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Metagenomic Diversity of Gut Microbiota of Gestational Diabetes Mellitus of Pregnant Women

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ABSTRACT

Gestational Diabetes Mellitus (GDM) is defined as a condition in which a woman without diabetes develops abnormal glucose tolerance that is first recognized during pregnancy. GDM is a significant public health problem with an incidence of 1.9 – 3.6% of all pregnancies in Indonesia. Additionally, women with GDM during pregnancy have a high risk of developing diabetes when they are not pregnant, such as type 2 diabetes (T2D). One alternative variable in the management of T2D globally is gut microbiota. Here, to find out the role of gut microbiota in pregnancy, we characterized the stools of 30 pregnant women, each consisting of fifteen GDM-detected pregnant women, and healthy pregnant women using metagenomic approach with genome analysis by directly isolating genomic DNA from the microbiota ecosystem that occupies the digestive tract. DNA sequencing results were analyzed by MEGA 6 software with the BLASTn algorithm in NCBI. Thus fifteen GDM-detected showed high nucleotide sequence homology with the *Proteobacteria* at phylum level, and *Escherichia, Orchobacterium, Cronobacter, Shigella, Salmonella, Enterobacter, Klebsiella, Kosakonia, Vibrio* dan *Gamma-Proteobacterium* at genus level compared to the healthy pregnant women which found by *Firmicutes* at phylum level and *Ruminococcus, Clostridium, Clostridiales, Lachnospiraceae, Roseburia, Weisella, Eubacterium at* genus level had a higher abundance in healthy pregnant women. In this result, we found also one of the fifteen healthy pregnant women showed differential abundance with enrichment of *Prevotella* species. Gut microbiota of GDM-diagnosed pregnant women has more varied composition, and dominated by the phylum *Proteobacteria* than in normal pregnant women.

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BACKGROUND

One of seven births is affected by gestational diabetes mellitus (GDM), this occurs because metabolic and immunological changes occur designated by increased insulin resistance and abnormal glucose tolerance during pregnancy. The GDM is a significant public health problem with an incidence of 1.9 – 3.6% of all pregnancies in Indonesia (Crusell *et al.*, 2018; Wang *et al.*, 2018).

Gestational diabetes mellitus (GDM) is a disease detected in the first recognized during pregnancy (Buchanan *et al.*, 2007; Wang *et al.*, 2018). The causes of gestational diabetes are complex which can be caused by a combination of genetic, health, and lifestyle factors, some of which have not been identified yet. While, the gut microbiota is shaped by environmental factors, such as host genetics, daily food, and the immune system which particularly may have substantial effects on the gut microbiota composition (Salzman *et al.*, 2010; Wu *et al.*, 2011). This might cause a closely related change in composition of the gut microbiota with gestational diabetes.

Recently, several studies of gut microbiota have been linked to type-2 diabetes and results have been shown that patients with metabolic disorders such as obesity and type-2 diabetes contrast in fecal microbiota composition from normal individuals (Soderborg *et al.*, 2016a). The difference in microbiota composition of the metabolic diseases above becomes a possibility used as one of health management treatment (Santacruz *et al.*, 2010). That has been known the gestational diabetes mellitus is able to continue to type 2 diabetes mellitus when the mother has given birth (Koren *et al.*, 2012), this case is still unknown whether the cause could be due to changes in the gut microbiota. Therefore, this study would like to confirm whether similarity of a composition of gut microbiota can also be involved in gestational diabetes mellitus and type-2 diabetes as well.

MATERIALS AND METHODS

Ethics Statement

This study was approved by the Research Ethical Committee of Medical Faculty, Hasanuddin University, Makassar, Indonesia. Written informed consent was recorded from all the pregnant participants in this study. Information about the participant includes age, clinical manifestation, history of the disease, and laboratory findings were collected.

Study population and design

We collaborated in collecting samples at Community Health Center of Antang district in Makassar pregnant women who were referred due to the presence of risk factors for GDM in their third trimester (21–36 gestational weeks) with pre-pregnancy BMI ≥ 27 kg/m². In total, 30 women were invited to participate in the project, which consisting of fifteen GDM-detected pregnant women, and healthy pregnant women were included in the study.

Measurements and derived traits

Pre-pregnancy weight was declared by the participants according to the weight storied in their pregnancy health records. Body mass index known by BMI was counted by dividing the weight in kilograms by the square of height in meters. Normal weight was defined as BMI < 25 kg/m2, overweight as $25 < X < 30$ kg/m2 and obesity as BMI 30 kg/m²(Gomez-Arango *et al.*, 2016). Weight gain during pregnancy was calculated as the difference in measured weight obtained at the first visit and pre-pregnancy weight.

Blood pressure was noted which gestational hypertension was determined as the development of systolic blood pressure ≥ 140 mmHg, and diastolic blood pressure ≥ 90 mmHg. Then the measurement continued after 20 weeks of gestation. Height and weight were measured to the nearest 0.5 cm and 0.1 kg respectively without shoes (Koren *et al.*, 2012).

Microbiome analyses

Preparations of data fecal samples were collected at home by the participants following a standardized procedure including antiseptic handling, collection in sterile tubes. The samples were transferred to the laboratory on dry ice within 48h of collection and stored at -18°C until DNA extraction. Thirty women provided fecal samples at baseline.

Approximately 200μl of fecal was added to 800μl of DNAzol® reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA) in a 1.5 ml tube and preserved at room temperature for DNA analyses. Genomic DNA was extracted from the DNAzol® mixture according to the manufacturer's instructions with some modifications (Kamaruddin et al., 2020). Briefly, the sample was treated by 2 freeze and thaw cycles and continual overnight proteinase K (final concentration 0.4 mg/ml) digestion at 55°C before being subjected to the standard ethanol precipitation protocol for DNAzol®. The final DNA precipitate was resuspended in 80μl of 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA and preserved at -20°C until use as previous method (Kamaruddin *et al.*, 2014; Kamaruddin *et al.*, 2020).

Variable regions V1-V2 of the 16S rRNA gene were amplified using the UNIVERSAL1/UNIVERSAL2 primer pair with slight modifications. The PCR primer set was UNIVERSAL-1 (5´-CCA GCA GCC GCG GTA ATA CG-3´) and UNIVERSAL-2 (5´-ATC GG(C/T) TAC CTT GTT ACG ACT TC-3´). The PCRs were performed in a final volume of 10 or 20μl (the first and second PCR, respectively) using gSYNC™DNA Extraction Kit, including 20μl of absolute ethanol, 4 μl of W1 buffer, 6 μl of wash buffer, 1μl of pre-heated elution buffer, and 2μl of GSB buffer. Roughly 1.5 μg of genomic DNA or 1.0μl of the first PCR solution as templates, each primer at 0.2μM, each deoxynucleoside triphosphate (dNTP) at 2.5 mM, and 2.5 mM $MgCl_2$ under the following conditions: a denaturation step of 94°C for 1 min followed by 30 cycles of 90°C for 30 sec, annealing at 55°C for 15 sec, and extension at 69°C for 30 or 20 sec. The post-extension was finished at 69°C for 5 min. Both the first and second PCR reactions were performed in the same volume of a 10μl reaction mixture using enzyme Kappa, 1 μg of the extracted sample DNA or 0.5μl of the first PCR solution as the template, each dNTP at 0.8 mM, and each primer at 0.3 μM under the following conditions: a denaturing step 94°C for 30 sec followed by 30 cycles of 98°C for 10 sec, annealing at 55°C for 5 sec in both the first and the second PCR, and extension at 69°C for 81 or 55 sec, with post-extension at 69°C for 1 min. The PCR products were electrophoresed on 2% agarose gels with 0.2 μg/ml ethidium bromide and visualized on a Gel DocTM EZ UV transilluminator (BioRad Laboratories, Tokyo, Japan).

DNA sequencing analysis

The target bands were then ejected from the gel and purified using the FastGene® gel/PCR extraction kit (Nippon Genetics, Tokyo, Japan) according to the manufacturer's instructions. The direct sequencing of each purified PCR product was conducted with the ABI Prism BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies Japan, Tokyo, Japan) on an Applied Biosystems 3130 Genetic Analyzer (Life Technologies). All nucleotide sequences were confirmed using both the forward and reverse reading data, and ambiguous nucleotides were precisely confirmed by conducting the sequence procedures repeatedly.

Sequence identification and phylogenetic analysis

In total, 20 reference sequences of the V1-V2 regions of the 16S rRNA gene of microbiome were obtained from the DNA data bank of NCBI using a homology search with 30 sample sequences data in this study (Table 1). The 50 total sequences targeting 996 bp of the U1/U2 locus with MEGA5 (Tamura *et al.*, 2011).

RESULTS AND DISCUSSION
Description of the participant characteristic

The characteristics of the participants in this study are summarized in Table 1. In this study, the average age of GDM patients was 26-30 years age range (40%), while the average age of healthy pregnant women was 31-35 years age range and 36-40 years age range each in 33.3%. The parity of GDM dominant at gravida 2-3 (60%), compared to other parties, for primigravida (20%) and gravida >5 (20%).

GDM was diagnosed in 15 of 30 women due to BMI and blood glucose. As expected, markers of glucose and insulin homeostasis were higher in the GDM group. Women with GDM had indications of diminished beta cell function as shown by lower insulinogenic index and disposition index. The women diagnosed with GDM had higher glucose, more than 100 mg/dl was 60% compared to the healthy pregnant women was only 40%. The pre-pregnancy BMI of GDM had obesity level was in 40% vs 13.3%.

Taxonomic biomarkers of gestational diabetes

Compositionally, the gut microbiota of the total group of pregnant women was dominated by members of the three major bacterial phyla *Proteobacteria, Firmicutes*, and *Bacteroidetes*, which on average accounted for ~ 90% of all reads (Tabel 2). At the genus level, the composition followed a typical Asian pattern with *Proteobacteria* as the predominant genus in women with gestational diabetes.

Using linear discriminant analyses, we identified phylum *Proteobacteria* and several subordinate taxa as taxonomic biomarkers of GDM: genus *Enterobacter* and the parent family *Enterobacteriaceae* and parent order *Enterobacteriales*; genus *Salmonella* and the parent family *Enterobacteriaceae* and parent order *Enterobacteriales*; genus *Shigella*, also of order *Enterobacteriales*. Within the phylum *Proteobacteria*, the genus *Kosakonia* was a biomarker of GDM. Within *Firmicutes*, the genus *Weisella* and parent family *Leuconostocaceae*, genus *Lachnospiraceae* and genus *Ruminococcus* were biomarkers of GDM, whereas the genera *Roseburia*, *Prevotellamassilia* and *Alloprevotella* were markers of normal glucose regulation.

The partial confounding effect of BMI

Overweight and obesity are known risk factors for GDM (Saminan, 2012), and increased BMI has been associated with gut microbiota disruption in both pregnant and non-pregnant women (Santacruz *et al.*, 2010). In light of this and the overrepresentation of obesity among women with gestational diabetes (40 vs. 13.3%) of women with normal glucose regulation in our study, we looked for microbial biomarkers of excess body weight in order to assess the potential confounding effect of BMI. Comparing pregnant women with pre-pregnancy BMI within the overweight range (25–29.9 kg/m2) to women with normal (< 25 kg/m2) pre-pregnancy BMI, we identified genus *Cronobacter* as biomarkers of overweight, whereas *Ruminococcus* and all parent taxa within *Ochrobactrum* were markers of normal weight, as were the genera *Eggerthella* and *Sporobacter*, as well as genera *Ethanoligenens* and *Clostridium* XVIII (*Erysipelotrichaceae*). Comparing pregnant women with pre-pregnancy obesity (BMI ≥ 30 kg/m2) to women with normal (< 25 kg/m2) pre-pregnancy BMI identified the genera *Lachnospiraceae*, *Acidaminococcus* and *Ruminococcus* (*Lachnospiraceae*) as markers of obesity and the genera *Roseburia, Eubacterium*, and *Clostridium*, as markers of normal weight.

Table 1. Participant characteristics of GDM and normal pregnant women in the third-trimester pregnancy

	GDM (n = 15) (%)	Normal (n =15) (%)
Descriptive		
Age (years):		
20 – 25	6.7	13.3
26 – 30	40	20.0
31 – 35	20	33.3
36 – 40	33.3	33.3
Parities (person):		
Primigravida	20	6.7
Gravida 2-5	60	80
Gravida >5	20	13.3
BMI (kg/m²):		
Normal	33.3	66.7
Overweight	26.7	20
Obesity	40	13.3
Glucose (mg/dl):		
< 100	40	60
> 100	60	40
Previous child with birth weight (gr):		
< 2500	26.7	20
2500 – 4500	73.3	80

Data presented as mean (SD)

Members of the phylum *Proteobacteria* have a low abundance in the gut of healthy humans. In our study of women with GDM has been dominated by *Proteobacteria* and healthy pregnant women verified by *Firmicutes* and *Bacteriodetes*. Even the GDM diagnosed in late pregnancy is associated with an aberrant gut microbial composition at the time of diagnosis. Gut microbial communities are known to be affected by diet and weight in non-pregnant hosts(Boerner and Sarvetnick, 2011; Ferrocino *et al.*, 2018). The intestinal microbiota during pregnancy is associated with pre-pregnancy BMI as well as weight gain (Soderborg *et al.*, 2016b). When regulating for pre-pregnancy BMI, we similarly displayed relationships between body composition and gut microbiota also during pregnancy. Our results confirm previous findings with enrichment of genera *Lachnospiraceae, Acidaminococcus* and *Ruminococcus* (*Lachnospiraceae*) as markers of obese pregnant women and the genera *Roseburia, Eubacterium*, and *Clostridium* enriched in normal-weight pregnant women. There was no overlap in taxonomic biomarkers of GDM with markers of overweight or obesity even though overweight is a risk factor for the development of GDM. In our study, species assigned to *Roseburia* and *Eubacterium* were associated with low weight gain and Lactobacillus were associated with higher weight gain during pregnancy.

This finding is concurrent with the findings in nonpregnant individuals as strains of *Lactobacillus reuteri* have previously been associated with obesity. Of the taxa differentially abundant in women with and without GDM, only two were significantly correlated with pre-pregnancy BMI and only weakly so, indicating that the observed compositional differences were not strongly confounded by prepregnancy BMI (Alfadhli, 2015). To our knowledge, this is the first study to explore the gut microbiota composition at the time of GDM diagnosis in Makassar. A few studies with few participants have examined gut microbiota either week before or after GDM diagnosis(Qin *et al.*, 2010). A limitation of our study was that we did not have fecal samples before pregnancy. Also, our next idea of subspecies occurrence with divergent functionalities appeal to shotgun-based sequencing studies of the intestinal microbiome in GDM(Gomez-Arango *et al.*, 2016).

Table 2. Bacteria general associated with GDM status and healthy pregnant women

Genus	Species	GDM Pathogen	GDM Commensal	Healthy Pathogen	Healthy Commensal
Escherichia:	*Escherichia colli*	+			+
Ochrobactrum:	*Ochrobactrum intermedium*	+			
	Ochrobactrum sp.	+			
	Ochrobactrum anthropi	+			
	Ochrobactrum pseudintermedium	+			
Shigella:	*Shigella sp.*	+			
	Shigella sonnei	+			+
	Shigella flexneri	+			
Enterobacter:	*Enterobacter cloacae*	+			
	Atlantibacter subterranean	+			
	Enterobacter sp.	+			
Enterobacter Bacterium:	*Pantoea ananatis*	+			
	Pantoea agglomerans				
Vibrio	*Vibrio orientalis*	+			
	Vibrio crassostreae	+			
Enterobacteriaceae Bacterium	*Enterobacteriacae bakterium*	+			
Klebsiella:	*Klebsiella sp.*	+			
	Klebsiella pneumoniae subsp. pneumoniae	+			
Cronobacter:	*Cronobacter sakazakii*	+			
Kosakonia:	*Kosakonia oryzae*	+			
	Kosakonia oryziphila	+			
Salmonella:	*Salmonella enterica*	+		+	
Gamma-Proteobacterium:	*Gamma- proteobacterium*	+			
Ruminococcus	*Ruminococcus sp.*				+
	Ruminococcus faecis				+
	Ruminococcus torques				+
Clostridium:	*Clostridia bacterium*	+		+	
	Clostridium sp	+		+	
Clostridiales	*Clostridiales bacterium*		+		+
Lachnospiraceae:	*Lachnospiraceae bacterium*		+		+
Roseburia:	*Roseburia sp.*				+
	Roseburia faecis				+
Lactobacillus:	*Lactobacillus fermentum*	+			
Streptococcus:	*Streptococcus gallolyticus*	+			
Weisella:	*Weissella confuse*	+			
	Weissella cibaria	+			
	Weissella koreensis	+			
	Weissella sp.	+			
Eubacterium rectale	*Eubacterium rectale*				+
Prevotella:	*Prevotellamassilia timonensis*				+
	Alloprevotella sp.				+
Bacillus	*Bacillus sp.*		+		+
Firmicutes	*Firmicutes Bacterium*		+		+

CONCLUSION

Gestational diabetic mellitus (GDM) diagnosed in the third trimester of pregnancy is associated with a disrupted gut microbiota composition compared with healthy pregnant women. The gut microbiota composition of women with GDM had been dominated by the phylum *Proteobacteria*. While the *Proteobacteria* has a low abundance in the gut of healthy humans.

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CONFLICT OF INTEREST

We have no conflict of interest related to this work.

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