Microbiota-Dependent Metabolomic Changes After Nutritional Intervention During Pregnancy

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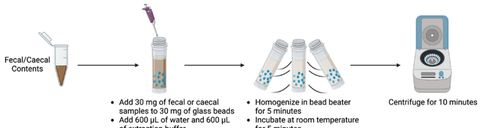
Introduction

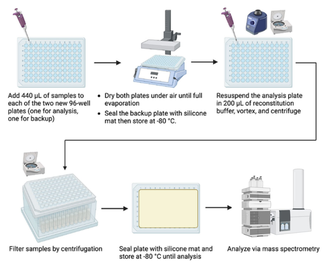
Maternal and infant undernutrition is a major problem in many lower and middle-income countries. A lack of balanced nutrition during pregnancy affects both maternal health and proper fetal growth, leading to low birth weight and premature birth in infants. Recent studies highlight the critical role in infant development played by the maternal microbiome, which shifts dramatically throughout pregnancy. Diet is known to be a profound lever for changing the gut microbiome, however changes to interventions are often varied within populations. Therefore, understanding how the microbiome shifts compositionally and metabolically during interventions that target pregnancy offers a novel approach to improve birth outcomes and maternal health in developing populations through appropriately targeted nutritional supplements. The metabolites acted upon or produced by the microbiota also contribute greatly to the environment sensed by the fetus during pregnancy. Many MDMs have been shown to play a role in a variety of functions, including as signaling molecules and immune factors. While metagenomics is a powerful tool, it is difficult to utilize to monitor and predict health outcomes in real time, especially in developing countries. Metabolites produced by the microbiota, however, can potentially serve as effective biomarkers that can be monitored in a medical setting or developed into assays. Additionally, by knowing which bacterial species produce these metabolites, they can be more easily studied in animal models and serve as potential markers of specific microbes or biological processes. The large-scale goals for this project are two-fold: 1) to understand how nutritional intervention during pregnancy impacts the gut microbiota (and therefore MDMs) of mothers, and 2) to understand global associations between the gut microbiota and infant health outcomes as a function of nutritional intervention. This project aims to address these goals through the lens of the metabolites produced by the microbiome, and how they change as a result of these interventions, and throughout pregnancy.

Methods

Previously the lab has developed an MDM-focused library of 850+ metabolites for analysis of untargeted metabolomics data from biological samples (Han and Van Truren, *et. al. Nature.* 2021). Study participants either received a nutritional intervention during their pregnancy or continued with an existing dietary program. Fecal samples were collected prior to the interventions and periodically throughout the pregnancy, then flash frozen. Samples were analyzed using metagenomics for strain identification and using LC-MS for untargeted metabolomics analysis.

Sample Preparation

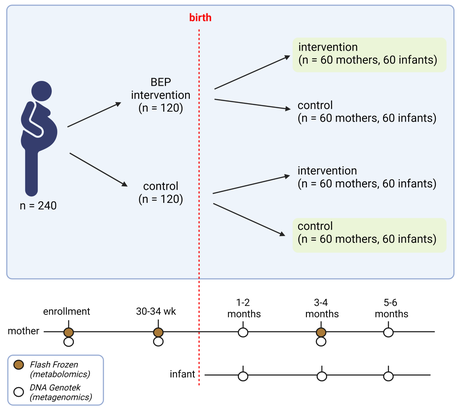




Data Acquisition

Extracted metabolites were analyzed via LC-MS in reverse phase in both positive and negative mode, as well as HILIC positive mode. Data were then converted to a vender-neutral format using MS-Dial and compared against a previously-collected microbial-metabolite specific in-house library for identification using exact mass and retention time. Internal standards were used for normalization.

Instruments:	Agilent LC/Q-TOF with Infinity II LC
C18+ Mobile Phases:	[H_2O (A) MeOH (B)]+ 0.1% FA
C18- Mobile Phases:	[H_2O (A) MeOH (B)] + 6.5 mM AmBic
HILIC+ Mobile Phases:	[H_2O (A) + ACN (B)] + 0.125% FA + 10 mM AF



Sample Cohort (MISAME)

In collaboration with the Gates Foundation, samples were collected from individuals in undernourished populations with high rates of preterm birth and negative maternal or infant health outcomes. A cohort of 240 pregnant mothers in Burkina Faso were recruited, half of whom received a nutritional intervention of a balanced-energy fortified protein supplement during pregnancy. Fecal samples were collected for both metabolomics and metagenomics throughout pregnancy (time of enrollment and late 3rd trimester) and post-pregnancy/lactation. Metagenomics shows clear species differences between the infants and the mothers at the time of birth. Health data for both mother and infant were collected throughout this time frame and show that the nutritional intervention statistically improved most infant and maternal health outcomes measured (see table below).

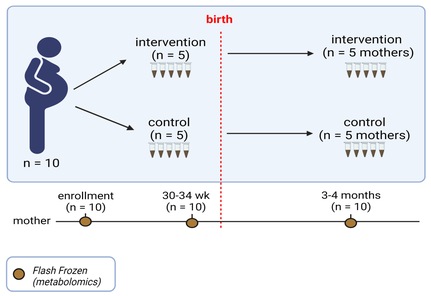
Health Outcome Data

Health Outcome	Effect	Significance
Gestation duration	+0.2 weeks	P = 0.01
Birth weight	+50.1 g	P = 0.019
Birth length	+0.2 cm	P = 0.044
Arm circumference (maternal)	+0.86 mm	P = 0.025
Low birth weight prevalence	~3.95% reduction	P = 0.007

De Kok, *et. al.* PLOS Medicine. (2022)

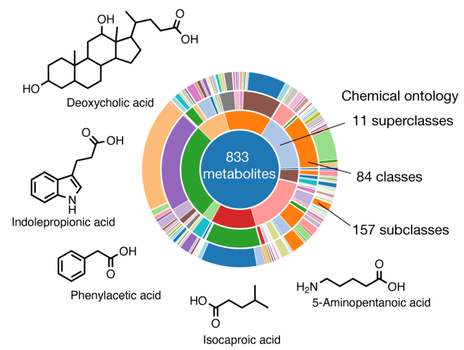
Results

Pilot Study Design
5 samples/group, 2 conditions, 3 time points



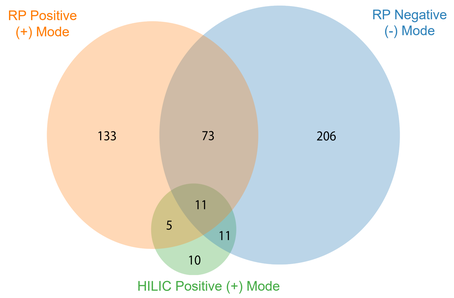
To test the untargeted metabolomics pipeline and microbial-derived metabolite library, an initial pilot study was conducted on these samples. A small sample set of this cohort was analyzed using untargeted metabolomics, with samples from 10 individuals. These individuals represent both diet groups (nutritional intervention or existing diet) for an n of 5 per group. Samples from the time of enrollment, the third trimester, and post-pregnancy were analyzed for each individual to allow tracking of longitudinal metabolite changes. In total, 30 samples were analyzed in triplicate for the data shown.

A gut-microorganism-focused metabolomics pipeline elucidates metabolomic changes from biological samples using a standard library



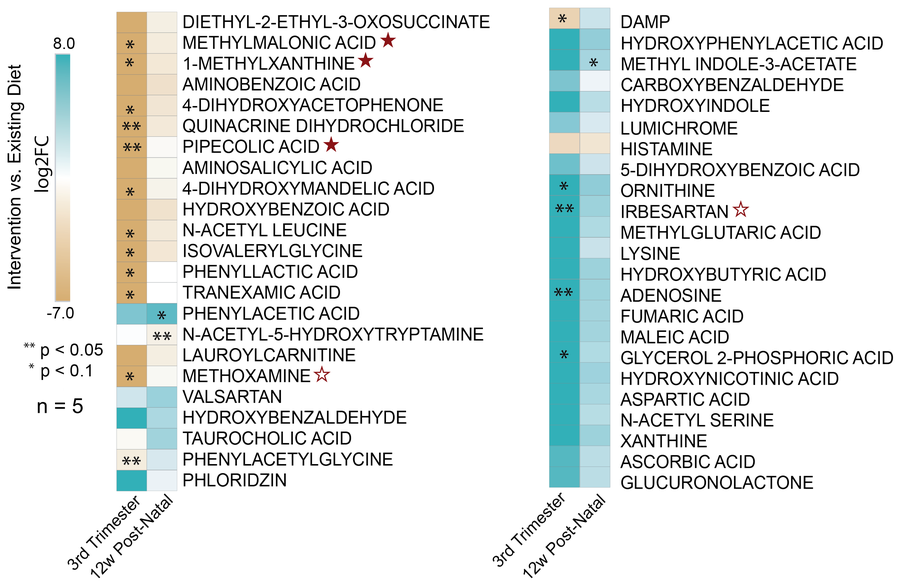
This study utilized an 850+ metabolite in-house library for gut-microorganism-focused metabolites to aid in identification of untargeted metabolomics data. This pipeline allows analysis of various biological samples (urine, feces, serum, bacterial supernatant) based on *m/z* and retention time. (Han and Van Truren, *et. al. Nature.* (2021); Han, Guiberson, Sonnenburg. Protocol Exchange. *In Revision.*)

Utilizing 3 modalities per sample allows broad metabolite coverage



The current metabolomics pipeline includes analyzing samples in three modalities: C18 (RP) positive and negative mode, and HILIC positive mode, to maximize coverage of metabolites of interest. In the pilot study of 30 human fecal samples we were able to detect a total of 449 metabolites that could be identified from our library. Above is a Venn diagram showing which modalities metabolites were detected in. These data highlight the importance of a broad untargeted method to capture a wide range of metabolites with varying chemical properties, and the plethora that can be identified from our library.

Metabolite abundances change dramatically and significantly in women receiving a nutritional intervention during pregnancy



A small cohort of maternal fecal samples (5 per condition; existing diet or BEP intervention) were analyzed using the untargeted metabolomics pipeline and relative abundances of metabolites were compared. All samples were normalized for extraction efficiency and ionization efficiency through internal standards. Some metabolites had higher abundance in women who received the intervention (blue) while some decreased in abundance in women receiving the intervention (brown). The two groups were compared at the third trimester, and 12 weeks postnatally (left and right columns, respectively). Many of these metabolite changes were significantly changed due to the intervention, especially when compared at the third trimester. Certain metabolites screened in infants as markers of metabolic disorders (**pipecolic acid, methylmalonic acid, 1-methylxanthine**) are decreased in mothers on the intervention, as well as compounds linked to maternal health such as **methoxamine** which is a marker of increased blood pressure. **Irbesartan**, a high blood pressure medicine frequently used as an anti-malarial drug, was found to be higher in women receiving an intervention. These findings from such a small initial cohort indicate the applicability and versatility of utilizing metabolomics to monitor infant and maternal, as well as overall gut, health.

★ Metabolite with known link to an infant health outcome
☆ Metabolite with known link to a maternal health outcome

Conclusions

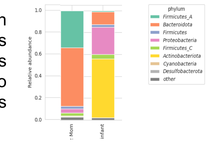
This study analyzed a small pilot cohort of samples from a nutritional intervention study in Burkina Faso using an in-house metabolite library and an untargeted metabolomics pipeline. Pregnant women in Burkina Faso were either given a fortified protein supplement or continued their existing diet during pregnancy to determine changes to the microbiome resulting from dietary interventions. From these 30 preliminary samples, we were able to detect and identify over 400 metabolites from our microbial-derived metabolite library by utilizing three analysis modalities to maximize metabolite coverage. Within these 400+ metabolites, many changed in relative abundance as a result of diet, including some with known negative health implications for infants. Interestingly, most of these health-associated metabolites were decreased in women receiving the intervention. For instance, methylmalonic acid (MMA) is commonly screened for as a marker of the metabolic disorder methylmalonic acidosis. The decreased abundance of MMA in women receiving nutritional supplementation suggests that potentially maternal undernourishment may be a risk factor for methylmalonic acidosis or other metabolic disorders, which can be mitigated through dietary intervention. Similar trends are seen for pipecolic acid and 1-methylxanthine, once again suggesting that this nutritional intervention could reduce infant health risks through modulating the maternal microbiome.

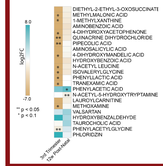
This pilot study highlights the benefits and power of this tri-modality untargeted metabolomics approach when combined with a metabolite standard library, with future analyses focusing on targeted analysis approaches. Additionally, by studying a generally undernourished population we are able to see large scale impacts of diet during pregnancy through this unique sample cohort. These data can help guide diagnosis and treatment of individuals at high risk for negative birth outcomes in the future based on metabolite profiling.

Future Directions

Future collaboration will allow us to further improve our metabolomics pipeline through the use of a Q Exactive instrument for analysis. This will allow for the addition of a HILIC- mode, polarity switching to reduce analysis time, and MS2-level analyses.

Investigate correlations between metabolomics data with metagenomics sequencing data for these samples, as well as individual health outcome data, to identify metabolite predictive biomarkers of disease.





After finding key metabolites that can be predictive for certain health outcomes, we will develop targeted quantitative assays to determine concentrations in fecal samples with the ultimate goal of developing in-country assays for metabolic markers.

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