Obstetrics; *Maternal-Fetal Medicine and Perinatology*

Analysis of Vaginal Microbiome in Women with or Without Episodes of Spontaneous Abortion in Eastern Nigeria

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ABSTRACT

OBJECTIVES: Spontaneous abortion (miscarriage) is a common adverse pregnancy outcome earth-wide, and has remained a challenge in Nigeria. This study aimed at comparing the vaginal microbiome of women who have had episodes of spontaneous abortion with those who have not experienced any incident - in order to find out any possible role of vaginal microbiota in spontaneous abortion.

STUDY DESIGN: High vaginal swab samples were collected from the vagina fornix of 6 women of reproductive age, with a history of recurrent spontaneous abortion, as well as those without such history (non-spontaneous abortion). The samples were analyzed and interpreted by standard metagenomic and bioinformatic techniques.

RESULTS: The following phyla were encountered in spontaneous abortion and non-spontaneous abortion, respectively: Firmicutes (69.4%, 94.9%), Actinobacteria (12.7%, 1.1%), Bacteroidetes (9.5%, 2.8%), Proteobacteria (7.9%, 0.3%), Chloroflexi (0.2%, 0.0%), Fusobacteria (0.2%, 0.0%), Tenericutes (0.02%, 1.0%). There was more bacterial diversity in spontaneous abortion (H=2.34856) than in Non-spontaneous abortion (H=0.61384), with evenness (EH) of 0.60668 and 0.24703, respectively. On the contrary, Lactobacillus had more relative abundance in non-spontaneous abortion (83%) than spontaneous abortion (23.5%). The following genera (among others) occurred exclusively in spontaneous abortion: Enterococcus (relative abundance=26%), Peptostreptococcus (5.1%), Anaerococcus (2.4%), Dialister (2.1%), Streptococcus (1.9%), Megasphaera (1.3%), Mobiluncus (1.0%), Peptinophilus (0.9%), and Veillonella (0.7%). The efficiency of taxonomic identification, using the operational taxonomic unit clustering method, declined, downstream, from family to species levels.

CONCLUSION: Recurrent spontaneous abortion appears to be associated with low vaginal Lactobacillus abundance and high bacterial diversity. We recommend that the current operational taxonomic unit -based sequence taxonomic analysis technique be reviewed.

Keywords: Eastern nigeria, Spontaneous abortion, Vaginal microbiome

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Introduction

The human body is estimated to contain as many bacterial cells as human cells, which represents a magnitude of about 10^{13} bacterial cells; these organisms inhabit different parts of the body, such as the gastrointestinal tract, vagina, breast, skin, and oral cavity - with or without adverse consequences (1). The complex bacterial community that resides in the body, along with its total genomic materials, is referred to as the body microbiome. During the past decade, the microbiome has been identified as a major contributor to human health (1-4); the vaginal microbiome has a significant role in women's reproductive health. Imbalances in the microbiota, also referred to as "dysbiosis", may lead to several undesirable conditions, including negative reproductive outcomes (5).

The microbiota of a healthy vagina is defined as *Lactobacillus*-dominated; the dominant *Lactobacillus* creates an acidic environment that protects the vagina against infec-

tion by pathogenic organisms (6). Vaginal communities where *Lactobacilli* are either unstable or not dominant are dysbiotic and engender overgrowth of other species often implicated in many female reproductive tract disorders (5,7).

One of the commonly reported negative reproductive outcomes in Nigeria is spontaneous abortion (8), commonly referred to as miscarriage (4), and which is believed to be associated with the following identifiable risk factors: febrile illnesses such as malaria, urinary tract or lower genital tract infections, smoking, alcohol ingestion, advanced maternal age, increasing parity, increasing paternal age, previous miscarriages, smoking, obesity, among others (9-11). Several authors have included vaginal dysbiosis as a risk factor in spontaneous abortion (SA) (12,13). No work emanating from our locality has investigated the taxonomic profile of vaginal microbiome in SA, hence the need for this study.

Material and Method

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. A simple random sampling technique was used in the recruitment of subjects.

The study was reviewed and approved by the ethics committee of Federal Teaching Hospital Abakaliki, Ebonyi State, Nigeria (reference number: FETHA/REC/Vol. 2/2018/084 - dated July 31, 2018) as well as Federal Medical Centre, Owerri, Imo State (reference: number FMC/OW/HREC/226, dated September 18, 2018). The study was conducted in accordance with the Declaration of Helsinki.

A total of four sexually active women (of reproductive age), with episodes of SA, were recruited into the study; SA (or miscarriage) was defined as unplanned (spontaneous) loss of pregnancy before the fetus reaches viability; if the pregnancy losses occurred more than two consecutive times (before 20 months of gestation, in each case) it was considered as recurrent SA.

All participants signed informed written consent before being enrolled in the study and were served interviewer-administered questionnaires. Information sought through the questionnaires included: age, history of antibiotic treatment, smoking and alcohol consumption habits, obesity, symptoms of vaginal infection, organ transplant, and use of steroid hormone, among others. Women who were menstruating, or having vaginal bleeding, those with a known history of vaginal infection, organ transplant, human immunodeficiency virus (HIV) infection, and diabetes mellitus, were excluded. Also excluded were women who had received antimicrobial therapy within the previous six months, and those using any form of vaginal suppository formulations, including prebiotics or probiotic preparations. Two premenopausal women with regular menstrual periods, who did not have any of the exclusion criteria, and had carried pregnancy to term, were enrolled in comparison.

Vaginal samples were collected from each subject, using the Norgen Microbiome collection and preservation kit (Cat no 45690). On the whole, High Vaginal Swab (HVS) samples were collected from a total number of six women - 4 with episodes of (SA) and 2 healthy women, who were not experiencing SA (Non-SA).

Metagenomic Sequencing

The DNA from each of the HVS samples was isolated, extracted, and purified using the Norgen microbiome isolation kit (Cat No. 64100), according to manufacturers' instructions. The purity and quantity of the DNA were 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 hypervariable regions of 16S rRNA, using paired-end universal primer 341F, 5'-CCTACGGGNGGCWGCAG-3' (14) and 785R, 5'-GACTACHVGGGTATCTAATCC-3'(15). Samples were barcoded with a unique combination of forward and reverse indexes, allowing for the 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-Rad iCycler qPCR kit on a Bio-Rad MyiQ machine, 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 the Illumina Base space pipeline for quality check. Reads with an average Q-score greater than 30 (Q score >30) were filtered. Sequenced data were processed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline (http://qiime.sourceforge.net/) (16). 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 (17). The valid reads obtained from Illumina MiSeq sequences were normalized to 1000 for comparison of community diversity. Reads with 97% similarity were then clustered into operational taxonomic units (OTUs) (18). The Greengenes database and ribosomal database project (RDP) classifier were used to assign the effective sequence tags to different phylogenetic bacterial taxa (19). Diversity indices and statistical analyses were by standard methods (20).

Results

The demographic information on the subjects recruited for this study is shown in table I.

Table I: *Demographic information on study participants*

Demographic information	Particulars of Subjects					
	#1	#2	#3	#4	#5	#6
Age	38	32	40	35	42	34
State of origin	Anambra	Imo	Ebonyi	Abia	Imo	Ebonyi
Marital Status	Married	Married	Married	Married	Married	Married
Level of Education	Tertiary	Secondary	Secondary	Secondary	Tertiary	Secondary
Profession	Trader	Trader	Teacher	Tailor	Teacher	Technician
Employment	Trading	Trading	Teaching	Sewing	Teaching	Civil servant
Alcohol/tobacco intake	No	No	No	No	No	No
HIV positivity	No	No	No	No	No	No
History of recurrent abortion	Yes	Yes	Yes	Yes	None	None
Recent antibiotics therapy	None	None	None	None	None	None
History of organ transplant	None	None	None	None	None	None

Of a total of 112,196 high-quality sequence reads (Mean=18,699; SD=5009.405) generated, 87,541 were assigned to the phylum "*Fermicutes*" (giving a relative abundance of 78.0%); *Actinobacteria* (9,838 reads and relative abundance of 8.8%) was the next in abundance, as shown in table II.

In all, a total of seven taxonomic phyla were identified in vaginal samples of women who experienced episodes of SA, while five were identified in those who had no history of SA (Non-SA). In both groups, *Fermicutes* was the dominant phylum (SA=69.4%, Non-SA=94.9%); other phyla present were, respectively, as follows: *Actinobacteria* (12.7%, 1.1%), *Bacteroidetes* (9.5%, 2.8%), *Chloroflexi* (0.2%, 0.0%), *Fusobacteria* (0.2%, 0.0%), *Proteobacteria* (7.9%, 0.3%), and *Tenericutes* (0.02%, 1.0%), as expressed in figure 1.

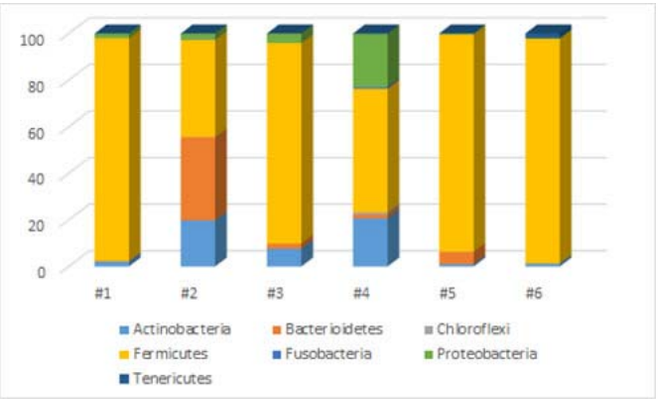

Figure 1: *Chart showing relative abundances of bacterial phyla in vaginal samples from all recruited women in Eastern Nigeria*
#1-4: From women with episodes of spontaneous abortion
#5-6: From women not having a spontaneous abortion

Table II: *Sequence reads and relative abundances of phyla identified in samples from the vagina of women with episodes of spontaneous abortion and in those without abortion*

S/No	OTUs Identified at Phylum level	Sequence reads and relative abundance in vaginal samples from women with:								
		SA					Non-SA			Over-all (#1-6)
		# 1	#2	#3	# 4	Total (% of Reads)	#5	#6	Total (% of Reads)	Total (% of Reads)
1	*Actinobacteria*	250	3427	2078	3667	9422 (12.7%)	199	217	416 (1.1%)	9838 (8.8%)
2	*Bacteroidetes*	24	6180	602	284	7090 (9.5%)	1033	18	1051 (2.8%)	8141 (7.3%)
3	*Chloroflexi*	0.0	17	0.0	160	177 (0.2%)	0.0	0.0	0.0 (0.0%)	117 (0.1%)
4	*Fermicutes*	11390	7218	23539	9408	51555 (69.4%)	18545	17441	35986 (94.9%)	87541 (78.0%)
5	*Fusobacteria*	0.0	35	0.0	124	159 (0.2%)	0.0	0.0	0.0 (0.0%)	159 (0.1%)
6	*Proteobacteria*	238	433	1121	4057	5849 (7.9%)	79	36	115 (0.3%)	5964 (5.3%)
7	*Tenericutes*	0.0	0.0	0.0	18	18 (<0.1%)	0.0	362	362 (1.0%)	380 (0.3%)
	Total Sequence Reads	**11902**	**17310**	**27339**	**17717**	**74,268**	**19855**	**18073**	**37,928**	**112,196**
	No. of phyla represented (n = 7)	4	6	4	7		4	5		7

OTUs: Operational taxonomic units, SA: Spontaneous abortion, Non-SA: Not having spontaneous abortion

Table III shows the number of times each taxonomic category occurred; more taxonomic groups were identified in SA samples, compared with non-SA samples, as follows: Phylum (SA=7, Non-SA=5), Class (15, 8), Order (24, 10), Family (54, 20), Genus (68, 16), and species (26, 11), respectively. Five of 60 (8%) OTUs were not classifiable at the taxonomic level of "Family"; similarly, 29 of 99 (29%) could not be classified at genus levels, while 87 of 118 (74%) were unclassifiable at the species level (Table III).

Figure 2 contains pie charts showing bacterial community distribution between SA (SAI - SAIII) and Non-SA (Non-SAI - Non-SAIII) at taxonomic levels of "Class", "Order", and "Family". (In the charts, only the top 10 taxa were illustrated - the remaining taxa were condensed into one group designated as "Others").

Table IV shows the genera represented in the vaginal samples. *Lactobacillus* was the most abundant of the genera in both SA (3/4; 23.5%) and non-SA (2/2; 83%), followed by *Prevotella* (SA:4/4, 9%; Non-SA:2/2, 2.6%), and *Corynebacterium* (SA:4/4, 2.2%; Non-SA:2/2, 0.4%).

Some of the genera that were exclusively present in SA included: *Enterococcus* (with a relative abundance of 26%), *Peptostreptococcus* (5.1%), *Anaerococcu*s (2.4%), *Dialister* (2.1%), *Arthrobacter* (1.4%), *Streptococcus* (1.9%), *Megaphaera* (1.3%), *Pseudomonas* (1.2%), *Mobiluncus* (1.0%), *Bacillus* (0.9%), *Peptoniphilus* (0.9%), *Veillonella* (0.7%), and *Rhodomonas* (0.7%), etc. On the other hand, *Clostridium* (9.1%) was exclusive to non-SA; *Bifidobacterium* occurred more abundantly in SA than non-SA (2.6% and 0.1%, respectively) as was also the case with *Gardnerella* (2.1% and 0.1%, respectively), as shown in Table IV. There was more evenness of distribution in SA samples (EH=0.60668) than in Non-SA (EH=0.24703); Sorenson's similarity coefficient (CC) of 0.23333 was recorded between SA and Non-SA samples.

We noted more microbial diversity in SA (Shannon diversity index (H)=2.34856) than in non-SA (H=0.61384) – t=147.13703 (p<0.001), as shown in figure 3.

Of 70 OTUs identified at the genus level, only a total of 12 (17%) could fully be identified at the species level, as shown in table V. Species of *Lactobacillus* represented in the vagina of participants, and their relative abundances were as follows: *L. iners* (SA=14.2%; Non-SA=30.2%), *L. reuteri* (SA=0.1%; non-SA=0.1%). *L. coleohominis* (SA=0.0%; Non-SA=0.2%), *Lactobacillus spp.* (SA=9.2%; non-SA=52.5%) -see Table V. As can also be seen in table 5, of the 23.5% relative abundance of *Lactobacilli* in SA, 9.2 (40%) could not be identified up to species level; similarly, 52.5 of 83 *Lactobacilli* (63%), from Non-SA samples, could not be speciated (Table V). Overall,

Table III: *Number of times operational taxonomic units were identified, at various taxonomic levels, in the vagina of women with spontaneous abortion and those without non-spontaneous abortion in Eastern Nigeria*

Taxonomic level	Identity status and number of OTUs encountered in Samples (#1- 6)							Distribution of OTUs in SA and Non-SA			Total No. in Strata	
	Identity status of OTUs	#1	#2	#3	#4	#5	#6	Sh.	ESA	ENSA	Total	SA	Non-SA
Phylum	Identified	4	6	4	7	4	5	5	2	0	7	7	5
	Unclassified	0	0	0	0	0	0	0	0	0	0	0	0
	Total	**4**	**6**	**4**	**7**	**4**	**5**	**5**	**2**	**0**	**7**	**7**	**5**
Class	Identified	7	11	8	15	8	8	8	7	0	15	15	8
	Unclassified	0	0	0	0	0	0	0	0	0	0	0	0
	Total	**7**	**11**	**8**	**15**	**8**	**8**	**8**	**7**	**0**	**15**	**15**	**8**
Order	Identified	10	15	10	24	8	9	10	14	0	24	24	10
	Unclassified	0	0	0	0	0	0	0	0	0	0	0	0
	Total	**10**	**15**	**10**	**24**	**8**	**9**	**10**	**14**	**0**	**24**	**24**	**10**
Family	Identified	15	25	20	54	14	13	19	35	1	55	54	20
	Unclassified	1	2	3	2	0	1	1	4	0	5	5	1
	Total	**16**	**27**	**23**	**56**	**14**	**14**	**20**	**39**	**1**	**60**	**59**	**21**
Genus	Identified	14	28	20	57	12	9	14	54	2	70	68	16
	Unclassified	5	8	6	37	4	1	4	24	1	29	28	5
	Total	**19**	**36**	**26**	**94**	**16**	**10**	**18**	**78**	**3**	**99**	**96**	**21**
Species	Identified	3	10	7	20	11	3	7	19	4	30	26	11
	Unclassified	17	29	20	69	10	8	11	72	1	84	83	12
	Total	**20**	**39**	**27**	**89**	**21**	**11**	**18**	**91**	**5**	**114**	**109**	**23**

OTUs: Operational taxonomic units, SA: Spontaneous abortion, Non-SA: Not having spontaneous abortion, Sh: Shared (present in both SA and Non-SA), ESA: Exclusively present in SA, ENSA: Exclusively present in Non-SA

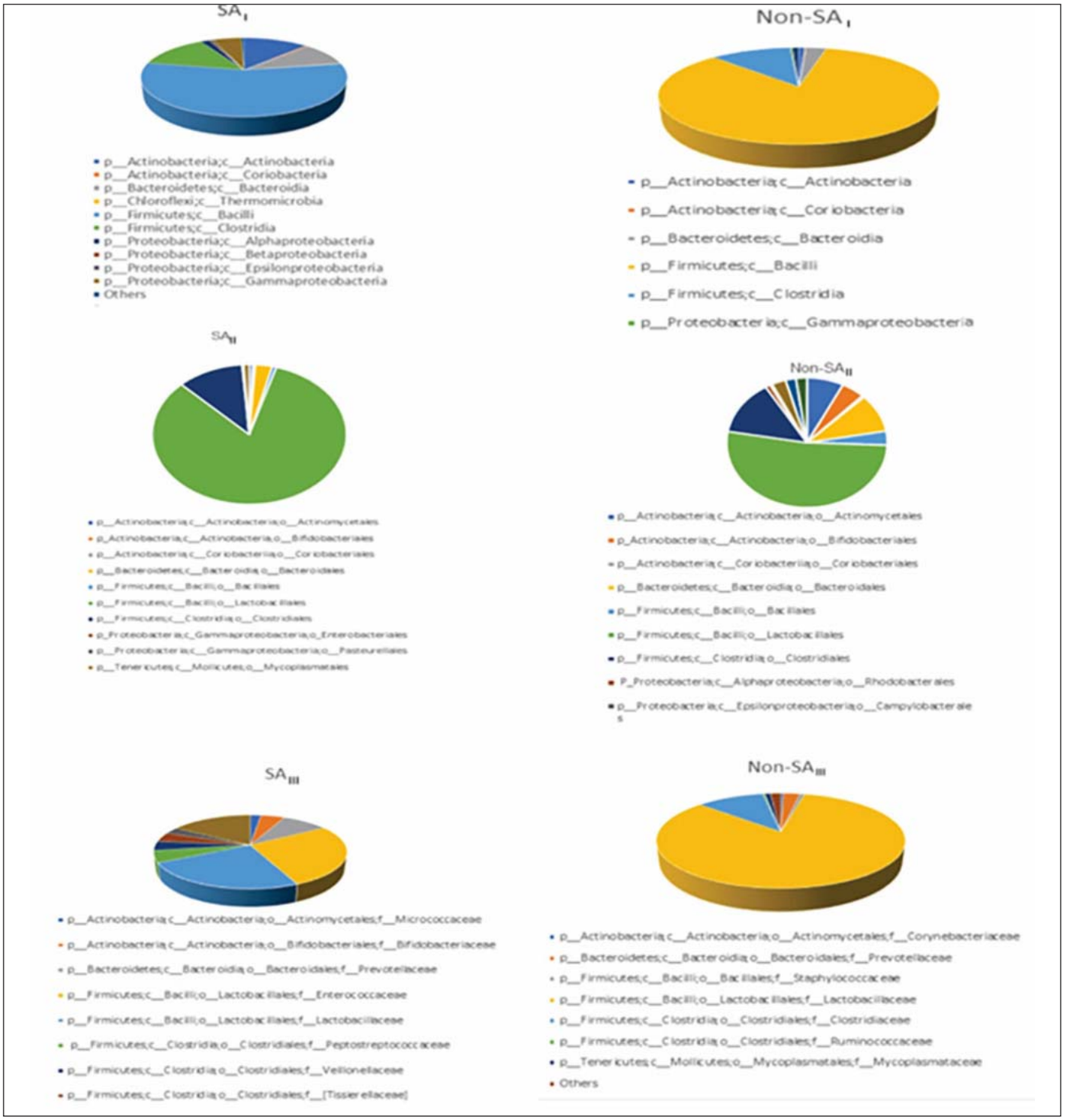

Figure 2: *Pie charts showing the classification of operational taxonomic units at Class, Order, and Family taxonomic categories in vaginal samples from women with spontaneous abortion or without non-spontaneous abortion in Eastern Nigeria*
SAI and Non-SAI: Classification of SA and Non-SA samples at "Class" taxonomic level, SAII and Non-SAII: Classification of SA and Non-SA samples at "Order" taxonomic level, SAIII and Non-SAIII: Classification of SA and Non-SA samples at the "Family" taxonomic level

the genus *Lactobacillus* was significantly more abundant in SA (23.5%) than in non-SA (83%) - t=2.4932; $p<0.05$.

In addition, 2.4 of 2.6 (85%) of OTUs corresponding to *Bifidobacterium* could not be speciated. The unclassifiable genera were distributed to their corresponding phyla, as follows: *Actinobacteria* (16.86%), *Bacteroidetes* (0.0%), *Chloroflexi* (2.68%), *Fermicutes* (27.20%), *Fusobacterium* (0.0%), *Proteobacterium* (54.79%), *Tenericutes* (0.0%). Figure 4 is a Pie chart showing the distribution of unclassifiable genera among the phyla represented in the vagina of women in Eastern Nigeria.

Table IV: *Bacterial community distribution, at the genus level, in samples from the vagina of women with episodes of spontaneous abortion and those not having an abortion*

		Relative Abundance (%) of OTUs in:	
		SA	**Non-SA**
A.	**Shared Genera**		
1.	*Lactobacillus*	23.5	83.0
2.	*Prevotella*	9.0	2.6
3.	*Staphylococcus*	1.6	0.7
4.	*Bifidobacterium*	2.6	0.1
5.	*Corynebacterium*	2.2	0.4
6.	*Gardnerella*	2.1	0.1
7.	*Haemophilus*	0.1	0.1
B.	**Genera Exclusive to women with SA**		
1.	*Enterococcus*	26.0	0.0
2.	*Peptostreptococcus*	5.1	0.0
3.	*Anaerococcus*	2.4	0.0
4.	*Dialister*	2.1	0.0
5.	*Streptococcus*	1.9	0.0
6.	*Arthrobacter*	1.4	0.0
7.	*Megasphaera*	1.3	0.0
8.	*Pseudomonas*	1.2	0.0
9.	*Mobiluncus*	1.0	0.0
10.	*Peptoniphilus*	0.9	0.0
11.	*Bacillus*	0.9	0.0
12.	*Rhodococcus*	0.7	0.0
13.	*Veillonella*	0.7	0.0
14.	*Pophyromonas*	0.5	0.0
15.	*Parococcus*	0.5	0.0
16.	*Acinetobacter*	0.5	0.0
17.	*Campylobacter*	0.5	0.0
18.	*Aerococcus*	0.3	0.0
19.	*Sutterella*	0.3	0.0
20.	*Finegoldia*	0.3	0.0
21.	*Micrococcus*	0.3	0.0
22.	*Dietzia*	0.3	0.0
23.	*Sneathia*	0.2	0.0
24.	*Atopobium*	0.2	0.0
25.	*Bulleidia*	0.2	0.0
26.	Others (n=33)	3.5	0.0
C	**Genera Exclusive to women not having SA (Non-SA)**		
1.	*Clostridium*	0.0	9.1
2.	*Ureaplasma*	0.0	0.9
3.	Others (n=3)	0.0	0.6

Discussion

Earlier reports (21,22) suggested that significant differences exist in average microbial diversity between women who experienced SA and those who successfully carried the pregnancy to term. In our study, we noted that the Shannon diversity index was much higher among SA samples, compared with non-SA (t=147.13703; $p<0.001$), showing that the vagina of SA patients had significantly more diversity of bacterial genera and species. Also, as can be seen from the Shannon

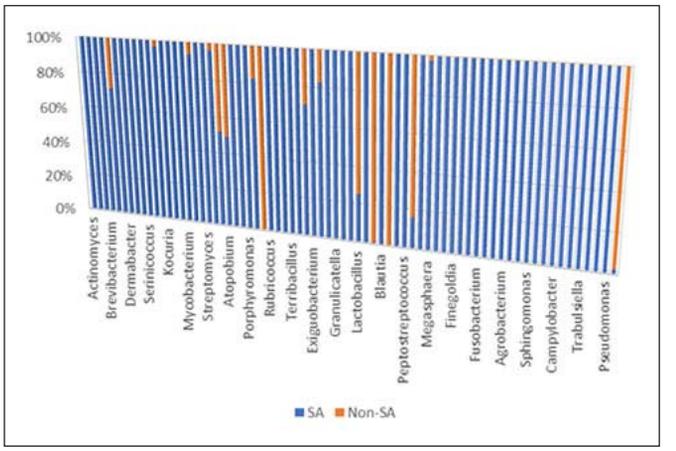

Figure 3: *Chart expressing the diversity of bacterial genera in the vagina of women with (SA) and without (Non-SA) spontaneous abortion in Eastern Nigeria*

Table V: *Operational taxonomic units identified at species levels and their relative abundances in the vagina of women experiencing spontaneous abortion and those who experienced no episodes in Eastern Nigeria*

S/No.	Species encountered	Relative abundance (%) in:	
		SA	Non-SA
1	*Bifidobacterium;s*	2.2	0.0
2	*Bifidobacterium;s adolescentis*	0.4	0.0
3	*Bifidobacterium;s longum*	0.0	0.1
4	*Prevotella;s*	9.0	1.1
5	*Prevotella;s copri*	0.0	1.2
6	*Prevotella;s stercorea*	0.0	0.3
7	*Lactobacillus;s*	9.2	52.5
8	*Lactobacillus;s coleohominis*	0.0	0.2
9	*Lactobacillus;s iners*	14.2	30.2
10	*Lactobacillus;s reuteri*	0.1	0.1
11	*Arthrobacter;s psychrolactophilus*	1.3	0.0
13	*Clostridium;s perfringens*	0.0	9.1
14	*Streptococcus;s*	0.7	0.0
15	*Streptococcus;s anginosus*	1.2	0.0
16	*Peptostreptococcus;s anaerobius*	5.1	0.0
17	*Veillonella;s dispar*	0.7	0.0

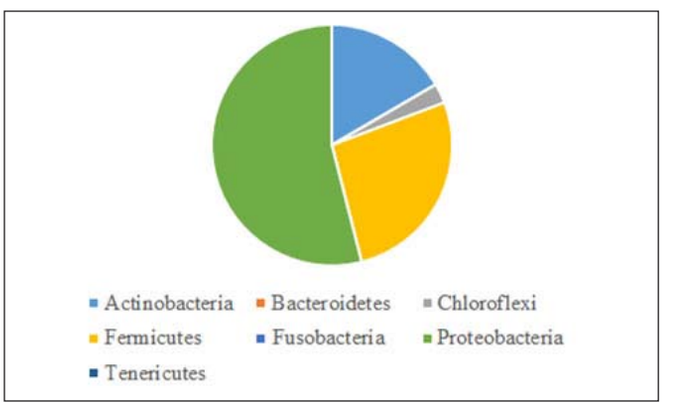

Figure 4: *A pie chart showing the distribution of unclassifiable genera/species among the phyla represented in the vagina of women in Eastern Nigeria*

Equitability Index (EH) values (SA=0.60668; non-SA=0.24703), women with SA had more species evenness in their vagina than those of non-SA (it should be pointed out that a community with low evenness contains few dominant species, whereas one with high evenness contains diverse species that are uniformly distributed in abundance). This shows that the vagina of women with SA had significantly more diverse species and that those species were more uniformly distributed in abundance than was the case with non-SA vaginal communities. This also shows that the vagina of non-SA women, unlike those of SA, was dominated by a few bacterial species. The dominant bacterium in non-SA was *Lactobacillus*, and this organism (*Lactobacillus*) was significantly more abundant in non-SA communities than in SA (t=2.4932; p<0.05). *Lactobacilli* are known to produce substances that limit the growth of other bacteria in the vagina, thereby promoting good vaginal health (23). Therefore, the relatively low abundance of *Lactobacillus* in the vagina of SA patients (compared with non-SA) must have resulted in the production of comparatively fewer amounts of antibacterial chemicals, thereby providing a more conducive environment for diverse bacterial species to thrive. A vaginal microbiota is considered unbalanced (dysbiotic) when the diversity is high (as seen in SA cases); such disturbed vaginal composition (dysbiosis) can lead to negative reproductive outcomes (5,7).

Based on this study, it can be concluded that SA is associated with reduced *Lactobacillus* levels and increased diversity of vaginal microbiota. This is possibly due to the fact that a reduced level of vaginal *Lactobacilli* may predispose the vagina to colonization by diverse groups of bacterial genera and species, which may, in turn, induce immunological pressures that could result in the premature ejection of the developing fetus - before the fetus attains viability; this could be attributed to the fact that presence of infecting organisms can lead to consequent activation of pattern-recognition receptors (PPRs) and release of inflammatory mediators that could ultimately stimulate uterine contractions, leading to the eventual ejection of the unviable fetus (22,24).

We also noted a low similarity coefficient between the vagina of SA and non-SA women (CC=0.23333). Sorenson's similarity coefficient (CC) ranges in value between 0 and 1; the lower the value between two communities, the less the communities have in common. This shows that the bacterial community in SA was quite dissimilar to those in non-SA, and further suggests that the vaginal microbiome profile may have a role in negative reproductive outcomes, such as SA. The apparent involvement of vaginal dysbiosis as a risk factor in SA (as noted in this study) agrees with the report of earlier authors (25), who attributed miscarriages to reduced concentration of hydrogen peroxide-producing *Lactobacilli* and the presence of bacterial vaginosis. The use of probiotics or prebiotic preparations may be an effective way to address such a dysbiotic state and restore the integrity of the vagina.

Ravel et al. (6) placed black women in vaginal community type IV - a type lacking Lactobacillus-dominated microbiota communities. However, the result of this study showed that the relative abundance of vaginal *Lactobacilli* was higher than the level that defines the community state type IV that is ascribed to African women. This difference is not surprising, because the black population investigated by Ravel et al. (6) were not African residents but black women in the diaspora; the vaginal flora of such "westernized" African women might not be truly reflective of those of non-diaspora African women, especially as cultural and personal (westernized) practices can influence the body microbiota.

Most of the genera we found to be exclusive to SA (i.e. *Sneathia, Megaphaera, Dialister, Peptostreptococcus, Gardnerella*, and other facultative anaerobes encountered) have previously been associated with vaginal dysbiosis by other authors (22,26). Also, the facultative anaerobes identified (exclusively or more abundantly in SA) in this study included: *Anaerococcus, Gardnerella, Peptostreptococcus Megasphaera, Dialister, Prevotella,* and *Peptoniphilus,* among others. These bacteria have been found to be among the causative agents of pelvic inflammatory diseases (PID), fallopian tube blockade, and miscarriage (27). It has been shown that SA is one of the most common adverse outcomes of pregnancy, affecting an estimated 12-24% of known pregnancies (4). Deaths caused by this condition can be avoided, or drastically reduced, if pregnancies at risk of SA are identified early and necessary interventions promptly administered. This can be achieved by monitoring the vagina of pregnant women for the dysbiotic state.

In the course of this study, we found out that some OTUs lacked classification at taxonomic levels of "Family" (5/60 or 8%), Genus (29/99 or 29%), and species (84/114 or 74%). Consequently, 39% of *Lactobacilli* OTUs in SA and 63% of those in Non-SA could not be speciated; this situation made it difficult to precisely determine all the species of vaginal *Lactobacilli* involved in health and disease among the subjects investigated. This was also the case with the genus *Bifidobacterium*, 85% of which could not be speciated. The high prevalence of unclassified OTUs (at genera and species levels) underscores the need to review the currently used 16S amplicon analysis method, which involves clustering sequences within arbitrarily chosen sequence similarity threshold (usually 97%) into the operational taxonomic units (OTUs), to delineate species; poorly clustered OTUs can have significant impacts on downstream analyses and can lead to overestimation of evolutionary similarity between pairs being aligned (28). Amplicon sequence variant (ASV), also referred to as exact sequence variants (ESVs), has been suggested as a better option for the current use of the operational taxonomic unit (29), and is hereby recommended. However, Werner et al. (30) are of the view that OTUs without genus/species information is frequently both more abundant and more represen-

tative of total diversity than are OTUs with genus /species names.

The fact that the efficiency of taxonomic identification of the OTU clustering method steadily declined, downstream, from family level (92%) to genus level (71%), and species level (26%) makes us believe that more family, genera, and species would have been revealed, if the sequence similarity threshold was set higher than 97%, or possibly if an alternative technique was adopted.

Conclusion/Recommendations

The results of this study tend to affirm that vaginal dysbiosis is a risk factor in SA. It is, therefore, recommended that vaginal microbiome examination be listed among routine investigations in pregnancy, especially for those with a history of miscarriage(s); the use of prebiotics or probiotic preparations is suggested for those whose vaginal analyses indicate dysbiosis. Based on our results, it is also recommended that the current sequence taxonomic analysis technique, involving a 97% similarity threshold, be reviewed. The inability to employ a larger sample size (due to technical limitations and the small size of funds) was a limitation of this study.

Declarations

Ethics approval and consent to participate: Availability of data and materials. The data supporting this study is available through the corresponding author upon reasonable request.

Conflict of interest: The authors declare no conflict of interest.

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