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## ***Lactobacillus plantarum* and *Lactobacillus fermentum* with Probiotic Potentials Isolated from the Vagina of Healthy Nigerian Women**

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**Abstract:** The search for Lactobacilli of African origin with probiotic properties formed the basis of the study. Two hundred and forty one premenopausal healthy (as defined by having no symptoms of vaginal infections and are HIV negative) Nigerian women provided vaginal swabs. The swabs were cultured on de Man Rogosa Sharpe (MRS) agar (pH 5.5) and incubated anaerobically at 37°C for 48 h. Microbial DNA was extracted from the colonies and directly from the swabs, amplified using Polymerase Chain Reaction (PCR) with *Lactobacillus* primers and processed by Denaturing Gradient Gel Electrophoresis (DGGE). DGGE bands were excised, re-amplified, purified, V2-V3 region of 16S rRNA gene sequenced with 3730xI ABI prism BigDye Terminator and sequence identification was by BLAST. *Lactobacillus* strains were tested for probiotic properties (H<sub>2</sub>O<sub>2</sub>, Pathogen inhibition and Biosurfactant production). Out of 241 swab samples cultured on MRS agar, only 24 (10%) samples had growth of *Lactobacillus species* at pH 4.5. *Lactobacillus gasseri* was isolated from 6 (3.3%) samples, followed by *L. plantarum* (2.4%), *L. vaginalis* (1.6%), *L. fermentum* (0.8%), *L. crispatus* (0.8%) and *L. rhamnosus* (0.8%). Two species, each of *Lactobacillus plantarum* and *L. fermentum* produced huge amount of biosurfactants. In addition they produced H<sub>2</sub>O<sub>2</sub>, inhibited the growth of intestinal and urogenital pathogens. The study presents a new understanding by culture method showing lactobacilli composition of the vagina of some Nigerian women. Two *Lactobacillus* species exhibited probiotic characteristics and clinical studies with these strains are now planned following investigation of the genomic regions encoding probiotic functionality.

**Key words:** Probiotics, *Lactobacillus plantarum*, *Lactobacillus fermentum*, Nigerian women, Bacterial vaginosis, HIV

### **INTRODUCTION**

The human vagina is a complex ecosystem containing an abundance of microorganisms (Tannock, 1995). It has been reported that more than 60 different bacterial species including aerobes, facultative anaerobes and obligate anaerobes colonize the healthy vagina (Hooton and Stamm, 1996; Redondo-Lopez *et al.*, 1990). Studies have shown that urogenital cells are covered by dense bacterial biofilms

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(Reid *et al.*, 1990), whose composition changes constantly, but in which members of the genus *Lactobacillus* predominate in 50 to 90% of women, at least until menopause (Reid *et al.*, 1996).

The dominant presence of lactobacilli in the urogenital microflora of healthy women and the obliteration of lactobacilli in patients who develop Urinary Tract Infections (UTI) (Stamey, 1973), Bacterial Vaginosis (BV) and many other genital infections have led to a focus on these bacteria.

Eastern Europeans, some of whom were apparently long-lived, consumed lactobacillus fermented dairy products as part of their daily diet. This was taken as proof of efficacy and milk fermented with the Bulgarian bacillus of Metchnikoff subsequently enjoyed some vogue in Western Europe till today (Tannock, 2004). Metchnikoff's theory thus gave birth to the concept of probiotics. However, in 2001, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) collaborated and defined probiotics as Live microorganisms which when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2001). Europe, has a highly developed, growing market; with probiotic yogurt in at least nine European countries (UK, France, Germany, Spain, Belgium, Netherlands, Denmark, Finland and Sweden) totaled 250 million kg in 1997, with France representing the largest with sales of 90 million kg, valued at US\$219 million (Hilliam, 1998).

Probiotics are rarely found in sub-Saharan Africa. In Nigeria, no probiotic products are available but conventional yogurts fermented with starter cultures of *Lactobacillus delbruekii var bulgaricus* and *Streptococcus thermophilus* abound in rural and urban cities. These organisms are designed to ferment milk and few if any pass into the gastrointestinal tract or confer health benefits beyond those of nutrition.

The concept of probiotic biotherapy is gaining an increasing momentum in developed countries, while the idea is yet to be grasped by the people of sub-saharan Africa, that are in dire need of probiotic benefits. The search for Lactobacilli of African origin with probiotic properties formed the basis of the study.

## MATERIALS AND METHODS

Two hundred and forty one premenopausal healthy (as defined by having no symptoms of vaginal infections and are HIV negative) Nigerian women provided vaginal swabs. The swabs were collected by clinicians, after approval by the ethical review board of the University of Benin. The women were attending reproductive health care centers in Benin City in June 2004 and the samples were taken after informed consent. 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 laboratory at the Lawson Health Research Institute, London, Ontario, Canada, for DNA extraction, sequencing and testing of probiotic characteristics.

### Isolation of Lactobacilli from Vaginal Swabs

The vaginal swabs were vigorously agitated in sterile 1 mL of Phosphate Buffered Saline (PBS), pH 7.1) to dislodge cells. Aliquots of each sample were plated in duplicates on MRS (de Man Rogosa Sharpe) agar plates which were prepared according to manufacturer's instructions. The pH of the MRS agar was adjusted to pH 4.5 with hydrochloric acid and the value determined with digital pH-meter (Ô240 pH/Temp Meter, Beckman, USA). The inoculated plates were incubated anaerobically by using the BBL™ GasPack system (Becton Dickinson, NY, USA) at 37°C for 48 h. Pale straw coloured colonies were identified as presumptive lactobacillus genus by Gram stain morphology and catalase negative test.

### DNA Preparation, PCR, DGGE and Sequencing of Isolated Lactobacilli

Briefly, an isolated presumptive lactobacillus colony was picked from the MRS agar plate and resuspended in 1 mL of autoclaved water in a microfuge tube. 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, centrifuged 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 sec and boiled at 100°C (Tekstir® Hot plate) for 8 min. The sample was vortexed for 10 seconds and centrifuged at 13000g for 3 min. The supernatant containing the DNA was stored at -20°C. PCR amplification, Denaturing Gradient Gel Electrophoresis (DGGE), sequencing of the re-amplified fragments were determined by dideoxy chain termination (Sequencing facility, John P. Roberts Research Institute, London, Ontario). Analysis of the partial 16S rRNA sequences was conducted using the GenBank DNA database and the BLAST algorithm (Altschul et al., 1990).

#### Testing for Hydrogen Peroxide Production (H<sub>2</sub>O<sub>2</sub>)

H<sub>2</sub>O<sub>2</sub> production by *Lactobacillus* strains was tested for by the method previously described by Eschenbach *et al.* (1989).

#### Inhibition Test Against Some Selected Pathogens

Six isolated hydrogen peroxide producing *Lactobacillus* strains were screened for their inhibition against selected pathogens such as *E. coli* o157: H7 and *Salmonella typhi*, *Staphylococcus aureus*, *Klebsiella aerogenes* and *Neisseria gonorrhoea*, using a modified version of the agar overlay technique as previously described (McGroarty and Reid, 1988).

#### Biosurfactant Production by Dialysis and Lyophilisation

Biosurfactant production was tested for six candidates; *Lactobacillus plantarum*, *Lactobacillus fermentum*, *L. gasseri*, *L. vaginalis*, *L. crispatus* and *L. rhamnosus*, in large quantities using 300 mL of overnight (18 h) cultures. Briefly, a colony of cells was picked from a precultured MRS plate and inoculated into 15 mL MRS broth. The broth was incubated at 37°C for 24 h. Thereafter, the incubated 15 mL broth containing the cells was added to 300 mL of MRS broth in flask and incubated at 37°C for 18 h. The cells were harvested by centrifugation (10,000 x g) for 5 min, at 10°C (cold room) washed twice in demineralized water by centrifugation (10,000 x g) for 10 min. The cells were re-suspended in 150 mL of Phosphate buffered saline (PBS), (pH 7.1). The bacteria were kept at room temperature for biosurfactant release with gentle stirring (Magnetic stirrer) for 2 h. Following biosurfactant release, the cells were pelleted by centrifugation (10,000 x g) in falcon tubes, for 15 min, at 10°C (cold room). The biosurfactant containing supernatant was filtered through a series of pore size filters (1.2, 0.8 µ, 0.4 µ and finally 0.2 µm) (Millipore). The biosurfactant was dialyzed against demineralized water at 4°C in a Spectrapor membrane tube (molecular weight cut off 6,000-8,000, Spectrum Medical Industries Inc., California, USA). The dialysate was lyophilized (freeze dried) and stored at -20°C.

## RESULTS

Out of the 241 swab samples cultured on MRS agar, only 24 (10%) samples had growth of *Lactobacillus* at pH 4.5. *Lactobacillus gasseri* (3.3%) was isolated from 8 samples, followed by *L. plantarum* (2.4%), *L. vaginalis* (1.6%), *L. fermentum* (0.8%), *L. crispatus* (0.8%) and *L. rhamnosus* (0.8%) (Table 1).

Table 1: *Lactobacillus* species isolated with culture dependent method on MRS agar plate and identified by DNA sequencing

No. of samples	Percentage	<i>Lactobacillus</i> species identified by BLAST
8	3.3	<i>Lactobacillus gasseri</i>
6	2.4	<i>Lactobacillus plantarum</i>
2	0.8	<i>Lactobacillus fermentum</i>
4	1.6	<i>Lactobacillus vaginalis</i>
2	0.8	<i>Lactobacillus rhamnosus</i>
2	0.8	<i>Lactobacillus crispatus</i>

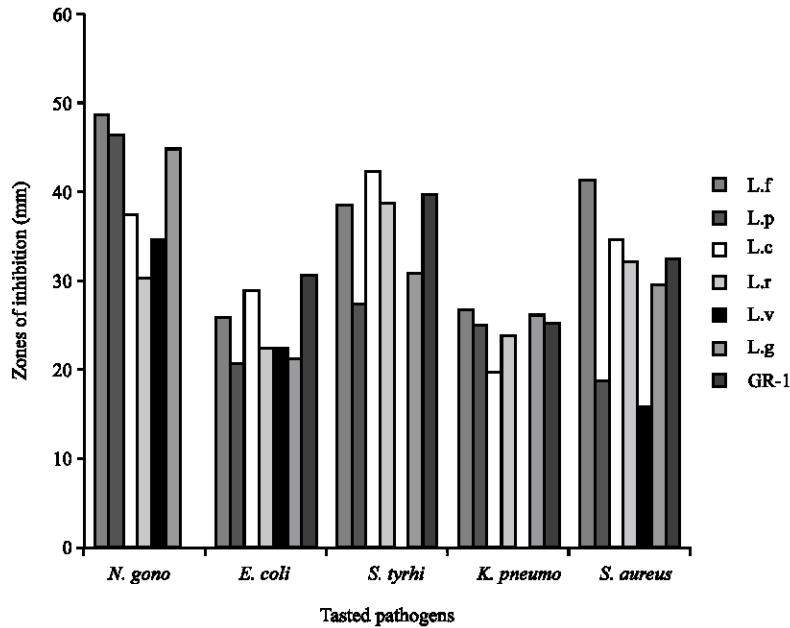


Fig. 1: Growth inhibition of intestinal and urogenital pathogens: zone of inhibition (mm) for *L. fermentum* (L.f), *L. plantarum* (L.p), *L. crispatus* (L.c), *L. rhamnosus* (L.r), *L. vaginalis* (L.v), *L. gasseri* (L.g). GR-1 (*Lactobacillus* probiotic)

Table 2: Summary of Biosurfactants and H<sub>2</sub>O<sub>2</sub> production by isolated *Lactobacillus* species

<i>Lactobacillus</i> species	Biosurfactant producer	Hydrogen peroxide producer
<i>Lactobacillus plantarum</i>	Positive	Positive***
<i>Lactobacillus fermentum</i>	Positive	Positive***
<i>Lactobacillus gasseri</i>	Negative	Positive
<i>Lactobacillus rhamnosus</i>	Negative	Negative
<i>Lactobacillus crispatus</i>	Negative	Negative
<i>Lactobacillus vaginalis</i>	Negative	Negative
<i>Lactobacillus</i> GR-1	Positive	Positive

\*\*\* *Lactobacillus plantarum* and *Lactobacillus fermentum* are the potential candidates. *Lactobacillus* GR-1 is a positive control for biosurfactant and hydrogen peroxide producer

Generally all the six *Lactobacillus* species tested exhibited various degrees of inhibition against the tested pathogens as shown in Fig 1. *Lactobacillus fermentum* (L.f) and *Lactobacillus plantarum* (L.p) had more inhibitory effect on *Neisseria gonorrhoea* with zones of inhibition above 40 mm. The inhibitory effects of all the *Lactobacillus* strains against *Escherichia coli* and *Klebsiella pneumoniae* did not go beyond 30 mm zone. *Lactobacillus fermentum*, *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus rhamnosus* and Probiotic GR-1 had growth inhibition zones against *Salmonella typhi* above 30 mm.

Among the six *Lactobacillus* strains, tested for hydrogen peroxide production (Table 2), only *Lactobacillus vaginalis* did not produce hydrogen peroxide. Out of 6 strains of *Lactobacillus plantarum*, 4 were strongly positive and 2 weakly positive for H<sub>2</sub>O<sub>2</sub> production. All the two strains of *Lactobacillus fermentum* were strongly positive for H<sub>2</sub>O<sub>2</sub> production. Of the 8 strains of *Lactobacillus gasseri*, 4 were strongly positive for H<sub>2</sub>O<sub>2</sub> production, 1 weakly positive and 3 were negative for H<sub>2</sub>O<sub>2</sub> production.

Six of the strains that were tested for hydrogen peroxide production were equally tested for biosurfactants production. Interestingly, two strains *Lactobacillus plantarum* and *Lactobacillus fermentum* were positive, while the rest of the tested strains were negative for biosurfactants (Table 2).

## DISCUSSION

The isolation of lactobacilli by cultural method has some limitations and identification has been uncertain because of unreliability of phenotypic classifications, which employ sugar fermentations. Our study revealed six lactobacillus species, with *Lactobacillus gasseri* predominating, contrary to previous studies, based on culture methods, indicating the predominance of *Lactobacillus acidophilus* (Redondo-Lopez *et al.*, 1990). However our recent published study showed that *Lactobacillus iners* is the predominant species colonizing the vagina of premenopausal Nigerian women using non-culture method involving 16S rRNA gene sequencing (Anukam *et al.*, 2006a). *Lactobacillus iners*, is a viable but non-culturable species when cultured in media used for the cultivation of Lactobacilli.

There is the hypothesis that commensal lactobacilli in the lower genital tract reduce the risk of gonococcal infection in women through the production of hydrogen peroxide (St.Amant *et al.*, 2002). The tested lactobacilli exhibited growth inhibition against all the tested pathogens. Although the specific mechanism of action involved in the inhibition of growth of the pathogens was not investigated. Other studies have suggested that the production of lactic acid may contribute to the inhibition. Besides, the approach of competitive exclusion seems to have potential in this regard. Zhao *et al.* (1998), tested a probiotic culture consisting of several non-pathogenic strains of *E. coli* isolated from the intestinal tracts of cattle for the prevention of *E. coli* O157: H7 colonization in young dairy calves. Their study revealed that probiotic cultures were able to reduce *E. coli* counts in the rumen and the colon.

The inhibition of pathogen growth demonstrated *in vitro* with some Lactobacillus strains in this study is important for the reduction of the risk of infection and improve recovery from diarrhea caused by *E. coli* O157: H7 and *Salmonella typhi* as well as urogenital infections. This finding supports previous studies on the ability of Lactobacilli to stimulate host defenses and reduce translocation and infectivity of *Salmonella typhimurium* (Reid *et al.*, 2002; Raza *et al.*, 1995).

The fact that not all lactobacilli possess properties that were required to colonize the vagina and inhibit urogenital pathogens needs to be emphasized. Only twelve Lactobacillus strains (Table 2) emerged with excellent production of hydrogen peroxide, thus supporting other studies that not all strains of lactobacilli produce hydrogen peroxide (Reid and Bruce, 2001). It should be noted that hydrogen peroxide production has been proposed as an explanation for the success of lactobacilli as vaginal colonizers and as a further mechanism by which lactobacilli may inhibit the growth of other genital pathogens (Eschenbach *et al.*, 1989). In one cross-sectional study (limited period of time) pregnant women colonized by hydrogen peroxide lactobacilli were less likely than women with hydrogen peroxide negative lactobacilli to have bacterial vaginosis, vulvo-vaginal candidiasis, vaginal trichomoniasis or vaginal colonization by other pathogens (Hillier *et al.*, 1992). Also the clinical relevance of hydrogen peroxide producing isolates of lactobacilli was suggested by Hawes *et al.* (1996). In a longitudinal study (long period of time) of non-pregnant women. After adjustment was made for douching and having multiple sex partners, it was shown that nonpregnant women lacking hydrogen peroxide producing vaginal lactobacilli were twice as likely to develop BV than were women colonized by hydrogen peroxide producing lactobacilli. Klebanoff *et al.* (1991), demonstrated *in vitro* that hydrogen peroxide produced by vaginal lactobacilli was the source of the bactericidal activity against *Gardnerella vaginalis* and *Prevotella bivia*.

Two strains *Lactobacillus plantarum* and *Lactobacillus fermentum* produced huge quantities of biosurfactant (Table 2) and were positive for hydrogen peroxide production. An important component of any lactobacillus strain that can serve as a probiotic is the production of biosurfactant, which inhibits adhesion of a range of uropathogens (Conway *et al.*, 1987; Reid and Bruce, 2001). This is based on the fact that strains of *L. plantarum* are known to be able to bind to vaginal cells (Bonetti *et al.*, 2003), produce bacteriocins (Nes *et al.*, 1996) and be good candidates for anti-infective therapy (Grangette *et al.*, 2004).

In a study on the potential of lactobacilli isolated from local ogi slurry in Nigeria, results indicated that *L. Plantarum* is a possible candidate as a probiotic (Oyetayo and Osho, 2004).

## CONCLUSIONS

The present study has shown two potential probiotic organisms of African origin that may be propagated, although further studies are needed to determine the safety and efficacy of the species towards conferring health benefits on the human host. As knowledge of probiotics is very poor among health care providers in Nigeria (Anukam *et al.*, 2006b), concerted research efforts are needed in Nigeria to support and embrace the probiotic concept for general health maintenance.

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