LACTOBACILLUS SPECIES: COLONIZATION AFTER TREATMENT FOR BACTERIAL VAGINOSIS (BV) AND THEIR INHIBITORY POTENTIAL TO BV ASSOCIATED BACTERIA

MATRIN NDONGA/MATU

A thesis submitted in partial fulfillment of the requirements for the award of the degree of Master of Science (Biotechnology) of Kenyatta University

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Matu, Martin Ndonga
Lactobacillus species:
DECLARATION

This thesis is my original work and has not been presented for a degree in any other university or any other award.

Martin Ndonga Matu

Signature Date 26/06/2008

We confirm that the work reported in this thesis was carried out by the candidate under our supervision

Dr. George O. Orinda
Department of Biochemistry and Biotechnology
Kenyatta University
P.O. Box 43844-00100
Nairobi

Signature Date 26/06/2008

Prof. Eliud N.M. Njagi
Department of Biochemistry and Biotechnology
Kenyatta University
P.O. Box 43844-00100
Nairobi

Signature Date 26/06/2008

Dr. Elizabeth A. Bukusi
Center for Microbiology Research
Kenya Medical Research Institute
P.O. Box 19464-00202, Nairobi

Signature Date 20/06/08
DEDICATION

To my wife Esther, son Reginald and daughter Sharleen.
You make life worth living.
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ABSTRACT

Maintenance of a normal vaginal flora is important in the prevention of sexually transmitted infections including gonorrhoea and Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome (HIV/AIDS). *Lactobacillus* species predominate a healthy vaginal flora and produce chemicals that are toxic to pathogenic microorganisms. Reduction in the number of *Lactobacilli* in the vagina may result in bacterial vaginosis (BV) characterized by adverse pregnancy outcomes including preterm birth, premature rupture of uterine membranes and amnionitis. BV has also been associated with increased risk of acquisition of HIV and although this condition can be easily treated with antibiotics, recurrence after treatment is common. The reasons for the recurrence have been linked to hygiene practices such as vaginal douching, multiple sexual partners, and other sexually transmitted infections (STIs). Evidence also suggests that failure to establish normal flora following antimicrobial therapy contributes to the recurrence. The main objective of the study was characterise *Lactobacillus* species isolated from vaginal tracts of women following BV treatment and to document their inhibitory capacity against *G. vaginalis*, *B. fragilis* spp, *P. bivia* and *Mobiluncus* spp. *Lactobacillus* spp were isolated from women treated for BV two months earlier to determine the diversity of the *Lactobacillus* species recolonising the vagina post treatment. *Lactobacilli* were also tested for hydrogen peroxide (H$_2$O$_2$) production and their inhibitory potential to pathogens associated with BV. The organisms were identified by morphologically and by biochemicals. H$_2$O$_2$ production was determined using tetramethylbenzidine agar and inhibitory activity by a deferred antagonism well assay in which *Lactobacilli* suspension was inoculated into wells cut in agar to which *Gardnerella vaginalis*, *Bacteroides fragilis*, *Prevotella bivia* and *Mobiluncus* spp suspension was seeded. The prevalence of BV among the Women tested was 38.3%. A wide diversity of *Lactobacilli* was identified with with *L. jensenii* being the most predominant *Lactobacilli* (18.4%). Thirty seven percent of the women were colonized by H$_2$O$_2$ producing *Lactobacilli*, and of these the majority of those with the H$_2$O$_2$ positive *Lactobacilli* had BV associated micro flora. When inhibitory potential of the *Lactobacilli* was tested, 23% were inhibitory to *G. vaginalis*, *P. bivia* and *Mobiluncus* spp. None of the *Lactobacilli* tested inhibited *B. fragilis*. Only about a third (37%) of the women were colonized with H$_2$O$_2$ positive *Lactobacilli* (LB). It was also observed that H$_2$O$_2$ positive LB were more inhibitory to indicator bacteria and therefore important in preventing growth of pathogens. Therefore, in a large proportion of women, H$_2$O$_2$ positive LB failed to recolonize following treatment and as a result remain unprotected from colonization with pathogens. In conclusion, the results of this study found that in spite of the *Lactobacillus* strains being capable of producing H$_2$O$_2$, acid and bacteriocins, not all strains producing each of these substances were inhibitory; for example, despite some strains producing H$_2$O$_2$ they were not inhibitory, some produced highly acidic culture supernatants yet they would not inhibit the indicator bacteria. Therefore we would suggest that there could be a synergistic action of these substances to make the overall effect. Based on the results of this study, it would be recommended that in management of BV, conventional treatment using antibiotics like metronindazole should be augmented by biotherapy using *Lactobacilli* producing inhibitory compounds to re-establish a normal vaginal microflora.
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LIST OF ABBREVIATIONS

AVF  Altered Vaginal Flora
BV   Bacterial vaginosis
DGGE Denaturing Gradient Gel Electrophoresis
DNA  Deoxyribonucleic Acid
H$_2$O$_2$ Hydrogen peroxide
HIV-I Human Immunodeficiency Virus Type I
OIF  Oil Immersion field
PFGE Pulse Fluid Gel Electrophoresis
PID  Pelvic Inflammatory Diseases
RAPD Randomly Amplified Polymorphic DNA
rRNA ribosomal Ribonucleic acid
SDS-PAGE Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
STD  Sexually Transmitted Disease
TGGE Temperature Gradient Gel Electrophoresis
TMB  Tetramethylbenzidine
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CHAPTER ONE
INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

*Lactobacillus* species are unambiguously present in the environment. They colonise plants, animals and humans (Collins *et al.*, 1991). In humans, *Lactobacilli* may colonise: urogenital tract, gastrointestinal tract and the oral cavity (Pavlova *et al.*, 2002; Horie *et al.*, 2002). *Lactobacilli* play an important role in the maintenance of a healthy vaginal and gastrointestinal tract of humans (Redondo-Lopez *et al.*, 1990). They have been found to contain antimicrobial compounds such as organic acids, hydrogen peroxide (H$_2$O$_2$) and bacteriocins that control undesirable microflora in both the gut and the vaginal tract (Oyetayo, 2004; Itoh *et al.*, 1995).

In healthy women, *Lactobacilli* are the dominant species in the vaginal microbiota (Eschenbach *et al.*, 1989; Antonio *et al.*, 2003). Certain predisposing factors such as vaginal douching, recent change of sexual partner, having multiple partners, certain types of contraceptives and taking antibiotics (like ampicillin or amoxicillin) results in a loss of *Lactobacilli*. This leads to overgrowth of other anaerobes and facultative anaerobes in the vaginal tract resulting to a condition called bacterial vaginosis (BV) (Wilson, 2004). BV is characterized by decreased hydrogen peroxide (H$_2$O$_2$) producing *Lactobacilli* and increased concentrations of facultative and strictly anaerobic bacteria including *Gardnerella vaginalis*, *Bacteriodes* species and *Prevotella* species. BV is also accompanied by a shift in vaginal microflora from the predominantly *Lactobacilli* flora to 100-fold increase in gram variable coccobacilli consistent with *G. vaginalis* or *Bacteroides* species (Hillier and Holmes, 1999). BV is also associated with various...
Antibiotic therapy with metronidazole or clindamycin has been shown to eliminate BV associated bacteria. However, there is a high recurrence rate of BV with up to 50% of treated patients experiencing recurrent infection (Hillier et al., 1993a). Some authors speculate that most relapses are having multiple partners (Wilson, 2004), while others suggest recurrence occur as a result of failure to establish normal flora following antimicrobial therapy (Hillier et al., 1993). For this reason, this study was designed to address this issue by characterizing Lactobacillus spp from vaginal tracts of women post BV treatment and assess their inhibitory effect against G. vaginalis, B. fragilis spp, P. bivia and Mobiluncus spp.

1.2 Literature Review

1.2.1 Description of Lactobacilli

Hillier and Holmes (1999) described a non-motile bacillus that he considered to be normal flora of vaginal tract of pregnant women. They isolated both facultative and strictly anaerobic microbes showing a mixture of microorganisms including Lactobacilli that constituted a normal vaginal flora. Lactobacillus species which dominates the normal vaginal flora are gram positive, facultative anaerobic or strict anaerobic, non-motile, non-spore-forming and fermentative organotrophs (Donders et al., 2000). They are usually straight, although they can form
spiral or coccobacillary forms under certain conditions. They are often found in pairs or chains of varying length (Altermann et al., 2005). Their sizes range from 0.7-1.1 x 2.0-4.0 μm and constitute members of the industrially important lactic acid bacteria. They are acid tolerant, cannot synthesize porphyrins, and possess a strictly fermentative metabolism with lactic acid as the major metabolic end product (Axelsson, 1998). Within this genus of lactic acid bacteria is a diverse assemblage of gram positive, catalase negative, indole negative organisms that include over 25 species (Collins et al., 1991).

1.2.2 Classification of *Lactobacillus* spp

*Lactobacilli* are classified as lactic acid bacteria, and derive almost all their energy from the conversion of glucose to lactate during homo lactic fermentation. In this process 85-90% of the sugar utilized is converted to lactic acid. They generate ATP by nonoxidative substrate-level phosphorylation (Altermann et al., 2005). They fall under the following taxonomic levels: Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; *Lactobacillus*. They are further categorised into the following distinct species; *L. fermentum*, *L. acidophilus* *L. plantanum*, *L. brevis*, *L. jensenii*, *L. vaginalis*, *L. dubreickii*, *L. salivarium* (Redondo-lopez et al., 1990). *Lactobacillus acidophilus* is a heterogeneous group constituting most of the healthy vaginal *Lactobacillus* flora (Vasquez et al., 2002). This is further divided into deoxyribonucleic acid (DNA) homology groups A and B. Group A includes; *L. acidophilus* (A1), *L. crispatus* (A2), *L. amylovoruos* (A3), *L. gallinarum* (A4), while group B constitutes *L. gasseri* (B1) and *L. johnsonii* (B2) (Johnson et al., 1980). According to DNA-DNA hybridization method, *L. crispatus*, *L. gasseri* and *L. jensenii* are the most common species found in the vagina (Giorgi et al., 1987; Antonio et al., 1999; Song et al., 1999). The less common species include *L.
ruminis, L. oris, L. reuteri and L. vaginalis. A new species, L. iners also colonizes the human vagina (Falsen et al., 1999).

1.2.3 Identification of Lactobacillus

1.2.3.1 Phenotyping

*Lactobacillus* species has been traditionally identified based on carbohydrate fermentation, morphology, gram staining (Tynkkynen et al., 1999) and highly standardized whole cell protein patterns obtained by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Pot et al., 1993). Other tests include biochemical methods such as catalase, nitrate reduction, indole production, aerotolerance and carbohydrate profiles (Jousimies-somer et al., 2002). Telcoplanin and vancomycin resistance can also be used for *Lactobacillus* species identification (Felten et al., 1999).

1.2.3.2 Molecular techniques

There have been significant advances in the recent years in the characterization and identification of bacteria by molecular methods. *Lactobacilli* have received particular attention due to their probiotics properties (Reid et al., 2001) and the application of genomic analyses has advanced the taxonomy of *Lactobacilli*. Based on DNA homology studies, the previous *L. acidophilus* species has been divided into six DNA homology groups that could not be distinguished biochemically (Johnson et al., 1980). These homology groups were characterized later as six distinct species: *L. acidophilus, L. crispatus, L. amylovorus, L. gallinarum, L. gasseri* and *L. johnsonii* (Du Plessis and Dicks 1995). DNA-DNA hybridization is one method that provides more resolution than 16S rDNA sequencing, and the 70% criterion (Wayne et al., 1987) has been a cornerstone for describing a bacterial species. In spite of these values, the method is not popular. Major
disadvantages are the laborious nature of pairwise cross-hybridizations, the requirement for isotope use, and the impossibility of establishing a central database. At present, more advanced genotypic methods are available to study microbial taxonomy. Most molecular methods for used detection and identification of Lactobacillus species are based on PCR amplification of nucleotide sequence of 16S ribosomal RNA (rRNA). The PCR products are then separated by suitable methods such as denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) of 16S amplicons (Walter et al., 2001).

Cloning and sequencing of the 16S ribosomal DNA (rDNA) pools in a population sample provides a method for obtaining sequence-level information on uncultivable bacteria. The use of 16S rRNA phylogenetic sequence analysis is now accepted as a reliable molecular method for identification of microbial communities (Collins et al., 1991). Suau et al. (1999) analyzed the sequence of 284 16S rDNA clones derived from one faecal sample and classified the clones into 82 molecular species, using 98% similarity criteria for a species. Direct cloning approach gives an idea of the total microbiota present in a complex population and also facilitates determination of species-level differences between bacterial populations, as shown by the discovery of novel phylotypes (Becker et al., 2002). Study on the diversity of vaginal Lactobacilli from women in different countries, based on 16S rRNA gene sequences, has been done (Pavlova et al., 2002). Approaches based on cloning are, however, rather tedious and are not optimal for analysis of large numbers of samples. Like all PCR-dependent methods, construction of clone libraries may be prone to biasing, possibly leading to falsification of the library structure (Bonnet et al., 2002). Restriction profiling 16S-23S rRNA is another molecular method for characterizing microbes. It uses in silico predictions of restriction patterns of 16S-23S rRNA short intergenic spacer of
selected *Lactobacillus* species and other related bacteria frequently isolated from the same sources as *Lactobacilli*. In this method PCR amplification of 16S-23S rRNA intergenic spacers is followed by digestion with a set of restriction enzymes with 6 bases recognition sequences. The restricted fragments are visualized by gel electrophoresis. This technique is faster and relatively cheap (Moreira et al., 2005).

Although for most bacteria different patterns can be predicted, some species present identical patterns and make it hard to differentiate the related strains (Moreira et al., 2005). Terminal restriction fragment length polymorphism (T-RFLP) is based on endonuclease digestion of PCR-amplified DNA and capillary electrophoresis analysis of the terminal restriction fragment (TRF) containing a fluorescent label. Terminal restriction patterns have been used to analyse marine bacterioplankton communities (Moeseneder et al., 2001) as well as faecal bacteria (Kaplan et al., 2001). The method is, however, limited by the choice of primers, which can, with their different affinities, dramatically change the patterns observed, while another problem is the TRF length overlap by phylogenetically distant bacteria (Kaplan et al., 2001).

In Pulsed Field Gel Electrophoresis (PFGE) and ribotyping analysis, *Lactobacillus* spp genomic DNA is digested with restriction enzymes. In PFGE (Schwartz and Cantor, 1984), rare-cutting enzymes are used and large genomic fragments are separated. PFGE analysis alone, performed with two or three appropriate enzymes, can be used for reliable strain typing. In several *Lactobacillus* studies, PFGE has been shown to be a powerful method for strain typing (Bjorkroth et al., 1996; Ferrero et al., 1996), and therefore useful in epidemiological studies (Tenover et al., 1995). It is however, laborious and expensive method and therefore it is not possible to analyze a large of samples. In ribotyping (Stull
et al., 1988), rRNA genes and/or their spacer regions are used as probes that hybridize with genomic restriction fragments. Ribotyping can be done automatically (RiboPrinter) and is therefore easily applied, but the equipment is rather expensive (Tynkkynen et al., 1999). Randomly Amplified Polymorphic DNA (RAPD) analysis is also used for identification of Lactobacillus spp. In RAPD analysis, short arbitrary sequences are used as primers in PCR, which yields strain-specific amplification product patterns. RAPD analysis is a rapid and cheap method, but careful optimization is needed to ensure the repeatability of the results (Bjorkroth et al., 1996). Southern -type hybridization can also be used for Lactobacillus spp identification. This technique uses DNA restricted with BglII (restriction enzyme) and probed with Lactobacillus plantarum genes of pyrimidine de novo pathway (Bringel et al., 1996). Among these molecular techniques, the 16S RNA analysis has been accepted as a more reliable method (Pavlova et al., 2002; Collins et al., 1991).

1.2.4 Economic importance of Lactobacillus

Lactobacilli are widely distributed in humans. They are adjuvants against gastrointestinal and urogenital disorders and dietary supplements as well as biological food processors in view of their fermentative properties (Amsel et al., 1983). Disruption of Lactobacillus flora has been associated with many urogenital infections afflicting people worldwide (Zhong et al., 1998). Many people resort to taking food products containing Lactobacillus as means of trying to maintain a healthy intestinal and in some cases vaginal flora (Zhong et al., 1998). Doderlein is quoted to have first used the Lactobacilli to treat gonorrhoea among commercial sex workers in 1892 (Hillier and Holmes, 1999). In the past two decades, the application of probiotics for prevention and management of gastrointestinal disorders has received much interest (Ouwehand et al., 2002). Probiotics are live
microorganisms which, when administered in adequate amounts, confer a health benefit to the host (FAO/WHO, 2001; Reid et al., 2001). Lactobacilli are commonly used as probiotics (De Vuyst et al., 2004). Well-designed clinical trials support the effectiveness of probiotics in keeping the human vagina and colon healthy (Gill and Guarner, 2004).

1.2.5 Role of Lactobacillus in the vaginal tract

Lactobacilli are widespread commensal bacteria in human and animal microflora (Felten et al., 1999). They are the predominant microorganism in normal microbial flora of vagina and intestines. They are habitats of intestinal flora together with other obligatory anaerobic bacteria (Hans et al., 2002). Lactobacilli are believed to play an important role in stabilization of the micro-flora by providing an important microbial defence of vaginal and intestinal colonization by exogenous pathogenic microorganisms (Fujisawa et al., 1992). They are therefore considered important for maintenance of a healthy gastrointestinal and urogenital tract (Zhong et al., 1998). Lactobacilli restrict the growth of pathogens through several mechanisms as shown in figure 1. First, they produce antimicrobial compounds including lactic acid (Oyetayo, 2004), Hydrogen peroxide (Pascual et al., 2006; Klebanoff et al., 1991; Eschenbach et al., 1989) and bacteriocins (Alpay et al., 2003; Sobel, 1999). In addition, Lactobacilli exert a protective role against pathogens by competition for nutrients and exclusion of the pathogens to adhesion on host cells (Vesterlund et al., 2006; Reid and Burton, 2002).

In vitro, Lactobacilli are lethal to various microorganisms including BV associated organisms such as G. vaginalis, Bacteroides spp, Prevotella bivia and Mobiluncus spp (McLean and Rosenstein, 2000). It has been documented that Lactobacilli can competitively inhibit other pathogens from colonising and subsequently
inhibits them from causing infection (Piyawan et al., 2006). *Lactobacilli* are believed to interfere with pathogens by competitive exclusion of genitourinary pathogens from receptors present on the surface of the genitourinary epithelium (Boris et al., 1997; Aslim and Kilic 2006). Secondly, *Lactobacilli* coaggregate with some uropathogenic bacteria and produce antimicrobial compounds that inhibit the growth of the pathogens (Boris et al., 1997).

**Figure 1:** *Lactobacilli* by products of metabolism with an antagonistic effect against urogenital pathogens. The biosurfactants inhibit adhesion; the acids, bacteriocins, and hydrogen peroxide inhibit growth; and the coaggregation molecules block the spread of the pathogens (Reid and Burton, 2002).

Among the antimicrobial compounds produced is lactic acid that is produced from the metabolism of carbohydrates that helps to maintain the vaginal pH at <4.5, thereby creating an inhospitable environment for growth of most endogenic bacteria (Sobel, 1999; Alpay et al., 2003; Kaewsrichan et al., 2006). There is little glucose or glycogen in premenarchal girls. During adolescence, glycogen is deposited in the vaginal epithelium under the influence of estrogen (Hillier and Holmes, 1999). Glycogen is metabolized by vaginal epithelial cells to glucose, which then serves as a substrate for *Lactobacillus* to convert glucose to lactic acid. *Lactobacilli* produce lactic acid from glucose keeping the
vagina at an acidic pH in the reproductive age. Vaginal microflora of women is directly related to the level of estrogen (Hiller and Holmes, 1999).

*Lactobacillus* spp in addition to producing lactic acid also produce $\text{H}_2\text{O}_2$ that is toxic to a wide variety of microorganisms. The $\text{H}_2\text{O}_2$ interacts with peroxidases produced by host along with halide ion. The product of this reaction is a potent oxidant, toxic to many bacteria (Hiller and Holmes, 1999). The $\text{H}_2\text{O}_2$ formed by *Lactobacilli* may inhibit or kill other members of microbiota particularly those that lack or have low levels of catalase peroxidase. Hydrogen peroxide is required for peroxidase catalysed microbial antagonism (Eschenbach *et al.*, 1989). The $\text{H}_2\text{O}_2$ producing strains play a role in controlling the microenvironment of vagina and inhibiting the overgrowth of potentially pathogenic microbes including HIV-1 (Harold *et al.*, 1999).

Bacteriocins and bacteriocin-like substances production by vaginal *Lactobacilli* has largely been demonstrated (Sobel, 1999; Alpay *et al.*, 2003; Kaewsrichan *et al.*, 2006; Piyawan *et al.*, 2006). Bacteriocins are bactericidal proteinaceous molecules produced by bacteria. They are classified into three classes according to their size, microbial target and mechanisms of action and immunity (Jack *et al.*, 1995). However, the term ‘bacteriocin-like compound’ is applied to antagonistic substances that do not fit the typical criteria of bacteriocins. They have been reported to inhibit a wide range of both gram-positive and gram-negative bacteria as well as fungi (McGroarty, 1993).

### 1.2.6 Bacterial Vaginosis (BV)

When pathogenic bacteria outnumber the bacteria that form the normal flora comprising predominantly of the *Lactobacillus* spp, there is an imbalance in the bacterial organisms
that exist in the vagina. Instead, bacteria such as *Bacteroides* spp, *Gardnerella vaginalis*, *Mobiluncus* spp and *Mycoplasma hominis* predominate and result in causing BV. BV is accompanied by decreased vaginal acidity, low concentration of *Lactobacilli* and 100-fold or more increase in facultative and anaerobic bacteria including *Gardnerella vaginalis*, *Bacteroides* species and *Mobiluncus* species. BV produces symptoms of increased quantities of malodorous vaginal discharge (Goldenberg *et al.*, 1996), of elevated pH (>4.5), fishy odour on addition of 10 % potassium hydroxide (KOH) and presence of clue cells (Hillier and Holmes, 1999).

1.2.7 Epidemiology of BV

Prevalence of BV varies widely because of differing diagnostic criteria (Goldenberg *et al.*, 1996) and the population studied (Mc Lean and Rosenstein, 2000). A recent study in the United States showed that prevalence was 16.6% among pregnant women at 23-26 weeks gestation. Prevalence varies widely with ethnicity from a low prevalence of 6.1% in Asians to 8.8% in Caucasian women, 15.9% in Hispanics and 22.7% African-American women (Goldenberg *et al.*, 1996). In Morocco, a prevalence of 19% was found among women attending family planning clinics and 24% among those attending primary health care (Ryan *et al.*, 1998). Prevalence of 11% has been reported in a gynaecological outpatient clinic in United Kingdom (Mc Lean and Rosenstein, 2000). In Lima Peru, BV was found in 25% of asymptomatic women and 37% women with symptoms of vaginal discharge seen in gynaecology clinic. Women attending sexually transmitted disease clinics (STD) have high prevalence than those visiting the health facilities for primary health care and family planning. Female commercial sex workers in Thailand showed 33% prevalence compared to 16% pregnant women (Hiller and Holmes, 1999). The
highest prevalence of BV was reported in rural Ugandan women with a prevalence of
51% (Sewankambo et al., 1997).

1.2.8 Acquisition of BV

Women with 3 or more partners have been shown to have a relative risk of about 1.7
times for acquisition of BV compared to women with one or no partners (Barbone et al.,
1990) and an increased risk with having two or more partners in the prior three months
(Avonts et al., 1990). This suggests a heterosexual mode of transmission. Hammerschlag
et al. (1985) examined eight girls aged 5-12 years for BY at their initial visit for
evaluation of sexual abuse and again two or more weeks later. None of the girls had clue
cells or fishy odour on addition of 10% KOH at initial visit. At the follow up visit 2 girls
had clue cells only, two girls had odour only, 4 had both odour and clue cells therefore
based on this criterion 50% developed BV within this period of alleged sexual abuse.
Jenny et al. (1990) examined 204 girls within 72 hours of rape and again two weeks after
the initial visit, 12.8% of 204 subjects had BV on the initial visit and an additional 19.5%
developed BV between first and second examination.

Berger et al. (1995) diagnosed BV in 28.7% of 101 participants in study of lesbians and
their partners seeking care for vaginal symptoms. BV was present in 8 of 11 women
whose partners had BV; the likelihood of a partner having BV was 19.7 times greater if
the index case had BV (p<0.008; 95% confidence interval CI). Fetchers et al. (2000) in a
retrospective cross-sectional study of 1408 women lesbians and 1,423 controls found BV
more common among the former (odds ratio 1.7, p=0.001).
Schwebke et al. (1999) had patients use diaries and make smears from self collected vaginal fluid to correlate microbial flora with behavioural activity. Gram stains were interpreted using Nugent’s criteria. Women with unstable flora recorded $3.5 \pm 0.9$ episodes of receptive oral sex per month compared to women with stable flora. Risk of acquisition of BV has also been associated with vaginal douching. Schwebke et al. (2004) demonstrated a positive association of douching and risk of BV acquisition. Increased frequency of douching has been associated with increased prevalence of BV. Watcharotone et al. (2004) showed that there is significantly increased prevalence of BV among those women who used douching inside the vagina.

The use of contraceptives has also been associated with BV. Many studies have demonstrated a positive association between intrauterine contraceptive device (IUD) uses with increased prevalence of BV compared to non-IUD users (Demirezen et al., 2005; Joesoef et al., 2001; Hodoglugil et al., 2000; Calzolari et al., 2000; Ceruti et al., 1994; Elhag et al., 1988). However, oral contraceptives and condom use have been associated with reduced rates of BV (Watcharotone et al., 2004; Calzolari et al., 2000; Ceruti et al., 1994). Shoubnikova et al. (1997) in a study on relationship between contraceptive use and bacterial vaginosis found a negative association between BV and oral contraceptives and condom use. Eschenbach et al. (2000) found that the vaginal flora remain unchanged after OC use for a period of two months. Spermicide use have not been found to have effect in development of BV (McGroarty et al., 1994); they have been reported to kill a wide range of bacteria and viruses including HIV in vitro and to protect in vivo from infection by gonorrhoea, chlamydia and pelvic inflammatory disease (Kirkman and Chantler, 1993). Diaphragm use has been associated with increased incidence of candidal
infections, urinary tract infections, and bacterial vaginosis (Tagg, 1995; Kirkman and Chantler, 1993).

1.2.9 Pathogenesis of BV

There are multiple factors that have been shown to contribute to the pathogenesis of BV. The protective effect of \( \mathrm{H}_2\mathrm{O}_2 \) producing \textit{Lactobacillus} has been shown. Hydrogen peroxide combines with halide to kill \textit{Prevotella bivia} and \textit{G. vaginalis in vitro} and the concentration of both analytes in vaginal fluid are sufficient to be functional \textit{in situ} (Klebanoff \textit{et al.}, 1991). BV is so called because it is characterised by increased vaginal discharge without concomitant inflammatory cells. Sturm (1989) reported inhibition of chemotaxis by succinic acid that is produced by \textit{P. bivia}, \textit{Bacteroides fragilis} and \textit{Mobiluncus} spp and therefore the vaginal discharge produced in BV do not contain inflammatory cells. \textit{Gardnerella vaginalis} produces a haemolysin (Gvh) with cytolytic activity that can be demonstrated \textit{in vitro}. This toxin elicits a specific Immunoglobulin A (IgA) antibody response in only a subset of women with BV. Pybus and Onderdonk (1997) showed that there is synergistic relationship between \textit{P. bivia} amino acids breakdown to produce ammonia and consumption of ammonia by \textit{G. vaginalis} to produce amino acids. Kilic \textit{et al.} (2001) has documented the presence of bacteriophage among vaginal \textit{Lactobacilli} in United States and Turkey, 30(28%) of 107 USA strains and 37 (36%) of 102 Turkish strains of \textit{Lactobacilli} had lysogenic phage for the overall rate of 32%, 4 genetic types were found among the USA isolates and three among the Turkish. These temperate phages were able to cause lytic infection in multiple strains of \textit{Lactobacilli}. 
Figure 2: Suggested flow diagram for pathogenesis of bacterial vaginosis (Spiegel, 2002).

Figure 2 shows the proposed pathogenesis of BV (Spiegel, 2002). It has been suggested that pathogenesis of BV therefore involves lysis of endogenous *Lactobacilli* by bacteriophage. Removal of this source of H$_2$O$_2$ and lactate leads to overgrowth of *Mobiluncus* spp and *Prevotella* spp both of which produce succinic acid which inhibits chemotaxis of white blood cells (WBCs). *Mobiluncus* spp produces trimethylamine the source of fishy odour. *Prevotella bivia* degrades amino acids to produce ammonia that *G.vaginalis* uses to produce amino acids. The increased ammonia and decreased lactic acid to elevated vaginal fluid pH characteristic of BV. *Prevotella bivia* and some strains of *P. disiens* and *G.vaginalis* produce sialidases that cleaves mucin leading to a
homogenous discharge and allowing for enhanced bacterial adhesion on vaginal epithelial cells to form clue cells (Spiegel, 2002).

1.2.10 BV Sequelae

BV has been associated with various gynaecological and obstetric complications including pelvic inflammatory diseases (PID), endometritis (Sewankambo, *et al.*, 1997), preterm birth, premature rupture of membranes, choriamnionitis and low birth weights (McLean and Rosenstein, 2000). It has also been shown to be a risk factor for non-obstetrical infection including postpartum infections, cuff cellulites, post-abortal and neonatal scalp abscesses, and non-peurperal breast abscesses (Hillier and Holmes, 1999; Spiegel, 1993). Flora associated with BV has been recovered from the placenta, chorioamnion and amniotic fluid of patients with clinical disease. Goldenberg *et al.* (1996) accounts for 40% attributable risk for spontaneous birth at less than 32 weeks gestation of pregnancy to BV. Increase of vaginal pH to alkalinity is a risk factor for preterm delivery. Pregnant women with disturbed vaginal flora associated with BV have increased amnionitis postpartum endometritis and preterm delivery, whereas, women with predominant *Lactobacilli* flora have a low risk of preterm delivery. Abnormal microbiological flora of lower genital tract during pregnancy and cervical-vaginal infections are therefore causes of perinatal complications (Martius *et al.*, 1988). Camargo *et al.* (2005) in a study on pregnant women showed that treatment of BV significantly reduces the rates of premature birth and other perinatal complications regardless of the history of previous preterm delivery.

There is growing literature on the relationship between BV and the risk of acquiring HIV-1 infection (Moodley *et al.*, 2002; Cu-Uvin *et al.*, 2001). *Gardnerella vaginalis* has been
shown to activate HIV expression in monocytes and some T-cells (Hashemi et al., 1999). Cohen et al. (1995) in a cross-sectional study of female sex workers in Thailand found a positive association of clinical diagnosis of BV and HIV-1 seropositivity. Royce et al. (1999) in another study done in North Carolina showed increased prevalence of HIV-1 with increase in vaginal flora score. Harold et al. (1999) showed an inverse relationship between vaginal colonization with Lactobacillus species and risk of acquisition of HIV-1 infection and N. gonorrhoea in Kenya. BV affects the aspects of HIV infectivity (infectiousness and susceptibility) by directly affecting the survival of HIV in genital tract or by prompting immune activation (proliferation of CD4+ cells or production of cytokines). It has been observed that pregnant HIV-seronegative women with clinically defined BV are 1.5-2.3 times likely to seroconvert during postnatal or prenatal period as compared to those without BV (Goldenberg et al., 1996). A low vaginal pH may inhibit lymphocyte activation and therefore decrease in HIV target cells in the vagina (Ryan et al., 1998). Conversely, elevated pH makes the vagina more conducive to HIV survival and adherence (Sanchez et al., 1998). Bacterial vaginosis raises the levels of intravaginal interleukin 10, which increases susceptibility of macrophages to HIV (Hallen et al., 1987). Findings show that a heat stable protein produced by G. vaginalis increases production (replication) of HIV by HIV infected cells to as much as 77 fold (Cohen et al., 1995) and that Mycoplasma hominis is the most potent inducer of HIV-1 expression among several bacterial and fungal species studied (Sewankambo et al., 1997). Bacterial vaginosis has recently been associated with increased herpes simplex type 2 (HSV-2) shedding in the vaginal tract and therefore that of HIV-1 (Cherpes et al., 2005).
1.2.11 Sampling and Diagnosis of BV

1.2.11.1 Sampling and physical examination

Discharge is collected from the posterior fornix, taking care to avoid the cervix. A minimum of 2 swabs are submitted to the laboratory. The vaginal sample can either be self-collected or collected by a clinician. Few studies have evaluated self-sampling techniques for diagnosis of BV. Wilkinson et al. (1997) compared specimens collected on tampons by the subjects and high vaginal swab (HVS) collected by the clinician. Twenty-four women with gram stain diagnosis of BV on specimen collected by Clinician, 22 had similar gram stain as the tampon collected specimens a specificity and sensitivity of 100% and 88.3% respectively.

Figure 3: Obtaining a sample of vaginal discharge (http://www.afraidtoask.com)

Diagnosis is made by physical examination with observation made with the naked eye and nose and also carrying out a pelvic examination. A sample of the vaginal discharge is obtained from posterior fornix (figure 3) and viewed under the microscope, either stained
or in special lighting, to determine the absence of *Lactobacilli* and presence of *Gardnerella* spp to confirm by microscopic examination. The vaginal fluid is also tested for change in pH (decreased acidity) and whether the vaginal cells have the classic appearance of 'clue cells. The sample is mixed with potassium hydroxide and produces a strong fishy odor when the BV associated bacteria are present (http://herpes-coldsores.com/std/bacterial_vaginosis.htm).

### 1.2.11.2 Nugent’s criteria

BV can be diagnosed in the laboratory by gram stain of vaginal fluid using method devised by Nugent *et al.* (1991). Gram stain detects changes in vaginal flora from normal to BV (Spiegel *et al*., 1983). Facultative *Lactobacillus* species (large gram positive rods) predominate in a healthy vagina. In BV, *Lactobacillus* species morphotypes are diminished or undetectable microscopically and have been replaced by large numbers of small gram variable (*G. vaginalis*) and small gram negative short rods including *Bacteroides* species, *Prevotella* species, *Porphyromonas* species with or without curved gram variable rods (*Mobiluncus species*). Gram-positive cocci in chains and fusiform gram-negative rods are occasionally observed (Nugent *et al*., 1991). A scoring system was developed to provide a more standardized method for interpretation of gram stain, the bacterial morphology are quantified under oil immersion field (OIF) as 0+ (none) seen, 1+ (<1/OIF) seen, 2+ (1 – 5/OIF) seen, 3+ (6 – 30/OIF) seen and 4+ (>30/OIF) seen and a score assigned to each of the three groups. The score of each of the three morphotypes is summed and a score of ‘0 – 3’ is interpreted as normal, ‘4 – 6’ as ‘intermediate'/altered vaginal flora (AVF) and ‘7 – 10’ as BV (Nugent *et al*., 1991) (Figure 4).
Culture for BV diagnosis is not recommended because *G. vaginalis* do not serve as a marker for BV and are present in the vagina of more than 60% of women with normal examination (Schwebke *et al.* 1997).

### 1.2.11.3 Amsel criteria

BV is clinically diagnosed by presence of three of the following signs: Greyish, frothy, adherent and homogenous discharge, vaginal fluid pH > 4.5, fishy odour on addition of 10% KOH to the discharge (Positive Whiff Test) or presence of clue cells (figure 5) (Spiegel, 2002; Briselden and Hillier, 1994; Amsel *et al.*, 1983). The fishy odour is due to presence of amines in the vaginal fluid that become volatile at increased pH. Trimethylamine is likely to be the source of odour though putrescine and cadaverine may also be present (Chen *et al.*, 1979).
1.2.12 Treatment and management of BV

1.2.12.1 Conventional treatment

BV can be difficult to cure using either conventional or alternative treatments (http://herpes-coldsores.com/std/bacterial_vaginosis.htm). The primary goal for treating BV is to relieve signs and symptoms of infection and also to reduce abortion, PID and other complications. Conventional treatment involves use of antibiotics to clear the BV associated bacteria. These antibiotics include ampicillin, ceftriaxone, clindamycin, tetracycline and metronidazole taken orally or as intravaginal forms. The most common therapy is metronidazole available in oral (pill) form or in a gel that can be applied into the vagina. Clindamycin is also available in oral and intravaginal form (http://www.4woman.gov). Although it is uncertain if the infection is sexually transmitted, sexually active women appear to suffer from the infection more than other
women. Evidence suggests heterosexual transmission as one way of BV acquisition and transmission. Several factors are recommended to prevent acquisition and transmission of BV; abstinence from sex until the infection is cured and all symptoms have ceased, informing any sex partners so treatment may be undertaken, limit sexual relationships to a single, uninfected partner and regular use of condoms (http://herpes-coldsores.com/std/bacterial_vaginosis.htm).

1.2.12.2 Biotherapy

Biotherapy is management of diseases using live organisms such as probiotics (Ellis and Verma, 2000). Recent studies have emphasized the importance of a healthy, *Lactobacilli* dominated flora not only to prevent sexually transmitted diseases and preterm labour, but also to maintain the quality of life of women (Ellis and Verma, 2000). *Lactobacilli* play a role in the stabilisation of vaginal microflora by providing important microbial defence against vaginal colonisation by exogenous pathogenic micro-organisms such as *Gadnerella vaginalis, Bacteroides* spp, *Prevotella bivia* and *Mobiluncus* spp (Song et al., 1999). In an attempt to develop a non-chemotherapeutic means to restore and maintain a healthy urogenital tract, probiotic therapy using *Lactobacilli* has been considered, and there is evidence to indicate that certain strains can be effective when inserted directly into the vagina or when ascending from the rectum after oral ingestion (Reid and Bruce, 2001; Reid et al., 2001a). Evidence suggest that administration of selected microorganisms including non-patho-genic yeast and several genera of bacteria, such as *Lactobacilli* and bifidobacteria, is beneficial in the prevention and treatment of certain intestinal infections and possibly also vaginal infection (Elmer et al., 1996). The concept that *Lactobacilli* might be useful in displacing and replacing harmful microorganisms on mucosal surfaces dates back to 1908 (Reid et al., 2001). There have been attempts to
restore normal flora by recolonising with *Lactobacilli* (Hallen *et al*., 1992). However, the correct choice of *Lactobacilli* strains has not always been used (McLean and Rosenstein, 2000).

The first study on potential beneficial effect was carried out by Hilton *et al*. (1992) who examined whether daily ingestion of yogurt containing *Lactobacillus acidophilus* prevents vulvovaginal candidal infection. They found that daily ingestion of 8 ounces of yogurt containing *L. acidophilus* decreased candidal colonization and infection. However this study was criticized because previous *in vitro* studies examining the adherence of *Lactobacillus* spp to normal human vaginal epithelial cells found that *L. acidophilus* isolated from yogurt showed a significantly lower adherence than did *Lactobacillus* species from vaginal sources thus, commercial yogurt may not be a reliable way to deliver *Lactobacilli* (Wood *et al*., 1985). Evidence indicates that a daily oral dose of $10^8$ viable probiotic *Lactobacilli* can restore and maintain the urogenital health of women (Reid *et al*., 2001a). Consequently, there has been growing interest in the use of *Lactobacilli* of human origin as probiotics against urogenital tract infections. However the potential use of vaginal lactobacilli isolated from healthy women as probiotics has not been examined in detail (Aslim and Kilic, 2006). Although the available results concerning the effectiveness of the administration of *Lactobacilli* for the treatment of BV are mostly positive, it cannot yet be concluded definitively that probiotics are useful for this purpose. Several trials have found no significant difference in the cure rate of BV and in the number of vaginal *Lactobacilli* after intra-vaginal instillation of *Lactobacilli* when compared with the effect of a placebo or oestrogen. Thus, further studies of women with BV, in which *Lactobacilli* probiotic are
compared either with a placebo or metronidazole, need to be conducted before reaching definitive conclusions as to whether probiotics represent an effective and safe method for treating women with BV (Falagas et al., 2007).

1.2.13 Lactobacilli in normal and in disturbed (BV) flora

It has been reported that different Lactobacillus species predominate the vaginal tract and that Lactobacillus crispatus (Vallor et al. 2001; Song et al., 1999) and L. jensenii (Vallor et al. 2001; Antonio et al., 1999) are the predominant species. In a separate study, Lactobacillus acidophilus has been shown to be the predominant vaginal Lactobacillus species in women with normal vaginal flora (Eschenbach et al., 1989). The predominant species in normal women are strong H₂O₂ producers, with over 70% of the isolates testing positive for H₂O₂ while women with BV flora have are colonised predominantly by either either non-H₂O₂ producing Lactobacilli or not colonized by Lactobacilli (Beigi et al., 2005). Lactobacilli that produce H₂O₂ have been shown to inhibit various microorganisms in vitro, including Gardnerella vaginalis, anaerobes, Neisseria gonorrhoeae, and HIV (Skarin and Sylwan, 1986; Klebanoff and Coombs, 1991; Klebanoff et al., 1991; McLean and Rosenstein, 2000). To our knowledge, no study has been reported to characterize Lactobacilli following BV treatment; the current study
aimed to identify and characterize *Lactobacilli* isolated two months after treatment of women with BV and relate the results to the current BV status.

1.3 Problem statement

Antibiotic therapy with metronidazole or clindamycin has been shown to eliminate BV associated bacteria. However, there is a high recurrence of BV with up to 50% of treated patients experiencing recurrent infection (Hillier *et al.*, 1993a). Some authors speculate that most relapses are related to new sexual contacts and having multiple partners (Wilson, 2004). Evidence suggests that failure to establish normal flora following antimicrobial therapy contributes to the recurrence (Hillier *et al.*, 1993). It is therefore critical to look for factors that may be responsible for the recurrence, and the remedy for the same. Recurrence may result from re-colonization of the vagina by *Lactobacilli* that do not produce protective compounds hence allowing re-colonization by pathogenic microbes.

1.4 Justification

Oral metronidazole is the currently recommended treatment of choice for BV and has been shown to eliminate BV associated pathogens (McLean and Rosenstein, 2000). Studies record a BV cure rate of 80-90% in treatment trials after a week, but about 15-30% recurrent rates occur within three months (Larsson, 1992). Reasons for the extremely high BV recurrences are not well established. Recurrence may result from failure to re-establish a normal flora following treatment (McLean and Rosenstein, 2000). Little data exist on *Lactobacillus* species that recolonise the vagina and their antagonistic role to pathogenic bacteria associated with BV in Kenya. This study sought to identify and characterise the recolonising *Lactobacilli* after treatment of BV. The study also
investigated the production of H$_2$O$_2$ and *in vitro* inhibition potential of *Lactobacilli* isolated from vaginal tracts of Kenyan women on bacteria that have been associated with BV.

1.5 Research questions

i Bacterial vaginosis is recurrent, even after antimicrobial treatment. Are the re-colonising *Lactobacillus* spp a factor in the recurrence?

ii Is the prevalence of H$_2$O$_2$ producing *Lactobacilli* in this group similar to those reported in other parts of the World?

iii Is the inhibitory potential of *Lactobacillus*, related to vaginal flora status?

1.6 Hypothesis

*Lactobacilli* re-colonising women post BV antibiotic treatment do not produce hydrogen peroxide or inhibit growth of pathogenic bacteria.

1.7 Objectives

1.7.1 General objective

To characterise *Lactobacillus* species isolated from vaginal tracts of women post BV treatment and to document their inhibitory capacity against *G.vaginalis, B.fragilis* spp, *P.bivia* and *Mobiluncus* spp.

1.7.2 Specific objectives

i. To determine the prevalence of BV after BV treatment and isolate *Lactobacilli* in women with different vaginal status.
ii. To determine the diversity of *Lactobacillus* spp colonising the vaginal tract in women after BV treatment.

iii. To assess the prevalence of H$_2$O$_2$ producing species among the *Lactobacilli*.

iv. To assess the inhibitory potential of the *Lactobacillus* spp to BV associated organisms.
CHAPTER TWO

MATERIALS AND METHODS

2.1 Study site and population

Samples were taken from patients attending sexually transmitted infection (STI) clinic at the Special Treatment Center (Casino) of Nairobi City council at River road, Nairobi. These participants had been diagnosed with BV, enrolled in a separate randomised controlled trial study and treated for BV with oral metronindazole 500mg twice daily for 7 days. Women enrolled in the study were aged between 18 and 45 years and had come to the clinic to seek for STI treatment. Vaginal samples were collected two months after treatment, *Lactobacillus* species isolated and diagnosis of BV carried out by Nugent method.

2.2 Sample size

The sample size was calculated based on the prevalence of H$_2$O$_2$ producing *Lactobacilli* in Kenya at 11% (Harold et al., 1999), using formulae by Lwanga and Lemeshow (1991):

$$N = \frac{z^2 \times p \times (1-p)}{d^2}$$

Where; $N$ = minimum sample size

$z = 1.96$ (standard error)

$P = 11\%$ (prevalence H$_2$O$_2$ producing *Lactobacillus* species in Kenyan women)

$d^2 = 0.05$ (absolute precision)

$$N = \frac{1.96^2 \times 0.11 \times (1-0.11)}{0.05^2}$$

N=150; Therefore a minimum 150 isolates were required for the study.

A total of 158 isolates were tested from 82 women who had been treated for BV two months earlier.
2.3 Study design
This was a descriptive cross-sectional study in which *Lactobacillus* spp were isolated from vaginal fluid samples taken from female subjects enrolled in a Randomised Controlled Trial at two month visits.

2.4 Specimen collection and processing

2.4.1 Collection and transport of vaginal swabs
A clinical officer performed general vaginal examination on all participants and two vaginal swabs were obtained, one for gram stain diagnosis of BV and another for isolation of *Lactobacillus* spp. The swab was collected and transported as described by Jousimies-Somer *et al.* (2002). Briefly, a sterile speculum was inserted into the vagina and gently opened to enable visualization of the posterior fornix. A sterile dacron swab was inserted into the vaginal introitus and rotated 3 times, two swabs were obtained; one for *Lactobacillus* culture and another for preparing a smear for gram stain diagnosis of BV. The swab for culture was placed into anaerobic transport media for culture while the other swab was rolled gently on a clean slide along the length of the slide for gram stain diagnosis of BV. The slide was placed in a slide box. Culture swab together with the slide box were placed in a biohazard bag and transported to the Anaerobic laboratory at Center for Microbiology Research, Kenya Medical Research Institute, Nairobi for further processing.

2.4.2 Diagnosis of BV by gram's staining method
Smears of vaginal fluid were allowed to air dry and fixed by flooding the slide with methanol for 1 minute before gram staining by Preston and Morrel (1962) modified method. The slide was then flooded with the primary gram stain (crystal violet) solution
for 30 seconds, followed by iodine solution for a minute, iodine-acetone decolourizer for up to 10 seconds and finally with the counter stain (basic fuchsin) solution for 30 seconds. Rinsing with running tap water was done after application of each of the reagents used in the staining process. Procedure for preparation of the stains is described in appendix 2 (ii). Stained smears were allowed to air dry and examined microscopically by 1000x magnification. They were then read and interpreted by Nugent’s method (Nugent et al., 1991). Bacteria were described by colour and morphology; gram-positive organisms retained the crystal violet dye and appear purple while gram-negative organisms lose the crystal violet dye and appear red/pink.

BV was diagnosed by a microbiological method described by Nugent et al. (1991). Gram stained smears of vaginal fluid were examined and bacterial cells appearing as short gram positive/variable or negative rods were scored as *G. vaginalis/Bacteroides* morphotypes, those appearing as curved gram positive or negative rods, counted as *Mobiluncus* morphotypes, while those that appear as short/long parallel sided gram positive rods scored as *Lactobacillus* morphotypes. Bacterial morphotypes corresponding to *G. vaginalis/Bacteroides, Mobiluncus and Lactobacillus* were counted in ten 1000x microscopic fields were averaged. Bacterial cell counts were assigned each a score and the total score for the three bacterial morphotypes were interpreted as follows; “0-3”: normal, “4-6”: intermediate and “7-10”: BV (Table 1).
Table 1: Nugent's scoring for diagnosis of BV

<table>
<thead>
<tr>
<th>Score</th>
<th><em>Lactobacillus</em> morphotypes</th>
<th><em>Gardnerella/Bacteroides</em> morphotypes</th>
<th><em>Mobiluncus</em> (Curved rods) morphotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>More than 30/OIF</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>6 to 30/OIF</td>
<td>Less than 1/OIF</td>
<td>Less than to 5/OIF</td>
</tr>
<tr>
<td>2</td>
<td>1 to 5/OIF</td>
<td>1 to 5/OIF</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Less than 1/OIF</td>
<td>6 to 30/OIF</td>
<td>5 or more/OIF</td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td>More than 30/OIF</td>
<td></td>
</tr>
</tbody>
</table>

OIF - Oil Immersion Field

2.5 Isolation and reidentification of indicator (BV associated) bacteria;

*G. vaginalis, Bacteroides fragilis, Mobiluncus spp and Prevotella bivia*

The organisms that were tested for inhibition were isolated from a different study and stored at -70°C in a deep freezer in trypticase soy/glycerol mixture. For the purpose of this study, they were subcultured in fresh media and identity confirmed by gram stain and biochemical tests (Catalase test, gram stain method, Wee Tabs test, KVC and SPS disks test are described elsewhere in the text). Designated flow charts were used to identify the test organisms (Appendix 2)

2.5.1 *G. vaginalis*

*G. vaginalis* identity was confirmed by showing pleomorphic gram variable rod on gram stain, negative catalase, and sensitivity to sodium polyanethol sulfonate (SPS) potency disks and positive hippurate hydrolysis test as described below. Catalase test and SPS disk
test are described later in the text under *Lactobacillus* spp identification. The flow chart used for identification of *G. vaginalis* is shown in appendix 2 (i).

**i) Hippurate hydrolysis test**

This test determines if the organism produces the enzyme hippuricase. The end products of hydrolysis of hippuric acid by hippuricase include glycine and benzoic acid. Glycine is deaminated by the oxidizing agent, ninhydrin and the latter reduced to form a purple coloured product. This test is valuable when examining Streptococci, *Gardnerella vaginalis* and *Mobiluncus* species.

Sterile distilled water (0.4ml) was put in a 12x75 mm test tube and a heavy inoculum of the test organism added to make a milky/opaque suspension. One tablet containing hippurate was added and incubated for at least 5 hours at 35-37°C. After incubation, 0.2 ml of diluted ninhydrin reagent was added and reincubated for additional 10 minutes then observed for colour change. Positive test appears deep purple while a negative test appears colourless. *G. vaginalis* and *Mobiluncus* spp are both positive for hippurate.

**2.5.2 Bacteroides fragilis**

*Bacteroides fragilis* was confirmed by staining as gram-negative rods, growth in bacteroides bile esculin (BBE) agar as described below and resistance to Kanamycin (1mg), vancomycin (5μg) and colistin (10μg) impregnated disks. They were identified to species level by Wee Tabs test (see below) and the flow chart used for identification of *B. fragilis* is shown in appendix 2 (iv and v).
i) **BBE growth**

Bacteroides bile esculin (BBE) agar is selective for organisms capable of growing in presence of bile and to hydrolyze esculin into esculetin and dextrose. The medium separates members of the *Bacteroides fragilis* group from other anaerobic gram negative rods such as *Prevotella* and *Porphyromonas* group. Members of the *B. fragilis* group cause a black pigment to form on the normally pale medium. BBE also contains gentamicin that inhibits most members of *Enterobacteriaceae* family. The BBE plate was inoculated with test organism and incubated anaerobically at 35°C for 48-72 hours or until growth is obtained. Blackening usually occurs within 8 hours. Members of *Bacteroides fragilis* group grow in this medium and cause blackening while growth for members of *Prevotella* group is inhibited.

### 2.5.3 *Prevotella bivia*

*Prevotella bivia* was confirmed by their resistance to kanamycin (1mg), vancomycin (5μg), and colistin (10μg) impregnated disks as described under tests for identification of *Lactobacillus* spp, appearance as gram-negative rods and negative growth on BBE as described in the section for identification of *Bacteroides fragilis* (see 2.5.2). They were identified to species level by Wee Tabs test. The flow chart used for identification of *P. bivia* is shown in appendix 2 (iv and vi).

### 2.5.4 *Mobiluncus* species

*Mobiluncus* species was confirmed by appearance as curved gram variable rods, positive hippurate test and identity confirmed by Rapid test (Rapid ANA, biomereiux).
Rapid ANA panel system contains several reaction cavities molded into the periphery of plastic disposable tray. An example of a RapID-ANA system panel is shown in appendix 3 (iii). Reaction cavities contain dehydrated reagents and trays allow simultaneous inoculation of each cavity with a predetermined amount of inoculum. This system consists of a plastic tray with 10 wells containing dehydrated substrates including p-nitrophenylphosphate, o-nitrophenyl-β-D-galactoside, p-nitrophenyl-α-D-galactoside, p-nitrophenyl-β-D-glucoside, p-nitrophenyl-α-D-galactoside, p-nitrophenyl-α-D-fucoside, p-nitrophenyl-N-acetylglucosaminide, leucylglycyl-β-naphthylamide, glycyl-β-naphthylamide, prolyl-β-naphthylamide, phenylalanyl-β-naphthylamide, arginyl-β-naphthylamide, seryl-β-3-naphthylamide, pyrrolidonyl-β-naphthylamide, triphenyltetrazolium, arginine, trehalose, and tryptophan.

Pure culture from a Brucella agar plate was suspended in 1 ml of inoculation fluid which contained 0.75% KCl and 0.05% CaCl₂ and poured into the tray to rehydrate the test reagents and initiate the reaction. The tray was slowly tilted, permitting an even distribution of inoculum into all wells. Trays were incubated at 35°C for 4 h in air. The first 10 reactions as listed above were read visually for a color change before the addition of reagents. Cinnamaldehyde reagent was added to the tryptophan well. The "RapID-ANA Reagent" (0.1% sodium dodecyl sulfate, 0.01% D-phenyl-4-imethylamino acrolein, 1% methyl cellosolve, 1% acetic acid, 0.1% hydrochloric acid, 1% surfactant) was added to the first seven wells, which were observed for a subsequent colour change. A six-digit biotype number was generated and referred to a data base manual for identification.
2.6 Processing of vaginal fluid for isolation of *Lactobacillus* spp

Specimen was diluted $10^{-1}$ and $10^{-2}$ with yeast extract; the swab placed in 0.9ml of sterile yeast extract and shaken on a vortex. 100µl of this was transferred to a snap cap tube with 0.9ml yeast extract to make a $10^{-2}$ dilution. 100µl from each of the two dilutions was inoculated onto Columbia colistin nalidixic acid (CNA), brucella and Rogosa agar labeled appropriately. Plates were incubated at 35-37°C for 5 days under anaerobic conditions as shown in appendix 3 (ii). Anaerobic incubations were done either using chamber system (Forma Scientific) or anaerobic boxes (Anaero-Pack system, Mitsubishi Gas Chemical Co., Inc.). After the incubation period, plates were removed from the chamber/anaerobic boxes and examined for colonial morphologies. Photographs of the media used are shown in appendix 3 (i). Description of each colony type on every plate was recorded. Each colony type was subcultured onto brucella plate and stained by grams method to describe the cell appearance, then identified using methods described under section 2.6.1 below.

2.6.1 Identification and characterization of *Lactobacillus* species

2.6.1.1 Identification by Biochemical tests

Elimination flow chart (Appendix 2, i and ii) was used for every gram-positive rod to identify *Lactobacillus* and eliminate non-*Lactobacillus* spp using biochemical tests. The biochemical tests done to identify the *Lactobacilli* to species level included; test for indole production, catalase, nitrate reduction, and preformed enzyme tests (Key scientific, Round Rock, Texas). They were further characterized by carbohydrate fermentation test (described below). Identification tests were performed as described in section i) to iv) below.
i) **Indole production**

The indole test determines the activity of tryptophanase an enzyme produced by some bacteria such as various anaerobes including members of the *Prevotella* spp, *Porphyromonas* (e.g. *P. assacharolytica*) and *Bacteroides* genera (e.g. *B. thetaitamicron*). Hydrolysis of tryptophan releases indole which is detected by the reaction of indole with cinnamaldehyde to produce a blue color. A Brucella plate was streaked heavily with *Lactobacillus* spp (same was done for *Bacteroides* spp, *Prevotella* spp). Presterilized disks were placed on the heavy area of the inoculum. Plates were incubated until good growth was observed. A drop of cinnamaldehyde reagent was added on the disk and observed for color change. *Lactobacillus* spp, *B. fragilis* and *P. bivia* test negative for indole and the disk appear clear or orange. The disk appears bluish if the organism is indole positive.

ii) **Catalase production**

Catalase is one of the several enzymes that degrade hydrogen peroxide to water and oxygen. Presence of the enzyme in bacteria is detected by adding H$_2$O$_2$ to a culture of test organism and observing for formation of bubbles of oxygen (positive test). This test is useful for differentiating species of bacteria based on the ability of the organism to produce the enzyme catalase. It is particularly useful for distinguishing between *Staphylococcus* species and *Streptococcus* species and for distinguishing between *Corynebacterium* and *Lactobacillus* species. Colonies of test organisms were inoculated onto brucella agar (without blood) and incubated for 24-48 hours. Using an applicator stick center of the colonies was touched and smear made on a glass slide. A drop of 15% H$_2$O$_2$ solution was added onto the smear.
and observed for immediate bubbling (positive reaction). *Lactobacillus* spp and *Prevotella* spp do not produce catalase while *Bacteroides* spp test positive for catalase.

iii) **Nitrate reduction test**

The nitrate reduction test determines the ability of organisms to reduce nitrate to nitrite. Reduction of nitrate to nitrite is determined by adding sulfanilic acid and α-naphthylamine. Sulfanilic acid and nitrite forms a diazonium salt that couple with α-naphthylamine to form a red dye. Occasionally the addition of zinc is necessary to ensure that the reaction is really negative and that nitrate has not been reduced beyond nitrite.

A brucella plate was streaked heavily colonies of the *Lactobacillus* spp (same was done for *Bacteroides* spp, *Prevotella* spp). Nitrate disks were placed on the heavy area of the inoculum. Plates were incubated until good growth was observed. A drop of sulfanilic acid followed by a drop of alpha-naphthylamine reagent was added on the disk and observed for color change. If no colour develops, an applicator stick was dipped into zinc dust and the amount that adhered onto the stick was transferred to the disk. Red color change was observed for upto five minutes. Rapid development of a red color within 2-3 minutes after addition of sulfanilic acid and α-naphthylamine or No color change after addition of zinc was interpreted as positive nitrate reduction test while no color change within 2-3 minutes after addition of the reagents or a red colour change within 5 minutes of addition of zinc dust was interpreted as negative test. *Lactobacillus* spp, *B. fragilis* and *P. bivia* test negative for nitrate reduction.
iv) **Antibacterial potency disks for identification of bacteria**

Special potency disks containing three different antimicrobial agents are used to
determine susceptibility of an isolate to these agents and to aid in differentiation of
organisms.

A heavy inoculum of test organism was spread over the surface of brucella agar. For
identification of *Lactobacillus* spp, potency disks containing vancomycin (30µg) were
placed on the inoculated surface of the agar. Disks containing sodium polyanethol
sulfonate (SPS) were used for identification of *G. vaginalis* isolate while for *Bacteroides*
and *Prevotella* spp, three (KVC) disks (containing 5µg vancomycin, 1mg kanamycin and
10 µg colistin) were placed as far apart as possible in the area of the plate where
confluent growth was expected. The plates were incubated at 35°C anaerobically for 48-
72 hours. The results for inhibition for each disk was recorded and used for identification.

Most strains of *Lactobacillus* spp are resistant to vancomycin (30µg). *G. vaginalis* strains
are sensitive to SPS while *Bacteroides fragilis* and *Prevotella bivia* are resistant to
vancomycin (5µg), kanamycin (1mg) and colistin (10 µg).

v) **Wee Tabs® for detection of preformed enzymes**

This test was used to confirm identity of *Lactobacilli* that were identified by carbohydrate
fermentation test described below. Wee Tabs® are tablets that contain various enzymes in
neutral substrates. Several types of tests are available: single tests that are combined with
nitrophenol or aminopeptidase, double tests that combine nitrophenol and
aminopeptidase, and triple tests that combine nitrophenol, methylumbelliferyl and
aminopeptidase. All Wee Tabs are inoculated and treated alike. The results from these are
combined and used with prepared flowcharts or with reference manuals for definitive
identification of facultative and anaerobic organisms. Nitrophenol tests contain substrate
bound to nitrophenol; upon hydrolysis of the substrate, nitrophenol is released and is visible as a yellow compound. Methylumbelliferyl based tests contain substrate bound to methylumbelliferyl; upon hydrolysis of the substrate methylumbelliferone is released and can be observed under a wood’s lamp. Aminopeptidase tests contain substrate bound to arylamide; upon hydrolysis of the substrate, free beta-napthylamide is released which is detected by addition of aminopeptidase reagent (PEP, cinnamaldehyde).

Six drops of sterile distilled water were placed in 12x75 mm test tube and inoculated heavily with loopful of test bacterial colonies in pure culture to make a milky and opaque suspension. One tablet of the desired substrate was added and incubated for at least 2 hours at 35°C-37°C in room air. For nitrophenol based test appearance of yellow colour at any time during the reaction was considered positive. For methylumbelliferyl based tests, the tube was observed for florescence by holding the tube under ultra violet (UV) light (Wood’s lamp). Bright bluish-green florescence was considered positive. For aminopeptidase based tests, two drops of PEP reagent was added and incubated for upto 15 minutes. Red, blue or purple colour was considered positive. If dual or triple tests were used, the nitrophenol based test was read first, followed by fluorescence and finally the aminopeptidase based test.
Table 2: Examples of Some Wee Tab tests used for identification

<table>
<thead>
<tr>
<th>Single tests (nitrophenol bases)</th>
<th>Single tests (Aminopeptidase based)</th>
<th>Double tests (nitrophenol/ Aminopeptidase)</th>
<th>Triple tests (nitrophenol/ methylumbelliferyl/Aminopeptidase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yellow color</td>
<td>Add PEP; examine; red color</td>
<td>Yellow color; then add PEP; observe red color</td>
<td>Yellow color; then fluorescence; then add PEP; observe red color</td>
</tr>
<tr>
<td>PO4</td>
<td>Arginase</td>
<td>BGLU/ARG</td>
<td>NAG/BGLU/PRO</td>
</tr>
<tr>
<td>A-GAL</td>
<td>Proline aminopeptidase (PRO)</td>
<td>ONPG/PYR</td>
<td>AGLU/ a-GAL /LGY</td>
</tr>
</tbody>
</table>

$p$-nitrophenylphosphate (PO4), $p$-nitrophenyl-β-D-glucoside (BGLU), $p$-nitrophenyl-α-D-galactoside (α-GAL), $p$-nitrophenyl-N-acetylglucosaminide (NAG), leucylglycyl-β-naphthylamide (LGY), arginyl-β-naphthylamide (ARG), pyrrolidonyl-β-naphthylamide (PYR), arginine (ARG), Ortho-nitrophenyl-b-D-galactopyranoside (ONPG) (Key Scientific, Round Rock, Texas)

2.6.1.2 Identification of Lactobacillus spp by CHO Fermentation

*Lactobacillus* spp were tested for ability to ferment the following sugars; lactose, cellobiose, rhamnose, mannitol, sucrose, melezitose, xylose, raffinose and arabinose as described by Jousimies-Somer *et al.* (2002). Microtiter plate method was used for the carbohydrate fermentation test. The microtiter fermentation test for broth cultures was done by the method of Davies *et al.*, 1971) as follows:- One hundred and fifty microliter of tryptone broth containing Bromo Thymol blue was added to eight wells across a 96 wells microtiter plate. 25μl of the appropriate carbohydrate solution was added per well. 75μl of an 18-24 hour culture suspension of each *Lactobacillus* spp in Brain heart infusion broth was added to all eight wells containing different carbohydrates. The plates were sealed with microtiter pressure-stick adhesive covers and incubated for 18 to 24 hour at 37°C anaerobically, (CO2 incubation could not be used as an acid reaction would occur in all wells). Organisms in the wells that turned yellow after incubation were scored as positive for fermentation while those that remained green were scored as negative. The
Lactobacillus spp were identified using flow chart in appendix (iii). Wells containing dextrose alone were inoculated with *L. acidophilus* ATCC 4356 probiotic strain were used as positive control for fermentation while well containing tryptone broth and each of the respective carbohydrates without bacterial inoculation served as negative control. Those strains that could not be identified by biochemical tests, Wee Tabs and CHO fermentation was identified by RapID ANA system (described above).

### 2.7 Hydrogen peroxide production

Tetramethylbenzine (TMB) agar is used to test for production of H$_2$O$_2$ by *Lactobacillus* spp. The agar contains brucella agar, benzidine, horseradish peroxidase, hemin, vitamin K$_1$ and starch. Horseradish peroxidase in TMB agar hydroyses H$_2$O$_2$ produced by *Lactobacillus* spp to release water and oxygen. The oxygen oxidizes benzidine in the medium to produce a bluish pigment. Colonies of H$_2$O$_2$ producing species appear bluish on the test agar plates while the rest appear creamish. The method of Eschenbach et al. (1989) was used for this assay. A single colony from pure culture of bacteria isolates confirmed by conventional method as *Lactobacilli* was inoculated on the freshly prepared TMB and incubated anaerobically for 3 days at 37°C. After the incubation period, agar plates were removed and exposed to ambient air for up to 30 minutes. H$_2$O$_2$ production was indicated by production bluish pigmentation on colonies of H$_2$O$_2$ positive strains. A probiotic H$_2$O$_2$ producing strain, *L. acidophilus* ATCC 4356 was used as a control for H$_2$O$_2$ production.

### 2.8 Measuring pH

Prior to inoculating the *Lactobacillus* spp culture supernatant to the wells of the antagonism plate, the amount of acid produced by *Lactobacilli* was measured indirectly
by determining pH of the supernatant using a pH meter (Corning pH meter 610A). One ml of supernatant of an overnight culture of Lactobacillus spp was put in a glass container. The pH meter was calibrated according to manufacturer’s directions by using three buffers (pH 4, pH 7 and pH 10). The knob of the meter was placed in the supernatant after rinsing with distilled water, and the pH read was directly from the meter and recorded. The pH of L. acidophilus ATCC 4356 supernatant probiotic strain was similarly taken.

2.9 Assay for inhibitory activity of Lactobacillus spp culture supernatants

2.9.1 Preparation of culture supernatants

Pure colonies of Lactobacilli were inoculated into brain heart infusion broth and incubated overnight. The Lactobacilli culture was centrifuged at 10,000 revolutions per minute for 5 minutes, and then the supernatant was used for the assays described below. L. acidophilus ATCC 4356 probiotic strain was used as a control for inhibition before and following physical and chemical treatment of the Lactobacilli culture supernatants.

2.9.1 Procedure for well-antagonism assay

Antibacterial activity of Lactobacillus isolates was assessed by a deferred well-antagonism assay described by modified McLean and Rosenstein (2000). Well antagonism assay, tests for ability of an organism to inhibit growth of other organisms when seeded together in an agar media. It is commonly used to test for ability of Lactobacillus spp to inhibit growth of various bacteria types. The test bacteria (B. fragilis, G. vaginalis, Prevotella bivia and Mobiluncus spp) were isolated in pure culture and used to make a seeding suspension containing approximately 1x10^7 colony forming units (cfu/ml). A sterile swab was used to swab culture plates of the indicator bacteria; this was suspended in 3mls of yeast extract to make a bacterial suspension equivalent to 0.5
MacFarland solutions. The suspension was seeded evenly using a sterile swab onto freshly prepared brucella agar in which 7mm diameter wells were bored using a cork borer. One hundred microliter (μl) of overnight broth culture of *Lactobacillus* spp was added to the wells. Plates were incubated anaerobically at 37°C for 48 hrs either in anaerobic chamber system model 1025 (Forma Scientific) or in rectangular jars with gas generating kit (Anaero-Pack system, Mitsubishi Gas Chemical Co., Inc.). Zones of inhibition were measured from the outer edge of bacterial growth. Results were interpreted as sensitive, if there was an observable zone of inhibition. Zone sizes were associated to inhibitory capacity of the individual *Lactobacillus* species, the larger the zone size, the stronger the inhibitory potential. *L. acidophilus* ATCC 4356 probiotic strain was used as a control for inhibition prior and following physical and chemical treatment of the *Lactobacilli* culture supernatants.

2.9.2 Characterization of inhibitory substances

The culture supernatants of the *Lactobacillus* spp that showed antibacterial activity to indicator bacteria in the well antagonism assay were assayed for acids and bacteriocins using a method described by Jin et al. (1996).

2.9.2.1 Assay for bacteriocins

Bacteriocins from *Lactobacillus* spp are proteinaceous compounds, which have inhibitory effect against closely related species and other gram-positive bacteria. Bacteriocins form the pores in the membrane of sensitive cells and deplete the transmembrane potential and/or the pH gradient, resulting in the leakage of cellular materials. The inhibitory effect of bacteriocin is influenced by the phospholipids composition of the target strains and the pH of the environment (Zalan et al. 2005).
To assay for bacteriocins, aliquots of culture supernatant were treated with trypsin from beef extract (BDH, Co. Inc.), pepsin and proteinase K solution each at a final concentration of 1 mg/ml for 60 minutes at room temperature. To 1 ml of the culture supernatant, 100 μl of each of the three enzyme solutions was added and allowed to stand at room temperature for 1 minute. The treated supernatant was then tested for antagonistic activity to indicator bacteria by well antagonism assay described above (2.9.1). 100 μl of the enzyme treated culture supernatant was transferred into the wells, in agar plates prior seeded with indicator bacteria and inhibition observed after incubation for 24 hrs at 37°C. Zones of inhibition were measured as in the above assay.

2.9.2.2 Effect of pH on antimicrobial activity
Culture supernatants of an overnight culture of *Lactobacillus* spp were neutralized to pH 7.2 by addition of 1N NaOH. To 2 ml of the culture supernatant, 1N NaOH was added dropwise to bring the pH to 7.2. 100 μl of the treated supernatant was used for antagonism assay to test inhibitory activity to the selected organisms BV associated (indicator) organisms by the method described above (see 2.9.1).

2.9.2.3 Effect of heat on antimicrobial activity
Two millilitre aliquots of the overnight culture supernatant of inhibitory *Lactobacilli* were put in a test tube and heated in a hot water bath at 100°C for 30 minutes. To test heat sensitivity of inhibitory substances, 100 μl of heated culture supernatant was added to wells and the residual activity was assayed by well antagonism assay using the same indicator organisms as used in the well antagonism assay described above (see 2.9.1).
2.10 Data management and analysis

The biochemicals data, H₂O₂ production data and data on inhibitory potential of *Lactobacillus* was entered in Microsoft excel and filemaker pro and recorded in a notebook in the laboratory KEMRI-CMR-PID laboratory. Data was analysed by InStat version 3.0 for windows statistical software (GraphPad Software, Inc., San Diego, USA). Categorical data such as comparison of inhibition and production of H₂O₂ by the *Lactobacilli* was analysed by chi-square test or Fisher’s exact tests. Continuous data was analysed using student’s t-test, while data with multiple comparisons was analysed by Analysis of Variance (ANOVA) and Tukey Kramer multiple comparison test. Results were presented in figures and tables.
CHAPTER THREE

RESULTS

3.1 Prevalence of BV in women two months following treatment

Of the 107 women sampled, 82 had *Lactobacillus* spp while no *Lactobacillus* spp isolated was from culture of 25 women. The latter were included to enable an assessment of the relationship between the presence or absence of *Lactobacillus* spp identified on gram stain and those isolated by culture. When vaginal flora was categorized by Nugent’s method after staining the vaginal fluid of the women by gram’s method, more women had BV (38.3%), than those with intermediate (28.1%) or normal vaginal flora (33.6%). Among those women who had BV, 4.9% had a score of 9 and 10 each, while 51.2% and 39% had scores of 8 and 7, respectively (mode=8). About 30% of the women who had intermediate vaginal flora had scores of 6 while 36.7% and 33.3% had scores of 5 and 4, respectively. Among the women with normal vaginal flora 25% had a score of 3 while 30.6%, 22.2% (8/36) and 22.2% had scores of 2, 1 and 0, respectively (mode=2; Table 3).
Table 3: Variation in vaginal states among sampled women using Nugent’s criteria

<table>
<thead>
<tr>
<th>Vaginal state*</th>
<th>Percentage Prevalence per category</th>
<th>Nugent’s Score</th>
<th>Percentage Prevalence in each value in Nugent’s scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial vaginosis</td>
<td>38.3</td>
<td>10</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>51.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>39</td>
</tr>
<tr>
<td>Intermediate</td>
<td>28.1</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>36.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>33.3</td>
</tr>
<tr>
<td>Normal</td>
<td>33.6</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>30.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>22.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>22.2</td>
</tr>
</tbody>
</table>

Gram stained smears of 107 women were examined and scored for bacterial vaginosis in the Nugent’s 10 value scoring scale, where increase in the value indicate vaginal abnormality; 0-3 is normal vaginal flora, 4-6 intermediate (altered vaginal flora) and 7-10 is BV (abnormal flora). The prevalence values are in percentages. *vaginal flora of the women whether they had BV or not.
3.2 Isolation rate of *Lactobacillus* spp among women with different vaginal status

Overall, 76.6% of the women sampled had vaginal colonization with at least one strain of *Lactobacilli*. Among these, *Lactobacillus* spp were isolated from 70.7% of the women who had BV and from 76.6% and 83.3% of the women who had intermediate and normal vaginal tract, respectively. Among women who had BV, *Lactobacillus* spp were isolated from 1 of the two women with Nugent’s score of 10. Similarly, *Lactobacilli* were isolated in 100%, 66.7% and 75% of women with scores of 9, 8 and 7, respectively. Therefore, *Lactobacillus* spp were isolated in women with BV, including one woman in whom *Lactobacilli* were not seen on gram stain (Nugent’s score of 10) examination. Among the women with intermediate scores, *Lactobacilli* were isolated in 100%, 72.7% and 60% of women who had Nugent’s score of 6, 5 and 4, respectively. Of those who had normal vaginal flora, *Lactobacilli* were isolated in vaginal fluids of 88.9%, 81.8%, 100%, and 62.5% women with scores of 3, 2, 1 and 0, respectively (Table 3).

Table 4 shows the distribution of women with and without *Lactobacillus* spp with different Nugent’s scores in the 0-10 score system. Generally, *Lactobacilli* were isolated in more of the women with normal vaginal flora. However, there was no significant difference between those women who had *Lactobacillus* spp and those who did not have between the different categories of vaginal flora ($\chi^2 = 1.551, p = 0.67$).
### Table 4: Number of women colonized with *Lactobacillus* species

<table>
<thead>
<tr>
<th>Vaginal status</th>
<th>Sample size</th>
<th>Percentage colonized by <em>Lactobacillus</em> spp</th>
<th>Percentage not colonized by <em>Lactobacillus</em> spp</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial vaginosis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>21</td>
<td>66.7</td>
<td>33.3</td>
</tr>
<tr>
<td>7</td>
<td>16</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>41</strong></td>
<td><strong>70.7</strong></td>
<td><strong>29.3</strong></td>
</tr>
<tr>
<td><strong>Intermediate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>9</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>11</td>
<td>72.7</td>
<td>27.3</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>30</strong></td>
<td><strong>76.7</strong></td>
<td><strong>23.3</strong></td>
</tr>
<tr>
<td><strong>Normal</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>88.9</td>
<td>11.1</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>81.8</td>
<td>18.2</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>8</td>
<td>62.5</td>
<td>37.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>36</strong></td>
<td><strong>83.3</strong></td>
<td><strong>16.7</strong></td>
</tr>
</tbody>
</table>

Percentage of women with or without *Lactobacillus* spp isolated from their vaginal fluid was distributed for each of the values in Nugent scoring scale. Sample size indicates number of women who had vaginal flora scored as indicated by value in the vaginal status (Nugent’s) scale.
3.3 Hydrogen peroxide producing *Lactobacillus* spp among sampled women

Production of H$_2$O$_2$ by the *Lactobacillus* isolated among the women was assessed using TMB agar as shown in Figure 6.

![Figure 6: Growth and color production by H$_2$O$_2$ producing *Lactobacillus* on TMB agar. A-back view of the Petri-dish, B-front side of the Petri-dish](image)

Of the 82 women who had *Lactobacilli* isolated from their vaginal fluid, 37.8% had H$_2$O$_2$ producing *Lactobacillus* spp while 62.2% had non-H$_2$O$_2$ producing *Lactobacillus* spp. Among those with H$_2$O$_2$ producing *Lactobacillus* spp, 38.7% had a BV flora while 25.8% and 35.5% had intermediate and normal flora, respectively by Nugent’s scoring method (Figure 7). Seventeen of 51 women with non-H$_2$O$_2$ *Lactobacillus* spp had BV while 29.4% and 37.3% had intermediate and normal flora, respectively. H$_2$O$_2$ producing *Lactobacillus* spp were isolated in more women with BV than other vaginal flora while non-H$_2$O$_2$ producing *Lactobacillus* spp were isolated in more women with normal vaginal flora. However, there was no significant difference between those who had H$_2$O$_2$ producing or non- H$_2$O$_2$ producing *Lactobacillus* spp among the women with different categories of vaginal flora ($\chi^2 = 2.256, p = 0.32$; Table 5; Figure 7).
Figure 7: Percentage distribution of H$_2$O$_2$ positive and negative Lactobacillus spp in women with different status of vaginal flora

About 96.8% out of 31 women with H$_2$O$_2$ Lactobacillus spp, had facultative anaerobic Lactobacillus spp, 3.2 had a mixture of both strict and facultative anaerobic Lactobacillus spp and none of these women was found colonized by strict anaerobic Lactobacillus spp alone (Table 5). 88.2% of the women with non-H$_2$O$_2$ Lactobacillus spp had facultative anaerobic Lactobacillus spp while 5.9% had both strict and facultative anaerobes and strict anaerobic Lactobacillus spp alone each. A significant difference was found between women with different vaginal flora status colonized with facultative anaerobes and strict anaerobes ($p < 0.001$) and those colonized by both facultative anaerobes and strict anaerobes, and facultative anaerobes only ($p < 0.001$) but no significant difference between those colonized by both facultative anaerobes and strict anaerobes, and strict anaerobes alone ($p > 0.05$).
Table 5: Hydrogen peroxide producing *Lactobacillus* spp in women with different categories of vaginal flora

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Normal (%)</th>
<th>Intermediate (%)</th>
<th>BV (%)</th>
<th>Total N=82</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=30</td>
<td>N=23</td>
<td>N=29</td>
<td></td>
</tr>
<tr>
<td><strong>H₂O₂ producers</strong></td>
<td>35.5</td>
<td>25.8</td>
<td>38.7</td>
<td>37.8</td>
</tr>
<tr>
<td>Facultative anaerobes</td>
<td>36.7</td>
<td>26.7</td>
<td>36.7</td>
<td>96.8</td>
</tr>
<tr>
<td>Facultative anaerobes + anaerobes</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>3.2</td>
</tr>
<tr>
<td>Anaerobes only</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Non-H₂O₂ producers only</strong></td>
<td>37.3</td>
<td>29.4</td>
<td>33.3</td>
<td>62.2</td>
</tr>
<tr>
<td>Facultative anaerobes</td>
<td>33.3</td>
<td>31.1</td>
<td>35.6</td>
<td>88.2</td>
</tr>
<tr>
<td>Facultative anaerobes + anaerobes</td>
<td>66.7</td>
<td>33.3</td>
<td>0</td>
<td>5.8</td>
</tr>
<tr>
<td>Anaerobes only</td>
<td>66.7</td>
<td>0</td>
<td>33.3</td>
<td>5.9</td>
</tr>
</tbody>
</table>

Isolated *Lactobacilli* from women with different vaginal flora were categorised into strict anaerobes and facultative anaerobes; some women had both strict anaerobic and facultative anaerobic *Lactobacilli*.

3.4 Media of primary isolation of the *Lactobacillus* spp

Out of 158 species of *Lactobacillus* spp, 31% were isolated primarily on Brucella Medium while 20.2% and 48.7% were recovered from Columbia Colistin Nalidixic (CNA) and Rogosa agar, respectively. More of the isolates tested were recovered from Rogosa than from each of the other two media (Table 6).
Table 6: Effect of media of primary isolation on production of H2O2 production

<table>
<thead>
<tr>
<th>Media</th>
<th>Hydorgen Peroxide</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (%)</td>
<td>Negative (%)</td>
<td>Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brucella</td>
<td>31</td>
<td>69</td>
<td>31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNA*</td>
<td>50</td>
<td>50</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rogosa**</td>
<td>22</td>
<td>78</td>
<td>77</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Columbia Nalidixic Acid agar; ** De man Rogosa Sharpe agar. Total is percentage of the Lactobacillus spp growing on each medium.

Thirty one percent of Lactobacilli isolates from brucella agar produced H2O2 in vitro. Similarly, 50% and 22 of those recovered from CNA and Rogosa were H2O2 positive. Among those that did not produce H2O2, 69%, 50% and 78% were isolated from Brucella, CNA and Rogosa agar, respectively. Significantly, more Lactobacillus spp isolated from CNA were H2O2 producing, as compared to those from each of the other two media ($\chi^2 = 8.335, p = 0.02$; Figure 8).

![Figure 8: Production of H2O2 by Lactobacillus spp on different media](image)
3.5 Relationship between presence of *Lactobacillus* spp in gram stain smears and culture isolation

Overall, 73.8% vaginal gram stained smears samples had at least 1 bacterial cell morphologically resembling *Lactobacilli* (*Lactobacillus* morphotype) per 1000x magnification microscopic field. Among these, 35.4% had *Lactobacillus* morphotype cell count of over 30 per oil immersion microscopic field (OIF), 22.8%, 19% and 22.8% had 6 to 30, 1 to 5 and less than one cell(s) counted per OIF, respectively. Overall, the number of women with > 30 per OIF *Lactobacilli* cells count in gram stain was higher than each of those with less counts ($t = 6.982$, df=3, $p$ (2-tailed)=0.01).

Of the women who had >30 per OIF *Lactobacilli* cell count on gram stain, 82.1% had *Lactobacillus* spp isolated from the cultures. Similarly, 83.3%, 73.3% and 72.2% of those with *Lactobacillus* cell counts of 6-30, 1-5 and <1 per OIF, respectively, had *Lactobacillus* isolated from culture of vaginal fluid. 21.5%, of women with at least one *Lactobacillus*, morphotype in gram stained smear did not have *Lactobacillus* spp isolated from their vaginal fluid (Table 4). More of the women with gram stain score of > 30 per OIF, had *Lactobacillus* spp isolated from culture; however there was no statistical difference between gram stain count and isolation ($\chi^2 = 1.636$, $p = 0.80$).

About 86.2% of the women sampled did not have any *Lactobacillus* spp cell morphotype counted in the gram stain of vaginal fluid. Despite the fact that no *Lactobacillus* morphotype was seen in the gram stain, *Lactobacillus* spp were isolated in vaginal fluid of 71.4% of these women, but none was recovered in 28.6% of them. In general there was no correlation between presence of *Lactobacillus* spp in the gram stain and isolation from the culture specimen (spearman correlation = 0.579, $p$ (2-tailed) = 0.42; Table 7).
Table 7: Recovery of *Lactobacillus* in culture of vaginal fluid in women with different quantities of *Lactobacillus* cell morphotypes in gram stained smears of vaginal fluid

<table>
<thead>
<tr>
<th>Lactobacillus spp count in gram stain (per OIF)</th>
<th>Sample size</th>
<th>Women with <em>Lactobacillus</em> spp isolated (%)</th>
<th>Women with <em>Lactobacillus</em> spp (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(0)&gt;30</td>
<td>28</td>
<td>82.1</td>
<td>17.9</td>
</tr>
<tr>
<td>1 (6-30)</td>
<td>18</td>
<td>83.3</td>
<td>16.7</td>
</tr>
<tr>
<td>2 (1-5)</td>
<td>15</td>
<td>73.3</td>
<td>26.7</td>
</tr>
<tr>
<td>3 (&lt;1)</td>
<td>18</td>
<td>72.2</td>
<td>27.8</td>
</tr>
<tr>
<td>4 (none)</td>
<td>28</td>
<td>71.4</td>
<td>28.6</td>
</tr>
</tbody>
</table>

3.6 Prevalence of *Lactobacillus* strains

A total of 158 different *Lactobacillus* strains were isolated from samples of 82 women. Among the *Lactobacillus* spp isolated, 14.6% were *L. acidophilus*, 12.6% *L. fermentum*, 14.6% *L. iners*, 18.4% *L. jensenii*, 2.5% *L. brevis*, 1.9% *L. casei*, 3.2% were *L. cateniforme*, 0.6% were *L. crispatus*, 3.2% were *L. minutus*, 12.6% were *L. plantanarum*, 1.3% were *L. paracasei*, 5.8% were *L. paraplantanaum*, 3.2% were *L. rhamnosum*, 1.9% were *L. salivarian* and 3.8% were *L. vaginalis*. *L. jensenii* was significantly more abundant than each of the other *Lactobacillus* species (t = 4.29, p (2-tailed) = 0.001) (Figure 9).
Table 7 shows the prevalence of *Lactobacillus* species and their distribution in different vaginal status. 41.1% *Lactobacillus* species were isolated from women with normal vaginal flora, 31.6% and 27.2% were isolated from BV and intermediate vaginal flora, respectively. The distribution of *Lactobacillus* species isolated from women with BV ranged from 2% for *L. brevis* to 20% for *L. acidophilus* and *L. jensenii* each. In women with intermediate vaginal flora *Lactobacillus* spp ranged from 2.3% *L. rhamnosum*, *L. crispatus*, *L. paracasei* and *L. casei* each to 27.9% *L. plantanarum*. Those isolated from women with normal vaginal flora ranged from 1.5% for *L. salivarian* and *L. paracasei* each, to 20% for *L. jensenii*. There was no significant difference in the distribution of
Lactobacillus spp isolated in women with different categories of vaginal flora (p=0.32, Table 8).

Table 8: Prevalence of Lactobacillus species among sampled women

<table>
<thead>
<tr>
<th>Lactobacillus species</th>
<th>BV (%)</th>
<th>Intermediate (%)</th>
<th>Normal (%)</th>
<th>% Prevalence of each species</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. acidophilus</td>
<td>20</td>
<td>4.7</td>
<td>16.9</td>
<td>14.6</td>
</tr>
<tr>
<td>L. brevis</td>
<td>2</td>
<td>0</td>
<td>6.7</td>
<td>2.5</td>
</tr>
<tr>
<td>L. casei</td>
<td>4</td>
<td>2.3</td>
<td>0</td>
<td>1.9</td>
</tr>
<tr>
<td>L. cateniforme</td>
<td>4</td>
<td>0</td>
<td>6.7</td>
<td>3.2</td>
</tr>
<tr>
<td>L. crispatus</td>
<td>0</td>
<td>2.3</td>
<td>0</td>
<td>0.6</td>
</tr>
<tr>
<td>L. fermentum</td>
<td>6</td>
<td>11.6</td>
<td>18.5</td>
<td>12.6</td>
</tr>
<tr>
<td>L. iners</td>
<td>12</td>
<td>16.3</td>
<td>15.4</td>
<td>14.6</td>
</tr>
<tr>
<td>L. jensenii</td>
<td>20</td>
<td>13.9</td>
<td>20</td>
<td>18.4</td>
</tr>
<tr>
<td>L. minutus</td>
<td>6</td>
<td>0</td>
<td>3.1</td>
<td>3.2</td>
</tr>
<tr>
<td>L. plantanarum</td>
<td>12</td>
<td>27.9</td>
<td>3.1</td>
<td>12.6</td>
</tr>
<tr>
<td>L. paracasei</td>
<td>0</td>
<td>1 (2.3)</td>
<td>1.5</td>
<td>1.3</td>
</tr>
<tr>
<td>L. paraplantarum</td>
<td>8</td>
<td>4.7</td>
<td>6.7</td>
<td>5.7</td>
</tr>
<tr>
<td>L. rhamnosum</td>
<td>0</td>
<td>2.3</td>
<td>6.2</td>
<td>3.2</td>
</tr>
<tr>
<td>L. salivarian</td>
<td>0</td>
<td>4.7</td>
<td>1.5</td>
<td>1.9</td>
</tr>
<tr>
<td>L. vaginalis</td>
<td>6</td>
<td>6.9</td>
<td>0</td>
<td>3.8</td>
</tr>
<tr>
<td><strong>Total n (%)</strong></td>
<td><strong>50 (31.6)</strong></td>
<td><strong>43 (27.2)</strong></td>
<td><strong>65 (41.1)</strong></td>
<td><strong>158</strong></td>
</tr>
</tbody>
</table>

The above table shows percentage distribution of each species of Lactobacilli isolated in women with BV, normal flora or intermediate flora.
3.7 Inhibition of BV associated organisms by *Lactobacillus* spp

Culture supernatants obtained from 158 *Lactobacillus* spp isolated from vaginal samples of 82 women were tested for antibacterial activity against BV associated bacteria. Antibacterial activity of *Lactobacillus* isolates was assessed by a modified deferred antagonism well assay described by McLean and Rosenstein (2000) (Figure 10).

![Figure 10: Example of zone of inhibition of indicator organisms by *Lactobacillus* spp inoculated into the well](image)

The supernatants were assayed against one strain of *G. vaginalis*, *P. bivia*, *B. fragilis* and *Mobiluncus* spp. None of the *Lactobacillus* spp tested inhibited growth of *B. fragilis*, 23% inhibited growth of *P. bivia*, 28% *G. vaginalis*, and 29% inhibited growth of *Mobiluncus* spp. More *Lactobacillus* spp strains inhibited *Mobiluncus* spp than the other two susceptible indicator bacteria. This variation in inhibitory response was statistically significant ($\chi^2=55.410, p<0.001$; Figure 11).
Out of 158 isolates of *Lactobacillus*, 31% inhibited the three susceptible BV associated bacteria. The inhibitory *Lactobacillus* spp produced zones of inhibition of growth of 1-8mm for *Mobiluncus* spp, 1.5-8mm for *G. vaginalis* and 1.5-7mm for *P. bivia*. *L. acidophilus* ATCC 4356 probiotic strain produced zones of inhibition of 6.0mm and 5.0mm to *Mobiluncus* spp and *G. vaginalis* each and *P. bivia* respectively. The control strain was not inhibitory to *B. fragilis*. The *G. vaginalis* was more susceptible to inhibition than the other two susceptible indicator bacteria; the mean zone of inhibition (mm) was 5.2±3.0 for *G. vaginalis* as compared to 4.8±1.8 mm and 3.0±1.3 for *Mobiluncus* spp and *P. bivia*, respectively. There was significant difference in the zones of inhibition produced
by *Lactobacillus* spp on different indicator bacteria (*t* = 6.405, *p* (2 tailed) = 0.02; Figure 12).

![Figure 12: Mean inhibition of BV-associated bacteria by the *Lactobacillus* isolates](image)

There was a significant difference between the inhibitory *Lactobacillus* spp that inhibited each indicator bacteria independently i.e *G. vaginalis*, *Mobiluncus* spp and *P. bivia*, and those that inhibited all three indicator bacteria was assessed. Among the *Lactobacillus* spp that were inhibitory to *G. vaginalis*, 42.2% were isolated from women with normal flora, 20% and 37.8% from women with intermediate and BV flora as categorized by Nugent’s method. The inhibitory *Lactobacillus* spp from women with normal flora were more abundant than those from women with intermediate and BV flora. 41.3% of the *Lactobacillus* spp that were
inhibitory to *Mobiluncus* spp were isolated from women with normal flora, 21.7% and 37% were from intermediate and BV flora, respectively. 45.9%, 16.2% and 37.8% of those that were inhibitory to *P. bivia* were isolated from women with normal, intermediate and BV flora, respectively. Those *Lactobacilli* isolated from women with normal flora were more inhibitory than those in each of the other categories of vaginal flora (Table 9).

There was no significant difference between inhibition of *G. vaginalis* and *Mobiluncus* spp (Tukey-Kramer multiple comparison test; p > 0.05); a significant difference was observed between the inhibition of *G. vaginalis* and *P. bivia* (p<0.01) and *Mobiluncus* spp and *P. bivia* (p<0.01) by *Lactobacilli* from women with different status of vaginal flora. 48.5%, 15.2% and 36.4% of the *Lactobacillus* spp strains that inhibited all three susceptible indicator BV associated bacteria were isolated from normal, intermediate and BV flora respectively. More of *Lactobacilli* isolated from women with normal flora were inhibitory to each individual indicator bacteria and to all three indicator bacteria together. There was a significant difference between the status of the flora in which *Lactobacilli* were isolated and the inhibition of indicator strains (ANOVA, p = 0.004).

### Table 9: Association between vaginal status of *Lactobacillus* spp source and inhibition

<table>
<thead>
<tr>
<th>Indicator strains inhibited (%)</th>
<th><em>G. vaginalis</em> n=45</th>
<th><em>Mobiluncus</em> spp n=46</th>
<th><em>P. bivia</em> n=37</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vaginal flora status</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lactobacilli</em> from</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal women</td>
<td>42.2</td>
<td>41.3</td>
<td>45.9</td>
</tr>
<tr>
<td><em>Lactobacilli</em> from</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>women with Intermediate score</td>
<td>20</td>
<td>21.7</td>
<td>16.2</td>
</tr>
<tr>
<td><em>Lactobacilli</em> from</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>women with BV</td>
<td>37.8</td>
<td>37</td>
<td>37.8</td>
</tr>
</tbody>
</table>
3.9 Influence of pH of the supernatant on inhibition of indicator bacteria

The pH of the supernatant was measured before use in the inhibition assay. The pH ranged between 2.62 and 6.71, with a mean (±SD) pH of 4.82±1.1. Of these, 33.5% produced highly acid pH (2-3.99), 20.9%, 16.5% and 29.1% produced cultures with moderately acidic pH (4-4.99), slightly acidic pH (5-5.99) and very slightly acidic pH (6-6.99), respectively. *L. acidophilus* ATCC 4356 supernatant had a pH of 3.4. Majority of the strains produced highly acidic supernatants.

Of the *Lactobacilli* culture supernatants that inhibited *G. vaginalis*, 49.1% (26/53) were highly acidic, 42.4%) were moderately acidic, 19.2% were slightly acidic and 2.3% was very slightly acidic. More of the highly acidic *Lactobacillus* spp culture supernatants inhibited the growth of *G. vaginalis*.

Among those that inhibited *Mobiluncus* spp, 47.2%, 45.4%, 19.2% and 2.3% of the *Lactobacillus* spp culture supernatants had highly acidic, moderately acidic, slightly acidic and very slightly acidic pH, respectively. 33.9%, 39.4%, 19.2% and 2.3% *Lactobacillus* spp that produced supernatants that were highly acidic, moderately acidic, slightly acidic and very slightly acidic, respectively, inhibited *P. bivia*.

Generally, there was an inverse relationship between inhibition of *G. vaginalis* and *Mobiluncus* spp, and decreasing acidity of the supernatant. Highly acidic supernatants were mostly inhibitory while only one supernatant with pH of between 6-6.99 was inhibitory to the indicator bacteria. The difference in the inhibition of the three susceptible indicator bacteria by supernatant at different pH levels was significantly different (ANOVA, \( p < 0.001 \); Table 10).
Table 10: Relationship between pH of supernatant and growth inhibition of BV-associated bacteria

<table>
<thead>
<tr>
<th>pH of supernatant n(%)</th>
<th>Indicator bacteria inhibited</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G. vaginalis (%)</td>
<td>P. bivia (%)</td>
</tr>
<tr>
<td>Highly Acidic**** n=53 (33.5)</td>
<td>49.1</td>
<td>33.9</td>
</tr>
<tr>
<td>Moderately acidic*** n=33 (20.9)</td>
<td>42.4</td>
<td>39.4</td>
</tr>
<tr>
<td>Slightly acidic** n=26 (16.5)</td>
<td>19.2</td>
<td>19.2</td>
</tr>
<tr>
<td>Very slightly acidic* n=46 (29.1)</td>
<td>2.3</td>
<td>2.3</td>
</tr>
</tbody>
</table>

*pH range of 6-6.99, **pH=5-5.99, ***pH=4-4.99, ****pH=2-3.99

3.10 Sensitivity of the inhibitory substance to treatment by physical and chemical agents

Sixteen of 33 Lactobacillus spp that inhibited three of the indicator bacteria were randomly selected for further analysis. Sensitivity to physical and chemical substance was evaluated. The supernatants were boiled at 100°C for 30 minutes, treated with proteases and pH raised to 7.2. None of culture supernatants tested inhibited *P. bivia* (i.e. boiling for 30”, adjustment of pH to 7.2 and proteases treatment), but some of the supernatants continued to inhibit *G. vaginalis* and *Mobiluncus* producing smaller zones of inhibition compared to the untreated supernatant (Table 11). Following boiling, raising pH to 7.2 and proteases treatment respectively, *L. acidophilus* ATCC 4356 supernatant produced zones of inhibition of 4.0mm, 5.0mm and 5.0 to *G. vaginalis*, 4.0mm, 4.5 and 4.5mm to *Mobiluncus* spp and 4.0mm for each treatment to *P. bivia*. After boiling the supernatant, raising pH to 7.2 and treating with proteases, the inhibitory supernatants produced mean inhibition zone of 3.11mm, 3.2mm and 3.0mm to *G. vaginalis*, respectively. Mean zones of inhibition of 2.1mm and 2.0 mm was produced following treatment of the culture.
supernatants with proteases and raising pH, none of the *Lactobacilli* supernatants inhibited *Mobiluncus* spp following boiling. There was significant reduction in the mean zones of inhibition after chemical and physical treatment (ANOVA using transformed means; \( p = 0.0025 \))

**Table 11:** Mean inhibition zone among the 16 selected inhibitory *Lactobacillus* spp after treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th><em>G. vaginalis</em></th>
<th><em>Mobiluncus spp</em></th>
<th><em>P. bivia</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>4.31</td>
<td>4.09</td>
<td>2.96</td>
</tr>
<tr>
<td>Boiling</td>
<td>3.11</td>
<td>0*</td>
<td>0*</td>
</tr>
<tr>
<td>pH 7.2</td>
<td>3.2</td>
<td>2</td>
<td>0*</td>
</tr>
<tr>
<td>Proteases</td>
<td>3</td>
<td>2.1</td>
<td>0*</td>
</tr>
</tbody>
</table>

* = Indicator bacteria not inhibited

After treatment with pepsin, trypsin and proteinase K, culture supernatants from 62.5% *Lactobacillus* spp showed inhibitory activity against *G. vaginalis* and *Mobiluncus* spp each. Following neutralization of the culture supernatant to pH 7.2, 62.5% showed activity against *G. vaginalis* and 12.5% against *Mobiluncus* spp, while 56.3% of the heat treated supernatants were inhibitory to *G. vaginalis* (Table 12). The difference in the resistance of the inhibitory substances to inactivation by treatment with different agents was not significant (one way ANOVA using transformed values; \( p = 0.0580 \)).
Table 12: Effect of proteases (pepsin, trypsin and proteinase K), pH and heat treatment on antibacterial activity

<table>
<thead>
<tr>
<th>Protease treatment</th>
<th>Heating at 100°C for 30min</th>
<th>pH of supernatant to 7.2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=16 (%)</td>
<td>n=16(%)</td>
</tr>
<tr>
<td><em>G. vaginalis</em></td>
<td>62.5</td>
<td>56.3</td>
</tr>
<tr>
<td><em>P. bivia</em></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Mobiluncus spp</em></td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

3.11 Hydrogen Peroxide production and its influence on growth inhibition of indicator strains among the isolated *Lactobacillus* spp

Among the *Lactobacillus* spp isolated, 30.4% of the species produced H$_2$O$_2$, 69.6% were non- H$_2$O$_2$ producing. 37.5% of the H$_2$O$_2$ producing *Lactobacillus* spp inhibited three susceptible indicator strains (*G. vaginalis*, *P. bivia* and *Mobiluncus* spp) and only 14.5% non-H$_2$O$_2$ producing strains were inhibitory. Significantly, more H$_2$O$_2$ positive *Lactobacillus* spp than the non-H$_2$O$_2$ producing strains were inhibitory (Fisher’s exact test; p (two-sided) = 0.0027, Odds Ratio (O.R.) = 3.525, 95% Confidence Interval (CI): 1.601 to 7.760 (using the approximation of Woolf.). Of the 48 H$_2$O$_2$ producing *Lactobacillus*, 100% *L. acidophilus*, 66.7% *L.minutus*, and *L.paraplantarum* each, 50% *L. vaginalis* and *L. Plantanarum* each were inhibitory. Similarly, 30%, 50%, 20% of *L. jensenii*, *L. salivarian* and *L. fermentum*, respectively were also inhibitory. In contrast, *L. iners*, *L. brevis*, *L. casei*, *L. cateneforme*, *L. crispatus*, *L. paracasei* and *L. rhamnosus* though producing H$_2$O$_2$ were not inhibitory.
Of the 110 non-H$_2$O$_2$ producing *Lactobacillus* spp, 38.8%, 15.8%, 50%, 33.3%, 16.7%, 6.7% and 6.1% of *L. acidophilus, L. jensenii, L. casei, L. rhamnosum* and *L. brevis* each, *L. paraplantanarum, L. fermentum* and *L. plantanarum* were inhibitory. In contrast, *L. catenaforme, L. crispatus, L. iners, L. minutus, L. paracasei, L. rhamnosum* and *L. vaginalis*, that were non-H$_2$O$_2$ producing did not inhibit any of the indicator strains (Table 13).

Table 13: Prevalence of H$_2$O$_2$ production and inhibition among the *Lactobacillus* spp

<table>
<thead>
<tr>
<th><em>Lactobacillus</em> spp</th>
<th>Number of isolated</th>
<th>H$_2$O$_2$ Positive (%)</th>
<th>H$_2$O$_2$ Positive Inhibitory (%)</th>
<th>H$_2$O$_2$ negative (%)</th>
<th>H$_2$O$_2$ negative Inhibitory (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. acidophilus</em></td>
<td>23</td>
<td>21.7</td>
<td>38.5</td>
<td>78.3</td>
<td>38.8</td>
</tr>
<tr>
<td><em>L. brevis</em></td>
<td>4</td>
<td>25</td>
<td>0</td>
<td>75</td>
<td>33.3</td>
</tr>
<tr>
<td><em>L. casei</em></td>
<td>3</td>
<td>33.3</td>
<td>0</td>
<td>66.7</td>
<td>50</td>
</tr>
<tr>
<td><em>L. catenaforme</em></td>
<td>5</td>
<td>40</td>
<td>0</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td><em>L. crispatus</em></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td><em>L. fermentum</em></td>
<td>20</td>
<td>25</td>
<td>20</td>
<td>75</td>
<td>6.7</td>
</tr>
<tr>
<td><em>L. iners</em></td>
<td>23</td>
<td>21.7</td>
<td>0</td>
<td>78.3</td>
<td>0</td>
</tr>
<tr>
<td><em>L. jensenii</em></td>
<td>29</td>
<td>34.5</td>
<td>30</td>
<td>65.5</td>
<td>15.8</td>
</tr>
<tr>
<td><em>L. minutus</em></td>
<td>5</td>
<td>60</td>
<td>66.7</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td><em>L. plantanarum</em></td>
<td>20</td>
<td>20</td>
<td>50</td>
<td>60</td>
<td>6.1</td>
</tr>
<tr>
<td><em>L. paracasei</em></td>
<td>2</td>
<td>50</td>
<td>0</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td><em>L. paraplantanarum</em></td>
<td>9</td>
<td>33.3</td>
<td>66.7</td>
<td>66.7</td>
<td>16.7</td>
</tr>
<tr>
<td><em>L. rhamnosum</em></td>
<td>5</td>
<td>40</td>
<td>0</td>
<td>60</td>
<td>33.3</td>
</tr>
<tr>
<td><em>L. salivarian</em></td>
<td>3</td>
<td>66.7</td>
<td>50</td>
<td>33.3</td>
<td>0</td>
</tr>
<tr>
<td><em>L. vaginalis</em></td>
<td>6</td>
<td>66.7</td>
<td>50</td>
<td>33.3</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>158</strong></td>
<td><strong>48 (30.4)</strong></td>
<td><strong>18 (37.5)</strong></td>
<td><strong>110 (69.6)</strong></td>
<td><strong>16 (14.5)</strong></td>
</tr>
</tbody>
</table>
3.12 Comparison of inhibitory capacity by obligate and facultative anaerobic *Lactobacillus* spp

Of the 158 *Lactobacillus* spp strains isolated, 3.1% were strictly (obligatory) anaerobic, while 96.8% were facultative anaerobes. None of the obligate anaerobes showed inhibition to any of the indicator organisms. In contrast, 21.6% of the facultative anaerobic *Lactobacillus* spp were inhibitory to the three susceptible indicator BV associated bacteria, and the rest, 78.4% were not. There was no significant difference in inhibition between facultative anaerobes and anaerobic *Lactobacillus* spp (Fischer's exact; \( p = 0.58 \), OR = 3.1, 95% CI 0.2 to 56.8, using the approximation of Woolf; Figure 13).

![Figure 13: Influence of atmosphere on H$_2$O$_2$ production](image-url)
CHAPTER FOUR
DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 Discussion

The present study sought to identify and characterise the recolonising *Lactobacilli* in the vagina of women two months following treatment for BV. The *Lactobacilli* isolated were tested for production of H$_2$O$_2$ and for inhibitory potential against some bacterial that are associated with BV as indicator organisms. The indicator organisms used were *G. vaginalis*, *B. fragilis*, *P. bivia* and *Mobiluncus* spp. The inhibitory substances were characterized by treatment of the *Lactobacilli* culture supernatants by chemical and physical treatment using proteases and NaOH and boiling at 100°C respectively. The pH of the supernatants was further measured using pH meter before use in the inhibition assay.

Prevalence of BV among the 107 women sampled was 38.8%. 76.6% (82/107) of the sampled women had *Lactobacillus* spp isolated in culture, 37.8% of these had H$_2$O$_2$ producing *Lactobacilli*. Women with higher *Lactobacillus* morphotype (>30/0IF) count had a higher rate of *Lactobacilli* isolation from culture. *Lactobacilli* were isolated from vaginal fluid of 71.4% of 28 women whose gram of vaginal fluid did not show presence of *Lactobacillus* morphotype; no correlation was found between presence of *Lactobacilli* in gram stain and their isolation in culture. One hundred and fifty eight *Lactobacilli* were isolated from 82 women, among these *Lactobacillus jensenii* was the predominant strain (18.4%). 21% of the 158 *Lactobacillus* strains isolated inhibited three BV associated bacteria (*P. bivia*, *G. vaginalis* and *Mobiluncus* spp) and none of the tested strains inhibited the growth of *B. fragilis*. More acidic culture supernatants were more inhibitory
to the three indicator strains. Reduction in the inhibitory potential was found after treating the supernatants with proteases, NaOH to pH 7.2 and boiling at 100°C for 30 minutes.

Bacterial vaginosis (BV) is one of the most common infections among sexually active women that is responsive to antibiotics. It is present in at least 15% of the sexually active population; this makes BV 3–4 times more common than urinary tract infections, many times more common than Trichomonas vaginalis infection (Eschenbach, 2007). It is associated with serious sequelae such as spontaneous abortion, preterm delivery, and increased susceptibility to HIV and other sexually transmitted infections (STI) (Taha et al., 1998; Sewankambo et al., 1997; McGregor et al., 1995).

In this study, the prevalence of BV at two months after treatment for BV using metronidazole 500mg twice daily for 7 days was 38.8%. In other studies carried out elsewhere in Kenya, Harold et al. (1999) reported a BV prevalence of 33% two months, following treatment with oral metronidazole in a group of female sex workers in Mombasa. Elsewhere, Bradshaw et al. (2006) found a BV prevalence rate of 23% and 33% at one and three months respectively, following treatment with oral metronidazole for 7 days. Results of this study are comparable to those of Harold et al. (1999) carried out on population of women in Kenya and those of Bradshaw et al. (2006) carried out on white population in the United States. This is contrary to the expected racial differences that have been attributed to increase in prevalence and in recurrence of BV among black ethnic groups (Hay et al., 1994; Goldenberg et al., 1996).

Among this selected sample of 107 women who were treated for BV, and then a repeat sample taken at two months, more than one third (38.3%) had BV. These findings
indicated that either the women had not recovered after treatment with oral metronindazole or they had a relapse of BV. Treatment failure may result after treatment with metronindazole or clindamycin. Eschenbach (2007) suggested that failure to respond to antibiotic treatment in women with BV may result due to the resistance of BV associated bacteria to metronindazole and clindamycin. Treatment with metronindazole or clindamycin administered orally or intra-vaginally, has been followed by frequent recurrences of BV following successful treatment (Bradshaw et al., 2006). This may be attributed to failure of re-colonization by ‘protective’ i.e H₂O₂ positive inhibitory Lactobacilli, sexual behaviour of the women such as having multiple partners or recent change of partners and certain hygiene practices such as vaginal douching.

The vaginal biota has been widely studied and diverse species of Lactobacillus have been identified as the predominant biota. There have been no reports on the predominant Lactobacilli in vaginas of women in the sub-Saharan Africa where BV is highly prevalent. In the present study, a large diversity of Lactobacilli was identified. Among the Lactobacillus spp isolated were; L. jensenii, L. acidophilus, L. iners, L. fermentum, L. brevis, L. casei, L. cateniforme, L. crispatus, L. minutus, L. plantanarum, L. paracasei, L. paraplantanaum, L. rhamnosum, L. salivarian and L. vaginalis. L. jensenii was the most significantly abundant compare to other Lactobacillus species (p= 0.001). There was no particular species that could be associated with the vaginal flora as L. jensenii was the predominant species in populations of women with and without BV. Anukam et al. (2005) identified L. iners, L. gasseri, L. plantarum, L. crispatus, L. vaginalis and L. rhamnosus from vaginal swabs of women with BV. Antonio et al. (1999) reported the identification of strains of Lactobacillus ruminis, L. reuteri, L. oris and L. vaginalis. Boris et al. (1998) identified L. acidophilus, L. plantarum, L. fermentum, L. brevis, L. casei subsp. casei in a
group of women. Eschenbach et al. (1989) identified *Lactobacillus catenaforme, L. helveticus* and *L. salivarius*. In general, *L. crispatus, L.jensenii, L. acidophilus* and *L. fermentum* have been reported to be the predominant species in vaginal tracts of women with normal flora in majority white population. In different populations of women, *L. gasseri* has been reported to be the predominant strain of *Lactobacillus* colonizing the vagina. Aslim and Kilic (2006) reported *L. gasseri* as the predominant strain in Turkish women, while Song et al., (1999) reported *L. crispatus* and *L. gasseri* as the predominant species colonizing the vaginas of Japanese women. In the present study, *L. jensenii, L. acidophilus* and *L. iners* were the predominant *Lactobacillus* spp, agreeing with results reported in the other studies despite racial differences in many studies across the world. These results would therefore suggest that racial differences do not affect the vaginal *Lactobacillus* spp diversity.

Although majority of the isolates were facultative anaerobic *Lactobacilli*, 5 obligatory anaerobic strains of *Lactobacilli* were isolated in this study with two being H$_2$O$_2$ producers. This is contrary to other studies where either no anaerobic *Lactobacilli* were isolated (Pascual et al., 2006), or the anaerobic *Lactobacilli* did not produce H$_2$O$_2$ (Eschenbach et al., 1989).

When *Lactobacillus* bacterial morphotypes counted in the gram stain smear of the vaginal fluids from the women were compared with isolation rate of *Lactobacillus* spp in culture, a significant number of women with high *Lactobacilli* morphotype count per OIF (>30/OIF) had *Lactobacillus* spp. *Lactobacilli* were recovered in 71% of women with no *Lactobacilli* morphotype counted in the gram stain of their vaginal fluid. These findings indicate that the presence of *Lactobacillus* morphotypes in gram stain is does not
necessarily confirm protective *Lactobacillus* spp are present in the vaginal tract. Twenty-two percent of women who had at least one *Lactobacillus* morphotype in gram stain of their vaginal fluid did not have *Lactobacillus* spp isolated in culture. Wilson and Blitchington (1996) reported that cultivability of bacteria range between 10%-50% and therefore many uncultivable microorganisms remain unidentified but it is known that that cultivation approaches are biased due to inability of some bacteria to grow on artificial media (Simpson et al., 2000). It is therefore possible that the organisms counted as *Lactobacillus* morphotype in gram stain were either not *Lactobacillus* or if they were, they are not cultivable. It is also be a possible that the culture technique was not appropriate; either the culture was properly performed resulting to failure of growth perhaps due to bad incubation conditions, or the transport of the specimen from the source was inappropriate resulting to loss of bacterial viability at the time of culture. It may be important to relate the morphology in gram stain with the actual identity of the organisms by use of more sensitive methods, such as Pulsed field gel electrophoresis and 16S rDNA sequencing.

*Klebanoff* et al. (1991) demonstrated the importance of H2O2 producing *Lactobacilli* in vagina of most normal women. The study showed the role of H2O2 and chloride in prevention of infection by genital pathogens and demonstrated that the H2O2 producing *Lactobacilli* could be replaced by H2O2. Hillier et al. (1992) confirmed these findings and reported that the H2O2 producing *Lactobacillus* spp is inversely correlated to infection by genital pathogens.

In this study, H2O2 positive *Lactobacilli* were recovered in over one third of women who had BV and were higher than in those without BV. In other studies, Rosenstein *et al.*
(1997) isolated H$_2$O$_2$-producing *Lactobacilli* from 91.7% of pregnant women whose microbiota was indicative of BV. Alvarez-Olmos *et al.* (2004) in a separate study among reported isolation of H$_2$O$_2$-producing *Lactobacilli* from 85% women with BV, and from 15% women without BV. Hillier *et al.* (1993) recovered H$_2$O$_2$ producing *Lactobacilli* in only 5% of women with BV, while Nagy *et al.* (1991) recovered H$_2$O$_2$ producing *Lactobacilli* in 23% of women with BV flora. Results from this study are similar with the findings of Rosenstein *et al.* (1997) and Alvarez-Olmos *et al.* (2004) and also support the suggestion by Rosenstein *et al.* (1997) that abnormal bacteria begin to appear and increase before disappearance of H$_2$O$_2$ positive *Lactobacilli* and that BV may develop in some women despite the presence of H$_2$O$_2$-producing strains of *Lactobacilli*. It can further be suggested that other factors, as yet unidentified, might be conducive to the appearance of abnormal bacterial flora with progression to vaginosis. Overgrowth of pathogenic bacteria may therefore be important than the reduction of H$_2$O$_2$ producing *Lactobacilli*. Nagy *et al.* (1991) showed that some strains of *G. vaginalis*, *Bacteroides* spp, *Mobiluncus* spp and *Peptostreptococcus* spp would inhibit *in vitro* growth of some strains of different *Lactobacillus* spp. It may therefore be possible that these BV associated bacteria would inhibit *in vivo* growth of *Lactobacilli* and recolonize the vagina.

Nagy *et al.* (1991) associated the production of H$_2$O$_2$ with the origin of the strain (vagina of women with or without BV) and found that more H$_2$O$_2$ producing strains were isolated in women with normal vagina. This study found no such association and majority of the women with H$_2$O$_2$ producing *Lactobacilli* had BV flora. This may be explained by the fact that the population used in this study was composed of women who originally had BV and were treated two months earlier, and they had either not been re-colonized by the
correct' *Lactobacilli* or the H$_2$O$_2$ producing *Lactobacilli* did not protect them against the BV associated bacteria.

Lactic acid bacteria have been shown to inhibit the *in vitro* growth of many pathogens and have been used as probiotics in both humans and animals to treat a broad range of gastrointestinal and/or vaginal disorders (Falagas *et al*., 2007; Piyawan *et al*., 2006; Karaoglu *et al*., 2003; Rolfe, 2000). *Lactobacilli* have been found to be protective against vaginal infections and several mechanisms of protection have been suggested. The inhibition of growth have been attributed to production of organic acids such as lactic acid (Oyetayo, 2004), H$_2$O$_2$ production (Kaewsrichan *et al*., 2006), bacteriocins (Karaoglu *et al*., 2003) and competitive inhibition of the pathogens to adhesion and nutrient by *Lactobacilli* (Vesterlund *et al*., 2006; Reid and Burton, 2002). The natural inhibition of some common pathogenic bacteria by *Lactobacilli* may be important in understanding the initiation of vaginal infections or bacterial vaginosis associated with an unexplained decrease in vaginal *Lactobacilli* (Karaoglu *et al*., 2003).

In this only 21% of the *Lactobacilli* strains inhibited growth of *P. bivia*, 28% the growth of *G. vaginalis*, and 29% the growth of *Mobiluncus* spp. None of the tested strains inhibited *B. fragilis* (*B. fragilis* was resistant to inhibition by all strains of *Lactobacilli* tested). The cause of the resistance by *B. fragilis* to inhibition by *Lactobacilli* is unclear but it may be partly associated with their ability to produce catalase, which may hydrolyze H$_2$O$_2$ produced by *Lactobacilli*; it may also be speculated that they produced other substances such as enzymes that inactivate the inhibitory agents produced by *Lactobacilli*. McLean and Rosenstein (2000) tested 60 vaginal isolates of *Lactobacilli* their ability to inhibit the growth of *G. vaginalis, Bacteroides* spp, and *Prevotella bivia* and found all the
tested *Lactobacilli* inhibitory to each of the indicator bacteria. These results contradict the findings of the current study where only a fraction (33/158) of the *Lactobacilli* was inhibitory to the indicator bacteria and non inhibited *B. fragilis*. It is possible that since *Lactobacilli* in the current study were isolated from women who originally had BV, the recolonising *Lactobacilli* had less protective qualities and this may further explain the reason for the high rate of BV recurrence in women in Sub-Saharan Africa. The *Lactobacilli* tested in this study produced zones of inhibition of growth in the range 1.5-8mm, with *G. vaginalis* being the most susceptible to *in vitro* inhibition by *Lactobacilli*. These findings agree with those reported by McLean and Rosenstein (2000).

Lactic acid produced by vaginal *Lactobacilli* contributes to the maintenance of a low vaginal pH and high redox potential which can inhibit growth of other bacteria (Oyetayo, 2004; McLean and Rosenstein, 2000; Holmes *et al.*, 1985). In this study, *Lactobacilli* isolates that produced highly acidic supernatant (pH 2.62-3.99) were more inhibitory. McLean and Rosenstein (2000) observed that four of the strains tested that produced more acidic culture were the most inhibitory which is supported by findings of the present study therefore demonstrating the role of organic acids in inhibition of growth of pathogens.

In this study, the inhibitory compounds produced by the test isolates showed a reduction in the inhibitory activity to *G. vaginalis* and to a greater extent to *Mobiluncus* spp, and a complete loss of inhibitory activity to *P. bivia*. This further confirms the role of organic acid in *in vitro* inhibition of the bacterial vaginosis associated bacteria.

In this study, treatment of the supernatants by heating at 100°C (meant to test for bacteriocins) was detrimental to the inhibitory compounds as only 56.3% of the culture
supernatant were *G. vaginalis* and none were inhibitory either by *P. bivia* and *Mobiluncus* spp. These findings demonstrate the possible contribution of heat labile compounds (bacteriocins) in *in vitro* inhibition of the BV associated bacteria. Ogunbanwo *et al.* (2003) found that inhibitory compounds produced by the test isolates in their study were heat stable. McLean and McGroarty (1996) demonstrated that heat treatment of supernatants had no effect on the sizes of inhibition zones. These results contradict the results of the present study which demonstrates a possible role of heat labile compounds in inhibition.

This study observed that the inhibitory potential of culture supernatants of the *Lactobacilli* were not completely destroyed after treatment with three proteases (proteinase K, pepsin and trypsin). However, there was a reduction in the inhibitory activity of the treated supernatants to the indicator strains. Karaoglu *et al.* (2003) observed that inhibitory activity of *Lactobacilli* isolated was completely destroyed by treatment with proteinase K and trypsin while Ogunbanwo *et al.* (2003) reported that antimicrobial activity was lost or unstable after treatment with proteolytic enzymes. Oyetayo (2004) on the other hand found that the inhibitory zones remained unchanged when the supernatant was treated with trypsin, indicating that bacteriocin was not responsible for the inhibition of the bacteria (Oyetayo 2004). Findings of this study agree with the findings of Karaoglu *et al.* (2003) and Ogunbanwo *et al.* (2003 and suggests possible the role of bacteriocins in the inhibition of the test bacteria.

From these findings it was clear that more than one substance was involved in the inhibition, and the heat labile compounds (bacteriocins) had a greater role in the inhibition of growth of these indicator bacteria. This is because, after heating at 100°C, only few
strains of *Lactobacilli* still had some inhibitory potential to *G. vaginalis* only, and no activity on *P. bivia* and *Mobiluncus* spp.

*G. vaginalis* was the most susceptible, followed by *Mobiluncus* spp while *B. fragilis* was the most resistant to inhibition by the *Lactobacillus* spp tested. The former two are gram positive and gram variable respectively. The antibacterial effect of the isolates was demonstrated against both gram positive and negative bacteria but *Lactobacilli* showed stronger antibacterial effect against gram positive/variable bacteria (*G. vaginalis* and *Mobiluncus* spp) than gram negative strains (*P. bivia* and *B. fragilis*). This observation agrees with the report of Gilliland and Speck (1977) that *Lactobacilli* are more inhibitory to gram positive than gram negative bacteria. It was also noted that different bacteria are inhibited by different concentrations of inhibitory substance.
4.2 Conclusions

Following results of this study, the following conclusions are made:

- BV was found in over a third of the women indicating that BV is still highly recurrent following antibiotic therapy.

- Large diversity of *Lactobacilli* was found among the women with *L. Jensenii*, *L. acidophilus* and *L. iners* being the predominant species; these would be useful in future studies for designing probiotics for BV management in Kenya.

- Two months following treatment of women for BV, only about a third were colonized with H$_2$O$_2$ positive *Lactobacilli* (LB) as a result many women remained unprotected following treatment and hence prone to recurrent BV.

- Only a fifth of the *Lactobacilli* were inhibitory to BV associated bacteria and therefore majority of the women are recolonized by unprotective *Lactobacillus* strains. This partially explains why BV would recur among the women.

- It is necessary for the metabolic product of *Lactobacillus* spp (bacteriocins, hydrogen peroxide and acid) to augment each to be effective inhibitors of growth of vaginal pathogens.

- When selecting a *Lactobacilli* strain for use in bacteriotherapy, a strain producing all the indicated inhibitory substances is desirable.

- This study did not find a significant difference in the number inhibitory *Lactobacillus* and those producing H$_2$O$_2$ and therefore accept the hypothesis.
4.3 Recommendations

This study recommends:

- Further studies to confirm resistance of many strains of *B. fragilis* using different stains of *Lactobacilli* and recommend appropriate treatment.

- Further studies involving a combination of conventional therapy (metronidazole/clindamycin) and biotherapy using *Lactobacilli* with good inhibitory capacity to BV pathogens

- Use of more sensitive methods, such as 16S rRNA typing, Pulsed Field gel Electrophoresis (PFGE) for *Lactobacilli* identification and confirmation of presence or absence of *Lactobacilli* in vaginal fluid.

4.4 Limitations

- The population of women sampled was highly selective (two months post BV treated women) and therefore results may not be generalized to represent Kenyan women.

- Another problem encountered was the natural fastidious nature of the anaerobes used in this study as indicator bacteria. Sometimes it was difficult to grow continuously in culture and would keep re-isolating them from stock media. This delayed some set experiments.
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Appendix 1

Preparation and Concentration of Reagents, Chemicals and Media

Preparation of culture media

Brucella blood agar containing 5% sheep blood vitamin K1 and Hemin

To prepare Brucella agar base:
Prepare the Brucella agar base by adding the specified amount of Brucella agar base to distilled water.
Mix thoroughly and heat with frequent agitation. Boil for 1 min to ensure complete solution of ingredients.

To prepare vitamin K1 stock solution
Dissolve 0.15mls of vitamin K1 (sigma) in 30mls of 95% ethanol.
NB: Care must be taken to ensure full amount is pipetted before dissolving it in ethanol because its difficult to pipette. Store in dark container or wrap tube with foil at 4°C, shelf life is 1 month.

To prepare hemin solution
Dissolve 0.5 g hemin in 10mls of 1N sodium hydroxide (NaOH).
Bring volume to 100mls with distilled water.
Sterilise by autoclaving. Concentration of final solution is 5mg/ml.
Use 1ml/liter to supplement anaerobic media.
Store in dark container or wrap tube with foil at 4°C.

To prepare Brucella agar
Dissolve Brucella agar base by boiling as directed by manufacturer.
Following boiling, but not before autoclaving add appropriate amounts of vitamin K1 and hemin and mix well.
Autoclave at 121°C for 15 min. after autoclaving, cool to 50°C and add sheep blood.
Mix well and avoid creating bubbles. Dispense in petridishes.

Quality control
Check the performance of the complete medium with pure culture of stable control organisms producing known desired reactions.

<table>
<thead>
<tr>
<th>Organism</th>
<th>desired result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroides fragilis</td>
<td>growth</td>
</tr>
<tr>
<td>Peptostreptococcus anaerobius</td>
<td>growth</td>
</tr>
</tbody>
</table>

Incubate one additional plate per batch to determine the sterility of the media.
Columbia Colistin-Nalidixic acid (CNA) agar containing 5% sheep blood

To prepare columbia agar base:
Prepare the columbia agar base by adding the specified amount of columbia agar base to distilled water.
Mix thoroughly and heat with frequent agitation.
Boil for 1 min to ensure complete solution of ingredients.
To prepare colistin
Dissolve specified amount of colistin in a small volume of water. N.B 2mg=0.0020; 5mg=0.005; etc.

To prepare nalidixic acid
Dissolve specified amount of nalidixic acid in a small volume of 1N NaOH and dilute with a small volume of distilled water. For 1 litre of CNA agar, 4 drops of NaOH can be used to dissolve the nalidixic acid.
NB: colistin and nalidixic acid should be weighed any time CNA is prepared. No stock solutions should be made.

To prepare CNA agar
Dissolve columbia agar base as directed by manufacturer.
Following boiling, but not before autoclaving add appropriate amounts of colistin and nalidixic acid and mix well.
Autoclave at 121 °C for 15 min.
After autoclaving, cool to 50 °C, and add sheep blood.
Mix well and avoid creating bubbles.
Dispense in petridishes and Store at 2-8 °C for up to 4 weeks in plastic bags.

Quality control
Check the performance of the complete medium with pure culture of stable control organisms producing known desired reactions. Use the following stock cultures and incubate plates as directed for 24-48 hrs at 35 °C at appropriate atmosphere.

<table>
<thead>
<tr>
<th>Organism</th>
<th>desired result</th>
<th>culture number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus (aerobically)</td>
<td>growth</td>
<td>ATCC 25923</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>inhibited</td>
<td>ATCC 12453</td>
</tr>
<tr>
<td>Propionibacterium acnes (anaerobically)</td>
<td>growth</td>
<td></td>
</tr>
<tr>
<td>Bacteroides fragilis</td>
<td>inhibited</td>
<td></td>
</tr>
</tbody>
</table>

Incubate one additional plate per batch anaerobically and one additional plate per batch aerobically to determine the sterility of the media.
Rogosa SL Agar

<table>
<thead>
<tr>
<th>To Prepare</th>
<th>Rogosa Agar</th>
<th>Distilled H₂O</th>
<th>Glacial Acetic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 mLs</td>
<td>37.5 grams</td>
<td>500 mLs</td>
<td>0.66 mLs</td>
</tr>
<tr>
<td>1 liter</td>
<td>75 grams</td>
<td>1 liter</td>
<td>1.32 mLs</td>
</tr>
<tr>
<td>2 liters</td>
<td>150 grams</td>
<td>2 liters</td>
<td>2.64 mLs</td>
</tr>
</tbody>
</table>

To Prepare Rogosa agar:
Suspend Rogosa SL agar in distilled water and boil to dissolve completely.
Add glacial acetic acid.
Mix well and boil for 2 to 3 minutes. DO NOT AUTOCLAVE.

Storage Instructions:
Prepared media may be stored at 2 to 8 degrees Celsius for one month. Each lot needs to be dated with preparation date.

Quality Control:
Check the performance of the media with pure cultures of stable control organisms producing the desired results.

<table>
<thead>
<tr>
<th>Atmosphere</th>
<th>Organism</th>
<th>Desired Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaerobic</td>
<td><em>Lactobacillus</em></td>
<td>Growth</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>Group B Strep</td>
<td>No Growth</td>
</tr>
</tbody>
</table>

Incubate one uninnoculated plate to serve as sterile control

Yeast Extract

To prepare Yeast Extract Liquid for specimen dilution
Prepare the Yeast Extract Liquid by adding the specified amounts of Yeast Extract powder to the specified volume of distilled water.
Mix thoroughly with frequent agitation
Autoclave at 121 °C for 15 minutes
After autoclaving, place tubes into anaerobic chamber; loosen caps and allow them to cool in chamber
After cooling, tighten caps and remove from chamber
Refrigerate at 4 °C until needed

Storage instructions
Prepared media may be stored at 2-8 °C for up to 6 months in screw caps. Each lot should be dated
Quality control of Yeast Extract Liquid
Check performance of the complete medium with pure cultures of stable control organisms producing known desired reactions. Inoculate one tube for each of the following stock cultures. Incubate tubes as directed for 24-48 hours at 35°C

**Incubate one uninnoculated tube to serve as sterile control**

Carbohydrate fermentation test agar

**To prepare fermentation basal test medium**
Prepare the basal test medium by adding the specified amount of fermentation basal medium to the specified volume of distilled water.
Mix thoroughly, and heat with frequent agitation
Boil for 1 minute to ensure complete solution of ingredients.
Adjust the volume of the finished product as needed with distilled water.
Dispense in 100ml amounts into screw cap bottles.
After autoclaving, let cool to 50°C before using with desired sugars.

**Preparation of sugars**
To prepare sugars, prepare 10% solution in distilled water (e.g. 10g of glucose in 100ml of distilled water).
Sugars must be sterilised by filtration.
Transfer to sterile screw cap bottles.
NB: sugars may be stored for up to 1 year. Check sugars carefully for contamination and/or precipitation before using.

Quality control
Check the performance of the complete medium with pure culture of stable control organisms producing known desired reactions. Inoculate one tube of basal medium and one tube of sugar for each stock culture. Use the following stock cultures and incubate plates as directed for 24-72 hrs at 35°C in room air.

**Organism** | **culture number** | **sugar** | **desired result**
--- | --- | --- | ---
*Escherichia coli* | ATCC 25922 | Glucose | Growth; positive
*Pseudomonas aeruginosa* | ATCC 27853 | Glucose | Growth; positive (top)

**Incubate one uninnoculated tube to serve as sterile control**

Bile Esculin Agar

**To prepare Bile Esculin agar base**
Prepare Bile Esculin agar base by adding the specified amounts of Bile Esculin agar base to the specified volume of distilled water.
Mix thoroughly and heat with frequent agitation
Boil for one minute to ensure complete solution of ingredients
Adjust volume of finished product as needed using distilled water
Dispense 4ml amounts into 13x100mm screw cap tubes
Autoclave at 121°C for 15 minutes
After autoclaving, place tubes in a slanted position and allow to cool
Storage instructions
Prepared media may be stored at 2-8 °C for up to 6 months in screw caps. Each lot should be dated.

Quality control of Bile Esculin agar
Check performance of the complete medium with pure cultures of stable control organisms producing known desired reactions. Inoculate one tube for each of the following stock cultures. Incubate tubes as directed for 24-48 hours at 35 °C.

<table>
<thead>
<tr>
<th>Atmosphere</th>
<th>Organism</th>
<th>Culture #</th>
<th>Desired result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Room air</td>
<td>E. faecalis</td>
<td>ATCC 29212</td>
<td>Growth, BE positive (black)</td>
</tr>
<tr>
<td>Room air</td>
<td>Group A Streptococcus</td>
<td>ATCC 19615</td>
<td>Inhibited; BE negative</td>
</tr>
</tbody>
</table>

Incubate one uninoculated tube to serve as sterile control.
ii Preparation of reagents

Gram stain Reagents

Ammonium oxalate-crystal violet
Crystal violet powder 20 grams
Methylated Spirit 200 mls
Ammonium oxalate 8 grams
Distilled water 800 mls
Dissolve the crystal violet powder with the methylated spirit. Then add the ammonium oxalate solution. Stir to mix thoroughly and then filter the solution into a clean bottle. Make a new label recording the date of preparation and your initials. Store at room temperature for 1 year.

Iodine solution
Iodine 10 grams
Potassium iodide 20 grams
Distilled water 1000 mls
Dissolve the iodine and potassium iodide in the water. Mix thoroughly before filtering into a clean bottle. Make a new label recording the date of preparation and your initials. Store at room temperature for 1 year.

Liquor iodifortis (BP)
Iodine 10 grams
Potassium iodide 6 grams
Methylated spirits 90 mls
Distilled water 10 mls
Dissolve the iodine and potassium iodide in the methylated spirits. Add the water and mix thoroughly. Make a label recording the date of preparation and your initials. Store in a clean bottle at room temperature for 1 year.

Iodine-acetone
Liquor iodifortis 35 mls
Acetone 965 mls
Mix the solutions well. Make a label recording the date of preparation and your initials. Store in a clean bottle for 6 months.

Basic Fuchsin stain
Basic fuchsin 1.0 grams
Distilled water 100 mls
Dissolve the basic fuchsin in the water. Mix thoroughly before filtering into a clean bottle. Make a label recording the preparation date and your initials. Store at room temperature for 1 year.

Quality Control

<table>
<thead>
<tr>
<th>Organism</th>
<th>desired result</th>
<th>culture number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>gram positive</td>
<td>ATCC 25923</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>gram positive</td>
<td>ATCC 25922</td>
</tr>
</tbody>
</table>
Appendix 2

i Flow diagram for identification of *Lactobacillus* spp and *G. vaginalis* by Biochemicals

Work up of aerobic Gram positive rods
ii Flow diagram for identification of *Lactobacillus* spp by Wee Tabs

**Anaerobic Gram Positive Non-Sporforming Bacilli using Wee Tabs (K1464 and K1283)**

[Diagram of the flow chart for identification of *Lactobacillus* spp using Wee Tabs (K1464 and K1283). The diagram includes various steps and outcomes for different tests and reactions.]
iii Flow diagram for identification of *Lactobacillus* spp by CHO fermentation

```
Strict Anaerobe
  +-----------------+------------------+
  | Yes             | No               |
  +-----------------+------------------+
  | Cellibiose      | Lactose          |
  +-----------------+------------------+
  | Neg, L. uli     | Pos, L. catenefome|
  +-----------------+------------------+
  | Pos, DO RAF     | Neg, DO Succ     |
  +-----------------+------------------+
  | Neg, L. rham    | Pos, L. paraplan |
  +-----------------+------------------+
  | Neg, DO Raf.     | Pos, DO Raf.     |
  +-----------------+------------------+
  | Neg, Do Celle    | Pos, DO Mel      |
  +-----------------+------------------+
  | Pos, L. vag      | Neg, L. crisp.   |
  +-----------------+------------------+
  | L. leich        | Pos, L. acido    |
  +-----------------+------------------+
  Pos, L. para Sb para | Neg, L. casei   |
  +-----------------+------------------+
  | Pos, L.paracasei subspecies paracasei | Neg, L. brevis |
  +-----------------+------------------+
  | L. salivarius   | Pos, DO Xylo    |
  +-----------------+------------------+
  | L. paraplanarum | Pos, L. planta  |
  +-----------------+------------------+
  | L. iners        | Neg, L. oris    |
  +-----------------+------------------+
  | L. fermentum    | Pos, L. vaginalis |
  +-----------------+------------------+
  | L. crispatus    | Neg, L. casei   |
  +-----------------+------------------+
  | L. paracasei    | Neg, L. brevis  |
  +-----------------+------------------+
  | L. paraplantarum | Pos, DO Xylo    |
  +-----------------+------------------+
  | L. jensenii     | Neg, L. oris    |
  +-----------------+------------------+
  | L. leichmannii  | L. fermentum    |
  +-----------------+------------------+
```

Flow diagram for identification of Anaerobic Gram Negative rods by biochemicals
Flow diagram for identification of *Bacteroides* spp by Wee Tabs

Organisms that grow well on BBE

![Flow diagram](image-url)
vi Flow diagram for identification of *Prevotella* spp by Wee Tabs

*Prevotella* sp. using Wee Tabs (K1464)
vii Flow diagram for identification *Mobiluncus* spp by biochemicals and Wee Tabs enzymes

**Workup of Anaerobic Gram Negative (or Gram variable) Curved Rods**

- Gram stain of anaerobic culture shows Gram variable or Gram negative rods; curved, motile, non-sporoforming rods with tapered ends, occurring singly or in pairs, with a gullwing appearance

- **Oxidase**
  - Positive
    - *Campylobacter* species
      - See p. 154 Isenberg 1998
  - Negative
    - *Mobiluncus*
      - Confirm with starch hydrolysis

- **Hippurate**
  - Positive
    - Nitrate
      - Positive
        - *M. curtisi subsp. holmesii*
      - Negative
        - *M. curtisi subsp. curtisi*
  - Negative
    - Rapid ANA
      - *M. mulieri*
Appendix 3

iii Photos of agar plate media, identification and incubation systems used

Media

‘A’-Columbia colistin Nalidixic acid (CNA) agar, ‘B’- Man Rogosa Sharpe (MRS) agar, ‘C’ - Brucella agar, ‘D’ – Tetramethylbenzidine (TMB) agar
iv Anaerobic Incubation systems

Anaerobic Incubation systems; 'A'-Anaerobic boxes (used with gas generating kits), 'B'-Anaerobic chamber system (used with mixed gases)

AnaeroPack gas generating kit (used with the anaerobic box (above))
Identification systems: from left; RapID ANA panel, Carbohydrate fermentation microtiter plate system