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Lactobacillus Species Isolates and Vaginal Pro-Inflammatory Cytokine Levels in Women of Reproductive Age Attending Nnamdi Azikiwe University Teaching Hospital (NAUTH), Nnewi

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ABSTRACT

The association between vaginal Lactobacillus spp and regulation of pro-inflammatory cytokines have been reviewed. This study was done to evaluate the relationship between the presence of Lactobacillus spp isolated in the vagina of women of reproductive age resident in Nnewi and vaginal tissue pro-inflammatory cytokines (TNF-α and IFN-ɣ) levels. A total of 220 women of reproductive age (pregnant and non-pregnant women) aged between 18-45 years who attended Obstetrics and Gynaecology clinics at Nnamdi Azikiwe University Teaching Hospital (NAUTH) Nnewi and had been classified according to their clinical Bacterial Vaginosis (BV)- status, were enrolled for the study. Two vaginal swabs samples were obtained from each woman. The first swab was used for cultivation and isolation of *Lactobacillus* spp, while the second swab used for evaluation of pro-inflammatory cytokines (TNF-α and IFN-ɣ). Within the study population, women who were noted to be non-pregnant and clinically BV-positive had significantly higher mean levels of Pro-inflammatory cytokines (PC); TNF-α (57.90±27.26 pg/ml) and IFN-ɣ (168.98±100 pg/ml) compared non-pregnant women who were BV-negative with TNF-α (13.48±4.67 pg/ml) and IFN-ɣ (29.56 pg/ml). Comparison between both groups showed statistically significant difference ($p < 0.05$). Generally, pregnant women had lower mean levels of PC; TNF-α (24.68±21.69 pg/ml) and IFN-ɣ (82.35±35.74 pg/ml) compared to non-pregnant women with TNF-α (36.79±29.90) and IFN-ɣ (102.71±100.86) regardless of their BV-status ($p > 0.05$). Non-pregnant participants in which Lactobacillus iners were isolated had the highest up-regulation of PC; TNF-α (55.80 pg/ml) and IFN-ɣ (149.9 pg/ml) compared with other women in the same group who had *Lactobacillus crispatus* and *Lactobacillus acidophilus* isolated from their vaginal samples. In pregnant women, subjects with *Lactobacillus plantarum* isolated had the highest up-regulation of PC; TNF-α (75.63 pg/ml) and IFN-ɣ (163.63 pg/ml) compared to women who had other Lactobacillus spp isolated from their vaginal swabs. It is inferred by this study the presence of *L.iners* has a remarkable association with the up-regulation of pro-inflammatory cytokines which promote pathological process in the vaginal disease states. The potential for the deployment of *L.iners* as a promising potential algorithm and biomarker in the detection of vaginosis and vaginitis is noted in this study.

Keywords: Cytokines, non-pregnant, pregnant, pro-inflammatory.

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I. Introduction

Pro-inflammatory cytokines are positive mediators of inflammation. In a wide variety of infections, such molecules are released as a host response due to inflammasome activation, this is popularly known as the **pro-inflammatory cytokine response**. Cytokines with a pro-inflammatory function are elevated in the infected tissue. Over time, some of them increase systemically. Recent research has shown the importance of the pro-inflammatory cytokine response in causing a state of insulin resistance. The infusion of TNF-α has been shown to produce insulin resistance in vivo [1]. Pro-inflammatory cytokines are produced predominantly by activated macrophages and are involved in the up-regulation of inflammatory reactions.

There is abundant evidence that certain pro-inflammatory cytokines TNF-α and IFN-ɣ are significantly elevated in the

cervico-vaginal mucosa in women with Bacterial Vaginosis (BV) and idiopathic infertility [2], [3]. Similarly, later reports by [4] have also noted the strong links between BV and upregulation of pro-inflammatory cytokines in women of reproductive age in South Africa. Age, education, and occupational factors have also been noted be associated with the prevalence of BV in women of reproductive age in Nigeria [5]. Reference [6] have also studied the structure of the human vaginal mucosa and espoused its important role in the defending the immune system. In a previous study done in the United States by [7] to assess cytokines levels and *Lactobacillus* species in both pre-menopausal and post-menopausal women, the latter group exhibited a greater assortment of *Lactobacillus* species, and a higher level of several pro-inflammatory cytokines as well as one anti-inflammatory cytokine. That finding was also consistent with vaginal epithelial or mucosal damage, and a shift in local immune function observed in post-menopausal women. The study offered key evidence into the direct correlation between *Lactobacilli* presence and cytokine production in pre-menopausal and post-menopausal women.

II. Methodology

A. Study Setting and Design

The study was carried out at Nnamdi Azikiwe University Teaching Hospital (NAUTH), Nnewi, Anambra state. NAUTH is a tertiary health institution established in 1991. It is located at Nnewi, a town that shares boundary with Ichi, Oraifite, Ozubulu and Nnobi all in Anambra state of Nigeria. NAUTH is primarily established for specialized clinical service, teaching, and research purpose.

This was a cross-sectional study where vaginal samples were collected from BV-categorized women of reproductive age for the isolation of *Lactobacillus* spp and evaluation of vaginal tissue cytokines. Systematic random sampling technique was applied in selecting the subjects.

B. Study Population, Sample Size Calculation and Subject Selection/Recruitment

The study population was composed of women of reproductive age who had been documented to be either BV-positive or BV-negative while attending O and G clinic. The women enrolled in this study were aged between 18-45 years.

Sample size for this research work is determined through the use of the Daniel's sample size determination formula [6].

$$N= Z^2PQ/ D^2$$

where
N = minimum sample size,
Z = the level of statistical significance of the expected result, in this case 1.96 for 95% confidence interval,
P = prevalence rate of PID in Nigerian women = 0.17 (17%) [9],
Q = 1-P,
D = maximum allowable error which is normally put at 0.05.
Therefore,

$$n = [(1.96)2 \times 0.17(1-0.17)]/ (0.05)^2$$
$$= 3.8416 \times 0.14/ 0.0025$$
$$= 217 \text{ participants}$$

The subjects were women who attended Obstetrics and Gynaecology clinics in NAUTH between June 2021 and September 2021. The subjects were women who were either non-pregnant or pregnant at any trimester. Women who had vaginal bleeding, had been previously diagnosed with Human Immunodeficiency Virus (HIV) or were on antibiotics were excluded from the study. After agreeing to participate, a well-structured questionnaire and an informed consent form were administered to each subject.

C. Ethical Approval

This study represents a part of a wider research conducted in NAUTH, Nnewi. Ethical approval for the study was officially obtained from the Nnamdi Azikiwe University Teaching Hospital Health Research Ethics Committee (NAUTHHREC) in their letter dated 29th, June 2021 with reference number NAUTH/CS/66/VOL.14/VER3/ 121/2021/039.

Subjects' participation in the study was voluntary after they were properly informed on the nature, merits and aim of the study before sample collection. Subjects were enrolled into study only after reading and signing the informed consent form.

D. Specimen Collection

Each subject was clinically examined and categorized by the attending clinician in the O and G clinic as either BV-positive and BV-negative. High vaginal swab (HVS) specimens collected with sterile cotton-tipped swabs which were pre-labelled with the participants' study identification numbers. Collection of the HVS sample from these consenting participants was done with the aid of a speculum.

E. Demographic and Clinical Data Collection

A well-structured questionnaire which took into consideration demographic information and factors that could influence of Lactobacillus isolation and pro-inflammatory cytokine evaluation such as BV status. Essentially, information on age, marital status, trimester, educational status, occupational status, parity, use of antibiotics and polyherbals were collected and collated for this study. Participants of antibiotics and polyherbals were not considered for inclusion in the study.

F. Laboratory Testing of Specimens

Laboratory testing of specimen was done in the Medical Laboratory complex of NAUTH, Nnewi.

G. Cultural Identification of Lactobacillus spp and Related Genera (LAB)

- The Lactic acid bacteria were isolated culturally by employing the Dehydrated de Man, Rogosa and Sharpe MRS media (Himedia™).
- After preparation and sterilization, the MRS agar was dispensed from the glass flasks into different sets of sterile Petri dishes (Silver Health, UK), which were inoculated and incubated at 37 °C for 48 hours.
- The single colonies of LAB isolates were observed for their colony morphology and some biochemical tests such as Gram staining, catalase and oxidase tests were also executed.

- *Lactobacillus* spp. isolates produced small, irregular and round shape with shiny whitish cream or brownish coloured colonies morphologically on the MRS Agar [10].
- For isolation of the highly fastidious *Lactobacillus iners*, the MRS media was incorporated with 5% nystatin, further enriched with 5% blood, and incubated anaerobically for 48 hours at 37 °C. After enrichment, inoculation on Tryptone Soy Agar and blood agar was also done concurrently for differentiation [11]-[13]. Isolation of *L. iners* produced non-pigmented colonies smaller than other *Lactobacillus* spp [14].

H. Analytical Profile Index (API) 50 CHL Medium

Further biochemical analysis was done to done to identify and speciate *Lactobacillus* and related genera (apart from *L.iners*) with the aid of the API 50 CHL medium.

- A suspension of the inoculum containing the isolated bacteria using an ampule was made using MRS broth.
- The homogenized inoculum was then transferred to fill the tubes on the API 50 CHL Medium (Biomerieux, France).
- The tubes were then covered with mineral oil. Incubation was done aerobically at 37 °C for 48 hours. Acids are produced from the fermentation of the carbohydrates on the prepared API 50 CH strips incubation.
- After 48 hours of incubation, positive tests which correspond to acidification were revealed by the indicator changing to yellow.
- Biochemical profile identification was done using the chart provided to identify and differentiate the species and the results recorded on result sheets.

1) Cytokine Evaluation of Human Tumor Necrosis Factor-alpha (TNF-α) and Human Interferon-gamma (IFN-ᵞ) of Vaginal Tissue samples

- Vaginal swab samples were collected using sterile swab sticks, it was then aliquoted and sent to the laboratory where it frozen pending analysis for cytokines evaluation.
- During ELISA evaluation it was thawed, vortex vigorously and the swab squeezed on the wall of the tube to maximize elution and analyzed for pro-inflammatory cytokines TNF-α and IFN-ᵞ using the Pro-Human Immunoassay evaluation kit (Accubind Inc, USA).
- Standards were added to standard wells, black wells were set with standard, and sample added at 10ul dilution together with 40ul sample dilution buffer.
- Enzyme conjugate reagents were then added at 100 ul to each well except the blank well. Incubation was done at 37 °C for 60 minutes.
- Washing was done with the 20-fold diluted wash solution for a total of 5 times. After washing, complete removal of liquid was done.
- Subsequently, 50ul of chromogen solution A and 50 ul of chromogen solution B was added to each well and gently mixed. Incubation was done for 15 minutes at 37 °C.
- Afterwards, a 50 ul Stop solution was added to each well, changing the colour of the wells from blue to yellow and optical density read at 450 nm using a micro-titer plate reader within 15 minutes obtain values.

III. RESULTS

In Table I, Pro-inflammatory cytokines TNF-α and IFN-ᵞ were assayed and analyzed. The mean value of TNF-α in Non-pregnant without BV was 13.48±4.67 pg/ml while the mean value for non-pregnant women with BV was 57.90±27.26 pg/ml. There were significant differences in the mean values of the TNF-α of the two groups of women ($p<0.001$). The pregnant women in their first trimester had mean value 70.68±35.19 pg/ml, pregnant women in their second trimester have 47.61±24.53 pg/ml while pregnant women in their third trimesters had 13.35±3.29 pg/ml. For pro-inflammatory cytokine IFN-ᵞ, non-pregnant women without BV had a mean value of 29.56±7.49 pg/ml while non-pregnant women with BV had a significant higher mean value of 168.98±100.49 pg/ml. In Table II, cumulative analysis of vaginal tissue cytokine TNF-α showed that non-pregnant women had 36.79±29.90 pg/ml as mean values while pregnant women had a mean value of 24.68±21.69 pg/ml. Analysis of mean difference between non-pregnant and pregnant groups of women show significant difference ($p<0.001$). IFN-ᵞ had a mean value of 102.71±100.86 pg/ml in non-pregnant women while pregnant women had mean value of 82.35±35.74 pg/ml. The mean difference between the two groups for the IFN-ᵞ value was also significant ($p<0.001$).

Fig. 1 showed a graphical analysis of mean levels of pro-inflammatory cytokine TNF-α in non-pregnant women with different *Lactobacillus* isolates obtained from their vaginal samples. Non-pregnant women had vaginal microbiota that were less rich in *Lactobacillus spp* compared to pregnant women.

Women who had *Lactobacillus iners* as dominant isolates had the highest level of mean TNF-α recorded from their vaginal tissue cytokines at 55.8 pg/ml, women with *Lactobacillus crispatus* as dominant isolates had a mean TNF-α of 44.52 pg/ml, while women with *Lactobacillus acidophilus* had the lowest mean value of TNF-α of 35.4 pg/ml. Fig. 2 showed a graphical representation of mean levels of vaginal tissue pro-inflammatory cytokine IFN-ᵞ in non-pregnant women in relation to *Lactobacillus spp* isolated in the vaginal microbiota. Participants in the group with *L. iners* as dominant isolates had the highest mean IFN-ᵞ at 149.97 pg/ml, in participants with *Lactobacillus acidophilus* isolates the Mean IFN-ᵞ was 106.65 pg/ml, cytokine was further downregulated at the lowest level in women with *L.crispatus* as the dominant Lactobacillus species with a mean value of 53.23 pg/ml.

Fig. 3 showed mean levels of vaginal tissues pro-inflammatory cytokines TNF-α in pregnant women in connection to different Lactobacillus species isolated. The cytokine was upregulated highest in women with *L. plantarum* as dominant isolate at 75.63 pg/ml, participants with *L. crispatus* as dominant isolate had 32.27 pg/ml as mean TNF-α, in women with *Lactobacillus acidophilus* detected the cytokine was further down-regulated at 29.15 pg/ml. Mean levels of the cytokine in participants with *L. iners* isolated in their vaginal microbiota was 18.85 pg/ml. Women with *L. reuteri, L.vaginalis, L. mucosae and L. rhamanosus* mean TNF-α of 17.85 pg/ml, 13.29 pg/ml, 13.00 pg/ml and 10.75 pg/ml respectively.

Fig. 4 showed mean levels of vaginal tissues pro-inflammatory cytokines IFN-ɣ in pregnant women in connection to different Lactobacillus species isolated. The cytokine was also upregulated highest in women with *L. plantarum* as the dominant isolate at 106.63 pg/ml while 88.85 pg/ml was recorded in participants with *L. crispatus* as the dominant *Lactobacillus spp* isolated. In participants with *L. acidophilus* 83.51 pg/ml was the mean cytokine value recorded. Partipants with *L. reuteri* as dominant Lactobacillus spp had 82.40 pg/ml as the mean IFN-ɣ cytokine level. Pregnant women with *L iners, L. rhamnosus, L. mucosae and L. vaginalis* had 74.81 pg/ml, 71.02 pg/ml, 67.66 pg/ml and 66.42 pg/ml respectively.

TABLE I: COMPARISON OF PRO-INFLAMMATORY CYTOKINES BETWEEN NON-PREGNANT WOMEN WITHOUT BV AND NON-PREGNANT WITH BV

Variable	Study group (mean±SD) (pg/ml)		P value
	Non pregnant BV-Negative	Non pregnant BV-Positive	
Cytokines TNF- α	13.48±4.67	57.90±27.26	0.001*
Cytokines TNF-ɣ	29.56±7.49	168.98±100.49	

*=significant p-value <0.05

TABLE II: COMPARISON OF VAGINAL PRO-INFLAMMATORY TISSUE CYTOKINES AMONG THE PREGNANT AND NON-PREGNANT GROUPS OF WOMEN

Variable	Study group (mean±SD) (pg/ml)		P value
	Non pregnant	Non pregnant	
TNF- α	36.79±29.90	24.68±21.69	0.001*
TNF-ɣ	102.71±100.86	82.35±35.74	

*=significant p-value <0.05

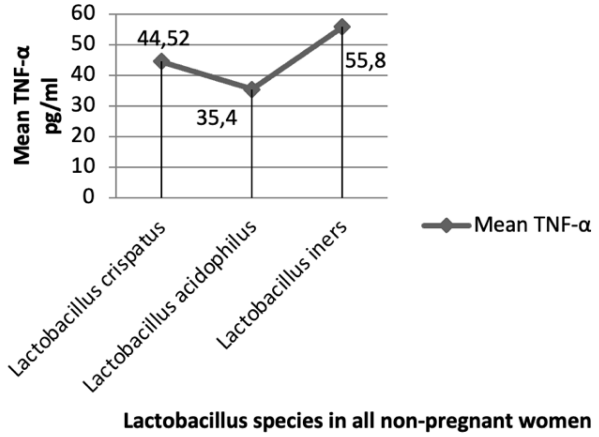

Fig. 1. Levels of vaginal tissue pro-inflammatory cytokine TNF-α in relation to *Lactobacillus* species isolated in non-pregnant women.

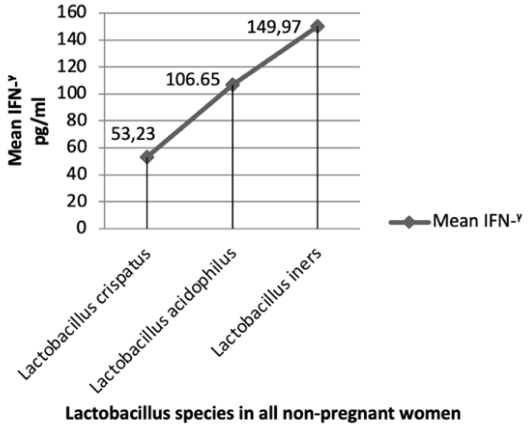

Fig. 2. Levels of vaginal tissue pro-inflammatory cytokine IFN-ɣ in relation to *Lactobacillus* species isolated in non-pregnant women.

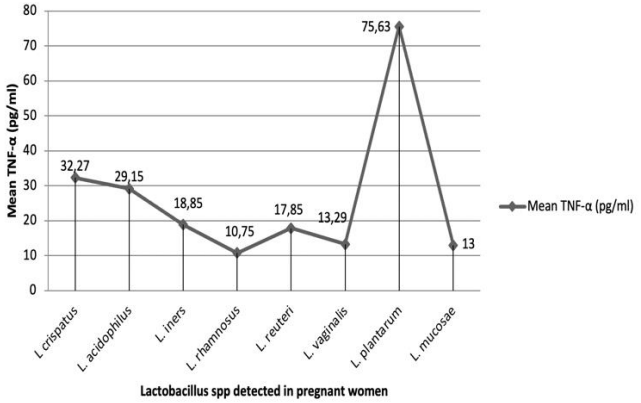

Fig. 3. Levels of vaginal tissue pro-inflammatory cytokine TNF-α in relation to *Lactobacillus* species isolated in pregnant women.

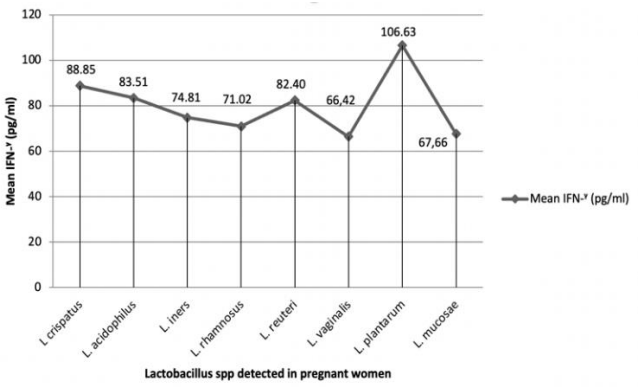

Fig. 4. Mean levels of vaginal tissue pro-inflammatory cytokine IFN-ɣ in relation to *Lactobacillus* species isolated in pregnant women.

IV. DISCUSSION

In this report the mean concentration of vaginal tissue pro-inflammatory cytokines was significantly higher in non-pregnant women diagnosed with BV compared with non-pregnant women without BV. This result is not dissimilar with the reported findings of [4] who studied and compared vaginal pro-inflammatory cytokine levels in South African women of reproductive age who had untreated BV with those who were treated for BV. Also, similar previous study executed by [2] had also showed that TNF-α and IFN-ɣ are significantly elevated in the cervico-vaginal mucosa in women with BV and idiopathic infertility. Research done in Canada by [15] also showed that *Lactobacillus rhamnosus* attenuated the release of pro-inflammatory cytokines TNF-α and IFN-ɣ in concurrence with our findings.

In pregnant women, pro-inflammatory cytokine levels were generally down-regulated compared to the non-pregnant women and this can be attributed to the higher level of colonization of *Lactobacillus* species in pregnancy compared to the non-pregnant women. Moreover, it can also be argued that the routine supplements administered to pregnant participants in study also helps to promote anti-inflammatory ability. Folic acid and Vitamin B complex recommended during antenatal care have been understood to promote tissue repair, negate and suppress pro-inflammatory processes [16], [17]. Reports from this study is also supported by previous findings from [7] who showed significant difference levels of pro-inflammatory cytokines in women with Lactobacillus-dominant vaginal microbiota and those with Lactobacillus-deficient vaginal microbiota.

Futhermore, cytokine level was highest in non-pregnant women who had *Lactobacillus iners* as the dominant *Lactobacillus* specie detected in their vagina microbiota, supporting earlier findings by [18] and [19] who suggested that *L. iners* had greater pathogenic potential in the vagina microenvironment compared to other *Lactobacillus* species. [20] also noted negative levels of inflammatory cytokine response in women with optimal vaginal microbiota rich in other *Lactobacillus* species.

Remarkably, the pro-inflammatory cytokines where higher in the vaginal tissues of pregnant women with *Lactobacillus plantarum* chiefly isolated in their samples. The propensity of this bacteria to induce and upregulate the production of pro-inflammatory cytokines have been documented in a study by [21], while strains of *L. reuteri* showed anti-inflammatory potentials. In another experimental study with attenuated *Lactobacillus plantarum* strains, the bacteria were noted to be remarkably associated with a raise in TNF-α levels [22]. The link between *L. plantarum* and causation of meningoencephalitis in geriatric, immuno-compromised and cancer patients was also documented in earlier study by [23]. The result of this study of this also by a previous study [24] who noted the significant ability of *L. plantarum* to show a higher propensity to upregulate TNF-α and IFN-ᵞ in human peripheral blood cells compared to *L. acidophilus*. Furthermore, [25] also confirmed the promotion and increase of pro-inflammatory cytokines TNF-α and IFN-ᵞ levels by *Lactobacillus plantarum* in support of the result of the study. Reference [26] have also found significant levels of interplay between microbiota, metabolites and immunity in women with BV in support of our findings.

V. Conclusion

This study revealed that *L. iners* was the *Lactobacillus* spp linked with the highest up-regulation of pro-inflammation in non-pregnancy compared to another detected *Lactobacillus* spp. In pregnancy, *L. plantarum* was linked with the highest up-regulation of pro-inflammation than other *Lactobacillus* species. This study infers that the above-mentioned *Lactobacillus* species may possess notable pathological potential in non-pregnant and pregnant women respectively.

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Conflict of Interest

Authors declare that they do not have any conflict of interest.

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