Supplemental Materials

Landscape of the gut mycobiome dynamics during pregnancy and its relationship with host metabolism and pregnancy health

This file includes:

Methods

(1) Study population

(2) Sample collection

(3) Questionnaires and clinical data collection

(4) Bioinformatic analyses

(5) Statistical analysis

Supplemental Figure S1 to S2

(1) Supplemental Figure S1. Supplementary Table S1: Characteristics of demographic, clinical and dietary factors stratified by early-pregnancy fungal enterotypes.

(2) Supplemental Figure S2. The association between changes in consumption of food groups during pregnancy and alterations in gut fungal richness.

Fu Y, *et al. Gut* 2024;0:1–11. doi: 10.1136/gutjnl-2024-332260

Methods

Study population

The current study was based on the Tongji-Huaxi-Shuangliu Birth Cohort (THSBC), in which pregnant women aged 18 to 41 years were recruited during the early pregnancy when they presented to antenatal care clinics in in a local maternal and child health hospital during their early pregnancy. Exclusion criteria were 1) receiving infertility treatment (e.g., in vitro fertilization or intrauterine insemination); 2) reporting severe chronic or infectious diseases (e.g., cancer, HIV infection, or tuberculosis); or 3) were unable to or refused to sign the informed consent. The THSBC study was approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (No. [2017](S225)-1), and informed consent was obtained from all participants.

In the present analysis, we included 4800 participants who had available ITS2 sequencing data, dietary information and clinical records during their pregnancy. This dataset enables us to comprehensively profile the gut mycobiome among pregnant women and investigate potential determinants contributing to the variations of gut mycobiome. To examine how pregnancy impacts the gut mycobiome over time and investigate their potential associations with host metabolism, we established a sub-cohort of 1059 participants, which included 514 women who gave birth to preterm (n=240), low birthweight (n=137), or macrosomia (n=216) infants, as well as 545 randomly selected participants who did not experience the above three adverse pregnancy outcomes.

ITS2 sequencing was performed for all the 4800 participants, while the shotgun metagenomics sequencing was performed for T1 samples within the established sub-cohort (n=1059). Additionally, within the sub-cohort, 750 and 748 participants had ITS2 and 16S sequencing data available, respectively, for all trimesters. We also repeatedly measured serum metabolome throughout each trimester of pregnancy for participants in this selected sub-cohort using an LC-ESI-MS/MS system.

Fu Y, *et al. Gut* 2024;0:1–11. doi: 10.1136/gutjnl-2024-332260

Sample collection

Stool samples were collected and stored in ice boxes at the hospital or home by the participants under instructions and then transferred to the hospital to store at −40 °C within 24 hours. A detailed standard operating procedure was given to the pregnant women for instructions on fecal sample collection, temporary storage, and transportation to the hospital. Stocks of frozen fecal samples were transported by dry ice every 2 to 3 months to the laboratory in Huazhong University of Science and Technology and stored at -80 °C before further processing.

Questionnaires and clinical data collection

All participants completed a set of structured questionnaires on sociodemographic information, lifestyle and behaviors (such as cigarette smoking and alcohol drinking), diet (including recent daily intakes of major food groups), history of pregnancy and births, history of diseases and medications, and family history of diseases. Anthropometric and blood pressure measurements were collected on site using devices according to standard protocols. Body mass index (BMI) was calculated by dividing the weight in kilograms by the square of height in meters. Underweight, overweight and obesity was defined as BMI<18.5, BMI ≥ 24 kg/m^2 and BMI ≥ 28 kg/m^2, respectively. Pregnancy complications (e.g., gestational diabetes mellitus) and birth outcomes were extracted from the electronic clinical records.

Bioinformatic analyses

Gut mycobiome analysis using ITS2 rRNA gene sequencing data

Microbial DNA was extracted using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) according to manufacturer's protocols. The final DNA concentration and purification were determined by NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA), and DNA quality was checked by 1% agarose gel electrophoresis. The ITS2 hypervariable regions of the fungal ITS rRNA gene were amplified with primers ITS3F: GCATCGATGAAGAACGCAGC and ITS4R: TCCTCCGCTTATTGATATGC by

thermocycler PCR system (GeneAmp 9700, ABI, USA). Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 250) on an Illumina NovaSeq platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

The mean sequencing depth and its standard deviation for all samples were 118,177 and 7955, respectively. It is worth noting that within the sub-cohort analyzed longitudinally, the number of sequencing reads remained relatively consistent across trimesters. Specifically, the mean (SD) sequencing depths were 117,906 (8350), 118,882 (6125), and 116,688 (9198) for samples collected during the first, second, and third trimester of pregnancy, respectively. The demultiplexed ITS2 sequences were denoised and grouped into amplicon sequence variants (ASVs; i.e., 100% exact sequence match) using DADA2.[1] During the process, marker gene Illumina sequence data and low-quality regions of the sequences were detected and filtered. We trimmed 28 bases (primer and barcode) from the beginning of the sequences. We also truncated the sequences at the 245 bases as the quality dropped around position 245 (median of quality score <30). The ASV features that were presented in only one sample were excluded as suggested by the Qiime2 tutorial, based on the suspicion that these may not represent real biological diversity but rather PCR or sequencing errors. The individual ASVs were taxonomically classified based on the UNITE (version 8.2, 99%) database using the VSEARCH tool wrapped in QIIME2 (version 2021.2).[2] α-diversity analysis was conducted through the q2-diversity plugin at the sampling depth of 10000. α-diversity was estimated by Shannon's diversity index (or Shannon; a quantitative measure of community richness and evenness), Observed Features (or Richness; a qualitative measure of community richness), and Faith's PD (or Faith's Phylogenetic Diversity; a qualitative measure of community richness that incorporates phylogenetic relationships between the observed features).

Gut bacteria analysis using 16S rRNA gene sequencing data

For the 16S analysis, raw sequencing reads were merge-paired, quality filtered and

analyzed using QIIME2 (version 2021.2). As described above, we used DADA2 denoised-paired plugin in QIIME2 to process the fastq files. We filtered the features that were present in only a single sample. The taxonomies of ASVs were subsequently determined using the Naive Bayes classifier trained on the Sliva_138 99% reference database. α-diversity analysis was conducted at the sampling depth of 10000. A diversity of the gut bacteria was estimated by the indices the same as ITS2 data.

Microbial functional profiling using metagenome data

Microbial DNA extractions were carried out by a standardized CTAB procedure. DNA concentration was measured using Qubit dsDNA Assay Kit in Qubit 2.0 Fluorometer (Life Technologies, CA, USA). For DNA library preparation, a total amount of 1μg DNA per sample was used. In addition, the NEBNext Ultra DNA Library Prep Kit (NEB, USA) was used following manufacturer's recommendations and index codes were added to attribute sequences to each sample. The DNA samples were fragmented by sonication to a size of approximately 350 bp. Then, the DNA fragments were end-polished, A-tailed, and ligated with the full-length adaptor for Illumina sequencing with further PCR amplification. Thereafter, PCR products were purified (AMPure XP system) and libraries were analyzed for size distribution by Agilent2100 Bioanalyzer and quantified using real-time PCR. The clustering of the index-coded samples was performed on a cBot Cluster Generation System according to the manufacturer's instructions. Lastly, sequencing was performed using the Illumina NovaSeq platform at Shanghai Personal Biotechnology Co. Ltd. (Shanghai, China) and 150 bp paired-end reads were generated.

Next, raw sequencing reads were first quality-controlled with KneadData toolkit (v0.10.0): 1) to trim the reads by quality score from the 5′ end and 3′ end with a quality threshold of 20; 2) removed read pairs when either read was < 50 bp, contained "N" bases or quality score mean below 30; and 3) deduplicate the reads. Reads aligning to the human genome (H. sapiens, UCSC hg38) were removed via KneadData integrated with Bowtie2 tool (v2.4.5).

Functional profiling was performed with HUMAnN3 (v3.0.1), which maps sample reads against the sample-specific reference database to quantify gene presence and abundance in a species-stratified manner, with unmapped reads further used in a translated search against Uniref90 to include taxonomically unclassified but functionally distinct gene family abundances. We extracted the Uniref90 gene families of gut bacteria for downstream analyses. The Uniref90 gene families were then converted into relative abundances of unstratified pathway.

Serum metabolomics profiling

The sample extracts were analyzed using an LC-ESI-MS/MS system (UPLC, ExionLC AD, https://sciex.com.cn/; MS, QTRAP® System, https://sciex.com/) at Wuhan Metware Biotechnology Co., Ltd. (Wuhan, China). LIT and triple quadrupole (QQQ) scans were acquired on a triple quadrupole-linear ion trap mass spectrometer (QTRAP), QTRAP® LC-MS/MS System, equipped with an ESI Turbo Ion-Spray interface, operating in positive and negative ion mode and controlled by Analyst 1.6.3 software (Sciex). Instrument tuning and mass calibration were performed with 10 and 100 μmol/L polypropylene glycol solutions in QQQ and LIT modes, respectively. QQQ scans were acquired as MRM experiments with collision gas (nitrogen) set to 5 psi. Declustering potential (DP) and collision energy (CE) for individual MRM transitions was done with further DP and CE optimization. A specific set of MRM transitions were monitored for each period according to the metabolites eluted within this period. The mass spectrum data were processed using Software Analyst 1.6.3. The metabolite identification was conducted by referencing standards in self-built metware database and public databases. The identified metabolites were matched with the parent ion mass-to-charge ratio, the fragment ion mass-to-charge ratio as well as retention time of their corresponding standards. The accuracy of metabolite characterization was classified into three levels, depending on the presence of isotope internal standards or matching score with the secondary mass spectrometry. The matching score>0.7 indicates the level 1 accuracy of metabolite characterization, while 0.5-0.7 and <0.5 indicate the level 2 and level 3 accuracy, respectively.

Statistical analysis

Determinants of gut fungal composition among pregnant women

The gut mycobiome data were analyzed at the genus level. We investigated the determinants of the gut fungal composition using the data collected from 4800 participants during the first trimester, with each participant contributing only 1 stool sample. We used the *vegdist* function in the R package *vegan* to calculate the gut fungal Bray-Curtis dissimilarity matrix. The contribution of 20 environmental variables (including demographics, physiologic traits, diseases, and habitual dietary intakes) to fungal community variation was determined by PERMANOVA analysis using the function adonis2 in *vegan*.[3] We applied a complete data analysis strategy which excluded 19 participants with missing values for at least one of the environmental variables. The 4800 samples included in this analysis were sequenced in two separate batches. Therefore, we included the batch information as a covariate in the model, to adjust for potential batch effects. The p value was determined through 999 permutations.

Gut fungal and bacterial enterotype clustering

The fecal samples (T1, n=4800; T2, n=890; T3, n=850) of ITS2 amplification were clustered into fungal enterotypes by using a partitioning around medoid (PAM) clustering method as those previously described.[4] Briefly, the samples were grouped into clusters with partitioning around medoid (PAM) based on the between-sample Bray–Curtis distance calculated at genus-level. The optimal number of clusters was determined by the silhouette index. The driver genus of each enterotype was determined as the genus with the highest relative abundance in the enterotype. The fecal samples of 16S amplification were clustered into bacterial enterotypes by using the method as that for bacterial enterotype.

Dynamics of within-sample α diversity throughout each trimester of pregnancy

This analysis was conducted in the established sub-cohort of 750 participants who had available gut fungi α diversity data for each trimester of pregnancy. We utilized

paired t-tests to assess the statistical significance of the changes in fungal α diversity between T1 and T2, as well as between T2 and T3 independently.

Among the 750 participants included in the study to profile changes in gut fungal richness from T1 to T3, data on the estimation of consumption changes in food groups from T1 to T3 based on the FFQ were available for 639 participants. These food groups consisted of rice, steamed bread, noodles, vegetables, meat, eggs, milk, and fruit. The weight of each food group consumed per day was quantified based on the FFQ. We applied a linear regression model to estimate the association between the changes in richness from T1 to T3 and the respective food group. As covariates, we incorporated age, pre-pregnancy BMI, interval time between sample collections, parity, and gravidity. An FDR<0.05 was considered statistically significant.

Loss rate calculations and discriminative genera identification

Utilizing the available repeated measurements of gut fungi throughout pregnancy within the designated sub-cohort (n=750), we elucidated the loss rate for each fungal genus as the host underwent progression from T1 to T3. The loss rate for each fungal genus was determined by quantifying the proportion of the decline in frequency observed between T1 and T3.

To assess the gut fungi enriched or depleted during early or late pregnancy, we conducted an analysis using paired t test analysis. This analysis was based on the examination of 465 genera that were detected at either T1 or T3. We transformed the taxa data using the centered log-ratio (CLR) method to address the compositional nature of the mycobiome data before we perform the paired t test analysis. To determine statistical significance, a false discovery rate (FDR)-adjusted p-value threshold of less than 0.05 was used.

Additionally, we used these 465 fungal genera to construct a machine learning framework of LightGBM for predicting the trimester that the samples belong to.[5] The construction of prediction model was based on Scikit-learn (v0.15.2), and ten-

fold cross validation (CV) was applied. To evaluate binary classification performance, receiver operating characteristic (ROC) curve analyses were conducted using the R package pROC. We used the SHAP (Shapley Additive exPlanations) to interpret predictions and the importance of each fungal genus to the prediction model is represented using Shapley values.[6]

Quantification of intra-individual gut fungi compositional alterations

We applied the *vegdist* function from the R package *vegan* to calculate the Bray-Curtis distance based on gut fungal genus-level composition to assess intra-individual and inter-individual dissimilarities, respectively.[3] The sub-cohort, consisting of 750 participants, was included in this analysis, as they had relevant data throughout each trimester of pregnancy. To determine the intra-individual distance, paired data was used, with the gut mycobiome data at T1 serving as the reference. Subsequently, a Bray-Curtis distance value was calculated for each participant in the sub-cohort, reflecting the extent of gut fungal compositional alterations.

We fitted a multivariate regression model to examine the associations of pre-pregnancy overweight status (category variable) or pre-pregnancy BMI (continuous variable, z-score transformed) with the extent of gut fungal compositional alteration within the established sub-cohort. The model was adjusted for potential confounders including age, time interval between sample collection, parity, antibiotics use and pregnancy complications. To examine the potential influence of gut fungal compositional alteration on adverse birth outcomes, multivariate regression models were constructed for preterm delivery, low birthweight, and macrosomia. The aforementioned potential confounders, along with the extent of gut fungal compositional alteration, were considered as exposure variables in these models.

With regard to the divergence between individuals, the inter-individual distance was evaluated at various time points (i.e., T1, T2 or T3), separately. At each time point (e.g., T1 or T3), we calculate the average Bray-Curtis distance for each participant compared to all other participants. Thus, at each time point, each participant

possessed a distance value to reflect the similarity of her gut fungal composition with others.

Dynamic trajectory of the core gut fungal genera and their relationship with host health status

We conducted a longitudinal trajectory analysis for each core fungal genus in the established sub-cohort comprising 750 participants. For each core fungi, every participant had three measurements recorded at different time points, namely T1, T2, and T3. Therefore, the trajectory of a genus within an individual participant could be characterized by a vector consisting of three component values corresponding to these time points. Thereafter, we applied the *vegdist* function from the R package *vegan* to calculate the Canberra distance metric.[3] We performed PERMANOVA analysis using the function adonis2 in *vegan*, to assess the association of between-individual variation in the trajectory of each fungal genus with pre-pregnancy overweight status or adverse birth outcomes. The p value was determined through 999 permutations and an FDR-adjusted p value of less than 0.05 was considered indicative of statistical significance.

Network analysis among gut fungal enterotype, functional pathways and host serum metabolites

The network analysis was conducted among the participants who had available metagenome data during the first trimester of pregnancy. After excluding 35 participants who had antibiotics exposure within 2weeks before stool sample collection, this analysis included a total of 1001 women. We firstly performed Kruskal-Wallis test to identify pathways whose distribution varied across fungal enterotypes. Thereafter, we performed post-hoc pair-wise comparison to defined which enterotype was enrich with the identified pathways. We fitted multivariate regression models to examine the associations of identified pathways with host serum metabolites. Here, we adjusted for potential confounders including age, gestation week, parity and pre-pregnancy BMI. Both the fungi and pathway data were

standardized using z-score before the regression analysis. An FDR-adjusted p value of less than 0.05 was considered indicative of statistical significance.

Covarying relationship between gut fungi alterations and host metabolic changes

We investigated the relationship between alterations in gut fungi and changes in host metabolism among 709 participants. These participants had available gut mycobiome sequencing data as well as serum metabolome data during each trimester of pregnancy. To address the compositional nature of the mycobiome data, we first applied the centered log-ratio (CLR) method to transform the taxa data. Next, we calculated the changes in each core fungus from T1 to T3 for each participant. Additionally, we calculated the changes in signal intensity of each serum metabolite from T1 to T3 for each participant. This enabled us to construct a matrix of gut fungi alterations and a matrix of host metabolic changes. To investigate the overall relationship between gut fungi alterations and host metabolic changes, we conducted Procrustes analysis in R using the 'vegan' R package. Procrustes. The p-value was generated based on 999 permutations.[3]

Furthermore, we explored the covarying relationship between individual fungal genera and individual serum metabolites. For this analysis, we applied pairwise Spearman correlation analysis to each genus-serum metabolite pair in the aforementioned dataset. We considered a false discovery rate (FDR)-adjusted p-value of less than 0.05 as indicative of statistical significance.

Pre-pregnancy overweight status impacts the metabolic changes during pregnancy

For those participants with available serum metabolomics data throughout each trimester of pregnancy, we had constructed a matrix of host metabolic changes. Based on this matrix, the *vegdist* function from the R package *vegan* was utilized to calculate the Canberra distance metric.[3] To assess the contribution of pre-pregnancy overweight to the variation in metabolic changes between individuals, a

PERMANOVA analysis was conducted using the *adonis2* function from the vegan package.[3] The significance of the results was determined using 999 permutations, and a false discovery rate (FDR)-adjusted p-value of less than 0.05 was considered statistically significant.

To identify the distinct metabolic alterations in underweight and overweight pregnant women, we classified the participants into three groups based on their pre-pregnancy weight status: underweight, normal weight, and overweight/obese. Paired t-tests were conducted for each serum metabolite measured at T1 and T3 within each group. A FDR-adjusted p-value of less than 0.05 was considered statistically significant. Metabolic changes that were observed as significant solely among the underweight group, but not in the other groups, were defined as unique metabolic changes in pregnant women who were underweight prior to pregnancy. Similarly, significant metabolic changes observed solely among the overweight/obese group were defined as unique metabolic changes in pregnant women who were overweight/obese prior to pregnancy.

Clinical impact of the gut mycobiome during pregnancy

Logistic regression was used to evaluate the association between each core fungal genus, specifically measured during the first trimester of pregnancy, and the occurrence of pregnancy complications (GDM and PIH), as well as adverse birth outcomes (e.g., preterm delivery, macrosomia, and low birthweight). The model included age, pre-pregnancy BMI, parity as covariates. Specifically, when examining GDM or PIH as exposures, the model was adjusted for these variables. Additionally, when fungal genera were included as exposures, the fungal genera data were z-score standardized and the model was further adjusted for the gestational week corresponding to stool sample collection and the batch of sequencing.

The analysis pertaining to pregnancy complications was conducted among 4606 participants who possessed gut fungi sequencing data at T1 and information regarding

pregnancy complications. Likewise, the analysis concerning birth outcomes was performed among 4656 participants who had gut fungi sequencing data at T1 and information on birth outcomes. In both analyses, a complete data analysis strategy was implemented for variables with an exceptionally low occurrence of missing values, which consequently excluded 3 participants. For the variable "gestational week at stool sample collection," which was missing for 278 participants, a multiple imputation strategy was employed to impute these missing values. As a result, the logistic regression model for pregnancy complications consisted of 4603 participants, while the logistic regression model for adverse birth outcomes encompassed 4653 participants.

For pregnancy complications that exhibited significant associations with both gut fungi and adverse birth outcomes, we conducted mediation analysis to investigate the potential mediation effect of pregnancy complications on the link between the mycobiome and adverse birth outcomes. All statistical analyses were performed using Stata version 15 or R version 4.0.2.

References

1 Callahan BJ, McMurdie PJ, Rosen MJ, et al. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13:581–3.

2 Bolyen E, Rideout JR, Dillon MR, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol. 2019;37:852–7.

3. Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Sólymos, P., Stevens, M.H.H. & Wagner, H., vegan: Community Ecology Package, 2012, Software. http://CRAN.R-project.org/package=vegan.

4. Arumugam M, Raes J, Pelletier E, et al. Enterotypes of the human gut microbiome. Nature. 2011;473:174–80.

5. Ke, G., Meng, Q., Finley, T., Wang, T., Chen, W., Ma, W., Ye, Q., Liu T.Y. (2017) LightGBM: a highly efficient gradient boosting decision tree. NIPS'17: Proceedings of the 31st International Conference on Neural Information Processing Systems.

3149–3157.

6. Lundberg, S.M., Lee, S.I. (2017) A unified approach to interpreting model predictions. NIPS'17: Proceedings of the 31st International Conference on Neural Information Processing Systems. 4768–4777.

Table S1: Characteristics of demographic, clinical and dietary factors stratified by early-pregnancy fungal enterotypes

	Candida-dominated enterotype (n=907)	*Aspergillus*-dominated enterotype (n=2723)	*Saccharomyces*-dominated enterotype (n=1170)	*p**
Age (year)	26.4 (3.6)	26.3 (3.6)	26.5 (3.5)	0.246
Pre-pregnancy BMI	20.8 (2.9)	20.9 (2.9)	21.0 (3.1)	0.343
Gestation week (wk)	10.2 (2.1)	10.2 (2.0)	10.1 (2.0)	0.374
Time to delivery (day)	198.2 (16.1)	198.4 (15.5)	198.5 (15.6)	0.940
Parity				0.013
Primiparous	54.0%	57.3%	60.5%	
Overweight status				0.249
Overweight or obese	21.8	19.2	19.7	
Normal weight	65.1	67.6	65.3	
Underweight	13.1	13.3	15	
Steam bread consumption				<0.001
Ever (during the past 1 year)	46.9%	47.3%	54.2%	
Drinking				0.070
Ever (during the past 1 year)	22.4%	22.0%	18.9%	
Tea consumption				0.024
Ever (during the past 1 year)	34.0%	33.7%	29.5%	
Coffee consumption				0.057
Ever (during the past 1 year)	24.6%	26.6%	23.1%	
Milk consumption				0.564
never	19.2%	21.0%	19.7%	
<1 / day	42.9%	42.9%	42.1%	
⩾1/day	38.0%	36.1%	38.2%	

* One-way ANOVA was applied to examine the significance of difference between groups for continuous variables, while chi-square test was used for category variables.

Table S2. The association between changes in consumption of food groups during pregnancy and alterations in gut fungal richness*

Exposure (changes in consumption of food groups)	Beta coefficient	95% CI	p	FDR
Rice (per 50g/day)	-3.20	(-5.75, -0.64)	0.014	0.11
Steamed bread (per 1 bread/day)	-6.38	(-12.9, 0.14)	0.055	0.15
Noodle (per 50g/day)	-5.06	(-10.63, 0.50)	0.074	0.15
Vegetables (per 50g/day)	1.20	(-9.82, 3.22)	0.243	0.39
Meat (per 50g/day)	1.45	(-3.48, 6.37)	0.564	0.64
Egg (per 1 egg/day)	7.33	(0.26, 14.41)	0.042	0.15
Milk (per 50g/day)	0.01	(-0.01, 0.03)	0.51	0.64
Fruit (per 50g/day)	-0.30	(-1.89, 1.29)	0.712	0.71

* Covariates included in the regression model: age, pre-pregnancy BMI, interval time between sample collections, parity, and gravidity.
639 out of the 750 with available data on the estimation of consumption changes in food groups from T1 to T3 based on the FFQ were included.