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ORIGINAL ARTICLE

# The phenotypic and genotypic antibiotic susceptibility of vaginal *Lactobacillus* with potential probiotic properties isolated from healthy women in northern Iran

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## ABSTRACT

**Background and Objectives:** This study assesses the antibiotic susceptibility of vaginal *Lactobacillus* strains and provides data for determining the prevalence of certain antibiotic resistance genes in the new strains of lactobacilli serving as probiotics and selected from healthy women in northern Iran.

**Materials and Methods:** One hundred premenopausal non-pregnant women in the reproductive age range of 22-50 years participated in this study. The potential probiotic vaginal lactobacilli used in the study included *Lactobacillus crispatus* (34.2%), *Lactobacillus gasseri* (26.3%), *Lactobacillus johnsonii* (10.5%), *Lactobacillus acidophilus* (15.7%) and *Lactobacillus jensenii* (13.1%). The phenotypic antibiotic susceptibility of the strains was determined by E test and DNA extraction and PCR were performed to examine the antibiotic resistance genes.

**Results:** 38 potential probiotic vaginal lactobacilli were isolated. All the strains of lactobacilli were resistant to metronidazole and trimethoprim/sulfamethoxazole and all of the strains were susceptible to ampicillin and chloramphenicol antibiotics. The results showed that *erm*B, *erm*C, and *erm*A genes were observed in the strains of *Lactobacillus acidophilus*. Metronidazole resistance (*nim*) gene was also found in one strain of *Lactobacillus crispatus* and *Lactobacillus johnsonii*. The aminoglycoside resistance (*aac6'-aph2''*) gene was observed in 8% of the strains. Also, *tet*M, *tet*K and *tet*W genes were found in more than 80% of the *Lactobacillus* strains.

**Conclusion:** The antimicrobial susceptibility of vaginal lactobacilli is an important criterion for establishing whether or not the organism is a probiotic. A high level of resistance to clinical antibiotics, such as metronidazole and aminoglycosides, was demonstrated. Antibiotic resistant genes also appeared widely in vaginal lactobacilli.

**Keywords:** Antibiotic susceptibility; Iranian women; Probiotic; Resistance genes; Vaginal *Lactobacillus*

## INTRODUCTION

The widespread use of antibiotics has led to the emergence, evolution and spread of antibiotic resistance in pathogenic and non-pathogenic bacteria associated with humans, animals and the environment (1). The rapid spread of antibiotic resistance among pathogens and the lack of new antibiotics are a major global health concern (2). The antibiotics used to treat infections in humans are usually the same ones used in veterinary medicine, which leads to the rapid spread of antibiotic resistance genes

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among bacteria associated with the food chain (3). The antibiotic resistance genes in probiotic strains and their transfer through plasmids and transposons to pathogenic bacteria constitute a potential risk to human health (4). Some lactic acid bacteria act as a reservoir for antibiotic resistance genes, which may in turn be transferred to pathogenic bacteria causing different infections (5). Bacteria that are used as probiotics must be free of transferable antibiotic resistance genes (6). The antimicrobial susceptibility of vaginal lactobacilli is an important criterion for establishing whether the organism is a probiotic (7). Antibiotics have different mechanisms of action in the destruction of microorganisms; some antibiotics, such as streptomycin, chloramphenicol, tetracycline and clarithromycin, prevent protein synthesis, while others, such as rifampicin and cotrimoxazole, prevent mRNA production (8). *Lactobacillus* are usually sensitive to penicillin, but they are more resistant to cephalosporins (9). Data suggests that the resistance of lactobacilli to aminoglycosides is often high (10). Meanwhile, several genes contributing to resistance to macrolide antibiotics, such as erythromycin, have been observed in lactobacilli. The most common resistance factors against lactobacilli, however, are tetracycline resistance genes (11). In humans, the *Lactobacillus* species normally exists in the vagina, and because of their protective and probiotic properties, recent research has paid considerable attention to vaginal *Lactobacillus* species (12). Approximately, 70% of the total bacteria isolated from healthy females are vaginal lactobacilli. It is therefore imperative to identify and determine *Lactobacillus* strains and their antibiotic resistance profile as well as toxicity to human organs. The present study thus seeks to determine the antibiotic susceptibility and screen some antibiotic resistance genes of *Lactobacillus* strains isolated from the vaginal samples of healthy women in northern Iran.

## MATERIALS AND METHODS

**Study groups and sampling.** One hundred healthy females referred to gynecology clinics for routine gynecological consultations in Amol, Iran, were recruited for this study. The inclusion criteria were: Age 22-50 years (premenopausal), no history of sexually transmitted diseases, immunodeficiency, vaginitis or vaginal candidiasis infection, and not being menopausal or having vaginal bleeding. The exclusion criteria were: Pregnancy or use of antibiotics or antifungal compounds, vaginal medications or suppositories or contraceptive spermicides over the past 60 days. Sterile swabs were used for sampling the exocervix and the side of the vagina; then, the swab was put into MRS broth medium (Liofilchem, Italy) and immediately incubated at 37°C for 24 hours.

**Purification of *Lactobacillus* isolates.** After incubation, the samples were cultured on MRS agar (Liofilchem, Italy) and incubated at 37°C in an anaerobic jar using Anaerocult C (Merck, Germany) for 24-48 hours. Then, Gram staining reaction, colony morphology and catalase test were used to identify and select *Lactobacillus* single colonies grown on MRS agar. Total Gram-positive bacilli and negative catalase test results were identified as lactobacilli and stored in MRS broth containing 10% (w/v) glycerol at -20°C for further investigations. The phenotypic characteristic of some vaginal samples was yeast or cocci, and *Lactobacillus* did not grow after 48-72 hours on MRS agar incubation at 37°C; therefore, they were excluded from the study. A total of 38 vaginal *Lactobacillus* strains were isolated and selected.

**Antibiotic susceptibility testing and determining the minimum inhibitory concentration.** For antibiotic susceptibility, the minimum inhibitory concentration (MICS) was determined by E test (Liofilchem, Italy). Three to five colonies were picked up from fresh culture grown on MRS agar medium and a McFarland standard 0.5 turbidity in sterile 0.85% NaCl solution was prepared. The suspensions were cultivated on Mueller Hinton agar medium (Merck, Germany) and left to dry before applying the E test strips. The E test strips were then placed on each plate. Afterwards, the plates were incubated at 37ºC for 48 hours under anaerobic conditions before the final results were recorded. MICS were read directly from the test strip according to the manufacturer's instructions. The strains showing MICS less than EFSA breakpoints were taken to be susceptible and the others were taken as resistant (13).

The E test strips contained these antibiotics; tetracycline (0.016-256 µg/ml), amoxicillin (0.016-256 µg/ml), kanamycin (0.016-256 µg/ml), vancomycin (0.016-256 µg/ml), ciprofloxacin (0.002-32 µg/ml), erythromycin (0.016-256 µg/ml), penicillin (0.016-256 µg/ml), chloramphenicol (0.016-256 µg/ml),

ampicillin (0.016-256 µg/ml), trimethoprim/sulfamethoxazole (0.002-32 µg/ml) and metronidazole (0.016-256 µg/ml).

**Molecular identification of *Lactobacillus* isolates.** Bacterial DNA was extracted from all the isolated strains using High Pure PCR template preparation kit (Roche, Germany). The quality and quantity of the extracted DNA were evaluated with a nanodrop spectrophotometer (Thermo Fisher Scientific, USA) and electrophoresis on 0.8% agarose gel.

The sequences of the 16S/23S ribosomal RNA intergenic spacer region of phylogenetically-related species were retrieved from GenBank (www.ncbi.nlm.nih.gov). DNA fragments encoding 16S rRNA were amplified using specific primer sequences, including forward (5'-CTCAAAACTAAACAAAGTTTC -3') and reverse (5'- CTTGTACACACCGCCCGTCA -3') primers, which confirmed the *Lactobacillus* genus. The PCR reaction mix with a total volume of 25 µl con- sisted of buffer (2.5 µl), 0.25 µl Taq DNA polymerase,
0.8 µl of each primer and 2 µl of DNA. A DNA-free vial was used as the negative control. PCR was performed by SimpliAmp Thermal Cycler (ABI, USA) using the following schedule: Initial denaturation at 95°C for 5 minutes followed by 35 cycles, each consisting of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute and a final elongation step at 72°C for 5 minutes. A 100-bp molecular mass marker (SinaClon, Iran) was used for assessing the size of the PCR products. The PCR product was electrophoresed in 1% (W/V) agarose gel in TBE buffer at 100 V for 45 minutes and stained with ethidium bromide, and the gel was visualized with a UV transilluminator (UVitec, UK).

**Sequencing.** The PCR product was sent to Niagen Noor Company (Tehran, Iran) along with the 16S rRNA primer for sequencing. The chromatograms were edited using Chromas version 3.1 software. After sequencing the amplified fragment of the 16S rRNA gene, the isolated strains were compared with other sequences in GenBank on NCBI website using BLAST software.

**Detection of antimicrobial resistance genes.** Total DNA of *Lactobacillus* strains was extracted using High Pure PCR template preparation kit (Roche, Germany) according to the manufacturer's protocol. The PCR assay mix (total volume, 50 ml) contained 20 pmol of each primer (Table 1), 1× PCR buffer, and 2U Taq DNA polymerase. A 50-ng portion of purified total DNA was used as a template. For each antibiotic resistance gene specific primers designed by Allele ID version 6.01 software. All the *Lactobacillus* strains were tested for the presence of the tetracycline efflux genes (*tet*K), and *tet* genes encoding ribosomal protection proteins (RPP), i.e., *tet*M and *tet*W, were detected with specific primers. Polymerase chain reaction amplicons were performed for *tet* genes in

**Table 1.** Primer sequences in the study

| Target Gene | Primer | Sequence | PCR product bp |
|---|---|---|---|
| *tet*W | F | 5'-CAGCCAGCCACACCATCCATATC-3' | 322 |
| | R | 5'-TGCGTCCCTGATTCCTTCAATGC-3' | |
| *tet*M | F | 5'-TGAACATCATAGACACGCCAGGAC-3' | 121 |
| | R | 5'-CGAGTTTGTGCTTGTACGCCATC-3' | |
| *tet*K | F | 5'-AGCCCACCAGAAAACAAACCAAG-3' | 457 |
| | R | 5'-TAGGATCTGCTGCATTCCCTTCAC-3' | |
| *erm*C | F | 5'-GAGGTGTAATTTCGTAACTGCCATTG-3' | 368 |
| | R | 5'-GTGAGCTATTCACTTTAGGTTTAGGATG-3' | |
| *erm*B | F | 5'-AAAGGGCATTTAACGACGAAACTG-3' | 435 |
| | R | 5'-ATCTGGAACATCTGTGGTATGGC-3' | |
| *Erm*A | F | 5'-TCTTATCGTTGAGAAGGGATTTGC-3' | 145 |
| | R | 5'-TACAGAGTCTACACTTGGCTTAGG-3' | |
| *aac6'-aph2"* | F | 5'-GCCACACTATCATAACCACTACCG-3' | 226 |
| | R | 5'-ATCCAAGAGCAATAAGGGCATACC-3' | |
| *Nim* | F | 5'-TGCTTCCTTGCCTCGTTCTCATC-3' | 157 |
| | R | 5'-TTCAACAACAATCCTGCCACCTTG-3' | |

SimpliAmp Thermal Cycler (ABI, USA) with the following schedule: Initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 1 min, annealing at 56°C for 1 min and at 72°C for 2 min, and a final extension at 72°C for 10 min. The amplification specifications for genes associated with resistance to macrolides (*erm*A, *erm*B, *erm*C), metronidazole (*nim*), and aminoglycosides (*aac6'-aph2''* bifunctional gene) were as follows: 94°C for 5 min, followed by 25 cycles of 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min, and a final elongation step at 72°C for 7 min. Amplicons were electrophoresed on 1.5% agarose gel.

**Statistical analysis.** The collected data of the current study were analyzed using SPSS software, version 22. The Chi-square test was used to compare and assess the significance of antibiotic resistance among the *Lactobacillus* isolates.

**Ethical considerations.** All the participants were given sufficient information about the process of the study and written consent was obtained from all of them. Also, the study protocol was approved by the Ethics Committee of Ayatollah Amoli Azad University (ID: IR.IAU.AMOL.REC.1400.051).

## RESULTS

**Isolation of *Lactobacillus* strains from the vagina.** The isolated *Lactobacillus* strains cultured on MRS agar medium were investigated and their phenotype properties were recorded, such as colony color (white to milky). A total of 38 vaginal lactic acid bacteria isolated from reproductive-aged women were selected. They were Gram-positive, rod shaped, catalase negative. All the isolated vaginal *Lactobacillus* strains were finally identified by sequencing the 16S rRNA gene and compared with sequences available in GenBank database using BLAST software on NCBI and five species were identified for them.

The results showed that the identified strains belonged to *L. crispatus* (LC1 to LC13), *L. gasseri* (LG 1 to LG 10), *L. acidophilus* (LA 1 to LA 6), *L. jensenii* (LJe 1 to LJe 5) and *L. johnsonii* (LJh 1 to LJh 4).

**Nucleotide sequence accession number.** The DDBJ-EMBL GenBank accession numbers of the spacer sequences used in this study are listed in (Table 2).

**Table 2.** NCBI GenBank accession numbers of *Lactobacillus* strains sequence

| Accession Number | Species | Isolates Label | NCBI GenBank Strain name |
|---|---|---|---|
| OR320567 | *L. acidophilus* | LA1 | A1HZA |
| OR326956 | *L. acidophilus* | LA3 | A3HZA |
| OR326955 | *L. crispatus* | LC1 | C1HZA |
| OR220801 | *L. crispatus* | LC2 | C2HZA |
| OR220199 | *L. crispatus* | LC3 | C3HZA |
| OR220200 | *L. crispatus* | LC4 | C4HZA |
| OR220201 | *L. crispatus* | LC5 | C5HZA |
| OR220202 | *L. crispatus* | LC6 | C6HZA |
| OR220203 | *L. crispatus* | LC7 | C7HZA |
| OR229087 | *L. crispatus* | LC8 | C8HZA |
| OR229088 | *L. crispatus* | LC9 | C9HZA |
| OR229089 | *L. crispatus* | LC11 | C11HZA |
| OR229090 | *L. crispatus* | LC12 | C12HZA |
| OR220204 | *L. crispatus* | LC13 | C13HZa |
| OR326958 | *L. gasseri* | LG1 | G1HZA |
| OR326957 | *L. gasseri* | LG2 | G2HZA |
| OR268776 | *L. gasseri* | LG3 | G3HZA |
| OR268773 | *L. gasseri* | LG4 | G4HZA |
| OR220205 | *L. gasseri* | LG5 | G5HZA |
| OR220195 | *L. gasseri* | LG6 | G6HZA |
| OR220196 | *L. gasseri* | LG7 | G7HZA |
| OR220197 | *L. gasseri* | LG8 | G8HZA |
| OR268777 | *L. gasseri* | LG9 | G9HZA |
| OR220198 | *L. gasseri* | LG10 | G10HZA |
| OR268775 | *L. jensenii* | LJe1 | J1HZA |
| OR220568 | *L . jensenii* | LJe2 | J2HZA |
| OR220569 | *L. jensenii* | LJe3 | J3HZA |
| OR268778 | *L. jensenii* | LJe4 | J4HZA |
| OR220801 | *L. jensenii* | LJe5 | J5HZA |
| OR220564 | *L. johnsonii* | LJh1 | Jh1HZA |
| OR268774 | *L. johnsonii* | LJh2 | Jh2HZA |
| OR220565 | *L. johnsonii* | LJh3 | Jh3HZA |
| OR220567 | *L. johnsonii* | LJh4 | Jh4HZA |

**Phenotypic antibiotic resistance of the *Lactobacillus* strains.** After 24 to 48 hours, the plates containing Mueller Hinton medium were observed with E test strips, and the antibiotic susceptibility was measured by the concentration gradient of the non-growth zone based on the EFSA standard (Table 3). Our results revealed that all the strains were resistant to metronidazole (≥256 µg/ml) and trimethoprim/sulfamethoxazole (≥ 32 µg/ml). Also, except the isolated *L. acidophilus* (one strain = LA6) and *L. crispatus* (three strains: LC2, LC3 and LC12) and *L. gasseri* (one strain = LG1), all the strains were resistant to

**Table 3.** MIC values in µg/ml of antibiotics against vaginal *Lactobacillus* strains

| Strain | TC | Er | K | Van | MTZ | CIP | P | AMX | SXT | AMP | CHL |
|---|---|---|---|---|---|---|---|---|---|---|---|
| LC1 | 16 | 0.016 | 32 | 192 | ≥256 | 32 | 2 | 1.5 | ≥32 | 0.25 | 1.5 |
| LC2 | 1.5 | 256 | 192 | 0.5 | ≥256 | 32 | 0.5 | 1 | ≥32 | 0.25 | 1 |
| LC3 | 1.5 | 0.016 | 192 | 192 | ≥256 | 24 | 3 | 1 | ≥32 | 1 | 1.5 |
| LC4 | 256 | 192 | 192 | 0.125 | ≥256 | 0.75 | 0.25 | 0.25 | ≥32 | 0.25 | 0.75 |
| LC5 | 192 | 128 | 128 | 0.5 | ≥256 | 1.5 | 32 | 0.25 | ≥32 | 0.75 | 1 |
| LC6 | 128 | 96 | 128 | 192 | ≥256 | 24 | 192 | 1 | ≥32 | 1 | 0.75 |
| LC7 | 128 | 192 | 192 | 0.125 | ≥256 | 32 | 1 | 1 | ≥32 | 1 | 1 |
| LC8 | 256 | 128 | 192 | 1 | ≥256 | 32 | 192 | 1 | ≥32 | 1 | 1 |
| LC9 | 192 | 256 | 256 | 1.5 | ≥256 | 24 | 192 | 192 | ≥32 | 1 | 1.5 |
| LC10 | 128 | 128 | 256 | 1 | ≥256 | 0.64 | 192 | 3 | ≥32 | 0.75 | 0.75 |
| LC11 | 128 | 1.5 | 128 | 1.5 | ≥256 | 24 | 1.5 | 1.5 | ≥32 | 0.75 | 0.75 |
| LC12 | 1.5 | 3 | 128 | 0.38 | ≥256 | 0.25 | 2 | 0.25 | ≥32 | 0.75 | 1 |
| LC13 | 128 | 128 | 128 | 192 | ≥256 | 24 | 0.75 | 1 | ≥32 | 0.75 | 0.25 |
| LG1 | 1.5 | 0.016 | 192 | 0.5 | ≥256 | 32 | 0.5 | 0.25 | ≥32 | 0.25 | 1 |
| LG2 | 16 | 4 | 256 | 2 | ≥256 | 32 | 1.5 | 0.75 | ≥32 | 0.25 | 1 |
| LG3 | 12 | 4 | 128 | 192 | ≥256 | 2 | 2 | 1 | ≥32 | 1 | 0.25 |
| LG4 | 256 | 0.016 | 192 | 1.5 | ≥256 | 24 | 1.5 | 0.125 | ≥32 | 0.125 | 1 |
| LG5 | 256 | 256 | 256 | 192 | ≥256 | 32 | 256 | 192 | ≥32 | 0.25 | 1 |
| LG6 | 256 | 256 | 256 | 192 | ≥256 | 0.75 | 1 | 0.19 | ≥32 | 1 | 1 |
| LG7 | 192 | 0.19 | 128 | 0.125 | ≥256 | 24 | 1 | 1 | ≥32 | 0.25 | 1 |
| LG8 | 192 | 192 | 128 | 128 | ≥256 | 24 | 1.5 | 1 | ≥32 | 1 | 0.25 |
| LG9 | 192 | 256 | 192 | 192 | ≥256 | 24 | 256 | 192 | ≥32 | 1 | 1.5 |
| LG10 | 128 | 192 | 192 | 128 | ≥256 | 24 | 192 | 2 | ≥32 | 1 | 1.5 |
| LA1 | 192 | 192 | 128 | 192 | ≥256 | 32 | 192 | 192 | ≥32 | 1 | 1 |
| LA2 | 256 | 256 | 256 | 192 | ≥256 | 32 | 1 | 192 | ≥32 | 0.75 | 1 |
| LA3 | 192 | 0.5 | 0.75 | 32 | ≥256 | 24 | 0.5 | 192 | ≥32 | 0.25 | 0.75 |
| LA4 | 256 | 0.25 | 0.25 | 0.125 | ≥256 | 32 | 192 | 256 | ≥32 | 0.25 | 1 |
| LA5 | 128 | 128 | 0.75 | 192 | ≥256 | 32 | 1.5 | 1.5 | ≥32 | 0.25 | 1 |
| LA6 | 1.5 | 0.19 | 0.25 | 192 | ≥256 | 1 | 1.5 | 0.5 | ≥32 | 0.75 | 0.75 |
| LJe1 | 16 | 256 | 256 | 192 | ≥256 | 1 | 128 | 0.75 | ≥32 | 1.5 | 1 |
| LJe2 | 192 | 192 | 192 | 0.75 | ≥256 | 32 | 0.75 | 0.75 | ≥32 | 1.5 | 1.5 |
| LJe3 | 128 | 256 | 192 | 1 | ≥256 | 32 | 1 | 192 | ≥32 | 1 | 1 |
| LJe4 | 128 | 192 | 128 | 1.5 | ≥256 | 24 | 1.5 | 0.75 | ≥32 | 1 | 1 |
| LJe5 | 192 | 128 | 128 | 1.5 | ≥256 | 24 | 1 | 1.5 | ≥32 | 1 | 1 |
| LJh1 | 256 | 192 | 256 | 0.75 | ≥256 | 24 | 0.75 | 0.75 | ≥32 | 1 | 0.75 |
| LJh2 | 192 | 0.016 | 192 | 0.75 | ≥256 | 24 | 0.016 | 0.75 | ≥32 | 0.75 | 0.25 |
| LJh3 | 256 | 256 | 192 | 1 | ≥256 | 24 | 1 | 1 | ≥32 | 1 | 0.75 |
| LJh4 | 128 | 256 | 128 | 0.75 | ≥256 | 32 | 192 | 192 | ≥32 | 0.75 | 0.75 |

Key: CHL= chloramphenicol, AMP= Ampicillin, SXT= Trimethoprim/sulfamethoxazole, AMX= Amoxicillin, P= Penicillin, CIP=Ciprofloxacin, MTZ= Metronidazole, VAN= Vancomycin, K= Kanamycin, E r= Erythromycin, TC= Tetracycline

tetracycline. The results showed that only four strains of *L. acidophilus* were susceptible to kanamycin and the other strains expressed resistance to this antibiotic (32-256 µg/ml). Moderate resistance to ciprofloxacin was observed in the *Lactobacillus* strains (24-32 µg/ml). The findings showed that more than 50% of the strains were susceptible to vancomycin, while 60% of them were susceptible to penicillin and amoxicillin. In the *L. jensenii* all the strains were resistant to erythromycin (192-256 µg/ml). We observed all *Lac-*

*tobacillus* strains were susceptible to ampicillin and chloramphenicol antibiotics.

**Antibiotic resistance genes.** The results of this study also revealed that *erm*B, *erm*C, and *erm*A genes were present in the *Lactobacillus acidophilus* strains. In the *Lactobacillus crispatus* strains, only *erm*B and *erm*C genes were observed, and *erm*A was not found in them; however, *erm*A gene was found in the *Lactobacillus johnsonii* isolates (Fig. 1). In 70% of the vaginal *Lactobacillus* strains, *tet*M gene was detected. Furthermore, *nim* gene was present in one strain of *L. crispatus* (LC2) and *L. johnsonii* (LJh 3). The kanamycin resistance gene (*aac6'-aph2"*) was not found in the *L. crispatus* and *L. gasseri* strains. (Figs. 2 and 3) present further details about *tet* and *aac6'-aph2"* genes. Based on the results, it seems that six *Lactobacillus* strains (LC8, LG4, LG6, L Je2, LJe3, and LA1) isolated from the vagina of healthy women in our region did not carry these antibiotic resistance genes.

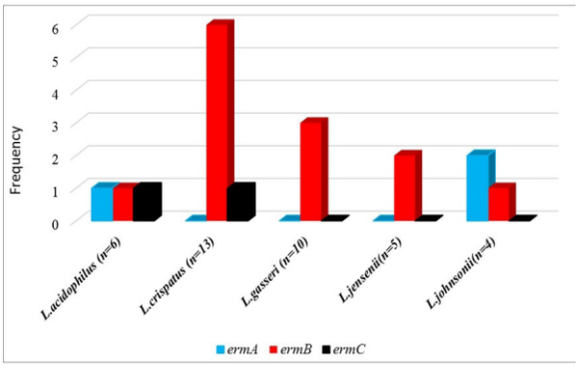


**Fig. 1.** Frequency of antibiotic resistance genes *erm*C, *erm*B, *erm*A in the *Lactobacillus* species

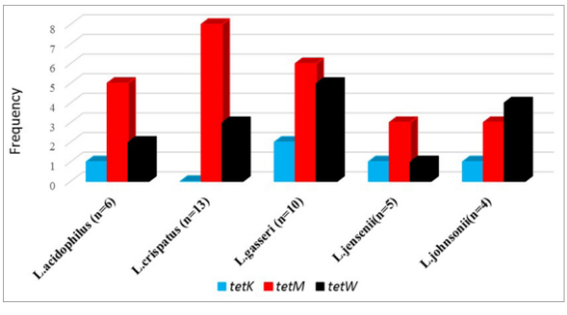


**Fig. 2.** Frequency of antibiotic resistance genes *tet*W, *tet*M, and *tet*K in the *Lactobacillus* species

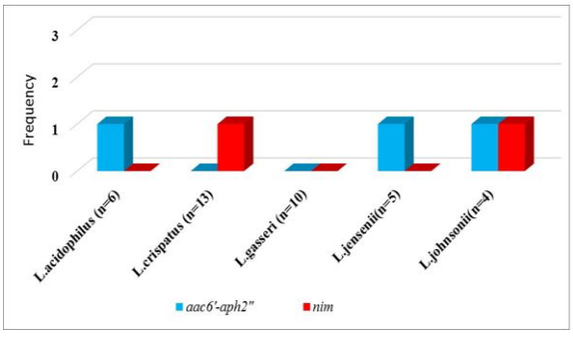


**Fig. 3.** Frequency of antibiotic resistance genes *aac6'-aph2"* and nim in the *Lactobacillus* species

## DISCUSSION

Antibiotic resistance is one of the major challenges faced by health systems across the world that imposes huge costs on societies. Probiotics can be an alternative of antibiotics due to their positive effects on human health (14). Meanwhile, probiotic strains should be susceptible to antibiotics, and if they do not contain infectious antibiotic resistance genes, they become resistant to antibiotics (15, 16). Another advantage of antibiotic-resistant probiotics for patients is that they can be prescribed without antibiotics (17). The antibiotic resistance was found especially in *Lactobacillus* strains and other LAB is not intrinsic, and the resistance of some LAB may possibly be plasmid-encoded (18).

The resistance of probiotics to antibiotics as one of the important characteristics of these microorganisms, and the investigation of this resistance or susceptibility to antibiotics determines the safety of probiotics (19). A number of studies have investigated the antibiotic resistance of vaginal lactobacilli (20-22). Štšepetova et al. showed the high resistance of vaginal lactobacilli from the *Lactobacillus acidophilus* group (including *L. crispatus*, *L. gasseri* and *L. jensenii*) to metronidazole and sulfonamides (trimethoprim sulfamethoxazole). They also found a high level of resistance to aminoglycosides (kanamycin) and quinolones (norfloxacin) (21). The tetracycline resistance genes *tet*M and *tet*K as well as the erythromycin resistance gene *erm*B were the most frequently-detected genes in vaginal *Lactobacillus* species (21). In the present study, these genes were also detected in all vaginal *Lactobacillus* strains. Although some of the tested strains were phenotypically susceptible to erythromycin, *erm*B gene was detected in most of the strains. The higher prevalence of *erm*B could be related to its frequent localization

on mobilizable plasmids or mobile conjugative transposons (23). To display phenotypic resistance, the association of several genes is mandatory (24). The strains tested in this study showed a high resistance to erythromycin and tetracycline; these observations were in contrast to the data reported by Danielsen and Wind, while Hoque et al. found that the *Lactobacillus* species is highly resistant to tetracycline (25, 26). Meanwhile, antibiotic resistance genes that code for tetracycline and erythromycin have been detected in different *Lactobacillus* species isolated from fermented foods and probiotics (4, 10, 27).

Our results showed that all the isolated strains were resistant to metronidazole. In the study by Simoes et al. the concentrations of all the strains were between 128 and 256 µg/ml of metronidazole which stimulated the growth of vaginal *Lactobacillus* species (20). The lack of hydrogenase activity in lactobacilli may be responsible for their resistance to metronidazole. Intrinsic resistance to metronidazole is a positive feature that supports the use of these strains as vaginal probiotics, because metronidazole is also the agent of choice for the treatment of bacterial vaginosis and trichomoniasis (28). Similarly, Pino et al. reported a high level of resistance to metronidazole (22). In the study by Kiray et al. all the strains were resistant to ciprofloxacin, gentamicin, tobramycin, amikacin, netilmicin, and cefoperazone (29). Generally, *Lactobacillus* species have a high intrinsic resistance to vancomycin, cefoxitin, metronidazole, nitrofurantoin, and sulfadiazine as well as antibiotics that inhibit the synthesis of proteins, such as chloramphenicol, erythromycin, clindamycin, and tetracyclines (30).

Consequently, lactobacilli can be prescribed both during and after antibiotic treatment. Our study was the first to evaluate the tetracycline resistance genes (*tet*K, *tet*M, and *tet*W), erythromycin resistance genes (*erm*A, *erm*B, and *erm*C), kanamycin resistance gene (*aac6'-aph2''*), and metronidazole resistance gene (*nim*) in *Lactobacillus* strains isolated from the vagina of women in the northern regions of Iran. According to the results, *erm*B gene was present in most *Lactobacillus* strains, and *erm*A and *erm*C genes were also seen in *L. acidophilus* isolates. The most common tetracycline resistance genes (*tet*K, *tet*M, and *tet*W) were present in the strains of *L. acidophilus, L. gasseri, L. jensenii* and *L. johnsonii*. The resistance gene of metronidazole (*nim*) was also identified in one strain of *L. crispatus* and *L. johnsonii,* while the aminoglycosides resistance gene (kanamycin *aac6'-aph2''*) was not seen in the strains of *L. crispatus* and *L. gasseri*. The study by Sirichoat et al. did not look for any of the aminoglycoside resistance genes with PCR, including the widespread *aac6'-aph2''* and *aad*E genes (31). Further, they reported that resistance to aminoglycosides may occur based on several mechanisms, which include (i) enzymatic modification and inactivation of the antibiotics mediated by aminoglycoside acetyltransferases, nucleotidyltransferases, or phosphotransferases, (ii) decreased permeability and (iii) modifications of the 30S ribosomal subunit interfering with the binding of this class of antibiotics (31). In the case of kanamycin, the MIC values measured in this study were very high, as consistent with the results reported by Hutt et al. (24), and in contrast to the results reported by Kassa et al. who found that all the strains were susceptible to kanamycin (32). Zhou et al. showed a high resistance to kanamycin for certain lactobacilli (16). Similarly, lactobacilli showed intrinsic aminoglycoside resistance that leads to membrane impermeability due to efflux pump mechanisms. Intrinsic resistance to aminoglycosides has been observed in *Lactobacillus* species in various studies (33, 34). In our study, only 13% of the *Lactobacillus* strains were susceptible to tetracycline, while the other strains had resistance to this antibiotic. In contrast, Sirichoat et al. detected that all *Lactobacillus* strains were susceptible to tetracycline (31). It has been previously reported that *tet*M is the most common tetracycline resistance gene. This gene has been found in members of *L. acidophilus, L. gasseri,* and *L. crispatus* species (35). In addition, chromosomal mutations leading to antibiotic resistance phenotypes have been described in lactobacilli (10). In the present study, the *erm*B gene was present in most isolated lactobacilli. This gene has already been identified in various lactobacilli. Among others, *erm*B has been seen in *L. reuteri, L. fermentum, L. casei, L. plantarum, L. acidophilus, L. gasseri, L. rhamnosus* and *L. johnsonii* species. The resistance to erythromycin in the human vaginal lactobacilli was also attributed to the transition mutation in the V region of 23S rRNA codifying gene (36).

Previous studies have shown that many *Lactobacillus* species are resistant to vancomycin (21). Nevertheless, homofermentative lactobacilli may be susceptible to vancomycin. Also, the reported vaginal *L. acidophilus* group was found to be susceptible

to vancomycin (21). Resistance to vancomycin is the most important source of concern, because it is one of the broad-spectrum antibiotics that is greatly effective against clinical infections caused by multidrug-resistant pathogens (29). In our study, all the strains of *L. johnsonii* were found to be susceptible to vancomycin, while 45% of the *Lactobacillus* strains were resistant to vancomycin. It was then reported that vancomycin is connected to peptidoglycan precursors on the cytoplasmic cell membrane and binds to the D-alanine/D-alanine penta-peptide terminus, while preventing the polymerization of peptidoglycan precursors (37). In our study, vaginal *Lactobacillus* species exhibited multiple resistances, while multiple resistances were not common for intestinal lactobacilli (38). Inactive antibiotic resistance genes might reflect a hazard and can be easily reactivated, leaving the restored gene stable afterwards. Therefore, the use of strains harboring such genes in food and feed systems should be avoided (31).

## CONCLUSION

The antimicrobial susceptibility of vaginal lactobacilli is an important criterion for establishing whether or not the organism is a probiotic. To be safe, a probiotic bacterium should not contain antibiotic resistance genes to normal microbiota. To prevent the transfer of antibiotic resistance to natural microflora, probiotics should not have any resistance genes. Since most probiotics are used in functional foods, the presence of antibiotic resistance determinants in their genomes should be systematically screened. Future studies should focus on analyzing either the plasmid or chromosomal DNA of certain *Lactobacillus* isolates and assessing the proteins encoded by antibiotic resistance genes in new strains of vaginal lactobacilli. It is necessary for probiotic producer companies to perform antibiotic susceptibility testing for a large number of antibiotics before using the microorganism as a probiotic and to analyze the whole genome sequence for antibiotic resistance genes.

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