

CORRELATION OF H₂O₂ PRODUCTION OF **LACTOBACILLUS SPECIES** WITH NUGENT SCORES OF YOUNG NIGERIAN WOMEN

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ABSTRACT

Aim: To correlate H₂O₂ production of **Lactobacillus species** with the Nugent scores of young Nigerian women in order to assess their vaginal health.

Study design: Cross-sectional study.

Place and Duration of Study: Department of Biochemistry and Department of Medical Microbiology, College of Medicine of the University of Lagos, between May and August 2009.

Methodology: Ninety-seven isolates of **Lactobacillus** from eighty-two women with Bacterial vaginosis (BV) and fifteen women without BV were used for the study. BV was diagnosed using Nugent scoring method. Lactobacilli were isolated using MRS agar and categorized into facultative anaerobes and strict anaerobes. Hydrogen peroxide was detected and

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measured by titration using dilute sulphuric acid and reaction stopped with potassium permanganate.

Results: Out of 97 isolates studied, 76 (78%) were facultative anaerobes, while 21 (22%) were strict anaerobes. The facultative anaerobes were obtained from 11 of 15 women with BV and 65 of 82 women without BV. Forty-nine (50.51%) of the 97 isolates produced H₂O₂. Forty-four of the H₂O₂ producers were from women without BV while five were from women with BV. Majority (67%) of the strains obtained from women with BV were non-hydrogen peroxide producing. Proportion of H₂O₂ producing *Lactobacillus* by Nugent score were 70%, 43% and 33% in negative, intermediate and BV Nugent scores respectively. There was no significant difference between the mean concentrations of H₂O₂ production in the various Nugent scores.

Conclusion: The overall rate of hydrogen peroxide production was low. While the rates of hydrogen peroxide production correlated with Nugent scores, being highest in negative Nugent scores and lowest with BV scores, the concentration of hydrogen peroxide produced had no association with Nugent scores. The Nigerian women studied might have a relatively high susceptibility rate to vaginal infections.

Keywords: *Lactobacillus*, Nugent score, Bacterial vaginosis, hydrogen peroxide production, MRS agar (deMan Rogosa Sharpe)

INTRODUCTION

Lactobacilli are the predominant bacteria in the vagina of premenopausal women. They occur as either facultative anaerobe, which grow more abundantly in the presence of carbon dioxide or as strict anaerobes¹. Their presence is believed to regulate the growth of other vaginal bacteria. This is because they produce compounds such as lactic acid and hydrogen

32 peroxide which inhibit their growth. The presence of lactic acid helps to reduce the vaginal
33 pH, making it acidic and this selects for aciduric organisms, while the production of hydrogen
34 peroxide inhibits the growth of organisms that lack enzymes such as peroxidase and
35 catalase².

36 The absence or reduction of hydrogen peroxide producing **lactobacilli** in the vagina could
37 therefore lead to colonization of the vagina by other **bacterial species**, which are normally
38 present in small numbers¹⁻⁴. Such organisms include *Gardnerella vaginalis* and some
39 anaerobes, the overgrowth of which is associated with biochemical changes including
40 increased concentration of diamines, polyamines, organic acids and enzymes such as
41 mucinases, sialidases and collagenases in vaginal fluid.³⁻⁴ These biochemical end products
42 cause a further increase in pH and a vaginal discharge with an unpleasant odour⁴. The
43 condition, which is called bacterial vaginosis (BV) is asymptomatic in many women, but has
44 also been associated with a number of obstetric and gynaecologic complications including
45 endometritis, pelvic inflammatory disease, miscarriages, premature rupture of membranes
46 and preterm birth^{5,6}. The diagnosis of BV is therefore important whether it is symptomatic or
47 not. This has led to the validation and acceptance of Nugent score especially for the
48 asymptomatic woman⁷.

49
50 The Nugent scoring system (zero to 10) is a weighted combination of bacterial morphotypes
51 present in the vagina depending on the presence or absence of BV. The morphotypes
52 assessed include *Lactobacillus* (large gram-positive rods), *Gardnerella vaginalis* and
53 *Bacteroides species* (small gram-variable or gram-negative rods), and ***Mobiluncus species***
54 (curved gram-variable rods). The total score is the sum of the weighted quantity of the three
55 bacterial morphotypes. Scores 0 - 3, obtained when the biota is predominantly ***Lactobacillus***
56 is accepted as negative for BV while scores 7 -10 which relatively devoid of **lactobacillus** and
57 may show the ***Mobiluncus spp*** morphotype, is accepted as positive for BV⁷ Some studies
58 have shown that not all strains of ***Lactobacillus*** produce hydrogen peroxide and their

presence does not necessarily indicate vaginal protection^{6,8}. This may explain why at Nugent scores 7 and 8, which are diagnostic of BV may also be associated with the presence of *Lactobacillus* on microscopy⁸. In order to assess the vaginal health of women and speculate susceptibility to reproductive infections, this study was done to detect hydrogen peroxide production in some *Lactobacillus species* previously isolated, quantify the produced H₂O₂ and to correlate the amounts produced with Nugent score 0 to 10.

2. METHODS

Between May and August 2009, hydrogen peroxide production and quantification was carried out by titration method at the Department of Biochemistry, College of Medicine of the University of Lagos, Nigeria, on 97 isolates of *Lactobacillus* obtained from the Medical Microbiology Department of the same institution. It was approved by the Health Research Ethics Committee of the Lagos University Teaching Hospital, Lagos, Nigeria.

2.1 Sources and identification of Isolates

Ninety seven women attending the cytology clinic of Lagos University Teaching Hospital (LUTH), a nearby community clinic and students of the LUTH school of nursing were divided into three groups on the basis of their Nugent score into BV (Nugent score 7-10), Intermediate (4-6) and Negative (1-3)⁹. Ninety seven isolates of *Lactobacillus* had been cultured from high vaginal swabs from the women and stored in 10% glycerol stock. Two vaginal swabs were collected from each woman and cultured on two plates of MRS (deMan, Rogosa and Sharpe) agar and two plates of blood agar (prepared from Brain Heart infusion broth to which 7% of sheep blood was added). A pair of MRS and blood agar was incubated at 37°C in 5-10% carbon dioxide for 24 hours and the other pair in anaerobic jar for 48 hours. Anaerobic environment was created by gaspak technique using anaerogen (Oxoid). All facultative anaerobes and anaerobic isolates were identified as *Lactobacillus species* if

they were Gram positive bacilli and catalase negative. If more than one strain from one vaginal swab was characterized as, they were stored and later investigated for H₂O₂ production. Isolates subcultured from glycerol broth onto MRS agar and blood agar, and incubated at 37°C in 5-10% carbon dioxide jar and anaerobically for 24-48 hours were re-identified as *Lactobacillus species* based on Gram staining and catalase reaction.

2.2 Quantitative Analysis of Hydrogen Peroxide

The test is based on the principle that 1ml of 0.1N potassium permanganate is equivalent to 1.070mg of hydrogen peroxide and therefore at the endpoint the volume of potassium permanganate used is equivalent to the H₂O₂ produced. To 5ml of MRS broth culture of each test organism, 5ml of 0.1M dilute sulphuric acid was added and titrated with 0.1N potassium permanganate. The endpoint was reached when the sample was decolourised. The negative control was broth without lactobacilli. The volume of hydrogen peroxide produced by the organism was then compared with the Nugent score in the source.

Where more than one strain of *Lactobacillus* was isolated from a vaginal swab, all were tested for H₂O₂ production. The isolates were taken as H₂O₂ producing if at least one strain produced H₂O₂ and non-H₂O₂ producing if none produced H₂O₂. The volumes of hydrogen peroxide produced by the organism were compared with the Nugent scores in the source. Means were compared using the ANOVA and Spearman's correlation coefficient and a p value of < 0.05 was considered significant.

3. RESULTS

Fifteen of the 97 women studied had BV. Two of the women had two strains of lactobacilli isolated from each of them, but only the H₂O₂ producing strains were included in the analysis. While two of them produced H₂O₂ (one from each woman), all four were aerobic. Of the 97 lactobacilli analysed, 76 (78%) were facultative anaerobes, while 21 (22%) were

strict anaerobes. Forty nine (50.51%) of the 97 isolates produced H₂O₂ and forty four (89.79%) of these were isolated from women without BV. The proportion of H₂O₂ producers by Nugent scores were 70%, 43% and 33% in negative, intermediate and BV Nugent scores respectively (Table 1). There was a statistically significant difference between the proportions of H₂O₂ producers between negative and intermediate Nugent scores and also between intermediate score and BV score (χ^2 (1, n=49)=41.543, p<0.05), (χ^2 (1, n=15)=6.429, p<0.05)

Table 1: Proportion of hydrogen peroxide producing *Lactobacillus* species in women with and without BV

H ₂ O ₂ PRODUCERS	NO BV (82)		
	NEGATIVE NUGENT SCORE (N = 33)	INTERMEDIATE NUGENT SCORE (N = 49)	BV (N = 15)
FACULTATIVE LACTOBACILLI (N =35/76)	18/28	13/37	4/11
ANAEROBIC LACTOBACILLI (N =14/21)	5/5	8/12	1/4
TOTAL (49)	23 (70%)	21 (43%)	5 (33%)

As shown in Table 1, majority of the strains obtained from women with BV did not produce hydrogen peroxide. Also shown is that prevalence of H₂O₂ producing lactobacilli decreased with increasing Nugent score, and non-H₂O₂ producers increased with increasing Nugent score. (r = -0.746, p<0.05)

There was no statistically significant difference in the mean concentrations of H₂O₂ produced by the obligate anaerobes with respect to Nugent scores. Although the mean concentration

132 of H₂O₂ produced by facultative **lactobacilli** in the intermediate was higher than the negative
 133 score, this did not correlate with presence or absence of BV (Tables 2). **Using Spearman**
 134 **correlation coefficient, no association was observed between the Nugent scores and the**
 135 **individual concentrations of either the facultative anaerobes (r = -0.375) or strict anaerobes (r**
 136 **= 0.449)**

137 **Table 2: Correlation of mean concentrations of H₂O₂ produced by with Nugent scores**

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139 **Facultative **lactobacilli****

	Negative Score	Intermediate Score	BV Score
	(n = 18)	(n=13)	(n = 4)
Mean	0.1415	0.2586	0.1659
Std Deviation	0.0018	0.0027	0.0013
(F _{2, 32} = 2.616; p > 0.05)			

140 **Anaerobic **lactobacilli****

	Negative Score	Intermediate Score	BV Score
	(n = 5)	(n=8)	(n = 1)
Mean	0.1648	0.1511	0.0535
Std Deviation	0.0028	0.0191	0.0061
(F _{2, 11} = 0.982; p > 0.05)			

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143 **DISCUSSION**

144 **This study shows that the proportion of H₂O₂ producing *Lactobacillus* decreases with**
 145 **increasing Nugent score, thus confirming that H₂O₂ producing lactobacilli generally protects**
 146 **the vagina against BV while non H₂O₂ producing lactobacilli do not. Other studies have also**
 147 **shown that the presence of H₂O₂ producing lactobacilli in the vagina protects it from BV. A**

148 cohort study by Hawes et al in 1996 found that acquisition of BV was independently
149 associated with either a lack of vaginal H₂O₂ producing lactobacilli or presence of only non-
150 H₂O₂ producing lactobacilli⁸. Another study by Valour et al in 2001 showed that loss of H₂O₂-
151 producing lactobacilli leads to an increased acquisition of bacterial vaginosis¹⁰.

152

153 The overall proportion of hydrogen peroxide producing lactobacillus was low. This is
154 worrying because it may imply that many women are susceptible to BV, especially since the
155 non - H₂O₂ producing lactobacilli were isolated more frequently from women with
156 intermediate scores (57%) and BV scores (67%), confirming that the absence of H₂O₂
157 producing lactobacilli constitutes a primary defect which increases susceptibility of the
158 vagina to overgrowth by abnormal biota present in BV^{3,11,12}. On the other hand, it appears
159 there were other factors that protected the vagina from BV where H₂O₂ was not present
160 since as many as 30% of isolates from 0-3 Nugent (negative) scores did not produce H₂O₂.
161 Recent studies have shown that H₂O₂ production is not sufficient to kill BV associated
162 bacteria^{13, 14}. Furthermore, H₂O₂ producing *Lactobacillus species* failed to prevent BV in 33%
163 of the women. Clearly, there are factors that determine in vivo production and effectiveness
164 that need to be understood. Of note is the finding by O'Hanlon et al that cervico-vaginal fluid
165 and semen inactivates the microbicidal activity of H₂O₂ produced by vaginal *Lactobacillus*¹³.
166 We speculated that a higher concentration of hydrogen peroxide would reduce the likelihood
167 of BV. Contrary to our expectation, this study shows that the concentration of H₂O₂ produced
168 has no correlation with Nugent score. Furthermore, there have been no known studies
169 linking concentration or amounts of H₂O₂ produced with Nugent scores.

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172 Species identification was not performed in this study. The most prevalent species of
173 *Lactobacillus* in the vagina are *L. crispatus*, *L. jensenii*, *L. iners* and *L. gasseri*. Vallor et al
174 found in 2001 that the production of H₂O₂ is closely linked with species¹⁰. Majority of the *L.*

175 *crispatus* and *jensenii* strains produce H₂O₂ and have been recommended as candidates for
176 vaginal probiotic for the prevention of BV and sexually transmitted infections¹⁰. The presence
177 of such H₂O₂ producing species was found to be a predictor for sustained long-term
178 colonization of the vagina¹⁰.

179
180 Vaginal *Lactobacilli* are either facultative anaerobes or strict anaerobes. In this study there
181 was a higher proportion of the facultative anaerobes compared with strict anaerobes
182 regardless of association with BV, which is in keeping with previous reports³. As many as 14
183 (66.67%) of the 21 anaerobic lactobacilli in this study produced H₂O₂. Although, this appears
184 unusual since obligate anaerobes did not produce H₂O₂ in previous studies. Whittenburg in
185 1964 had demonstrated that there was no correlation between H₂O₂ production and a
186 preference for anaerobic or aerobic growth conditions^{3,4}. Eschenbach *et. al.*, in 1989
187 however mentioned the possibility of the obligate anaerobes transiently producing small
188 amounts of H₂O₂³. It is therefore noteworthy that all the anaerobic lactobacilli isolated from
189 women with negative Nugent scores produced H₂O₂ while most of the ones isolated from
190 women with BV scores (75%) did not. Of particular note is the fact that the women with
191 intermediate scores provided the highest number of lactobacilli isolated, many of which were
192 obligate anaerobes, which constituted half of all obligate anaerobes isolated. These
193 anaerobes nevertheless produced H₂O₂. If indeed anaerobes only produce H₂O₂ transiently,
194 making carriers susceptible to BV, it could be that, for some reason, facultative anaerobes
195 become replaced by obligate anaerobes which produce H₂O₂ that cannot sustain protection
196 in the women, causing the biota to gradually progress to that of BV. However, the obligate
197 anaerobes analysed were quite few and were not identified to the species level, making it
198 difficult to make such an association. It is necessary to study the role if any of sensitivity to
199 oxygen, among other factors, in reducing the number of hydrogen peroxide producing
200 lactobacilli which is believed to precede overgrowth of the organisms associated with BV.

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204 4. CONCLUSION

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206 The overall rate of hydrogen peroxide production was low. The Nigerian women studied

207 may thus have a relatively high susceptibility rate to vaginal infections. While the proportion

208 of isolates producing hydrogen peroxide production correlated with Nugent scores, being

209 highest in negative Nugent scores and lowest with BV scores, the concentration of hydrogen

210 peroxide produced had no association with Nugent scores. Species identification of

211 Lactobacillus in the study population, among other information will further help in the

212 understanding of the factors that determine the H₂O₂ production and its effectiveness in vivo.

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215 AUTHORS' CONTRIBUTIONS

216 "Author A" designed the study and wrote the first draft of the manuscript, "Author B"
217 performed the statistical analysis, "Author B" and "Author C" managed the literature
218 searches, "Author B" and "Author E" managed the analysis of the study, "Author D" wrote
219 the protocol. All authors read and approved the final manuscript.

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