Clinical and Translational Report

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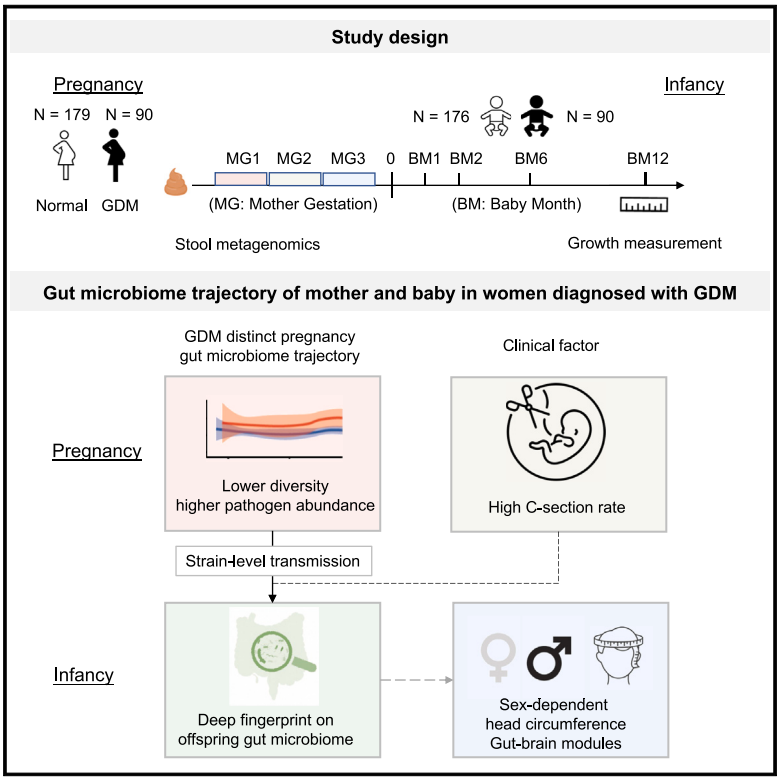
Maternal gestational diabetes mellitus associates with altered gut microbiome composition and head circumference abnormalities in male offspring

Graphical abstract



Highlights

- Gestational diabetes mellitus (GDM) mothers have distinct gut microbiomes during pregnancy
- GDM fingerprints on the offspring's gut microbiome are confounded by C-section
- GDM affects the offspring's head circumference growth in a sex-dependent manner
- The gut microbiome of GDM mothers with male fetuses has different gut-brain modules

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In brief

Gestational diabetes mellitus (GDM), defined as hyperglycemia during pregnancy, is the leading gestational complication worldwide. Assessing the fecal microbiome in 264 mother-baby pairs, Wang et al. demonstrate that GDM affects both maternal and infant gut microbiome composition and gut-brain modules, which are associated with infant head growth in sexual dimorphism.

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Maternal gestational diabetes mellitus associates with altered gut microbiome composition and head circumference abnormalities in male offspring

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SUMMARY

The impact of gestational diabetes mellitus (GDM) on maternal or infant microbiome trajectory remains poorly understood. Utilizing large-scale longitudinal fecal samples from 264 mother-baby dyads, we present the gut microbiome trajectory of the mothers throughout pregnancy and infants during the first year of life. GDM mothers had a distinct microbiome diversity and composition during the gestation period. GDM leaves fingerprints on the infant's gut microbiome, which are confounded by delivery mode. Further, *Clostridium* species positively correlate with a larger head circumference at month 12 in male offspring but not females. The gut microbiome of GDM mothers with male fetuses displays depleted gut-brain modules, including acetate synthesis I and degradation and glutamate synthesis II. The gut microbiome of female infants of GDM mothers has higher histamine degradation and dopamine degradation. Together, our integrative analysis indicates that GDM affects maternal and infant gut composition, which is associated with sexually dimorphic infant head growth.

INTRODUCTION

The gut microbiome is known to be essential in the perinatal health of mother and infant, which supports the developmental origins of the health and disease hypothesis. During pregnancy, host factors, such as changes in hormone levels, remodel the gut microbiome.[1–3] Third-trimester stool is associated with greater inflammation and energy content, and the host metabolism is similar to that in metabolic syndrome.[1] Nevertheless, it is still under debate whether and how pregnancy alters the gut microbiome trajectory.[4,5]

Gestational diabetes mellitus (GDM), defined as hyperglycemia during pregnancy, is the leading complication during pregnancy worldwide.[6] Notably, previous studies proved the causal role of the gut microbiome in the pathogenesis of GDM through human-to-mouse fecal transplant study,[7] which indicated the indispensable role of the gut microbiome in GDM early prediction, diagnosis, and stratification.[7,8] However, given the low resolution of the GDM gut microbiome's dynamic change during the whole pregnancy, it is necessary to uncover the roles of the maternal gut microbiome in GDM from a prospective longitudinal cohort.

GDM could increase the risk of long-term complications in children, including cardiometabolic risk,[9–11] metabolic syndrome,[12,13] and neurodevelopmental disorder.[14,15] In mouse models, chemical-induced GDM could induce offspring behavior

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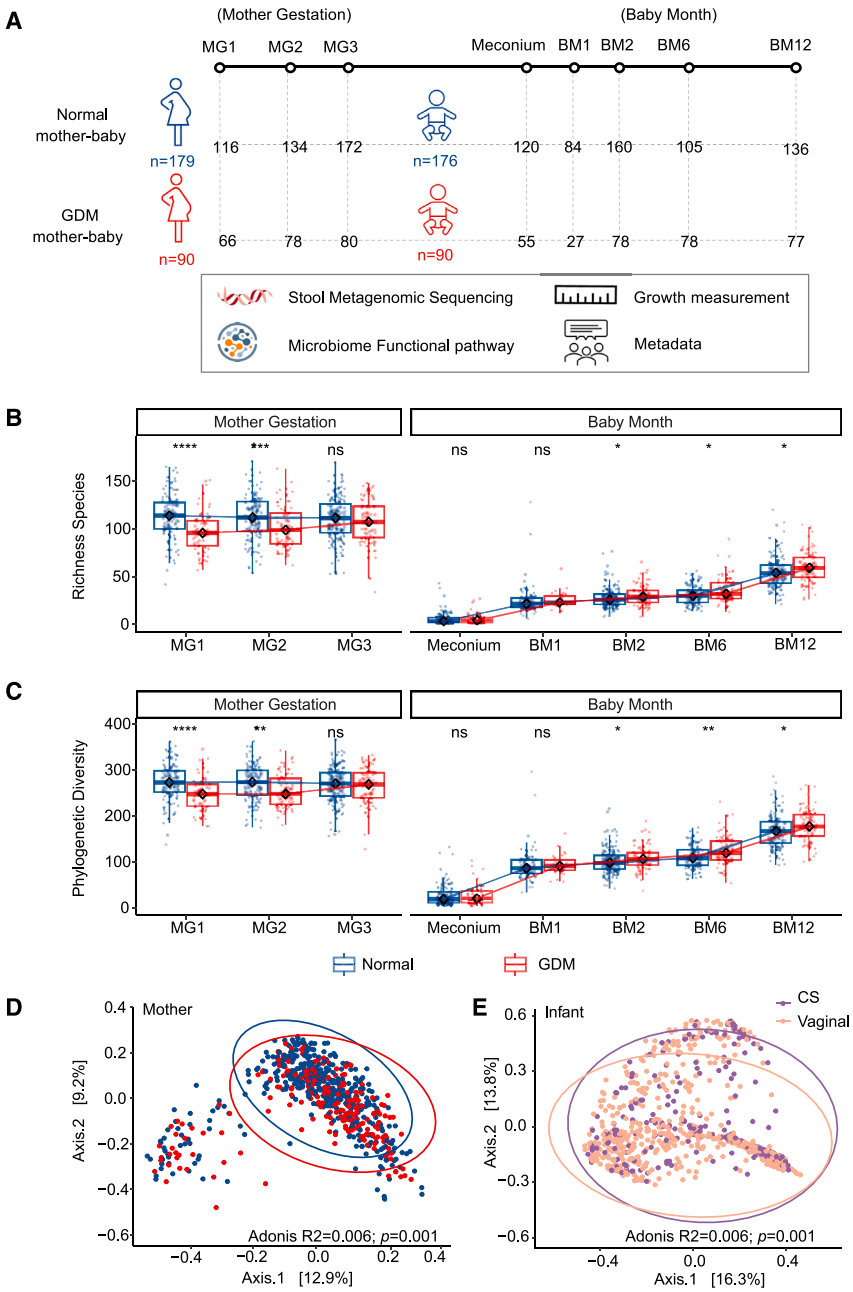

Figure 1. Longitudinal metagenomic profiles of 264 mother-baby dyads from the first gestational periods of mothers to the first year of life of infants in the MOMmy cohort

(A) Study design: 269 pregnant women recruited for the study collected a stool sample and clinical and obstetric history at each gestational period. After delivery, 266 babies were followed up with serial stool samples, extensive exposure metadata, and growth measurement at each time point. See also Table 1.

(B and C) Microbial alpha diversity: (A) richness and (B) phylogenetic diversity at MG1 and MG2 were lower in mothers diagnosed with GDM later. *p* values were evaluated by the Wilcoxon test. Data are represented as median ± interquartile range (IQR). * indicated $p < 0.05$, ** indicated $p < 0.01$, *** indicated $p < 0.001$, **** indicated $p < 0.0001$. See also Figures S1A–S1E.

(D) Maternal microbial beta diversity: species-level PCoA analysis revealed a significantly different profile between GDM and normal mothers after adjusted age and BMI before pregnancy. See also Figures S1F–S1H.

(E) Infant microbial beta diversity: species-level PCoA analysis revealed that delivery mode affected gut microbiome composition after considering the GDM group and antibiotic use. CS, C-section.

disorder, especially in males.[16] Retrospective studies demonstrated the correlation between early-life accelerated head circumference growth and infants later diagnosed with autism spectrum disorder (ASD).[17–19] Specifically, one study showed the abnormally accelerated head circumference during the first year of life in male ASD infants but not in female ASD infants.[17] Therefore, abnormal head growth in the first year of life may be useful as a minimally invasive parameter for the early detection of neurodevelopmental disorders.[17]

The gut microbiome of the mother and infant may impact infant neurodevelopment[20,21] via the gut-brain axis. GDM has been reported to affect the meconium microbiome composition,[13,22–24] but whether it is affected via vertical transmission,[25–27] and how it is confounded by clinical covariates, are less explored. Moreover, whether GDM impacts the developmental trajectory of the infant microbiome during the critical window of the first year of life,[28] and subsequently influences postnatal growth health in a sex-dependent manner, remains unknown.

To address these knowledge gaps, we analyzed a large set of metagenomic datasets with detailed clinical metadata to elucidate the maternal microbiome trajectory and assess the relationship between GDM and the maternal gut microbiome. Furthermore, we evaluated the effect of GDM on the offspring's gut microbiome and growth index during the first year of life and uncovered the linkage between the microbiome and the infant's head circumference growth in a sex-dependent manner.

RESULTS

Study population characteristics

The MOMmy (mother-infant microbiota transmission and its link to long-term health of baby) study is a prospective, longitudinal birth cohort from Hong Kong, China. GDM was diagnosed in 90 of 981 women from the MOMmy cohort. In total, 535 participants, comprising 269 pregnant women (90 with GDM, 179 normal) and 266 babies (90 born to mothers with GDM, 176 born to normal

Table 1. Demographic and clinical characteristics of mothers and infants

Characteristic	Normal mother[a] (N = 179)	Mother with GDM[a] (N = 90)	*p* value[b]
Age at recruitment (year)	32.0 (29.0, 35.0)	34.0 (31.0, 37.0)	<0.001
Region (Hong Kong)	176 (98%)	88 (98%)	0.500
Marital status (married)	162 (91%)	88 (98%)	0.048
Number of children in house (*n* = 0)	118 (66%)	49 (54%)	0.028
Education	-	-	0.100
High school or below	58 (32%)	31 (34%)	-
Bachelor's degree	110 (61%)	47 (52%)	-
Master's or above	11 (6.1%)	12 (13%)	-
Working status	-	-	0.400
Full-time	138 (77%)	66 (73%)	-
Housewife	31 (17%)	14 (16%)	-
Part-time	6 (3.4%)	7 (7.8%)	-
Others	4 (2.2%)	3 (3.3%)	-
Baseline any disease	0 (0%)	17 (19%)	-
Digest	0 (0%)	2 (2.2%)	-
Immune	0 (0%)	2 (2.2%)	-
Allergy	0 (0%)	1 (1.1%)	-
Hypertension	0 (0%)	1 (1.1%)	-
Hyperlipidemia	0 (0%)	1 (1.1%)	-
Psychological disorders	0 (0%)	2 (2.2%)	-
Pre-pregnancy overweight or obesity (BMI > 23 kg/m^2)	31 (19%)	36 (42%)	<0.001
Other maternal complications	-	-	-
Gestational hypertension	0 (0%)	5 (5.6%)	-
Gestational sepsis	0 (0%)	1 (1.1%)	-
Characteristic	Infants born to normal mothers[a] (N = 176)	Infants born to mothers with GDM[a] (N = 90)	*p* value[b]
Sex (male)	77 (43%)	54 (60%)	0.012
Gestational age (weeks)	39.14 (38.29, 40.00)	38.43 (38, 39.14)	<0.001
Preterm birth	0 (0%)	8 (8.9%)	-
Delivered by C-section	43 (24%)	33 (37%)	0.037
Intrapartum antibiotic prophylaxis use	91 (52%)	59 (66%)	0.031
Birth weight (g)	3,075 (2,825, 3,325)	3,162 (2,930, 3,408)	0.140
Neonatal complications	58 (33%)	49 (54%)	<0.001
Hypoglycemia	5 (2.8%)	23 (26%)	<0.001
Neonatal infection	11 (6.2%)	6 (6.7%)	0.900
Meconium aspiration syndrome	1 (0.6%)	0 (0%)	>0.900
Jaundice	30 (17%)	21 (23%)	0.200
Neonatal respiratory distress syndrome	0 (0%)	2 (2.2%)	0.110
Other neonatal complications	28 (16%)	19 (21%)	0.300
Neonatal SBCU/NICU admission	56 (32%)	49 (54%)	<0.001
Exclusive breastfeeding before discharge	89 (51%)	18 (20%)	<0.001

[a]Median (IQR); *n* (%).
[b]Wilcoxon rank sum test; Fisher's exact test; Pearson's chi-squared test.SCBU/NICU, special care baby unit or neonatal intensive care unit.

mothers) were included in this study (Figure 1A). We sequenced 1,566 stool samples from 264 mother-baby pairs, with an average sequence depth of 5.74 Gb per sample after removing the host and contamination-matched reads (Table S1A).

The characteristics of study participants are shown in Table 1. Mothers with GDM were more likely to be at a more advanced age at childbearing ($p < 0.001$) and overweight or obese (OWOB) before pregnancy ($p < 0.001$). A higher proportion of males ($p = 0.009$), higher cesarean rates ($p = 0.037$), and intrapartum antibiotic prophylaxis (IAP) usage ($p = 0.031$) were observed in babies born to GDM mothers. Also, babies born to GDM mothers had a lower breastfeeding rate before hospital discharge.

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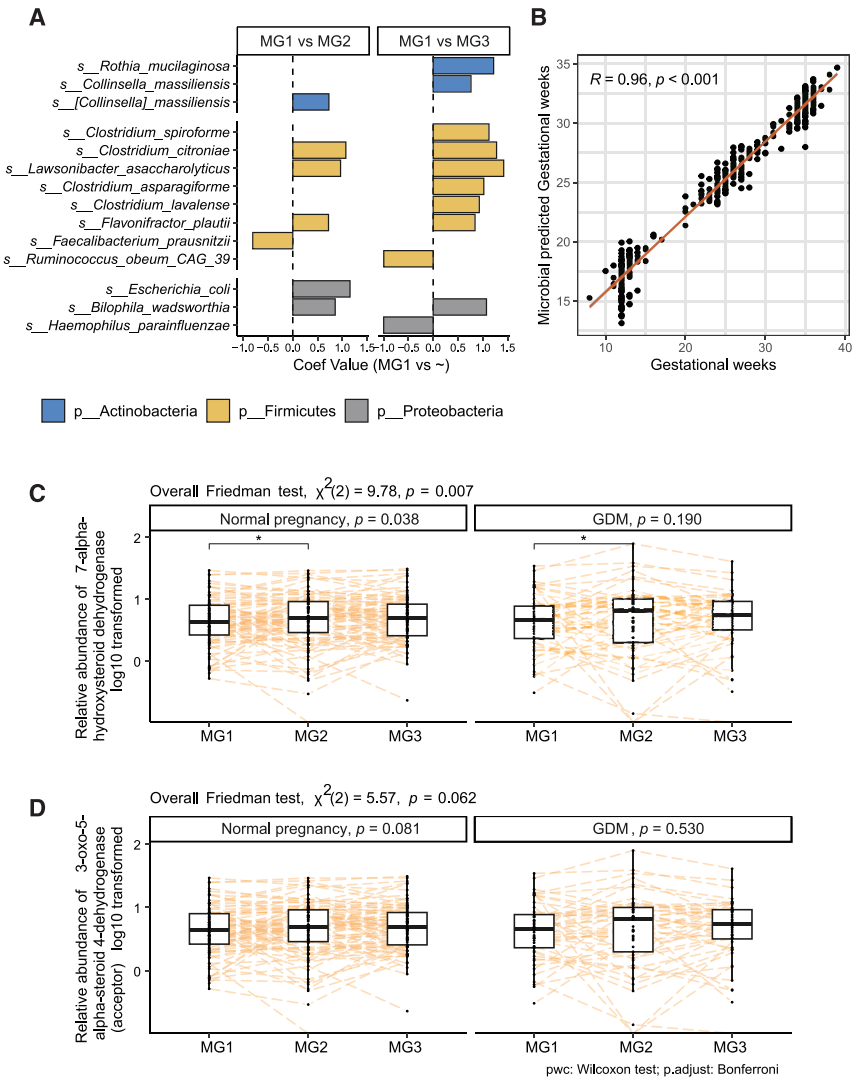

Figure 2. Partial convergence of gut microbiome during pregnancy relates to adaption to hormonal changes

(A) Differential species between MG2/MG3 and MG1 in normal pregnant women. FDR < 0.25. Coef value < 0 indicated the species enriched in MG1, and coef value > 0 indicated the species enriched in MG2/MG3. See also Figures S2C–S2E. Coef: the model cofficient value (effect size).

(B) Microbial predicted gestational weeks versus gestational age in normal pregnancy. R and *p* values were calculated based on Spearman correlation analysis. See also Figure S2F.

(C and D) The relative abundance of two gut hydroxysteroid dehydrogenase changes during three gestational periods. The Friedman test was used to calculate the overall *p* values and *p* values in each group. Wilcoxon test was used for post hoc analysis. * indicated $p < 0.05$. Data are represented as median ± IQR.

Altered microbial diversity and composition in women with GDM and their offspring

Compared with normal women, women with GDM had significantly lower richness and phylogenetic diversity in their gut microbiota in mother gestational period (MG)1 and 2, but not 3 (Figures 1B and 1C). These differences were not confounded by sequence depth (Table S1B). This result could be explained by the increasing richness and phylogenetic diversity in the GDM at MG3 but not in the normal group when compared with MG2 or MG1 (Figures S1A and S1B).

Further, older age was implicated with lower bacterial richness and phylogenetic diversity after adjusting the pre-pregnancy overweight in all 269 subjects or the 179 normal women alone (Figure S1C). Mediation analysis confirmed that gut microbiome richness at MG1 was involved in the association between advanced age at childbearing and GDM development (Figure S1D). Further, a sensitivity analysis that excluded the subjects with the lowest quartile age found that higher gut bacterial richness and phylogenetic diversity play a protective role in GDM development in mothers older than 30 (Figure S1E). Surprisingly, infants born to mothers with GDM demonstrated higher richness and phylogenetic diversity, with a difference in age from baby month (BM)2 to BM12 ($p < 0.05$, Figures 1B and 1C; Table S1C).

For bacterial composition, species-level principal coordinate analysis (PCoA) revealed an altered overall gut microbiota configuration in GDM compared with normal mothers, after adjusting age and BMI before pregnancy and stratifying by 3 gestation periods (R = 0.006, $p = 0.001$, Figure 1D), especially in MG2 (Figures S1F–S1H). There was no difference in beta diversity of gut microbiome between infants born to GDM mothers and control in the first year after adjusting delivery mode and antibiotic use. Delivery mode, one of the modifiers of the GDM infant gut microbiome born via C-section, significantly affected the infant's gut microbiome during the first year of life (R = 0.006, $p = 0.001$, Figure 1E).

Partial convergence of gut microbiome during pregnancy relates to adaption to hormonal changes

Possibly reflecting changes in hormones, the gut microbiome similarity of MG3-MG2 was higher than that of MG3-MG1 in the same subjects, and the MG2-MG2 or MG3-MG3 similarity was higher than that of MG1-MG1 in two unrelated females (Figures S2A and S2B, $p < 0.001$), either in the normal pregnancy or GDM group.

Differential species were detected across the three gestation periods in the normal group (Figure 2A). The relative abundance of two butyrate producers (*Flavonifractor plautii* and *Lawsonibacter saccharolytic*),[29] one bile-tolerant bacteria (*Bilophila wadsworthia*),[30] and *Clostridium citroniae* were consistently increased during MG2 and MG3 compared with MG1 (Figures S2C–S2E). In a microbial gestational age model, the gestational age (weeks) of the samples collected positively correlated with the predicted microbial gestational age (Figure 2B, R = 0.96, $p < 0.001$). *Bilophila*

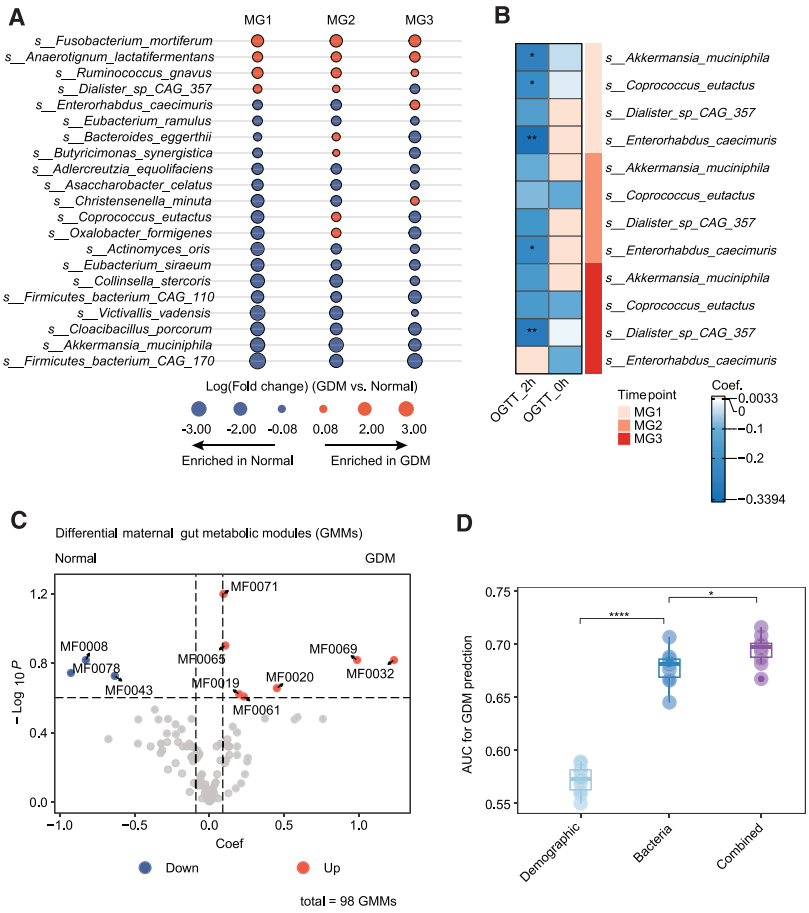

Figure 3. The impact of GDM on females' gut microbiome and its association with host glucose status during pregnancy

(A) Longitudinal differential species between GDM and normal pregnant women during the gestational period. FDR < 0.25. See also Table S2A.

(B) The associations between GDM-related species with OGTT at each gestational period. * represented $p < 0.05$ based on Spearman correlation analysis. The box color indicates the Rho value.

(C) Volcano plot of the longitudinal differential gut metabolic modules (GMMs) of GDM and normal pregnant women during pregnancy. FDR < 0.25. See also Table S2B.

(D) Boxplot showed the AUC scores for GDM prediction based on three vectors in the training set. Data are represented as median ± IQR. p values were evaluated by the Wilcoxon test.

wadsworthia and *Flavonifractor plautii* were both included in the top 20 important variables in this model (Figure S2F).

Pregnant women experience dramatic increases in estrogen and progesterone, and hydroxysteroid dehydrogenase (HSD) has been reported to be involved in hormone metabolism.[2,31] Here, we found that the key enzyme involved in bile acid metabolism, 7-alpha- HSD, was increased during either normal or GDM pregnancy (Figure 2C), which was consistent with the increase of bile-tolerant bacteria (*Bilophila wadsworthi*).[30] Moreover, 3-oxo-5-alpha-steroid 4-dehydrogenase (acceptor), which is able to convert progesterone into its corresponding 5-alpha-3-oxosteroids, tended to increase in MG2 compared with MG1 (Figure 2D). These results suggested partial convergence of the gut microbiome during late pregnancy, which may relate to adaption to hormonal changes.

GDM dampens the maternal gut microbiome trajectory

We next compared the longitudinal differential species between GDM and the normal group during pregnancy, and 21 species were identified (Figures 3A and S3A; Table S2A, false discovery rate [FDR] < 0.25). Sensitivity analysis using Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) was further performed. The relative abundance of species depleted in GDM, including *Akkermansia muciniphila*, *Coprococcus eutactus*, and *Enterorhabdus caecimuris* at MG1, as well as *Dialister sp CAG 357* at MG3, negatively correlated with the 2 h oral glucose tolerance test (OGTT) levels (Figure 3B).

Furthermore, seven gut metabolic modules (GMMs) longitudinally increased and three depleted in the guts of GDM subjects (Figure 3C, FDR < 0.25). Notably, two enriched energy metabolism GMMs, including MF0071 pentose phosphate pathway (non-oxidative branch) and MF0065 *Bifidobacterium* shunt at MG1, were positively correlated with 2 h OGTT (R = 0.313, R = 0.318, respectively, FDR < 0.25, Table S2B), perhaps due to excessive energy extraction by the gut microbiome. Together, dysbiosis in the gut microbiome correlates with glucose intolerance and affects maternal metabolic health during pregnancy.

Furthermore, Figure 3D showed that a combined prediction model based on all gut bacterial species and host risk factors at MG1 could differentiate GDM onset in later pregnancy, with an area under the receiver operating characteristic curve (AUC) of 0.70. Specifically, females diagnosed with GDM were characterized by a lower relative abundance of two equol producers, *Adlercreutzia equolifacien* and *Asaccharobacter celatus*, and a higher relative abundance of the pathogen *Parabacteroides distasonis* (Figure S3B) at MG1. Briefly, a combination of GDM risk factors and gut microbial factors could provide a higher early-discrimination ability for GDM risk stratification.

GDM affected the taxonomy and network of the infant gut microbiome and strain transmission

The gut microbiome of infants born to GDM mothers demonstrated higher richness and phylogenetic diversity than its counterparts from BM2 to 12, especially within the Firmicutes phylum (Figure 4A; Table S1C). In the univariate analysis, three species (*Rothia mucilaginosa*, *Collinsella stercoris*, and *Collinsella massiliensis*) were significantly decreased and one was enriched (*Enterococcus faecalis*) in the GDM mother and baby gut microbiome with the same trend (Table S3A), which displayed that the taxonomic differences between infants born to GDM mothers

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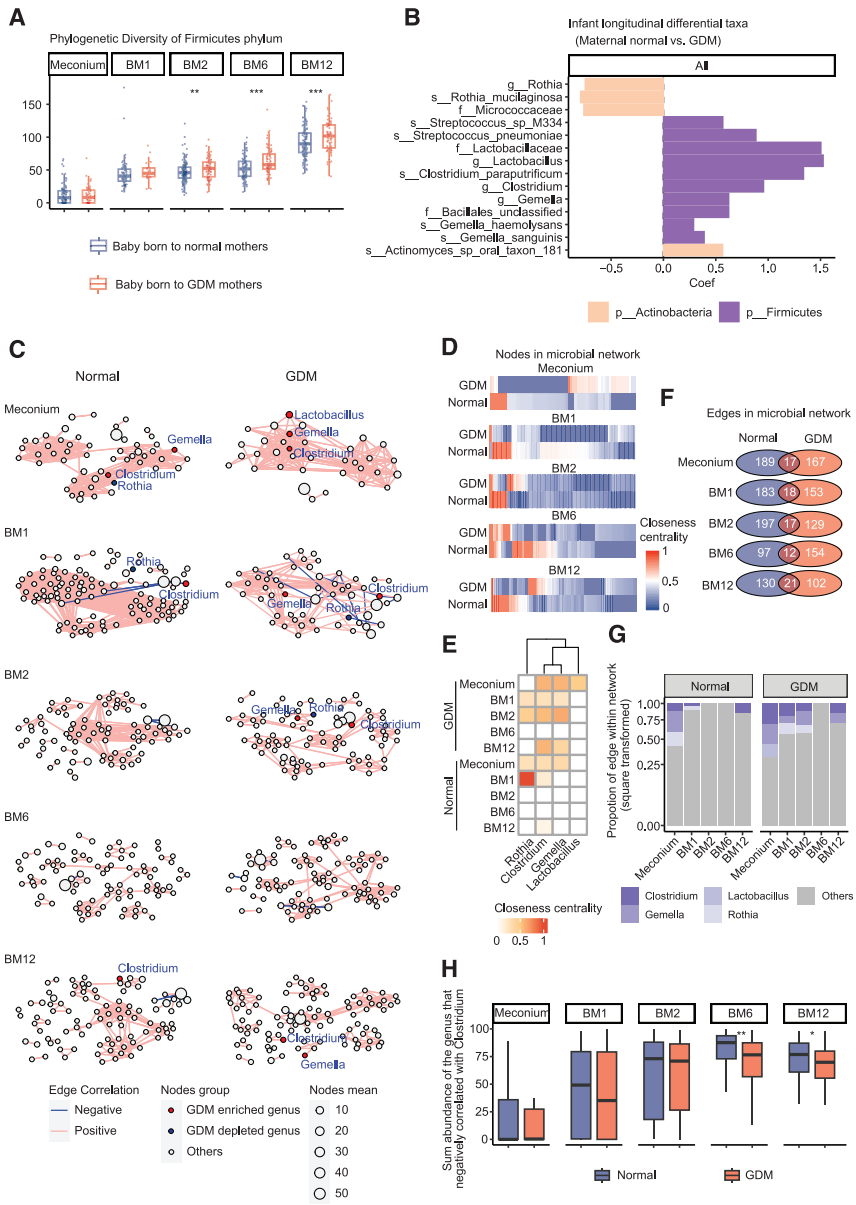

Figure 4. GDM affected the taxonomy and network of the infant gut microbiome

(A) The phylogenetic diversity of Firmicutes phylum of the infant microbial community within samples during the first year of life and compared between babies born to GDM and normal mothers. Data are represented as median ± IQR. See also Table S1C.

(B) Longitudinal differential taxa between infants born to GDM and normal mothers during the first year of life. The color indicated the phylum of different bacterial features. FDR < 0.25. Coef: the model coefficient value (effect size).

(C) GDM influences dynamic changes of the genus-level microbial network in early life. Pairwise, Spearman's rank correlations were applied at each time point for two groups ($R > 0.40$, $p < 0.05$).

(D) The centralities (closeness) of nodes in the infant microbial networks between the GDM and the normal group. Each column represents a genus that appeared in the corresponding microbial network.

(E) The centralities (closeness) of four differential genera within each microbial network.

(F) The number of unique and shared edges in the infant microbial networks between GDM and normal group.

(G) Proportion of four differential genera-related edges within each microbial network.

(H) The sum relative abundance of the genus negatively correlated with the *Clostridium* genus in the infant's gut at BM6 and BM12. Data are represented as median ± IQR.

* indicated $p < 0.05$, ** indicated $p < 0.01$, *** indicated $p < 0.001$.

and controls reflected differences in the maternal microbiome. As mentioned previously, GDM infants were characterized by higher C-section rates and less breastfeeding before discharge, which were important factors associated with the infant gut microbiome.[32] The impact of GDM on the offspring gut microbiome was partially confounded by the delivery mode and feeding modes ($p < 0.05$, Figures S4A and S4B). After adjusting for potential confounders, GDM was still deeply fingerprinted on the infant's gut microbiome at BM2 and 12 but was not at other time points especially enriched two potential pathogenic bacteria, including *Streptococcus pneumonoiae* and *Klebsiella quasipneumoniae* at BM12 (FDR < 0.25, Figure S4C). Similarly, in the longitudinal analysis, 15 infant bacteria features were associated with GDM (FDR < 0.25), especially the enriched *Clostridium* and *Lactobacillus* genus from Firmicutes phylum in the gut of infants born to GDM mothers (Figure 4B; Table S3B).

To reveal the microbial co-occurrence network of GDM and control infants, we assessed bacterial genus ecological interactions (Figure 4C). First, the closeness centrality of genera was distinct between the two groups (Figure 4D). Three enriched genera (*Clostridium*, *Gemella*, and *Lactobacillus*) in the GDM infants' gut community had higher closeness centrality in the microbial network of the GDM infants' gut, which indicates the importance of these taxa. The GDM-depleted genus (*Rothia*) had higher closeness centrality in the microbial network of normal infants, especially meconium and BM1 (Figure 4E; Table S3C). Second, hundreds of edges were specific to normal or GDM infants, with few overlaps (Figure 4F). Further, the interactions between four differential genera (between GDM and normal infants) and other genera were checked. More GDM infant differential genera-related edges could be detected in GDM infant gut compared with the control, especially three enriched genera (Figure 4G; Table S3D). Of note, *Gemella* and *Clostridium* positively correlated with each other in the meconium of the GDM infants' network (Table S3D). Also, *Clostridium* positively correlated with *Klebsiella* in the meconium of infants born to normal mothers. In addition, *Clostridium* negatively correlated with three genera (including *Bifidobacterium*, *Bacteroides*, and *Collinsella*

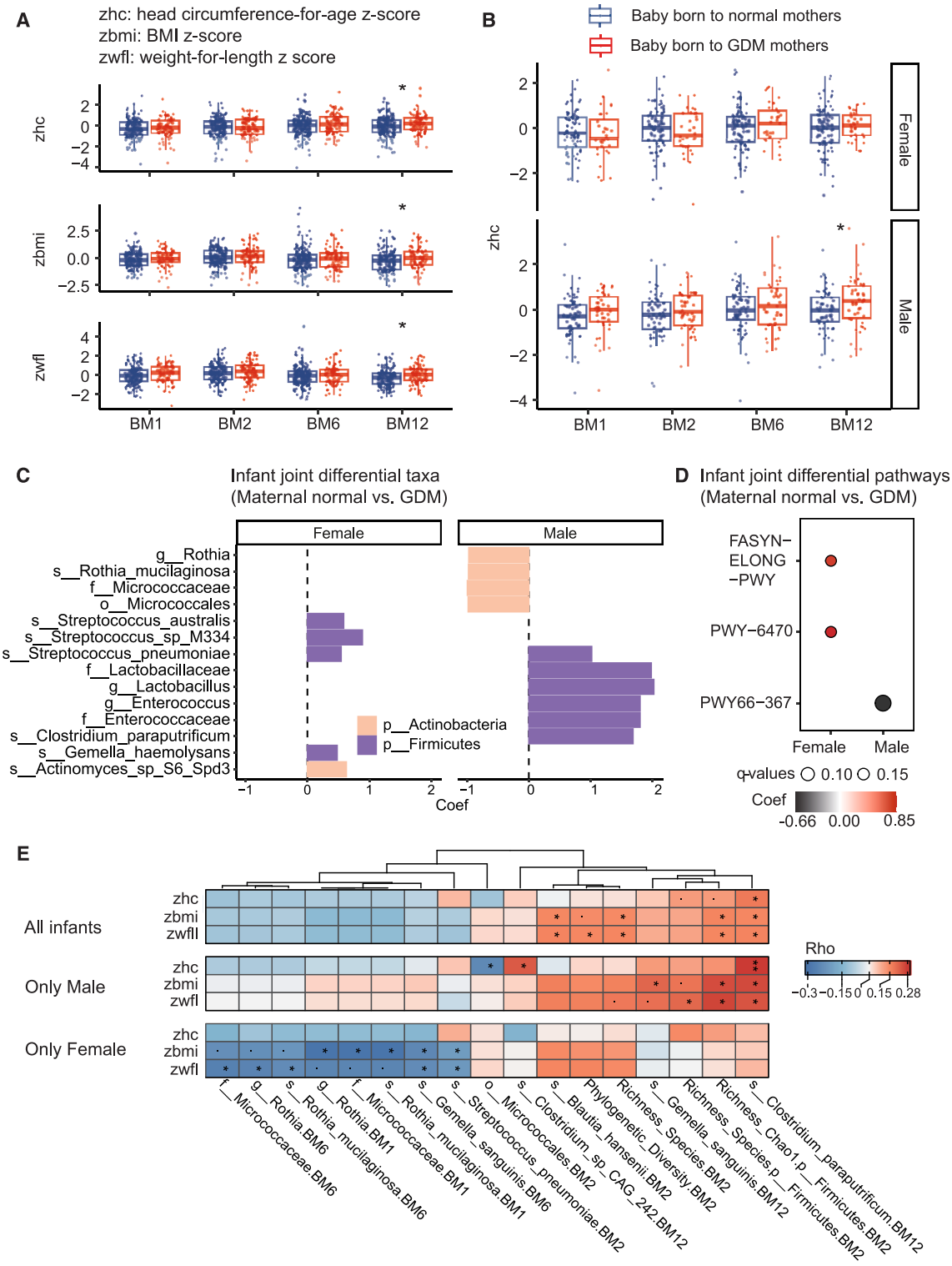

Figure 5. Gut microbiome associated with the impact of GDM on the infant growth index during the first year of life

(A) Impact of GDM on infant growth indexes during the first year of life. See also Figures S6A–S6D and Table S5A.

(B) Impact of GDM on infant zhc indexes during the first year of life after sub-grouping sex of the baby. Statistical significance was assessed using Wilcoxon rank-sum tests at each time point. Data are represented as median ± IQR.

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at BM1 or BM2) (Table S3D), and the sum abundance of these three potentially beneficial genera was significantly lower in the GDM group at BM6 and BM12 (Figure 4H). The above ecological network results indicated that GDM not only affects taxa in the infant gut microbiome but also impacts gut microbial interaction.

Considering that the maternal gut microbiome is the major source of the infant's gut microbiome, we analyzed strain-level transmission between MG3 and infants. C-section and IAP usage could significantly disturb the mother-baby strain transmission rate (Figures S5A and S5B), especially within the Actinobacteria and Bacteroidetes phyla (Figures S5C and S5D; Table S4A). Although the GDM group was characterized by a higher C-section rate and IAP usage, GDM did not affect the strain transmission rate (Figure S4D). Only the number of transmission strains within the Firmicutes at month 6 was marginally higher in GDM mother-baby pairs than normal pairs (p = 0.050, FDR = 0.259) (Table S4A). Importantly, GDM infants tended to have higher transmissibility of a potential pathogen, *Klebsiella quasipneumoniae*, at BM6 (p = 0.067, FDR = 0.259, Table S4B). As shown in Figure S5E, 6 out of 7 of the transmitted species detected only in the GDM group were from the Firmicutes phylum, including potential pathogens *Clostridium sp CAG 242* and *Enterococcus gallinarum*. Furthermore, the transmission of *Fusobacterium mortiferum*, which had a higher abundance in mothers with GDM during gestation, was only detected in GDM mother-baby pairs. To sum up, marginal evidence was detected about the effects of GDM on gut microbiome transmission between mother-baby pairs in our cohort.

We additionally evaluated carbohydrate-active enzymes in infants' gut microbiomes, potentially reflecting the utilization of carbohydrates within breast milk. One human milk oligosaccharide, (HMOs)-relevant glycoside hydrolases (GHs) 2,[28] was depleted in the gut microbiome of infants born to GDM mothers compared with the control at BM2 (Figure S5F, FDR < 0.25), which might reflect lower HMO levels in the breastmilk of GDM mothers and is consistent with one recent study.[33]

Gut microbiome associated with the impact of GDM on infants' growth index during the first year of life

The early-life gut microbiome could affect the child's growth and development and, thus, we were interested in differential bacterial species that lead to different growth in GDM infants. Here, GDM-exposed infants were characterized by larger head-circumference-for-age Z score (zhc), BMI Z score (zbmi), and weight-for-length Z score (zwfl) than control at BM12 (Figure 5A). The head circumference growth rate (calculated between BM1 and BM12) was significantly higher in infants born to GDM mothers (Figures S6A–S6D, $p < 0.05$), suggesting that GDM infants had accelerated head growth within the first year of life. In the univariable analysis, only GDM and maternal OWOB before pregnancy were related to BM12 growth outcomes ($p < 0.05$) (Table S5A); in particular, infants born to mothers with OWOB and GDM have higher zwfl, zbmi, and zhc.

After sub-grouping according to baby's sex, only GDM-exposed male infants were characterized with larger zhc than control at BM12 (Figure 5B), implying a sex-dependent effect. Likewise, GDM had a distinct longitudinal impact on the male offspring's gut microbiome (Figure 5C; Table S3B) and pathway (Figure 5D). Males born to GDM mothers were characterized by increased *Clostridium paraputrificum* (Firmicutes phylum), whereas females born to GDM mothers were characterized by enriched *Actinomyces sp S6 Spd3* from the Actinobacteria (Figure 5C). Such a sexual dimorphism of the gut microbiome was validated in two cohorts from America[34] and Europe.[35] GDM had a distinct fingerprint in the male offspring's gut microbiome after adjusting for potential confounders (Figure S6E). Specifically, we found that male infants born to GDM mothers were characterized by depleted *Bifidobacterium bifidum* and enriched species from the Firmicutes and Proteobacteria phyla. Further, the beta diversity between the MOMmy cohort and the validation cohorts showed that gut microbiome composition was different even in the healthy group (Figure S6F), which indicated that different markers might be due to cohort differences.

To better understand whether the gut microbiota affects the association between GDM and child growth, we analyzed the relationship between differential bacterial features in GDM infants and growth Z scores at BM12, either in all infants or subgroups of the babies. The Micrococcaceae order depleted in GDM male infants was negatively correlated with the zhc at BM12. Remarkably, the relative abundance of *Clostridium sp CAG 242*, one bacterial species whose strain transmission was detected only in the GDM group, was positively correlated with the zhc of male infants at BM12 but not females. A similar trend was found in the richness of Chao 1 of Firmicutes in the infant's gut at BM2. Moreover, *Clostridium paraputrificum* at BM12 was significantly positively associated with three higher infant growth indexes at BM12 in all infants and only-male infants datasets (Figure 5E) but not the female infant population. Further, 23 metagenome-assembled genomes (MAGs) of *Clostridium paraputrificum* were recovered via assembly and binning. Consistent with the Metaphla3-based abundance result, GDM infants had a higher recovered rate of *Clostridium paraputrificum* MAGs than did control infants (GDM:14/90, control: 9/167, OR = 3.40, $p < 0.01$, Fisher test). Male GDM infants had higher *Clostridium paraputrificum* MAG recovered rates than control male infants (GDM male: 9/54, control male: 4/77, OR = 3.613, p = 0.039, Fisher test), but this was not significant in GDM infant females (GDM female: 5/36, control female: 5/99, OR = 3.003, p = 0.1304, Fisher test). We checked the KEGG Orthology (KO) overlap between the MAGs recovered from four groups of infants (Figure S6G). A total of 1,328 KOs were shared among four groups of infants (Table S5B). Among them, six resistant genes (K15973, MarR family transcriptional regulator, 2-MHQ, and catechol-resistance regulon repressor; K18218, tetracycline resistance efflux pump; K18220, ribosomal protection tetracycline resistance protein; K18346, vancomycin resistance protein

(C and D) Longitudinal differential taxa (C) and functional pathways (D) between infants born to GDM and normal mothers during the first year of life after sub-grouping sex of the baby. FDR < 0.25. Coef > 0 indicated the enriched taxa or pathways in GDM infants. Coef: the model coefficient value (effect size).
(E) The correlation between differential bacterial features in GDM infants and growth Z scores at BM12 in all infants, only male, or only female infants. *represented $p < 0.05$, and dot represented $p < 0.1$ based on Spearman correlation analysis. FDR < 0.25. The color indicates the correlation Rho value.

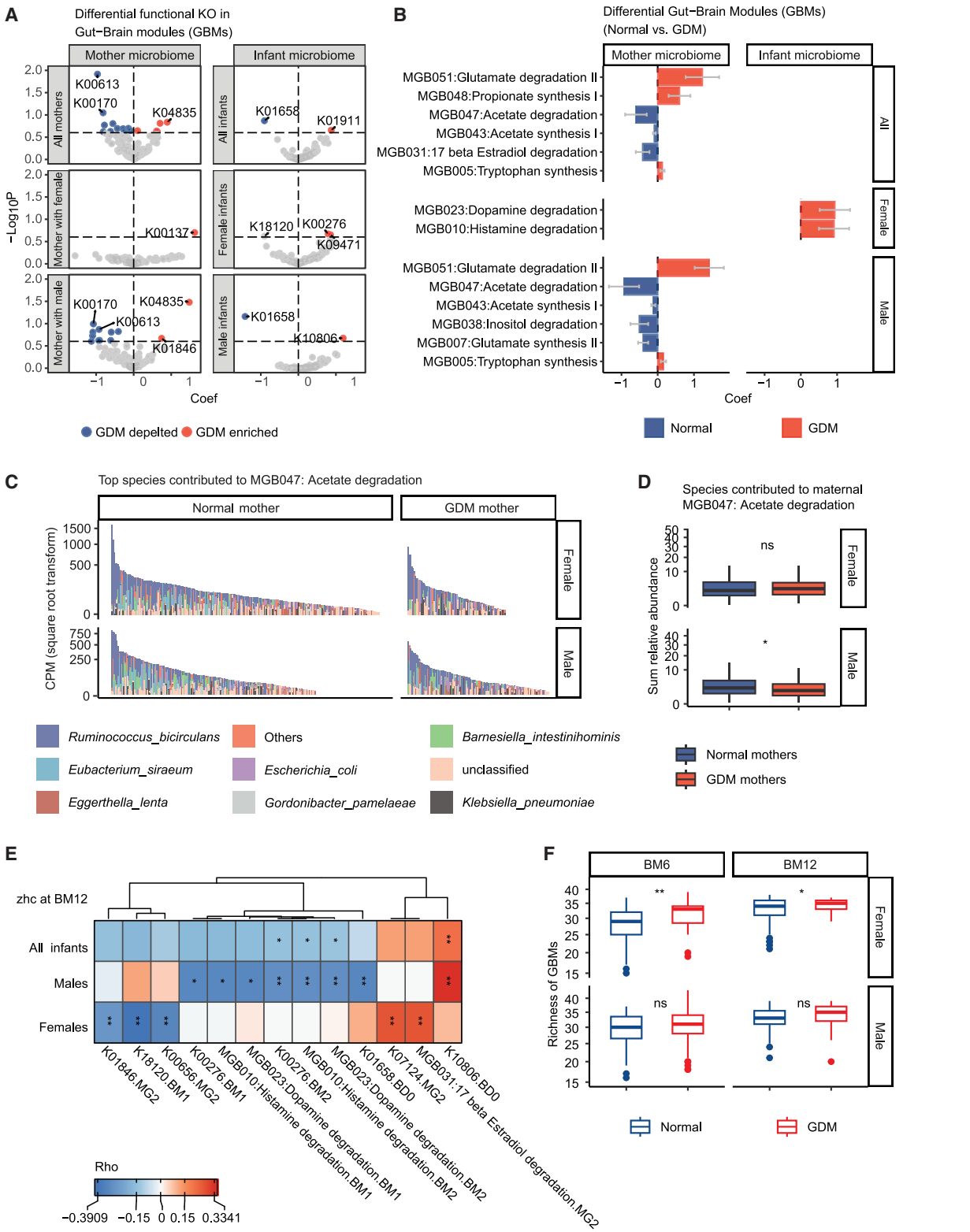

Figure 6. Associations of gut microbiota neuroactive potential with head growth of GDM offspring in a sex-dependent manner

(A) Volcano plot of the longitudinal differential KO involving gut-brain modules (GBMs) in GDM and normal mothers during pregnancy or infants during the first year of life separately. FDR < 0.25.

(B) Longitudinal differential GBMs in GDM and normal mothers during pregnancy or infants during the first year of life separately. FDR < 0.25. Coef: the model coefficient value (effect size).

(legend continued on next page)

VanW; K21744, MerR family transcriptional regulator, thiopeptide resistance regulator; and K23775, MarR family transcriptional regulator, organic hydroperoxide resistance regulator) were identified, which reflected the pathogenic features of this species. A total of 59 KOs were only detected in MAGs of GDM males. Notably, K00382 (dihydrolipoyl dehydrogenase [EC:1.8.1.4]) is involved in the isovaleric acid synthesis I (KADH pathway), one of the gut-brain modules (GBMs).

Additionally, four differential KOs were shared between GDM mother-baby pairs, including 2 protein families: genetic information processing KOs (K02518 and K21907) enriched GDM mothers and infants compared with their counterparts (Table S6). Interestingly, the abundance of K21907 at meconium was positively correlated with zhc at month 12 in male infants but not females (Figure S6H). To sum up, GDM impacts infant head growth in a sex-dependent manner, which is associated with the gut microbiome.

Associations of gut microbiota neuroactive potential with head growth of GDM offspring in a sex-dependent manner

Further, the GBMs framework[36] was applied to determine whether neuroactive compound metabolism[36] is associated with head development in a sex-dependent manner. Of note, several differential KOs (10 depleted and two enriched) (Figure 6A, FDR < 0.25) and GBMs (four depleted and two enriched) (Figure 6B, FDR < 0.25) were detected in GDM mothers carrying male fetuses compared with their normal counterparts, after adjusting for potential confounders (Table S7). Specifically, MGB043 (acetate synthesis I), MGB047 (acetate degradation), and MGB007 (glutamate synthesis II) were depleted in the GDM mothers with male infants (Figure 6B). Consistently, the total relative abundance of species that contributed to MGB047 (acetate degradation) was lower in GDM mothers with male fetuses and, ranked 2nd, *Eubacterium siraeum* was also a depleted species in GDM mothers (Figures 6C and 6D). Only 1 KO (K00137) involved in GABA synthesis II was enriched in the gut microbiome of GDM mothers carrying female fetuses compared with their normal counterparts (Figure 6A, FDR < 0.25). Briefly, these results indicated that male fetuses of GDM mothers experienced distinct prenatal gut-brain axes.

In the gut microbiome of GDM male infants, K01658 related to tryptophan synthesis was depleted, and its abundance in the meconium sample negatively correlated with zhc at BM12 only in male offspring but not females (Figure 6E). In contrast, K10806 involved in isovaleric acid synthesis I (KADH pathway) was enriched in GDM male offspring in comparison with their corresponding control infants (Figure 6A), and its abundance in the meconium sample positively correlated with zhc at month 12 in male offspring but not female (Figure 6E). In female infants, the richness of GBMs was higher in GDM than in control at BM6 and BM12 (Figure 6F). Of note, histamine degradation, dopamine degradation, and K00276 (primary-amine oxidase, a key enzyme involved in histamine degradation and dopamine degradation) increased in the gut microbiome of female infants born to GDM mothers compared with the female control group (Figures 6A, 6B, and S7A), and was also enriched in the infants born to GDM mothers when compared with healthy ones in the validation cohorts (Figures S7B and S7C). This might indicate increased availability of the corresponding neuroactive molecules in the gut. Also, GDM female offspring showed a significant positive association with KOs in GBMs, such as K09471 (gamma-glutamyl putrescine oxidase), which is a key component in GABA synthesis I. Interestingly, gut abundance of these two GBMs and their related K00276 at BM2 or BM1 in male infants was negatively correlated with zhc at BM12 (Figure 6E) separately. Briefly, female offspring of GDM mothers have a distinguishable gut microbiome with neuroactive potential. Taken together, these results indicated that GDM affects their offspring's gut microbiome with neuroactive potential.

DISCUSSION

In this study, we investigated the gut microbiome profiles of mothers during pregnancy and infants during the first year of life from a large-scale longitudinal cohort of 264 mother-baby dyads with 1,566 total metagenomic sequence stool data in Hong Kong. We identified several maternal bacteria features associated with GDM status and glycemia levels during whole gestational periods. To our knowledge, this is a pioneer study in identifying that GDM is deeply fingerprinted on the gut microbiomes of male offspring up to 12 months of age. GDM affects the offspring's head circumference growth in a sex-dependent manner, which, in the prenatal and infancy gut microbiome, is implicated with neuroactive potential.

Here, GDM affected the offspring's microbiome during the first year of life, after adjusting for potential confounders. The microbiome changes were reflected by enrichment in *Clostridium* from Firmicutes phylum and the potential pathogen *Klebsiella quasipneumoniae* from Proteobacteria. It is potentially possible that GDM increased the likelihood of C-section birth and IAP usage, which disrupted the transmission strain within the Actinobacteria and Bacteroides phylum and thus left a niche[25–27] for the non-common transmitted strain belonging to Firmicutes[37] in GDM mother-baby pairs. However, only marginal evidence was detected about the effects of GDM on gut microbiome transmission between mother-baby pairs in our cohort, which may be due to the strain detection requiring sufficient marker genes for each species in each metagenomic sample and thus losing part of the samples involved in the phylogenetic tree construction for transmission analysis.

It is essential to understand the impact of GDM on infant growth. Here, the richness of Chao 1 of Firmicutes in the infant's

(C) Bacterial species contributing to MGB047 in the maternal gut, using species-stratified data. The top contributing species were shown separately, and other species collapsed into a single group.
(D) The sum abundance of species contributing to MGB047 in the maternal gut after sub-group sex of the baby.
(E) The correlation between differential GBM features in the GDM group and infant zhc at BM12 in all infants, only male, or only female infants. * represented $p < 0.05$ based on Spearman correlation analysis. The box color indicates the Rho value. FDR < 0.25.
(F) The richness of GBMs in infant gut microbiome after sub-group sex of the baby. Data are represented as median ± IQR. * indicated $p < 0.05$, ** indicated $p < 0.01$.

gut at BM2 positively correlated with zbmi and zwfl at BM12, which also accords with an earlier observation in which gut microbiome and delivery mode mediated intergenerational overweight and obesity from mother to offspring.[38] Besides the obesity risk, GDM-exposed male infants tended to have larger zhc than control at BM12. Head circumference is the earliest validated marker for neurodevelopment,[21] and abnormal accelerated growth of it has been linked with ASD.[17–19] Furthermore, neurodevelopment was significantly delayed up to 4 years of age among boys born to GDM mothers in the Japanese epidemiology cohort,[39] but whether the gut microbiome modulates brain development of GDM infants is poorly understood.

The maternal prenatal gut microbiome was relevant to the children's neurodevelopment in the first year of life.[40] For example, acetate synthesis or degradation was depleted in the GDM mothers with males. Maternal and placental metabolome could be affected by maternal gut microbiota (i.e., acetate),[41] and acetate can cross the blood-brain barrier and play a role in the GABA metabolic and neuronal-glial cycle of glutamate-glutamine metabolic coupling in the hypothalamus.[42] In addition, tryptophan plays a significant role in regulating the growth and development of the fetus. Here, the enriched tryptophan synthesis in the gut of GDM mothers with male fetuses may be a compensatory result to maintain normoglycemia[43] because the metabolites of L-tryptophan could increase beta-cell function during insulin-resistant status.[44] To sum up, male infants born to mothers with GDM may have distinct neurodevelopment due to their exposure to the disordered prenatal gut microbiome with neuroactive potential.

It is well-recognized that a mother's physiology can impact fetal metabolism. However, increasing studies have shown that the fetus can influence maternal physiology in several potential perspectives. First, women carrying a male fetus have a higher likelihood of developing GDM,[45] and one possible mechanism is that the male fetus may impact the placentally derived hormones or proteins involved in β-cell compensation[45] and insulin resistance.[46] Additionally, pregnancy-related hormonal changes could modulate gut microbiota.[2] Thus, variations in hormone levels between pregnancies with male and female fetuses may contribute to differences in the maternal gut microbiome's functional potential. Second, a possible contributory factor involves the Y chromosome carried by the male fetus; thus, maternal immune response are divergent based on fetal sex. Specifically, women carrying a male fetus were characterized by increases in levels of proinflammatory cytokines and proangiogenic growth factors, while women carrying a female fetus were characterized by increases in the expression of regulatory cytokines.[47] The low-grade inflammation at intestinal mucosal surfaces of pregnant women could lead to changes in the gut microbiome.[1] Our study builds upon previous observations by demonstrating, in a more specific manner, that gut microbiome GBMs varied among GDM mothers with male fetuses compared with their control counterparts. However, further research is needed to better understand the changing maternal gut microbiome in relation to fetal sex.

Sex-specific microbiome fingerprints are also reflected in growth variation. *Clostridia* species have been linked with autism via the production of clostridial toxins, which may have pathological effects on the central nervous system.[48] Six resistant genes could be commonly detected in the MAGs of *Clostridium paraputrificum*. The gut microbiota of children with ASD was reported by an increase in antibiotic resistance genes in the resistome, which could serve as possible predictors of ASD.[49] K00382 is involved in isovaleric acid synthesis I (KADH pathway), and higher fecal levels of isovaleric acids have been detected in children with ASD.[50] Further, the microbial network showed that the *Clostridium* genus negatively correlated with potentially beneficial genera (including *Bacteroides*). *Bacteroides* have been linked with enhanced neurodevelopment,[21] which further reflects the harmful effect of the enriched *Clostridium* genus. Lastly, one enzyme, K21907, enriched in GDM mother-baby pairs and positively correlated with the zhc of males, was mainly encoded by potential pathogens, including *Escherichia coli* and *Klebsiella pneumoniae*, which may be because the glutamate-dependent acid resistance system helps the pathogens overcome the extreme gastric acidic condition.

The impact of GDM on offspring neurodevelopmental sex differences should be considered not only for brain damage but also for brain protection.[39] Dopamine and histamine are essential neurotransmitters. Gut microbiota contains intrinsic enzymatic activity highly involved in dopamine metabolism, facilitating dopamine synthesis and its metabolite breakdown.[51] Dysfunction of dopaminergic signaling may lead to a series of developmental disorders.[52] These GBMs might protect females born to GDM mothers from abnormal zhc at BM12. Further work is warranted to follow up on the cognitive and brain development of children born to GDM mothers and validate whether the microbiome mediates it in sexual dimorphism.

Hosts remodel the gut microbiome during pregnancy,[1,2,4] and we found a converged trend in the gut microbiome during late pregnancy related to bile acid and hormone-related enzymes. Recently, another paper also indicated dramatically increased steroids and steroid derivatives (such as gonadal hormone metabolites and intermediates of primary bile acid biosynthesis) at delivery.[30] Further studies are warranted to quantify gut metabolite changes during pregnancy and investigate their relationship to gut microbiota.

Several studies[7,8,53] using the gut microbiome before diagnosis predicted GDM development. Likewise, we built a prediction model suggesting that the gut microbiome was a useful tool for early GDM risk stratification. Interestingly, two equol (nonsteroidal estrogen) producers, *Adlercreutzia equolifacien*[54] and *Asaccharobacter celatus*, were depleted in the GDM subjects. S-equol has been reported as a potential anti-diabetic agent,[55] which could promote glucose-induced insulin secretion from pancreatic β cells and have a protective effect on streptozotocin-induced hyperglycemia by increasing β cell function in male mice.[56] Therefore, the depletion of two equol producers in the gut of GDM women may partially account for insulin deficiency and further result in hyperglycemia, which may have the potential to alleviate GDM.

There are several strengths in our study. First, we screened healthy, normal pregnant females as a GDM control to rule out the influence of other baseline diseases or pregnancy complications on the gut microbiome. Second, longitudinal maternal samples beginning in early pregnancy allowed us to assess maternal microbiota dynamics leading up to and following birth, providing strain resolution to detect gut microbiome transmission between

mother-baby pairs. Third, we systematically investigate the gut microbiome of GDM-exposed children during the first year of life and further link it with the growth of children. Our study's limitations include the limited GDM case sample size at MG1, which was not big enough for us to build a prediction model with higher accuracy. In addition, further animal experimental studies are necessary to establish causality and other mechanisms. Further study is needed using multiple omics data (placenta for the epigenetic sequence[57] together with metagenomic dataset) to see whether GDM affects infant development and whether there are secondary links with gut microbiome colonization. Moreover, MAG reconstruction is complex and challenging via short-read sequencing technologies.[58,59] Genome assemblies from short-read sequences are highly fragmented,[60,61] especially from diverse communities, which makes it difficult to recover high-quality MAGs for all species from metagenomic data. Further studies utilizing long-read sequencing technologies may improve binning and enable the acquisition of whole-genome sequencing.[62]

Conclusions

In conclusion, our study characterized the gut microbiome dysbiosis of GDM mothers from MG1, before GDM was diagnosed, compared with the normal group. Further, we confirmed deep GDM fingerprints on the infant's gut microbiome till BM12, which is potentially associated with the head growth of children in a sex-dependent manner, indicating the importance of the prenatal and early-life gut microbiome on long-term child health. Future research is necessary to understand the long-term consequence of over-growth in these infants and whether microbiotal modulation serves as a potential approach to mitigate and prevent these risks.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.chom.2024.06.005.

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AUTHOR CONTRIBUTIONS

Conceptualization, L.Z., S.C.N., and S.W.; methodology, S.W., Y.L., W.X., S.Y., B.Q., L.L., and C.P.C.; software, Y.L., and J.Z.; formal analysis, S.W., and Y.L.; investigation, J.Y.C., K.L.I., Y.M.W., P.K.C., Y.K.L., and W.H.B.K.; resources, W.H.T., J.Y.L.C., T.F.L., and T.Y.L.; data curation, S.W. and Y.L.; writing – original draft, S.W.; writing – review & editing, L.Z, S.C.N., F.K.L.C., H.M.T., Y.P., W.H.T., T.F.L., E.B.C., D.T.R., E.C.C., and all other authors; visualization, S.W. and Y.L.; supervision, L.Z. and S.C.N.; project administration, F.K.L.C., S.C.N., L.Z., and H.M.T.; funding acquisition, F.K.L.C. and S.C.N.

DECLARATION OF INTERESTS

F.K.L.C. is a board member of CUHK Medical Centre. He is a co-founder, non-executive board chairman, and shareholder of GenieBiome Ltd. He receives patent royalties through his affiliated institutions. He has received fees as an advisor and honoraria as a speaker for EISAI Co. Ltd, AstraZeneca, Pfizer Inc., Takeda Pharmaceutical Co., and Takeda (China) Holdings Co. Ltd. S.C.N. has served as an advisory board member for Pfizer, Ferring, Janssen, and AbbVie and received honoraria as a speaker for Ferring, Tillotts, Menarini, Janssen, AbbVie, and Takeda. S.C.N. has received research grants through her affiliated institutions from Olympus, Ferring, and AbbVie. S.C.N. is a scientific co-founder and shareholder of GenieBiome Ltd. S.C.N. receives patent royalties through her affiliated institutions. F.K.L.C., S.C.N., L.Z., and H.M.T. are named inventors of patent applications held by the CUHK and MagIC that cover the therapeutic and diagnostic use of the microbiome.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Mothers and Infants stool samples	Prince of Wales Hospital, Hong Kong SAR, China	MOMmy (The MOther-infant Microbiota transmission and its link to long terM health of babY) cohort (NCT04117321).
Critical commercial assays		
Stool nucleic acid preservatives	Norgen	Cat#28330
Qiagen DNeasy PowerSoil Pro Kit	Qiagen	Cat#47016
Nextera XT DNA Library Preparation kit	Illumina	Cat#FC-131-1096
ZymoBIOMICS Microbial Community Standard	ZYMO	Cat#D6300
UltraPureTM distilled water	Invitrogen	Cat# 10-977-015
Deposited data		
Metagenomic sequencing data	This paper	PRJNA1049511
Software and algorithms		
R Statistical Software v4.1.3	R Core Team	https://www.r-project.org; RRID: SCR_001905
Trimmomatic v0.39	Bolger et al.[63]	http://www.usadellab.org/cms/?page=trimmomatic; RRID: SCR_011848
Bowtie2 v2.4.2	Langmead and Salzberg[64]	https://bowtie-bio.sourceforge.net/bowtie2/index.shtml; RRID: SCR_016368
Kneaddata v0.10.0	Huttenhower Lab	https://huttenhower.sph.harvard.edu/kneaddata/
MetaPhlAn v3.0.13	Beghini et al.[65]	https://huttenhower.sph.harvard.edu/metaphlan/
StrainPhlAn v3.0.13	Beghini et al.[65]	https://huttenhower.sph.harvard.edu/metaphlan/
Humann v3.0.0	Beghini et al.[65]	https://huttenhower.sph.harvard.edu/humann/; RRID: SCR_014620
Metapi v3.0.0	Zhu et al.[66]	https://github.com/ohmeta/metapi
MEGAHIT v1.2.9	Li et al.[67]	https://github.com/voutcn/megahit; RRID: SCR_018551
Minimap v2.24	Li et al.[68]	https://github.com/lh3/minimap2; RRID:SCR_018550
MetaBAT2 v2.15	Kang et al.[69]	https://bitbucket.org/berkeleylab/metabat/src/master/; RRID: SCR_019134
VAMB v3.0.9	Nissen et al.[70]	https://github.com/RasmussenLab/vamb
CheckM v1.2.2	Parks et al.[71]	https://github.com/Ecogenomics/CheckM; RRID: SCR_016646
GTDBTk v2.3.2	Chaumeil et al.[72]	https://github.com/Ecogenomics/GTDBTk
KOfamscan v1.3.0	Aramaki et al.[73]	https://github.com/takaram/kofam_scan
random forest v.4.7-1.1	Liaw and Wiener[74]	https://cran.r-project.org/web/packages/randomForest/index.html
ggtree v.3.2.1	Xu et al.[75]	https://github.com/YuLab-SMU/ggtree
Phyloseq v1.38.0	McMurdie and Holmes[76]	https://github.com/joey711/phyloseq
vegan v2.6-2	Oksanen et al.[77]	https://cran.r-project.org/web/packages/vegan/index.html
stats v 4.1.3	R Core Team[78]	https://stat.ethz.ch/R-manual/R-devel/library/stats/html/00Index.html
psych v.2.2.5	Revelle[79]	https://CRAN.R-project.org/package=psych
MaAsLin2 v.1.8.0	Mallick et al.[80]	https://huttenhower.sph.harvard.edu/maaslin/
anthro v 1.0.0	World Health Organization	https://cran.r-project.org/web/packages/anthro/index.html

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Lin Zhang (linzhang@cuhk.edu.hk).

Materials availability

This study did not generate new reagents.

Data and code availability

Quality-controlled and human DNA-removed sequence data have been deposited into the NCBI Sequence Read Archive database under BioProjects (PRJNA1049511). This paper does not generate new code. Additional data in this study are available from the lead contact upon reasonable request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Experimental Design and Cohort Recruitment

Pregnant women and infants (both GDM group and normal control) were selected from the MOMmy (The MOther-infant Microbiota transmission and its link to long terM health of babY) cohort (NCT04117321). The MOMmy cohort study was approved by the Joint Chinese University of Hong Kong – New Territories East Cluster Clinical Research Ethics Committee (the Joint CUHK-NTEC CREC 2019.243). Pregnant women presenting to the antenatal clinics of the Prince of Wales Hospital at the Chinese University of Hong Kong for prenatal care were invited to participate in this study. The cohort of women in the present analysis was enrolled between September 2019 and July 2021. A group of healthy normal pregnancy females was screened out by excluding pregnant women with any baseline disease, any pregnancy complications (GDM, gestational hypertension, acute infection, general anesthesia surgery, and gestational sepsis, etc.), and who deliver preterm babies in MOMmy cohort.

GDM was diagnosed at gestation period 2 (24-28 weeks of gestation) when the woman's fasting plasma glucose (PG) was 5.1-6.9 mmol/l or 2-hour PG after a 75-gram glucose load at OGTT: 8.5-11.0 mmol/l. This is modified from the WHO criteria without the 1-hour PG being adopted by the Hospital Authority (HA) of Hong Kong. The OGTT information was retrospectively retrieved from the HA computerized record. Women who have been diagnosed with type 1 or type 2 diabetes mellitus prior to pregnancy were excluded from this study.

METHOD DETAILS

Clinical information

Measured height (in cm) and self-reported pre-pregnancy weight (in kg) were collected at recruitment by questionnaires and used to compute the pre-pregnancy BMI (weight [kg]/height [m2]). Variables that are fixed through time (for example, mothers' height, mothers' pre-pregnancy weight, total gestational age, birth mode, and baby sex) are described on the basis of a subject and are thus constant for all samples from a given mother-infant pair. Other variables were categorized to reflect exposure in relation to time (for example, antibiotics use, feeding mode, and disease status) and therefore are on a per-sample basis. The growth indexes (head circumference, weight, and length) of infants were measured at each follow-up time point.

Sample collection

The parents collected their stool samples at home or in the delivery hospital. The nurse collected the meconium sample in the delivery hospital, and the mothers collected the other infant stool samples at home. The samples collected at home were stored in the household's freezer (4°C). The samples were then shipped on dry ice to the MOMmy biobank, where the samples were aliquoted and stored at -80°C until shipping to the lab for further processing. Stool nucleic acid preservatives (Norgen, Cat#28330) were used in this study to minimize the potential effect of sample storage on the microbiota composition.[81]

Gestational weeks of sample collection were calculated with the following formula: 280 days (40 weeks) - (Expected date of delivery - Date of sample collection). We collected three stool samples from each participant during their pregnancy, first at early gestation, at the time of booking antenatal visit (12.5 ± 0.097 weeks, mean ± SE), second at middle gestation (25.3 ± 0.19 weeks), and third at late gestation (34.8 ± 0.079 weeks). If the collection dates of two random samples differ by less than three weeks, we would only keep the one with the most distant time between it and the remaining sample (kept 2 out of 3 instead of all) for the categorical analyses.[82] We used the formula above to identify the time point of sample collection for subjects with missing samples. All available stool samples were included in continuous gestational weeks analysis. Finally, a total of 646 maternal stool samples were collected at three gestation periods (mother gestational period 1, MG1; mother gestational period 2, MG2; mother gestational period 3, MG3), and 920 infants' stool samples were collected from birth till one-year-old (Day 0, meconium; month1, BM1; month2, BM2 month 6, BM6; month 12, BM12). We performed shotgun metagenomic sequencing for the fecal bacteria profile.

DNA Extraction and Sequencing

DNA Extraction

Stool samples stored in a preservative were weighted 0.28-0.32 g per tube for further DNA extraction. DNA extractions of stool samples were carried out using the protocol of Qiagen DNeasy PowerSoil Pro Kit (Qiagen, Cat#47016, 250 reactions). Briefly, the power beads were transferred to a 2 mL tube containing a stool sample, and lysis buffer was added. The tube was placed into TissueLyser II (Qiagen, Cat#85300) for homogenization. CD2 solution was added to remove the PCR inhibitor and then centrifuged. The supernatant was transferred to another tube, added solution CD3, and loaded to the MB spin column to bind DNA in the filter membrane. Other reagents were washed with solution EA and C5. Finally, DNA was eluted into another 2 mL tube. In parallel, positive control (a microbial community standard (ZYMO, Cat#D6300)) and negative controls (UltraPureTM distilled water (Invitrogen, Cat# 10-977-015)) were included for each kit as well as each extraction batch. DNA quality will be checked using gel electrophoresis to determine DNA shearing and using Nanodrop OneC Spectrophotometer to determine DNA quality. Qualified DNA samples were stored at -80°C for further library construction.

Metagenome Library Construction

Metagenomic DNA concentrations were measured using Qubit (ThermoFisher) with the Qubit dsDNA HS Assay Kit (ThermoFisher). Illumina sequencing libraries were prepared from 100-250 pg DNA using the Nextera XT DNA Library Preparation kit (Illumina) according to the manufacturer's recommended protocol, with reaction volumes scaled accordingly. Insert sizes and concentrations for each pooled library were determined using an Agilent Bioanalyzer DNA 1000 kit (Agilent Technologies). WGS libraries were sequenced on the Illumina Novaseq 6000 platform with 150 bp paired-end reads.

Metagenome Quality Control and Pre-processing

Reads were quality controlled by trimming low-quality bases and removing reads shorter than 60 nucleotides. We identified and filtered out potential human contamination using the Trimmomatic (v0.39)[63] and Kneaddata (v0.10.0) with the hg38 human reference genome. We got an average of 6.575 Gb of sequence per sample after removing contaminating reads.

Species-Level Profiling and Functional Profiling

Quality-controlled samples were profiled taxonomically using MetaPhlAn3 (v3.0.13)[65] following Bowtie2 (v2.4.2)[64] alignment to the MetaPhlAn3 unique marker database. To annotate the function of gut microbiome genes, we applied Humann (3.0.0) to all metagenomic samples after filtering out reads mapping to human reference. All bacteria abundance analyses are based on MetaPhlAn3 results except for transmission analysis with clarity as below. To further identify gut microbiome Carbohydrate-Active enzymes (CAZy), a file linking CAZy to human UniRef90 results was retrieved from a previous publication (1) (filename ''map_cazy_uniref90.txt.gz'').[65,83,84] Analysis of Gut-Brain Modules (GBMs)[36] and Gut-Metabolic Modules (GMMs)[85] were performed as previously described. Briefly, the UniRef gene families that were detected by HUMAnN3[65] were mapped to KEGG Orthogroups (KOs) using the humann_regroup_table function, and the abundances of KOs were normalized using the humann_renorm_table function. Further, the KOs were stratified using the humann_split_stratified_table function. Next, these KOs were further mapped to GBMs and GMMs and calculated using the Gomixer tool.[86]

GDM prediction model at MG1

The early gut microbiome prediction model for later GDM onset was established with the random forest. Considering there are well-known GDM risk factors, we constructed three data frames: 1. Only include demographic risk factors (age, pre-pregnancy BMI group, sex of baby, number of children in the house, marital status, education, work status, race, smoke, husband smoke, alcohol intake, diet, dining out frequency daily, stool bristol score); 2. Only included gut bacteria species at MG1 before GDM was diagnosed; 3. Combined demographic factors and gut bacteria species. Each data frame was separated into a 70% testing set and a 30% training set. And performed 5 times cross-validation and repeated 10 times in the random forest model. Area Under the Receiver Operating Characteristic curve (AUC) was calculated to check the performance of each model in distinguishing between GDM and normal pregnancy at MG1. The top 20 important variables were defined based on the beta-IncNodepurity from the random forest model (v.4.7-1.1).[74]

Calculation of gut microbiota gestational age

The relative abundance of all species in training samples (a subgroup of 179 normal pregnant women, total of 422 samples during pregnancy) was fit against its corresponding gestational weeks using the randomForest package (v 4.7–1.1) in R to build up a gut microbiota gestational age model, as was reported previously.[28,87]

Analysis of transmission of the maternal gut microbiome

Strain characterization was performed using StrainPhlan (v.3.0.13)[65] based on pre-processed metagenomic to further characterize the single-nucleotide-variant (SNVs). In detail, StrainPhlAn was run with the default parameters to extract the consensus marker gene for species that could profile in stain level and generate alignments for each sample. Next, a phylogenetic tree was constructed with RaxML for a specific species. The genetic distance was received with ggtree (v.3.2.1)[75] in R, and this distance difference between paired samples was normalized by dividing the median value of the corresponding tree of each species. transmission between mother-baby pairs was defined if the normalized genetic distance was below the threshold of 0.1, as used by others.[27,32,88] Specifically, a normalized genetic distance smaller than 0.1 represented identical strains (possible transmitted strains), while the others (value larger than 0.1) represented distinct strains.

Strain transmission rates were calculated as the number of strains transmission between two samples divided by the number of shared species profiled by StrainPhlAn (number of transmission strains/number of shared species). The same calculation was used to assess same-individual strain retention between two time points in longitudinal datasets. Strain acquisition rates by the offspring

were defined as the proportion of strains profiled in the offspring transmitted from mothers, thus putatively originating from her. Species transmissibility was defined as the number of strain transmission events detected for a species divided by the total potential number of strain transmission events based on the presence of a strain-level profile by StrainPhlAn3.[89] We mainly assessed strain transmission across the following modes: related mother-infant (defined between mothers and their offspring). Unless specified, the transmission between mother-infant pairs was evaluated using mothers' stool samples during late pregnancy, which was the closest to delivery time.

Validation cohort

We used the metaphlan3 profile result of the curatedMetagenomicData R package[90] and filtered out two cohorts with healthy infants' and GDM infants' gut metagenomic sequence data. Considering the limited available GDM infant gut metagenomic sequencing sample size, we combined the two validation cohorts to check the GDM fingerprint on the gut microbiome in different sexes of babies. Finally, a total of 591 stool samples (550 samples of healthy control infants and 41 samples of infants born to GDM) collected from the first year of life were used for validation. We extracted the gene family profile to validate the findings of gut-brain modules (GBMs). The gene families were regrouped to KEGG Orthology (KO), used for the calculation of gut-brain modules (GBMs).

Metagenome-assembled genomes (MAGs) of Clostridium paraputrificum

We performed de novo assembly and identified the *Clostridium* species for downstream analysis. Metapi (v3.0.0) was applied.[66] Briefly, the high-quality reads were assembled using MEGAHIT (v1.2.9)[67] with the parameter "–min-contig 100". To facilitate genome recovery, reads of samples from the same infants collected at multiple time points during the first year of life were co-assembled. Reads were mapped back to the resulting contigs using minimap (v2.24-r1122)[68] with default parameters. Binning of contigs into metagenome-assembled genomes (MAGs) was done using two automatic binning programs: MetaBAT2 (v2.15)[69] and VAMB (v3.0.9)[70] with parameter "-m 2000 –minfasta 200000" based on the coverage matrix with a multi-split binning strategy implemented in VAMB. High- (completeness > 90% and contamination < 5%) or medium-quality (completeness > 50% and contamination < 10%) prokaryotic MAGs (pMAGs) were identified by CheckM (v1.2.2),[71] consistent with Minimum Information about a Metagenome-Assembled Genome (MIMAG) standard.[91] The taxonomic assignment of these representative bacterial species-level MAGs was performed by GTDBTk (v2.3.2)[72] based on the GTDB database (release214).[92] MAGs of *Clostridium paraputrificum* were filtered out, and the protein profiling was searched against the curated KEGG Ortholog (KO) database using KOfamscan (v1.3.0).[73]

QUANTIFICATION AND STATISTICAL ANALYSIS

Metaphlan data from metagenomics were imported into R (v 4.1.3). Alpha diversity metrics, richness (number of observed species) and diversity, were calculated using the phyloseq package (v1.38.0).[76] Principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarities was performed using the vegan package (v2.6-2).[77] Associations between gut microbial community composition and the GDM group were assessed using permutational multivariate analysis of variance (PERMANOVA), in which maternal analysis considered the GDM group, age and BMI before pregnancy and infant analysis considered GDM group, delivery mode, and antibiotic use and stratifying by each time point.

The alpha diversity between different groups was compared using the Wilcoxon rank-sum test or paired Wilcoxon rank-sum test if applicable.

The association between gut richness species at MG1 and age was evaluated with the glm in the stats package (v 4.1.3),[78] adjusting the pre-pregnancy overweight group. Generalized linear models (GLM) for binominal outcomes (normal pregnancy and GDM development at MG2 or MG3) were applied to determine the odd ratio value of the gut richness at MG1. GLMs were also constructed to investigate modification effects while adjusting for potential confounders identified in univariable analysis. Further, mediation analysis was performed with the psych package (v.2.2.5) psych::mediate function.[79]

The longitudinal joint association was used to explore the differences in species-level taxonomy and functional pathways and GBMs between normal pregnant women and GDM subjects during the whole gestation period using MaAsLin2 (v.1.8.0),[80] including each mother subject's ID as the random effect and adjusting potential confounders, including maternal age, gestational age, and pre-pregnancy BMI. The association between maternal differential bacteria species and OGTT level was explored with Spearman correlation.

Two approaches were performed to explore the longitudinal associations between infant microbial features (i.e., microbial species and functional pathways) and host phenotypes (e.g., GDM status, delivery mode, IAP usage, etc.). First, based on a combination of data collected from five time points during the first year of life, the overall associations were estimated using MaAsLin2 with linear mixed models (joint association). This model included each infant subject's ID as the random effect and adjusted for potential covariates, including sex of baby, sample collection day, delivery mode, IAP usage, breastfeeding mode, and antibiotic use within 3 months as fixed effects. Second, the dynamic associations were checked through sub-group infants' samples at five-time points, and the other set was similar to the first one. The first joint approach was also applied to explore the longitudinal associations between infant microbial features (bacteria species, GBMs, and GBMs-related KO) and GDM status through sub-group infants' sex and adjust potential covariates except for the sex of the baby.

In the validation cohort, a similar approach was adopted. The analysis included each infant subject's ID as the random effect and adjusted for potential covariates, including study, sex of baby, sample collection day, delivery mode, and current antibiotic use as fixed effects. After sub-grouping infants' sex, all potential covariates were adjusted except for the sex of the baby.

The genus level-microbial network was analyzed by pairwise Spearman's rank correlations at each time point ($R > 0.40$, $p < 0.05$, ggClusterNet 0.1.0). The network metrics of nodes (degree, closeness centrality, and betweenness centrality)[93] were checked.

Transmission analysis

The statistical significance of transmission rates between infants born to normal or GDM mothers was assessed using a two-sided Fisher's exact or Chi-Square test for each species. Statistical significance of the number of strains transmission within the different phylum between mother (MG3)-baby pairs stratified by different clinical factors was assessed using a two-sided Fisher's exact test.

Growth analysis

Participant growth was measured on every scheduled clinic visit (approximately similar to the stool collection schedule). Age-specific head circumference-for-age z-score, BMI z-score, and weight-for-length z-score were calculated using the anthro R package (v 1.0.0), based on World Health Organization Child Growth Standards. Besides, the relative growth rate of head circumference was calculated in two ways: (BM12-BM1)/BM1 or (BM12-BM1)/ months. The univariable analysis was performed to check the association between potential clinical factors (Maternal age, antibiotic use of infants within 3 months, exclusive breastfeeding duration, delivery mode, exclusive formula feed duration, GDM Group, IAP, feeding status before discharge, maternal BMI group before pregnancy, maternal smoke status before pregnancy, maternal smoke during pregnancy) and the growth outcomes of infants with the glm in the stats package (v 4.1.3). The association between infant GDM group differential features (bacteria species, GBMs, and GBMs-related KO) at specific time points and growth indexes at BM12 was explored with Spearman correlation in three scenarios (all infants, only male, and only female). The *p*-value was adjusted with Benjamini & Hochberg. $P < 0.05$ and FDR < 0.25 was considered significant. Only the marker features associated with growth outcomes results in at least one condition ($p < 0.05$, FDR < 0.25) were used for visualization.