Article presented at 1st Int Conf Med Sci of North Technical University /Kirkuk, Iraq (1st ICMS 2023)

Original Article

Docking of Vaginal *Lactobacillus* isolates as a Potential Bactericidal Agent in Kirkuk 2022

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Abstract

Background: Because the female genital tract is a complex microbial community that plays various roles in disease development. Alternatively, *Lactobacilli* in the vaginal environment play an important role in maintaining a healthy vaginal ecosystem by preventing pathogenic overgrowth. **Objective:** Therefore, this study was suggested to isolate vaginal *Lactobacilli* with antibacterial activity. **Materials and Methods:** Two hundred and sixty women participated in this study aged between 15 and 50 years divided into two cohorts of bacterial vaginosis (BV): infected and healthy individuals. BV-isolates isolated and identified according to microbiological and biochemical assays. Accordingly, *Lactobacillus* isolates were further identified through genomic DNA sequencing of the 16S rRNA, and the results were statistically analyzed. **Results:** A significant increase ($P < 0.05$) in the BV-infected women in the age range of 21–30 and 31–40 was recorded, with the percentages of 40.62% and 31.88%, respectively. Alternatively, a significant decrease ($P > 0.05$) in the percentages of vaginal *Lactobacillus* species between women of Cohort 1 and Cohort 2 recorded the ratios of 3.125% and 31% validating *Lactobacilli* presence as a sign of a healthy vaginal environment. Accordingly, four *Lactobacillus* isolates were assigned for their broad spectrum of antibacterial activity against several pathogenic bacteria. Moreover, results of the partial DNA sequencing revealed the isolation of four *Limosilactobacillus fermentum* strains registered at the National Center for Biotechnology Information (NCBI) under specific gene bank accession numbers. **Conclusion:** Effective *Lactobacillus* species with potential bactericidal activity validates vaginal health.

Keywords: 16S rRNA sequencing, bacterial vaginosis, bactericidal activity, *Limosilactobacillus fermentum*

Introduction

Bacterial strains are known to play critical immunological roles on all mucosal surfaces, including the female reproductive tract.[1] Therefore, maintaining vaginal health is critical in protecting women from adverse urogenital and reproductive health outcomes.[2] Thus, the principle of many investigators aimed to isolate healthy vaginal microbiota that defends the host against pathogenic colonization.[3-5] Interestingly, it was found that *Lactobacillus* species dominate the healthy vaginal microbiota, where these bacteria form a critical line of defense against pathogenic bacteria.[6-8] They are Gram's-positive rods, ovoid with no endosporestested negative for oxidase, gelatin, casein hydrolysis, catalase, nitrate reduction, hydrogen sulfide, and Voges Proskauer.[9,10] In addition, *Lactobacilli* stabilize the vaginal ecosystem by maintaining the normal vaginal pH around 3.8–4.2 by producing lactic acid, which prevents the overgrowth of unwanted microorganisms such as pathogenic bacteria and *Candida*.[11] Thus, any change in the ecology of the vaginal delicate system can easily harm the tissues and lead to severe infections, especially during the reproductive phase of a woman's life.[12,13]

Bacterial vaginosis (BV), which affects 70% of reproductive-age women at some point in their lives, is one of the most common infections during this stage.[14-16] Many BV

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Quick Response Code:

Website: https://journals.lww.com/mjby

DOI: 10.4103/MJBL.MJBL_179_22

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Submission: 16-Aug-2022 **Accepted:** 02-Sep-2022 **Published:** 29-Mar-2024

How to cite this article: Thaker HM, Tawfeeq AA, Ali AA. Docking of vaginal *Lactobacillus* isolates as a potential bactericidal agent in Kirkuk 2022. Med J Babylon 2023;20:S136-40.

cases are asymptomatic or present with only malodorous vaginal discharge and no inflammatory symptoms, increasing the risk of sexually transmitted disease, human immunodeficiency virus (HIV), and a variety of other negative reproductive and pregnancy outcomes.[17-20]

Therefore, this research was suggested to isolate and identify *Lactobacillus* strains with bactericidal activity in the vagina.

Materials and Methods

Study cohorts

A total of 260 women participated in this study in the age range of 15–50 years for the period from December 2021 to April 2022 after signing a consent to participate. They were divided into two groups according to their health index:

Cohort 1

It involved 160 women attendees of the maternity, gynecological, and children's hospital in Al-Nasr district/ Kirkuk /Iraq diagnosed with BV.

Cohort 2

It included 100 asymptomatic healthy women referred by gynecologists who had a clear history of vaginitis and urinary tract infections and met the criteria outlined in the procedure described in Happel *et al*.[2]. They were not receiving oral or local antimicrobial therapy within a month during sample collection.

Sampling collection

High vaginal swabs were collected according to the procedure cited in Parolin *et al.*[7] by inserting a sterile unlubricated speculum into the vagina and specimens were collected from the lateral vaginal wall and posterior fornix using two sterile cotton swabs. Swabs were carefully removed to avoid contamination with the microflora of the vulva and introits and coded for direct examination of pH and bacterial isolation.

Sample processing

Identification of bacterial vaginosis samples

A total of 160 vaginal swabs were directly inoculated onto blood agar, MacConkey agar/Himedia/India, and incubated at 37°C for 24–72 h for macroscopic, biochemical, and antibiotic susceptibility tests according to the procedure mentioned in Al-Wandawy *et al.*[20]. Stock cultures were inoculated into a slant of nutrient agar/ Himedia/India, and kept in the refrigerator at a temperature of 4ºC as mentioned in Mohamed and Thwani[21].

Identification of vaginal Lactobacillus samples

For each 106 vaginal swab, a plate of De Man, Rogosa, Sharpe (MRS/Himedia/India) solid medium was prepared according to Al-Mayah *et al.*[22] and Tawfeeq and Abbas.[23] Then, cultivated plates were incubated in aerobic conditions at 37°C for 48 h a CO_2 incubator. In addition, a single colony from each pure *Lactobacillus* culture was selected for the morphological and biochemical identification tests as cited in Fahem *et al.*[9].

Confirmation of *Lactobacillus* species by 16S rRNA partial gene sequence

Genomic DNA was isolated from *Lactobacillus* pure cultures according to the protocol cited in Fahem *et al.*[9] following manufacturer's instruction (Presto Mini gDNA Kit of Geneaid/Taiwan); then DNA quantity of the DNA was determined by Quantus Fluorometer (Promega/ USA). After that, the gene sequence was amplified with a thermal cycler (Thermo Fisher Scientific) using one set of bacterial universal primers (Macrogen, Korea) with the sequences of 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) and products were analyzed with electrophoresis (OWL Electrophoresis System/Thermo Fisher Scientific) by running the samples on 1.5% agarose gel (Promega).

Following that, the amplification products were Sanger sequenced at Macrogen/Korea, and the 16S rRNA sequences were aligned to National Centre for Biotechnology Information (NCBI) database sequences using the Basic Local Alignment Search Tool (BLAST) algorithm, with the highest percentage identity match of over 99% being chosen as the species identity, followed by NCBI registration of the isolates using the methodology cited in Fahem *et al.*[9]

Statistical analysis

Results were statistically analyzed using the mini-tab statistical program and the *t* test and *F*-test. The arithmetic means were compared according to the Duncan test at the probability level of 0.05.

Ethical approval

The research was carried out in accordance with the ethical principles outlined in the Helsinki Declaration. Before taking the sample, the patient's verbal and analytical approval were obtained. To obtain this approval, a local ethics committee reviewed and approved the study protocol, subject information, and consent form using document number 3657 (including the number and dated November 11, 2021).

Results

This study aimed to identify vaginal *Lactobacillus* species with bactericidal activity against BV in Kirkuk during 2021–2022. Consequently, two hundred and sixty females were included in this study which involved women with BV- Cohort 1, as shown in Table 1, whereas Cohort 2

Table 1: Age distribution of bacterial vaginosis (BV) among Cohort 1 women during the study period

Cohort 1 age groups	Number of vaginal swabs samples	Percentage of BV-infected women
15–20	23	14.38
21–30	65	40.62
31–40	51	31.88
41–50	21	13.12
Total	160	100%

Table 2: Number of *Lactobacillus* species among Cohort 1 and 2 participants of the study

Study age group	Cohort 1 Number of vaginal swabs	No. of Lb. isolates/age group	Cohort 2 Number of vaginal swabs	No. of Lb isolates/age group
15–20	23	2	25	11
21–30	65	1	25	5
31–40	51	1	25	7
41–50	21	1	25	8
Total	160	5	100	31

members were considered as the contributors of vaginal *Lactobacillus* samples.

Results of Table 2 showed a significant decrease ($P > 0.05$) in the percentages of vaginal *Lactobacillus* species between women of Cohorts 1 and 2 recording the ratios of 3.125% and 31%, respectively.

Discussion

BV is one of the most common causes of reproductive tract infections that reduces the immunity and makes the vaginal tract a major focus of pathogens.[1] Untreated infections place a great burden on the health of the infected woman and probably her partner, especially in the asymptomatic cases.[18,19,24] Still, treatment with antibiotics is not a great solution due to its influence on the normal flora, vaginal tissues, and the inability to consume during pregnancy.[24,25] On the contrary, vaginal *Lactobacillus* species is associated with immune quiescence and protection against BV-related pathogens.[26-28]

Careful examination of this [Table 1] showed a significant increase ($P < 0.05$) in the BV-infected women in the age range of 21–30 and 31–40 recording the percentages of 40.62% and 31.88%, respectively. However, BV was lower at younger ages and in women older than 40s. These findings agreed with the findings of Ranjit *et al.*,[16] where they showed that the 30–40 year age group had the highest number of BV cases (8.8%), whereas the 10–20 and 50–60 year age groups had the lowest number of BV cases (1.3%).The same was documented by Joyisa *et al.*,[19] and Al-Wandawy *et al.*,[20] suggesting that BV was age-related infection.

Nevertheless, the results of Table 1 showed a significant increase ($P < 0.05$) in the BV incidence rate higher than the results of Al-Wandawy *et al.* in Kirkuk in 2020 where they displayed a percentage of 17.28%–24.69% at the same age range and at the same city signifying an elevation in the BV-cases within two years.

Yet, for many years, BV has received little attention, as it is considered to be a trivial disease.[18-24] But it started to gain more interest lately as this infection started to increase the risk of acquiring human immunodeficiency virus[18] and other sexually transmitted infections (STI), such as gonorrhea, trichomoniasis, and herpes simplex virus type 2 (HSV-2),[24] as well as miscarriage, preterm labor and wound infections in pregnant women.[19,29,30] It also raises HIV viral shedding.[1]

Meanwhile, one of the most important factors leading to the increase of the BV- incidence rate is the reduction of *Lactobacilli*,[8,13,28] which might explain the significant increase of BV rate in this study than the results of Al-Wandawy *et al.*,[20] and is confirmed by the results displayed in Table 2. Therefore, the presence of *Lactobacilli* is regarded as a sign of a healthy vaginal environment due to their ability to inhibit opportunistic vaginal microorganisms.[26-28]

However, it was also noticed in Table 2 that the number of vaginal *Lactobacillus* was lowered as well in Cohort 2 vaginal samples where a significant worldwide decrease in the vaginal *Lactobacillus* strains was confirmed by the latest researches.[29,31,32] This reduction was probably due to viral infections and antimicrobial abuse which alters the vaginal microbiome by downregulating host mucosal innate peptides normally used by *Lactobacilli* as amino-acid sources.[32]

Still, not all *Lactobacillus* strains express antimicrobial properties with the same intensity; on the contrary, there are substantial differences among species and among strains from a single species.[29] Therefore, the antibacterial activity of the isolates was tested against a number of vaginosis bacterial isolates and other common bacteria where obtained results exposed four strains *Lactobacillus* with a potential bactericidal activity as clarified in Figure 1.

Accordingly, the species of the four *Lactobacillus* isolates were subjected to confirmation by 16S rRNA partial gene sequence (PGS) as shown in Figure 2.

Results of the partial DNA sequencing revealed the isolation of four *Limosilactobacillus fermentum* strains with potential bactericidal activity against a number of pathogenic bacteria which were mainly isolated from the healthy women of Cohort 2. However, the *Lactobacillus* isolates of Cohort 1 women (BV-participants) were mostly mixed cultures difficult to purify with very poor antibacterial activity [Table 3]. This result however show

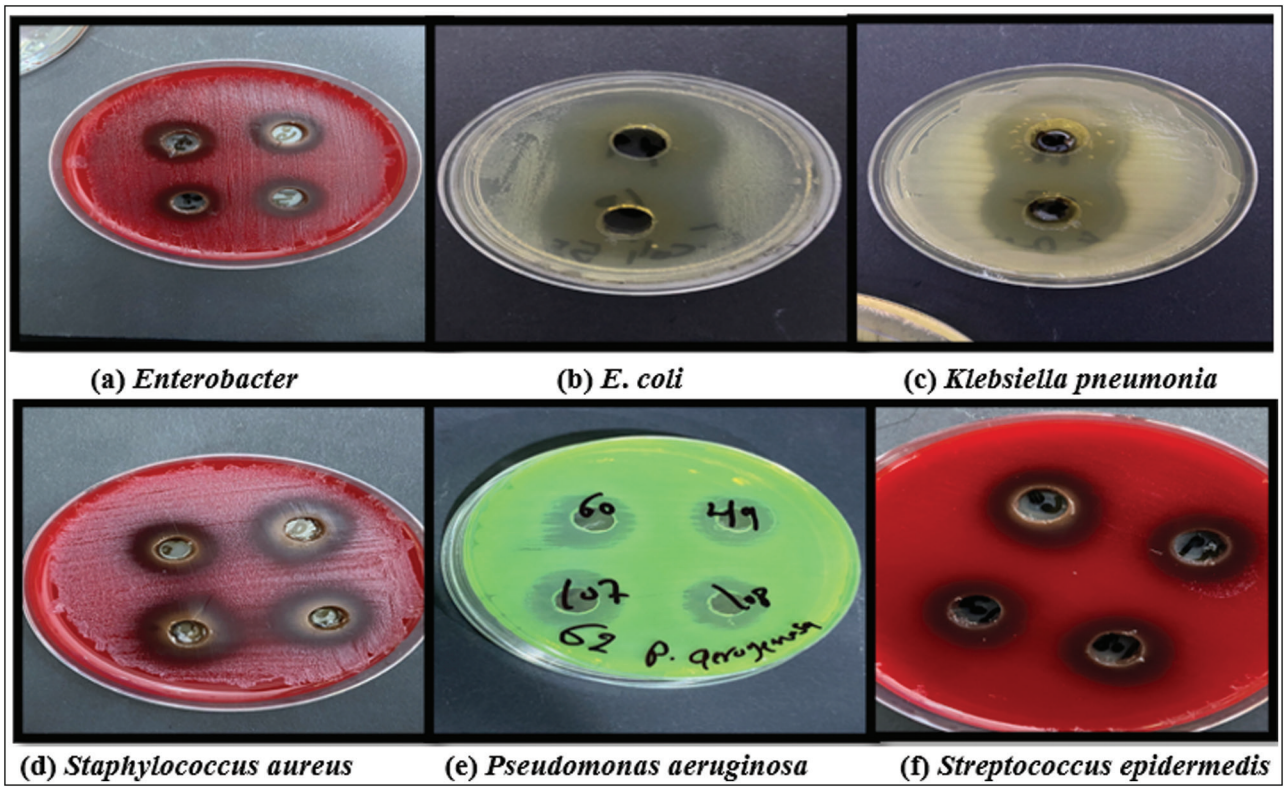

Figure 1: Antibacterial activity of Cohort 2 vaginal *Lactobacillus* species isolated through the study

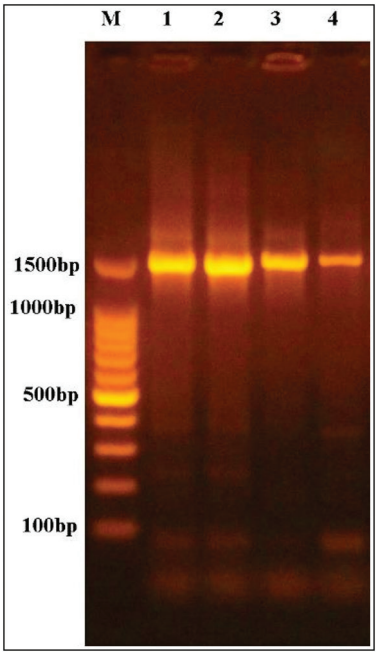

Figure 2: Gel electrophoresis image of the amplification of 16S rRNA gene of four *Lactobacillus* species isolated in the study fractionated on 1.5% agarose gel electrophoresis stained with ethidium bromide, where Lane M resembles (100 bp marker) and Lanes 1–4 resemble *Lactobacillus* isolates of the study

an inverse relationship between presence of *Lactobacilli* and the bacteria that causes vaginities and agreed with the findings obtained by several researchers who found that *Lactobacilli* constitute an important part of the urogenital tract microbiota, and these indigenous bacteria considered as a natural resistant factor against potential pathogenic microorganisms.[29-32] On the contrary, the uniqueness of *Limosilactobacillus fermentum* strain was mentioned by many recent investigators as an elevator of protection against BV,[33] anti-inflammatory lowering oxidative stress in vaginal tissues[34] with antiobesity and cholesterol-lowering effects.[35-37]

Table 3: Species and accession number of the vaginal *Lactobacillus* isolates in the National Library of Medicine/ National Center of Biotechnology Information (NCBI)/USA

Lactobacilius isolate no. in the study cohort	Lactobacillus isolate definition by the NCBI	Lactobacillus isolate accession number in the NCBI	Gene length /bp
Cohort 2-15/6	*Limosilactobacillus fermentum IQ/NTU-1*	Gen Bank: ON682345.1	1370
Cohort 2-30/2	*Limosilactobacillus fermentum HA21*	Gen Bank: ON682345.1	1370
Cohort 2-40/1	*Limosilactobacillus fermentum HA22*	Gen Bank: ON682345.1	1370
Cohort 2-45/3	*Limosilactobacillus fermentum*	Gen Bank: ON682345.1	1370

Conclusion

Effective *Lactobacillus* species with potential bactericidal activity validates vaginal health.

Financial support and sponsorship

Not applicable.

Conflicts of interest

There are no conflicts of interest.

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