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Research Paper

Metagenomic Analysis of Vaginal Microbiome of Post Menopausal Women Living In South Easthern, Nigeria

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ABSTRACT
Results obtained from several authors regarding the association of serum oestrogen with vaginal lactobacillus abundance and bacteria diversity have remained conflicting. Several researches have also attributed vulvo vaginal atrophy (VVA) observed in post menopausal women, the low level of vaginal lactobacillus species, increase in bacteria diversity, increase in vaginal pH to low oestrogen level while some researchers have disputed this. This research is therefore aimed at analysing the vaginal microbiome of post menopausal women living in south eastern, Nigeria and comparing the results with their serum oestrogen level to ascertain their relationship. A total of eight high vaginal swab samples collected from the vaginal fornix and eight serum samples were collected from post menopausal women with and without vulvo vaginal atrophy. The samples' DNA was 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. Chimeras were removed and sequence reads with 97% similarity were assigned to the same operational taxonomic unit (OTU) using CHIME and Quantity Insight into Microbial Ecology (QIIME) respectively. National centre for biotechnology information (NCBI) database was used to identify the OTUS. Shannon diversity index and CHAO1 were used to estimate the alpha and beta diversity indices of the OTUs. The enzyme linked immunosorbent assay (ELISA) technique was used to analyze the serum samples for hormonal assay. The results obtained showed that postmenopausal women generally have low lactobacillus species and high bacteria diversity. Also this results show that serum oestrogen level does not have a direct relationship with vaginal microbiota abundance and diversity. Conclusively, at menopause, vaginal lactobacillus level declines with increase in bacteria diversity. This reduction may not have a direct link with serum oestrogen level.

Key Words- , Diversity, Microbiome, menopause, Oestrogen, postmenopausal.

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I. INTRODUCTION

As women approach menopause, oestrogen reduction can lead to vulvo vaginal atrophy (VVA) (Mac Bride *et al*., 2010). Several studies have estimated that 25–50% of postmenopausal women experience VVA which can include vulvo vaginal symptoms of burning on urination, bleeding after intercourse, painful sexual intercourse, and vaginal discharge, soreness, itching or burning sensation (Tan *et al*.,2012) Some vulvo vaginal symptoms can be alleviated by use of vaginal lubricants (Sturdee and Panay, 2010). However, emerging data suggest that lubricants may adversely affect the vaginal epithelium, lamina propria and the vaginal microbiota (Brotman *et al*., 2010; Wolf, 2012). New interventions are needed to reduce vulvo vaginal symptoms caused by VVA in postmenopausal women (Hansen and Eyster, 2012).

One approach to VVA that has not been well explored is harnessing the protective features of the vaginal microbiome (Hummelen *et al*., 2011). The vaginal microbiota play an important role in preventing colonization by pathogenic organisms, including sexually transmitted and urinary tract infectious agents, and broadly act to maintain a woman's gynaecological and reproductive health (Brotman *et al*., 2010; Cohen *et al*., 2012,). The predominant connection between the vaginal microbiome and menopause is through the influential

action of oestrogen. Oestrogen contributes to vaginal epithelium maturation through deposition of glycogen in the vaginal epithelium (Sturdee and Panay, 2010).

Vulvo vaginal Atrophy,(VVA) a symptom associated with menopause, including vaginal dryness and discomfort occur in 45–77% of women and cause significant distress *(*Minkin *et al*., 2015). Few studies have evaluated risk factors for vaginal symptoms in menopausal women. In a study of 32 postmenopausal women, those with greater evidence of genitourinary atrophy on examination had lower abundance of *Lactobacillus species*, and a more diverse community of vaginal microbes (Hummelen *et al*., 2011). However, overall severity of patient-reported symptoms was significantly lower than severity of observed atrophy, and did not correlate well with exam findings.

Several researches have been carried out on vaginal microbiome of pre and postmenopausal women with different findings (Mitchell *et al*., 2017). These findings show that the communities of women who receive hormonal therapy (HT) often resemble those of premenopausal women by essentially restoring high proportions of *Lactobacillus,* but the means by which this occurs is not understood (Mitchell *et al*., 2017).

Studies using both culture and molecular methods have revealed that postmenopausal women are less likely than premenopausal women to have vaginal colonization with *Lactobacillus* bacterial species (Brotman *et al*., 2014). This has been attributed to decreased serum oestrogen, which reduces glycogen content in vaginal epithelial cells, and limits the energy source for *lactobacilli.* While all postmenopausal women experience a drop in serum estradiol, not all lose vaginal *lactobacill*i (Brotman *et al*., 2014). Recent data have shown that free glycogen in vaginal fluid, which is liberated from epithelial cells by enzymes like α-amylase, is associated with *Lactobacillus* colonization in both pre- and postmenopausal women, suggesting glycogen may be a mediating factor for *Lactobacillus* presence *(*Mirmonsef *et al*., 2014).

The vaginal microbiota is not dominated by *Lactobacillus* throughout a woman's lifetime. Indeed, in childhood, anaerobes and *Escherichia coli* predominate *(Brotman et al 2013).* After puberty, the oestrogen rise leads to the production and accumulation of glycogen, which is essential for *Lactobacillus* growth and the colonization of the vaginal epithelium; the dominance of *Lactobacillus* is maintained during the reproductive years. Finally, after menopause, the proportion of *Lactobacillus* species decreases again due to the drop in endogenous oestrogen. Interestingly, the *Lactobacillus* content, as well as a low vaginal pH, is maintained in women receiving hormonal replacement therapy during menopause *(Brotman et al., 2013).*

Among the endogenous factors known to contribute to microbiome changes are hormonal changes during the menstrual cycle. These changes are associated with shifts in vaginal bacterial content, with menses representing the phase in which the microbiome is more diverse, while the oestradiol and progesterone peaks are more bacterially stable times *(Gajer et al.,* 2012). Adequate oestrogen levels are important for a multitude of functions outside of its reproductive role. Specific to the microbiome, oestrogen and oestrogen-like compounds prevent the loss of and promote growth and proliferation of beneficial bacteria (Hummelen *et al*., 2011).

The predominant connection between the vaginal microbiome and menopause is through the influential action of oestrogen. Oestrogen contributes to vaginal epithelium maturation through deposition of glycogen in the vaginal epithelium (Sturdee and Panay, 2010)

Rebecca *et al.* found significant associations between vaginal bacterial composition with both menopause stage and signs of vaginal atrophy. They identified a novel community assemblage (CST IV-A), which was highly associated with signs of VVA and was predominantly found among postmenopausal women. CST IV-A is a low *Lactobacillus* state and is typified by a higher abundance of genera including *Anaerococcus, Peptoniphilus and Prevotella,* which could be playing a putative role in the clinical presentation of VVA. (Rebecca *et al*., 2014)

Rebecca *et al.,* utilized 16S rRNA gene sequencing to characterize the vaginal microbiota of 87 postmenopausal women and found an inverse correlation between *Lactobacillus* and dryness as well as increased bacterial diversity in women experiencing moderate to severe vaginal dryness. Among participants not reporting symptoms, *L. iners* and *L. crispatus* were generally the most abundant (Rebecca *et al.,* 2014). Giang *et al*., 2016 found that *lactobacilli* were prominent members of vaginal communities of most healthy post-menopausal women. They constituted more than half of communities in 55.2% of the women enrolled in the study and more than 0.1% of all bacteria in 83% of the subjects. Other bacteria such as *Gardnerella*, *Lactobacillales*, *Prevotella* and *Atopobium* were also common and found in 82.8%, 79.3%, 75.9% and 65.5% of communities, respectively, but on average occurred in lower proportions (16.7%, 0.7%, 7.4% and 2.7%, respectively)(Giang *et al.,*2016). Considering the conflicting results obtained by these researchers, it is therefore necessary to analyze the vaginal microbiome of Igbo women vis a vis their serum oestrogen level to ascertain their relationship

II. MATERIALS AND METHODS

2.1 Study Design. This was a cross-sectional study involving women of different age groups attending Obstetrics and Gynaecology clinics with complaints of recurrent spontaneous abortions with or without clinical symptoms of infection. Simple random sampling technique was used for the recruitment of subjects.

Ethical 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.

2.2 Inclusion Criteria: Post menopausal women with or without symptoms of vulvo vaginal atrophy, were enrolled

2.3 Exclusion Criteria: Premenopausal women, women with known history of organ transplant or HIV infection and women who were currently applying any form of steroid hormone.

2.4 Sample Collection

A total number of eight High Vaginal Swab (HVS) samples were collected from eight participants, four participants from Owerri and four from Abakaliki, Ebonyi state. Post menopausal women within the age range of fifty six and eighty years were recruited. High vaginal swab samples collected from their vaginal fornix using Norgen Microbiome collection and preservation kit (Cat no 45690) and serum samples were preserved for analysis.

2.5 DNA Extraction and Metagenomic Sequencing:

The DNA from each of the HVS samples was isolated, extracted and purified using Norgen microbiome isolation kit (Cat No. 64100) according to manufacturers' instructions. The purity and quantity of the DNA was checked using a Nano-Spectrophotometer (Model ND 2000, Thermo Scientific)**.** Polymerase Chain Reaction (PCR) was carried out on the DNA extracts to amplify V3-V4 hyper variable regions of 16S rRNA using paired end universal primer 341F, 5'-CCTACGGGNGGCWGCAG-3'(Herlemann et al., 2011) and 785R, 5'-GACTACHVGGGTATCTAATCC-3'(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. Sequencing was carried out in a pair-end modality on the Illumina NextSeq 500 platform.

16S rRNA metagenomics sequence analysis: Raw sequence reads were demultiplexed using Illumina's BCL2FASTQ algorithm. The paired-end sequence FASTQ reads were imported into Illumina Base space pipeline for quality check. Reads with an average Q-score greater than 30 (Q score >30) were filtered. Furthermore, Sequenced data were processed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline (http://qiime.sourceforge.net/) (Caporaso *et al., 2010*). The sequences with the same barcode were assigned to the same sample, and then the barcode and primer sequences were removed. The chimeric sequences were removed from aligned sequences using the UCHIME algorithm (Edgar *et al.*, 2011). The valid reads obtained from Illumina MiSeq sequences were normalized to 1000 for comparison of community diversity. The reads were then clustered into operational taxonomic units (OTUs, 97% similarity) (Edgar *et al.*, 2010). The Green genes data base and Ribosomal Database Project (RDP) Classifier were used to assign the effective sequence tags into different phylogenetic bacterial taxa (Ya Qin *et al*., 2016). The diversity indices, Alpha-diversity was calculated for species richness by ACE, and Chao1 method, (Chao & Bunge, 2002) while Beta diversity indices were calculated by Shannon, Non-parametric indices to indicate the community diversity. Statistical analysis was performed using the SPSS 21.0 software package. (SPSS Inc., Chicago, IL,USA). The tests of significance were performed using a two-sided Student's two-sample t-test. The nonparametric p-values were calculated using Monte Carlo permutations. The serum fertility hormone levels were assayed using a research kit, Melsin Medical Co. LTD, ELISA Research kit, Cat No EKHU-1704,

III. RESULTS AND DISCUSSION

The 0perational Taxonomic Unit (OTU) observed in participants with Vulvo vaginal atrophy and those without vulvo vaginal atrophy (VVA) is as represented in table 1. *Lactobacillus* species abundance is generally low among this age group, although, participants without vulvo vaginal atrophy have higher percentage of *Lactobacillus species*, higher Shannon diversity index, evenness, and effective number of species (ENS) than those with VVA. Details of this, is as shown in table 1.

Table1. Diversity, evenness and ENS of postmenopausal women with and without VVA in Eastern Nigeria.

OTUs	Diversity Indices	
	Women withVVA (C1)	Women without VVA (C2)
Enterococcus	0.02692	0
L.iners	0	0.12067

Lactobacillus	0.02382	0.09147
Prevotella	0.30359	0.22581
Peptoniphilus	0.02593	0.02594
Veillonella dispar	0	0.01305
Anaerococcus	0.01099	0.04333
Corynebacterium	0.31332	0.21758
Bifidobacterium	0.04214	0
Gardnerella	0.16674	0.06461
Dialister	0.04891	0.02671
Streptococcus	0	0.14567
Megasphaera	0	0
Staphylococcus aureus	0	0.01662
Sneathia	0.01099	0
Coriobacteriaceae	0	0
Mobilincus	0.02664	0.00854
Total no. Of species /strata	11	12
Shannon Diversity index	1.78202	2.09234
Evennes (E_H)	0.74316	0.84202
ENS	5.9	8.1

Charts showing the different OTUs obtained from women with Vulvovaginal atrophy and those without atrophy. Samples FN-P 15, 16, 17 and 18 are from women with VVA while samples FN-P19,20,21 and 22 are from women without VVA. Generally, there is high bacterial diversity of bacteria among postmenopausal age group, effective number of species being 5.9 and 8.1 for postmenopausal with VVA and postmenopausal without VVA respectively. Fig 1a,b and c show the relationship between the OTUs of these two categories of women.

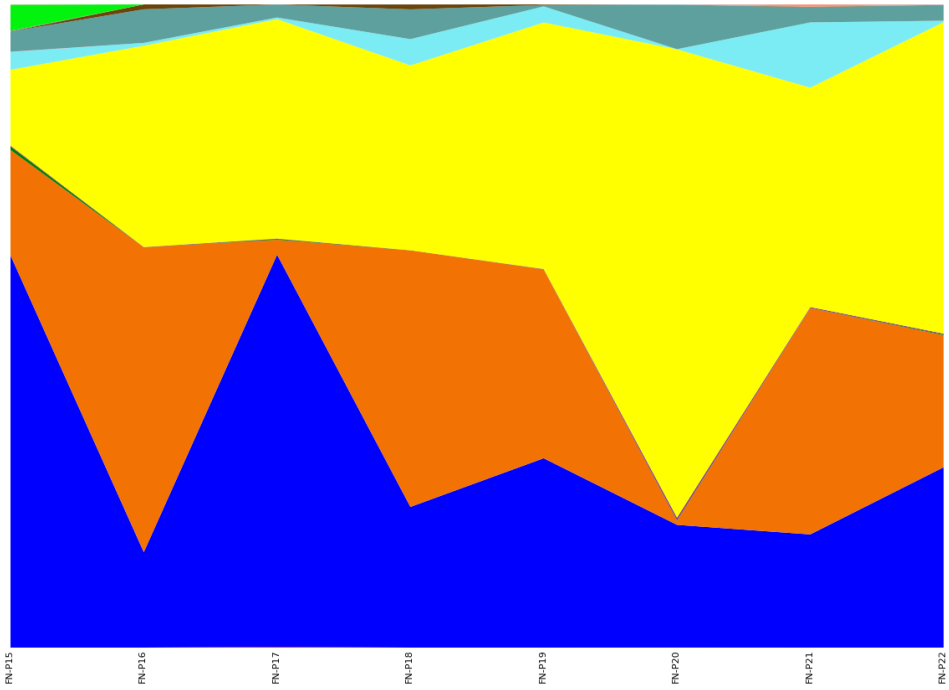


Fig1a: Chart showing the different OTUs obtained from individual women with Vulvovaginal atrophy and those without atrophy

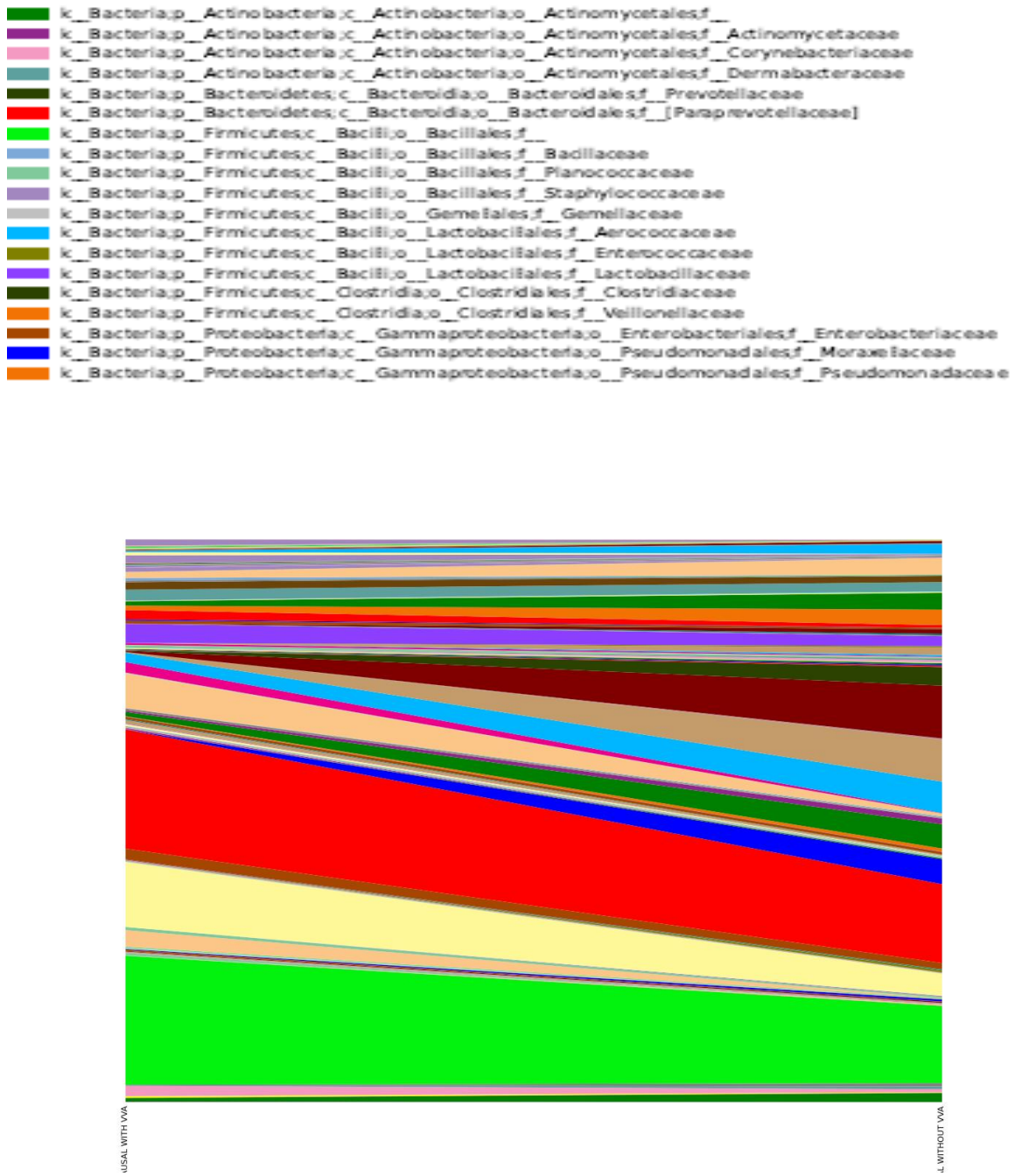


Fig 1b: Charts showing the different OTUs obtained from women with Vulvovaginal atrophy and those without atrophy

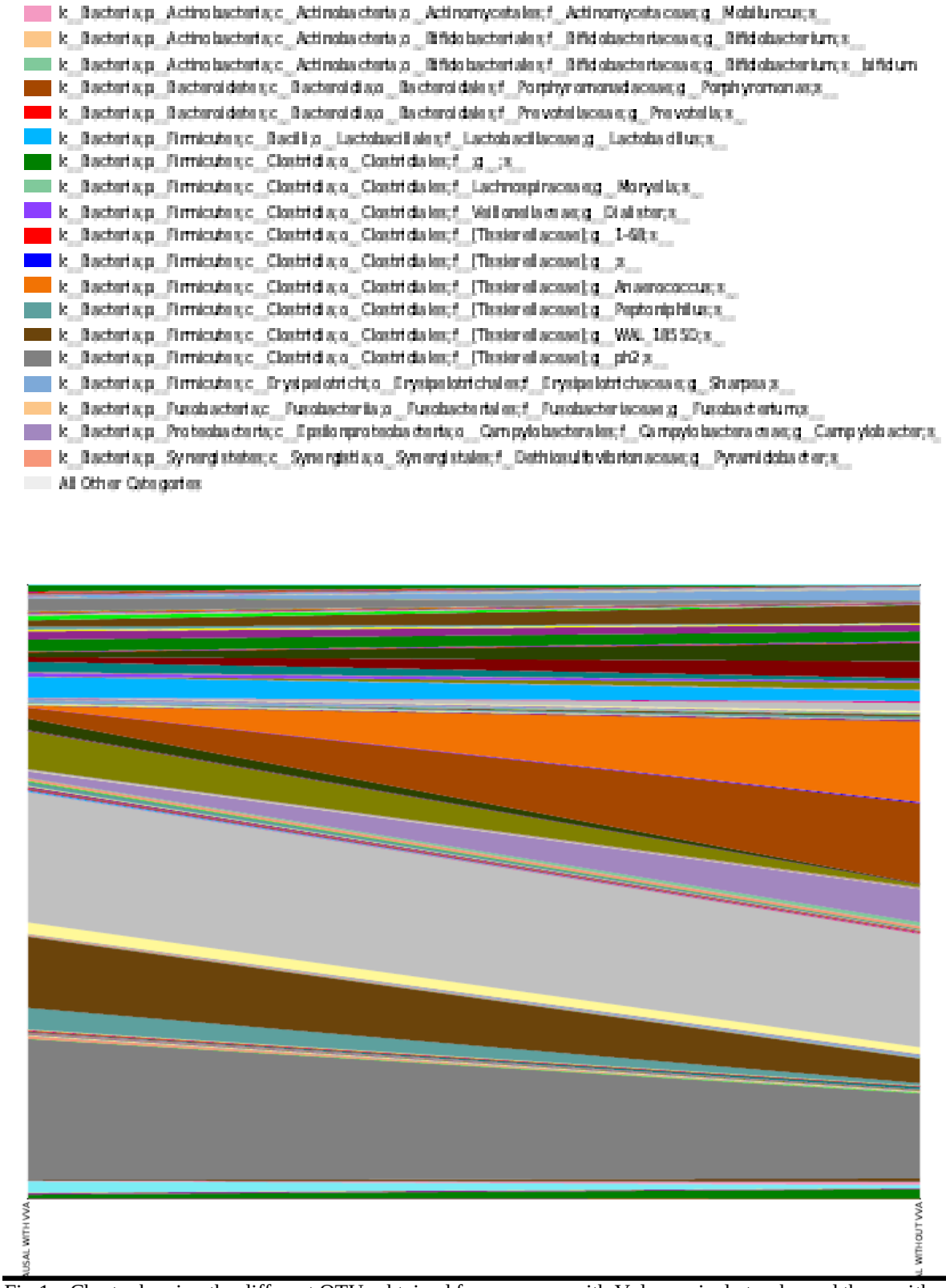
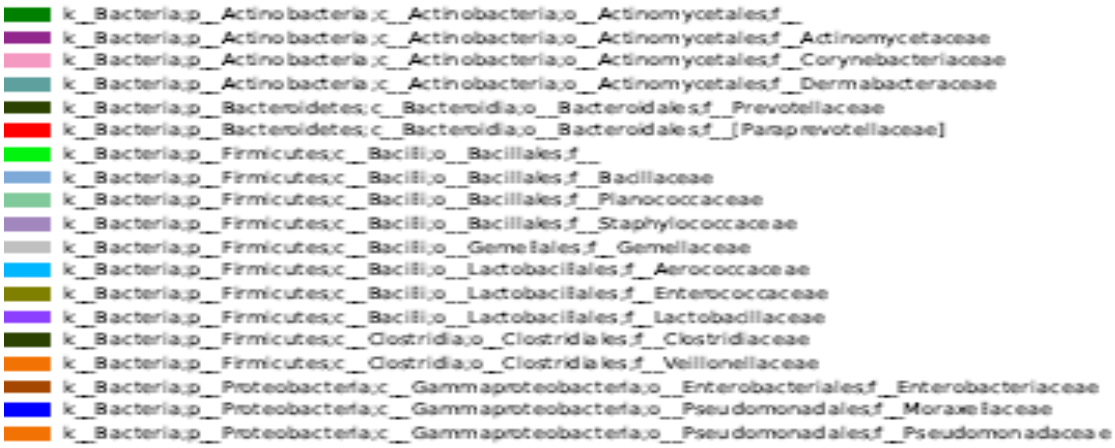


Fig 1c: Charts showing the different OTUs obtained from women with Vulvovaginal atrophy and those without atrophy



The box plots representing the OTUs of postmenopausal women with and without VVA is as shown in fig 2. The box plots show a clear visual difference between postmenopausal women with and without VVA. The box plots show that the postmenopausal women without VVA have higher values than postmenopausal women with VVA.

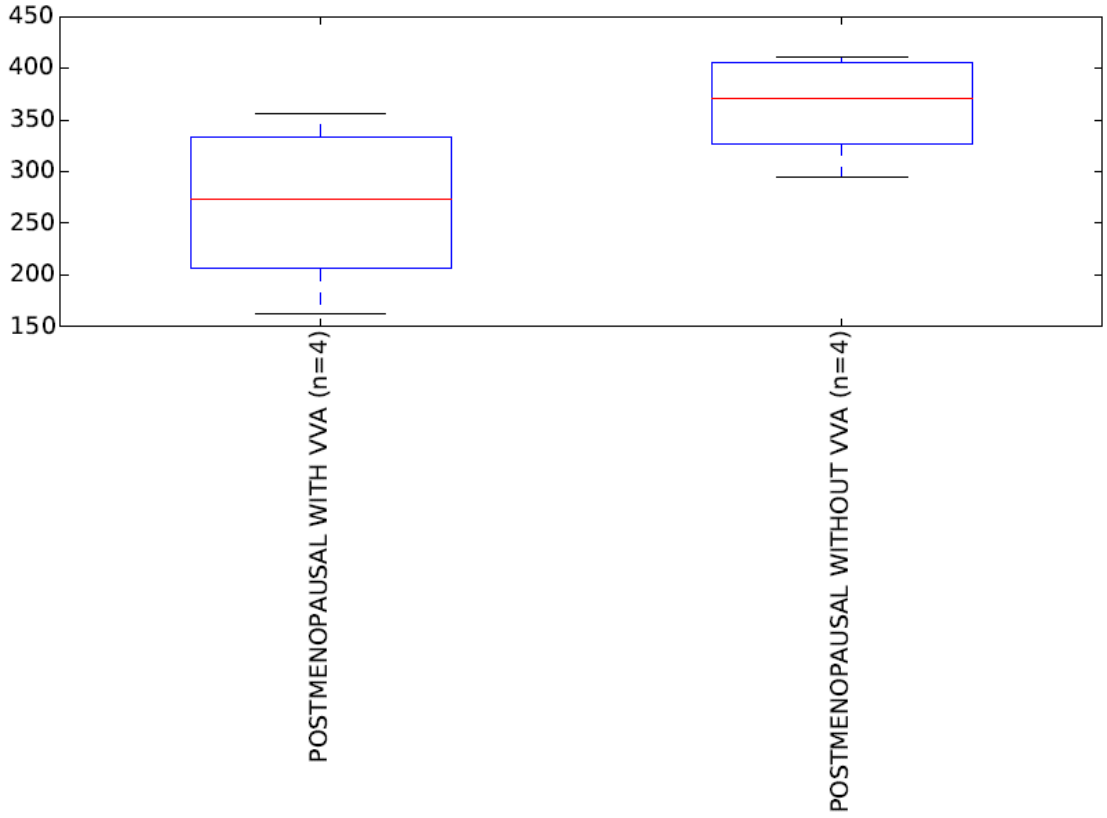


Fig2. Box plots showing the distribution and comparative analysis of the different OTUs from postmenopausal women with and without vulvo vaginal atrophy.

Serum level of fertility hormones of 8 post menopausal participants.

The results obtained from the hormonal assay show that, of all women tested in this group,(N=8), only 1 (12.5%) were found to have slightly lower FSH level than the reference range (ref range 0.5-10iu/L), (12.5%) of women had oestradiol (E2) lower than reference range (ref.range 2-50pmol/L) while 100% of women had both Progesterone and Leutenizing hormone within their reference range.(50-1500pm/ml and 1.5-55miu/ml)

respectively. All the respondents had very low level of *lactobacillus* species and high bacteria diversity. Details of result is as shown in table 2a and b

Table 2a Serum level of fertility hormones of 8 post menopausal participants.

SAMPLE	*STRATA*	*DESCRIPTION*	*SAMPLE NUMBER*	*PROG*	*OESTROGEN*	*L H*	*FSH*
15	C1	Post menopausal with VVA	IMO 7	134.2	2.435	3.805	1.056
16		‘’	IMO 33	127.1	2.793	4.491	0.653
17		‘’	AB 29	360.7	20.55	7.968	2.755
18		‘’	AB 4	128.2	1.963	3.343	0.706
19	C2	Post menopausal without VVA	IMO 1	107.4	2.138	3.330	0.494
20		‘’	IMO 22				
21		‘’	AB 5	156.1	3.122	3.952	0.883
22		‘’	AB 15	167.9	5.473	4.123	1.292

Table 2b: Bacteria diversity and abundance of participants with and without VVA

BACTERIA DIVERSITY	PARTICIPANTS WITH	PARTICIPANTS WITHOUT VVA
	C1	C2
Enterococcus	1.91	0
Lactobacillus iners	0	7.77
Lactobacillus	1.69	5.89
Prevotella	21.54	14.54
Peptoniphilus	1.84	1.67
Veillonella dispar	0	0.84
Anaerococcus	0.78	2.79
Corynebacterium	22.23	14.01
Bifidobacterium	2.99	0
Gardnerella	11.83	4.16
Dialister	3.47	1.72
Streptococcus	0	9.38
Megasphaera	0	0
Staphylococcus aureus	0	1.07
Sneathia	0.78	0
Coriobacteriaceae	0	0
Mobiluncus	1.89	0.55

IV. DISCUSSION

Vulvo vaginal atrophy has been known as a clinical condition which presents its self with vaginal dryness, painful urination, painful sexual intercourse and bleeding after intercourse, soreness, vaginal discharge, itching and burning sensation, which cause some level of distress and discomfort among postmenopausal women. Low level of serum estrogens in menopausal and postmenopausal women has long been thought to be associated with these clinical presentations. (Ravel *et al*., 2011, Brothman *et al*., 2014). This condition has been attributed to low vaginal *lactobacillus* level resulting from low serum oestrogen level, low epithelial glycogen level and invariably low lactic acid presence in the vagina. (Ravel *et al*., 2011),(Brothman *et al*., 2014). The low *lactobacillus* level and high bacteria diversity obtained from all postmenopausal women in this study is in agreement with the result obtained by Karol *et al*., (2019) and Hummelton *et al*., (2011) who recorded low *lactobacillus* abundance and high bacteria diversity among postmenopausal women. This result therefore suggests that vaginal microbiome of postmenopausal Igbo women is similar to those of women from other parts of the world. This suggests that age may have an important role in influencing the diversity of vaginal microbiota. This seems to suggest that absence of significant level of vaginal *lactobacillus* can lead to increased chances of vaginal colonization by more pathogenic microorganisms which can lead to bacteria vaginosis.

The results obtained from this study showed that both postmenopausal women with evidence of vulvo vaginal atrophy and those without evidence of vaginal atrophy had diverse array of bacteria, including *Mobilincus, Dialister, Megasphaera, Corynebacterium* and *Prevotella.* These bacteria have been known to cause bacteria vaginosis and sexually transmitted infections which may lead to premature rupture of membrane in pregnancy, spontaneous abortion and sometimes infertility in premenopausal women. This finding agrees with the work done by Hummelton *et al., (2011),* Brotman *et al., (2014)* and Rebecca *et al* (2014) who independently discovered high bacteria diversity among postmenopausal women who showed evidence of genitourinary atrophy. The presence of high bacteria diversity among this age group may be as a result of possible increase in the vaginal pH giving room to the influx of more pathogenic and facultative anaerobes.

Lactobacillus species which were thought to provide a defence mechanism against invading pathogenic organisms due to their ability to produce lactic acid, which makes the vaginal environment uncomfortable for

more pathogenic organisms were low in all menopausal and post menopausal women. The low *lactobacillus* level was attributed to low level of serum oestrogen However, this result indicated no difference in serum oestrogen level among these categories of women (post menopausal women with or without VVA), and only 12.5% had a level slightly lower than normal. These findings do not agree with the previous results obtained by some researchers indicating that all postmenopausal women experience a drop in their serum oestrogen. (Ravel *et al*., 2011, Brothman *et al*., 2014). From this result, one can therefore infer that the high bacteria diversity and low *Lactobacillus species* in postmenopausal women may not only be as a result of low serum oestrogen level, but other factors like stress and immunity may play active roles. It has been known that the immunity of an individual reduces with age. Thymus gland, B-cell and natural killer (NK) cells reduce in size at middle age up to about 15% in size thereby reducing the involvement of these cells in fighting infections. Also, the activities of macrophages reduce as individuals age. All these may contribute to the low immunological response which an individual experiences as he ages which invariably may lead to increase level of facultative anaerobes in the vagina of such elderly person.

V. CONCLUSION

Based on this result, postmenopausal women generally, have high bacteria diversity and low *lactobacillus* species level with no evidence of serum oestrogen involvement. Although there were observable differences in vaginal microbiome diversity and abundance among women with and those without vulvo vaginal atrophy, the difference was not statistically significant.

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