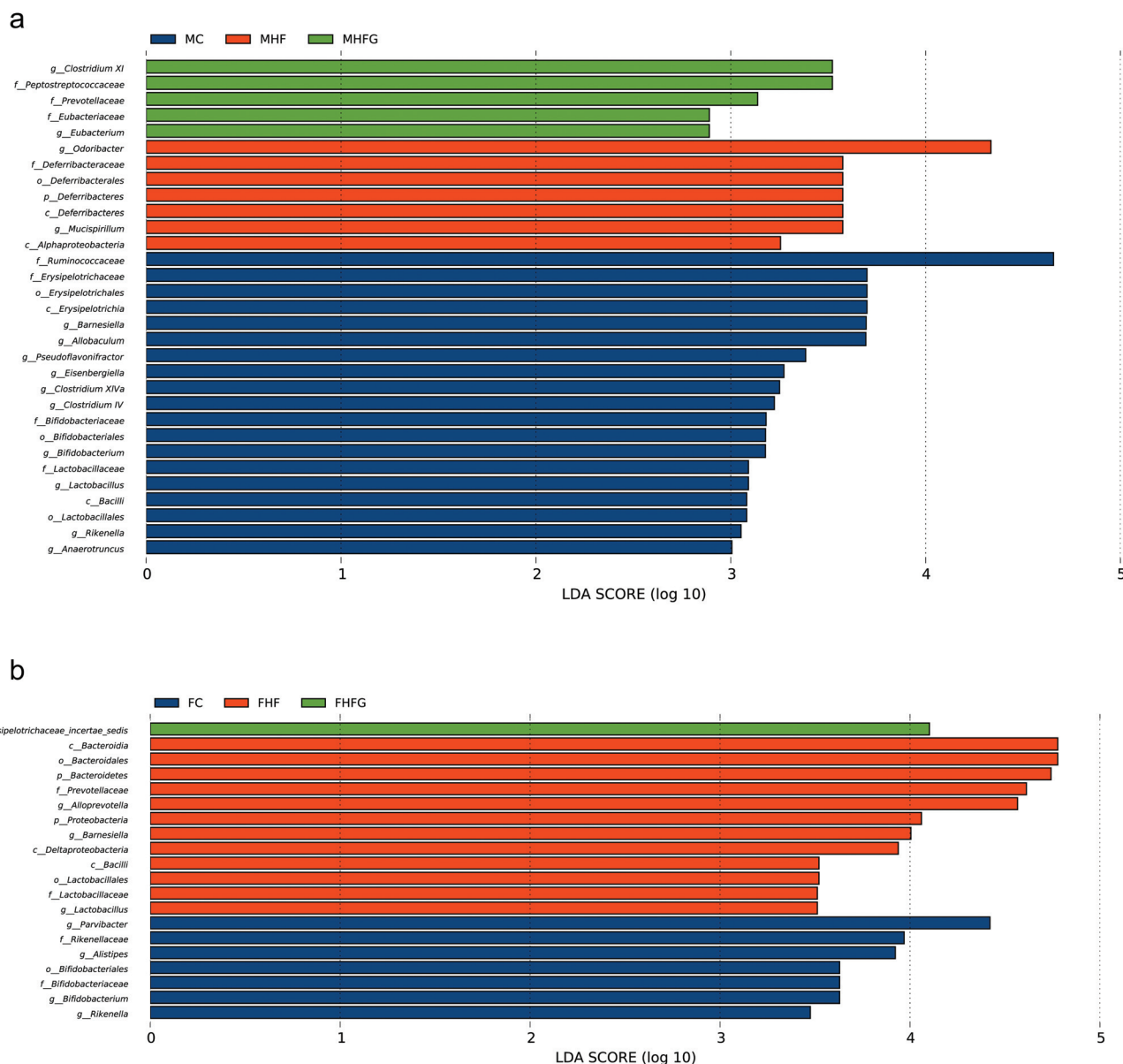


Fig. 7 LEfSe analysis of the different gut microbiota from the phylum level down to the genus level ($n = 7-8$ per group). (a) Among three groups in dams; and (b) among three groups in female offspring. MC, normal control diet in dams; MHF, high-fat diet in dams; MHFG, high-fat diet with genistein in dams. FC, female offspring of normal control diet fed dams; FHF, female offspring of high-fat diet fed dams; and FHFG, female offspring of high-fat diet with genistein fed dams.

plementation could increase the levels of short-chain fatty acids while reducing the abundance of *Odoribacter* in the feces of mice.³³ Consistent with these studies, our research also indicated that *Odoribacter* was an unfavorable species for metabolism, which was further confirmed by the positively correlation between *Odoribacter* and maternal blood glucose levels. *Mucispirillum* is Gram-negative bacteria and is inhabited in the intestinal mucus layer. However, it is an important mucin degrader,^{34,35} and could influence the intestinal permeability. The relationship between the abundance of *Mucispirillum* and intestinal inflammation has been confirmed.^{36,37} Xiao *et al.* showed that *Paederia scandens*

extracts could improve arthritis through reducing *Mucispirillum*.³⁸ The beneficial effects of dietary fiber, inulin and probiotic supplementation on metabolism and inflammation in diet-induced obese mice or db/db mice have also been reported to be associated with the decrease of *Mucispirillum*.³⁹⁻⁴¹ Our study also showed that significantly increased *Mucispirillum* induced by the high-fat diet was positively correlated with the maternal FBG levels. Nevertheless, perinatal genistein consumption dramatically reduced the unfavorable species elevated by the high-fat diet, which might be associated with the improvement of metabolism in dams.



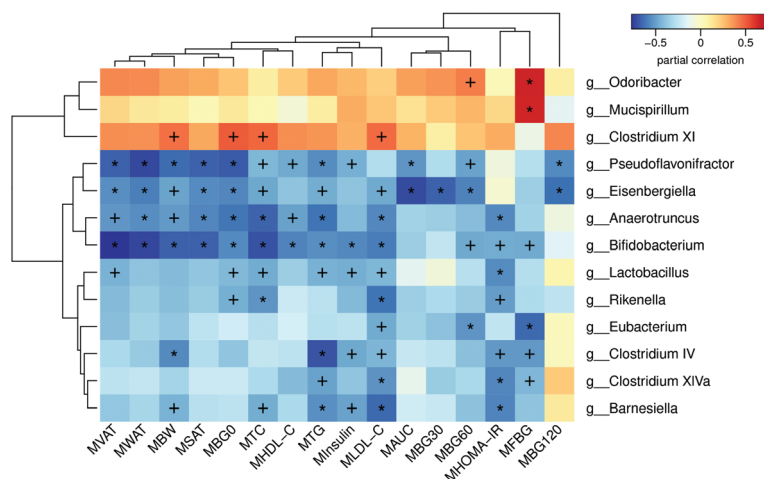


Fig. 8 Heatmap of Spearman correlation analysis between the altered genera and metabolic parameters in dams. MBG0, blood glucose level at 0 min of OGTT; MBG30, blood glucose level at 30 min of OGTT; MBG60, blood glucose level at 60 min of OGTT; MBG120, blood glucose level at 120 min of OGTT; MAUC, area under the curve of the OGTT; MFBG, fasting blood glucose at the first week of pregnancy; MHOMA-IR, the homeostasis model assessment of insulin resistance; MTC, total cholesterol; MTG, triglyceride; MLDL-C, low-density lipoprotein cholesterol; MHDL-C, high-density lipoprotein cholesterol; MSAT, subcutaneous adipose tissue mass; MVAT, visceral adipose tissue mass; MWAT, white adipose tissue mass; MBW, body weight. Values were significantly correlated between the genera and metabolic parameters: $^+p < 0.05$; $^*p < 0.01$.

Table 1 Correlation analysis between the relative abundance of altered species at the genus level and metabolic parameters in adult female offspring

Genera	Parameters	r	p value
<i>Bifidobacterium</i>	Insulin	-0.6450	0.0009
	HOMA-IR	-0.6359	0.0011
	LDL-C	-0.5499	0.0066
	FFA	-0.4947	0.0164
	SAT	-0.4410	0.0352
	WAT	-0.4987	0.0154
<i>Erysipelotrichaceae_incertae_sedis</i>	BG0	-0.5345	0.0086
	Insulin	-0.4668	0.0247
	SAT	-0.4294	0.0409
<i>Alloprevotella</i>	WAT	-0.4283	0.0415
	TC	0.5927	0.0029
	SAT	0.4702	0.0236
	VAT	0.4413	0.0350
	VAT	0.4901	0.0176

HOMA-IR, homeostasis model assessment of insulin resistance; LDL-C, low-density lipoprotein cholesterol; FFA, free fatty acid; SAT, subcutaneous adipose tissue; WAT, white adipose tissue; BG, blood glucose; TC, total cholesterol; and VAT, visceral adipose tissue.

In addition, this study also showed that perinatal genistein supplementation markedly enriched *Allobaculum*, *Clostridium XIVa*, *Clostridium IV*, *Eubacterium* and *Barnesiella* in dams at weaning, which were all reported to be the short-chain fatty acid (SCFA) (mainly butyrate)-producing bacteria. These SCFA producers have been shown to have an important physiological function, especially for metabolism. The benefits of *Allobaculum* have been shown in several studies. High-fat diet significantly reduced the abundance of *Allobaculum*. In contrast, prebiotic, berberine, as well as berberine and metformin treatment could significantly increase SCFA-producing

Allobaculum and improve obesity induced by the high-fat diet, and accordingly, the intestinal SCFAs were also elevated.^{42–44}

Studies have demonstrated that most of the butyrate-producing species belong to the clostridial clusters XIVa and IV, including *Clostridium XIVa*, *Clostridium IV* and *Eubacterium*.^{45,46}

Evidence from both patients with obesity or T2DM^{47,48} and animals fed high-fat sucrose diets⁴⁹ showed that *Clostridium XIVa* and *Clostridium IV* were dramatically reduced, while losing weight could elevate the abundance of *Clostridium IV*.⁵⁰ Another clinical trial indicated that *Eubacterium* was significantly reduced in patients with gestational diabetes mellitus.⁵¹ In addition, conversion of acetate to butyrate has been reported to be one of the important roles of *Eubacterium* in improving metabolism.⁵² As a member of the *Barnesiellaceae* family, *Barnesiella* is also a SCFA producer.⁵³ It has recently been reported that multiple bioactive components, such as mannoglycosaccharides (MOS) from spent coffee grounds,⁵⁴ polyphenol-rich cranberry extracts,⁵⁵ *Grifola frondosa* polysaccharides,⁵⁶ polyunsaturated fatty acids from *Spirulina platensis*,⁵⁷ and decaffeinated green and black tea polyphenols,⁵⁸ might regulate glucose homeostasis, insulin sensitivity and lipid metabolism as well as modulate body weight through modifying gut microbiota, including increasing the abundance of *Barnesiella*. Moreover, improving T2DM with a traditional Chinese medicine, Xiexin Tang, was also associated with enriched *Barnesiella*.⁵³ Anti-inflammation might play an important role in the benefits of *Barnesiella*.^{53,59,60} Consistent with these findings, our study showed that the markedly enriched SCFA-producing bacteria in the MHFG group were negatively correlated with the glucose and lipid metabolic parameters as well as body weights in dams.

Therefore, our study indicated that the beneficial effects of perinatal genistein intake on metabolism in dams was associ-



ated with the significant enrichment of SCFA (mainly butyrate)-producing bacteria. Most of these bacteria could produce butyrate and play important roles in the host health. Butyrate is one of the SCFAs resulting from the fermentation of dietary fiber in the gastrointestinal tract by the microbiota. Butyrate is not only the main energy supply of the colonic epithelium thus promoting its growth, but it also mitigates inflammatory responses through activation of its target G-protein coupled receptors and the histone deacetylase inhibitor activity, potentially protecting the gut permeability.⁶¹ In addition, it could pass through the enterocytes and into the circulation.⁶² Thus, the butyrate could also reach and affect the peripheral tissues. The benefits of butyrate supplementation against obesity and diabetes were shown to be associated with promoting mitochondrial adaptation by increasing β -oxidation of fatty acids and inhibiting ectopic lipid deposition and inflammation.⁶¹ However, in the present study, the levels of butyrate and the specific mechanism that decipher the metabolic benefits of butyrate-producing bacteria are unclear and need further exploration.

The transgenerational metabolic protection of maternal genistein intake against high-fat diet was also accompanied by changed gut microbiota in adult female offspring, including decreased *Alloprevotella*, while exhibiting enriched *Erysipelotrichaceae_incertae_sedis* from the *Erysipelotrichaceae* family. *Alloprevotella* was considered a harmful species, and weight loss was associated with the decrease of *Alloprevotella* in obese patients.⁶³ Subsequently, Kang *et al.*⁶⁴ also showed that supplementation of dietary fiber-konjac flour could significantly improve metabolism and inflammation, as well as reduce fat mass in high-fat fed mice, and was related to the decreased abundance of *Alloprevotella*. In this study, we showed that maternal genistein intake transgenerationally programmed an improvement of metabolism and reduction of fat mass in adult female offspring and decreased *Alloprevotella*, which was positively correlated with the TC and fat mass. Although the function of *Erysipelotrichaceae_incertae_sedis* is still unclear, this research indicated that it was beneficial bacteria and had negative correlation with the blood glucose levels, insulin levels and fat mass in female offspring. Therefore, this study demonstrated that the significant enrichment of *Erysipelotrichaceae_incertae_sedis* while a decrease of *Alloprevotella* might play a crucial role in the transgenerational metabolic benefits of perinatal genistein consumption in adult female offspring.

Furthermore, *Bifidobacterium* is another genus which plays crucial roles in the metabolic benefits of perinatal genistein consumption in both dams and adult female offspring in this study, and was significantly reduced in the high-fat diet fed mice and was negatively correlated with the glucose and lipid metabolic parameters. Although the abundance of *Bifidobacterium* is relatively low in adults, it has disproportionately large impacts in the host health. It has been widely accepted that *Bifidobacterium* has multiple functions, including the production of antioxidants and acids, maturation of the immune system, maintenance of immune homeostasis,

and protection of the gut barrier.⁶⁵ Although the beneficial effects of *Bifidobacterium* on metabolism have been extensively researched, to our knowledge, this is the first study to show the relationship between the metabolic benefits of genistein consumption and *Bifidobacterium*, especially the transgenerational effects.

5. Conclusions

Overall, perinatal genistein consumption during 3 weeks prior to mating and throughout pregnancy and lactation significantly counteracted the deleterious effects of high-fat diet on glucose and lipid metabolism in both dams and adult female offspring. Markedly enriched SCFA (mainly butyrate)-producing bacteria and reduced harmful species were obviously associated with the metabolic benefits of perinatal genistein intake in mothers. A significant decrease of *Alloprevotella* and enrichment of *Erysipelotrichaceae_incertae_sedis* might play crucial roles in the protective effects of maternal genistein intake on female offspring. In addition, *Bifidobacterium* was associated with the metabolic improvement in both dams and female offspring. To the best of our knowledge, this is the first study to indicate that gut microbiota might be a crucial factor in deciphering the metabolic benefits of perinatal genistein consumption in dams and adult female offspring. However, there are several limitations in this study. First, the fecal SCFA levels were not detected. Furthermore, the specific mechanism by which the gut microbiota mediates the transgenerational metabolic benefits of genistein supplementation remains largely unknown. Last, it is still unclear how maternal genistein intake programs the changes of the gut microbiome in later life of offspring and whether the maternal gut microbiota could influence the colonization and establishment of the microbial community in offspring, which require further investigation. All in all, our study might provide some evidence and new targets during early life for preventing the occurrence and development of metabolic disorders.

Abbreviations

HF	High-fat diet
FFA	Free fatty acid
T2DM	Type 2 diabetes mellitus
GDM	Gestational diabetes mellitus
DOHaD	Developmental origins of health and disease
SAT	Subcutaneous adipose tissue
VAT	Visceral adipose tissue
WAT	White adipose tissue
GWG	Gestational weight gain
FBG	Fasting glucose level
OGTT	Oral glucose tolerance test
BG	Blood glucose
AUC	Area under the curve
TC	Total cholesterol



TG	Triacylglycerol
HDL-C	High-density lipoprotein cholesterol
LDL-C	Low-density lipoprotein cholesterol
HOMA-IR	Homeostasis model assessment of insulin resistance
OTUs	Operational taxonomic units
PCoA	Principal coordinates analysis
ANOSIM	Analysis of similarities
LefSe	Linear discriminant analysis of the effect size
SCFA	Short-chain fatty acid

Conflicts of interest

There are no conflicts of interest to declare.

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