

Comparative Analysis of Vaginal *Lactobacillus* spp., Microbial Dysbiosis, and Antimicrobial Activity in Healthy and Infected Women

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Abstract: **Background:** The vaginal microbiota consists primarily of *Lactobacillus* species, which help maintain microbial balance through acid production and antimicrobial activity. The disruption of balance causes vaginal dysbiosis and infection. **Objective:** The aim of this study was to analyze the association of vaginal infection with the distribution of *Lactobacillus* species, vaginal pH, and antimicrobial activity. **Methods:** The study was designed as a case-control study. A total of 100 subjects, including 50 healthy subjects and 50 infected subjects, were included. Vaginal swabs were collected to assess pH, microbial isolation, and identification using the standard microbiology technique. The antimicrobial activity of *Lactobacillus* isolates was assessed using the agar well diffusion technique and peroxide production was assessed qualitatively. Chi-square test and independent t-test were used for the statistical analysis of the results. **Results:** The isolation of *Lactobacillus* was 90% in healthy women and 40% in infected women, with a statistically significant difference ($p < 0.001$). Infected women had increased vaginal pH (5.6 ± 0.5) as compared to healthy women (4.3 ± 0.3) ($p < 0.001$). *Lactobacillus crispatus* was the most frequent in healthy women and *Lactobacillus iners* was the most frequent in infected women. Pathogenic organisms, especially *Gardnerella vaginalis*, were statistically significantly more common in the infected group. The isolates taken from healthy women had the highest hydrogen peroxide production and antimicrobial activity. **Conclusions:** An increase in vaginal pH, a decrease in antimicrobial defenses, and overgrowth of pathogens are associated with the loss of functionally active *Lactobacillus* species. Composition and functional capacity are critical factors in determining vaginal health.

Keywords: Vaginal Microbiota, *Lactobacillus* spp, Vaginal pH, Antimicrobial Activity, Hydrogen Peroxide, Vaginal Dysbiosis, *Lactobacillus Crispatus*, *Gardnerella Vaginalis*.

1. INTRODUCTION

The vaginal microbiome is an important ecosystem that is involved in the reproductive health of a woman, as well as in the defense against infections and the susceptibility to them. While the presence of many species of bacteria in a mucosal surface is considered a sign of health, the opposite is the case for healthy women of childbearing age. Here, there is a low diversity microbiome and a predominance of *Lactobacillus* species [1, 2]. This phenomenon is the result of a finely tuned interaction of the host immune system with the microbiota, regulated by hormones, the presence of glycogen, and specific dietary preferences of the bacteria. The presence of vaginal dysbiosis (a reduction in number of *Lactobacillus* spp) is associated with many negative outcomes in gynecology and obstetrics, such as bacterial vaginosis (BV), STIs, infertility, and preterm birth [3, 4].

Vaginal *Lactobacillus* spp. not only dominate the vaginal ecosystem, but they also exert a tremendous influence in the development of the vaginal environment for their own survival and reproduction [5]. In the vaginal ecosystem, these species produce lactic acid, which leads to a vaginal pH of approximately 4.5. This pH inhibits the survival and growth of many pathogenic bacteria [5]. Lactic acid has both pH-dependent and pH-independent antimicrobial properties, which include the disruption of bacterial membranes and the alteration of the immune system of the vaginal epithelium [6]. In addition, some *Lactobacillus* strains also produce other antimicrobial compounds such as hydrogen peroxide, bacteriocins,

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and biosurfactants. These compounds, in addition to hydrogen peroxide, also inhibit the growth of some opportunistic pathogenic vaginal bacteria (e.g. *Gardnerella vaginalis*, *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*) [7, 8]. The presence of lactobacilli and their biofilm formation also provide physical and mechanical barriers to the epithelial surface of the mucosa and thereby enhance the mucosal surface barrier [9].

Vaginal dysbiosis is also known as bacterial vaginosis, which involves the reduction of *Lactobacillus* dominance and the increase of polymicrobial anaerobic communities [10]. However, evidence is supporting that not all species of *Lactobacillus* provide the same protective measures. Community state types that are dominated by *Lactobacillus crispatus* provide more stability in the microbiome and possess less inflammation in the clinical environment. Meanwhile, communities dominated by *Lactobacillus iners* appear more transitional and are often in a state of dysbiosis [11, 12]. These findings demonstrate the necessity of resolution at the species level and functional characterization of a microbiome as opposed to a singular reliance on the abundance at the genus level.

There have been microbiome sequencing technologies that have been developed recently, yet the functional biological understanding of the level of strain is why these technologies have yet to be applied in a certain way to assess the antimicrobial and ecological activities of the individual microbial strains. When combined with compositional analysis, the functional assessment of microbes, such as pathogenesis, pathogen inhibition, and the assessment of microbial cellular activities that lead to the generation of hydrogen peroxide, is complex and more effective [13,14]. In addition, situated (or relevant) data are needed since environmental variables of a region, as well as variables of total and health systems, are likely to be a controlling factor on the microbial communities' structure and function.

Consequently, the goal of this investigation is to thoroughly examine the ecology, and the function of vaginal *Lactobacillus* spp., in both healthy, and diseased, women. More specifically, we intend to (i) examine and compare the isolation rates of, and the distribution of species within, the *Lactobacillus* genus, (ii) analyze the relationship, if any, between the depletion of *Lactobacillus* and vaginal pH, and (iii) evaluate, and compare, the antimicrobial effects, and the capacity to produce Hydrogen Peroxide, of the isolated strains in relation to the overgrowth of pathogens. By focusing on both functional and compositional analyses, we hope to provide an answer to the mechanistic relations of the structure and the metabolic activity of the *Lactobacillus* community, in relation to vaginal microbiome homeostasis.

2. MATERIALS AND METHODS

Study Design and Population

A case-control study spanning six months was set up in Dhi Qar Province, Iraq. A total of 100 women of reproductive age were equally allocated to two groups. Healthy women (n = 50) had no clinical signs of vaginal infection, while infected women (n = 50) had signs of vaginitis or were clinically diagnosed with bacterial vaginosis. Women who had received antibiotics, antifungals, or probiotics 2–4 weeks prior were excluded from the study. All participants provided written informed consent.

Specimen Collection and pH Measurement

Vaginal swabs were obtained from aseptically sterile specula. Samples were collected from the lateral vaginal wall or posterior fornix. Vaginal pH was measured using narrow-range pH paper (3.8–5.5) or a calibrated pH meter immediately after collection.

Isolation and Identification of *Lactobacillus* spp

Swabs were inoculated onto de Man, Rogosa and Sharpe (MRS) agar and then incubated for 24–48 hours at 37°C in microaerophilic conditions. Presumptive *Lactobacillus* isolates were identified through the criteria of colony morphology, Gram staining (which showed the presence of Gram-positive rods), catalase negativity, and standard biochemical tests.

Isolation and Identification of Pathogens

Further swabs of the pathogens were sent to blood agar, MacConkey agar, and Sabouraud dextrose agar to try and isolate the bacterial and fungal pathogens. Identification was dependent upon colony morphology, gram staining and some basic biochemical tests.

Preparation of Cell-Free Supernatant (CFS)

Pure cultures of *Lactobacillus* isolates were developed in MRS broth and incubated at 37°C for 18-24 hours. These cultures were then subjected to centrifugation and the resulting supernatant was obtained through a 0.22 µm membrane filter to yield a sterile cell-free supernatant (CFS).

Assay of Antimicrobial Activity

Antimicrobial activity was done using the agar well diffusion method. Pathogenic isolates were standardized to 0.5 McFarland and spread on to Mueller–Hinton agar (or suitable media). The W CFS were then added to the wells and the zones of inhibition were recorded after 18-24 hours of incubation at 37°C .

Production of Hydrogen Peroxide

The production of hydrogen peroxide was done through qualitative screening method (e.g. TMB peroxidase agar or similar). The isolates were determined to be H₂O₂ positive or Negative based upon production of colour .

Statistical Analysis:

These results were analysed using a computer statistical programme. For comparison of variables, the Chi-square test was done for categorical data and the independent t-test was done for continuous data. The p value of < 0.05 was said to be statistically significant.

3. RESULTS

The present study provides evidence on the relationship between the presence of *Lactobacillus* spp., vaginal acidity and antimicrobial activity with protection against vaginal infections. The study population comprised healthy and infected women in equal numbers (50 each) making comparison and statistical consistency valid.

A significantly greater isolation of *Lactobacillus* spp. was observed in healthy women (90%) than in infected women (40%) and this was statistically significant (p < 0.001). This shows that the absence of *Lactobacillus* is strongly correlated with vaginal infections. This absence was also correlated with an increase in the vaginal pH in infected women (5.6 ± 0.5) as compared to the healthy women (4.3 ± 0.3) (Table 3, Figure 3). This evidenced the important role *Lactobacillus* plays in the maintenance of acidity through the secretion of lactic acid.

More so, Table 4 (Figure 4) shows citational disparity in the species. *L. crispatus* was the most predominant species in healthy women (40%), and is of interest because of her positive acid and hydrogen peroxide production. Conversely, in infected women, *L. iners* was most prevalent (45%) (Table 5, Figure 5) which is an indicator of a shift to a more dysbiotic microbiota.

Table 6 (Figure 6) shows the frequencies of the pathogenic organisms that dysbiosis is affecting, with *Gardnerella vaginalis* in infected women (60%) being greater than *Gardnerella vaginalis* in women with 10% infection status (p < 0.001). This is relevant for the dysbiosis of the microflora. The fact that Table 7 (Figure 7) illustrates that the *Lactobacillus* from healthy women has far greater inhibitory activity (higher inhibition zones) than those from infected women demonstrates the relevant microorganisms.

Table 8 (Figure 8) also illustrates this phenomenon to an extent that the healthy isolates (66%) are far more of those that secrete hydrogen peroxide than those infected isolates (30%; p < 0.01). The data suggests that the absence of the protective species of *Lactobacillus*, in particular *L. crispatus*, leads to a rise in vaginal pH, a decrease in the secretion of anti-microbial agents, an increase in pathogenic microflora, and a vaginal infection. In this regard, *Lactobacillus* has a protective function. *Lactobacillus* is of great importance in maintaining the equilibrium in vaginal microflora.

Table 1: Distribution of Study Participants

Group	Number (n)	Percentage (%)
Healthy women	50	50%
Infected women	50	50%
Total	100	100%

Table 2: Isolation Rate of *Lactobacillus* spp. Among Study Groups

Group	Positive for <i>Lactobacillus</i> n (%)	Negative n (%)	P-value
Healthy (n=50)	45 (90%)	5 (10%)	
Infected (n=50)	20 (40%)	30 (60%)	<0.001*
Total	65 (65%)	35 (35%)	

*Significant difference using Chi-square test (p < 0.05)

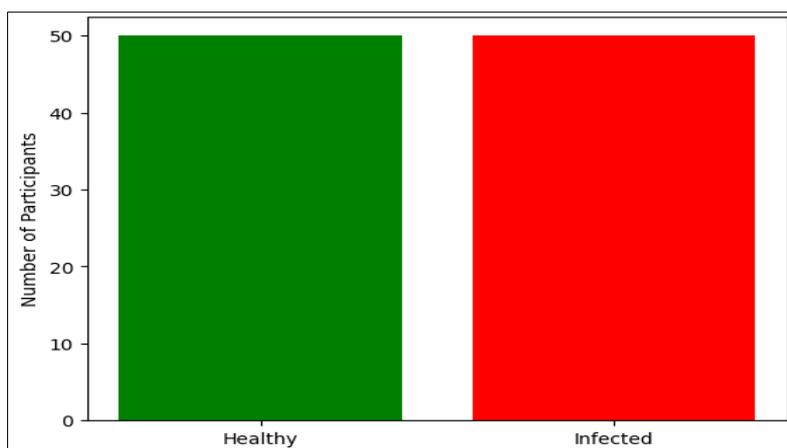


Figure 1: Distribution of Study Participants

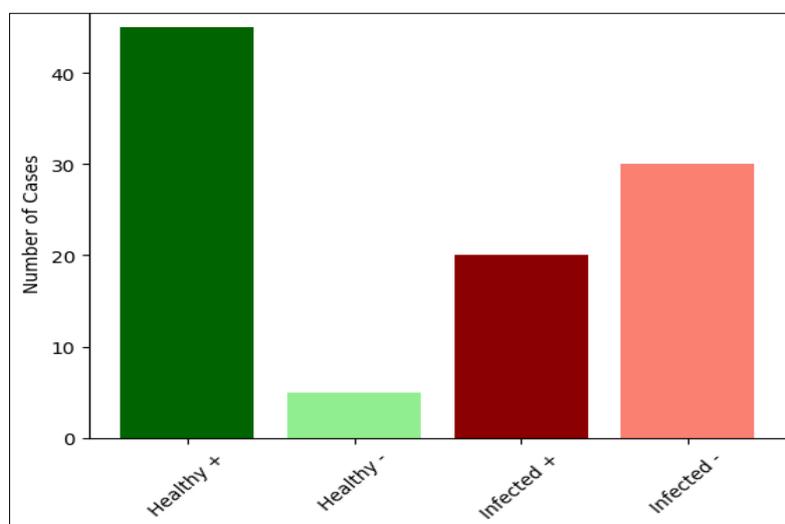


Figure 2: Isolation Rate of Lactobacillus spp. Among Study Groups

Table 3: Mean vaginal pH in Study Groups

Group	Mean pH ± SD	P-value
Healthy	4.3 ± 0.3	
Infected	5.6 ± 0.5	<0.001*

*Significant difference using Independent t-test

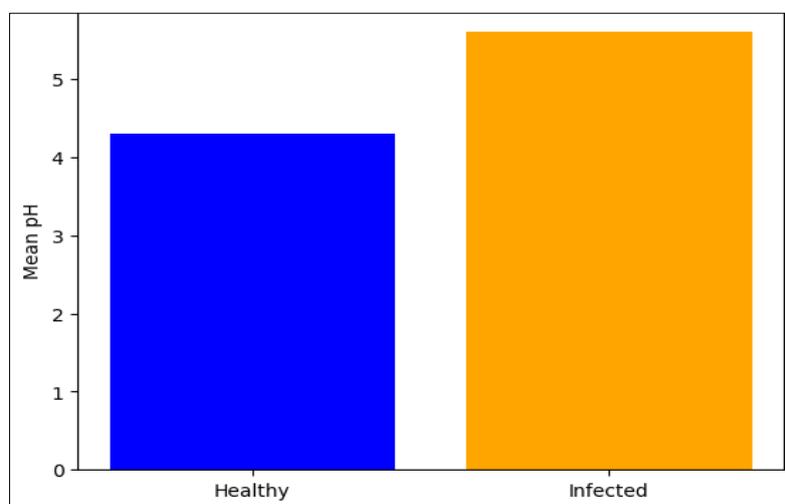


Figure 3: Mean vaginal pH in Study Groups

Table 4: Distribution of Lactobacillus Species Isolated from Healthy Women (n=45)

Species	Number (n)	Percentage (%)
L. crispatus	19	40%
L. jensenii	10	22%
L. gasseri	8	18%
L. iners	6	16%
Others	2	4%
Total	45	100%

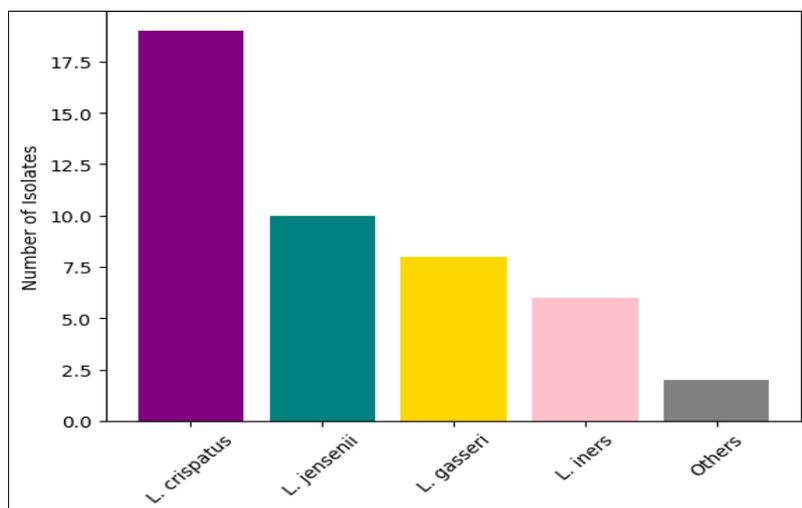


Figure 4: Distribution of Lactobacillus Species Isolated from Healthy Women (n=45)

Table 5: Distribution of Lactobacillus Species Isolated from Infected Women (n=20)

Species	Number (n)	Percentage (%)
L. iners	9	45%
L. gasseri	5	25%
L. crispatus	3	15%
L. jensenii	2	10%
Others	1	5%
Total	20	100%

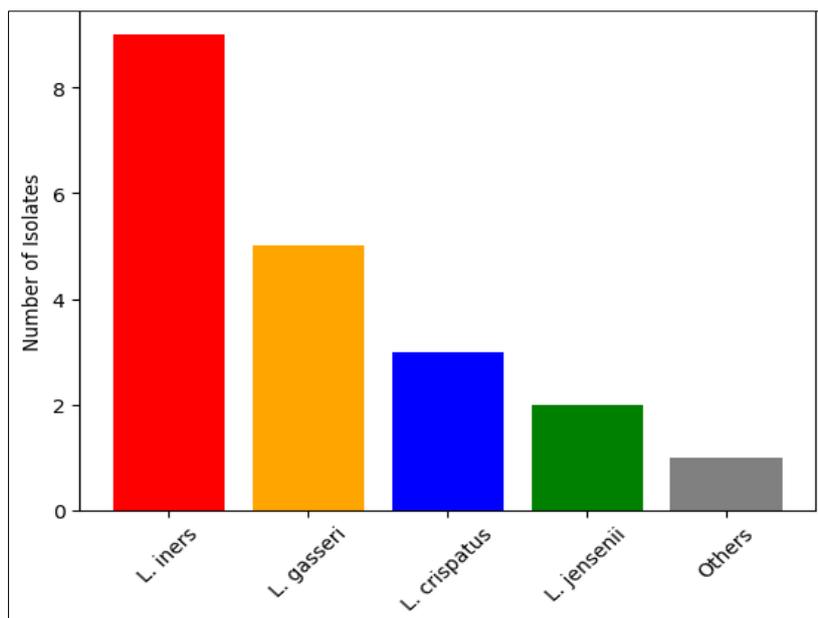


Figure 5: Distribution of Lactobacillus Species Isolated from Infected Women (n=20)

Table 6: Frequency of Pathogenic Bacteria Isolated from Study Groups

Pathogen	Healthy (n=50) n (%)	Infected (n=50) n (%)	P-value
Gardnerella vaginalis	5 (10%)	30 (60%)	<0.001*
E. coli	4 (8%)	18 (36%)	<0.01*
Staphylococcus aureus	3 (6%)	12 (24%)	<0.05*
Candida albicans	6 (12%)	15 (30%)	<0.05*
Pseudomonas aeruginosa	1 (2%)	6 (12%)	<0.05*

*Significant difference using Chi-square test

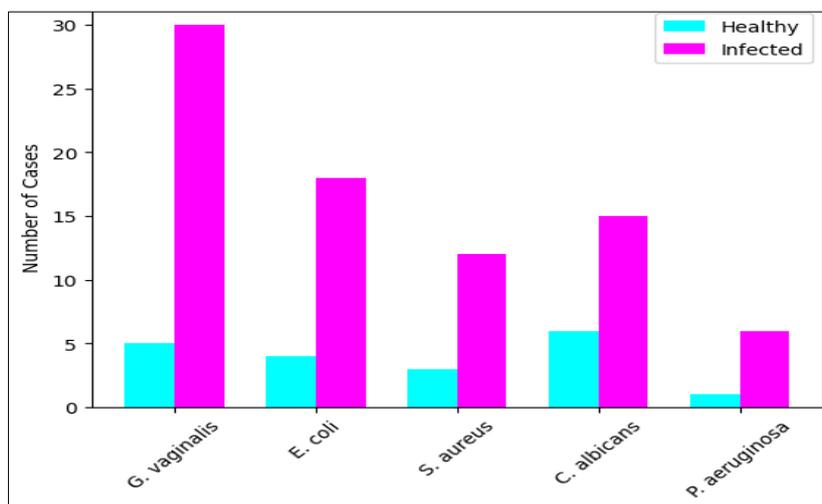


Figure 6: Frequency of Pathogenic Bacteria Isolated from Study Groups

Table 7: Mean Diameter of Inhibition Zones (mm) of Lactobacillus Isolates Against Pathogens

Pathogen	Healthy isolates (Mean ± SD) mm	Infected isolates (Mean ± SD) mm	P-value
G. vaginalis	18 ± 4	12 ± 3	<0.01*
S. aureus	16 ± 4	11 ± 3	<0.05*
E. coli	14 ± 3	10 ± 3	<0.05*
P. aeruginosa	10 ± 3	7 ± 2	0.06
C. albicans	12 ± 4	8 ± 3	<0.05*

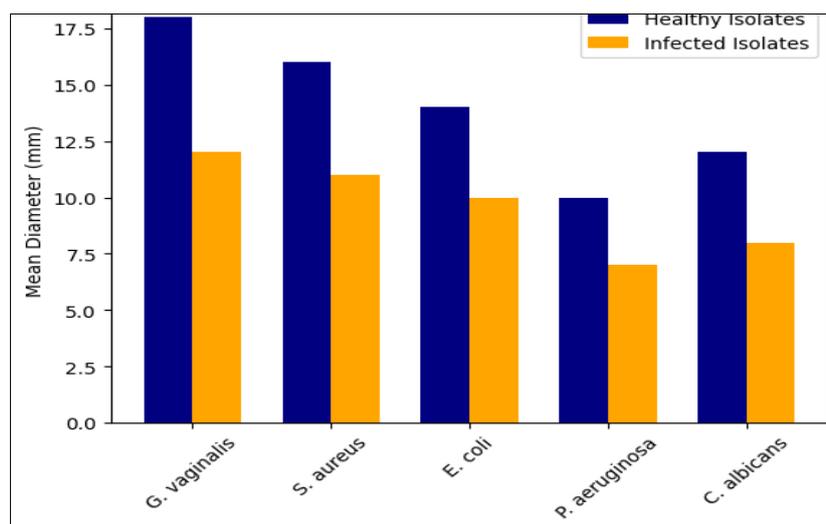


Figure 7: Mean Diameter of Inhibition Zones (mm) of Lactobacillus Isolates Against Pathogens

Table 8: Hydrogen Peroxide Production among Lactobacillus Isolates

Group	H ₂ O ₂ Positive n (%)	H ₂ O ₂ Negative n (%)	P-value
Healthy isolates (n=45)	30 (66%)	15 (34%)	
Infected isolates (n=20)	6 (30%)	14 (70%)	<0.01*

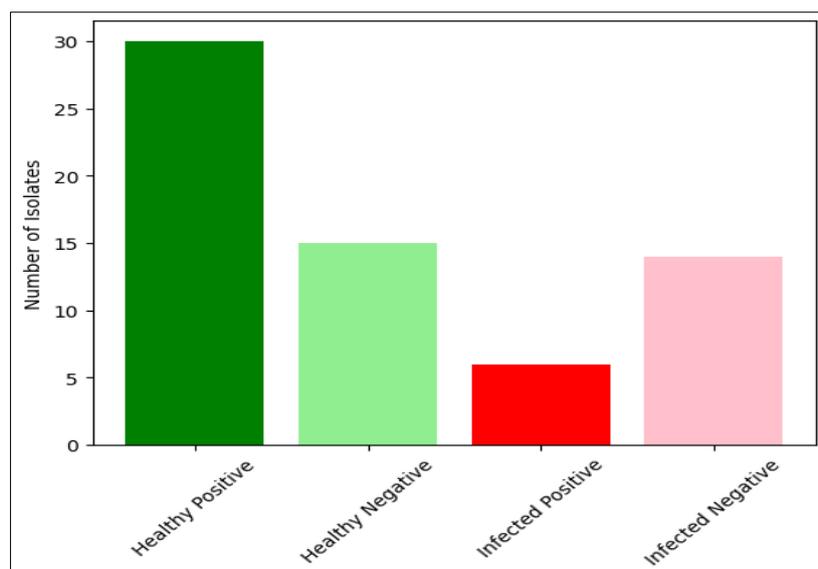


Figure 8: Hydrogen Peroxide Production among Lactobacillus Isolates

4. DISCUSSION

The current research underscores the importance of *Lactobacillus* spp. in sustaining the vaginal ecosystem and preventing bacterial overgrowth and infection. Significantly greater isolation of *Lactobacillus* spp. from healthy women (90%) compared to infected women (40%) (Table 2, Figure 2) underscores the importance of *Lactobacillus* spp. in promoting vaginal health. *Lactobacillus*-dominated vaginal microbiomes are well-established as healthy vaginal ecosystems [15, 16].

The absence and/or disruption of *Lactobacillus* of vaginal microbiomes has a well-documented association with bacterial vaginosis (BV), aerobic vaginitis, and a heightened risk of acquiring sexually transmitted infections. The significant reductions in *Lactobacillus* spp. in the infected cohort of this study is a clear representation of dysbiosis [15-17]. The infection correlates with the elevation in vaginal pH (Table 3, Figure 3). As previously stated, in the normal physiological condition, the pH of the vagina is maintained at 3.8 to 4.5 [18]. This is due to the presence of the *Lactobacillus* species which metabolize the glycogen derived sugars into lactic acid. In the case of vaginal pH 5.6 ± 0.5 , the increased pH is due to the infection (19, 20). In most cases, the vaginal pH environment that is ideal for the pathogenic *Gardnerella vaginalis* is usually above 4.5 [19, 20]. This infection is a consequence of the absence of *Lactobacillus* species of the acid-producing type and corroborates the proliferation of *Lactobacillus* species [17-20].

Healthy and infected women also presented remarkable changes at the species level. *Lactobacillus crispatus* dominated in the healthy participants (Table 4, Figure 4), while the most frequent species in the infected women was *Lactobacillus iners* (Table 5, Figure 5). This difference is of some biological importance. *Lactobacillus crispatus* is one of the most protective vaginal species, associated with strong lactic acid production, and the secretion of hydrogen peroxide and bacteriocins, as well as increased epithelial adhesive capacity, and is also associated with increased colonization resistance to invading microorganisms [21-25].

In comparison, *L. iners* has been considered to have dual behavior, as it can remain in both healthy and dysbiotic environments, but its persistence is associated with a decreased quantity of antimicrobial metabolites, and it may not provide comparable protective effects. Some genomic studies indicate that *L. iners* may have a smaller genome and limited biosynthetic potential than *L. crispatus*, which would account for its decreased vaginal defense functionality. The dominance of *L. crispatus* in healthy women and the domination of *L. iners* in infected women reflects not only the quantitative decline of *Lactobacillus* but also the qualitative alteration in the microbial ecosystem [26, 27].

The presence of pathogenic bacteria in infected women is statistically significant (Table 6, Figure 6) and further confirmation of the dysbiosis model. Of particular interest is the increase in *Gardnerella vaginalis* of 60% ($p < 0.001$) relative to 10% in the control group. *G. vaginalis* is well known to contribute to the pathogenesis of BV and the formation of associated biofilms. *G. vaginalis* can compromise the integrity of the epithelium, produce cytotoxic agents, and promote the formation of polymicrobial biofilms that are resistant to the host's immune system [28, 29].

The increased *E. coli*, *Staphylococcus aureus*, *Candida albicans*, and *Pseudomonas aeruginosa* is further evidence that the loss of *Lactobacillus* associated colonization resistance has been replaced by an expansion of various opportunistic

species. Under normal circumstances, *Lactobacillus* spp. will inhibit adhesion of pathogens via competitive exclusion, co-aggregation and the production of biosurfactants. When the ecological balance is disrupted in this manner, pathogens are able to access and occupy epithelial receptor sites and proliferate [30, 31].

Functional antimicrobial testing demonstrated that inhibitory activities of *Lactobacillus* isolates obtained from healthy women were statistically significant for most evaluated pathogens (Table 7, Figure 7). The largest inhibition zones suggest that the isolates may have produced greater amounts of antimicrobial agents, including lactic acid, hydrogen peroxide, and bacteriocins. This functional superiority underlies the fact that protection is not provided solely based on the numbers of antagonistic bacteria but on metabolic activity and strain-related features. The lesser inhibitory activities among the infected women may reflect changed strain composition, reduced antimicrobial gene expression, or stress conditions leading to reduced metabolism or altered environmental conditions. The inhibition against *Pseudomonas aeruginosa* seems to expose intrinsic resistance mechanisms [32-34], such as efflux pumps and biofilm formation.

Table 8 and Figure 8 depict hydrogen peroxide production among isolates from healthy women (66%) and infected women (30%) and its notable difference in production. The production of hydrogen peroxide by *Lactobacillus* strains is protective, as hydrogen peroxide causes oxidative stress to anaerobic pathogens and increases the killing power of the immune system. Although the production of hydrogen peroxide by *Lactobacillus* strains is an indicator, the exact concentration of hydrogen peroxide in vivo in the vagina is a debate among researchers and is a good indicator of the robustness of a *Lactobacillus* strain. The lower proportion of H₂O₂ producing isolates in infected women probably contributes to their impaired antimicrobial defense and increased survival of pathogens [35-39].

Aside from their microbial activities, *Lactobacillus* spp. also have an immune-modulating effect on the host. They have the ability to induce the synthesis of anti-inflammatory cytokines, modulate the signaling of Toll-like receptors, and improve the integrity of epithelial tight junctions. Hence, the absence of *Lactobacillus* may not only cause an overgrowth of pathogens, but also disturb the local immune system, increase inflammation, and aggravate tissue destruction. This multi-faceted protective role shows the intricate nature of vaginal microbial ecology [40, 41].

The findings of this study corroborate the emerging CSTs concept in microbiome studies, where *L. crispatus*-dominated vaginal microbiota are recognized as healthy, while communities lacking *Lactobacillus* are classified as unhealthy and are related to infections and negative reproductive consequences. These findings also strengthen the evidence based reasoning for probiotic therapies aimed at restoring the dominance of *Lactobacillus*, in particular, those *Lactobacillus* strains that are characterized by strong acid and hydrogen peroxide (H₂O₂) production [38-41].

5. CONCLUSION

This research indicates that vaginal health is positively correlated with presence and functional activity of *Lactobacillus* species. In afflicted individuals, *Lactobacillus* species were significantly deficient, vaginal pH was elevated, and pathogens were present in increased numbers, reinforcing the hypothesis of the dominant role of lactobacilli in microbial homeostasis. Marked species-level diversity was demonstrated by the presence of *L. crispatus* in the majority of healthy females and increased incidence of *L. iners* in unhealthy females, suggesting the presence of protective effects was species-specific. Functionally, the lactobacilli isolates from healthy females demonstrated superior metabolic output of H₂O₂ and increased production of anti-microbial compounds, reinforcing the hypothesis of metabolic competence as primary in driving pathogen suppression. These results strongly confirm that the vaginal *Lactobacillus* community's composition and functional antimicrobial activity are primary factors of vaginal homeostasis and are the basis of subsequent probiotic and microbiome treatments.

6. Ethical Responsibilities of Authors

The author, irrefutably, takes responsibility for the content. Furthermore, the author will not publish the content elsewhere. The author has done no fabrication or manipulation of the content. The author has received no other submissions or no other publications. The author provides the full submission to the ethics standards of the institutional research committee and the 1964 Helsinki Declaration. The author has received ethical clearance from the University of Sumer, Institutional Review Board. The author has received written informed consent from the participants.

7. Disclosure and Conflict of Interest:

Conflict of Interest: The authors declare no competing interests with respect to this research.

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