



Molecular Characterisation and Antibiogram of Vaginal Flora from Students Attending a Tertiary Institution in Port Harcourt, Rivers State, Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The microbiome of the vagina is characterized by a community bacteria playing important roles in the overall health status of the female genital tract. This study was conducted to isolate and characterize bacteria from the female genital tract and as well evaluate the antibiotics susceptibility pattern of the vaginal bacterial isolates. For this purpose, a total of fifty (50) vaginal swab samples were collected (using sterile swab sticks) from females attending a tertiary institution in Port Harcourt, Rivers State, Nigeria, and subjected to standard bacteriological analysis. Antibiotics sensitivity analysis was carried out using the modified Kirby Bauer disc diffusion method. A total of 160 bacterial isolates were obtained from the subjects of different age brackets in the study population. Molecular identification based on the nucleic acid sequence of the bacterial isolates revealed the isolates to be *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Bacillus flexus* and *Lysinibacillus macrolides*. The result further showed that *Escherichia coli* was the most occurring bacterial isolate. Also, female subjects within the age bracket 21-23 years recorded the highest number of bacterial isolates (67) and 24-26 years had the least number of bacterial isolates (36). The antibiotic sensitivity analysis revealed that *Escherichia coli* and *Staphylococcus aureus* were resistant to 50% of the antibiotics tested, whereas *Klebsiella*

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pneumoniae was resistant to all (100% of) the antibiotics tested. The study has revealed that the vaginal microbiome of healthy female subjects is characterised by diverse species of bacteria, including opportunistic bacterial pathogens. The study therefore, recommended that regular screening for bacterial vaginosis as well as personal hygiene, sensitization programs to improve knowledge of women, should be encouraged.

Keywords: Antibiotics sensitivity; female genital tract; molecular characterization; tertiary institution; vaginal flora.

1. INTRODUCTION

In healthy women, the vaginal environment is characterised by the presence of various species of bacteria mostly in the genus, *Lactobacillus*. Five community state categories (CSTs) have been described based on the relative abundance and species of *Lactobacillus* found in vaginal microbial populations [1]. *Lactobacillus*-dominant communities are thought to be beneficial for obvious reasons, including the reduction of inflammation and prevention of colonisation by pathogens and thereby helps to maintain a stable microenvironment [2]. Some species of *Lactobacillus* have been found to be associated with these beneficial roles. *L. crispatus*, for example, produces the most lactic acid among *Lactobacilli* in the vaginal canal and can help prevent infection with other microbes without causing inflammation, making it extremely beneficial [1,3-5]. *Lactobacillus iners* populations, on the other hand, are often dysfunctional and are usually associated with anaerobic microbes such as *Gardnerella*, *Ureaplasma*, and *Prevotella* species, among other bacteria, that have the potential to cause bacterial vaginosis [6].

The vaginal flora's composition varies in response to exogenous and endogenous influences [7] such as the various stages of the menstrual cycle [7-8], gestation, the use of contraceptives, the use of antibiotics [9], the frequency of sexual activity [10], and the use of showers or deodorant items, and or other drugs with immune-suppressive properties. Intra-vaginal activities such as vaginal wiping, vaginal washing and inserting substances into the vagina, are carried out by women for various reasons including for genital hygiene, treatment of sexually transmitted diseases, and the enhancement of sexual pleasure (to prevent dry sex) [11], and have all been found to influence the micro flora of the vagina.

The decrease in *Lactobacilli* and increase in facultative and anaerobic bacteria may lead to some pathological syndromes characterized by changes in the characteristics of vaginal fluid such as thickness and odour of the discharge [12-13].

Bacterial vaginosis (BV) is an infection that primarily affects black women in Sub-Saharan Africa [14]. It is found in 10-40% of women worldwide and is most common in women who engage in multiple sex, those of low-income status and lower levels of education, among women who smoke cigarettes [15]. It is a polymicrobial syndrome characterised by the loss of normal vaginal flora predominantly hydrogen-peroxidase producing *Lactobacillus* spp. and the increase in the number of other bacterial species in vaginal fluid [16-17]. The common complications associated with this disease include the potential of causing late miscarriages, preterm births, and preterm premature rupture of membranes, pelvic inflammatory disease, and increased risk of HIV infections [18].

Several recent studies using molecular methods have identified mixed species of vaginal flora that were previously unidentified using cultural methods, such as BV-associated bacteria (BVAB). One explanation for this is that the sequencing techniques that have aided research into the bacterial microbiome are either inapplicable or ineffective for these microbes. Viruses, for example, are a diverse group of microbes that, unlike bacteria, lack a conserved gene that can be used to characterize the viral population using amplicons [19]. Some of these molecular methods include use of PCR based technique to probe bacterial isolates from vaginal swabs for identification based on 16S rRNA determination [20].

Treatment and management of bacterial infection of the vagina depends on the efficacy of the antimicrobial agent. Antibiotics susceptibility testing is not only important in determining the

potentials of conventional antibiotics in the control of bacteria-associated vaginal infection but also helps to evaluate exposures to antibiotics, as frequent use of antibiotics results in the emergence of drug resistant bacterial populations. This study was therefore conducted to isolate and characterize vaginal bacterial species, using molecular techniques, and to as well determine the susceptibility pattern of the isolates to conventional antibiotics. The findings could be utilized as a supportive tool in the confirmation of vaginal bacterial flora.

2. MATERIALS AND METHODS

2.1 Study Design

A cross-sectional (prevalence) study design was adopted to investigate the presence of bacterial species in the genital tract of female students attending a tertiary institution in Port Harcourt, Rivers, State, Nigeria. The study sampled fifty (50) female subjects using the convenience sampling method, which covered female students of different age groups.

2.2 Collection of Samples

High Vaginal Swab (HVS) samples were collected with the aid of a speculum, from subjects attending a tertiary institution in Port Harcourt, Rivers State, Nigeria. The transportation of the samples was done as described by Sampson and George [21]. Those on antibiotics and on their menstrual cycle were excluded in the study.

2.3 Methods of Isolation

Vaginal bacterial flora were isolated using standard microbiological procedures. This involved streaking the sterile swab sticks on freshly prepared Nutrient agar, Mannitol Salt Agar, MacConkey Agar, Eosin methylene blue agar and De Man, Rogosa and Sharpe (MRS) agar plates, and incubated at 37°C for 24 hours. Distinct colonies were subsequently sub cultured onto freshly prepared sterile nutrient agar plates to obtain pure cultures.

2.4 Antibiotics Sensitivity Testing by the Kirby Bauer Disk Diffusion Methods

The sensitivity of the respective isolates was evaluated using the Kirby Bauer Disk Diffusion Method. This was done by spreading bacterial

suspensions, whose turbidity was equivalent to 0.5 McFarland's Turbidity Standard, on the surface of the Petri dishes which contained already prepared Mueller Hinton agar. The impregnated antimicrobial discs were placed evenly on the surface of the inoculated plate and incubated as previously described [22-26]. The diameter of each zone of inhibition was measured in millimeters using a ruler on the underside of the plate and each zone was observed, interpreted and recorded in line with the standards of Clinical and Laboratory Standards Institute (CLSI) [27].

2.5 Molecular Identification of the Isolates

To confirm identity, the following tests were performed on each isolate. The DNA of the isolates was extracted using the boiling method [28], and was quantified using the Nano drop 1000 spectrophotometer [29]. The 16srRNA amplification was carried out using an ABI 9700 Applied Bio-system thermal cycler [28].

Sequencing was carried out using the Big-Dye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria, South Africa. This method was as described by Srinivasan et al. [30]. Phylogenetic analysis was done using downloaded sequences (similar sequences) from the National Center for Biotechnology Information (NCBI) data base using BLASTN prior to the edition of the obtained sequences using the bioinformatics algorithm trace editor.

2.6 DNA Extraction by Boiling Methods

DNA extraction is the separation of DNA from proteins, membranes, and other cellular materials contained in a cell. A 0.5ml of overnight broth cultures of the investigated bacterial isolates in Luria Bertani (LB) was placed in properly labelled Eppendorf tubes and were centrifuged at 14000 rpm for 3 min. The DNA, after the vortex and centrifugation processes, remained at the base of the tubes, following the decantation of supernatant fluid. The cells were re-suspended in 500 µl of normal saline and heated for 20 minutes at 95°C. The hot bacterial suspension was then cooled on ice before being spun at 14000rpm for around 3 minutes. The DNA-containing supernatant was transferred to a 1.5 ml micro-centrifuge tube and stored at -20°C for down-stream reactions [28].

2.7 DNA Quantification

DNA quantification is a procedure for determining the concentration and purity of DNA. The Nanodrop 1000 spectrophotometer was used to measure the concentration of the extracted genomic DNA. The Nanodrop spectrophotometer employs Beer Lambert's theory, which is used to assess the quality and quantity of genomic DNA. The app was launched by double-clicking on the Nanodrop button. The equipment was then blanked with regular saline and initialized with 2 µl of sterile distilled water. This involved 2 microliters of collected DNA loaded onto the lower pedestal to determine the concentration of the sample, and the upper pedestal was lowered to make contact with the DNA on the lower pedestal. The concentration of DNA was determined by pressing the "measure" button [29].

2.8 16S rRNA Amplification

An ABI 9700 Applied Biosystems Thermal Cycler was used to perform the 16srRNA amplification. On an ABI 9700 Applied Biosystem, the isolates' 16s rRNA area was amplified for 35 cycles at a final volume of 40 micro-litres using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' (forward primer) and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' (reverse primer) The DNA cocktail involved the Taq polymerase, DNTPs, MgCl, primers at a concentration of 0.5 µM, extracted DNA as template, Buffer 1X, and water. The PCR conditions included denaturation at 95°C for 5 minutes; denaturation at 95°C for 30 seconds; annealing at 52°C for 30 seconds; extension at 72°C for 30 seconds for 35 cycles and final extension at 72°C for 5 minutes. The final PCR product was resolved on a 1% agarose gel at 130V for 30 minutes and visualized with a blue light trans-illuminator [28].

2.9 Sequencing

Sequencing analysis was performed at Inqaba Biotechnical Pty Ltd, South Africa, using the Big-Dye Terminator kit on a 3510 ABI sequencer at a final volume of 10 µl, as described by Srinivasan et al., (2015) [30]. The components of the kit included 0.25ul BigDye® terminator v1.1/v3.1, 2.25ul 5 x BigDye sequencing buffer, 10uM Primer PCR primer, and a 2-10ng PCR template per 100 base pairs. The sequencing condition however, involved 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min.

2.10 Phylogenetic Analysis

This was accomplished by using BLASTN to download sequences (similar sequences) from the National Centre for Biotechnology Information (NCBI) data base, followed by the Trace edit bioinformatics algorithm to edit the collected sequences. MAFFT was used to align these sequences. The evolutionary history was inferred using MEGA 6.0's Neighbor-Joining process [31]. The evolutionary history of the taxa studied was represented by a bootstrap consensus tree inferred from 500 replicates [32]. The Jukes-Cantor method was used to determine the evolutionary distances of the identified species [33].

3. RESULTS

3.1 Occurrence of Bacterial Species in the Vaginal Samples

Bacterial isolates from the vagina in the study population is presented in Table 1. The result showed that 160 isolates belonging to five (5) genera were seen on the basis of their colonial, morphological and biochemical characteristics. The isolates were identified as *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Bacillus flexus* and *Lysinibacillus macroides*. A total of 48 *E. coli* isolates were obtained from the female genital tract of subjects in the study population. The data also revealed 38 *Staphylococcus aureus*, 10 *Klebsiella pneumoniae*, 42 *Bacillus flexus*, and 22 *Lysinibacillus macroides* isolates. The percentage occurrence of the isolates showed that *E. coli* had the highest percentage occurrence while *Klebsiella pneumoniae* had the lowest (Table 1).

3.2 Distribution of the Bacterial Isolates in Different Age Groups in the Study Population

A total of fifty-seven (57) bacterial isolates were isolated from the vagina of females within the age bracket of 18-20. Also, 67 bacterial isolates were isolated from the vagina of female within the age bracket 21-22 while 36 bacterial isolates were obtained from the vagina of female within age bracket 24-26. Furthermore, the result revealed that the age bracket 21-22 had the highest occurrence of bacterial isolates while age bracket 24-26 had the lowest occurrence of bacterial isolates as shown in Table 2.

3.3 Antibiotics Sensitivity Pattern of Vaginal Bacterial Isolates

The result of the antibiotic susceptibility pattern of the bacterial isolates is shown in Fig 1. The data showed that *E. coli* and *Staphylococcus aureus* were resistant to 50% of the antibiotics tested. While *E.coli* was resistant to Amoxicillin, clavulanate, Cefotaxime, Cefuroxime, Impenem cilaststin, Nitrofuratoin, Ampiclox, *Staphylococcus aureus* was resistant to Amoxicillin clavulanate Cefotaxime, Cefuroxime, Impenem/celaststin, Cefexime, Ceftriaxonsulbactarm. Also, *Klebsiella*

pneumoniae was susceptible to 100% of the antibiotics tested; Cefuroxime, Amoxicillin clavulanate, cefotaxime, Impenem cilaststin, ofloxacin, Gentamycin, Nalidixic acid, Nitrofuratoin, Levofloxacin, Ceftriaxone sulbactarm, Ampliclox, cefexime. *Bacillus flexus* was also susceptible to 50% of the antibiotics tested; Ciprofloxacin, Levofloxacin, Ofloxacin, Azithromycin, Gentamycin, Erthromycin. *Lysinibacillus macroides* was on the other hand susceptible to 66.6% of the antibiotics tested; Ciprofloxacin, Levofloxacin, Ceftriaxone Sulbactarm, Cefotaxime, Ofloxacin, Azithromycin, Gentamycin, Erthromycin.

Table 1. Prevalence of the bacterial Isolates in the study population

Isolates	No of Isolates	(%)
<i>Escherichia coli</i>	48	30
<i>Staphylococcus aureus</i>	38	23.75
<i>Klebsiella pneumoniae</i>	10	6.25
<i>Bacillus flexus</i>	42	26.25
<i>Lysinibacillus macrolides</i>	22	13.75
Total	160	100%

Table 2. Pattern of bacteria Isolation from different age groups in the study population

Age	<i>E. coli</i> N=48	<i>Staphylococcus aureus</i> N = 38	<i>Klebsiella pneumoniae</i> N = 10	<i>Bacillus flexus</i> N = 42	<i>Lysinibacillus macrolides</i> N= 22	TOTAL
18-20	16	15	4	12	10	57
21-22	20	13	4	20	10	67
24-26	12	10	2	10	2	36
Total	100%	100%	100%	100%	100%	160

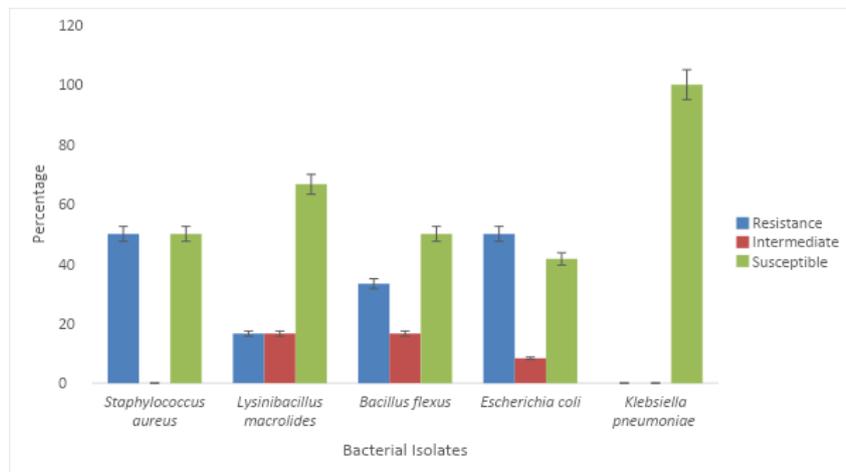


Fig. 1. Antibiotics sensitivity of the bacterial isolate

3.4 Gel Electrophoresis of the 16S rRNA and Phylogenetic Analysis

The Agarose gel electrophoresis of the amplified 16SrRNA gene of bacterial vaginal isolates before sequencing shows that Lanes B1-B5 represent the 16SrRNA gene bands (1500 bp) while Lane K represents the 100 bp molecular ladder (Plate1).

The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16S rRNA of the isolates within the *Escherichia*, *Klebsiella*, *Staphylococcus*, *Bacillus* and *Lysinibacillus* genera. The 16s rRNA sequence obtained from the isolates produced an exact match during the megablast search and revealed a closely relatedness to *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Bacillus flexus* and *Lysinibacillus macrolides* (Fig. 2).

4. DISCUSSION

This study revealed different bacteria that colonize the female genital tract. *E. coli* had the highest frequency of 30%. This is in agreement with the work of Bachir et al. [34]. Their reason was that *E. coli* is one of the most common nosocomial pathogens that cause urinary tract infections (UTIs) and enterocolitis. Furthermore, the result is in agreement with the work of

Heinonen and Miettinen, [35] who reported that vaginal colonization with *E. coli* is associated with various genitourinary, obstetric and neonatal complications, such as the severe form of pelvic inflammatory disease, urinary tract infections very-low-birth-weight infants and early-onset neonatal septicaemia and meningitis. The presence of *E. coli* in the vagina of this present study population may however, be as a result of poor practice of personal hygiene by females which includes improper washing of hands before and after defecating or not washing of hands at all, wearing of damp underwear, sharing of underwear, use of dirty public or personal toilet [23;36]. This is in agreement with the fact that the female anatomy has the vagina in proximity with the urinary channel and the anus, thereby making it prone to frequent contamination by pathogens of urinary and faecal origin.

The study also revealed the presence of other bacteria in the vagina, which included *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Bacillus flexus* and *Lysinibacillus macrolides*. This is in agreement with the work of Hacer et al. [37]. According to their report, the reason could be tired to bacterial vaginosis, which is known to be associated with a raised vaginal pH, mostly when the normal vaginal flora is replaced by mixed flora of aerobic, anaerobic and microaerophilic species. Aerobic vaginitis has been identified in

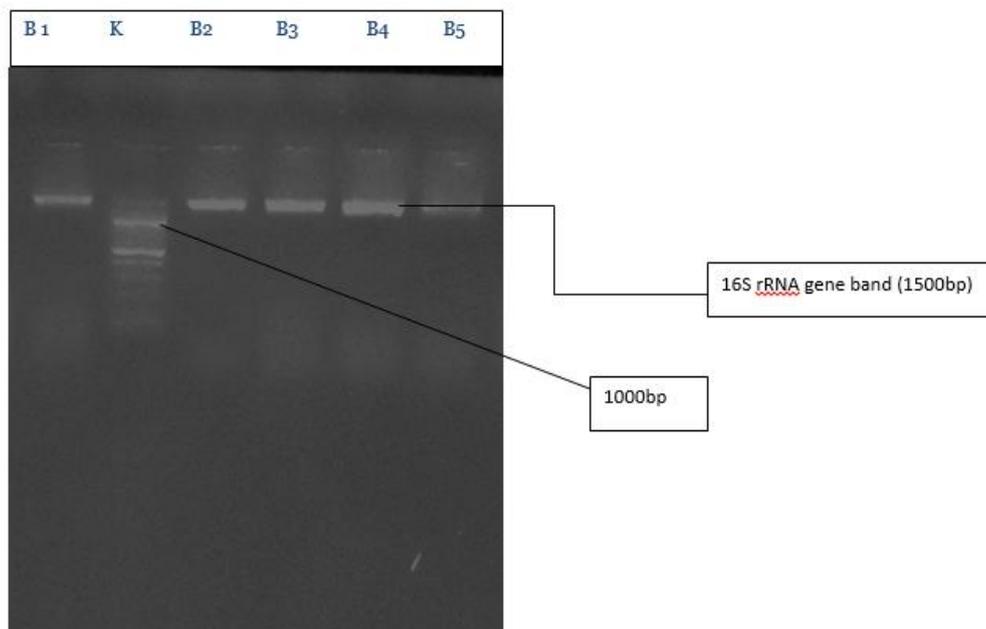


Plate 1. Agarose gel electrophoresis o the 16S rRNA gene of bacteria isolates
Lanes B1-B5 represent the 16SrRNA gene bands (1500bp), lane K represents the 1000bp molecular ladder

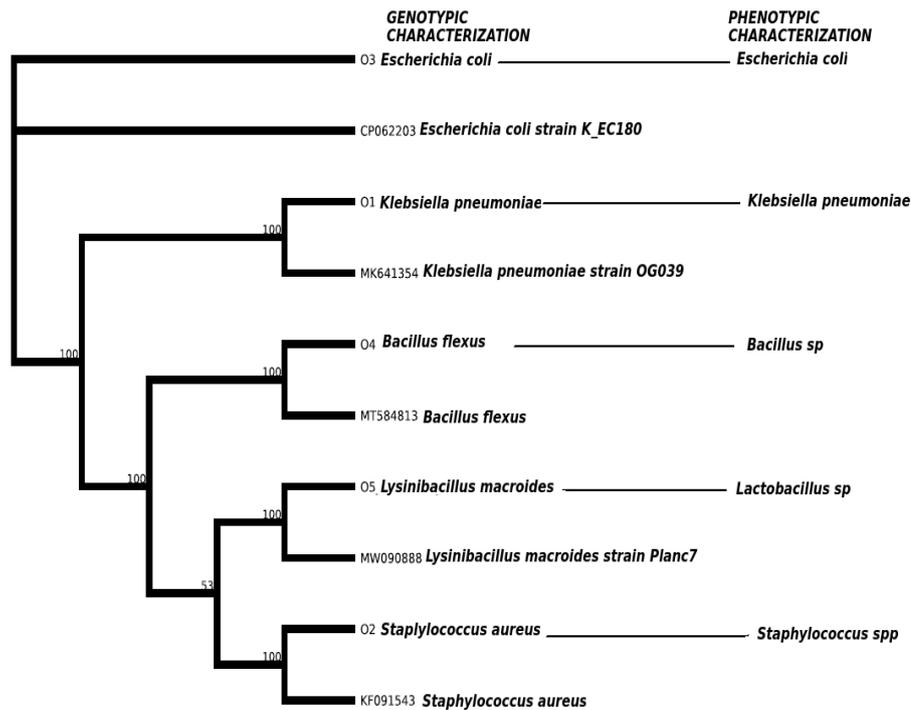


Fig. 2. Phylogenetic tree showing the evolutionary distance between the bacterial isolates

small proportion of women whose lactobacilli is dominated by facultative anaerobic or aerobic bacteria, especially *Staphylococcus aureus*, *Group B Streptococci*, *E. coli* and *Klebsiella* spp. [37-38]. The vaginal microbiota is known to be dominated by *Lactobacillus* species. This study has however, revealed that *Lysinibacillus macroides*, which is also known for antimicrobial activity, could colonize the vagina, as this was the first study to report its presence in the female genital tract. The presence of *Lysinibacillus macroides* may play an antimicrobial role in the female genital tract, as this antimicrobial potential has been reported by previous researchers [39].

The molecular identification of the bacterial species in this study has provided a reliable and important data set for understanding the pattern of bacterial colonization of the female genital tract, and at the same time enable the identification of bacterial agents like *Lysinibacillus macroides* that have not been previously isolated from the vagina. This thorough put method of bacterial identification, based on the bacterial unique nucleic acid sequence, has provided a good insight into the type of bacterial organisms lurking the vaginal microbiome. This implies that the female genital tract of a healthy female harbours bacteria genera of public health importance, as most of

the species are potential pathogens, mostly in immune-compromised conditions. The species identified to be associated with vagina in this study, are diverse not only in their disease causing potentials but also in terms of ecology. While *E. coli*, *Klebsiella* spp. are known to inhabit the colon and gut environment, *Bacillus flexus* and *Lysinibacillus macroides* on the other hand are known soil dwellers. *Staphylococcus* species is however, a normal flora of the skin and also found as predominant bacterial species in non-human environments such as soil, water and air. This therefore indicate that the species richness of the vagina maybe a function of the source of the bacterial organism. Having a healthy female genital tract will therefore require avoidance of practices that will expose this region to external sources of microbial invasion. Furthermore, maintaining a healthy immunological status is critical to the prevention of pathological disorders of the vagina.

The distribution of bacteria isolated from the vagina of different age bracket from this study showed that age bracket 21-22 had the highest percentage of bacterial population (41.87%). This may be due to lack of awareness, increased sexual activity and unhealthy practices, including lack of personal hygiene. From this study, the number of bacterial isolates increased with

increase in age, and later reduced between 24 – 26 age brackets. Age bracket 24-26 having the least percentage of bacterial isolates (22.5%) may be due to increased awareness and good knowledge of preventive measures and healthy personal hygiene practices. The Middle East and Central Asia guidelines in female genital hygiene also stressed on the importance of personal hygiene to ensure a healthy female genital tract [36]. These findings are in agreement with the findings of Chen et al., [40]. They had recommended in their review that women should be encouraged to choose a carefully formulated and clinically tested external wash that provides targeted antimicrobial and other health benefits without negatively impacting on the natural vulvovaginal microbiota.

While earlier researchers [21,41] have indicated the risk associated with the mycological profile of the female genital tract, this study has shown the bacterial community composition of the vagina and have noted that the composition varied amongst females of different age brackets, with species diversity being composed of bacterial groups with potentials to cause a pathological condition in humans, including bacterial vaginosis (BV). This therefore further buttresses the need for hygienic practices to maintain a healthy vaginal milieu.

The data from the antibiotics sensitivity result showed that the bacterial species were susceptible to most of the conventional antibiotics. This findings showed that any infection caused by these organisms, these drugs may be of therapeutic importance as the isolates showed little or no resistance to these drugs. This level of susceptibility of these isolates could possibly be a function of prior exposure of these bacterial agents to these antibiotics [23; 42]. It was further observed that *E. coli*, *Staphylococcus aureus*, *Lysinibacillus macroides* and *Bacillus flexus* were all resistant to Cefuroxime and Imepenem/celaststin in line with the findings of Riswan et al., [43], and implies that these drugs may not have any therapeutic value because the isolates may have developed resistance to the drugs.

5. CONCLUSION

From the study, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Bacillus flexus* and *Lysinibacillus macrolides* have been identified to be associated with the vaginal microbiome. Considering the bacteria present in

the vagina, it can be concluded that both pathogenic and nonpathogenic organisms are present in the vagina of humans, with females showing no sign or symptoms of pathogen presence. This study showed that bacterial isolates identified were resistant to some groups of antibiotics tested, and this can pose a serious public health problem for females associated with infection caused by these organisms. Adequate personal hygiene of humans should be adopted, and sensitization programs to improve knowledge of women should be encouraged.

DISCLAIMER

The products used for this research are commonly and predominantly used products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT AND ETHICAL APPROVAL

Informed consent obtained from respondents and ethical approval obtained from the ministry of health and preserved by authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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