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ANALYSIS OF VAGINAL MICROBIOME COMPOSITION OF IGBO WOMEN OF REPRODUCTIVE AGE WITH AND WITHOUT SYMPTOMS OF VAGINAL DYSBIOSIS

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ABSTRACT

The vagina of healthy asymptomatic reproductive aged woman is often dominated by species of *Lactobacillus* occupying over 90% of the bacteria composition. A shift from this (dysbiosis) may result to myriad of health complications. Vaginal dysbiosis is often diagnosed using clinical symptoms. Are all women without clinical manifestation of vaginal dysbiosis apparently healthy? Can the diagnosis of vaginal dysbiosis be based on clinical manifestations? This study is aimed at ascertaining the vaginal bacteria composition of women with and without clinical manifestations of vaginal dysbiosis using new generation sequencing (NGS) in order to ascertain the role of clinical symptoms in the diagnosis of vaginal dysbiosis. A total of 15 respondents were recruited for the study. Speculum assisted High vaginal swab (HVS) samples from posterior fornix of each respondent were collected, the DNA of the microorganisms from the HVS were extracted, purified, quantified and V3-V4 hyper variable region of 16S rRNA was amplified using paired end bar-coded universal primers which was thereafter sequenced using illumina miseq platform. Sequence reads were quality filtered and Chimeras were removed. Sequence reads with 97% similarity were assigned to the same operational taxonomic unit (OTU) using quantity insight into microbial ecology (QIIME). National centre for biotechnology information (NCBI) database was used to identify the OTUS. Total number of phylum obtained from women with symptoms were five with *Firmicutes* having the highest prevalence with relative abundance of 83.8%, Actinobacteria had 14.1%, Proteobacteria, 5.2%, Bacteriodetes 3.3% and Cyanobacteria recording 1.9%

while a total of six phyla were obtained from women without symptoms, with Firmicutes recording 61.2%, Fusobacteria 41.1%, Proteobacteria 27.0%, Bacteriodetes 15.5%, Actinobacteria 12.2% and Tenericus recording 2.0%. Hundred percent (100%) of symptomatic participants had high *lactobacillus species* abundance and low level of facultative anaerobes. Hundred percent (100%) of participants which were from symptomatic participants with clinical symptoms of infection had high *lactobacillus species* abundance while 36.4% (4/11) of asymptomatic participants had high level of *lactobacillus species* with 18.1% (2/11) having low lactobacillus species while 45.5% (5/11) had no *lactobacillus* species and high level of facultative anaerobes. Conclusively, clinical symptoms of vaginal dysbiosis do not depict the state of the diversity and abundance of vaginal microbiota of reproductive aged Igbo women hence cannot be used as an indicator of vaginal dysbiosis.

KEYWORDS: Dysbiosis, Microbiome, Microbiota, Symptoms, Vaginosis.

INTRODUCTION

1.1 Background

Vaginal microbiome of ‘’normal’’ reproductive age women is majorly composed of *lactobacillus species*. However, in dysbiosis, microbiome composition alters comprising of majorly facultative anaerobes with low *lactobacillus* species abundance (Ravel *et al*., 2001). Some women with vaginal dysbiosis are presented with symptoms like vaginal itching, thin, gray, white or green vaginal discharge, Foul-smelling "fishy" vaginal odour, burning during urination etc while some don’t. This condition has been discovered as one of the most common lower genital tract conditions, which occurs in 35% of women attending sexually transmitted infection (STI) clinics, 15% to 20% of pregnant women, and 5% to 15% of women attending gynecology clinics (Eschenbach, 1993). Clinical features which were first described by Gardner and Dukes (Gardner and Dukes, 1955), range from asymptomatic to an increased thin vaginal discharge with or without a fishy odour. So many pregnancy and/or gynecological complications are associated with bacterial vaginosis (Schwebke, 2003). Specifically, bacteria vaginosis (BV) has been associated with both sexually transmitted infections (STI) and non chlamydial, non gonococcal pelvic inflammatory diseases (Faro *et al*., 1993). Most importantly, vaginal dysbiosis may lead to bacteria vaginosis which in turn may be associated with an increased risk of acquisition of HIV (Martin *et al*., 1999), infections following termination of pregnancy, insertion of intrauterine devices, and hysterectomy, both vaginal and abdominal (Sewankambo *et al*., 1997). This syndrome has

been associated with serious pregnancy complications, including premature rupture of the membranes, preterm delivery and postpartum endometritis (Korn *et al*., 1995).

More so, vaginal cultures, which were previously used as primary laboratory tests were found to be of little value. Organisms which are associated with vaginal dysbiosis, including *Gardnerella vaginalis*, were recovered on laboratory media from 83% to 94% of women with clinical signs of BV, and were also recovered in 36% to 55% of asymptomatic women without clinical features (Hillier, 1993). However, cultural identification of other bacteria from vaginal specimens such as *Bacteroides* species, *Peptostreptococcus* species and *Mycoplasma hominis* has been evaluated, and found to be specific but insensitive and costly to the laboratory (Krohn, 1989). Other anaerobic bacteria strongly associated with BV, such as *Mobiluncus* species, are very difficult to recover by culture (Roberts, 1985), while normal vaginal *lactobacilli* are significantly reduced or absent. It is therefore important to base clinical diagnosis on methods that identify all bacteria in the vaginal specimen (Morgan *et al* 1996). Amsel's method of diagnosis of dysbiosis, which was based on the identification of at least 3 of his 4 criteria (a vaginal pH of greater than pH 4.5, the presence of clue cells in the vaginal fluid, a milky homogeneous vaginal discharge; and finally, the release of an amine (fishy) odour after addition of 10% potassium hydroxide to the vaginal fluid may not be appropriate in asymptomatic women (Morgan *et al.*, 1996).

Considering the various conditions associated with this common disorder, vaginal dysbiosis, and the inability of cultural technique in the isolation and identification of all possible bacteria associated with bacteria vaginosis, it is therefore necessary to use a more appropriate technique (metagenomics) using new generation sequencing, to analyse the vaginal bacteria composition of women with or without clinical evidence of vaginal dysbiosis in order to know the state of their vaginal microbiota so as to decipher if the use of clinical symptoms of vaginal dysbiosis alone in the treatment and management of bacteria vaginosis is diagnostic.

As stated earlier, some women with vaginal dysbiosis are presented with symptoms like vaginal itching, thin, gray, white or green vaginal discharge, Foul-smelling "fishy" vaginal odour, burning during urination etc while some don't. Do these clinical symptoms reflect the true state of the vagina? Can clinical symptoms alone be used in the treatment and management of vaginal dysbiosis? What is the vaginal microbiota composition of women with and without symptoms of vaginal dysbiosis? All these questions will be answered in this study. This research is therefore aimed at ascertaining the vaginal microbiome composition

and abundance of women with or without clinical evidence of vaginal dysbiosis with the view of understanding the role of clinical symptoms in the diagnosis, treatment and management of vaginal dysbiosis.

2.0 MATERIALS AND METHODS

2.1 Study Design

A cross-sectional study was used for this research where reproductive aged women with or without clinical symptoms of infection attending Obstetrics and Gynaecology (O and G) clinic were recruited using self reported questionnaire. Simple random sampling technique was used for the recruitment of subjects.

2.2 Ethical Approval

A study approval was sought and obtained from Ethics and Research Review committees Federal Medical Centre (FMC) Owerri Imo State with reference number FMC/OW/HREC/226 and Federal Teaching Hospital Abakaliki, (FETHA1), Ebonyi State with reference number 19/06/2018-27/07/2018.

2.3 Study Area

Samples were collected from Owerri in Imo State, Abakaliki in Ebonyi state and Nnewi in Anambra state.

2.4 Inclusion Criteria: Women with and without clinical symptoms of vaginal dysbiosis.

2.5 Exclusion Criteria: Women below age 20 (<20 years), women with known history of organ transplant or HIV infection, pregnant women, and women who are currently applying any form of steroid hormone. Also women who are not sexually active were excluded.

2.6 Study Population

A total number of fifteen High Vaginal Swab (HVS) samples were collected from 15 participants. The participants were patients attending routine medical check in Obstetrics and Gynaecology (O and G) clinic, Federal Medical Centre, Owerri, Imo state and Federal Teaching Hospital Abakaliki (FETHA 1), Ebonyi State. A total number of eight HVS samples were collected from eight participants in Owerri, Imo State, while five HVS samples were collected from five participants in Abakaliki, Ebonyi State and 2 samples collected from Nnewi, Anambra state. Women within the age range of twenty and forty five (20-45years)

were enrolled. They were classified as premenopausal women according to Stages of Reproductive Aging Workshop (STRAW,) criteria (Soules *et al.*, 2011).

2.7 Sample Collection

The vaginal samples were collected using Norgen Microbiome collection and preservation kit (Cat no 45690). The collected samples were quickly inserted into the kit, covered and stored at room temperature until it was processed.

Metagenomic sequencing. The deoxyribonucleic acid from each of the HVS samples was isolated, extracted and purified using Norgen microbiome isolation kit (Cat No. 64100) according to manufacturers' instructions. A Nano-Spectrophotometer was used to check the concentration and purity of DNA. Polymerase Chain Reaction (PCR) was carried out to amplify V3-V4 hyper variable regions of 16S rRNA using paired end universal primer 341F and 785R. (Herlemann *et al.*, 2011, Anna *et al.*, 2013. Samples were barcoded with a unique combination of forward and reverse indexes allowing for simultaneous processing of multiple samples. PCR products were pooled, column-purified, and size-selected through microfluidic DNA fractionation. Consolidated libraries were quantified by quantitative real-time PCR using the Kapa Bio-Radi Cycler qPCR kit on a Bio-Rad MyiQ before loading into the MiSeq sequencer where sequencing was performed. (Vasque *et al*., 2002.

16S rRNA metagenomics sequence analysis: Raw sequence reads were de-multiplexed using Illumina's BCL2FASTQ algorithm. Reads were filtered using an average Q-score > 30. The paired-end sequence FASTQ reads were imported into Illumina Basepace pipeline for quality check (QC) In addition; Ez Biocloud pipeline was employed for alpha and beta diversity estimation using PKSSU4.0 version database and Open reference UCLUST_MC2 for OTUs picking at 97% cut-off. Sequences were pre-screened using QIIME-UCLUST algorithms for at least 97% identity to ribosomal sequences from the RNA databases. (Jespers *et al*., 2017). Rarefaction to 1000 reads per sample was employed to calculate microbial diversity. Alpha-diversity was calculated for species richness by Chao1 method, while diversity indexes were calculated by Non-parametric, Shannon and Simpson indexes. Principle coordinates analysis (PCoA) with Jensen-Shannon divergence distance metrices were used to evaluate beta diversity between the different strata (Machado *et al*., 2016).

3.0 RESULTS AND DISCUSSION

3.1 Results

Demographic Characteristics of Respondents

The Socio- demographic characteristics of respondents in this study is as described in table 1 below. As observed, a total of fifteen respondents within age range 20-45years were recruited. Women from different states of origin, Imo (53.3%) Ebonyi,(33.3%), Anambra, (13.3%) were recruited. Both married and unmarried women with various occupations were recruited. Majority of whom are health workers. Women with different educational background were also recruited. Table 1 shows details of the demographic characteristics.

Table 1: Socio- demographic characteristics of respondents.

Demographic Characteristics	Prevalence (%)
Age	
20-29	4 (26.7)
30-39	7 (46.6)
40-49	4 (26.7)
Total	**15**
State of Origin	
Imo	8 (53.3)
Ebonyi	5 (33.4)
Anambra	2 (13.3)
Total	**15**
Educational Qualification	
Primary	0 (0)
Secondary	9 (60)
Tertiary	6 (40)
Occupation	
Trading	6 (40)
Teaching	7 (46.7)
Public servant	2 (13.3)
Clinical Presentations	
Symptomatic	4 (26.7)
Asymptomatic	11(73.3)
Douching	
Yes	5 (33.3)
No	10 (66.7)
Nature of menstrual period	
Regular	12 (80)
Irregular	3 (20)
Total	**15 (100)**

Bacteria Composition of Samples

Total number of phylum obtained from women with symptoms were five with *Firmicutes* having the highest prevalence with relative abundance of 83.8%, *Actinobacteria* had 14.1%, *Proteobacteria*, 5.2%, *Bacteriodetes* 3.3% and *Cyanobacteria* recording 1.9% while a total of six phyla were obtained from women without symptoms, with *Firmicutes* recording 61.2%, *Fusobacteria* 41.1%, *Proteobacteria*, 27.0%, *Bacteriodetes* 15.5%, *Actinobacteria* 12.2% and *Tenericus* recording 2.0%. Details of this is as recorded in figure 1 and 2.

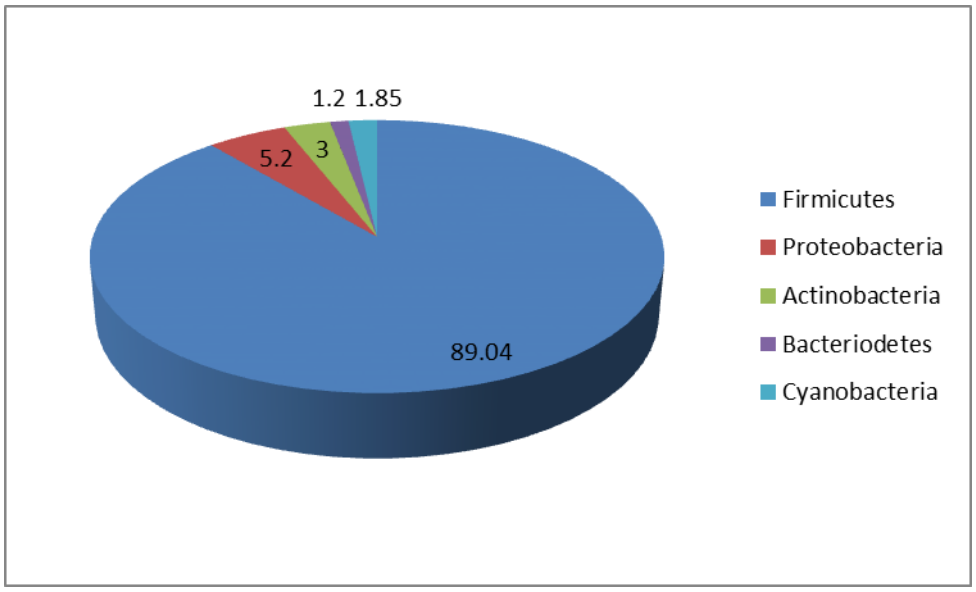

Fig. 1: The vaginal bacteria composition of Igbo women with clinical evidence of vaginal dysbiosis at Phylum level.

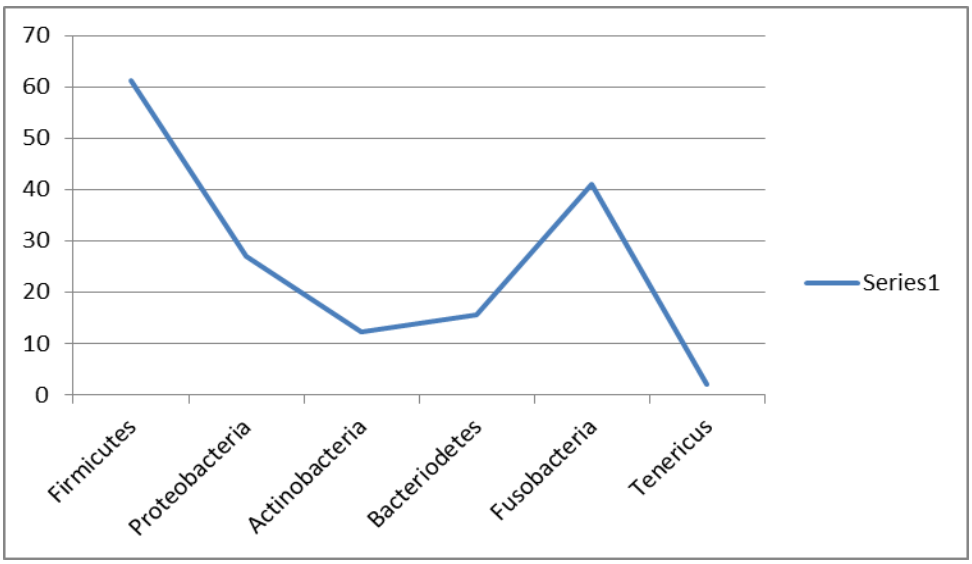

Fig. 2: The vaginal bacteria composition of Igbo women without clinical evidence of vaginal dysbiosis at Phylum level.

At genus level, a total of eight genera were obtained from symptomatic women with *Lactobacillus* having relative abundance of 66%, *Enterococcus*, 40.9% , *Gardnerella*,36.5%, *Prevotella*, 8.42%, *Dialister*, 2.2%, *Strptophyta*, 1.85%, *Corynebacteria*, 1.63% and *Bifidobacteria*, 1.4%, while a total of thirty seven genera were obtained from participants who were asymptomatic, with *Lactobacillus* having relative abundance of 61.2%. Others included *Gardnerella*,14.7%, *Prevotella*, 12.90%, *Sneathia*, 22.9%, *Porphyromonas*, 16.20%, *Corynebacteria*, 2.3%, *Peptostreptococcus*,10.5%, *Bifidibacteria*, 7.1%, *Streptococcus*, 29.32%, *Megasphaera*, 4.09%, *Peptoniphilus*, 2.12%, *Mobilincus*, 1.8%, *Campylobacter*, 1.7%, *Finegoldia*, 1.1%, *Enterococcus*, 68.1%, *Anaerococcus* 6.6%, *Escherichia*,8.0%, Veillonella, 19.3%, Bacillus, 6.9%, Pseudomonas, 6.7%, Staphylococcus, 5.7%, *Paenibacillus*, 4.5%, *Paracoccus*, 3.5%, *Arthrobacter*, 3.4%, *Acinetobacter*, 2.0%, *Rhodococcus*, 2.0%, *Klebsiella*, 1.3%, *Rhizobim*, 1.24%, *Pseudoxanthomonas*,1.2%, *Brevundimonas* 1.2%, *Aerococcus*, 8.2%, *Sacharofermentans*, 3.7%, *Clostridium Sensu_ Stricti*, 15.4%, *Ureaplasma*,1.9%, and *Howardella*,5.3%.

Hundred percent (100%) of participants which were from symptomatic participants with clinical symptoms of infection had high *lactobacillus species* abundance while 36.4% (4/11) of asymptomatic participants had high level of *lactobacillus species* with 18.1% (2/11) having low lactobacillus species while 45.5% (5/11) had no *lactobacillus* species. Asymptomatic participants had very high bacteria diversity while symptomatic participants had low bacteria diversity. Table2 and 3 has details of this result.

Table 2.0: Bacteria Diversity and Relative Abundance of Asymptomatic Igbo Women at genus level.

Genera	Sample 1	Sample 2	Sample 3	Sample 4	Relative Abundance (%)
Lactobacillus	87.6	56.1	67.6	52.5	66.0
Prevotella	-	-	6.87	1.55	8.4
Enterococcus	-	40.9	-	-	40.9%
Gardnerella	-	-	-	36.5	36.5
Corynebacteria	1.63	-	-	2.2	1.63
Dialister	-	2.2	-	-	-
Bifidobacteria	-	-	-	1.40	1.40
Streptophyta	-	-	-	1.9	1.9

Table 3.0: Bacteria Diversity and Relative Abundance of Asymptomatic Igbo Women at Genus Level.

Genera	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	Relative abundance
Lactobacillus	-	-	23.9	-	-	46.6	92.2	-	62.3	72.5	69.5	61.2
Prevotella	24.6	3.1	1.17	24.8	5.69	-	-	27.9	-	11.2	4.76	12.90
Enterococcus	-	68.1	-	-	-	-	-	-	-	-	-	68.1
Gardnerella	-	5.12	-	12.3	19.9	11.1	-	5.89	33.6	-	-	14.7
Corynebacteria	1.40	1.99	2.77	-	-	-	-	-	1.84	-	-	2.30
Dialister	6.07	-	-	-	3.17	-	-	3.00	-	-	-	4.10
Bifidobacteria	7.10	-	-	-	-	-	-	-	-	-	-	7.10
Porphoromonas	26.0	-	-	-	-	6.39	-	-	-	-	-	16.20
Peptostreptococcus	8.38	5.95	-	-	17.3	-	-	-	-	-	-	10.5
Streptococcus	6.77	-	-	-	45.1	-	-	-	-	-	-	29.32
Megasphaera	3.88	-	-	2.61	-	-	-	8.49	-	1.37	-	4.09
Peptoniphilus	3.16	-	-	-	-	-	-	-	-	1.08	-	2.12
Mobilincus	1.79	-	-	-	-	-	-	-	-	-	-	1.8
Campylobacter	1.72	-	-	-	-	-	-	-	-	-	-	1.7
Finegoldia	1.11	-	-	-	-	-	-	-	-	-	-	1.1
Anaerococcus	-	6.60	-	-	-	-	-	-	-	-	-	6.6
Escherichia	-	2.48	3.90	-	-	17.7	-	-	-	-	-	8.0
Veillonella	-	1.68	-	15.8	-	-	-	1.79	-	-	-	19.3
Bacillus	-	6.9	-	-	-	-	-	-	-	-	-	6.9
Pseudomonas	-	6.7	-	-	-	-	-	-	-	-	-	6.7
Staphylococcus	-	-	6.03	-	-	-	2.28	-	-	8.63	-	5.7
Paenibacillus	-	-	4.45	-	-	-	-	-	-	-	-	4.5
Paracoccus	-	-	3.48	-	-	-	-	-	-	-	-	3.5
Arthrobacter	-	-	3.38	-	-	-	-	-	-	-	-	3.4
Acinetobacter	-	-	1.99	-	-	-	-	-	-	-	-	2.0
Rhoococcus	-	-	1.97	-	-	-	-	-	-	-	-	2.0
Klebsiella	-	-	1.30	-	-	-	-	-	-	-	-	1.3
Rhizobium	-	-	1.24	-	-	-	-	-	-	-	-	1.24
Pseudoxanthomonas	-	-	1.23	-	-	-	-	-	-	-	-	1.2
Brevundimonas	-	-	1.21	-	-	-	-	-	-	-	-	1.2
Aerococcus	-	-	1.16	-	-	-	-	-	-	-	-	1.2
Sneathia	-	-	-	39.9	-	-	-	-	5.91	-	-	22.9
Saccharofermentans	-	-	-	1.53	-	-	-	-	5.91	-	-	3.7
Clostridium Sensu_Stricti	-	-	-	-	-	18.7	-	-	-	-	12.0	15.4
Ureaplasma	-	-	-	-	-	-	2.84	-	1.01	-	-	1.9
Howardella	-	-	-	-	-	-	-	-	-	-	5.28	5.3

Key: S= sample

3.2 DISCUSSION

Symptoms are clinical manifestations which represent some level of systemic anomally. Usually, clinical symptoms are utilized by clinicians and gynaecologist as an aid in knowing the physiological state of the internal organs of an individual. In some emergency cases,

clinical signs may be utilized in the quick diagnosis and management of infections including bacteria vaginosis (Amsel, 1983). However, some women are asymptomatic hence; there may be tendency for misdiagnosis. In this study, the researcher analyzed the vaginal composition of symptomatic women with clinical symptoms of vaginal dysbiosis and those from asymptomatic women without clinical symptoms to identify the role of symptom presentation in the diagnosis of vaginal dysbiosis.

The presence of these phyla, *Firmicutes, Actinobacteria, Proteobacteria* and *Bacteroidetes* at different levels in all participants (both symptomatic and asymptomatic) could mean that these phyla are normal vaginal flora when present at certain level. Only few participants had *Fusobacteria, Tenericutes* and *Cyanobacteria* which may suggest that the presence of these phyla may be opportunistic. This finding is in agreement with Ravel *et a*l., 2011 and Zhou *et al*., 2010, who independently, discovered that some asymptomatic reproductive age women have their vaginal microbiome composed of *Firmicutes, Proteobacteria*, and *Actinobacteria* among others.

High level of *Lactobacillus* in the vagina has been known to serve as host defence against invading pathogenic organisms in the vagina of healthy women through so many mechanisms including the production of lactic acids, hydrogen peroxides and bacteriocin which renders the vaginal micro environment unconducive for the invasion of more pathogenic facultative anaerobic microorganisms.

Lactobacillus species were found to be more abundant in symptomatic participants, where all participants (100%), had high level of *Lactobacillus species* than in asymptomatic participants where 36.4% had high *Lactobacillus* level, 18.1% had very low level with 45.5% having no *Lactobacillus species*. The low bacteria diversity and high lactobacillus species abundance among symptomatic participants may shows that symptoms are not specific and can occur irrespective of the diversity of vaginal microbiota. The low prevalence of *Lactobacillus species* in asymptomatic women and its high level in symptomatic women may suggest that clinical symptoms do not reflect the true state of vaginal microbiota and may be deficient in revealing appropriate information concerning the level of vaginal *lactobacillu*s composition, hence may not be a good indicator of vaginal dysbiosis. Again, lack of *Lactobacillus* species in some asymptomatic women may indicate that individuals can be having problems without clinical manifestation hence need vaginal microbiome analysis for proper diagnosis in personalized medicine. Also, the presence of *Bifidobacteria* and

streptococcus which are known probiotics in asymptomatic women without *lactobacillus* species may indicate that these bacteria may play some role in suppression of vaginal dysbiosis symptoms when used as probiotics.

This study revealed so many bacteria which are associated with bacteria vaginosis especially, among the asymptomatic participants. Such bacteria include *Sneathia, Mobilincus, Mycoplasma, Gardnerella* among others. These high bacteria diversity among asymptomatic women also may suggest that clinical symptoms do not reflect the true state of health of individuals. Also this study revealed that the participants with high *Lactobacillus* level had low bacteria diversity while reverse is the case for participants with low *Lactobacillus* level. This finding could mean that the presence of *Lactobacillus species* in the vagina confers some level of protection against microbial invasion. This finding is in agreement with findings from numerous researchers on the role of vaginal *Lactobacillus* in reproductive aged women.

It has also been known that normal healthy asymptomatic women have their vaginal microbiome occupied majorly by *Lactobacillus* species (Ravel *et al*., 2011). From this research, 33.3% of participants recorded no *Lactobacillus species*. 100% of these women were asymptomatic. This result could mean that the presences of vaginal *Lactobacillus* species alone may not be responsible for presence or absence of clinical symptoms of vaginal dysbiosis. Other factors like the immunity of the individual may play vital role. It could also mean that the driving force for clinical presentations (presence of symptoms) in a woman is not based on the presence or absence of vaginal *Lactobacillus* but the immunological state of the T-cells. Absence of symptoms therefore does not rule out bacteria vaginosis in a reproductive aged woman as asymptomatic women had bacteria associated with bacteria vaginosis including *Mobilincus, Gardnerella, Sreptopeptococcus* etc. It is therefore important to note that diagnosis of bacteria dysbiosis based on clinical presentations alone may be misleading.

4.0 CONCLUSION AND RECOMMENDATION

From the results obtained in this research, clinical symptoms do not reflect the diversity and abundance of vaginal microbiota of reproductive aged Igbo women hence cannot be used as an indicator of vaginal dysbiosis. Every woman of reproductive age attending gynaecology and antenatal clinics should therefore be tested for bacteria vaginosis irrespective of the absence of clinical symptoms.

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