Egyptian Journal of Medical Microbiology Volume 34 / No.3 / July 2025 99-107 Online ISSN: 2537-0979

ORIGINAL ARTICLE

Identification of *Lactobacillus* Species from Vaginal Secretions of Healthy and Women with Vaginitis by Molecular Methods

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Key words:
Lactobacillus, Microbiome, vaginal secretions, Multiplex Polymerase Chain Reaction, vaginitis

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ABSTRACT

Background*: The Lactobacillus species bear a high responsibility toward vaginal microbiota and, accordingly, play a very important role in the maintenance of vaginal health. In healthy women, Lactobacillus bacteria keep the vagina in an acid environment and helps in maintaining the microbiota balance.* ***Objective: To identify and compare Lactobacillus species*** *in vaginal secretions of healthy women and those with vaginitis, by using molecular techniques. M****ethodology****: The study was conducted in College of Medicine laboratories- University of Anbar and Private Specialized laboratories from November 2023 until October-2024. 234 specimens were collected from women with expected vaginitis, their ages ranged from 20-50 years they were selected from Al-Ramadi Teaching Hospital for maternity and pediatrics in Ramadi, Western Iraq. Genomic DNA extraction from vaginal secretions was performed using Presto™ Mini gDNA Bacteria Kit. The Nano drop instrument was used to calculate DNA purity and concentration. Multiplex Polymerase chain reaction (PCR) was carried out using Multiplex-PCR primer design for ten of Lactobacillus species. All Multiplex-PCR reactions were done in Applied Bio-system 2720 thermo cyclers, and amplification of target gene.* ***Results:*** *The results of sampling show that 148/234 of specimens were identified a vaginitis positive while 86/234 were negative. The molecular screening findings by Multiplex-PCR indicated that five species were identified as L. jensenii, L. acidophilusL. crispatus,L. iners, L. gasseri as common species in the vaginal microbiome.* ***Conclusion:*** *The analysis results show that the more frequent species in women case was L. crispatus comparing to the control group with p-value was 0.001, they were present in 26 samples of patients versus 11 samples of controls. The consistent presence of Lactobacillus iners across different types of vaginitis is a notable finding*

INTRODUCTION

Lactobacillus is a member of the Lactobacillaceae family, with 170 species and 17 subspecies[1]. The vaginal microflora of healthy women typically consists of a diverse array of aerobic and anaerobic microorganisms[2]. In the normal flora, lactobacilli species, referred to as Doderlein's bacillus, are prominent and exert a substantial influence on vaginal microbiota [3]. The release of organic acids by lactobacilli sustains a vaginal pH below 4.5, so establishing an unfavorable habitat for infections[4] .Besides acid generation, the amalgamation of hydrogen peroxide (H2O2) and bacteriocins inhibits the native pathogenic flora and preserves the local balance [5-7]. Generally, the indigenous microbiota in the vaginal environment is believed to maintain a symbiotic interaction with the host fungi, particularly *Candida* species, would join the mucosal layer of the vagina as commensals and participate in an important way in the complex vaginal ecosystem that is host to the variety of bacteria[7]. These differences in composition between microbiota and mycobiota in the reproductive-age female might be assumed to modulate temporal shifts in the community dynamics of the vagina [8]. Actually, this variability depends on hormonal changes, age, sexual behavior, and also the practice of antimicrobial therapy. Vaginal microbial dysbiosis leads to the overgrowth of opportunistic pathogens, thereby promoting disease initiation[9,10].

Vaginitis is a common condition that causes inflammation or infection of the vagina. Symptoms can include discharge, itching, irritation, pain, or changes in the vaginal environment[11]. Women of all ages can be affected, and the causes vary, including infections, hormonal changes, or allergic reactions[12]. The predisposing conditions include menstruation, pregnancy, sexual relations, vaginal douching, and the indiscriminate use of antibiotics, which can alter the microbial flora rapidly[5]. Disturbance in the vaginal microbiome is characterized by depletion of Lactobacillus species and overgrowth of non-Lactobacillus organisms. Overgrowth of anaerobic bacteria generally results in abnormal conditions such as bacterial vaginosis, sexually transmitted infections, and pregnancy-related problems[7].

A well-established symbiotic relationship exists between reproductive-age women and the *Lactobacillus* species inhabiting their vaginas, where Estrogen stimulates vaginal epithelial cells to produce glycogen, which serves as a food source for these beneficial bacteria. In turn, *Lactobacillus* bacteria ferment glycogen, producing lactic acid that lowers the vaginal pH, this acidic environment inhibits the growth of harmful microorganisms, maintaining a healthy balance within the vaginal ecosystem[13]. When women are not dominated by lactobacilli, it has been shown that they have increased susceptibility to many genital infections, including *Chlamydia trachomatis*, herpes and HIV [12]. The healthy vaginal microbiome is primarily composed of several Lactobacillus species, including *L. crispatus*, *L. jensenii*, *L. gasseri*, and *L. iners*. Each of these species possesses unique defense mechanisms. Different *Lactobacillus* species and strains demonstrate probiotic properties through various mechanisms. These include outcompeting other bacteria and providing protection against reproductive tract pathogens like *Chlamydia trachomatis*[14]. Vaginal pH is lowered through the fermentation of glucose, a process where glycogen breakdown products are converted into lactic acid under anaerobic conditions [15].

Traditional methods for detecting microbial imbalances, such as culture-based techniques, are limited by their time-consuming nature and inability to differentiate closely related bacterial species accurately[16]. Molecular detection methods, particularly those utilizing polymerase chain reaction (PCR), have become a powerful tool for identifying and quantifying microbial species with high specificity and sensitivity[17]. Molecular approaches, including 16S rRNA gene sequencing, metagenome sequencing, and denaturing gradient gel electrophoresis (DGGE), have been extensively utilised for the reliable and quick identification of bacterial species. 16S rRNA sequencing is frequently employed for the identification of bacteria, including Lactobacillus species, The 16S rRNA gene sequences of numerous *Lactobacillus* species exhibit excessive similarity, hindering their differentiation [18,19].

One study in Iraq reported according to Molecular Sequences alignment and in silico diagnosis using BLAST indicated that *Lactobacillus crispatus* comprised the majority of species type of *Lactobacillus* in agnail micro-biome followed by *L. gasseri*, *L. jensenii*, *L. acidophilus*. It was noticed that L. *crispatus* and *L. gasseri* showed a little variation which is expected due to inter individual and environment variation [20, 21]. On the other hand, a study published in 2010 reported the presence of eight major clusters of *lactobacillus* species in Tanzanian women[22,23]. Also, a study that investigated of common type of vaginal flora indicated that presence of four vaginal grades (*L. crispatus* is the most dominant *Lactobacillus* species followed by *L. jensenii*, *L. iners* and *L. gasseri* [24].

Multiplex PCR offers several benefits in the classification of *Lactobacillus* in vaginal samples. This advanced molecular technique allows for the simultaneous amplification of multiple target genes in a single reaction[18], such simultaneous detection of multiple species where Multiplex PCR enables the identification of several *Lactobacillus* species in a single assay and this way is considered a faster turnaround time compared to running separate PCR reactions for each species [25]. Also this technique aid in comprehensive microbiome profiling such Assessment of community composition whereas provides insights into the relative presence of various *Lactobacillus* species, aiding in understanding their role in vaginal health, and have the ability to detect shifts in *Lactobacillus* species dominance (e.g., replacement of *L. crispatus* by *L. iners*) can help predict conditions like bacterial vaginosis or the efficacy of probiotic interventions [26].

The current study depending to one step reaction of PCR (Multiplex-PCR) for 10 gene targeting as housekeeping gene to most common bacterial species type of vaginal environment to identify and compare *Lactobacillus* species in vaginal secretions of healthy women and those with vaginitis.

METHODOLOGY

Study design and Samples collection:

This work is a case control study which focuses on the molecular detection of *Lactobacillus* species in vaginal secretions from healthy women and those diagnosed with vaginitis. Understanding the variations in *Lactobacillus* species composition between these two groups can provide insights into the role these bacteria play in vaginal health and how their disruption may contribute to infections.

Seventy infected women with vaginitis were involved in this study, their ages range from 20-45 years they were selected from Al-Ramadi Teaching Hospital for Maternity and Pediatrics, they were selected by gynecology physicians regarding to the symptoms and laboratory diagnosis. Seventy healthy women without urinary and vaginal infections were also involved in this study as a control group. Vaginal swabs were taken from vaginal walls of healthy and infected women, the swabs were used for culture on MRS agar. Identification of *Lactobacilli* were done according microscopic examination and biochemical tests.

Molecular Identification of *Lactobacilli*:

DNA Extraction:

Genomic DNA extraction of the bacteria from human vaginal samples secretion was performed by using Presto™ Mini gDNA Bacteria Kit.

Calculating the Purity and Concentration of DNA

The Nano drop instrument was used to calculate DNA purity and concentration. Purity was assessed by the ratio of sample absorbance at 260 and 280 nanometers, and one microliter of each DNA sample was applied.

Primer Design and Preparation of primers solution

Multiplex Polymerase chain reaction (PCR) was carried out using Multiplex-PCR primer design for ten of *Lactobacillus* species-specific genes target as listed in Table(1). The lyophilized primers were dissolved in deionized distilled water (DDH2O) in the master tube to achieve 100 pmol/µl, and then 10 pmol/ µl was created as a working solution by transferring 10µl from the master tube to another tube and completing the volume to 100µl by adding DDH2O. Then saved at-20ºC.

Molecular Screening for *Lactobacillus* species

All Multiplex-PCR reactions were done in Applied Bio-system 2720 thermo cyclers, and amplification of target gene. Twenty five microliter of Multiplex-PCR amplification reaction contained 12.5 µl from OneTaq (NEB®) mastermix, 3 µl of DNA sample, 1.5µl 10 pmol/µl from each primer and 6.5 µl of free-nuclease water.

Table 1: Primer Sequences of *Lactobacillus* species and their size Gene

Bacteria Sp.	Primer	Oligonucleotide sequence (5'-3')	Tm(ºC)	Target gene	Expected size (bp)
L. jensenii	LjensF	**TGCTACAAAACCTGGTTCCAG**	58	*gapR*[a]	967
	LjensR	**AGCCATGTTTGACTCGGTGC**	61		
L. fermentum	LfermF	**TAGGTGGTGGTGGTCACAGT**	60	*mraW*[b]	814
	LfermR	**GCCCGGTGGTTAACCTTCAA**	61		
L. acidophilus	LacidF	**CGTGATAGGGCCATTTGTGC**	60	*htpX*[c]	646
	LacidR	**ACAAGCGTAAAACTGCGCTA**	59		
L. crispatus	LcrisF	**TGTTAGTAATCACCTTCGCGCTA**	60	*cbiQ1*[d]	474
	LcrisR	**TTTGCCCTTCGACATAGCCA**	60		
L. reuteri	LreutF	**CCATGCAGTCACAACAACCT**	59	*xerS*[e]	386
	LreutR	**GTCCCCGGTACATGTGTGAA**	60		
L. iners	LinerF	**CGTCACTCAATCATCGACCAGAA**	61	*folE*[f]	315
	LinerR	**GCCGTGCTTTAATAGCAACTGCT**	62		
L. casei	LcaseF	**TGGCATTGTCGGCTTAAATGG**	60	*atu*[g]	268
	LcaseR	**TGATGTTAAATGCTCAACCCGC**	60		
L. gasseri	LgassF	**CAACGGAACCCCGCGACTATGC**	66	*murQ*[h]	219
	LgassR	**GGCAGCATCTAAAACGCCAAGAC**	63		
L. plantarum	LplanF	**CGAGACAGCAATTCCTGCACTCG**	64	*apbE2*[i]	176
	LplanR	**CCTCAGAAACAGTCCGGTTGAC**	61		
L. rhamnosus	LrhamF	**ATTTAACCGCAAGTGGCAGC**	60	*aes*[j]	124
	LrhamR	AAATTGTGTGAACCGGCGTA	58		

Ethical Approval

The study was approved by Ethics Committee of the Al Anbar Medical Research University (approval number 21, January 4, 2024). All research participants supplied written informed permission.

Statistical Analysis:

All statistical analyses were performed using SPSS, version 26.0 (SPSS Inc., NY, USA).

RESULTS

The results of sampling show that 140/234 of specimens identify a vaginitis positive while 94/234 were negative (Figre1), depending on *Lactobacillus* colonies on MRS agar the *Lactobacillus* colony was typically small, round, and creamy white (Figure2). Gram staining reveals *Lactobacilli* as Gram-positive rods, they are long, slender, and sometimes slightly curved, distinguishing them from other vaginal flora like Gram-negative anaerobes or cocci.

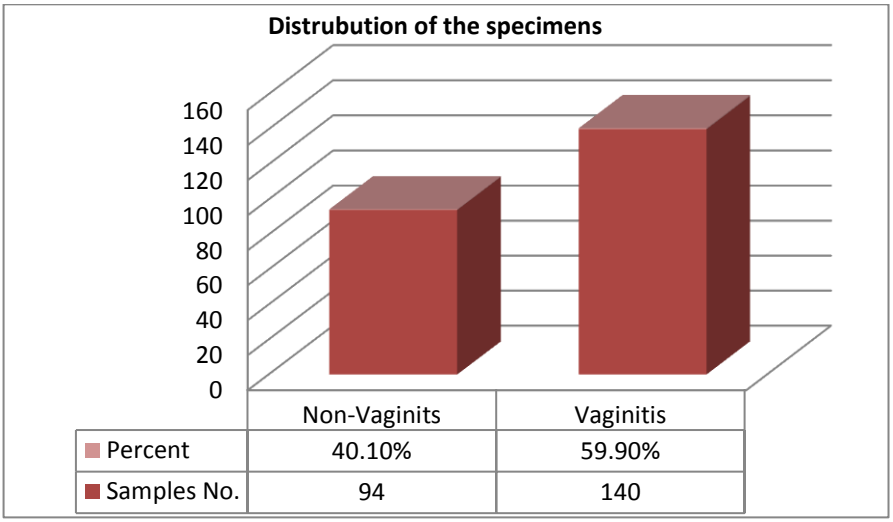

Fig. 1: Distribution of Cases with Vaginitis and Non- Vaginitis samples

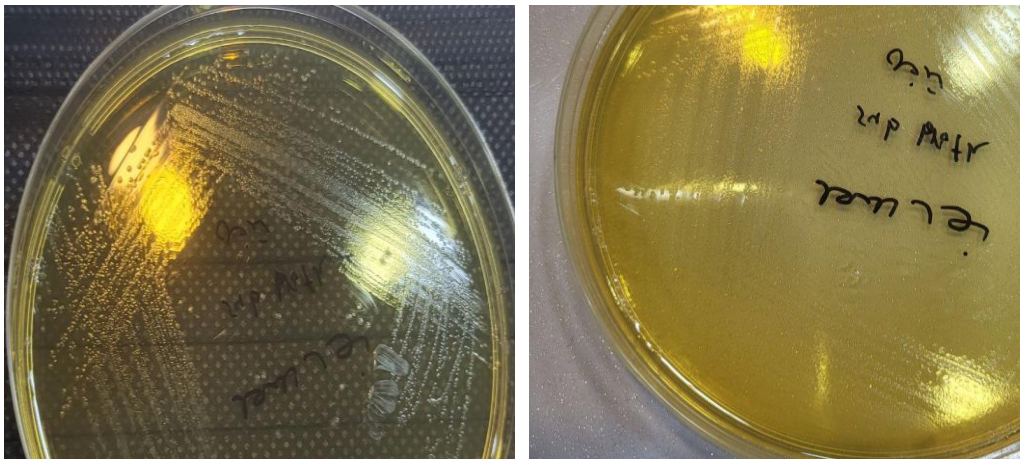

Fig. 2: Morphology culture of Lactobacillus sp. In MRS agar

The frequency of vaginitis cases were 140 positive samples were classified into four types as shown as shown in (Table 2).

Table 2: Frequency of Vaginitis Cases

Type of Vaginitis	Frequency	Percent	Chi- squre	P- value
Bacterial vaginosis	58	41.4	52.857	0.0001
Candidiasis	72	51.4		
Trichomoniasis	4	2.9		
Idiotype	6	4.3		
Total	140	100.0		

Molecular Detection

The nucleic acid (DNA) of bacterial was submitted to genomic extraction from the vaginal secretion samples. The purity and concentration of extracted DNA was measured by using Nano Drop device and the results ranged among (20.6 -134.4) ng/µl as a concentration while the purity ranged from (1.68 to 2.0). Extracted DNA was confirmed and analyzed by gel electrophoresis to ensure the presence of target DNA.

The molecular screening results by Multiplex-PCR as shown in (Figure 3) and presented in (Table 3) indicate that the presence of only five species of *lactobacilli*, the identified species were; *L. jensenii, L. acidophilusL. crispatus,L. iners, L. gasseri*.

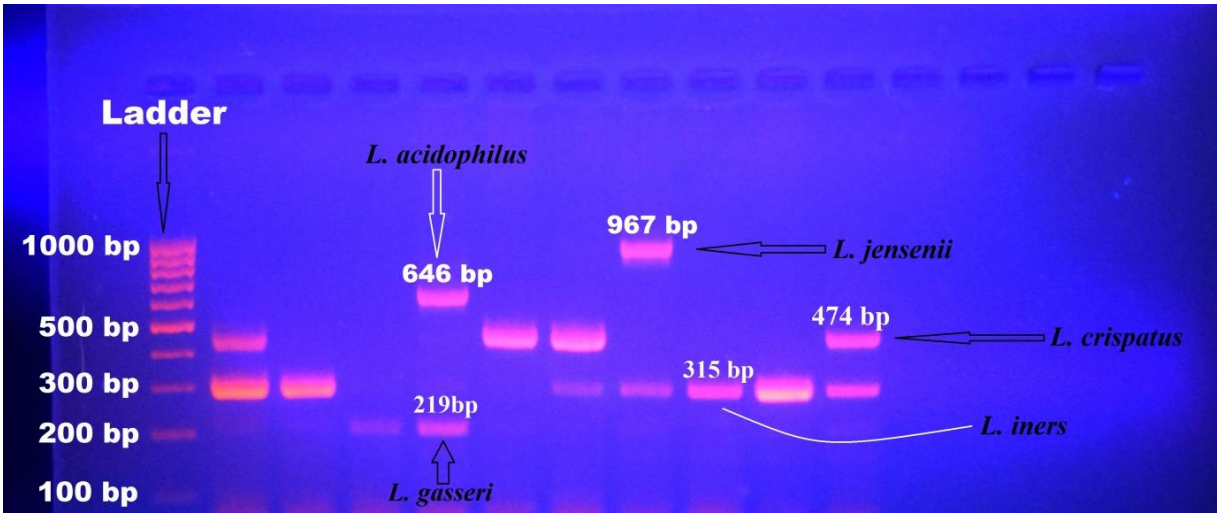

Fig. 3: Multiplex-PCR amplification fragments for the detection of *Lactobacillus* species (1.5%agarose, 5 V/cm2for 90min).

Table 3: Distribution of Lactobacillus species between patients and control groups

Factor			Data		Total	X^2	*P value*
			Positive	Negative			
L. jensenii	group	Patient	5	31	36	1.66	0.206 N.S
		control	8	28	36		
	Total		13	59	72		
L. acidophilus	group	Patient	0	36	36	1.01	0.314 N.S
		control	1	35	36		
	Total		1	71	72		
L. crispatus	group	Patient	26	10	36	12.51	0.001 S.
		control	11	25	36		
	Total		37	35	72		
L. iners	group	Patient	28	8	36	2.35	0.125 N.S
		control	22	14	36		
	Total		50	22	72		
L. gasseri	group	Patient	11	25	36	0.067	0.796 N.S
		control	10	26	36		
	Total		21	51	72		

The results showed that there was a non-significant variation in the distribution and frequency of the species of *Lactobacillus*. There was a complete absence of *L. acidophilus* in all cases of vaginitis form all age groups, other species like *L. jensenii and L. gasseri* showed reduction in frequency within patient with bacterial vaginosis and candidiasis (3.44% and 10.34%) as shown in (Table-4).

Table 4: Frequency of *Lactobacillus* spp according types of vaginitis with correlation between Type of vaginitis and Lactobacillus Spp.

Type of vaginitis	lactobacillus spp	No	%	X^2	p-value
Bacterial vaginosis 29	*L. jensenii*	1	3.44	14.920	0.093
	L. acidophilus	0	0		
	L. crispatus	6	20.68		
	L. iners	8	27.58		
	L. gasseri	3	10.34		
Candidiasis 36	*L. jensenii*	1	2.77		
	L. acidophilus	0			
	L. crispatus	15	41.66		
	L. iners	14	48.27		
	L. gasseri	4	11.11		
Trichomoniasis 2	*L. jensenii*	2	100		
	L. acidophilus	0			
	L. crispatus	2	100		
	L. iners	2	100		
	L. gasseri	2	100		
Idiotype 3	*L. jensenii*	1	25		
	L. acidophilus	0			
	L. crispatus	3	75		
	L. iners	4	100		
	L. gasseri	2	66.66		

DISCUSSION

In recent years, the role of *Lactobacillus* species in maintaining vaginal health has garnered significant attention, particularly in the context of vaginitis. Microscopic detection of vaginal secretions is a valuable diagnostic tool for assessing vaginal health and identifying infections, so the results indicated that 59.9% of specimens were positive as a vaginitis depending to microscopic examination. A high percentage of positive specimens of vaginal secretions was Candidiasis as shown in (table 2). The high incidence of positive specimens for Candidiasis is due to several factors: its common occurrence, the vaginal environment's suitability for its growth, and its frequent association with underlying predisposing conditions[27]. Bacterial vaginitis presented with frequency 58 case The high rate of positive BV (Bacterial Vaginosis) diagnoses is a result of several factors: its common occurrence, the relatively simple methods for detecting it, and the numerous factors that increase a woman's susceptibility. The disruption of the healthy balance of bacteria in the vagina and the resulting complications make BV a primary cause of abnormal vaginal discharge.

The molecular screening results indicated that presence of five species of *Lactobacillus* species, such as *L. jensenii*, *L. acidophilus*, *L. crispatus*, *L. iners*, and *L. gasseri*, as common species in the vaginal microbiome. This finding of just five species types under study indicated although that these consider a most common but also, they were specific target for Multiplex-PCR screening, that mean may be presence another species type when insert a more target than 10 species. All the identified species did not show significant differences between patients and control except *L. crispatus* with p-value was 0.001, they were found in 26 samples of patients versus 11 samples of control. The most frequent species in both patients and control was *L. iners*, they were found in 77.1% of patients and 62.8% of control. On the other hand the least species was *L. acidophilus* where they were found only one specimen of control group 2.5% and 0% of patients

The consistent presence of *Lactobacillus iners* across different types of vaginitis is a notable finding, suggesting its distinct behavior within the vaginal ecosystem. Unlike other Lactobacillus species, *L. iners* displays traits that favor its dominance in disrupted microbial communities, which are characteristic of various vaginitis conditions. While the complete absence of *Lactobacillus acidophilus* species in cases of vaginitis is a crucial observation. It signifies an imbalance in the typical vaginal bacterial community, which is essential for preserving vaginal health [28].

The analysis results show that the more frequent species in infected with Iraqi women was *L. crispatus* comparing to control group. This usually bodes well when the balance of *Lactobacillus crispatus* is higher in a patient than in the normal healthy range. This indicates that lactobacilli are in dominance within the vaginal microbiome, usually an indication of good

health within the vagina. Higher-than-normal levels of *L. crispatus* may signify resilience of the microbiome to infections-protective, hard, and not damaging [28].

On the other hand, According to study done by Karolina *et al*., [29] the vaginal microbiomes of Polish women were predominantly colonized by two *Lactobacillus* species: *L. gasseri* (found in 93% of cases) and *L. crispatus* (found in 83% of cases), the study also observed an increase in the abundance of *L. delbrueckii* and a decrease in the abundance of *L. gasseri* within these microbiomes. Similar results were obtained by De Backer *et al*. [30], who examined 71 vaginal swabs collected from Belgian women. They demonstrated that, among four *Lactobacillus* species (*L. crispatus*, *L. iners*, *L. jensenii*, and *L. gasseri*), *L. crispatus* was the most common (detected in 93% of the samples); *L. iners* was detected in 75% of swabs, *L. gasseri* in 73%, and *L. jensenii* in 46%. In a different study, *L. crispatus* was the predominant *Lactobacillus* species in both the samples from healthy women and from the women affected with bacterial vaginosis. Among 96 women in Florida, *L. iners* (55%), *L. crispatus* (29%), *L. gasseri* (13%), and *L. jensenii* (13%) were the predominant species present in the vaginal and cervical swabs (32). In another American study, *L. iners* was the most abundant species in healthy women and in those with bacterial vaginosis [32]. lactobacilli in Korean women were *L. crispatus* [33]. It is also noteworthy that the predominant lactobacilli in Japanese women were found to be *L. crispatus* (52.7%) and *L. gasseri* (20.8%) by DNA-DNA hybridization analysis [34].

The study found that *L. acidophilus* was only detected once in the vaginal microbiome of the control group. This is likely because L. acidophilus is better suited for the intestinal environment. Compared to species like *L. crispatus* and *L. iners*, it is less efficient at colonizing the vagina and producing an acidic pH, which is important for maintaining vaginal health, and current study reported to limited diversity in *Lactobacillus* species in the vaginal microbiome in Iraqi women, as compared to other populations, might be contributed by a combination of genetic, cultural, environmental, and lifestyle factors.

CONCLUSION

Our study reported understanding of vaginal microbiota dynamics, specifically the role of *Lactobacillus* species, to inform more effective clinical strategies for managing and preventing vaginitis. The consistent presence of *Lactobacillus iners* across different types of vaginitis is a notable finding. The study conclude that all of the identified species did not show significant differences between patients and control except *L. crispatus*.

Declarations:
Consent for publication: Not applicable
Availability of data and material: Data are available upon request.
Competing interests: The author(s) declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.
This manuscript has not been previously published and is not under consideration in another journal.
Funding: Authors did not receive any grants from funding agencies.

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