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Research Article

Qualitative and quantitative analysis of vaginal *Lactobacillus* species in relation to polymorphisms in the interleukin 1 gene complex

Behrouz Taheri[1], Mohammad-Hossein Modarressi[2,3], Zahra Farshadzadeh[4], Mojgan Karbakhsh[5], shahnaz barjani[3] and Elahe Motevaseli[*3,6,]

[1]Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran.
[2]Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran.
[3]Department of Molecular Medicine, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran.
[4]Department of Medical Microbiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran.
[5]Department of Community & Preventive Medicine, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran.
[6]Food Microbiology Research Center, Tehran University of Medical Sciences, Tehran, Iran
*Corresponding author: Elahe Motevaseli. E-mail: e_motevaseli@tums.ac.ir. Tel/Fax: +982143052000

ABSTRACT

The *Lactobacillus* species are the prevalent constituents of the healthy vaginal microbiota and protect their host from pathogens. These bacteria belong to probiotics defined as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host. The evaluation of association between host factors and the *Lactobacillus* species might provide useful information for developing criteria to select appropriate species for probiotic therapy. The present study was conducted to examine whether genetic polymorphisms in the interleukin 1 gene complex (*IL-1B-511*, *IL-1B+3954*, and *IL-RN*) are associated with the most common vaginal species of *Lactobacillus*. The detection and quantification of *Lactobacillus* species were performed using species-specific PCR and real-time PCR. Polymorphisms in the *IL-1B-511*, *IL-1B+3954*, and *IL-RN* genes were detected by RFLP-PCR. The prevalence and quantity of *L.iners* and quantity of total lactobacilli were significantly lower in carriers of the allele 2, *IL-1RN**2, compared to non-carrier ($p<0.05$). Association of *IL-RN* gene polymorphisms with *Lactobacillus* species might be useful in designing an optimal probiotic therapy through selection of an appropriate probiotic species of *Lactobacillus* for each woman considering her genotype. The observational findings of present study can be helpful in designing further confirmatory studies.

Key words: Interleukin-1B, interleukin-1 receptor antagonist, *Lactobacillus* species, Polymorphism, probiotic therapy

INTRODUCTION:

The *Lactobacillus* species are the prevalent constituents of the healthy vaginal microbiota and protect the their host from pathogens through several mechanisms including production of lactic acid, hydrogen peroxide and bacteriocins and

competitive inhibition of the adherence of bacterial pathogens to vaginal epithelial cells [1].

Lactobacillus species belong to a group of useful microorganisms which are called probiotics. Probiotics are live microorganisms that, when administered in adequate amounts, give up a health benefit to the host. Probiotic lactobacilli are used in treatment of vaginal infections such as bacterial vaginosis (BV) and candidiasis. A good vaginal colonization after probiotic administration increases effectiveness of treatment [2, 3]. Host factors such as age, skin color, pregnancy and menopausal status can influence the colonization ability of these probiotics [4, 5, 6]. Identification of further such factors could be useful for improving the efficacy of probiotic therapy.

Different compositions of *lactobacillus* species were reported from various regions of the world [4-7, 5-8]. Investigation of association between *lactobacillus* species and different host factors in various populations can identify host factors influencing vaginal colonization. A number of studies have revealed that the capacity of producing antimicrobial factors genetically variable among different individual affects the composition of vaginal flora and the response to pathogens. Polymorphisms in components of the innate immune system can explain variations in the capacity of producing antimicrobial factors. Hence, genetic polymorphisms that influence innate immune recognition or the response to infectious microorganisms could explain the genetic variability to vaginal flora alterations [5, 6] Although the association of polymorphisms in components of the innate immune system with composition of bacterial vaginosis-related flora such as vaginal *Gardnerella vaginalis*, anaerobic Gram-negative rods and *Ureaplasma urealyticum* was demonstrated in several researches [9, 10], no study was done to evaluate association of these polymorphisms with the composition of *Lactobacillus* species in healthy vagina. The IL-1 family including two agonists, IL-1α and IL-1β, a receptor antagonist, IL-1ra (gene symbol IL1RN), and two other receptors, IL-1R (I) and IL-1R (II) are responsible for the regulation of the innate immune system [11].

In standard antibiotic treatments of BV, because of microbial resistance and the destructive effect of antibiotics on the vaginal microbiota, the recurrence rates can be considerably high; therefore, it seems necessary to develop alternative or supplementary treatments such as probiotic therapy [12, 13]. The evaluation of association between polymorphisms in components of innate immune system and *Lactobacillus* species could be useful in developing criteria to select appropriate species for probiotic therapy. However, to date, there are no studies for evaluating prevalence and quantities *Lactobacillus* species in relation to SNPs in components of innate immune system**.** In this context present study was aimed to evaluate prevalence and quantities of *Lactobacillus* species in relation to polymorphisms in the *IL-1B-511, IL-1B+3954*, and *IL-RN* genes in healthy women.

MATERIALS AND METHODS:

Study population

After obtaining informed consent, vaginal samples were obtained from healthy fertile (regular cycles) women aged from 18 to 40 years at Gynecology Outpatient Clinic of Imam Khomeini Hospital affiliated to Tehran University of Medical Sciences in Tehran, Iran. In this study pregnancy, immunosuppression, menses during sample collection, current use of antibiotics or antifungals, and diagnosis of trichomoniasis, abnormal vaginal discharge, BV or intermediate vaginal microbiota according to Nugent scoring were considered as exclusion criteria for the sampling. These healthy women had no vaginal discharge or signs or symptoms of infections and had vaginal Gram-stained smears dominated by lactobacilli.

Sampling

Two vaginal swab samples were collected from each woman. One of them was used to make smear for gram staining procedure and Nugent

criteria and the second swab was diluted with 1ml sterile phosphate buffered saline (PBS) (pH 7.4) and stored at -20 °C until PCR.

DNA extraction

The vaginal samples diluted with PBS and stored at -20 °C were thawed; then centrifuged at 10 000 g for 10 min. The pellets were suspended in 200 ml lysis buffer (25% ultrapure sucrose, 5 mM Tris, 1 mM EDTA, pH 8.0) with 20 mg/ml lysozyme and incubated at 37 °C overnight; then 80 ml SDS (10%) and 5 ml RNase A (10 mg/ ml) (Qiagen) were added and incubated at 60 °C for 1 h and 20 ml Proteinase K was added and DNA Mini kit (Qiagen) was used in according to the instructions of the manufacturer and the DNA extracts were stored at -20 °C until PCR. Spectrophotometer was used for measuring purity and concentration of the isolated DNA.

Species-specific PCR

Lactobacillus species were identified by PCR assay using species-specific primers sets for 14 vaginal species. The PCR mixture consisted of 1× PCR Master Mix (Qiagen), 1 µl genomic DNA template, and 1 µl each of forward and reverse primers in a final volume of 25 µl. The primers, annealing temperatures and amplicon sizes are listed in previous study [7]. The PCR products were visualized after electrophoresis in 1.5% agarose gel and stained with ethidium bromide and visualized under UV light.

Genus- and species-specific real-time PCR

Real-time PCR using genus- and species-specific primer sets was used to quantify each of the five most prevalent *Lactobacillus* species and the total of lactobacilli in each sample. The conditions and the primers for real-time PCR used in present study were same as those used in the previous study [7]. For quantifying each *Lactobacillus* species, one standard curve was developed from the 10-fold serial dilution of genomic DNA extracted from reference strain of same species. The reference strains of *L.iners*(LMG 18914 ^{T}), *L.crispatus* (LMG 9479^{T}), *L.acidophilus* (LMG9433^{T}), *L.gasseri* (LMG 9203^{T}) and *L.jensenni* (LMG 6414^{T}) were used for preparing standard curve for quantifying the five most prevalent *L.iners, L.crispatus, L.acidophilus, L.gasseri* and *L.jensenni* , respectively. The DNA concentration of each reference strain was determined using NanoDrop 2000 (Thermo Scientific). The number of cells in each dilution was calculated according to the genome size of the bacterial species as described in previous study [14]. Amplification and detection of target DNA were carried out using an ABI step one ^{TM} (Applied Biosystems).

Analysis of genes polymorphism.

***IL-1B-511* gene polymorphism**. Identification of substitution of C by T at position -511 in the promoter region of the *IL-1B* gene that abolishes an *AvaI* site was performed by using PCR-RFLP. The primers 5· TGGCAT TGA TCT GGT TCA TC 3· (sense) and 5· GTT TAGGAATCT TCC CAC TT 3· (antisense) were used to amplify this region, according to previous study [15]. After initial denaturation at 94℃ for 5 min, the samples were subjected to 45 cycles of denaturation at 94℃for 1min, 55°C for 1 min and 72°C for 1 min, with a final extension step at 72℃for 7 min. The PCR products were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide. The overnight digestion of PCR product, 305 bp, was performed at 37°C with 0.13 U/ml *AvaI*. The intact and digested, 190 and 114 bp, fragments indicated allele 2 or variant allele and allele 1 or wild-type allele, respectively.

***IL-1B+3954* gene polymorphism**. The polymorphic site within exon 5 of the *IL-1B* gene that abolishes a *TaqI* site was identified by using PCR-RFLP. The primers 5· GTT GTC ATC AGA CTT TGACC 3· (sense) and 5· TTC AGT TCA TAT GGA CCA GA3· (antisense) were used to amplify this region, according to previous study [16]. After initial denaturation at 94℃ for 5 min, the samples were subjected to 45 cycles of denaturation at 94℃for 30 sec, 55°C for 30 sec

and 72°C for 30 sec, with a final extension step at 72°Cfor 7 min. The PCR products were analysed by electrophoresis on a 2% agarose gel stained with ethidium bromide. The digestion of PCR product, 249 bp, was performed for 5 h at 65°C with a Final 0.16 U/ml TaqI. The intact and digested, 135 and 114 bp, fragments indicated allele 2 or variant allele and allele 1 or wild-type allele, respectively.

***IL-1RN* gene polymorphism**. Analysis of the polymorphic region within the second intron of the *IL-1RN* gene containing a variable number of identical tandem repeats (VNTR) of 86 bp was performed by using PCR-RFLP. The primers 5▪ CTC AGC AAC ACT CCT AT 3▪ (sense) and 5▪ TCC TGG TCT GCA GGT AA 3▪ (antisense) were used to amplify this region, as described previously [17]. After initial denaturation at 94°C for 5 min, the samples were subjected to 45 cycles of denaturation at 94°Cfor 1 min, 56°C for 1 min and 72°C for 1 min, with a final extension step at 72°Cfor 5 min. The PCR products of 412 bp (allele 1 = four repeats), 240 bp (allele 2 = two repeats), 498 bp (allele 3 = five repeats), 326 bp (allele 4 = three repeats), 584 bp (allele 5 = six repeats) and 756 bp (allele 6 = eight repeats) were analysed by electrophoresis on a standard 2% agarose gel stained with ethidium bromide [18]. The allele 2, *IL-1RN**2, from six alleles were evaluated in relation to *Lactobacillus* species because this allele is associated with various chronic inflammatory conditions. In our study allele 2 has been presented as 2 and other five alleles as X.

Statistical analysis

Means of normally distributed bacterial quantities were compared using two-tailed unpaired *t* test. The chi-square(χ^2) or Fisher▪ s exact tests were used to compare distribution of the five most prevalent *Lactobacillus* species (*L.iners, L.crispatus, L.acidophilus, L.gasseri* and *L.jensenni*) among the *IL-1B-511, IL-1B+3954*, and *IL-RN* genotype groups. The statistical procedure was carried out at a significance level of 5% ($P < 0{\cdot}05$) by SPSS 20 software.

RESULTS:

Study population

Two hundred and fifty women were screened based on exclusion manifestations, and then 86 were excluded according to Nugent scoring. Totally 174 healthy women were investigated for determining *lactobacillus* species.

Prevalence of *Lactobacillus* species

Twelve *Lactobacillus* species were detected in 174 vagina samples by species specific PCR in present study. The five most prevalent species were *L.iners* (80.5%), *L.crispatus* (71.8%), *L.acidophilus* (38.5%), *L.gasseri* (24.7%) and *L.jensenni* (16%), followed by *L. rhamnosus* (2.9%), *L. paracasei* (2.9%), *L. fermentum* (2.3%), *L. plantarum* (2.3%), *L. salivarius* (1.1%), *L. reuteri* (1.1%) and *L. johnsonii* (1.1%). Sixteen percent of vagina samples had one *Lactobacillus* species, 43% had two, 32% had three and 9% had four. According to statistical analysis, *L.iners* was less prevalent among homozygous and heterozygous carriers of the allele 2, *IL-1RN**2, than homozygous carriers of the alleles X (p= 0.016). The prevalence of four other *Lactobacillus* species among other genotypes was not significantly different (Table 1). The number of each *IL-1B-511, IL-1B+3954*, and *IL-RN* genotype group is also shown in this table.

Quantities of *Lactobacillus* species

In order to compare the quantities of each *Lactobacillus* species and total species between *IL-1B-511, IL-1B+3954*, and *IL-RN* genotype groups, each SNP was grouped into carrier and non-carrier of polymorphism. The results of quantification of *Lactobacillus* species in relation to genotype groups of SNP in mentioned genes are shown in table 2. In this table mean quantities of each and total *Lactobacillus* species in each genotype group were expressed. Quantities of the total lactobacilli and *L.iners* were significantly lower in *IL-1RN**2 carriers than noncarriers

($p<0.05$). The differences between the amounts of four other *Lactobacillus* species in other genotypes were not significant.

DISCUSSION

In the present study, *Lactobacillus* species in the vaginal microbiota of healthy women were determined by PCR. In comparison with culture-independent methods such as PCR, culture-dependent methods have limitations for successful isolation of all species [19]. Accordingly in the past, *L. acidophilus and L. fermentum* were showed to be the most prevalent vaginal microbiota in healthy women that followed by *L. brevis, L. jensenii, L. casei*, and other species [20, 21]. But results of molecular method-based studies recently revealed that *L. crispatus, L. iners*, and *L. jensenii* were the most common lactobacilli isolates in healthy vaginal microbiota [22]. In our study the vaginal *Lactobacillus* species composition in healthy women had clear differences with some other communities. These differences were also found among various racial groups. For example, the dominant *Lactobacillus* species in healthy vagina of Indian women were *L. reuteri (32.5%), L. fermentum (25%),* and *L. salivarius* (16.25%) [4] and the prevalence of *L.crispatus, L. gasseri, L. jensenii,* and *L. iners* in the healthy Chinese women were 91%, 67%, 43% and 75%, respectively [19]. Yan and colleagues in their study in china revealed that the frequency of *L. crispatus, L. gasseri* and *L. iners* was higher in healthy women. In addition to studies mentioned above, a large number of them performed on the microbiota of healthy female genitalia showed various results in different populations around the world [4, 5]. Several studies demonstrated that host factors varying among different populations may affect the colonization ability of species [6, 7, 8]. To our knowledge, the evaluation of prevalence and quantity of *Lactobacillus* species in relation to the polymorphisms in one of components of innate immune system was performed for first time in present study. According to statistical analysis, the prevalence and quantity of *L.iners* and quantity of total lactobacilli were lower among allele 2 carriers than noncarriers. This negative association may be explained by findings of previous study that IL1RN*2-positive women had a high level of susceptibility to bacterial vaginosis and diminished lactobacilli colonization [11]. It is possible that the polymorphism in this molecule may indirectly influences the lactobacilli composition due to the impact on the susceptibility to pathogens [23]. Although women enrolled in this study were healthy, it is likely that some of them were infected before the sampling and subsequently the composition of species was changed. So the polymorphism in *IL-1RN* may indirectly influence the composition of *Lactobacillus* species through effect on infection susceptibility.

Regardless of affection mechanism, existence of relationship between *Lactobacillus* species and host factors could be a useful guideline for selecting therapeutic approach. The probiotics are extensively used to conserve the normal microbiota of the female genitalia. *Lactobacillus* species as Probiotics are used to treat vaginal infections such as candidiasis and bacterial vaginosis (BV) [2, 3]. In order to develop criteria to select appropriate *Lactobacillus* species for probiotic therapy, considering host factors associated with an increased rate and quantity of a particular species may be useful in long-term colonizing probiotic species. Given the possibility that a single polymorphism is not solely responsible for immunoregulation and numerous polymorphisms in multiple genes affect the net magnitude of the immune response, it is likely that other polymorphisms in other genes are associated with lactobacilli. A limitation of our study is that other genes and their interactions not included in this analysis may play a bigger effect or alter the interaction of *IL-1RN* polymorphism and *L. iners*. Several host factors such as age, skin color, pregnancy and menopausal status could be associated with the colonization ability of species [6, 7, 8]. In order to limit the effects of these factors in our study, all women were fertile, non-

pregnant and white. The means of ages in two groups of carrier of the allele 2 and noncarriers had not significant difference ($P > 0.05$) (dada not presented), so the effect of age on the colonization ability was limited. Our results revealed that *L. iners* may be not an appropriate probiotic species for carriers of the allele 2, *IL-1RN**2. Although lower prevalence and quantity of this species in carriers of the allele 2 compared to noncarrier is likely to be explained by low colonizing ability of this species among carriers of the allele 2, however further studies are needed to confirm that finding. Our study also revealed that further studies should be conducted to evaluate SNPs in other components of innate immune system in relation to *Lactobacillus* species because those may associate with colonization ability of species.

CONCLUSION

To our knowledge, this is the first study for investigating the relationship between polymorphism in component of innate immune system and prevalence and quantities vaginal *Lactobacillus* species. Evaluation of the relationship between the five most prevalent *Lactobacillus* species (*L.iners, L.crispatus, L.acidophilus, L.gasseri* and *L.jensenni*) and the *IL-1B-511*, *IL-1B+3954*, and *IL-RN* genes polymorphisms in this study, revealed that *L. iners* is less common in the carriers of allele 2, *IL-1RN**2, compared to noncarrier. Further studies are needed to confirm this relationship and to investigate other genes and their interactions. The evaluation of association between polymorphisms in components of innate immune system and *Lactobacillus* species could be useful in developing criteria to select appropriate species for probiotic therapy. The observational findings of present study can be helpful in designing further confirmatory studies.

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Conflict of Interest

The authors have no conflicts of interest.

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Tables

Table 1. Percentage of samples of each IL-1β- IL-1RN-SNP genotype group for which the tested species were detected

Lactobacillus	IL-1β (C-511T)		IL-1β (C+3954T)		IL-1RN	
species	CC	CT/TT	CC	CT/TT	XX	X2/22
Number of samples	159	15	156	18	151	23
*L.iners**	81.1	73.3	80.1	83.3	83.4	60.8
L.crispatus	71.7	73.3	72.4	66.7	72.2	69.5
L.acidophilus	39	33.3	37.8	44.5	39	34.8
L.gasseri	24.5	26.7	24.3	27.7	25.2	21.7
L.jensenni	16	13.3	16	16.7	16	13

X = an allele different from allele 2.

* *L.iners* was less prevalent among X2/22 carriers than among XX carriers (P=0.016)

Table 2. Quantitative determination of each *Lactobacillus* species in the different IL-1β- and IL-1RN-SNP genotype groups expressed as mean ± standard deviation (cells/ml)

Lactobacillus	IL-1β (C-511T)		IL-1β (C+3954T)		IL-1RN	
species	CC	CT/TT	CC	CT/TT	XX	X2/22
L.iners	8.12 ±1.61	8.43±1.22	7.83±0.92	8.36±1.10	8.52 ±1.31*	6.85 ±2.21*
L.crispatus	7.52 ±0.91	7.66 ±0.97	6.92 ±1.12	7.12 ±0.81	7.32 ±1.30	7.14 ±1.23
L.acidophilus	7.12 ±0.64	7.52 ±0.83	7.36 ±0.95	7.22 ±1.11	7.12 ±0.96	6.82 ±1.31
L.gasseri	6.82 ±1.21	6.22 ±1.45	7.12 ±0.71	7.42 ±0.48	6.82 ±0.51	6.62 ±0.77
L.jensenni	5.82 ±1.43	5.96 ±1.76	6.12 ±1.16	6.42 ±1.41	6.75 ±0.95	7.52 ±1.30
Total lactobacilli	8.43±1.37	8.88±0.83	8.92±1.89	8.56±1.34	8.84±0.84**	7.37±1.43**

X = an allele different from allele 2.

*: Low quantity of *L.iners* in X2/22 carriers than in XX (P<0.001). **: Low quantity of total lactobacilli in X2/22 carriers than in XX (P=0.02).