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Full Length Research Paper

16S rRNA gene sequence and phylogenetic tree of *lactobacillus* species from the vagina of healthy Nigerian women

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Lactobacilli are ubiquitous in nature and in humans they play a very significant role in the general health maintenance of the host. Identification of lactobacilli has previously been based on culture-dependent methods and recently molecular techniques involving gene sequencing are now the 'gold standard'. Scarce information exists in Africa on the real identity of *Lactobacillus* species, albeit phylogenetic distances among the species present in the human vagina. In this study, 185 vaginal swabs were collected from healthy premenopausal women (18 to 48 years). Bacterial DNA was extracted, amplified using PCR, with group specific *Lactobacillus* primers, and processed by denaturing gradient gel electrophoresis (DGGE). Phylogenetic tree was constructed with the sequences of the V2-V3 region of 16S rRNA gene. Results show two distinct divisions among the *Lactobacillus* species. The study presents a new understanding of the nature of the *Lactobacillus* vaginal microbiota of women in Nigeria, which may lead to the design of probiotic-lactobacilli for biotherapy.

Key words: Lactobacilli, gene sequence, phylogenetic tree, human vagina, Nigerian women.

INTRODUCTION

Lactobacilli play an important role in the maintenance of vaginal health of women (Redondo-Lopez, et al., 1990). In healthy women, lactobacilli are the dominant species in the vaginal microbiota **(**Keane et al., 1997). When lactobacilli are depleted due to antibiotics, douching or sexual practice, other pathogenic organisms such as Gram negative anaerobes takes over, which leads to a condition known as bacterial vaginosis (BV) (Sobel, 1997). The adverse effects of BV on pregnancy and acquisition of HIV is enormous to women in sub-Saharan Africa (Sewankambo et al, 1997). In the development of appropriate biotherapeutic remedy for BV such as probiotic- lactobacilli, the identification of the dominant vaginal lactobacilli present is very vital.

There have been significant advances in recent years in the characterization and identification of bacteria by molecular methods. Notably, *Lactobacillus* species that are commonly present in the human vagina have received considerable attention as a result of their probiotic properties (Reid, 1999). Most of the molecular techniques for lactobacilli identification are based on the nucleotide-nucleotide sequence of the V2-V3 region of the 16S rRNA gene. Polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) have been demonstrated to be suitable tools for the analysis of lactobacillus community of the human vagina because they allow the detection of species very quickly and economically (Burton et al., 2003). In the past, the nature of vaginal lactobacilli diversity has been limited due to reliance on culture-dependent methods. It has

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been estimated that more than 99% of micro- organisms observable in nature are not cultivable with the available standard techniques (Amann et al., 1995). For thirty-two years between 1928 and1960, the dominant lactobacillus species in the vagina was believed to be *Lactobacillus acidophilus* (Thomas, 1928; Rogosa and Sharpe, 1960). The taxonomy of lactobacilli has expanded as a result of genomic sequence analysis. Using DNA homology studies, *L. acidophilus* complex which previously cannot be distinguished biochemically has been subdivided into six distinct species; *L. acidophilus, L. crispatus, L. gasseri, L. gallinarum, L. amylivorus* and *L. johnsonii* (Du Plessis and Dicks, 1995). Previous studies indicated that colonization by *L. crispatus* or *L. jensenii* are the most common species of vaginal lactobacilli (Antonio et al., 1999). Only, recently has *Lactobacillus iners* been identified in the human vagina (Falsen et al., 1999). It was found in vaginal samples from Swedish women, but the most frequently occurring species were *L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii* (Vasquez et al., 2002). 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). In recent times, the concept of biodiversity in a particular ecosystem has been articulated through the use of phylogenetic tree maps based on gene sequences. 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), but the study did not include women from Africa. Therefore, the identity and diversity of these *Lactobacillus* populations remain largely obscure in Africa and the complex interactions of the various members of the vaginal microbiota are still poorly understood in our environment. The aim of the present study was to determine the genetic sequences and phylogenetic distances of V2- V3 region of 16S rRNA gene of vaginal lactobacilli from Nigerian women.

MATERIALS AND METHODS

Extraction of DNA

DNA was extracted from the bacteria present on 185 vaginal swabs of apparently healthy women, after informed consent in Benin City, using Instagene Matrix (Bio-Rad Laboratories, Ontario, Canada), according to manufacturer's instructions. Briefly swabs were vigorously agitated in 1 mL of phosphate buffered saline (PBS) (pH 7.1) to dislodge cells. The cells were pelleted by centrifugation (Eppendorf, Digital Centrifuge 5417C) at 10,000 g for 5 min, and later washed by re-suspending cells in PBS and centrifuging at 13,000 g for 3 min. The pellets were re-suspended in 200 µl Instagene Matrix, incubated for 20-30 min in a water bath (Isotemp®, Fisher Scientific, USA) at 55°C. The sample was vortexed for 10 s and boiled at 100°C (Tekstir® Hot plate) for 8 min. The sample was vortexed for 10 s and centrifuged at 13000 g for 3 min. The supernatant containing the DNA was stored at –20°C.

PCR

The amplification reactions of the DNA sample were carried out in 0.2 mL PCR single tube (Diamed, Lab. Supplies, Mississauga, Ontario, Canada) with hinged flat cap in a Thermocycler (Eppendorf Mastercycler). Each PCR consisted of 5.0 µL of 10X buffer (10 mM Tris-HCl, 50 mM KCl), 2.5 µL of $MgCl_2$ (50 mM), 1.0 µL dNTPs (5 mM each), 1.25 µL of glycerol (80%) (Sigma), 4.0 µL of bovine serum albumin (BSA) (10 mg/ml) (Sigma), 50 pmoles/µL of each primer, Lac-1 (AGCAGTAGGGAATCTTCCA) and Lac-2 GC(CGCCCGGGGCGCGCCCCGGGCGGCCCGGGGGCACCGG GGGATTYCACCGCTACAC) (Invitrogen™, Life Technologies), 0.2 µL of Platinum® Taq DNA polymerase (5 U/µL) (Invitrogen™, Life Technologies), 2.0 µL of the DNA template/sample, and sterile water (Fluka H_2O) to a volume of 50 µL. The PCR amplification has initial DNA denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, 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. 5 µL PCR product 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 (50 µl/L) and de-staining with TAE for 10 min. Gels were visualized by UV transillumination and recorded with Polaroid 667 instant film.

Denaturing gradient gel electrophoresis (DGGE)

In DGGE, the denaturing environment is created by a combination of uniform temperature, typically between 50 and 65^{o}C and a linear denaturant gradient formed with urea and formamide. Preparation of gel gradients and electrophoresis was carried out according to the manufacturer's instructions for the D-Code™ Universal Mutation Detection System (Bio-Rad Laboratories) . 20µL of sample amplified PCR products loaded into the wells and gels were run at 130 V for 3.5 to 4 h in TAE. Gels were visualized as previously described.

Each DGGE fragment of interest was excised from the gels with a sterile scalpel and placed into a 1 ml Eppendorf tube for washing. Cut bands were washed in PCR buffer and incubated in 35 µL of the same buffer overnight at 4^{o} C. 5 µL of the buffer solution containing the cut bands was used as template for PCR amplification. PCR re-amplification was conducted with *Lactobacillus* primers LGC-1 and LGC-2 (without the GC clamp). The amplification, annealing and extension, conditions were the same as described above. The DNA fragments from PCR reactions were purified using QIAquick purification Kit protocol (QIAGEN Inc. Mississauga, Ontario, Canada).

Sequencing and phylogenetic analysis

4 µL of the purified DNA mixture was added to 4µL of Big-Dye Terminator Reaction Mix (Applied Biosystems, USA), plus 1.6µL of primer and 0.4µL of de-mineralized H_2O. Sequences of the fragments, were determined by the automatic Big Dye (dideoxy chain terminator) sequencer ABIPRISM 3730*xl* , (Sequencing Facility, John P.Robarts Research Institute, London, Ontario).

Sequences were edited to exclude the PCR primer binding sites and manually corrected with Chromas 2.3 (Chromas version 2.3; www.technelysium.com.au.chromas.html). For identification of the closest relatives, newly determined sequences were compared to those available in the V2-V3 region of the 16S rRNA sequences using the GenBank DNA databases (Table 1) (www.ncbi.nih.gov) and the standard nucleotide-nucleotide BLAST algorithm (Altschul et al., 1990) . The identities of the relatives were determined on the basis of the highest GenBank accession number.

Table 1. *Lactobacillus* species Genbank accession number and nucleotide sequences of the V2-V3 region of the 16S rRNA gene.

***Lactobacillus* species**	**GenBank Accession Number**	**Nucleotide sequences of the V2-V3 region of the 16S rRNA gene**
L. iners clone FX181-4	**AY283275**	AAAGACTCTGTTGTTGGTGAAGAAGGACAGGGGTAGTAACTGACCTTTGTTTGACGGTAATCA ATTAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTG TCCGGATTTATTGGGCGTAAAGCGAGTGCAGGCGGCTCGATAAGTCTGATGTGAAAGCCTTC GGCTCAACCGGAGAATTGCATCAGAAACTGTCGAGCTTGAGTACAGAAGAGGAGAGTGGAAC TCCATGTGTACCGGTGAAATAAACCTATGTGTAT
L. iners clone FX177-4	AY283272	CACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTGGTGAAGAAGGACA GGGGTAGTAACTGACCTTTGTTTGACGGTAATCAATTAGAAAGTCACGGCTAACTACGTGCCA GCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGTG CAGGCGGCTCGATAAGTCTGATGTGAAAGCCTTCGGCTCAACCGGAGAATTGCATCAGAAAC TGTCGAGCTTGAGTACAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGAAATAAACGTAT ATATATGGAAGATTCCCTAC
L. gasseri	AF375930	CCGCTACACATGGAAGATTCCCTACTGCTACACATGGGTATTCCACCGCTACACATGGAAGAT TCGCTACTGCTACACTGGGTATTTCACCGAGTACGCTTGGAAGATTCGCATTTGCGAGGCTG GGGGAGCGTAGGTGGCCGTGACTTTCTAAGTGATTACCGTCAAATAGGGGCCAGTTACTACC TCTGTCTTTCTTCACTACCAACAGAGCTTTACGAGCCGAAACCCTTCTTCACTCGGGCGGCGT TGCTCCATCAGACTTTCGTCCATTGTGGAAGATTCCCTACTGCTA
L. suntoryeus	AY445815	ACCCAAGGGTTTCCATCAGACTTATCGAGCCGACTGCGCTCGCTTTACGCCCAATAAATCCG GACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGACTTTCTG GTTGATTACCGTCAAATAAAGGCCAGTTACTACCTCTGTCCTTCTTCACCAACAACAGAGCTTT ACGATCCGAAAACCTTCTTCACTCACGCGGCGTTGCTCCATCAGACTTGCGTCCATTGTGGAA GATTCCCTACTGCTAACATGGGTATTCCACC
L. rhamnosus	AY299488	GTTTCCNNGTTTCCGATGCACTTCCTCGGTTAAGCCGAGGGCTTTCACATCAGACTTAAAAAA CCGCCTGCGCTCGCTTTACGCCCAATAAATCCGGATAACGCTTGCCACCTACGTATTACCGC GGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTGGATACCGTCACGCCGACAACAGTTA CTCTGCCGACCATTCTTCTCCAACAACAGAGTTTTACGACCCGAAAGCCTTCTTCACTCACGC GGCGTTGCTCCATCAGACTTGCGTCCATTGTGGAAGATTCCCTACTGCTAA
L. crispatus	AY335504	AGAAAACAGTTTCCGATGCAGTTCCTCGGTTAAGCCGAGGGCTTTCACATCAGACTTATTCTT CCGCCTGCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTTGCCACCTACGTATTACCGC GGCTGCTGGCACGTAGTTAGCCGTGACTTTCTGGTTGATTACCGTCAAATAAAGGCCAGTTAC TACCTCTATCCTTCTTCACCAACAACAGAGCTTTACGATCCGAAAACCTTCTTCACTCACGCG GCGTTGCTCCATCAGACTTGCGTCCATTGTGGAAGATTCCCTACTGCTAA
L. helveticus	AY369116	CAGTTTCCGATGCAGTTCCTCGGTTAAGACGAGGGCTTTCACATCAGACTTATTCTTCCGCCT GCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGC TGGCACGTAGTTAGCCGTGACTTTCTGGTTGATTACCGTCAAATAAAGGCCAGTTACTACCTC TATCCTTCTTCACCAACAACAGAGCTTTACGATCCGAAAACCTTCTTCACTCACGCGGCGTTG CTCCATCAGACTTGCGTCCATTGTGGAAGATTCCCTACTGCTA
L. johnsonii	AE17206	CGAAGGCTTTCACATCAGACTTATTGAACCGCCTGCACTCGCTTTACGCCCAATAAATCCGGA CAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTGTTAGCCGTGACTTTCTAAGT AATTACCGTCAAATAAAGGCCAGTTACTACCTCTATCTTTCTTCACTACCAACAGAGCTTTACG AGCCGAAACCCTTCTTCACTCACGCGGCGTTGCTCCATCAGACTTTCGTCCATTGTGGAAGAT TCCCTACTGCTAA
L. vaginalis	AB158768	CACACCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTGGAGAAGAACGTAT CTGAGAGTAACTGTTCAGGTAGTGACGGTATCCAACCAGAAAGTCACGGCTAACTACGTGCC AGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGC GCAGGCGGTTGCTTAGGTCTGATGTGAAAGCCTTCGGCTTAACCGAAGAAGTGCATCGGAAA CCGGGCGACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGGAAT
L. fermentum	AY373589	ACACCGCGTGAGTGAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACACGTAT GAGAGTAACTGTTCATACGTTGACGGTATTTAACCAGAAAGTCACGGCTAACTACGTGCCAGC AGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGAGAGTGCAG GCGGTTTTCTAAGTCTGATGTGAAAGCCTTCGGCTTAACCGGAGAAGTGCATCGGAAACTGG ATAACTTGAGTGCAGAAGAGGGTAGTGGAACTCCATGTGTAGCGGTGGAATAA
L. plantarum	AY383631	AAGGNTTTCACATCAGACTTANNAAACCGCCTGCGCTCGCTTNNCGCCCAATAAATCCGGAC AACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTT AAATACCGNCAATACCTGAACAGTTACTCTCANATATGTTCTTCTTTAACAACAGAGTTTTACG AGCCGAAACCCTTCTTCACTCACGCGGCGTTGCTCCATCAGACTTTCGTCCATTGTGGAAGAT TCCCTACTGNTACNC

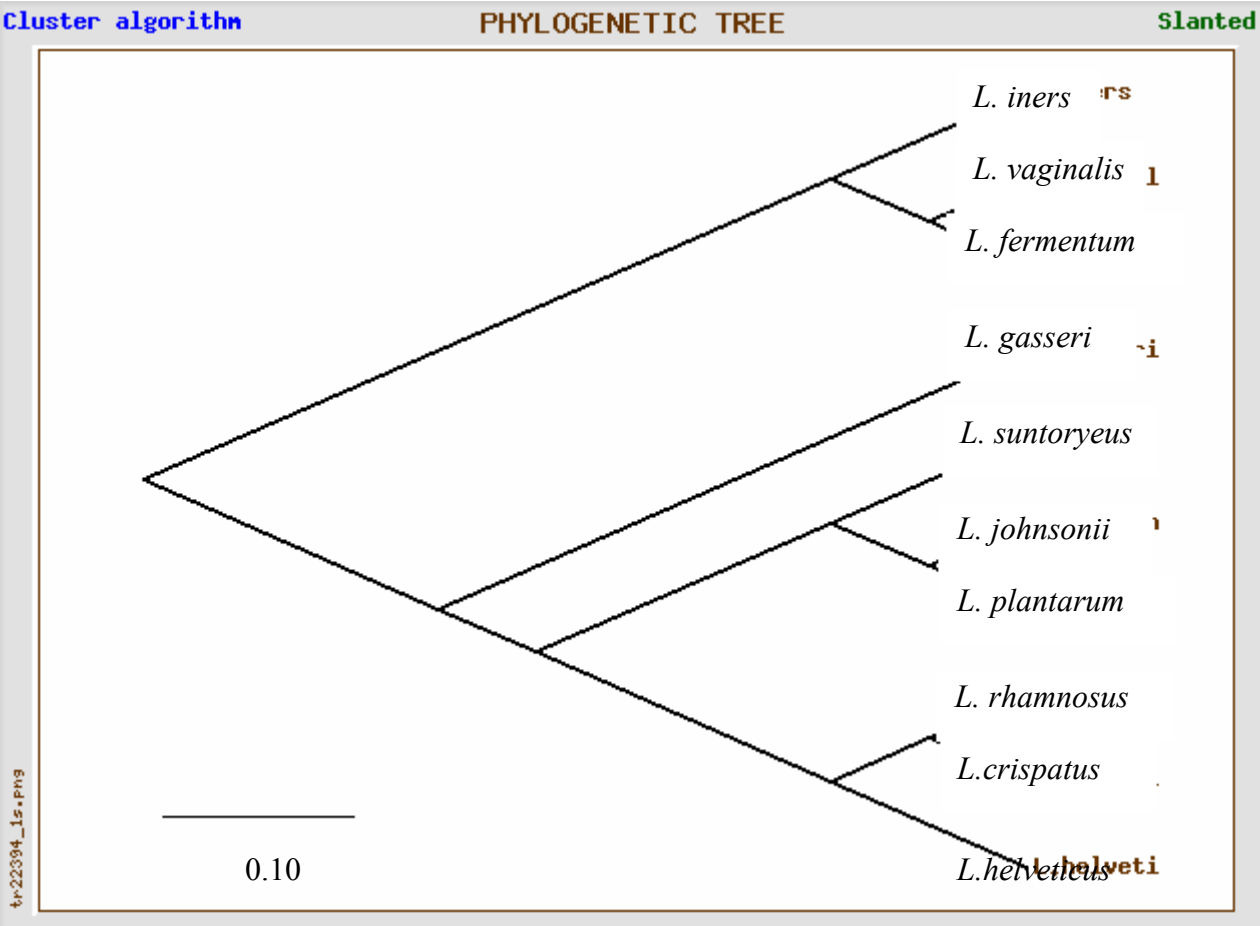

Figure 1. Phylogenetic tree based on the V2-V3 region of 16S rRNA gene sequences showing the relationships of the *Lactobacillus* species from Nigerian women.

Phylogenetic tree was constructed by the Tree Top-Phylogenetic Tree prediction program on the internet (http://www.gene-bee.msu.su/services/phtree_reduced.html).

RESULTS AND DISCUSSION

The edited nucleotide sequences shows that 118 (64%) of the samples were colonized by *L. iners* . This was followed by *L. gasseri* (8.3%), *L. plantarum* (7.0%), *L. suntoryeus* (7.0%), *L. crispatus* (4.0%), *L. rhamnosus* (2.7%), *L. vaginalis* (2.7%), *L. fermentum* (1.3%), *L. helveticus* (1.3%), and *L. johnsonii* (1.3%). Figure 1 show the phylogenetic tree based on the V2-V3 region of 16S rRNA gene sequences showing the relationships of the 10 *Lactobacillus* species from Nigerian women.

The subjects were most commonly colonized by the same *Lactobacillus* species, *L. iners,* as have also been observed in recent studies using PCR-DGGE and 16S rRNA gene sequence analysis in Caucasian women in Canada, USA and Sweden (Vasquez et al., 2002; Burton et al., 2003; Reid et al., 2004; Zhou et al., 2004). Indeed, this organism, which does not grow on Rogosa or MRS media traditionally used for lactobacilli recovery, was present in 64% of the samples. Our data revealed that *L. iners*, *L. gasseri*, and *L. plantarum* as the most frequently colonizing species, while the most frequently occurring species in Swedish were *L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii* (Vasquez et al., 2002). DNA homology studies of Antonio et al. (1999) and Song et al. (1999) also found that the most prevalent species of vaginal lactobacilli in women from Italy, United States (US) and Japan were *L. crispatus*, *L. gasseri*, and *L.jensenii*.

Previous studies conducted in the sixties to nineties had relied on biochemical and physiological properties to characterize Lactobacilli (Rogosa and Sharpe, 1960; Reid et al., 1996), or have depended on reference strains as a standard to identify clinical isolates by DNA-DNA hybridization (Antonio et al., 1999). These methods have limitations, as many of the reference strains were characterized with biochemical test, which may not be reliable. Besides, biochemical, physiological and DNA hybridization cannot establish the phylogenetic distances among the different *lactobacillus* species. Thus, it is

worth noting that the *Lactobacillus* species recovered from the Nigerian women were not unusual or different from those reported in recent studies of Caucasians. This would imply that if women in Canada, Finland, Sweden, Japan, responds favourably to probiotic lactobacilli administration, there should be no reason why Nigerian women could also not likewise benefit.

The phylogenetic tree depicted in Figure 1 shows the genetic distances of the various vaginal lactobacilli from the Nigerian women. There are two distinct divisions among the *Lactobacillus* species. *L. iners*, from both the cluster and topological algorithms, is closely related to *L .vaginalis* and *L. fermentum.* The work of Falsen et al. (1999) shows that *L .iners* was closely related to *L. gasseri* and *L. johnsonii*. *L. gasseri*, *L. suntoryeus*, *L. johnsonii*, *L. plantarum*, *L. rhamnosus*, *L. crispatus* and *L. helveticus* are in a more closely related. It should be noted that *L. iners*, *L .gasseri*, *L. johnsonii* and *L .crispatus* are homofermentative lactobacilli. These lactobacilli metabolize glucose only to lactic acid. While *L. fermentum*, *L. vaginalis* and *L .rhamnosus* are heterofermentative lactobacilli. They metabolize glucose to a mixture of lactic acid, acetic acid and carbon-dioxide. Based on the phylogenetic positions, our data shows that *L. rhamnosus* and *L. crispatus* are very much closely related than other species. The selection for a new probiotic *L. jensenii* vaccine against HIV was based on the fact that *L .jensenii* and/or *L. crispatus* was the most prevalent lactobacilli in Caucasian women (Chang et al., 2003). Although such remedy is highly needed in Africa, but our present study have shown that *L. iners* is the most prevalent among the women sampled in Nigeria, Africa's most populous country, and besides probiotic properties of *L. iners* are yet to be determined.

*L. suntoryeus***,** usually found in malted grains, which has not been associated with the human vagina was identified in 7% of the samples. This species is closely related to *L. plantarum* and *L. johnsonii* in the phylogenetic tree. This present findings indicate cross species colonization phenomenon, which is inconsistent with previous studies reporting species specificity amongst microbes (Tannock et al., 1982).

In summary, the study presents a new understanding of the nature of the *Lactobacillus* vaginal microbiota of women in Nigeria. In addition, the application of lactobacilli probiotics from western countries to treat African women at risk of BV is worthy of testing. Two examples of such strains are *L. rhamnosus* GR-1 and *L. reuteri* (formerly *fermentum*) RC-14, both of which were isolated and shown to have probiotic properties, and both of which have been shown since to colonize the vagina of Canadian women and reduce the risk of BV recurrence (Reid et al., 2001). Animal safety studies have been successfully performed with these strains in pregnant and non-pregnant rats (Anukam et al., 2004; Anukam et al., 2005]. Also encouraging animal data on a lactobacilli-based vaccine (Chang et al., 2003) lends credence to this approach.

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