Trop J Obstet Gynaecol, 22 (2), October 2005.

Association Between Absence of Vaginal Lactobacilli PCR Products and Nugent Scores Interpreted as Bacterial Vaginosis

Kingsley C. Anukam[1,2,4], Emmanuel O. Osazuwa[2], Ijeoma Ahonkhai[2] and Gregor Reid[1,3]

[1]Canadian Research & Development Centre for Probiotics, Lawson Health Research, Institute, 268 Grosvenor St., London, Ontario, Canada.; [2]Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Benin, Nigeria; [3]Department of Microbiology and Immunology and Surgery, University of Western, Ontario, London, Ontario, Canada and [4]Chidak Medical Diagnostic Laboratories, 200 M. M. Way, Benin City, Nigeria.

Abstract

Context: The most common cause of abnormal vaginal discharge in women of childbearing age is bacterial vaginosis (BV). This condition predisposes women to increased susceptibility to sexually transmitted diseases, including human immunodeficiency virus (HIV) infections and preterm birth. The diagnostic methods commonly adopted in the evaluation of patient samples for BV are Amsel criteria and Nugent score, the latter requiring microscopy and expert interpretation. The use of polymerase chain reaction (PCR) with group specific lactobacillus primers, in combination with Nugent criteria has not been widely used in developing countries such as Nigeria.

Objective: The aim of the present study was to examine the relationship between vaginal Lactobacilli using PCR with group specific lactobacillus primers and Nugent Score criteria for bacterial vaginosis.

Study Design, Setting, Subjects, and Methods: We evaluated 241 vaginal samples from apparently healthy premenopausal women in Benin City for the presence of Lactobacilli with group specific primers in a polymerase chain reaction master cycler and bacterial vaginosis using the Nugent score criteria.

Results: Of the 241 vaginal samples that were Gram stained for Nugent rating, 84 (34.8%) had Normal rating (0-3), 123 (51%) had Intermediate score (4-6), while 34 (14.2%) had Bacterial vaginosis, with Nugent score (7-10). There was a positive association between absence of lactobacilli PCR product and Nugent scores interpreted as BV (85.8% vs. 14.2%. $\chi^2 = 4.12$, P=0.05).

Conclusion: The study has demonstrated a strong relationship between bacterial vaginosis and absence or depletion of vaginal lactobacilli using molecular techniques.

Key words: Lactobacilli, bacterial vaginosis, Nugent score, polymerase chain reaction.

Introduction

BV formally referred to as non-specific vaginitis [1], is a major cause of urogenital disease in females. It has been defined as a mild infection of the lower female genital tract, characterized by the presence of three of four criteria to define urogenital disease: 1) release of an amine (putrescine, cadaverine, and trimethylamine) or a fishy odour after the addition of 10% potassium hydroxide, 2) a vaginal pH >4.5, 3) clue cells in the vaginal fluid, and 4) a milky homogenous, malodorous vaginal discharge [2, 3]. It is also characterized by a depletion of *Lactobacillus* spp and an overgrowth of diverse aerobic, anaerobic and micro-aerophilic species such as *Gardnerella vaginalis*, *prevotella spp.*, *Peptostreptococcus spp.*, *Mycoplasma hominis*, *Ureaplasma urealyticum*, *Mobiluncus spp* and *Atopobium vaginae* [4, 5]. The prevalence of BV varies in different parts of the world, e.g., 25% in a group of healthy Canadian women [6], 29.9% in Indonesia [7], 15% in rural Brazil [8] and 14.2% in healthy Nigerian women [9]. An estimated 25-30% women have BV at any given time, mostly without signs such as fishy-odour or discharge [10], and this rises to 85% in prostitute populations [11].

BV has been associated with various gynecological and obstetric complications including pelvic inflammatory disease (PID), post-caesarean delivery endometritis, chorioamnionitis and premature rupture of membranes, late miscarriage and pre-term labour [12]. A study of pregnant women demonstrated that women who were positive for BV on screening were five times more likely to experience preterm labour or late miscarriage than those without BV [13]. Recently links have been demonstrated between abnormal vaginal microbiota, lack of lactobacilli and HIV infection [14]. Of the diagnostic methods currently available, assessment of the clinical signs is the 'gold standard', but the signs are subtle and detection of the signs is very dependent on the acuity of the clinician evaluating the clinical signs. However, Nugent *et al* [15] described an improved standardized method of Gram stain interpretation. The criteria were designed to provide a scoring system to

Correspondence: K.C. Anukam, Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Benin, P.M.B. 1154, Benin City, Edo State, Nigeria.
***E-mail**: anukamkc@yahoo.com*

evaluate the alteration in vaginal microbiota from the normal to the abnormal BV state as a continuum rather than a dichotomy. Vaginal swabs smears are graded on a 10-point scale based on the presence or absence of Lactobacillus morphotypes. Recently, another method for the diagnosis of BV has been developed as a result of elevated bacterial sialidase activity associated with BV causing micro-organisms. The test known as BVBlue is a newly developed chromogenic point-of-care test for the diagnosis of BV, which is based on detection of increased vaginal fluid sialidase activity (>7.8 U). This new method is yet to be introduced in Nigeria, besides only three studies have evaluated its performance to date [16,17].

The use of polymerase chain reaction (PCR), which has not been widely used in sub-Saharan Africa offers the potential for rapid and reliable examination of the vaginal microbiota in clinical studies; this method has been used previously on bacterial microbiota isolated from other parts of the human body [18, 19]. The method allows numerous samples to be screened, because the microbial nucleic acid used as templates for PCR can be derived from human specimens, without the need for further culture enrichment. Primers designed to amplify DNA, using PCR, have been largely based on universal bacterial 16S ribosomal subunit gene sequences.

The goal of the present study was to examine the relationship between Lactobacilli vaginal microbiota of apparently healthy Nigerian women using polymerase chain reaction (PCR) with group specific lactobacillus primers and Nugent Score criteria for bacterial vaginosis (BV).

Materials and Methods

Study Subjects

Two hundred and forty one vaginal swabs were collected from premenopausal, healthy female volunteers. The age of the women ranged between 18 and 48 years. The swabs were packaged in ice-packs and subsequently transported via courier to the Lawson Health Research Institute Laboratory, London, Ontario, Canada, for bacterial DNA extraction and polymerase chain reaction (PCR) amplification.

Nugent Scores

Prior to DNA extraction, smears were made on microscope slides from the vaginal swab collected from each subject. The slides were Gram stained and scored by Nugent criteria [15]. A score of 0-10 was assigned considering the relative proportions of large gram positive rods (lactobacilli), small gram-negative or gram variable rods (*Bacteriodes*, *Prevotella*, or *Gardnerella* species) and curved gram-variable rods (*Mobiluncus* species). A score of 0-3 was interpreted as consistent with normal microbiota, a score of 4-6 as intermediate and a score of 7-10 was considered consistent with BV-like conditions.

Extraction of DNA from Bacteria on Vaginal Swabs

DNA was extracted from the bacteria present on vaginal swabs, using Instagene Matrix (Bio-Rad Laboratories, Ontario, Canada), according to the manufacturer's instructions. Briefly swabs were vigorously agitated in 1mL of phosphate buffered saline (PBS), (pH 7.1) to dislodge cells. The cells were pelleted by centrifugation (Eppendorf, Digital Centrifuge 5417C) at 10,000g for 5 minutes, and later washed by re-suspending cells in PBS, centrifuged at 13,000g for 3 minutes.

The pellets were re-suspended in 200µl Instagene Matrix, incubated for 20-30 minutes in a water bath (Isotemp®, Fisher Scientific, USA) at 55°C. The sample was vortexed for 10 seconds and boiled at 100°C (Tekstir® Hot plate) for 8 minutes. The sample was vortexed for 10 seconds and centrifuged at 13000g for 3 minutes. The supernatant containing the DNA was stored at 20°C.

PCR amplification of the DNA Template/Sample

The amplification reactions of the DNA template/sample were carried out in 0.2mL PCR single tube-RNase/DNase/pyrogen free (Diamed, Lab. Supplies, Mississauga, Ontario, Canada) with hinged flat cap in a Thermocycler (Eppendorf Mastercycler). Each PCR consisted of 5.0µL of 10 x buffer (No $MgCl_2$), 10mM Tris-HCl, and 50mM KCl), 2.5µL of $MgCl_2$ (50mM), 1.0µL dNTPs (5mM each), 1.25µL of glycerol (80%) (Sigma), 4.0µL of bovine serum albumin (BSA) (10mg/ml) (Sigma), 50 pmoles/µL of each primer, LGC-1, with the sequence; AGCAGTAGGGAATCTTCCA and LGC-2 GC with the sequence;
CGCCCGGGGCGCGCCCCGGGCGGCCCGGGGG CACCGGGGGATTYCACCGCTACAC,
(Invitrogen™, Life Technologies), 0.2µL of Platinum® *Taq* DNA polymerase (5U/µL)(Invitrogen™, Life Technologies), 2.0µL of the DNA template/sample, and sterile water (Fluka H_2O) to a volume of 50µL. The PCR amplification followed the LAC program in the Mastercycler, with initial DNA denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 1 min and elongation at 72°C for 1 min, which was followed by a final extension at 72°C for 10 min. To confirm amplicon production, the mixture (5µL PCR product and 2µL of loading buffer) was analyzed by electrophoresis (Bio-Rad) in 1.5% Ultrapure™ Agarose (Invitrogen, Life Technologies) gel, at 100 volts for 45 min, followed by staining with 1% solution of ethidium bromide (50µl/L) and de-staining with TAE (Tris glacial acetic acid and EDTA)

for 10 min. Gels were visualized by UV transillumination and recorded with Polaroid 667 instant film.

Statistical analysis

Nonparametric statistical method, Chi-Square (χ^2) was employed to test the association between presence of Lactobacilli PCR product and Nugent scores indicating Normal, and Bacterial vaginosis.

Results

Out of the 241 vaginal samples that were Gram stained for Nugent rating, 84 (34.8%) had 'Normal' rating (0-3) (Figure 1a). In this case, large gram-positive rods (lactobacillus morphotypes) dominated the entire field. Another 123 (51%) had 'Intermediate' score (4-6), while 34 (14.2%) had presumptive bacterial vaginosis (BV), with Nugent score (7-10). These were dominated by small gram-variable rods (*Gardnerella vaginalis* morphotypes), and curved gram-variable rods (*Mobiluncus spp* morphotypes) (Figure1b). However, all the 34 samples diagnosed with BV, had no PCR products for Lactobacilli as shown in Figure 2, while samples with normal Nugent scores had positive PCR products for Lactobacilli. The intermediate Nugent scores equally had lactobacillus PCR products, but the bands from the electrophoretic gels were faint. There was a positive association between absence of lactobacilli PCR product and Nugent scores interpreted as BV (85.8% vs. 14.2%. $\chi^2 = 4.12, P = 0.05$).

Figure 1a: A representative Normal Nugent Score (0-3) with *Lactobacillus* morphotypes predominating.

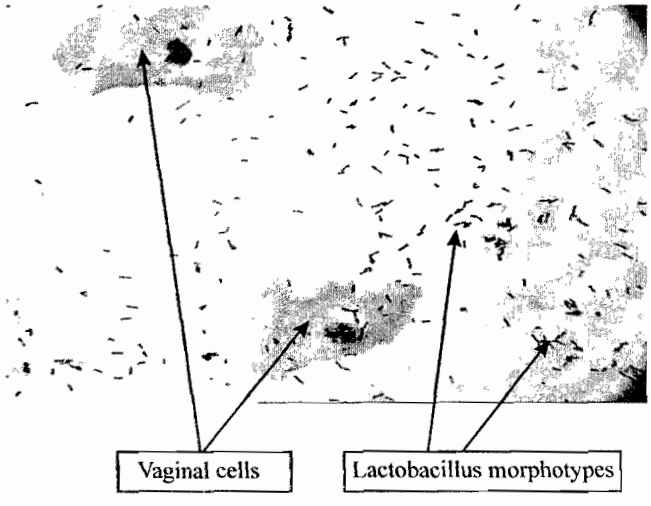


Figure 1b: A representative abnormal Nugent score (7-10) indicating BV.

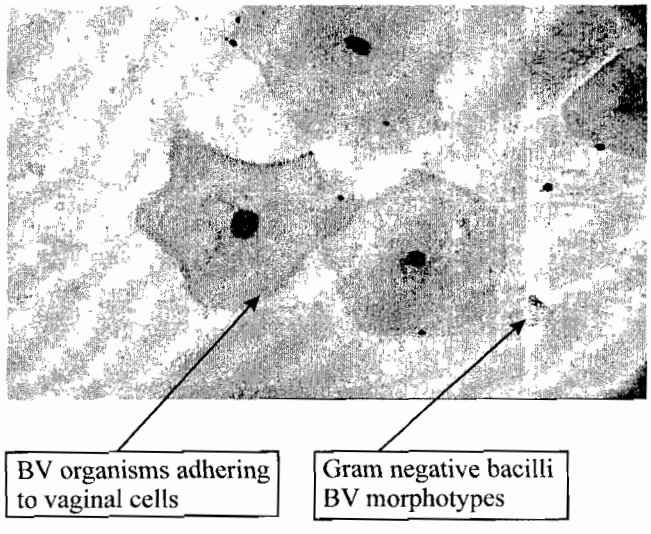
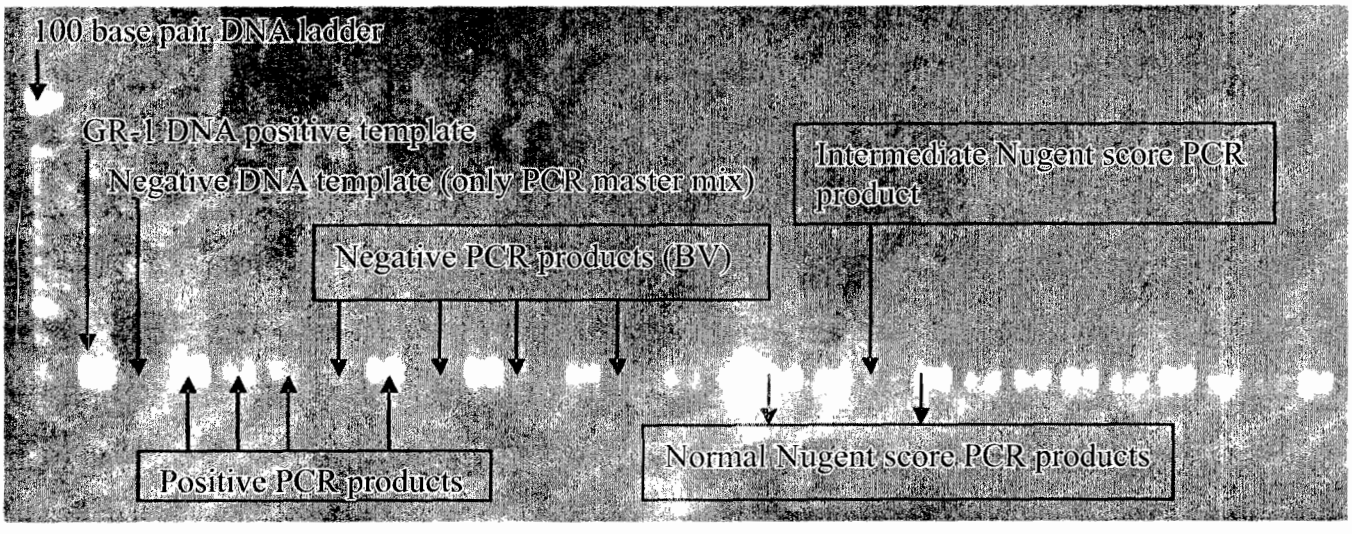


Figure 2: A representative electrophoretic agarose gel results of PCR products of amplified *Lactobacillus* DNA from the vaginal samples



Discussion

This is probably the first study using polymerase chain reaction (PCR) to examine the vaginal *Lactobacillus* microbiota of apparently healthy women in Nigeria, in association with bacterial vaginosis (BV). More than a third of the sampled population had normal Nugent rating with large Gram-positive rods, indicative of Lactobacilli. This is demonstrated by the presence of *Lactobacillus* PCR products using group specific Lactobacillus primers. This finding is in contrast with the previous study on the identification of vaginal *Lactobacillus* species, using culture followed by whole-chromosomal DNA probes and the demographic and microbiologic characteristics of women. The study indicated that vaginal colonization with Lactobacillus is associated with being Caucasian [20].

The description of the vaginal microbiota of BV patients and even normal women had been uncertain because of the unreliability of classic identification methods, which employ sugar fermentation and other phenotypic assays. Although the main concern of the study was not to identify the specific lactobacilli, but link presence of lactobacilli with normal Nugent rating. This was achieved with group specific nucleic acid probes for lactobacilli targeting the 16S rRNA genes. While the etiology of BV is diverse and still not completely clear, it is believed that the loss of lactobacilli is a major component of the condition and its affiliated complications. Anaerobic organisms such as Gardnerella vaginalis, Mobiluncus Mycoplasma hominis and Atopobium vaginae have been implicated in BV, along with group B streptococcus and Escherichia coli, but cultures are infrequently performed especially in our hospitals, and diagnosis is largely syndrome based. For example diagnostic testing for Mycoplasma is rarely done in most hospitals, and thus many cases of BV may be missed. For women who self-diagnose vaginal discharge, many incorrectly self-treat with over the counter antifungals for yeast vaginitis, when in fact they have BV. This may potentially lead to adverse consequences.

Paucity of information exists as regards identification even with culture methods albeit molecular techniques of *Lactobacillus* species colonizing the vagina of Nigerian women. This Lack of information may have led to the spurious speculation that Black women are not colonized by *Lactobacillus* species with protective capability. It could be argued that most clinical microbiologists in Nigeria focus their research efforts only on pathogens and pay little attention to the normal microbiota of the vagina.

A greater number of the women (51%) had intermediate Nugent score, indicating microbial fluctuations with few lactobacilli. This is demonstrated by the appearance of faint lactobacillus PCR products in the electrophoretic gels. Studies have shown that the vaginal microbiota is not very stable in some women and in many women during a given menstrual cycle, it changes from one dominated by lactobacilli to one with few or no lactobacilli and dominated by Gram negative anaerobes [21,22].

The level of BV detected in these Nigerian women was lower than that found in Canadian studies (14.2% versus 30%) [6], again counter to the presumption that this condition is inherently more common in black women. It would appear that factors such as douching, sexual practices or living conditions are more reflective of BV susceptibility than race itself. This is supported, by the finding of a BV of 25-31% amongst predominantly white lesbians [23, 24], although BV prevalence in sub-Saharan Africa have been reported in excess of 50% among pregnant women [25]. It should be noted that BV organisms produce multiple virulence factors including toxins and proteases that can degrade sIgA, and they have been found to induce inflammation and local damage, with a 19 fold increase in inflammatory cytokines reported, along with elevation in pH [26]. This inflammation is believed to be a factor associated with a significantly increased risk of sexually transmitted diseases (STDs) including HIV [27,28].

The significant depletion of lactobacilli ($p=0.05$) and the asymptomatology of BV is worrisome as women not detecting odor or discharge [29] do not realize that their vaginal microbiota is abnormal and they are now at higher risk of sexually transmitted diseases, including HIV [14]. Studies have also shown that the absence or depletion of lactobacilli in the vagina is associated with overgrowth of anaerobic pathogens that causes BV and this results in significantly increased risk for HIV as well as gonorrhea, chlamydia, and herpes simplex virus infections [30]. The vaginal microbiota consists of principally *Lactobacillus* species in healthy women [21], and our present study has shown that Lactobacillus absence has a strong association with BV.

The restoration of the depleted lactobacilli in BV patients by the use of probiotics is worth testing. Probiotics is defined as "live microorganisms which when administered in adequate amounts confer health benefits on the host" [31]. Vaginal insertion or oral administration of Lactobacillus probiotics have been successful in previous studies for the restoration of bacterial vaginosis [32,33].

The use of lactobacilli-probiotics for urogenital infections in sub-Saharan Africa, including Nigeria is yet to be implemented. The potential of probiotics to reduce bacterial vaginosis and other urogenital infections, leading to a vaginal flora dominated by lactobacilli and therefore less likely to become infected by HIV, [14,33] is worthy of pursuit in Nigeria. Our previous

study in an animal model has demonstrated nutritional appetite benefits of lactobacilli probiotics during pregnancy and improvements in weight of newborn pups [34]. On safety of probiotics, especially Urex cap-5 (GR-1 and RC-14), our earlier study has shown an excellent safety profile, with no significant alteration in the haematological parameters [35]

Also encouraging animal data on a lactobacilli-based vaccine [36] lends credence to this approach. Given the limitations of the existing HIV/AIDS prevention approaches, there is urgent need to extend the range of prevention methods available, particularly those that women can control. There is a resounding interest of probiotics with great appeal among university female students in Nigeria[37]. Probiotic products have the potential to strengthen HIV prevention efforts by allowing women, men, and children to use them for the maintenance of gastrointestinal and urogenital health.

References

1. Gardner HL, Dukes CD. Haemophilus vaginalis vaginitis: a newly defined specific infection previously classified "non-specific" vaginitis. *Am J Obster Gynecol*, 1955; 69: 962-976.
2. Amsel R, Totten PA, Spiegel CA, Chen KCS, Eschenbach D, Holmes KK. Non-specific vaginitis: Diagnostic criteria and microbial and epidemiologic associations. *Am J Med*, 1983; 74: 14-22.
3. Hillier SL. Diagnostic microbiology of bacterial vaginosis. *Am J Obster Gynecol*, 1993; 169: 455-459.
4. Rosenstein IJ, Morgan DJ, Sheehan M, Lamont RF, Taylor-Robinson D. Bacterial vaginosis in pregnancy: distribution of bacterial species in different gram-stain categories of the vaginal flora. *J Med Microbiol*, 1996; 45: 120-126.
5. Burton JP, Devillard E, Cadieux PA, Hammond, J.A., and Reid, G. Detection of *Atopobium vaginae* in post menopausal women by cultivation-independent methods warrants further investigation. *J Clin Microbiol*, 2004; 42: 1829-1831.
6. Reid G, Charbonneau D, Erb J, Kochanowski B, Beuerman D, Poehner R, *et al*. Oral use of *Lactobacillus rhamnosus* GR-1 and *L. fermentum* RC-14 significantly alters vaginal flora: randomized, placebo-controlled trial in 64 healthy women. *FEMS Immunol Med Microbiol*, 2003; 35: 131-134.
7. Joesoef MR, Karundeng A, Runtupalit C, Moran JS, Lewis JS, Ryan CA. High rate of bacterial vaginosis among women with intrauterine devices in Manado, Indonesia. *Contraception*, 2001; 64: 169-172.
8. de Lima Soares V, de Mesquita AM, Cavalcante FG, Silva ZP, Hora V, Diedrich T, *et al*. Sexually transmitted infections in a female population in rural north-east Brasil: prevalence,morbidity and risk factors. *Trop Med Int Health*, 2003; 8: 595-603.
9. Anukam KC, Osazuwa EO, Ahonkhai I, Reid G. Assessment of *Lactobacillus* species colonizing the vagina of apparently healthy Nigerian women, using PCR-DGGE and 16S rRNA gene sequencing. *World J Microbiol Biotechnol*, 2005. In Press.
10. Hillebrand L, Harmanli OH, Whiteman V, Khandelwal M. Urinary tract infections in pregnant women with bacterial vaginosis. *Am J Obstet Gynecol*, 2002; 186(5): 916-917.
11. Behets F, Andriamiadana J, Rasamilalao D. Sexually transmitted infections and associated socio demographic and behavioural factors in women seeking primary care suggest Madagascar's vulnerability to rapid HIV spread. *Trop Med Int Health*. 2001; 6(3): 202-11.
12. Sweet RL. New approaches for the treatment of bacterial vaginosis. *Am J Obstet Gynecol*, 1993; 169: 479-482.
13. Hay PE, Lamont RF, Taylor-Robionson D, Morgan DJ, Ison C, Pearson J. Abnormal bacterial colonization of the genital tract and subsequent preterm delivery and late miscarriage. *BMJ*, 1994; 308: 295-298.
14. Sewankambo N, Gray RH, Wawer MJ, Paxton L, McNaim D, Wabwire-Mangen F, *et al*. HIV-1 infection associated with abnormal vaginal flora morphology and bacterial vaginosis. *Lancet*, 1997; 350 (9077): 546-50.
15. Nugent RP, Krohn MA, Hillier SL. Reliability of diagnosing bacterial vaginosis is improved by a standardization method of Gram stain interpretation. *J Clin Microbiol*, 1991; 29: 297-301.
16. Myziuk L, Romanowski B, Johnson SC. BVBlue test for diagnosis of bacterial vaginosis. *J Clin Microbiol*, 2003; 41: 1925-1928.
17. Bradshaw CS, Morton AN, Garland SM, Horvath LB, Kuzevska I, Fairley CK. Evaluation of point-of-care test, BVBlue, and clinical and Laboratory criteria for diagnosis of bacterial vaginosis. *J Clin Microbiol*, 2005; 43: 1304-1308.
18. Satokari RM, Vaughan EE, Akkermans AD, Saarela M, De Vos WM. Polymerase chain reaction and denaturing gradient gel electrophoresis monitoring of fecal Bifidobacterium populations in a prebiotic and probiotic feeding trial. *Syst Appl Microbiol*, 2001; 24: 227-231.
19. Walter J, Tannock GW, Tilsala-Timisjarvi A, Rodtong S, Loach DM, Munro K, *et al*. Detection and identification of gastrointestinal Lactobacillus species by using denaturing gradient gel electrophoresis and species-specific PCR primers. *Appl Environ Microbiol*, 2000; 66: 297-303.
20. Antonio MA, Hawes SE, Hillier SL. The identification of vaginal *Lactobacillus* species and the demographic and microbiologic characteristics of women colonized by these species. *J Infect Dis*, 1999; 180: 1950-1956.
21. Keane FE, Ison CA, Taylor_Robinson D. A longitudinal study of the vaginal flora over a menstrual cycle. *Int J STD AIDS*, 1997; 8(8): 489-494.
22. Burton JP, Cadieux P, Reid G. Improved understanding of the bacterial vaginal microbiota of women before and after probiotic instillation. *Appl Environ Microbiol*, 2003; 69: 97-101.
23. Marrazzo JM, Koutsky LA, Eschenbach DA, Agnew K, Stine K, Hillier SL. Characterization of vaginal flora and bacterial vaginosis in women who have sex with women. *J Infect Dis*, 2004; 185(9): 1307-13
24. Bailey JV, Farquhar C, Owen C, Mangtani P. Sexually transmitted infections in women who have sex with women. *Sex Transm Infect*, 2004; 80(3): 244-6.
25. Govender L, Hoosen AA, Moodley J, Moodley P, Sturm AW. Bacterial vaginosis and associated infections in pregnancy. *Int J Gynecol Obstet*, 1996; 55: 23-28.
26. Cauci S, Guaschino S, De Aloysio D, et al. Interrelationships of interleukin-8 with Interleukin-1beta and neutrophils in vaginal fluid of healthy and bacterial vaginosis positive women. *Mol Hum Reprod*. 2003; 9(1): 53-58.
27. Taha TE, Hoover DR, Dallabetta GA, et al. Bacterial vaginosis and disturbances of vaginal flora:association with increased acquisition of HIV. *AIDS*, 1999; 12(13): 1699-1706.
28. Wiesenfeld HC, Hillier SL, Krohn MA, Landers, DV, Sweet RL. Bacterial vaginosis is a strong predictor of *Neisseria gonorroeae* and *Chlamydia trachomatis* infection. *Clin Infect Dis*, 2003; 36: 663-8.
29. Klebanoff MA, Schwebke JR, Zhang J, Nansel TR, Yu KF, Andrews WW. Vulvovaginal symptoms in women with bacterial vaginosis. *Obstet Gynecol*, 2004; 104(2): 267-72.. Cherpes TL, Meyn LA, Krohn MA, Lurie JG, Hillier SL. Association between acquisition of herpes simplex virus type 2 in women and bacterial vaginosis. *Clin Infect Dis*, 2003; 37(3): 319-325.
31. Reid G, Sanders ME, Gaskins HR, Gibson GR, Mercenier A, Rastall R, *et al*. New scientific paradigms for probiotics and prebiotics. *J Clin Gastroenterol*, 2003; 37(2): 105-18.
32. Reid G, Bruce AW, Fraser N, Heinemann C, Owen J, Henning B. *Oral probiotics can resolve urogenital infections. FEMS Microbiol Immunol*, 2001; 30: 49-52.
33. Reid G, Beuerman D, Heinemann C, Bruce AW. Probiotic *Lactobacillus* dose required to restore and maintain a normal vaginal flora. *FEMS Immunol Med Microbiol*, 2001; 32: 37-41.
34. Anukam KC, Osazuwa EO, Reid G. Improved appetite of pregnant rats and increased birth weight of newborns following feeding with probiotic *Lactobacillus rhamnosus* GR-1 and *L. fermentum* RC-14. *J Appl Res*, 2005; 5 (1): 46-52.
35. Anukam KC, Osazuwa EO, Reid G, Ozolua RI (2004). Feeding probiotic strains *Lactobacillus rhamnosus* GR-1 and *Lactobacillus fermentum* RC-14 does not significantly alter hematological parameters of Sprague-Dawley rats. *HAEMA Journal of Hellenic Society of Haematology*. 7(4): 497-501.
36. Chang CH, Simpson DA, Xu Q, Martin PK, Lagenaur LA, Schoolnik GK, *et al*. Inhibition of HIV infectivity by a natural human isolate of *Lactobacillus jensenii* engineered to express functional two domain CD4. *Proc Natl Acad Sci USA*. 2003; 100(20): 11672-11677.
37. Anukam KC, Osazuwa EO, Reid G, Katsivo MN. Receptivity for probiotic products among pre-menopausal female sudents in an African university. *Sex Transm Dis*, 2004; 31(8): 460-464.