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Original Article

In vitro adherence of *Lactobacillus* strains isolated from the vaginas of healthy Iranian women

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Abstract

Background: The lactobacilli are a part of the bacterial flora of the human vagina. Detection of normal *Lactobacillus* species in the vaginas of healthy women in different geographical locations, and evaluation of their specific properties, can aid in the selection of the best species for preventing sexually transmitted diseases in the future. This study was performed to isolate and identify the *Lactobacillus* species in the vaginas of healthy women and to evaluate the adherence of these lactobacilli to Vero and HeLa cell lines.
Methods: The study included 100 women. Bacteria were isolated from healthy women and purified. Phenotypic and biochemical tests were performed to identify the lactobacilli. The *Lactobacillus* species were detected by molecular methods using polymerase chain reaction amplification of the full length of the 16S rDNA of the isolated bacteria. Several isolates of each species were then selected to study their adherence to Vero and HeLa cell lines.
Results: Among the 50 samples taken from healthy women meeting the inclusion criteria, *Lactobacillus* species were identified in 33 (66%) samples. Of these lactobacilli, 14 isolates were *Lactobacillus crispatus*, six (18.2%) were *Lactobacillus gasseri*, nine (27%) were *Lactobacillus rhamnosus*, and the rest were either *Lactobacillus salivarius* (6%) or *Lactobacillus plantarum* (6%). *L. rhamnosus* showed the greatest adhesion to the cells when compared to the other tested species. All the lactobacilli isolated in this study showed a smaller capacity for cell adherence when compared with control species.
Conclusion: *L. crispatus*, *L. rhamnosus*, and *L. gasseri* were the dominant *Lactobacillus* species in the vaginas of healthy women in Iran. *L. rhamnosus* attached more readily to the cells than did the other species; therefore, this isolate is a good candidate for further studies on the potential health benefits and application of lactobacilli as probiotics.
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Keywords: 16S rDNA; bacterial adherence; *Lactobacillus*; vaginal microflora

1. Introduction

The vaginal microflora consists of many dynamic microbial agents that coexist in this organ and assist in promoting the health of their host. Lactobacilli are the predominant bacteria in the vaginas of healthy women.[1,2] Any disturbance in the composition of the normal flora of the vagina may lead to subsequent bacterial and viral infection of the vagina,

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especially by pathogens responsible for sexually transmitted diseases. Reduction in the numbers of lactobacilli in the vagina is considered a risk factor for the transmission of herpes simplex virus type 2 (HSV2), human papillomavirus (HPV), and human immunodeficiency virus.[3−5] The recognition of the significant role played by lactobacilli in the protection against genital infections and in the reduction of transmission of sexually transmitted diseases has suggested the use of this genus for the production of antibacterial and antiviral substances, or as probiotics for the prevention and treatment of vaginal infections.[6] Currently, > 20 species of lactobacilli have been documented in the normal vaginal microflora.[7] Therefore, investigation of the various species and types of strains that occur in women in different geographical areas is required to identify the best common species for prevention of vaginal infections in the future.[6]

The advances in molecular techniques have had a great impact on the classification of bacteria. One of the reliable molecular techniques for detection of bacterial species, including lactobacilli, is sequencing of the bacterial 16S rRNA.[7] Analysis of the 16S rRNA gene is one of the most powerful molecular tools for determining phylogenetic relationships among different microbiological agents.[8] The aim of the present study, given the importance of lactobacilli in female health, was to use the polymerase chain reaction (PCR) technique to amplify the full-length 16S rDNA as a means of isolating and identifying the species of the *Lactobacillus* genus in the vaginas of healthy women in Iran. Gene sequencing and analysis were performed using MEGA6 software (MEGA6, Tamura, Stecher, Peterson, Filipski, and Kumar 2013). In addition, because the ability of the different lactobacilli to adhere to epithelial cells and colonize mucosal tissues are prominent factors that allow them to compete with and remove pathogenic bacteria from vaginal tissues,[9] the adherence of the different isolates was also investigated.

2. Methods

2.1. Sampling

The study included 100 premenopausal women, aged 18−45 years, who attended private or public clinics in Iran. All participants were healthy women undergoing visits for routine cervical screening. None had any complaints of vaginal infection and their clinical examinations revealed no signs of vaginitis. Two cotton swabs were collected from the exocervix and the lateral vagina. One of the swabs was put into a transport medium (thioglycolate broth, Sigma−Aldrich, St. Louis, MO, USA) and incubated at 37°C for 24−48 hours. The women were confirmed free from vaginosis by smearing the other swab on a slide and air drying, followed by Gram staining. The samples were then evaluated according to their Nugent scores: 0−3 were interpreted as the presence of normal flora; 4−6 were considered to represent mild vaginitis and reduction of normal microbial flora; and 7−10 indicated manifestations of bacterial vaginitis.[10] Samples indicating mild or severe vaginitis were excluded from the study.

2.2. Cultivation of samples

The samples were incubated at 37°C and then subcultured on de Man, Rogosa, and Sharp (MRS) agar (Merck, Darmstadt, Germany) for 24−48 hours under microaerophilic conditions using a gas generating kit (Merck). Lactobacilli were determined based on colony morphology, Gram staining, and catalase and oxidase tests. Isolation of purified bacteria was performed by picking a single colony and subculturing on MRS agar plates. Gram-positive staining, and catalase- and oxidase-negative bacillus and coccobacillus isolates were stocked in MRS broth (Merck) containing 20% glycerol, and stored at −80°C until further investigation.

2.3. DNA extraction

Bacterial DNA was isolated by subculturing the colonies stocked at −80°C and growing them on MRS agar. A single colony was then suspended in 300 μL sterile distilled water and the bacterial genome was extracted using a DNA extraction kit (SinaClon, Tehran, Iran). The extracted DNA was stored at −20°C for use as a template for the PCR procedure.

2.4. 16S rDNA amplification

Universal primers FD1 and RD1 were used for amplification of the 16S rDNA. The extracted DNA was amplified in a final volume of 50 μL containing 5 μL 10 × PCR buffer (with 15mM $MgCl_2$), 1 μL dNTPs mixture (10mM), 1.5 μL of both sense and antisense primers (10 pmol/μL), 0.25 μL *Taq* DNA polymerase (5 IU/μL), 39.75 μL sterile UHQ H_2O, and 1 μL template DNA. The PCR was performed in a thermocycler (Bio-Rad, Hercules, CA, USA) using the following schedule: 95°C for 3 minutes as the initial denaturing step; 30 cycles consisting of 95°C for 45 seconds, 55°C for 45 seconds, and 72C for 2 minutes; and then one cycle of 72C for 5 minutes as the final extension step.[11] The PCR products were loaded onto a 1% agarose gel containing Safe Stain and subjected to electrophoresis for 45 minutes at 85 V. Following electrophoresis, the gel was visualized with a UV transilluminator (UVitec, Cambridge, UK). A 100-bp molecular mass marker (SinaClon) was used for judging the size of the PCR products.

2.5. Sequencing

The PCR products were sent to the Bioneer Company (Daejeon, Korea) and were directly sequenced using the forward and reverse primers used for the amplification step. The results of sequencing were obtained as ABI files, and the sequence of each product was assembled and edited using Codon Code Aligner 5.1.4 software (Codon Code Corporation) to ultimately obtain the full length of the 16S rDNA (1450 bp). The PCR products were identified using the BLAST algorithm to compare 36 sequences with the complete 16S rDNA gene of the reference sequences available in the NCBI database. For the phylogenetic analysis, samples with sequence similarities of ≥ 97% in their 16S rDNA sequences were considered to belong to the same

species.[12] Further phylogenetic analysis was conducted by inputting reference sequences into Mega6 software as follows: *Lactobacillus crispatus* ATCC 338220, *Lactobacillus gasseri* ATCC 33323, *Lactobacillus acidophilus* ATCC4356, *Lactobacillus ultunensis* AY253660, *Lactobacillus gallinarum* EF412985, *Lactobacillus helveticus* AB446394, *Lactobacillus iners* AY526083, *Lactobacillus johnsonii* FJ542293, and *Lactobacillus vaginalis* ATCC49540 from the *Lactobacillus acidophilus* group; *Lactobacillus casei* ATCC393 and *Lactobacillus rhamnosus* D16552 from the *L. casei* group; *Lactobacillus salivarius* ATCC11741, *Lactobacillus agilis* M58803, and *Lactobacillus hayakitensis* AB267406 from the *L. salivarius* group; and *Lactobacillus plantarum* ATCC14917 and *Lactobacillus pentusus* Lb3F2 from the *L. plantarum* group. Multiple sequence alignment was performed using the MUSCLE program. A phylogenetic tree was constructed using the maximum-likelihood algorithm.[13] The sequences were deposited in GenBank with accession numbers KP090100−KP090132.

2.6. *In vitro* cell adherence by isolated bacteria

Several bacterial strains belonging to various *Lactobacillus* species were selected to evaluate their ability to attach to Vero (African Green Monkey kidney epithelial) and HeLa (Human epithelial carcinoma) cells. The Vero and HeLa cell lines were cultured in 24-well plates with 10% fetal bovine serum for 24 hours until a monolayer of cells was formed in the wells. The broth from 18-hour cultures of the *Lactobacillus* isolates was centrifuged, and the pelleted bacteria were washed with phosphate-buffered saline (PBS) and resuspended in Dulbecco's modified Eagle's medium to a final concentration of 10^8 CFU/mL. The cell monolayers were washed with PBS and a bacterial suspension equal to multiplicity of infection of 100 was inoculated into each well, followed by incubation at 37°C for 4 hours. Each well was then washed with PBS three times to remove nonadhering bacteria from the well. The cell monolayers were detached from the plate using 0.5% trypsin and resuspended in Dulbecco's modified Eagle's medium. The numbers of viable bacteria attached to the cells were determined by inoculating serial 10-fold dilutions of the cell suspensions on MRS agar, incubating at 37°C for 48 hours, and then counting the bacterial colonies. Each experiment was performed in duplicate. *Lactobacillus brevis* ATCC 367 was used as a control.

2.7. Statistical analysis

Data analysis for adherence of *Lactobacillus* strains to Vero and HeLa cells was performed using the Kruskal−Wallis test. A p value < 0.05 was considered statistically significant.

3. Results

Based on Nugent score, 50 samples from the 100 participants of this study were excluded from the analysis because they did not show the criteria of a healthy vagina. The phenotypic characteristics of the other vaginal samples collected from healthy individuals led to isolation and purification of 36 bacterial isolates belonging to the genus *Lactobacillus*. Morphological analysis showed the isolates to be long and short bacilli or coccobacilli, Gram positive, non-spore-forming, and catalase negative.

The results of PCR amplification are shown in Fig. 1. The species of the isolated bacteria were identified by comparison of the amplified fragments with sequences deposited in GenBank. In this study, 33 of the sequences (66%) belonged to the genus *Lactobacillus* and another three (6%) were similar to *Enterococcus faecalis*. The isolated lactobacilli belonged to four different groups of the *Lactobacillus* genus. *L. acidophilus* comprised 20/33 (60.6%) of the samples, nine (27.3%) were *L. casei*, two (6%) were *L. plantarum,* and the remaining specimens (6%) were *L. salivarius*. BLAST analysis of the sequences with the sequences deposited in GenBank indicated that 14/33 isolates (42%) of the *L. acidophilus* group had 99% identity with *L. crispatus* and six samples (18.2%) were *L. gasseri*. Samples belonging to the *L. casei* group showed 99% identity with *L. rhamnosus*. Clusters of the isolated lactobacilli were obtained after phylogenetic analysis of their sequences (Fig. 2).

The different lactobacilli were evaluated for their adherence to Vero and HeLa cell lines. The initial bacterial counts were 10^8 CFU/mL. The bacteria were inoculated onto the cell lines and then the titer of the attached bacteria was determined again after 4 hours. The ratio of the bacteria after incubation with the cell lines to the original bacterial titer was an index of the population of bacteria that had adhered to the cells. The ability of the different lactobacilli to adhere to the cells varied greatly. *L. rhamnosus* strains adhered to the cells to a greater extent when compared with the other *Lactobacillus* species; *L. crispatus* showed intermediate adherence; and *L. gasseri*

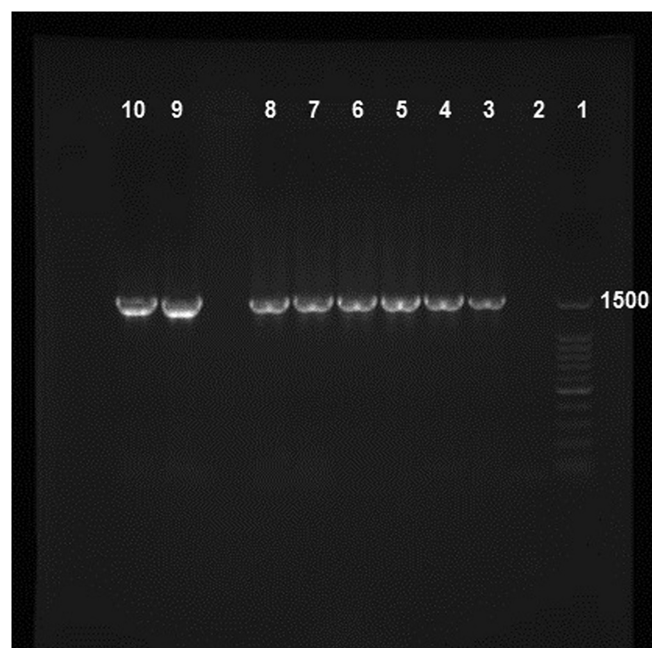

Fig. 1. Amplified sequences of the full 16S rDNA gene. Lane 1 = 100-bp marker; Lane 2 = negative control; Lane 3 = positive control; Lanes 3−10 = amplified segments of the 16S rDNA gene of the samples.

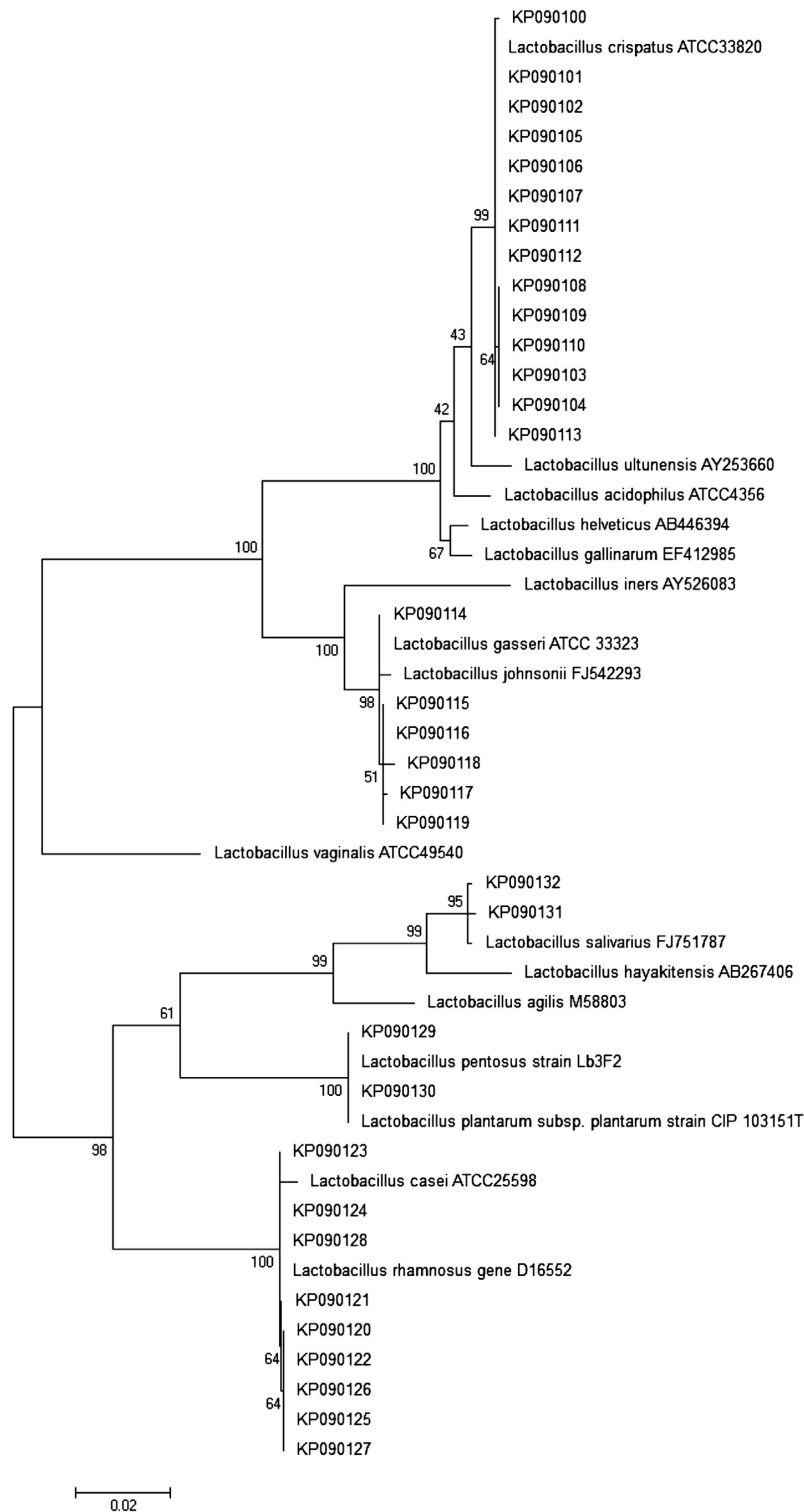

Fig. 2. Phylogenetic tree showing the positions of vaginal lactobacilli isolated from Iranian healthy women among the known *lactobacillus* species. It constructed based on the 16S rDNA sequences of the *Lactobacillus* species isolated in this study. The tree was drawn using the maximum likelihood method. The branch lengths are proportional to the genetic distance, and the numbers shown at the branch points indicate the bootstrap values. The data were subjected to 500 bootstrap replications. The reference sequences were obtained from GenBank at NCBI. The sequence accession numbers are KP090100−KP09032.

showed weak adherence. *L. brevis*, which was used as a control strain, had the greatest ability to adhere to the cells. All the tested lactobacilli showed a lower tendency to adhere to HeLa cells than to the Vero cell line (Fig. 3).

4. Discussion

In reproductive-age women, the occurrence of bacterial vaginitis is associated with an increased risk of upper genital

tract and sexually transmitted infections, as well as the acquisition of human immunodeficiency virus.[7] Lactobacilli are the predominant microflora of the healthy human vagina, and these bacteria decrease the vaginal colonization by other pathogenic bacteria through a variety of mechanisms, including the production of lactic acid, hydrogen peroxide, and bacteriocins.[2] Reduction of the pathogenic species in the vagina therefore requires recognition of the dominant lactobacilli in the vaginal microflora of healthy women. Identification of these lactobacilli was therefore the aim of the present study.

The predominant species of lactobacilli identified in our study were *L. crispatus*, *L. rhamnosus*, and *L. gasseri*, and the least common species were *L. salivarius* and *L. plantarum*. By contrast, reports from other countries indicated that *Lactobacillus jensenii*, *L. gasseri*, *L. crispatus*, and *L. iners* are generally the most prevalent microflora in the vaginas of healthy women.[14,15] The results of the present study are therefore in agreement with the previous reports, since *L. crispatus* and *L. gasseri* were among the most prevalent species in our cohort of Iranian women. Some species, however, differed from those previously reported for other areas of the world. For example, Vitali et al[16] used PCR-denaturing gradient gel electrophoresis and direct extraction of 16 rDNA from the vaginal samples obtained from Italian women, followed by PCR amplification of the v2−v3 region of the 16S rDNA. These researchers found that *L. acidophilus*, *L. gasseri*, *L. vaginalis*, and *L. iners* were the most prevalent species in the vaginal microflora of healthy women; these results were starkly different from ours. However, Vitali et al[16] performed their study directly on the collected samples without culturing them. Therefore, they were unlikely to have missed fastidious species of lactobacilli in their study. Jakobsson et al[17] have reported that some *Lactobacillus* species do not grow on MRS agar and require enriched media for their growth; *L. iners* is one of those species. No universal selection medium is available that will support the growth of all species of lactobacilli, which necessitates the application of culture-independent techniques for the precise detection of the lactobacilli in the vagina.

In India, *L. salivarius*, *L. plantarum*, *Lactobacillus reuteri*, and *Lactobacillus fermentum* have been reported as prevalent lactobacilli in the vagina,[18] which is different from the reports from other countries. The lifestyle, and especially the nutritional regimen, probably has some impact on the competition between, and vaginal colonization by, different lactobacilli. In 2013, Martínez-Peña and colleagues[19] determined the most dominant *Lactobacillus* species in the vaginas of Mexican women to be *L. gasseri*, *L. crispatus*, and *L. jensenii*; a similar result to ours. Taken together, the results of these various studies indicate that different factors, such as individual genetic makeup, nutrition, personal hygiene, and sexual activity, have some impact on the colonization of specific species of lactobacilli in the vaginas of healthy women.

In 2011, Amin et al[20] reported that the most prevalent *Lactobacillus* species colonizing the vaginas of women in our area (Ahvaz, Iran) was *L. acidophilus*. By contrast, in our study, *L. crispatus* and *L. gasseri*, which belong to the acidophilus group, were the predominant species of lactobacilli. The previous researchers used three pairs of specific primers for PCR detection of *Lactobacillus* species.[20] They did not amplify 16S rDNA. The discrepancy between our results and those of Amin et al[20] could therefore be attributed to the different techniques used in the two studies. Moreover, Amin et al[20] did not search for *L. rhamnosus*. The *L. acidophilus* group is highly heterogeneous and includes at least six separate species.[21] Differential detection of the members of the acidophilus group is possible by sequencing of 16S rDNA, random amplified polymorphic DNA−PCR, and restriction fragment length polymorphism.[17] In general, the discrepancies between the reported results for species of vaginal lactobacilli could be attributed to the laboratory methods used for the detection of these bacteria.

A bacterial strain with planned use as a potential probiotic needs to be selected based on its capacity to adhere to mucosal tissues, as this is a significant factor for regulation of the immune system and removal of pathogenic strains.[9] Several models, including Vero, CaCo, and HT29 cells, have been used to assess the ability of bacteria to attach to cells *in vitro*.[22,23] Vero and HeLa cells were used in the present study to evaluate the adherence of vaginal lactobacilli to these cells. We found that the various species of lactobacilli had different abilities to attach to the cells, as reported previously.[24,25] Previous studies have demonstrated that fimbriae on

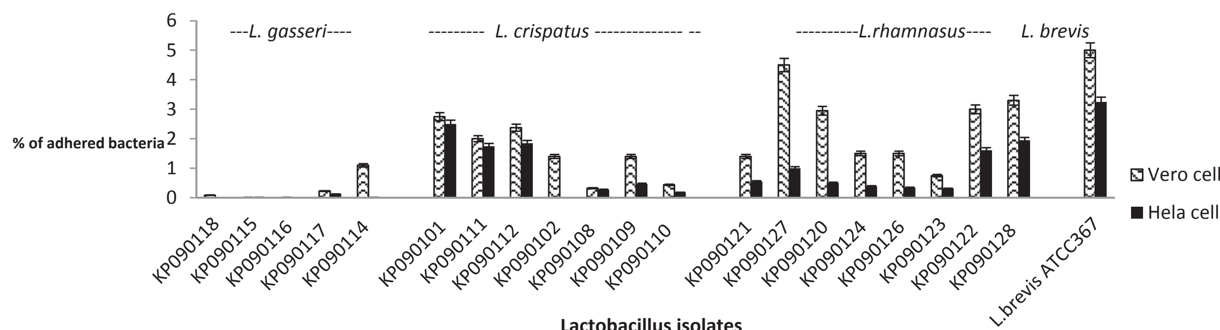

Fig. 3. The ability of different *Lactobacillus* strains to adhere to HeLa and Vero cells. A p value < 0.05, according to Kruskal−Wallis test, indicates a significant difference. The results are the means of three experiments.

the cell wall of some strains of lactobacilli, such as *L. rhamnosus*, enhances bacterial adherence to epithelial cells.[26] Similarly, the lectin-like protein in the cell wall of *L. plantarum* might be involved in adherence to human colon cells.[27] Other *Lactobacillus* surface factors, such as the s-layer proteins of *L. crispatus* and the lipoteichoic acid of *L. johnsonii*, might also play roles in adherence to epithelial cells.[28,29] Thus, different mechanisms could exist for different strains of lactobacilli for adherence to epithelial cells.

HeLa cells were originally derived from transformed epithelial cells of the human cervix, so lactobacilli were expected to adhere efficiently to these cells, but this was not the case in the present study. The transformation of these cells may have altered the nature of the cell structure, and some of the altered factors could reduce the ability of lactobacilli to adhere to the HeLa cells. This result confirms that the colonization of epithelial cells by bacteria is a complicated process.[26] In addition to the cell wall structure of lactobacilli, several host factors, such as cell receptors, soluble proteins secreted by the host cell, and electrostatic and hydrophobic forces, are involved in the adherence process.[30]

The most prevalent species found in the vaginas of healthy Iranian women in the present study were *L. crispatus*, *L. rhamnosus*, and *L. gasseri*. Nevertheless, in our opinion, other molecular techniques, such as fluorescence *in situ* hybridization,[31] should be used in combination with 16S rDNA sequencing of this genus to detect all the *Lactobacillus* species that make up the microflora of the vagina. Further analysis of the antimicrobial properties of *L. rhamnosus* and *L. crispatus* is also warranted, to more precisely illustrate their potential probiotic characteristics.

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