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Metagenomic Analysis of Intestinal Microbiota Derived from Stool Samples of Third Trimester Pregnant Women

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Abstract

Background: The human gut microbiota plays a unique role in metabolism, immunity, and nutrient absorption. It is known that an imbalance in the ratio of the phyla Firmicutes and Bacteroidetes has been associated with various chronic diseases, including inflammatory bowel disease and metabolic disorders. The Firmicutes/Bacteroidetes (F/B) ratio is a measure used to assess gut microbiota composition, which is believed to play a significant role in intestinal health and metabolism. Studies suggest that a higher F/B ratio is often associated with obesity and other metabolic conditions. **Objective:** This study aims to carry out metagenomic analysis of third trimester pregnant women's fecal samples to predict degenerative diseases using third generation whole genome sequencing, namely PromethION nanopore technology. **Methods:** Nanopore Technology: DNA sequencing of faecal samples from pregnant women in the third trimester was performed using the Oxford Nanopore Technology (ONT) device based on the LigationSequencing gDNA - Native Barcoding Kit 24 V14 and following its guidelines**. Results**: Based on the quality and quantity of total DNA reads from 20 stool samples from pregnant women, only 4 samples were continued for analysis (1A, 2A, 3A and 4A). The results of the abundance of intestinal microbiota based on the most abundant phylum are Proteobacteria, Bacteriodetes, and Firmicutes. **Conclusion:** Meanwhile, based on species abundance, it shows that *Prevotella copri* (phylum *Bacteriodetes*) is abundant in samples 2A, 3A and 4A, while in sample 1A the most abundant species is *Succinivirio dextrinosolvens* (phylum *Proteobacteria*). Bacteroidetes are a group of Gram-negative bacteria that are often found to increase in individuals with type 2 diabetes and obesity.

Keywords: Degenerative Diseases, Feses, Gut microbiota, Metagenomic

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Original Research Article

INTRODUCTION

Microbiota is a complex ecosystem comprising hundreds of billions of fungi, viruses, bacteria, and protozoa that reside within the body of their host, specifically humans (Perxachs & Real, 2019). Approximately 70% of the human microbiota is located in the intestine (Marisa, 2016). The colonization of intestinal microbiota initially occurs in utero through exposure to microbiota present in amniotic fluid and the placenta (Logor et al., 2021). Concurrent with human development, the composition of the intestinal microbiota undergoes changes. These alterations are influenced by various factors, including lifestyle, environmental conditions (place of residence), antibiotic usage, and consumption of diets low in fiber and high in fat and sugar (Venegas et al., 2019). Additional factors that can affect the state of the intestinal microbiota include gestational age, mode of delivery (vaginal or cesarean), and infant nutritional intake (Minarti, et al., 2020).

The composition of the gut microbiota is of significant importance during pregnancy due to its crucial role in nutrient absorption, immune system modulation, and protection against infection (Yang et al., 2020). Newbern et al (2011) posit that during gestation, numerous metabolic, immunological, and hormonal alterations can influence fetal development (Gorczyca et al., 2022). Kumar and Magon (2012) assert that the physiological changes occurring during pregnancy are primarily to support fetal growth and development (Nuriel-Ohayon et al., 2016). Alterations in the gut microbiota during pregnancy may contribute to the development of pregnancy-related disorders and have substantial implications for maternal and fetal health (Sinha et al., 2023). These modifications commence early in the first trimester (1-3 months of gestation), reach their peak at parturition, and revert to pre-pregnancy levels within a few weeks postpartum (Sajdel-Sulkowska, 2023). Pregnancy is characterized by an increase in pro-inflammatory gut bacteria and a concomitant rise in pro-inflammatory cytokines. These changes further enhance fat energy storage and ensure fetal growth (Sajdel-Sulkowska, 2023). The gut microbiota in pregnant women is predominantly composed of two main phyla, Firmicutes (78.8%) and Bacteroidetes (11.9%), followed by Actinobacteria (5.6%), Proteobacteria (1.8%), Verrucomicrobia (0.7%), and Archaea (0.6%) (Yang et al., 2020).

The diversity of healthy gut microbiota can be disrupted, leading to gut dysbiosis. This can be associated with the proliferation of pathogens and the depletion of commensal bacteria (Woodall et al. 2022). Gut microbiota dysbiosis refers to the disruption of the dynamic interaction between the host and microbial communities, as well as the bacterial imbalance between the ratio of aerobic and facultative anaerobic bacteria (Man et al. 2020). The occurrence of microbiota dysbiosis alters physiological and metabolic functions, thereby disrupting barrier function, causing changes in intestinal permeability, allowing bacterial products and inflammatory factors to enter the systemic circulation (Morimoto et al. 2023; C. Sun et al. 2023). The proportion of Firmicutes and Bacteroidetes increases in obese individuals compared to lean individuals and tends to decrease with weight loss, as consistently shown in numerous human studies. The concentration of Bacteroidetes in feces is positively correlated with body mass index (BMI), and the predominance of Bacteroidetes in overweight and obese individuals has been demonstrated. Firmicutes are more effective as an energy source than Bacteroidetes, thus promoting more efficient calorie absorption and weight gain. Other bacterial phyla also play a role in weight gain and obesity, such as Actinobacteria, which includes the genus Bifidobacterium and other genera. An increase in body mass index is associated with an increase in actinobacteria (Koliada et al. 2017).

The development of new sequencing technologies has facilitated the study of sequence variants present in the genome. Until recently, these could only be detected by comparative genomic hybridisation (CGH) or single nucleotide polymorphism (SNP) arrays. Sequencing of the 3rd generation offers new possibilities for the identification of sequence variants on a larger scale with two different approaches. One is based on short linked reads, as in the 10x Genomics and Hi-C approaches, and the

other is based on long read generation, as proposed by Pacific Biosciences and Oxford Nanopore Technologies (ONT). These approaches provide access to complex regions, which enhances their use for the improvement of genome assembly and the detection of structural variations in humans (Selle et al. 2022). Nanopores as single-molecule biosensors were originally developed for highly sensitive DNA sequencing and other label-free biomolecular sensing techniques. Nanopores have been used to stochastically sense and characterise DNA, RNA, peptides, proteins, metabolites and protein-DNA complexes at the single-molecule level. In particular, the success of nanopore-based DNA and RNA sequencing has stimulated a wide range of potential applications in a relatively simple, high throughput and label-free format (Selle et al. 2022).

This study aims to carry out metagenomic analysis of third trimester pregnant women's fecal samples to predict degenerative diseases using third generation whole genome sequencing, nanopore technology.

MATERIALS AND METHODS

Fecal Sampling

Stool samples were collected from March - June 2024 from third trimester pregnant women and after obtaining informed consent. The criteria for pregnant women used as participants in this study were pregnant women in the third trimester of pregnancy, no comorbid in their health status, and living in Bulukumba area. Each participant was given a labeled sterile container (sample code) to collect a stool sample a few days prior to delivery.

DNA Extraction

The principles of DNA extraction are lysis, precipitation and purification. Faecal samples are extracted to obtain pure DNA and this extraction process follows the steps of the FAVORGEN Biotech corp DNA extraction insert kit (Kamaruddin M, et al., 2020; 2023).

DNA Purification

Prepare two test tubes for the standards and one test tube for each sample, then add QubitTM working solution with 1:200 QubitTM reagent in QubitTM buffer to the working solution tube, then add 200ul of working solution for each standard and sample to the test tube. Vortex all tubes for 2-3 seconds. Incubate for 2 minutes at room temperature. Finally insert the tubes into the QubitTM Fluorometer and read the result.

Electrophoresis

Agarose gel was prepared with a concentration of 2% agarose weighed as much as 2 grams and then dissolved in 100 ml TAE buffer. The agarose was heated in a microwave oven. The hot and liquid gel was mixed with 2 µl of Safe Syber, homogenised and then poured onto the plate. The comb sheet was inserted while the agar was still hot and not solidified. The comb sheet was removed from the agarose gel when the agarose gel had solidified enough to form a well into which the sample of the extraction product was inserted. The solidified agarose gel was placed in a container containing TAE buffer solution.

DNA amplification results and markers are added to each well. The tank was closed and the electrophoresis run was started by connecting the electrophoresis unit to the power supply. The side containing the amplification results was given a negative temperature. The electrophoresis current was 100 volts for 60 minutes. In the process of electrophoresis amplification products used markers 1 kb DNA Ladder consists of fragments ranging from 100 to 3000.

Nanopore Sequencing

DNA sequencing of faecal samples from pregnant women in the third trimester was performed using the Oxford Nanopore Technology (ONT) device based on the Ligation Sequencing gDNA - Native Barcoding Kit 24 V14 and following its guidelines.

Statistical Analysis

Data analysis used Pavian software to display and analyze metagenomic data. Through the use of genome alignment viewers, Sankey flowcharts and comparison tables. In addition, classifiers such as

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KrakenUniq, Centrifuge, and MetaPhlAn are used to explore the data analyzed by Pavian, namely to see and understand the species present in a sample, and to compare species identification in different samples (Nurisyah et al. 2025; Breitwieser & Salzberg 2016),

Ethical Clearence

This study received ethical approval from the Health Research Ethics Committee of Makassar Health Polytechnic, Ministry of Health under number: 0363/OKEPK-PTKMS/III/2024.

RESULTS

Analyse of DNA quality and quantity using the Qubit Fluorometer

Samples are incubated according to the procedure and mixed with the working solution (2 minutes for Qubit® DNA and RNA Assay, 15 minutes for Qubit® Protein Assay). Based on the sample screening results, 5 samples with DNA amounts greater than 400ng were obtained as shown in Table 1.

Based on the quality and quantity of total DNA readings from 20 stool samples from pregnant women, only 4 samples were continued for analysis (1A, 2A, 3A and 4A). This is because a good amount of DNA is greater than 400ng, where in sample 1A (3564ng), 2A (1848ng), 3A (510.4ng) and 4A (536.8ng). The DNA quality analysis of samples 1A, 2A, 3A, and 4A were 8.1ng/uL; 21.0ng/uL; 5.8ng/uL; and 6.1ng/uL, respectively (Table 1).

Table 1. DNA Concentration using Qubit Fluorometer

ID Sample	DNA Concentration (ng/uL)	DNA amount (ng)
1A	8.1	3564
2A	21.0	1848
3A	5.8	510,4
4A	6.1	536,8
6A	9.8	862,4

DNA Sequencing using ONT PromethION

Sequencing with ONT PromethION resulted in a variable total number of raw reads in the samples from pregnant women in trimester 3. The sequencing quality of the samples showed that the raw reads ranged from 4 bytes to 39322 bytes. The number of nucleotide bases ranged from 15593 bytes to 160151301 bytes. The average length of the reads ranged from 3898.2 to 4569.4 base pairs, The average quality of the reads ranged from 10.9 to 14.3 and the GC value, which is the percentage of guanine and cytosine bases observed, ranged from 36.20 % to 46.18 %. (Table 2).

Table 2. Quality of raw sequence data

ID Sample	Raw Reads (bytes)	Number of Bases (bytes)	Mean read Length (basepairs)	GC (%)	Mean Qual
1A	17240	78776868.0	4569.4	39.46	14.3
2A	22681	103415167.0	4559.6	46.18	14.0
3A	7371	31058178.0	4213.6	44.36	14.3
4A	39322	160151301.0	4072.8	41.78	14.2
6A	4	15593.0	3898.2	36.20	10.9

Based on the quality and length of the reads, sample 6A showed low quality reads with only 4 bytes of raw reads and an average read length of 3898.2 bp, so sample 6A was not used in the gut microbiota diversity analysis.

Diversity of the gut microbiota

A dynamic complex of microorganisms found in the human gastrointestinal tract. It includes bacteria, archaea, viruses and protists, most of which are involved in symbiotic relationships with their hosts. The results of the gut microbiota genome sequence readings of each sample had an abundance from the smallest to the largest found bacteria as much as 31,206 to 2,777,030 Operational Taxonomy Units (OTU), chordates as much as 3,247 to 239,667 OTU, viruses from 8 to 940 OTU, fungi from 0 to 318 OTU and protozoa from 0 to 54 OTU. The results of the microbiota genome sequence read abundance of each sample are shown in Table 3.

Tabel 3. Results of microbiota genome sequence reads

ID Sample	1A	2A	3A	4A
Number of raw reads	1,815,349	3,484,756	2,135,017	283,179
Classified reads	93,90%	92,30%	92,50%	89,50%
Chordate reads	4,103	3,247	3,948	239,667
Fungi reads	0	318	206	0
Virus reads	37	940	55	8
Bacterial reads	764,486	2,777,030	1,866,067	31,206
Protozoan reads	0	54	6	0

Based on the Pavian tool to classify gut microbiota based on the diversity of each taxonomy of each sample, including phylum taxa and species in each sample.

The diversity of the gut microbiota in Figure 1 is visualised as a bar chart of 5 taxa ranked by phylum in all samples. The results showed differences in the number of phyla and relative abundance. The diversity of phyla in each sample was up to 5 phyla, in order of highest to lowest abundance.

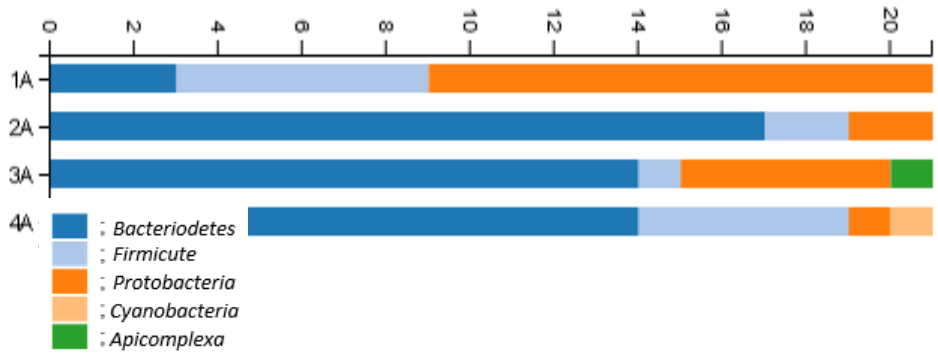

Figure 1. The abundance of gut microbiota derived from pregnant women in trimester 3

Figure 1 above shows the abundance of gut microbiota in samples from pregnant women in trimester 3 based on the most abundant phylum and has a high abundance of *Bacteriodetes*. However, in sample 1A, the most abundant phylum is the *Protobacteria*.

The percentages of the phylum taxa of the gut microbiota based on the relative abundance of each sample are, in sample 1A, *Bacteroidetes* (18.20%), *Firmicutes* (29.45%), and *Proteobacteria* (52.35%). Sample 2A consisted of *Bacteroidetes* (69.90%), *Firmicutes* (15.10%) and *Proteobacteria* (15.00%). Sample 3A consisted of *Bacteroidetes* (63.50%), *Firmicutes* (5.30%), *Proteobacteria* (25.90%) and *Apicomplexa* (5.30%). Sample 4A consisted of *Bacteroidetes* (63.50%), *Firmicutes* (25.90%), *Proteobacteria* (5.30%) and *Cyanobacteria* (5.30%).

Figure 2 below illustrates the results of mapping the gut microbiota at the species level. *Prevotella copri* was the predominant species in samples 2A, 3A, and 4A. In contrast, sample 1A was dominated by the species *Succinivibrio dextrinosolvent*.

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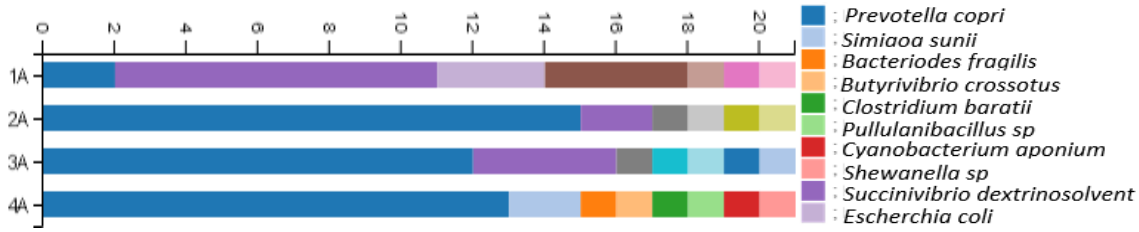

Figure 2. Mapping the gut microbiota at the species level

DISCUSSION

In this gut microbiota study, fecal samples were utilized because they can reveal changes in gut microbial composition (number of bacterial cells, composition of inhibitors) that vary. The results indicated that the most abundant phyla were Proteobacteria, Bacteroidetes, and Firmicutes shown in Figure 1. This finding aligns with the study by Tu et al. (2022), which demonstrated that the gut microbiota composition of pregnant women significantly differed from that of non-pregnant women. This alteration in the microbiota community is characterized by an increase in the abundance of the genus Bacteroidetes. The gut microbiota of pregnant women in the third trimester was significantly different from that of those in the first trimester, with changes in bacterial diversity, including an increase in Firmicutes and Clostridium during the first trimester, and an increase in Enterobacteriaceae and Streptococci in the third trimester (Song & Liu, 2023).

The most abundant species in this study was Prevotella copri. Currently, there are three classes of enterotypes, each defined by its dominant bacterial group: enterotype I, characterized by Bacteroides; enterotype II, characterized by Prevotella; and enterotype III, dominated by Ruminococcus (Figure 2). These three enterotypes have distinct and specific functions, producing energy from carbohydrates or proteins. Each individual is characterized by different enterotypes, which various factors, including diet can influence.

In the study by Yang et al. (2024), genera such as Ureaplasma, Streptococcus, Gemella, Veillonella, Prevotella, and Fusobacterium were also widely reported to be present in the placenta, and several studies have indicated that placental microbiota play a crucial role in adverse pregnancy outcomes such as preterm birth and fetal macrosomia.

Differences in age, diet, and environment among people worldwide significantly influence the patterns of gastrointestinal microbiota. While human microbiota are also present in the skin, lungs, urinary tract, and oral cavity, the gastrointestinal tract harbors the highest concentration of these microorganisms (Kurniawan et al., 2020). The abundance of Prevotella microbiota in pregnant women is partly attributed to dietary factors, as food plays a crucial role in shaping the microbiome (Hasibuan & Kolondam, 2017). Research by Fite et al. (2022) indicates that during pregnancy, the body experiences increased metabolic and physiological demands that should be met with adequate nutrition. However, many women lack sufficient nutrient intake at the time of conception to fulfill their body's needs.

This sequencing sample consists of third-trimester pregnant women living in rural areas whose diets are higher in fiber from vegetables (Minarti et al; (2020). A study by Simone et al. (2020) analyzed maternal gut microbiota in early pregnancy, comparing omnivorous diets with vegetarian diets. Women following a vegetarian diet exhibited an increase in bacterial groups involved in lipid synthesis, indicating changes in fermentation and the presence of bacterial species that produce significant amounts of short-chain fatty acids (enterotype II). Investigations of the gut microbiota in a sample of African women revealed a prevalence of enterotype II Prevotella, where a diet rich in vegetables and low in animal proteins and lipids promotes the growth of bacterial groups that degrade mucin-type glycoproteins covering the intestinal mucosa. In contrast, the diet of European populations, which is

rich in animal proteins and lipids, is associated with enterotype I (*Bacteroides*). *Prevotella* abundance is positively correlated with high dietary fiber intake (Suarez et al., 2022).

In samples 1A and 3A, the microbiota was notably abundant in the species *Succinivibrio dextrinosolvens*, which belongs to the phylum *Proteobacteria* (Figure 2). This phylum encompasses a wide variety of pathogenic genera, including *Escherichia coli*, *Salmonella, Vibrio, Yersinia, Legionella*, and many others (Lange-Enyedi et al., 2024). The prevalence of *Proteobacteria* in pregnant women has been associated with several physiological changes and immune responses that occur during pregnancy. Research indicates that fluctuations in hormone levels, immune modulation, and increased gut permeability contribute to elevated levels of *Proteobacteria* during this period. Specifically, in late pregnancy, the gut microbiota experiences alterations linked to inflammation and metabolic regulation, such as those involving *Proteobacteria*. These changes are believed to assist in meeting energy demands during pregnancy by enhancing nutrient absorption and modifying immune responses, although they also elevate markers of inflammation (Hudson et al., 2024).

Succinivibrio dextrinosolvens is involved in breaking down complex polysaccharides into fermentation products like acetate and succinate, which may promote gut health by supplying nutrients to other gut microbiota. Alterations in the gut microbiome are common during pregnancy, and evidence suggests that the presence of certain fermentative bacteria may impact maternal metabolism. Metabolites such as acetate and succinate could play a crucial role in providing additional energy during pregnancy (Amir et al., 2020).

In addition to the Succinivibrio dextrinosolvens species from the Proteobacteria phylum, Shiga toxin-producing *E. coli* (STEC) was also identified, one of the four well-known *E. coli* groups responsible for causing diarrhea in humans through the consumption of contaminated food or water or other fecal-oral routes (Hunt, 2020). This study is limited by having sufficient DNA from only four samples.

CONCLUSION

The results indicated that the most abundant phyla were Proteobacteria, Bacteroidetes, and Firmicutes. In some samples (1A and 3A), the microbiota was notably abundant in the species *Succinivibrio dextrinosolvens*, which belongs to the phylum *Proteobacteria Bacteroidetes* phylum, a group of anaerobic Gram-negative bacteria that do not form spores. The *Actinobacteria* phylum is also recognized for its role in inflammatory processes related to metabolic conditions. *Firmicutes* are anaerobic Gram-positive bacteria that form spores and ferment sugars. *Firmicutes* serve as a counterbalance to *Bacteroidetes* in gut microbiota imbalance (dysbiosis) and eubiosis (balance)

The ratio of *Firmicutes* to *Bacteroidetes* in the human gut microbiota is crucial for maintaining gut homeostasis. This ratio fluctuates throughout life and can be affected by diet and other factors.

Further studies should be on the analysis of the gut microbiota in samples of pregnant women from urban areas.

CONFLICT OF INTEREST

There is no conflict of interest in this research.

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