VAGINAL MICROBIOME IN THE THIRD TRIMESTER OF PREGNANCY AMONG WOMEN AT RAHIMA MOOSA MOTHER AND CHILD HOSPITAL

Simon E E I Emvula

This research reported is submitted to the University of the Witwatersrand Health Sciences Faculty in partial fulfilment of the degree of Master of Medicine in Obstetrics and Gynaecology

March 2021

DECLARATION

I, Simon E E I Emvula, hereby declare that this research is my work. I am submitting this research report for the degree Master of Medicine in Obstetrics and Gynaecology at the University of the Witwatersrand, Johannesburg. This research report has not been submitted before for any degree or examination at this or any other university.

This MMed research report is submitted in the format of a submissible research article. The article conforms to the author guidelines for the South African Journal of Obstetrics and Gynaecology.

Signed on 17/02/2021 in Johannesburg

Dedication

I dedicate this Masters to my mother, Meme Hertha Maria Emvula.

Presentation

1. 03/11/2020: Oral presentation at Chris Hani Baragwanath Academic Hospital.
2. 07/11/2020: Virtual presentation at the SAATOG research symposium.

Abstract

Background

The normal vaginal microbiome is an essential component in women's health. Obstetricians should be knowledgeable on the composition of the vaginal microbiome of healthy asymptomatic pregnant women and the microbiome's community state types (CST) that contribute to poor pregnancy outcomes.

Objectives

To describe the composition of the vaginal microbiome of healthy pregnant women and their demographics, and to evaluate short-term pregnancy outcomes.

Method

This was a prospective descriptive study. Vaginal swabs were obtained from 21 women in their late third trimester of pregnancy. The QIAamp DNA Mini Kit was used to extract the DNA. Polymerase chain reaction (PCR) with primers targeting the V3 and V4 region of the 16S rRNA gene was used to characterise the microbiome. The Greengenes database was used for the distribution of taxonomic categories.

Results

Lactobacillus species were identified in 95.2% (n=20) of participants. By fuzzy clustering of Pcoa Bray Curtis distances we identified three CSTs based on the dominant microbiome: CST-III n=10 (*L. iners* dominated), CST V n=5 (*L. jensenii* dominated), CST IV n=6 (*Gardnerella vaginalis* and anaerobes dominated). A high prevalence of *Atopobium vaginalis* (n=12) and *Finegoldia Magna* (n=16) was also noted. Obesity was associated with the prevalence of *Prevotella species* (p=0.048).

Conclusion

Lactobacillus were the predominant species of the microbial community. Obesity was associated with a high prevalence of *Prevotella species*. There are a significant number of women with unrecognised STI's and Group B Strep. These results may serve as a baseline for further microbiome studies during pregnancies, which could assist in predicting its ability to influence pregnancy outcome.

Keywords: Vaginal Microbiome, CST, Pregnancy, *Lactobacillus*

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List of Contents **Page**

List of figures

List of tables

LIST OF ABBREVIATIONS

ANC- Antenatal Clinic

ART-Anti retroviral treatment

BMI-Body Mass Index

CD4- Cell differentiation 4

CST- Community state type

DNA- Deoxyribonucleic acid

GBS- Group B Streptococcus

GCP- Good Clinical Practice

HIV- Human Immunodeficiency virus

IAP- Intrapartum Antibiotic Prophylaxis

ICH- International Conference on Harmonization

MMed - Master of Medicine

NHLS- National Health Laboratory Service

NICD-National Institutes for Communicable Diseases

NICU- Neonatal Intensive Care Unit

OTU- Operational Taxonomic Unit

PBS- Phosphate buffered saline

PCoA- Principal coordinate analysis

PID-Pelvic Inflammatory diseases

PPROM-Preterm premature rupture of membranes

PROM- Premature rupture of membranes

PTB- Preterm birth

QIIME- Quantitative Insights into Microbial Ecology.

Redcap- Research Electronic data capture

RMMCH-Rahima Moosa Mother and Child Hospital

RNA- Ribonucleic acid

STI- Sexual Transmitted Infections

UTI –Urinary tract infection

VM- Vaginal Microbiome

WITS- University of the Witwatersrand

Vaginal Microbiome in the Third Trimester of Pregnancy among Women at Rahima Moosa Mother and Child Hospital

S Emvula,[1] MB ChB, **A J Wise,**[1,2] MB BCh, FCOG (SA), MMed (O&G), Dip HIV Man (SA), ACHM (FPD), Cert Maternal Fetal Med (SA), **H Lombaard**,[1,2] MB ChB, FCOG (SA), MMed (O&G), PG Dip HSE

Corresponding author: *S E Emvula* (*sesemvula@gmail.com*)

Department affiliations:

[1]Obstetrics and Gynaecology Department, University of the Witwatersrand, Rahima Moosa Mother and Child Hospital, Cnr Fuel road and Oudtshoorn street, Coronation Ville, Johannesburg, South Africa, 2112

[2]Empilweni Services Research Unit, Rahima Moosa Mother and Child Hospital, University of the Witwatersrand, Johannesburg, South Africa

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Conclusion

Lactobacillus were the predominant species of the microbial community. Obesity was associated with a high prevalence of *Prevotella species*. There are a significant number of women with unrecognised STI's and Group B Strep. These results may serve as a baseline for further microbiome studies during pregnancies, which could assist in predicting its ability to influence pregnancy outcome.

Keywords: Vaginal Microbiome, CST, Pregnancy, *Lactobacillus*

INTRODUCTION

The normal vaginal microbiome is an essential component in women's health and may influence the pregnancy outcome. Alteration in the vaginal microbiome is associated with bacterial vaginosis, and increased risk for sexually transmitted infections (STI) and poor pregnancy outcome[1,2]. *Lactobacillus (L)* species dominate the vaginal microbiome in healthy women and are known to produce lactic acid[3–5]. Other microorganisms that can be part of the vaginal flora but in scanty amounts and are *Bifidobacterium, Gardnerella, Staphylococcus*, *Ureaplasma, Corynebacterium, Streptococcus, Bacteroid*es, *Escherichia* and *Candida* but these do not cause clinical symptoms[4,6]. There are more than 100 known *Lactobacillus* species of which 20 of them are identified in the vagina. However only four species: *L. jensenii, L. crispatus, L. gasseri and L. iners* are common[5]. Scanty *Lactobacillus* species in the vagina is associated with overgrowth of anaerobes and gram-negative bacteria[5,7,8]. This increases the susceptibility to urinary tract infection (UTI), chorioamnionitis, premature rupture of membrane, preterm labour, and puerperal sepsis[6,9].

Factors such as; age, smoking, alcohol consumption, geographical location, race, ethnicity, HIV infection, body mass index (BMI) and parity may influence the composition of the vaginal microbiome[5,10]. Culture-based analyses have been used to characterise the vaginal microbiome, but culturing has limitations as it depends on the ability of the microorganism to grow *in vitro*[11]. The use of culture-independent methods has amplified our understanding of the human microbiome by identifying taxas that are not culturable. The most commonly used culture independent method is the polymerase chain reaction (PCR) of the 16S ribosomal RNA (16S rRNA)[11]. This method involves amplifying and sequencing the bacterial 16S rRNA gene[1,5,12]. Groups of communities that have a similar microbial abundance and phylotype composition are called community state types (CSTs) in microbial ecology[5]. Most studies in the literature have described the vaginal microbiome to have five different CSTs according to the predominating species[4,5]. Four of these CSTs (CST I, CSTII, CST III, CST V) are dominated by the *Lactobacillus* spp: *L. crispatus* (CST I *), L. gasseri* (CST II), *L. iners* (CST III), *L. jensenii* (CST V) while CST IV is a heterogenous CST dominated by *Gardnerella,* anaerobes and gram-negative bacteria with sparse *Lactobacillus* species or completely lacking[7,12,13]. Women with CST IV may be asymptomatic, but because of the predominance of anaerobes and gram negative bacteria, this category is commonly linked with poor perinatal outcome[13].

Details about normal vaginal microbial composition in pregnancy within the South African population is still scanty. Therefore, this study aimed to characterise the composition of the vaginal microbiome in the third trimester of pregnancy and to describe an associated short term perinatal outcome. A better understanding of the composition of the vaginal microbial composition in pregnancy is essential to detect and manage pregnancy complications.

METHODS

This was a prospective observational study to characterise the vaginal microbiome during the third trimester of pregnancy. We enrolled 21 healthy pregnant women attending their routine antenatal care clinic at the Rahima Moosa Mother and Child Hospital (RMMCH), Johannesburg, South Africa, between January 2020 and May 2020. The hospital provides services to a diverse population, including a large proportion of non-South Africans (40%). In 2019 there was a total of 13 974 deliveries of which 8718 (62%) were normal vaginal deliveries (NVD) and 5 093 (37%) were caesarean sections (C/S), with a low birth weight (<2500g) of 14.6% and a perinatal mortality rate of 25.8 per 1000 births.

The study was conducted in line with the International Conference on Harmonization (ICH) Good Clinical Practice (GCP). Inclusion criteria were a gestational age of between 34-37 weeks, 18 years old and older and understood English. We excluded women who had received antibiotics within the two weeks before presentation, cervical cerclage in situ, history of antepartum haemorrhage, sexual activity within 48 hours prior to recruitment and symptomatic vaginal discharge. Upon enrolment, participants were given a questionnaire, and the results were recorded in the Redcap database.

Specimen Collection

Specimens were collected using a dry Copan flocked swabs (Copan Flock Technologies, Italy) with a sterile container. This was done by introducing the swab in the vaginal fornix and lateral walls through visual inspection after inserting a sterile plastic disposable speculum. Specimens were labelled, and a cooler box with ice packs (2-8°C) was used to transport the specimens to the National Health Laboratory Services (NHLS) at the University the Witwatersrand. All specimens were stored at -70°C until processed for DNA extraction.

DNA extraction

The stored swabs were thawed at room temperature for 30 minutes before DNA isolation. We added 1mL of sterile phosphate-buffered saline (PBS) to the swabs, incubated them for 2 hours at 25°C ± 5°C (room temperature) and mixed it with a vortex mixer at maximum speed for 5 minutes. A volume of 0.5mL of this bacterial seeded PBS was used for DNA extraction and was centrifuged. Cell lysis was commenced after mixing the centrifuged cell pellet with 50µL of lysozyme (10 mg/mL), 37.5µL mutanolysin (4000 U/ml; Sigma- Aldrich), 1µL lysostaphin, (150000 U/mL in sodium acetate) and 41µL TE50 buffer. This mixed solution was incubated at 37°C for one hour. Glass beads were added to disrupt the cells within the mixed solution by holding the tube for one minute on a vortex mixer. The QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) was used to process the resulting crude lysate according to the protocol issued by the manufacturer. The 16S rRNA amplification and pyrosequencing was done on the DNA.

DNA Sequencing and Bioinformatics

The protocol targeting the V3 and V4 regions of the 16S rRNA was used for sequencing. Amplicon primers for amplifying a single amplicon of approximately ~460bp were used (16S Amplicon PCR Forward Primer:
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG
16S Amplicon PCR Reverse Primer:
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATC)
Illumina adapter overhang nucleotides sequences were added to the gene-specific primers for compatibility with Illumina index and sequencing adapters. The V3 and V4 regions of the 16S rRNA were amplified using a limited cycle PCR during library preparation using the NextEra XT Library Preparation, followed by addition of dual-index barcodes and Illumina sequencing adapters to the amplicon target. The resulting reads were checked for quality, trimmed and analysed using the CLC Genomics Workbench version 20 (CLC, Bio-QIAGEN, Aarhus, Denmark). All reads with 97% similarity were clustered into operational taxonomic units (OTU) to represent species. Sequencing and amplicon library preparation and were performed at the National Institute for Communicable Diseases Sequencing Core Facility, Johannesburg, South Africa.

Data collected in Redcap were exported and entered into an Excel spreadsheet. Percentage and frequency tabulation were used to summarize all categorical variables. Mean, standard deviation (SD) and median were used to condense continuous variable. The x^2 and t-test were used to analyse relationships between each CST's, sociodemographic factors and pregnancy outcomes. Data were exported from Excel into Stata® 14.0 for analysis. A 5% significance level was used.

RESULTS

Cohort characteristics and pregnancy outcomes

We prospectively enrolled 21 black women who met the inclusion criteria and consented to participate in the study. The population demographics and pregnancy outcomes are summarised in Tables 1 and 2. Most of the participants were classified as obese (n=12) with a mean BMI of 30.76kg/m^2. None of the participants reported practising vaginal douching.

We observed an association between smoking (p=0.095) and alcohol consumption (p=0.115) with CST IV, although it was not statistically significant. One (4.8%) participant experienced preterm delivery and her vaginal microbiome belonged to CST III (*L. iners* dominated). There was one intrauterine foetal death (IUFD), delivered via NVD at 39 weeks of gestational age and the mother eventually developed puerperal sepsis (endometritis). *Escherichia coli* was cultured in her blood sample, and the microbiome was classified as CST IV (dominated by *G. vaginalis* and anaerobes) with 208 2055 reads in OTU (19.5% of total read in OTU). Unfortunately, the placenta was not sent for histopathology evaluation. None of the 20 neonates needed NICU admission.

Table 1: Cohort characteristics of the study population (n=21)

Characteristic	**Descriptive**
1.1 Maternal and pregnancy characteristics	
Age (years)	**(n=21)**
(Mean ± SD, Range)	32± 6.85(23-43)
18-25	6 (28%)
26-35	8 (38%)
36-49	7 (33%)
Body mass index (kg/m^2)	**(n=21)**

(Mean ± SD, Range)	30.76± 6.56 (20.3-46.4)	
Underweight (<18.5)	0 (0.00%)	
Normal Weight (18.51-24.9)	3 (12.3%)	
Overweight (25.0)	6 (28.6%)	
Obese (>30)	12 (57.1%)	
Ethnicity	**(n=21)**	
Black	21(100%)	
Parity	**(n=21)**	
para 0	2 (9.5%)	
para 1-4	19 (90.5%)	
Obstetric History for multiparous	**(n=19)**	
Previous caesarean section	3 (12.2%)	
Previous preterm Birth (<37 Weeks)	1 (4.76%)	
Previous Still birth	0 (0.0%)	
Previous Early Neonatal death	0 (0.0%)	
Grand multiparous (5 or more)	0 (0.0%)	
Gestational age at enrolment (weeks + days)	**(n=21)**	
(Mean ± SD, Range)	$35^{+2}\pm1^{+2}$ (34^{+2}-35^{+6})	
	Yes (n=21)	**No (n=21)**
Smoking	4 (19%)	17 (81%)
Occasional Alcohol use	3 (14.3%)	18 (85.7%)
HIV	9 (42.9%)	12 (57.1%)
1.2 Results of vaginal swabs		
CST	**(n=21)**	
III *(L. iners*)	10 (47.6%)	
V (*L.jensenii*)	5 (23.8%)	
IV (*Gardnerella* & anaerobes)	6 (28.57%)	
GBS	**5 (23.8%)**	

BMI= body mass index; CST= category state type; GBS= Group B Streptococcus;

HIV= Human immunodeficiency virus; SD=Standard Deviation

Table 2: Pregnancy outcome of the study population (n=21)

Characteristic	**Descriptive**
Gestational age at delivery	**(n=21)**
(Mean ± SD, Range)	$39^{+2}\pm1^{+1}(36^{+0}\text{-}41^{+0})$
Preterm birth (<37 weeks)	(n=21) 1 (4.8%)
Mode of delivery	**(n=21)**
Vaginal delivery	13(61.9%)
Caesarean section	8(38.1%)
Birth weight (grams)	**(n=21)**
(Mean ± SD, Range)	3179 ± 354.1 (2450-3760)
Low (<2500 g)	1 (4.8%)
Normal (2500–4000 g	20 (95.2%)
Large (>4000 g)	0(0.0%)
Neonate born Alive	**(n=21)**
Yes	20 (95.2%)
No	1 (4.8%)
Apgar score at 5 min	**(n=21)**
(Mean ± SD, Range)	8.97 ± 0.17 (8–9)
Neonate admitted to NICU	0
Puerperal sepsis	(n=21)1 (4.8%)

SD=Standard Deviation.

Pyrosequencing and OTU analysis

The analysis of the sequence read revealed a total of 1 7487 370 reads (ranging between 22 540-369 131 reads per sample). The sample needed to have 5 000 or more reads to pass the quality control, and all 21 samples passed.

Table 3 illustrates the prevalence of species detected in 21 participants and their combined abundance. *Lactobacillus* genus had the highest abundance, with 64.2% of the total OTU reads as represented in Figure 1 and Table 3. *L. iners* was the most prevalent species and was identified in 86.0% (n=18) of the participants. Other *Lactobacillus* species identified are listed in Table 2. *Gardnerella* was identified in 80.9% (n=17) of the participants, but its combined abundance was only 23.0% of the total read in OTU. The prevalence of GBS was 24.0% (n=5) and participants received intrapartum antibiotic prophylaxis (IAP) .

There was a high prevalence of mollicutes (*Ureaplasma* and *Mycoplasma*), while *Ureaplasma spp.* were identified in 76.2% (n=16) of the participants, but it only represented 0.13% of the total OTU reads.

Chlamydia trachomatis was identified in 33.0% (n=7) of the participants with a combined abundance of 0.07% (741) . Participants were notified and given a stat dose of Azithromycin.

Prevotella species were detected in 61.9% of the participants, and it had an association (p=0.048*)* with obese participants (BMI>30kg/m^2).

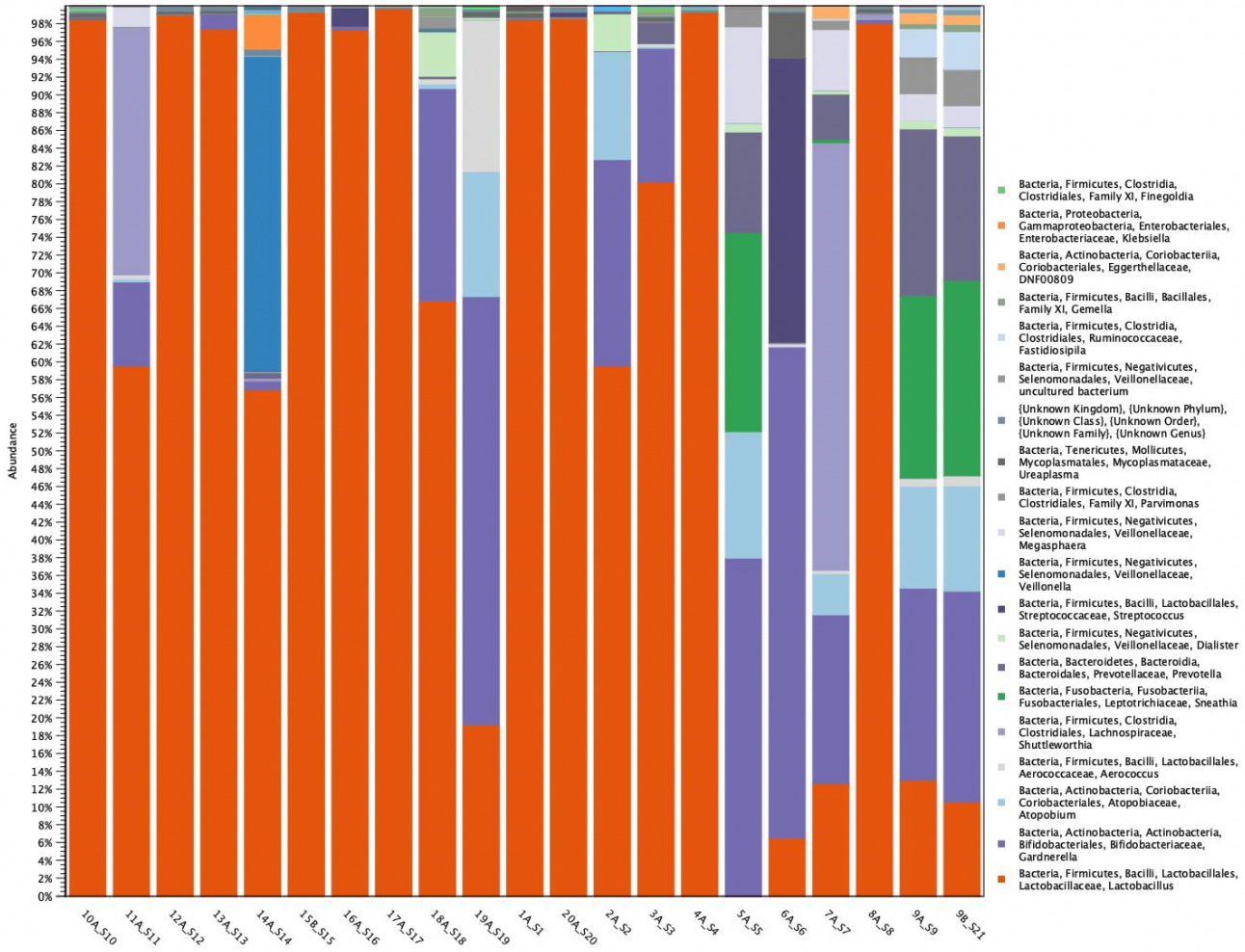

Figure 1: Abundance of different species and their distribution among the 21 participants.
On the X-axis = participants and Y-axis = microbial abundance (%).

Table 3: Prevalence of species detected in 21 participants and their combined abundance.

Species	Prevalence n (%) N=21	**Combined abundance**	**% of the total read**
Lactobacillus iners	18 (86%)	353 571	33%
Lactobacillus reuteri	5 (24%)	398	0.04%
Lactobacillus jensenii	2 (10%)	4479	0.42%
Lactobacillus sp	9 (42%)	312 846	29.4%
Streptococcus agalactiae	5 (24%)	7949	0.75%
Veillonellaceae bacterium	8 (38.1%)	753	0.07%
Klebsiella pneumonia	1 (4.7%)	435	0.04%
Aerococcus christensenii	8 (38.1%)	16 249	1.53%
Veillonellaceae bacterium	8 (38.1%)	3250	0.31%
Finegoldia magna	16 (76.2%)	319	0.03%
Corynebacterium sp.	5 (24%)	23	
Corynebacterium pseudogenitalium	6 (28.6%)	20	
Atopobium vaginae	12 (57%)	31 307	2.94%
Sneathia sanguinegens	5 (24%)	10 880	1.02%
Pseudomonas sp.	2 (9.5%)	2	
Clostridium perfringens	1 (4.7%)	72	
Prevotella bivia	13 (61.9)	914	0.09%
Prevotella buccalis	3 (14.2%)	65	
Prevotella amnii	3 (14.2)	1082	0.10%
Actinomyces hongkongensis	2 (9.5%)	43	
Megasphaera	7 (33.3%)	4185	0.39%
Mycobacterium sp	3 (14.2%)	58	
Chlamydia trachomatis	7 (33.3%)	741	0.07%
Ureaplasma	12 (57%)	450	0.04%
Bifidobacteriaceae	4 (19%)	36	
Shuttleworthia	8 (38.1%)	17 093	1.61%
Clostridium sp	4 (19%)	2422	0.23%
Gardnerella sp	17 (80.9%)	44 995	4.23%
Dialister sp	13 (61.9%)	13 643	1.28%

Mycoplasma sp	3 (14.2%)	56	
Gemella sp	5 (23%)	2126	0.20%
Solanum melongena (eggplant)	2 (9.5%)	41	

sp=species

The vaginal community state types analysis

By using fuzzy clustering of Pcoa Bray Curtis distances as the dissimilarity measure to form the clusters, we identified three CSTs (Figure 2) according to the dominant species in each cluster. Two were dominated by *Lactobacillus*: CST III (*L. iners* n=10) and CST V (*L. jensenii* n=5), and a heterogeneous one, CST IV (n=6), which was dominated by *Gardnerella vaginalis* and anaerobes.

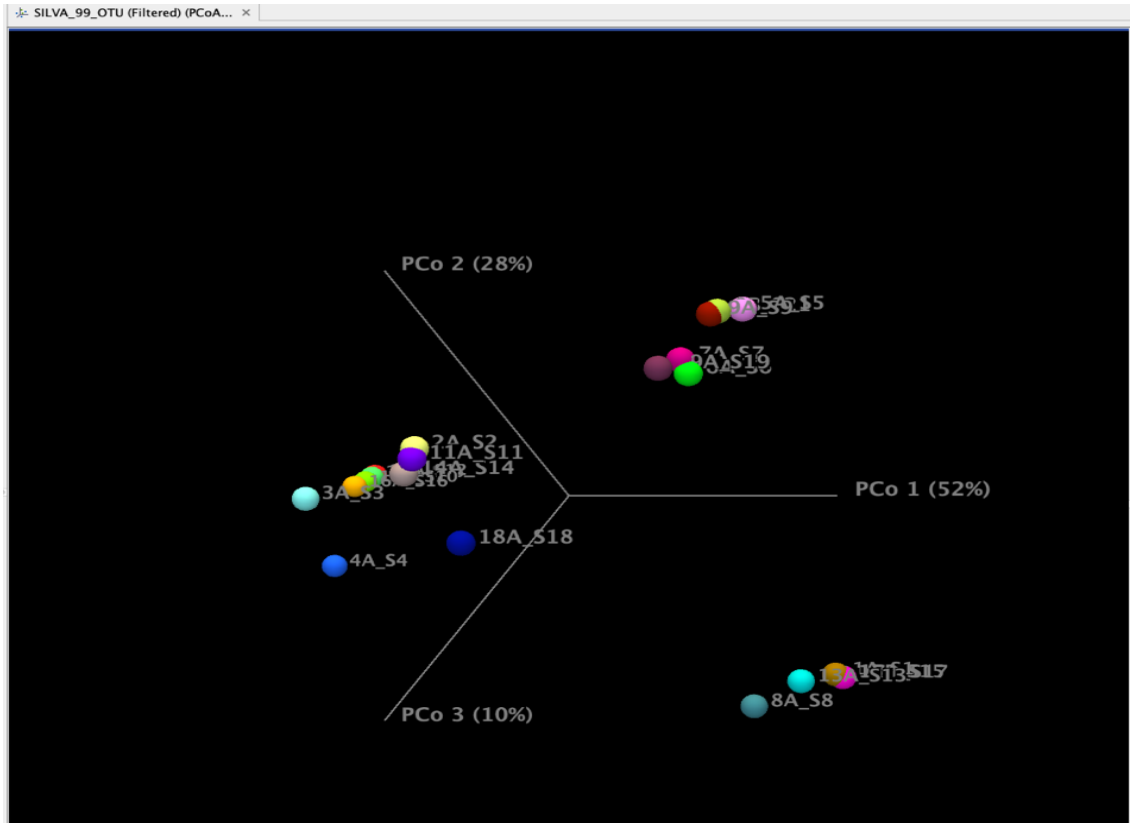

Figure 2: 3D beta diversity represented as PCoA, with colouring done according to the taxonomic abundance of species, depicting the three clusters.

DISCUSION

We characterised the vaginal microbiome of 21 healthy pregnant women in their third trimester of pregnancy. Rave et al., described five community state types: CST I (dominated by *L. crispatus*), CST II (dominated by *L. gasseri*), CST III (dominated by *L. iners*), CST V (dominated by *L. jensennii*) and CST IV (dominated by anaerobes and *G. vaginalis*)[5]. We identified three CSTs, namely CST III (*L. iners*), CST V (*L. jenseni*) and CST IV *(G. vaginalis*, & anaerobes). The microbiome of 71.4% of our participants were dominated by *Lactobacillus*, hence they are categorised in CST III and CST V, which correlates with the finding by Aagaard et al[12].

Lennard et al., investigated at the vaginal microbiome of 168 South African black women, and they observed three CSTs (CST III, CST II, CST IV)[14]. These CSTs were also found by Mugabe et al., who characterised the vaginal microbiome of 357 healthy pregnant women in Zimbabwe. Interestingly, in our cohort, we did not observe CST II (*L. crispatus)*; instead, we found CST V, dominated by *L. jensenii*[15]. *L. iners* is the most prevalent species, detected in 85.7% (n=18) of the participants in our studies. This reaffirms the findings by Fettweis et al., that concluded that *L. iners* is the most dominant species in black African women[7]. On the contrary, a study by Romero et al., on Northern American women of different ethnicity found a lower prevalence of *L. iners* in all participants irrespective of their races (blacks and Caucasians)[1]. They compared their results with studies done in Africa and observed a difference in the composition of the microbiome between African women and Northern American women, confirming the influence of geographical location on the microbiome's composition[1].

Our study shows a high prevalence of *G. vaginalis*, 85.0% (n=18) although its total abundance was only 4.0% of all reads in OTU and it only predominated in 23.0% (n=5) of the participant's microbiomes. Machado et al., found that *G.vaginalis* colonised 67.0% of Portuguese women but in less abundance and all women were asymptomatic[16], this was reaffirmed by Mugabe et al., in Zimbabwe who found that more than 40.0% of participants were classified as CST IV due to the high predominance of *G. vaginalis,* although they were asymptomatic. GBS prevalence in our study is 23.8%, which is almost equivalent to the prevalence found by Cutland et al., in pregnant women at the Chris Hani Baragwanath Academic Hospital, Soweto[17].

HIV prevalence in our study is 42.9% (n=9) which is a high compared to the national rate, which is approximately 30% [22]. Redelinghuys et al., found an association between HIV infection and dysbiosis.[18] There was no association between HIV infection and CST IV (p=0.163) in our study. This may be because eight of the nine HIV infected participants were on anti-retroviral treatment (ART) and were virally suppressed (viral load; lower than detectable). The one participant who was not on ART, had a CST IV microbiome. She had a high viral load (120000 copies/ml), low CD4 count (147 cells/mm^3) and of note she had a high bacterial load of anaerobes, which accounted for 21.0% of the total reads in OTUs of our cohort. Perhaps this indicates the influence of untreated HIV infection on the microbiome's composition as was previously discussed by Myer et al,[19].

One participant (4.8%) delivered prematurely. The same participant had a low-birth-weight baby, and her microbiome was classified as CST III (*L. iners).* Hence we could not find any association between these outcomes and her microbiome composition, unlike numerous studies that have reported an association between dysbiosis and preterm labour[9]. Of importance is a high prevalence of mollicutes (*Mycoplasmas* and *Ureaplasmas*), with a prevalence of 57.0% (n=12) in our study participants. However, it is not in significant abundance, representing only 0.04% of the total reads in OTU. This is similar to the prevalence found by Freitas et al., although it was reported to be associated with preterm labour in their study[19]. Mollicutes did not colonise our participant who had the preterm delivery.

We found a high prevalence of *Atopobium vaginalis* (57.0%) with 2.9% of total reads in OTU. It was recently discovered to be a lactic acid-producing microorganism associated with a healthy microbiome[14].

In 2010, Brotman et al., compared the microbiome of 20 smokers and 20 non-smokers and found that smokers had a less of an abundance of *Lactobacillus spp*. and where more prone to have dysbiosis (CST IV)[10]. We also found a correlation between smoking and CST IV, although it was not statistically significant(p=0.095). Of importance, we observed an association between alcohol consumption and CST IV, although it was also not statistically significant (p=0.115). Ravel et al., reported that the presence of *Prevotella* in the vagina positively affects the growth of anaerobes associated with bacterial vaginosis.[5] Si et al., investigated the microbiome of 544 pregnant Korean women and found that *Prevotella* is strongly associated with increased BMI[21].

Indeed in our study the prevalence of *Prevotella species* was 61.9% (n=13), and 57.1% of our participants were obese (BMI>30kg/m); thus we found an association (p=0.048) between obesity and the host *Prevotella* prevalence as previously described by Si et al,[21].

The strengths of our study are that it is the first such study done in South Africa on healthy asymptomatic pregnant women. We used a metagenomic approach (16S rRNA) which is more sensitive than culture-based methods. However, the study limitations are the sample size which was kept small due to high cost of molecular studies, and since there were few poor pregnancy outcomes it is difficult to associate them to a specific CST. Furthermore, all our participants were of the same ethnicity which could create bias as this doesn't reflect the racial distribution of the South African population.

CONCLUSION

L. species are the predominant member of the microbial community in our cohort. There is a positive association between increased BMI and *Prevotella spp*. prevalence. There are a significant number of women with unrecognised STI's and GBS. Further longitudinal studies are needed to compare the vaginal microbiome in pregnancy according to trimesters, with pregnancies with adverse outcomes.

Declaration. This study was done in partial fulfilment of an MMed (Obs&Gynae) degree.

Author contributions: HL and AW conceptualised the study, SE developed the protocol with input from HL and AW. SE recruited patients, collected data and did the DNA extraction. SE wrote the manuscript with input from HL and AW. All authors approved the manuscript for publication.

Ethics Considerations: The study received ethical approval from the human research ethics committee of the University of the Witwatersrand, protocol number: M190617, Authorization from the CEO of RMMCH and its registered with NHRD

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Conflicts of interest. S Emvula, and H Lombaard- Nil

A Wise – Received honoraria from Sanofi for presentations on maternal immunization and thromboprophylaxis – there is no relationship to this study

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Appendix A: Approved Research protocol

University of the Witwatersrand

Faculty of Health Science

School of Medicine

VAGINAL MICROBIOME IN THE THIRD TRIMESTER OF PREGNANCY AMONG WOMEN AT RAHIMA MOOSA MOTHER AND CHILD HOSPITAL

MMED

OBSTETRICS AND GYNECOLOGY

DR SIMON EBEN ESSER I EMVULA

2113356

Supervisors:

1.Dr Amy Wise

Senior Consultant,

Rahima Moosa Mother and Child Hospital

MBBCh, FCOG, MMed(O&G), Dip HIV Man, ACHM

2. Adj Prof Hennie Lombaard

Academic Head, Obstetrics and Gynecology

MBChB, FCOG, MMed(O&G)

ABBREVIATIONS

ACOG- American College of Obstetricians and Gynaecologists

ANC- Antenatal Clinic

EOGBS- Early onset of group B streptococcus

GBS-Group B Streptococcus

HIV- Human Immunodeficiency virus

IAP-Intrapartum Antibiotic Prophylaxis

NICD-National Institutes for Communicable Diseases

PID-Pelvic Inflammatory diseases

PPROM-Preterm premature rupture of membranes

PROM- Premature rupture of membranes

PTB- Preterm birth

RMMCH-Rahima Moosa Mother and Child Hospital

RCOG-Royal College of Obstetricians and Gynaecologists

STI- Sexual Transmitted Infections

UTI –Urinary tract infection

TABLE OF CONTENT

page

1.INTRODUCTION

The human body is composed of several microbiome species which reside on the different surface around it and play an important on the human physiology. This includes bacteria, fungus and viruses with different genetic material and resides in a different part of the human body without causing any harm. They play an important role in metabolism, digestion and immunity of humans(1).

This microbiome might be affected by several changes that happen in the human such as, weight gain, diet, hormonal change, immunity and metabolism. Pregnancy is associated with multiple physiological change in metabolism, endocrine function and immunity and thus it will affect the human microbiome(2).

The composition of the vaginal microbial community changes in different stages of life from birth, pre-puberty, puberty and post-menopause(3). The normal vaginal microbiota in healthy women is dominated by lactic acid-producing bacteria called *Lactobacillus species*(1). The presence of The *lactobacillus* make it difficult for the pathogenic bacteria to colonise the vagina and this is made possible by the abundant lactic acid produced by the *lactobacillus* which results in a low pH in the vagina and most pathogenic bacteria's are not active in an acidic environment(4).

During pregnancy, the vaginal microbiome is strongly dominated by the *lactobacillus,* and this reduces the risk of vaginal infection during pregnancy which might cause a poor maternal and neonatal outcome(5).

2. LITERATURE REVIEW

2.1 NORMAL VAGINAL FLORA

The human is a host to different microorganisms that play an important role in its physiology, nutrients and more importantly in the immune system(6). The female genital tract also hosts several microorganisms that participate in different functions. The normal vaginal flora is dominated by series of *Lactobacillus species* of bacteria's, which are lactic acid-producing organism that maintain an acidic pH around the vagina and in this way contribute to the immune system by preventing colonization by pathogenic organisms which can cause a condition called bacterial vaginosis and also increase the risk of acquiring sexually transmitted infections(7). *Lactobacillus* dominated vaginal microbiome is associated with reduced risk of vaginal infections, urinary tract infections and sexually transmitted infection(STI)(8). Hickey et al have reported that a normal vaginal microbiome will reduce the risk of HIV transmission(9).

Lactobacillus species form part of the normal vaginal flora and it was discovered by Doderlein in 1892 who initially started to study the composition of vaginal microbiome(10). Other microorganisms can also be part of the normal flora but in small quantity that they are unable to cause clinical symptoms and this is; *Staphylococcus*, *Ureaplasma, Corynebacterium, Streptococcus, Escherichia, Candida, Bifido Bacterium, Gardnerella, Bacteroid*es, (11). A vaginal microbiome which is dominated by *Lactobacillus species* is known as the normal vaginal flora(2).

Bacterial vaginosis is the most common type of vaginal infection in the world (2) and in non-pregnant woman its associated with pelvic inflammatory disease(PID), infertility, endometritis but in pregnant woman it can be associated with poor maternal and neonatal outcomes such as preterm premature rupture of membranes(PPROM), premature rupture of membranes(PROM), preterm labour(PTB), Puerperal sepsis, and neonatal sepsis(9).

The vaginal microbiome is influenced directly by the level of sex hormones, specifically oestrogen(12). Oestrogen influences the vaginal microbiome by increasing the production of glycogen in the epithelial cells of the vagina(10). *Lactobacillus species* of bacteria feed on glycogen to produce lactic acid and in this way maintain the low pH (pH=3.5 - 4) of the vagina(13).

Oestrogen level varies through different stages of the woman life span and this influences the composition of the vaginal microbiome(9). It's believed that the initial vaginal colonization in a new-born female neonate occurs at birth(14). During the first two weeks of life, their vaginal microbiome is dominated by *Lactobacillus species* and this is made possible by the maternal oestrogen which is still in the neonate's circulation(15), thus the neonates vaginal microbiome is as that of an adult in the reproductive age, and their vaginal environment is acidic due to the increase in the vaginal epithelial cells glycogen level(13). This will eventually change as the maternal oestrogen reduces in the neonate's, circulation and the pH will change to alkaline and will remain like that throughout childhood until puberty as the sex hormones(oestrogen) production will commence(16).

Ravel *et al*, have shown that the composition of the vaginal microbe is directly influenced by the oestrogen hormone as this influence the amount of glycogen produced by the epithelial cells of the vagina(13). It's not only the level of sex hormones that influence the vaginal microbe but other factors are known to influence the vaginal microbe and these are: use of antibiotics, showers and vaginal douches, frequency of sexual intercourse and use of immune depressing medication(10).

Lactobacillus species dominate the vaginal microbes in all healthy woman of reproductive age and there are four most common *Lactobacillus species: L. crispatus, L. gasseri, L. Iiners, L. Jjensenii*(16). In the event of low *Lactobacillus species* around the vagina, then the vaginal microbiome will be dominated by several anaerobic bacteria such as *Gardnerella vaginalis, Megasphaera, Prevotella, Atopobium vaginales, Mycoplasma hominis*; and these species are associated with bacterial vaginosis(7). By maintaining the vaginal environment acidic the overgrowth of other pathogenic microorganisms which can result in active infection will be prevented(11).

Bacterial vaginosis is a poly-microbial vaginal infection which can present in the form of a vaginal discharge syndrome characterised by malodourous discharge and this can be associated with poor obstetric outcomes including preterm labour(17).

2.2 VAGINAL MICROBIOME IN PREGNANCY

Pregnancy is associated with complex changes in the woman's physiology. There is a significant change in most systems. Significantly, in the immune system, for the foetus to accepted as an allograft, there is a significant change in the levels of the sex hormones(oestrogen and progesterone)(1). The hormonal changes that occur during pregnancy affect the vaginal microbiome, and this is characterised by a decrease in the variety and diversity of the microbe(11). The level of sex hormone increases progressively as the pregnancy advances and this will also affect the vaginal microbiome to be more lactobacillus dominated in the third trimester in comparison to the first and second trimester of pregnancy(12) and this contributes to the innate immune system as it prevents overgrowth of pathogenic microorganism(5).

Another microorganism which is widely found in a pregnant woman is *Candida Albican*s(10). Linhares et al found that *Candida* is tolerant to an acidic environment and its found in about 10% to 20% of health woman without causing any symptoms(10). Never the less in a study by Genovese et al (12) about vaginal microbe in 3rd trimester of pregnancy they found that 66% of the woman had candidiasis and it was found to be linked to preterm birth(12).

2.3 DEMOGRAPHIC INFLUENCES ON THE VAGINAL MICROBIOME

Hickey *et al* have indicated that other demographic features, such as race and ethnicity, geographic area, might influence the composition of the vaginal microbiome(9).

In the United State of America by Fettweis *et al* compared the vaginal microbiome in African American and those of Caucasian origin and they found that the woman of Caucasian origin are more likely to have a *Lactobacillus* dominated vaginal microbe and African American were found to have a more diversified Microbiomes(7). Furthermore, Hyman *et al* have occluded that race and ethnicity are an important determinant for vaginal microbiome and that preterm labour rate was 17. 5% in African American, 11.5% in Caucasian and 10.5%in Asian(18).

Serrano *et al* compared the diversity of microbe between a black and white woman and found that *lactobacillus inners* were the most dominant in both groups but in black women, *Gardnerella vaginalis* was the second most common while in white the second most common was *Lactobacillus Jjesenei* and *Gardnerella vaginalis* was third(7).

In United States of America Tabatabaei *et al* found the prevalence of the *Lactobacillus* to be 80.2% in white, 89% in Asians, 59.6 in Hispanics, and 61.9% in black women(15). A cohort study done in pregnant women from a rural area in Malawi involving 1017 postpartum women reported a high prevalence of *Gardnerella vaginalis* of 75.4% of the participant and *Lactobacillus* species was only found in 30.4% of the participants(4), this show how regional and ecological factor also plays an important role in the composition of the vaginal microbiome (4).

There are other factors such as smoking and alcohol use, body mass index (BMI), socioeconomic status, the vaginal douching practice also influences the character of the virginal microbe. In a study done by Serrano *et al*, it was found that smoking, alcohol intake and low-income were associated with a high risk of having bacterial vaginosis(7).

2. 4 ABNORMAL VAGINAL FLORA AND PREGNACY

Premature rupture of membranes and preterm premature rupture of membranes(PPROM) are two conditions that are likely to be linked to bacterial vaginosis during pregnancy(12). Premature rupture of the membrane is defined as rupture of membranes in a term pregnancy (>37weeks) before the onset of labour and PPROM is a rupture of membrane before 37 weeks of gestation age(5). Premature rupture of membrane and PPROM are caused by the ascending microorganism from the vagina through the cervix and this will infect the amnions (12). In Italy Genovese *et al*, found that PROM affected about 10%(12) of pregnancies and of which 85% go into spontaneous labour within 24 hours. Both PROM and PPROM are associated with neonatal and maternal complications, such as preterm labour, neonatal sepsis, chorioamnionitis and puerperal sepsis, the risk for caesarean section(6)12).
It's very important to treat and prevent bacterial vaginosis during the third trimester as it has been associated with preterm births(12)
Mycoplasma species *(M. genitalium, M. hominis, U. parvum, U. urealyticum)* are known to be one of the most common causes of bacterial vaginosis, it's reported to have a prevalence of *80%* in non-pregnant women although most of them are asymptomatic (24**).**
Ureaplasma urealyticum is the most pathogenic of all and is isolated in *62%-92%* of women with bacterial vaginosis.

Genital mycoplasmas are associated with pregnancy complications and are isolated in about *45%* of chorioamnionitis cases and frequently associated with pregnancy loss (24).
In a study done by Jim *et al*, it was found that the most commonly seen microorganism in a woman with vaginosis during pregnancy was, Group B streptococcus (4.0%), *Escherichia coli (3.8%), and Ureaplasma urealyticum*(5). It was also noted that these two bacteria were found in most babies with neonatal sepsis(5). Group B streptococcus is one of the most common causes o neonatal sepsis, and deferent health organisation have come up with several methods to prevent such. The American College of Obstetricians and Gynaecologists(ACOG) recommends universal GBS culture screening system(19) and RCOG recommends a risk-based screening method(20). Universal screening of GBS involves a culture-based screening in all pregnant woman between 34-37 weeks of gestational age(21). All woman that is GBS culture-positive should receive intrapartum antibiotic therapy against GBS. Risk factor-based screening involves identifying those woman with GBS and administration of intrapartum antibiotics to prevent early-onset Group B Streptococcus sepsis (EOGBS)(22).

Antibiotic use during pregnancy is associated with altering the normal vaginal microbiome which can have a significant change in the composition of vaginal microbiome. In Norway, Stockholm *et al*, compared antibiotic use in pregnancy to non-antibiotic use it was found that women who receive antibiotic therapy during pregnancy had a high risk of colonization by staphylococcus species in their vaginal specimens, and antibiotic use didn't have any effect on GBS rate(23). This is because antibiotic use also act on the *lactobacillus* and reducing its activity thus this will encourage overgrowth of pathogenic microorganism which may cause maternal or neonatal morbidity(23). In Malawi Doyle *et a*l, reported how the use of antibiotic therapy have reduced the risk of preterm delivery caused by ascending infection(24). In a study done in Europe, it was found that about 40% of pregnant woman have *Candida Albican*s and recommend that they should be treated with metronidazole or clotrimazole, as this doesn't affect the *lactobacillus species* and in this way, it will help restore the normal vaginal microbe(12).
Different vaginal hygienic process is also associated with interfering with the normal vaginal ecosystem. It has been reported vaginal douching practices interfere with the normal lactobacillus dominated flora and in most cases, it will result in overgrowth of pathogenic microorganism such *Gardnerella vaginalis* and other anaerobes(25).

Several studies were done in non-pregnant woman to compare the effect of smoking on the vaginal microbiome. In a study comparing the vaginal microbe of non-smoker and smoker woman, it was found that smoker has fewer Lactobacillus species and are more prone to bacterial vaginosis(26).
They also reported that cigarettes smoking have an anti-estrogenic effect and is also associated with vasoconstriction which reduces the vaginal wall perfusion, and all these factors contribute to low lactobacillus and favour overgrowth from pathogenic organism(26).
Increased BMI and obesity also have an impact on vaginal microbiomes(27).
In Korea Brotman *et al*, conducted a study involving 425 non-pregnant women it was found that the vaginal microbe of the obese participant was more dominated by *Prevotella* as opposed to the lactobacillus species as in those with normal BMI(26).

There was a high risk of preterm delivery in those woman with lactobacillus deficiency(15). Studies have shown that *Lactobacillus* deficiency is mostly associated with overgrowth of pathogenic organisms that associated with both neonatal and maternal morbidity. Its recommended that if abnormal vaginal flora is found during pregnancy it should be treated to reduce the risk of PPROM, PROM, preterm labour, chorioamnionitis, puerperal sepsis, and neonatal sepsis(12). More studies are needed to be conducted on the vaginal microbiome during pregnancy and information on the composition and characteristic of the vaginal microbiome in woman attending an antenatal clinic (ANC) at our hospital will assist in providing a baseline for future comparisons as interventions are implemented.

3. **AIMS AND OBJECTIVES**

3.1. AIMS

To evaluate the diversity and composition of the vaginal microbiome in 3rd trimester of pregnancy in woman attending antenatal clinic at Rahima Moosa Mother and Child hospital.

3.2. OBJECTIVES

1. To describe the diversity and composition of the vaginal microbiome in pregnant woman attending ANC at RMMCH
2. To describe the demographics of women screened for the vaginal microbiome
3. To evaluate short-term pregnancy outcomes

4. METHODS

4.1 STUDY DESIGN

This is a prospective descriptive study to investigate and describe the composition of the vaginal microbiome which will be carried out from 01 July 2019 to 31 January 2020.

4.2 SETTING

The study will be conducted in the Department of Obstetrics and Gynaecology at RMMCH, situated in the suburb of Coronationville, Johannesburg, South Africa. It's a teaching hospital specializing in maternal and paediatric care and it is affiliated to the University of the Witwatersrand. The Hospital was opened in 1944 and was established for Indian and Coloured people.
The hospital provides services to a diverse population which include a large proportion (40%) of non-South African. In 2017 there was a total of 12863 deliveries of which 7705 were vaginal deliveries and 4909 were caesarean section.

4.3 STUDY POPULATION

The pregnant woman attending antenatal clinic at the Rahima Moosa Mother and Child hospital and who are between 34 and 37 weeks of gestation age as determined in order of preference by (28):

- Early ultrasound(<24weeks)

- Late ultrasound
- Last normal menstrual period
- First abdominal palpation

4.3.1. INCLUSION CRITERIA

1. Gestational age between 34-37 weeks.
2. Informed consent
3. 18 years and above
4. Understand English

4.3.2. EXCLUSION CRITERIA

1. A patient who received antibiotic therapy 2 weeks before presentation.
2. A patient who has a cervical cerclage in situ
3. Current history of antepartum haemorrhage
4. Sexual activity in the past 48 hours

4.4 SAMPLING AND SAMPLE SIZE

A convenience sample of 100 patients will be recruited when the researcher is available. It is a descriptive study and so no sample size calculation has been made. The sample may be adjusted depending on funding.

4.5 MEASUREMENTS

Patients will be recruited at the antenatal clinic once they have met all inclusion criteria. The patient will be approached and provided with all the relevant information about the study. They will be asked if they would like to participate in the study. Once they have agreed to participate an informed consent will be explained in detail to them and then they can sign.

Once they have agreed to participate in relevant information regarding the pregnancy will be recorded and a questionnaire completed. In the questionnaire information will be collected regarding socio-economic information (employment, education level, marital status), demographic information, smoking history, alcohol intake, obstetric history (parity, previous IUFD, previous preterm labour), history of current pregnancy, antibiotic use in past two weeks, current history of per vaginal bleeding.

3.51. SPECIMEN COLLECTION

Patients will be provided with additional information about the genital swab. A general physical examination will be conducted including gynaecological examination, this includes the characteristics of the external genitalia, vulva vagina and cervix. The patient will be offered the option of having another female health care worker in the examination room as an escort.

Once they are comfortable a specimen will be collected from the vagina under direct visualization during a speculum examination. The speculum will be moistened with sterile saline as lubricants might alter the sample.

A high vaginal swab will be done, and a specimen is collected using a cotton wool swab. The swab will be inserted in the vaginal introits approximately 7 cm deep and it will be rotated around the vaginal wall at least five times to make sure we get an adequate specimen is obtained(6)**.** The specimen will be labelled correctly and will be sent to the NHLS laboratory at Rahima Moosa Hospital laboratory and then they will be transferred to the Microbiology Department at Helen Joseph Hospital. Patients will be given a follow up in two weeks for the culture results.

3.5.2 FOLLOW UP AND RESULTS

All patients will be given a follow up in two weeks after the specimen collection for the culture results review. Participants who have a positive culture for bacterial vaginosis but are asymptomatic will be counselled properly and explained to that recent evidence have proved that there is no need to treat asymptomatic patients (18). And for those that have positive culture test and with clinical symptoms will be offered treatment as per national guidelines (28). Those patients that have a positive culture for GBS, will be identified and it will be indicated in their antenatal card so that they receive intrapartum antibiotic therapy (IAP). As

per national guidelines, they will receive Ampicillin two grams intravenously as a stat dose and one gram six-hourly during labour (28). All participants will be followed up postpartum till discharge.

4.5.3 DATA CAPTURING AND MANAGEMENT

A screening log will be kept. The investigator will be using RedCap to capture the data.

5. ETHICAL CONSIDERATIONS

- Approval to conduct the study will be obtained from the Human Research Ethics Committee.
- The protocol was submitted to the postgraduate research committee of the Department of Obstetrics and Gynaecology of the University of the Witwatersrand.
- Approval will be sought from the Chief Executive Officer of Rahima Moosa Mother and Child Hospital.
- Consent will be obtained from participating mothers
- Any information obtained from the mother will be kept confidential at all times and will, where clinically relevant, this will be made part of her maternity case record,
- The study will be registered on the National Health Research Database

6. BUDGET

ITEM	COST
Printing of the questionnaire	R 500.00
Printing of the consent	R 500.00
Printing of the datasheet	R 500.00

Printing of dissertation	R200.00
Total cost	R 1700.00

All printing cost will be borne by the researcher

Operating funds (please itemize)	***Rands***
1. Sample collections and storage R 4.22 per sample	***R 422.00***
2. DNA extraction R78.30 per sample	***R 7830.00***
3. Open tray optimization R4.57 per sample	***R457.00***
4.Screening sample R 311.13 per sample	***R 31113.00***
Total	***R 39822.00***

Funding through both the NICD and the Postgraduate office is being sought.

8. BIAS

In the questionnaire we rely on recall information, some participant might withhold information, and this will give rise to recall bias.

9. TIMELINE AND PROJECT MANAGEMENT

TIMELINE AND PROJECT MANAGEMENT

ACTIVITY	November 2018	Jan-Feb 2019	March-june2019	July-Sep 2019	Oct-Dec 2019	January 2020	February 2020
Mmed submission	X						
Ethical Aspect			x				
Postgraduate Committee			x				
Permission from CEO/Lab			x				
Data collection				x	X		
Data processing					X		
Statistical analysis						X	
Publication and Preparation							X
Submission							X

10. REPORTING

- Feedback will be given to the Department of Obstetrics and Gynaecology of the finding study
- The results of the study will be presented to the department
- Dissertation

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Appendix B: Data collection sheet

Confidential

Demographic Information

Study ID	_____
Age	_____
Name and Surname _____	
Enrolment date _____	
Home Address	
Contact Number _____	
Date of Birth	_____
Race of participant	◯ Asian ◯ Black ◯ Coloured ◯ Indian ◯ White
Highest Education Level	◯ None ◯ Primary ◯ Secondary ◯ Tertiary
Marital Status	◯ Single ◯ Married ◯ Divorsed ◯ Stable relationship
Employment	◯ Unemployed ◯ Self-employed ◯ Employed
Dwelling Most of the year	◯ Urban ◯ Rural ◯ Semi-Urban

Obstetrics History

Study ID	_____
Gravidity	_____
Parity	_____
Number of Previous Miscariages	_____

Semester

	yes	No
1st trimester	◯	◯
2nd Trimester	◯	◯

Number of previous IUFD	_____
Previous NVD	_____
Previous Preterm birth	◯ Yes ◯ No
Have any of your baby died within 7 days?	◯ Yes ◯ No

Current Pregnancy

Study ID	_____
LNMP	_____
Estimate gestation Age(weeks)	_____
EDD	_____
Booking Bloods	_____
BMI	_____
Hiv Status	◯ Reactive ◯ Non Reactive
If HIV reactive: Cd4 VL ART regimen	_____
Antibiotic use in < 14 days	◯ Yes ◯ No
Any Vaginal discharge curently	◯ Yes ◯ No
If vaginal discharge, decribe	_____
Vaginal douche during pregnancy	◯ Yes ◯ No

Pregnancy Outcome

Study ID	_____
Mode of delivery	◯ NVD ◯ Cesarean section
Cesarean section Indication	◯ Previous cesarean section ◯ Fetal Distress ◯ APH ◯ CPD ◯ Failed AOL ◯ Prolonged Labour ◯ malpresentation ◯ Severe PET ◯ macrosomia ◯ Multiple pregnancy
Gestation age at delivery	_____
Baby born Alive	◯ Yes ◯ No
Birth weight	_____
Apgars score at 5 minute	_____
Neonate admitted to NICU	◯ Yes ◯ No
Maternal postpartum Complication	☐ Sepsis ☐ PPH
Describe maternal Complication	_____

PCR results:

Study ID	_____
Microbiome present	☐ Lactobacillus reuteri ☐ Lactobacilus jensenii ☐ Lactobacilus gasseri ☐ Lactobacius crispatus ☐ Lactobacillus iner ☐ Gemella ☐ Gardnerella Vaginalis ☐ Aerococcus christensenii ☐ Prevotella bivia ☐ Klebssiela pneumonie ☐ Ureaplasma ☐ Streptococcus agalactiae ☐ Sneathia sanguinegens ☐ Megasphaera ☐ Atophobium ☐ Chlamidia trachomatis ☐ Finegoldia magna ☐ Prevotella ☐ Veilonella bacterium ☐ pseudomonas ☐ Corynecacterias ☐ Enterobacteria ☐ Dialister ☐ Prevotella disiens ☐ clostridium ☐ Lactobacillus ☐ Bifindobacterium ☐ Streptococcus
Abundance of microorganisms	

Appendix C: Permission from RMMCH

GAUTENG PROVINCE
HEALTH
REPUBLIC OF SOUTH AFRICA

RAHIMA MOOSA MOTHER AND CHILD HOSPITAL

RAHIMA MOOSA MOTHER AND CHILD HOSPITAL
Tel: (011) 470 9031/9269
Fax: (011) 477 4117
Email : Frew.Benson@gauteng.gov.za

17 July 2019

Dr Simon Eben Esser Emvula
Dept of Obstetrics and Gynaecology
RMMCH

Re: VAGINAL MICROBIOME IN THE THIRD TRIMESTER OF PREGNACY AMONG WOMEN AT RAHIMA MOOSA MOTHER AND CHILD HOSPITAL

Dear Dr Emvula

Permission is granted for you to conduct the research as indicated in your request as per the title above.

The terms under which this permission is granted is contained in the Researcher Declaration form that you signed. Failure to comply with these conditions will result in the withdrawal of such permission.

Note that it is imperative that you notify the hospital of the actual start and end dates of your study by notifying the research coordinator for the hospital, Mrs Karen Marshall on Karen.Marshall@wits.ac.za

Should the study commence more than 12 months from receipt of this letter then the Researcher Declaration form needs to be re-signed prior to commencement of the research. Your are strongly advised to keep a signed copy of the declaration form so as to ensure that the terms of this agreement are complied with at all times.

Yours sincerely,

Executive Clinical Manager
Dr Frew Benson

ADDRESS: cnr. FUEL & OUDSTHOORN STREET CORONATIONVILLE 2093 / PRIVATE BAG X20 NEWCLARE 2112 JHB

Appendix D: Ethics clearance certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R14/49 Dr SEEI Emvula

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M190617

NAME: (Principal Investigator)	Dr SEEI Emvula
DEPARTMENT:	School of Clinical Medicine Department of Obstetrics and Gynaecology Rahima Moosa Mother and Child Hospital
PROJECT TITLE:	Vaginal microbiome in the third trimester of pregnancy amongst women at Rahima Moosa Mother and Child Hospital
DATE CONSIDERED:	2019/06/28
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr A Wise and Professor H Lombaard
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2019/08/26

This clearance certificate is valid for 5 years from the date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the 3rd Floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **June** and will therefore reports and re-certification will be due early in the month of **June** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

2019/09/05
Date

Appendix E: Journal Guidelines for South African Journal of Obstetrics and Gynaecology

Author Guidelines(SAJOG)

Preparation notes by article type

Manuscript preparation

Preparing an article for anonymous review

To ensure a fair and unbiased review process, all submissions are to include an anonymised version of the manuscript. The exceptions to this requirement are Correspondence, Book reviews and Obituary submissions.

Submitting a manuscript that needs additional blinding can slow down your review process, so please be sure to follow these simple guidelines as much as possible:

• An anonymous version should not contain any author, affiliation or particular institutional details that will enable identification.

• Please remove title page, acknowledgements, contact details, funding grants to a named person, and any running headers of author names.

• Mask self-citations by referring to your own work in third person.

General article format/layout

Submitted manuscripts that are not in the correct format specified in these guidelines will be returned to the author(s) for correction prior to being sent for review, which will delay publication.

General:

• Manuscripts must be written in UK English (this includes spelling).

• The manuscript must be in Microsoft Word or RTF document format. Text must be 1.5 line spaced, in 12-point Times New Roman font, and contain no unnecessary formatting (such as text in boxes). Pages and lines should be numbered consecutively.

• Please make your article concise, even if it is below the word limit.

• Qualifications, full affiliation (department, school/faculty, institution, city, country) and contact details of ALL authors must be provided in the manuscript and in the online submission process.

• Abbreviations should be spelt out when first used and thereafter used consistently, e.g. 'intravenous (IV)' or 'Department of Health (DoH)'.

• Numbers should be written as grouped per thousand-units, i.e. 4 000, 22 160.

• Quotes should be placed in single quotation marks: i.e. The respondent stated: '...'

• Round brackets (parentheses) should be used, as opposed to square brackets, which are reserved for denoting concentrations or insertions in direct quotes.

If you wish material to be in a box, simply indicate this in the text. You may use the table format –this is the only exception. Please DO NOT use fill, format lines and so on.

SAJOG is a general specialist obstetrics and gynaecology journal, therefore for articles involving genetics, it is the responsibility of authors to apply the following:

- Please ensure that all genes are in italics, and proteins/enzymes/hormones are not.
- Ensure that all genes are presented in the correct case e.g. TP53 not Tp53.

** NB: Copyeditors cannot be expected to pick up and correct errors wrt the above, although they will raise queries where concerned.

- Define all genes, proteins and related shorthand terms at first mention, e.g. '188del11' can be glossed as 'an 11 bp deletion at nucleotide 188.'
- Use the latest approved gene or protein symbol as appropriate:

o Human Gene Mapping Workshop (HGMW): genetic notations and symbols

o HUGO Gene Nomenclature Committee: approved gene symbols and nomenclature

o OMIM: Online Mendelian Inheritance in Man (MIM) nomenclature and instructions

o Bennet et al. Standardized human pedigree nomenclature: Update and assessment of the recommendations of the National Society of Genetic Counselors. J Genet Counsel 2008;17:424-433: standard human pedigree nomenclature.

Preparation notes by article type

Research

Guideline word limit: 3 000 words (excluding abstract and bibliography)

Research articles describe the background, methods, results and conclusions of an original research study. The article should contain the following sections: introduction, methods, results, discussion and conclusion, and should include a structured abstract (see below). The introduction should be concise – no more than three paragraphs – on the background to the research question, and must include references to other relevant published studies that clearly lay out the rationale for conducting the study. Some common reasons for conducting a study are: to fill a gap in the literature, a logical extension of previous work, or to answer an important question. If other papers related to the same study have been published previously, please make sure to refer to them specifically. Describe the study methods in as much detail as possible so that others would be able to replicate the study should they need

to. Where appropriate, sample size calculations should be included to demonstrate that the study is not underpowered. Results should describe the study sample as well as the findings from the study itself, but all interpretation of findings must be kept in the discussion section. The conclusion should briefly summarise the main message of the paper and provide recommendations for further study.

• May include up to 6 illustrations or tables.

• A max of 20 - 25references

Structured abstract

• This should be no more than 250words, with the following recommended headings:

o Background: why the study is being done and how it relates to other published work.

o Objectives: what the study intends to find out

o Methods: must include study design, number of participants, description of the research tools/instruments, any specific analyses that were done on the data.

o Results: first sentence must be brief population and sample description; outline the results according to the methods described. Primary outcomes must be described first, even if they are not the most significant findings of the study.

o Conclusion: must be supported by the data, include recommendations for further study/actions.

• Please ensure that the structured abstract is complete, accurate and clear and has been approved by all authors. It should be able to be intelligible to the reader without referral to the main body of the article.

• Do not include any references in the abstracts.

Illustrations/photos/scans

• If illustrations submitted have been published elsewhere, the author(s) should provide evidence of consent to republication obtained from the copyright holder.

• Figures must be numbered in Arabic numerals and referred to in the text e.g. '(Fig. 1)'.

• Each figure must have a caption/legend: Fig. 1. Description (any abbreviations in full).

• All images must be of high enough resolution/quality for print.

• All illustrations (graphs, diagrams, charts, etc.) must be in PDF form.

• Ensure all graph axes are labelled appropriately, with a heading/description and units (as necessary) indicated. Do not include decimal places if not necessary e.g. 0; 1.0; 2.0; 3.0; 4.0 etc.

• Each image must be attached individually as a 'supplementary file' upon submission (not solely embedded in the accompanying manuscript) and named Fig. 1, Fig. 2, etc.

Tables

• Tables should be constructed carefully and simply for intelligible data representation. Unnecessarily complicated tables are strongly discouraged.

• Embed/include each table in the manuscript Word file - do not provide separately as supplementary files.

• Number each table in Arabic numerals (Table 1, Table 2, etc.) consecutively as they are referred to in the text.

• Tables must be cell-based (i.e. not constructed with text boxes or tabs) and editable.

• Ensure each table has a concise title and column headings, and include units where necessary.

• Footnotes must be indicated with consecutive use of the following symbols: * † ‡ § ¶ || then ** †† ‡‡ etc.

Do not: Use [Enter] within a row to make ‘new rows’:

Rather:

Each row of data must have its own proper row:

Do not: use separate columns for n and %:

Rather:

Combine into one column, n (%):

Do not: have overlapping categories, e.g.:

Rather:

Use <> symbols or numbers that don’t overlap:

References

NB: Only complete, correctly formatted reference lists in Vancouver style will be accepted. If reference manager software is used, the reference list and citations in text are to be unformatted to plain text before submitting..

• Authors must verify references from original sources.

• Citations should be inserted in the text as superscript numbers between square brackets, e.g. These regulations are endorsed by the World Health Organization,[2] and others.[3,4-6]
• All references should be listed at the end of the article in numerical order of appearance in the Vancouver style (not alphabetical order).
• Approved abbreviations of journal titles must be used; see the List of Journals in Index Medicus.
• Names and initials of all authors should be given; if there are more than six authors, the first three names should be given followed by et al.
• Volume and issue numbers should be given.
• First and last page, in full, should be given e.g.: 1215-1217 not 1215-17.
• Wherever possible, references must be accompanied by a digital object identifier (DOI) link). Authors are encouraged to use the DOI lookup service offered by CrossRef:
o On the Crossref homepage, paste the article title into the 'Metadata search' box.
o Look for the correct, matching article in the list of results.
o Click Actions > Cite
o Alongside 'url =' copy the URL between { }.
o Provide as follows, e.g.: https://doi.org/10.7196/07294.937.98x

Some examples:

• Journal references: Price NC, Jacobs NN, Roberts DA, et al. Importance of asking about glaucoma. Stat Med 1998;289(1):350-355. http://dx.doi.org/10.1000/hgjr.182
• Book references: Jeffcoate N. Principles of Gynaecology. 4th ed. London: Butterworth, 1975:96-101.
• Chapter/section in a book: Weinstein L, Swartz MN. Pathogenic Properties of Invading Microorganisms. In: Sodeman WA, Sodeman WA, eds. Pathologic Physiology: Mechanisms of Disease. Philadelphia: WB Saunders, 1974:457-472.
• Internet references: World Health Organization. The World Health Report 2002 - Reducing Risks, Promoting Healthy Life. Geneva: WHO, 2002. http://www.who.int/whr/2002 (accessed 16 January 2010).
• Legal references
• Government Gazettes:

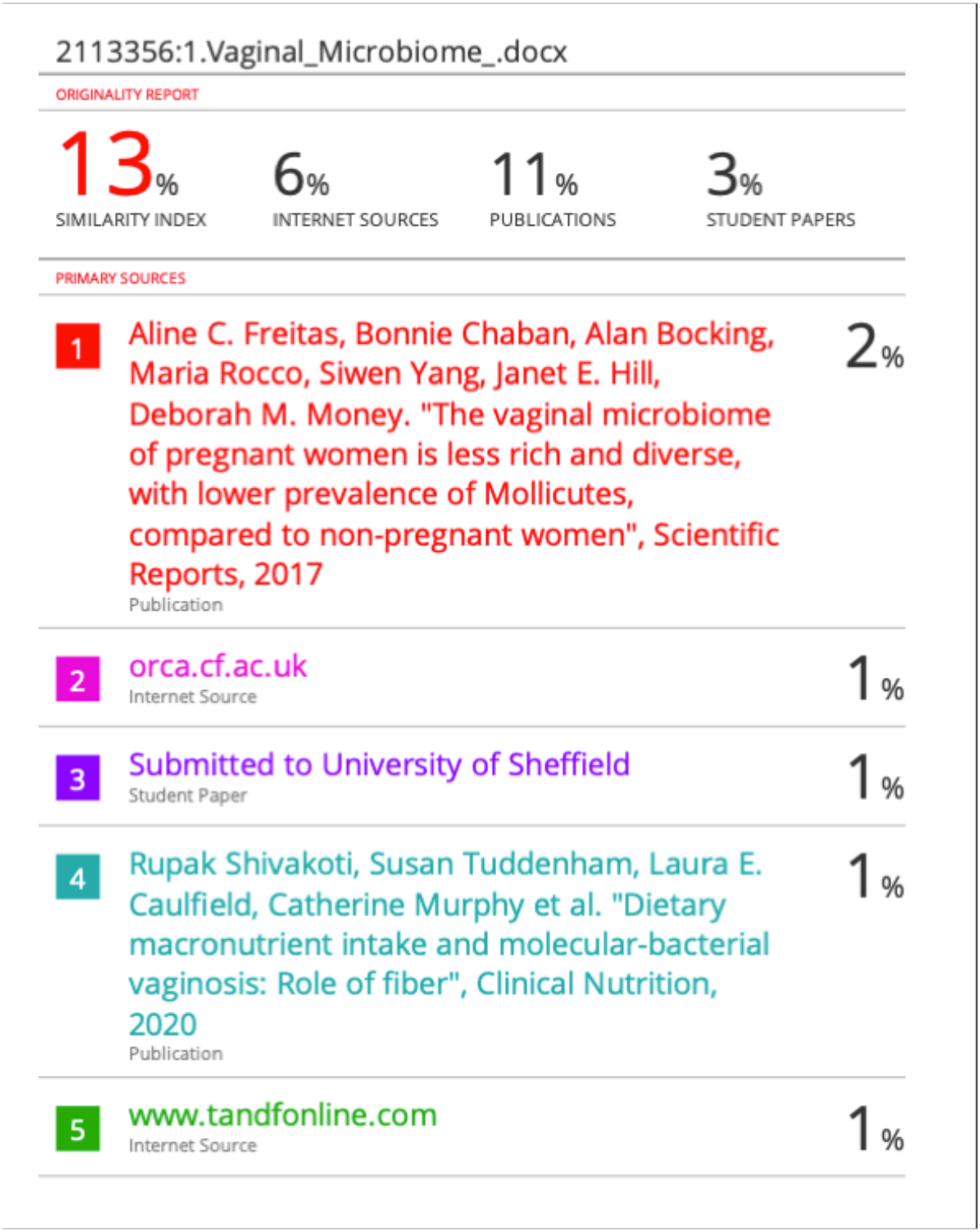
National Department of Health, South Africa. National Policy for Health Act, 1990 (Act No. 116 of 1990). Free primary health care services. Government Gazette No. 17507:1514. 1996. In this example, 17507 is the Gazette Number. This is followed by :1514 - this is the notice

number in this Gazette.

• Provincial Gazettes:

• Cited manuscripts that have been accepted but not yet published can be included as references followed by '(in press)'.

Appendix F: Turnitin plagiarism report



2113356:1.Vaginal_Microbiome_.docx

ORIGINALITY REPORT

13%	6%	11%	3%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Aline C. Freitas, Bonnie Chaban, Alan Bocking, Maria Rocco, Siwen Yang, Janet E. Hill, Deborah M. Money. "The vaginal microbiome of pregnant women is less rich and diverse, with lower prevalence of Mollicutes, compared to non-pregnant women", Scientific Reports, 2017 Publication	2%
2	orca.cf.ac.uk Internet Source	1%
3	Submitted to University of Sheffield Student Paper	1%
4	Rupak Shivakoti, Susan Tuddenham, Laura E. Caulfield, Catherine Murphy et al. "Dietary macronutrient intake and molecular-bacterial vaginosis: Role of fiber", Clinical Nutrition, 2020 Publication	1%
5	www.tandfonline.com Internet Source	1%

Appendix G: Plagiarism declaration

University of the Witwatersrand, Johannesburg

School of Clinical Medicine

SENATE PLAGIARISM POLICY

Declaration by Students

I Simon E E Emvula (Student number: 2113356) am a student registered for MMED (Obstetrics and Gynaecology)

in the year 2020. I hereby declare the following:

- **I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.**
- **I confirm that ALL the work submitted for assessment for the above course is my own unaided work except where I have explicitly indicated otherwise.**
- **I have followed the required conventions in referencing the thoughts and ideas of others.**
- **I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.**

Signature: _____ **Date:** 16/02/2021