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Evaluation of Vaginal Lactobacilli with Potential Probiotic Properties and Biotherapeutic Effects isolated from Healthy Turkish Women

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Abstract:
In this study, strains isolated from vaginal flora of healthy women were used. The anti-proliferative or cytotoxic effects of these strains on the HeLa [Human Cervical Carcinoma Cell] and Caco-2 [Human Colon Carcinoma Cell Line] cells after various in vitro tests to evaluate the probiotic potential of these strains were investigated. All strains were found to be resistant to low pH and high bile salt concentrations. It has been determined that vaginal strains have a broad spectrum of antimicrobial activity against urogenital pathogens and are resistant to frequently prescribed antibiotics for these pathogens. At the end of the 4th hours, all tested isolates had autoaggregation capacity between 63.6% and 85.2%. The strains were found to be well coagulated with microorganisms (*Escherichia coli* and *Candida albicans*) which cause vaginal and urogenital infections. In addition, the metabolites of L12, L13 and L19 strains had a significant antiproliferative effect (75-90% mortality rate) on the HeLa and Caco-2 cell lines. These strains with superior probiotic properties alone or with other strains having strong probiotic properties can be thought to contribute to the development of a new probiotic formulation to help improve maintain urogenital health and to reduce the clinical symptoms caused by vaginal infections.

Keywords: Probiotic, vaginal microbiota, urogenital infections, cervical cancer.

INTRODUCTION

Urogenital infections including bacterial vaginosis (BV), aerobic vaginitis, vulvovaginal candidiasis (VVC), sexually transmitted diseases and urinary tract infections and affect approximately one million women worldwide every year (Reid *et al*., 2013).

Lactobacilli species predominantly found in vaginal microbiota play an important role in protecting healthy women from genital and urogenital infections and preserving the natural balance of vaginal microbiota. Long-term studies suggest that some women with vaginal microbiota without lactobacilli have recurrent asymptomatic infections, and that in the majority of women, vaginal infections and urinary tract infections may cause serious symptoms within weeks or months (Stojanovic *et al*., 2012).

Lactobacilli are responsible for the protection of vaginal microbiota by different mechanisms. First of all, lactobacilli bacteria produce low vaginal pH with lactic acid and prevent the development of potential pathogenic microorganisms. Furthermore, lactobacilli inhibits the binding of pathogenic microorganisms by producing hydrogen peroxide, diacetyl, inhibitory enzymes, bacteriocin and bacteriocin-like antimicrobial agents and adhering to the receptors in the vaginal epithelial cells. In addition, they contain surface binding proteins that inhibit the binding of pathogenic microorganisms to vaginal epithelial cells by their coaggregation capabilities (Stojanovic, 2012; Stoyancheva *et al*., 2014; George *et al*., 2018).

The current definition of probiotics recommended by the World Health Organization and the Food and Agricultural Organization is 'live microorganisms that when administered in adequate amounts, confer a health benefit on the host' (FAO/WHO, 2002; Brazilya, 2016). Lactic acid bacteria constitute the most important group of probiotic microorganisms, but lactobacilli species are the most commonly used probiotic microorganisms (Önal *et al.,* 2005).

The use of probiotic products in the prevention and treatment of urogenital infections is considered to be an alternative method and it has been proven that, as a result of various studies, probiotic treatment has reduced or prevented the recurrent rate of BV even in high-risk patients ((Reid *et al*., 2013; Homayouni *et al*., 2014; Toma´s *et al*., 2011; Parma *et al*., 2014).

More than a quarter of women with urinary tract infections develop recurrent infections within six months. The widespread and irregular use of antibiotics for treatment increases the number of bacteria resistant to antibiotics. Therefore, it is considered that probiotic capsules or vaginal ovules may be a potential alternative, although in recent years there is a need for non-antibiotic methods that may be effective in protection and treatment (Verdenelli *et al*., 2014; Czaja *et al*., 2007; Caretto *et al*., 2017; Aragon *et al*., 2016). In this context, there is a need to identify strains of superior probiotic nature, which have the potential to reduce the symptoms of vaginal and urogenital infections, treatment and disease.

Not every microorganism is a probiotic and it is necessary to determine the probiotic properties of that microorganism by experimental and clinical studies in order to use a microorganism as a probiotic (Gulmez and Guven, 2002). As the mechanisms of action are better understood and the differences between the controlled studies and the strains with the same genetic structure are well established, the predicted probiotic effects of the strains will be proven (Schwebke, 2001).

In the present study was planned in this direction; firstly the probiotic properties of lactobacilli bacteria isolated from vaginal microbiota of healthy women in Kırşehir region were investigated by using universal criteria such as acid and bile tolerances, antimicrobial activities and antagonistic effects on pathogenic microorganisms, hydrogen peroxide (H_2O_2) production capabilities, autoaggregation and coaggregation capabilities, and binding capacity

to uroepithelial cells. Secondly, the anti-proliferative effect of these probiotic strains on HeLa and Caco-2 cells was investigated.

MATERIALS AND METHODS

Selection of *Lactobacilli* strains

In this study, 10 *Lactobacillus* spp. with superior probiotic properties were selected from among the strains isolated from vaginal regions of 60 healthy women who applied to Kırşehir Ahi Evran University Education and Research Hospital. Vaginal swabs were taken from voluntary patients, aged 18 - 45, without menopause, who were not protected by any birth control method and did not take antibiotics in the last 3 months. In this study, it was ensured that healthy patients who had vaginal swab samples to be read and signed the informed volunteer form.

All vaginal samples were inoculated onto MRS (De Man Rogosa Sharpe, Merck) agar and the plates were incubated at 37°C for 48 hours under anaerobic conditions. Lactobacilli were initially phenotypically identified using colony morphology, gram reactions and catalase activities, and API (Analytical Profile Index) 50 CHL test kit (BioMerieux, Inc., France) was used for biochemical identification. Gram positive and catalase negative colonies were selected and stored at -80°C in TSB medium containing 20% (v / v) glycerol (Schwebke, 2001).

Inoculation of API50 CHL strips was performed according to the manufacturer's recommendations. The presence or absence of carbohydrate fermentation in the strips was checked at 37°C for 48 hours after. The results were evaluated using the address https://apiweb.biomerieux.com/. 16S rDNA sequence analysis was used for the molecular identification of strains.

Identification of lactobacilli by 16S rDNA sequencing

Genomic DNA isolations of the strains were performed with Thermo Scientific GeneJET Genomic DNA Purification Kit (Kit No: # K0721). The repeat 16S rDNA sequence regions in DNA samples isolated - 27F forward (5 'AGA GTT TGA TCM TGG CTC AG3') and 1429R reverse (3 'GGT TAC CTT GTT ACG ACT T5') - were performed by using universal primers (Frank *et al*., 2008) and PCR process was conducted with Thermo Fisher Scientific Arctic Thermal Cycler 5020.

Low pH and high salt tolerance

The survival rates of the identified lactobacilli at low pH and high bile salt were measured by modification from previous studies (Maragkoudakis *et al.*, 2006). The cultures which were activated for 18 hours in MRS broth were centrifuged at 3000 xg for 15 minutes (4°C) and the cells were precipitated. The precipitate was washed twice with sterile phosphate buffered saline (phosphate-buffered saline (PBS) and resuspended again in phosphate buffer (pH 7.2) to 8.5 ± 9.1 log CFU/ml. 100 µl from the prepared PBS buffer was injected to MRS liquid media with pH 2.0, 2.5 and 3.0 and containing 0.3%, 0.5% and 1% (w/v) bile salt (oxgall, Sigma) and incubated at 37°C for 3 hours. Samples were collected from samples at hours 0 and 3 of incubation, and serial dilutions were performed up to 10^{-7} level and smears were cultivated in MRS solid media in 3 parallel forms. After incubation of the strains overnight at 37°C, colonies in the control and test groups were counted and their numbers were determined as log CFU/mL.

Survival rate (%) = (log cfu N1/log cfu N0) x 100

N1: The number of live microorganisms in the test group

N0: The number of live microorganisms in the control group

Antibiotic susceptibility assay of isolates

Antibiotic susceptibility of isolated strains was determined by disk diffusion method

(Georgieva *et al.*, 2015) against commonly used antibiotics (vancomycin, teicoplanin, penicillin, ampicillin, tetracycline, rifampicin, chloramphenicol, erythromycin, ciprofloxacin, gentamicin, cefazolin, clindamycin, tobramycin, amikacin, cefoperazone, aztreonam, streptomycin, netilmicin, imipenem, and ceftazidime). The density of the activated cultures was adjusted to 0.5 McF (625 nm absorbance = 0.08-0.1) in tubes containing sterile saline and homogenously spread to MRS agar plates with the drigalski spatula. The antibiotic discs were placed on the petri dishes with a disc dispenser and allowed to incubate at 37°C for 24 hours. At the end of the incubation period, the diameters of the zones around the antibiotic discs were measured by calliper in millimeters. The antibiotic susceptibility levels of the strains were evaluated according to the 2017 criteria set by EFSA (European Food Safety Authorit). Based on the standards, it was determined as Resistant (R), Semi-sensitive (I) and Sensitive (S)

Antimicrobial activity assay of isolates

In this study, the indicator microorganisms obtained from Microbiology Culture Collection, Faculty of Arts and Sciences and the clinical isolates obtained from the Microbiology Laboratory, Medical Faculty of Kırşehir Ahi Evran University were used respectively. In order to determine the efficiency of the inhibitory agents in supernatant fluids of the isolates, well diffusion method was used. *B. cereus* (709) Roma, *B. cereus* CU1065, *B. subtilis* (American Type Culture Collection- ATCC) 6633, *C. albicans* ATCC 90028, *C. albicans* 10098, *C. albicans* Y-1200-NIH, *C. tropicalis* ATCC 13803, *E. coli* ATCC 25922, *E. faecalis* ATCC 29212, *K. pneumoniae* ATCC 13883, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 29213, *Candida glabrata* AEÜ1, *E. coli* AEÜ2, *E. coli* AEÜ3 [Broad Spectrum Beta Lactamase positive (GSBL+)], *E. faecalis* AEÜ4, *K. pneumoniae* AEÜ5, *P. mirabilis* AEÜ6 and *S. aureus* AEÜ7 clinical isolates were used as indicator organisms.

The lactobacilli, which was activated successively twice in MRS broth (pH 6.5) at 37°C, was centrifuged at 10.000 x g for 10 min (4ºC). The supernatant resulting from the centrifugation was sterilized by microfiltration under sterile conditions through a 0.45 µm (Milipore, USA) disposable filter.

After pathogen test bacteria were activated for 18 hours at 37°C in the appropriate media, the final concentration of the cultures was distributed homogenously by a sterile drigalski spatula to Mueller Hinton Agar (Merck) and Potato Dextrose Agar (Merck) media at 1 x 109 CFU/mL. Wells (6 mm) were formed on the plates and 100 µL of sterile supernatants obtained from each isolate was transferred to these wells. After the plates were kept at room temperature for 2 hours, the bacteria were allowed to incubate at 37°C and yeast at 30°C for 24 hours. Antimicrobial activity was assessed by measuring the zone diameter (in mm) around the wells and taking the average of three studies (Nami *et al.*, 2014a; Maldonado *et al.*, 2012).

Detection of hydrogen peroxide production

A semi-quantitative method was used to determine the hydrogen peroxide production capabilities of lactobacilli (Gil *et al.*, 2010). The cultures that were activated overnight in MRS liquid broth were allowed to incubate at 37°C for 48 hours in MRS solid broth. Selected colonies (Macherey-Nagel Quantofix Peroxide, Germany) were treated with peroxidase-containing strips and the image obtained in different shades of blue on the test strip was compared with the manufacturer's recommended color scale. The results were evaluated in consideration of the negative test ranges of 1-3, 3-10, 10-30 and 30-100 mg/L indicated on the test kit.

Auto-aggregation and co-aggregation assays

The method practiced by Juárez Tomás et al. (2005) was applied for determining auto-agression characteristics of lactobacilli. The cultures activated overnight in MRS broth were centrifuged at 6000 x g for 15 minutes. After the

precipitate is washed twice with PBS buffer (pH 6.2), it was resuspended in the UV Spectrophotometer (Thermo Scientific Evolution 60S) with PBS at 600 nm and 0.6 optical density (OD). The same process was also prepared for use in the coaggregation study for *Candida albicans* ATCC 10231, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 strains. For each microorganism, cell suspensions (4 ml) (auto-aggregation) or each lactobacilli (2 ml) and pathogen (2 ml) from microorganisms were mixed in equal volumes and the resultant mix suspension (co-aggregation) was vortexed for 10 seconds and allowed to rest at room temperature for 4 hours. During the incubation, 0.1 ml was taken from the upper portion of cultures and diluted with 3.9 ml MRS liquid broth and culture ODs before (A_0) and after (A_4) the rest were read in UV spectrophotometer at 600 nm (A_{600}). Auto-aggression percentage was calculated based on the formula 1- (A_4 /A_0) x 100. The coaggregation percentage was calculated by the following formula:

$$\text{Co-aggregation}\,\% = \frac{(AX + AY/2) - A(X+Y)}{AX + AY/2} \times 100$$

x & y: 2 genera in the control tubes

x+y: Mixture

In order to observe the coaggregation study, 500 ul of each lactobacilli isolate was mixed with the suspension containing *C. albicans* ATCC 10231, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 cell fluids (500 ul) at the same concentration, and after a short vortexing, the samples were allowed to stir in the shaker (50 rpm) for 4 hours. Thereafter, a drop of gram suspension taken from this suspension was monitored under 100x magnification under the light microscope (Leica DM500). Specimens were classified by the density of bacteria clusters (+1 to +4) (Younes *et al.*, 2012; Santos *et al.*, 2016).

Determining enzyme profiles of vaginal lactobacilli

The cultures developed in MRS medium for 18 hours were centrifuged 10.000 g, 10 min, 4°C) and the precipitant was suspended with 2 mL of sterile saline (0.89% NaCl) solution and the cell density was adjusted to 5-6 McFarlanda. 65 µL of the suspension prepared in the wells of the enzyme impregnated strips in the API ZYM kit (Biomerieux, France) was added and the kits were allowed to incubate at 37°C for 4-5 hours. After incubation, 1 drop of ZYM A and 1 drop of ZYM B reagents were added drop wise to the wells and allowed to rest for 5 minutes and kept under 1000 W light source for 10 sec (Charteris *et al.*, 2001). The color changes and densities of the strips were evaluated according to the manufacturer's recommendations.

Adhesion to Uroepithelial Cells

Uroepithelial cells were obtained from the urine of healthy women with high epithelial cell density, who applied to Ahi Evran University Education and Research Hospital in Kırşehir. Uroepithelial cells and cultures activated overnight were washed twice with PBS and resuspended in PBS to achieve a cell density of 0.5 McF (625nm = 0.08-0.1). Uroepithelial cells without bacterial culture were used as negative control. Prepared bacterial cells and uroepithelial cells were mixed in equal volumes and allowed to incubate at 37°C for 3 hours. After incubation, the mixture was washed with PBS and then resuspended with PBS to eliminate bacteria that were not adhered to the epithelial cells. In order to observe the capability of the lactobacilli to adhere to epithelial cells, gram staining of the mixture suspended with PBS was performed and the preparations were observed at 100x magnification under the light microscope (Leica DM500). The degree of adhesion of bacterial cells to epithelial cells is ranked between +1 and +4 (Al-Zubaidy *et al.*, 2001).

Cancer cell lines and development conditions

HeLa and Caco-2 cell lines were used to determine the anticancer activity of the secretory metabolites of the vaginal isolates on tumor cells. Hela (ATCC CCL2) and Caco-2 (ATCC HTB-37) cell lines are commercially available. HeLa cells are routinely developed in RPMI 1640 medium containing 10% fetal bovine serum, gentamicin, penicillin, and streptomycin antibiotics whereas Caco-2 cells were developed in EMEM medium containing L glutamine, $NaHCO_3$, Na pyruvate, non-essential amino acid, 20% fetal bovine serum in an incubator with CO_2 (Nüve EC 160). All experiments were conducted at 37°C at 5% CO_2 atmosphere (Merghoub *et al.*, 2009).

During cell passage of cell cultures, as cell lines covered the culture dish their medium was taken by a pipette and the culture dish surface was washed with PBS. Wash buffer was then removed and 1-2 ml of 0.25% trypsin-EDTA solution was added depending on the type of cell to separate the cells from the culture dish surface and from each other for 5 minutes. 4-5 ml medium was added to inactivate trypsin and passages was applied at the desired level and cells were transferred to new culture media (Parsian *et al.*, 2016).

Preparing Culture Supernatants

After the strains with probiotic characteristics were developed at 37°C for 18 hours, they were centrifuged 5.000 g, 10 min, 4°C). The pH of the bacterial supernatants was adjusted to 7.2 (0.22 µm Milipore, USA). The lyophilization process of the sterilized supernatants was carried out at -58°C in the lyophilization device (Labfreez FD-10-R) (Haghshenas *et al.*, 2014).

XTT (Cell proliferation kit) test of vaginal lactobacilli

XTT (Biological Industries, Israel) kit was used to evaluate the anti-proliferative action of vaginal lactobacilli on HeLa and Caco-2 cells. Cells were cultured in 96-well plates overnight (24 h) in CO_2 incubator and then lyophilized culture supernatants were treated with cells for 72 hours. Relative growth amounts were determined by XTT agent (phenazine methosulphate) added to wells (Parsian *et al.,* 2016).

Statistical Analyses

A completely randomized experimental design was used with three replications in 10 x 2 and 6 x 4 factorial arrangements. One-way analysis of variance was also used. Tukey HSD and Dunnet multiple comparison tests were used to find out which group originated the difference between the groups. The normality assumption in the analyses was examined by Kolmogorov-Smirnov and Shapiro Wilk tests. Statistical analyses were performed using SPSS (version 20.0, SPSS Inc, USA) statistical package program. In the analyses, the significance level was determined as $p < 0.05$ and $p < 0.01$.

RESULTS

Idendification of isolates

In our study, ten lactobacilli strains with potential probiotic properties were selected among the strains isolated from the vaginal flora of healthy Turkish women aged 18-45. The selected strains are resistant to high bile salt and low pH environment, with good antimicrobial and antagonistic activity. Test bacteria selected according to morphological and biochemical characteristics of gram positive, catalase negative, bacilli appearance. Biochemical and molecular identification of strains were performed by sequence analysis of API-50 CHL assay and 16S rDNA gene regions, respectively. According to API 50 CHL test, *Lactobacillus plantarum*, *Lactobacillus rhamnosus* and *Lactobacillus paracasei* strains were compatible with 16S rDNA results. According to the results of 16S rDNA, 40% of the selected strains are *L.*

plantarum and 30% are *L. rhamnosus* while 10% are *L. acidophilus*, *L. paracasei* and one species can not be identified. Comparative table, strain numbers and NCBI (National Center for Biotechnology Information) gene bank numbers of selected strains according to 16S rDNA and API 50 CHL test kit are given in Table 1.

Table 1. Comparative table of selected isolates according to 16S rDNA and API 50 CHL test kit

Isolate number	API 50 CHL	16S rDNA	Genbank number
L12	*Lactobacillus rhamnosus*	*Lactobacillus rhamnosus*	MF155772
L13	*Lactobacillus rhamnosus*	*Lactobacillus rhamnosus*	MF155773
L14	*Lactobacillus gasseri*	*Lactobacillus acidophilus*	MF155774
L15	*Lactobacillus rhamnosus*	*Lactobacillus rhamnosus*	MF155775
L16	*Lactobacillus plantarum* 1	*Lactobacillus plantarum*	MF155776
L17	*Lactobacillus plantarum* 1	*Lactobacillus spp.*	MF155777
L18	*Lactobacillus plantarum* 1	*Lactobacillus plantarum*	MF155778
L19	*Lactobacillus plantarum* 1	*Lactobacillus plantarum*	MF155779
L20	*Lactobacillus paracasei*	*Lactobacillus paracasei*	MF155780
L21	*Lactobacillus paracasei ssp. paracasei* 1	*Lactobacillus plantarum*	MF155781

Low pH and high salt tolerance

Lactobacilli were isolated from vaginal swabs and their resistance to acid and bile salts were determined. All strains could not survive at the end of the hours 3 in the pH 2.0 environment. While the viability of the strains was 67.6% at pH 2.5, this rate increased to 89.3% at pH 3.0. As shown in Table S1 (Supplementary Table 1), the resistance rates of L16, L18 L19 and L21 strains to high bile salt media were found to be high, and all other strains were found to be resistant to different concentrations of bile salts (Table S1).

Antibiotic susceptibility

The selected isolates were found to be resistant to ciprofloxacin, gentamicin, tobramycin and amikacin antibiotics whereas all strains were observed to be susceptible toward tetracycline, erythromycin, penicillin, ampicillin, imipenem, chloramphenicol, antibiotics. It was determined that all strains were resistant against vancomycin and teicoplanin antibiotics from glycopeptide group except L14 and L21 strains. The antibiotic resistance profiles of the strains are given in Table S2.

Antimicrobial activity assay

In the study where the antagonistic activity of selected vaginal isolates on pathogens that cause urogenital infections and are clinically important was investigated. It was observed that all strains showed antagonistic activity against *C. glabrata* AEÜ1 ve *K. pneumoniae* AEÜ5 strains but none of the strains formed an inhibition zone against *C. tropicalis* ATCC 13803 and *P. mirabilis* AEÜ6 strain. It was determined that isolates showed antimicrobial activity against *E. coli* ATCC 25922, *E. faecalis* ATCC 29212, *B. subtilis* W168, *B. cereus* RSKK 709, *B. cereus* CU1065, *C. albicans* Y-1200-NIH and *E. coli* AEÜ3 (GSBL+). The inhibition zone diameters of the strains are shown in Table S3.

Detection of hydrogen peroxide production

In our study, the rate of H_2O_2 produced by vaginal lactobacilli strains was determined by a semiquantitative method. It was determined that L19 strains produced the highest level of H_2O_2 at 10 mg/L, while L12, L6, L17 and L21 strains produced 3 mg/L and other strains did not produce any H_2O_2.

Auto-aggregation and co-aggregation assays

As seen in Figure 1, at the end of the 4 h, it was determined that the average of all strains was 71.8% and the L16 strain had a very high autoaggregation capacity with 85.2 %. L21 strain was found to have the lowest autoaggregation capacity with a rate of 63.6 %. Autoaggregation of L15 strain at 1 and 4 h is given in Figure 2. In our coaggregation study on vaginal strains, it was determined that the strains, had good coaggregation with *C. albicans* and *E. coli* but their coaggregation capabilities with *P. aeruginosa* were found to be low. As shown in Table S4, strains with the best coaggregation characteristics were found to be L13, L16, L19 and L21.

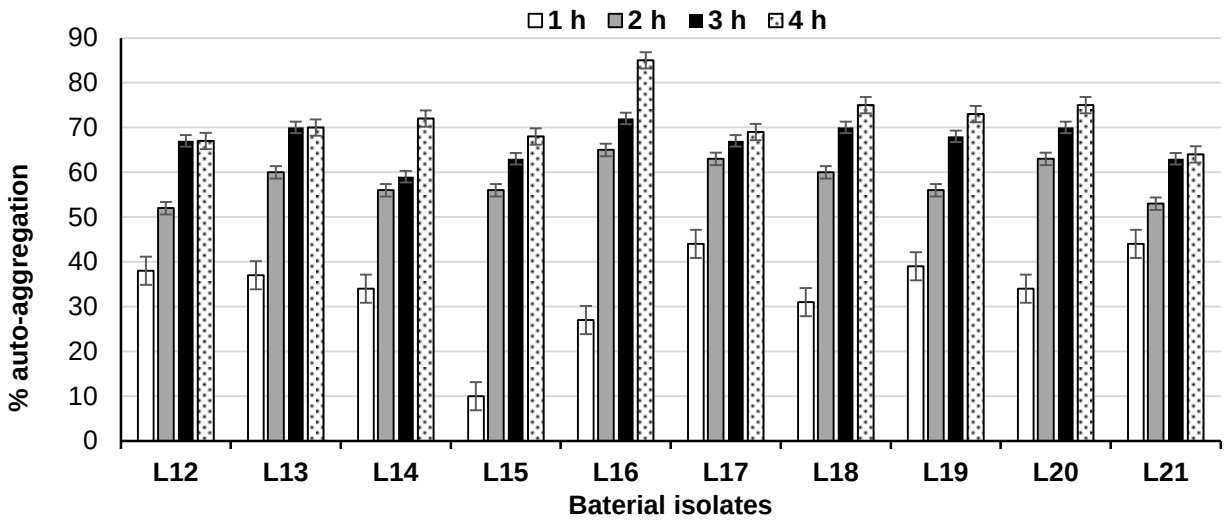

Fig. 1. Graphical representation of auto-aggregation percentage of vaginal lactobacilli

Adhesion to uroepithelial cells vaginal lactobacilli

Throughout this study, uroepithelial cells obtained from urine of healthy women were used. The adhesion degree of vaginal isolates to the uroepithelial cells at the end of the 3rd hours is shown in Table S4 and the binding images of the L16 and L19 strains are shown in Figure 3. As a result of this work, strains with the best binding rate to uroepithelial cells were determined as L16, L19 and L21.

Enzyme profiles of vaginal lactobacilli

According to the reaction results of the vaginal lactobacilli with the enzymes in the API ZYM test strips, most of the strains were found to have high levels of enzyme activity with leucine aramidase, valine arylamidase, acid phosphatase, naphthol -AS-BI-phosphohydrolase and α-glucosidase enzymes. It was also found out that in moderate reaction with esterase (C4), esterase lipase (C8), cystine arylamidase, β- galactosidase and β-glucosidase enzymes, the strains did not react with trypsin, α- mannosidase and β-glucuronidase enzymes.

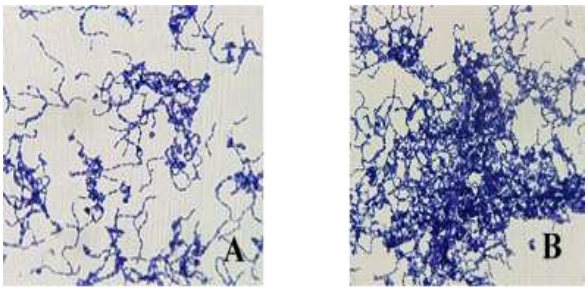

Fig. 2. Auto-aggregation of *Lactobacilli* spp. evaluated in a 4- hour study (A) and microphotographies (magnification of 100x) showing specifically auto-agregation of L15 assessed after 1h (A) and 4h (B) of incubation at room temperature.

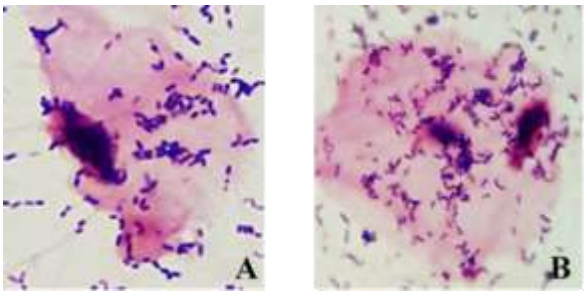

Fig. 3. Grams of L16 and L19 strains showing the degree of binding with uroepithelial cells at the end of the 3 hour; A (+3); L16, B (+4); L9.

The antiproliferative action of vaginal lactobacilli on HeLa and Caco-2 Cell lines

The antiproliferative effect of secretion metabolites of lactobacilli isolated from vaginal microbiota of healthy women on HeLa and Caco-2 cell lines were evaluated by XTT method (72 h). With LD_{50} (abbreviation of 50% lethal dose) program, in which the average lethal dose of a toxic substance is determined in toxicology. It was observed that L12 (LD_{50}: 0.009 g/ml), L13 (LD_{50}: 0.009 g/ml) and L19 (LD_{50}: 0.007 g/ml) strains created a significant antiproliferative action on HeLa. LD_{50} values showing the antiproliferative effects of vaginal lactobacilli on HeLa cells are given in Table S5.

As shown in Figure S1, approximately 90% death rate (mortality) was observed in the antiproliferative effect of the L19 strain at a dose of 0.07 g/ml and the L13 strain at 0.06 g/ml on the HeLa cells, however an approximately 75-80% death rate was observed in the antiproliferative effect on the HeLa cells of 0.004 g/ml dose of L12 metabolite.

The antiproliferative effect of L12, L13 and L19 strains on the Caco-2 cell line, which showed significant cytotoxic effect on HeLa cell line, while approximately 80% death rate was observed in the antiproliferative effect of 0.018 g/ml of L12 metabolite on Caco-2 cells, approximately 75% mortality rate was observed at a dose of 0.012 g/ml of L13 strain and 0.006 g/ml of L19 strain.

DISCUSSION

The effectiveness of probiotics in the treatment of urogenital infections and protection from recurrent vaginal infections has been proven by clinical researches (Borges *et al.*, 2014; Al-Ghazzewi *et al.*, 2016; Davar *et al.*, 2016). The studies carried out to determine the microorganisms with potential probiotic activity in vaginal microbiota have been continuing effectively.

In this study, 10 strains of lactobacilli selected from strains isolated from the vaginal walls of 60 healthy Turkish women were used. The most dominant species among the selected strains is *L. plantarum*.

In order to have a better effect on the health of probiotic microorganisms, it must first be resistant to the hard conditions of the stomach and small intestine. The low pH of the stomach and the antimicrobial effect of pepsins are known to act as an effective barrier to the entry of bacteria into the gastrointestinal system (Kaewnopparat *et al.*, 2013). Many lactobacilli strains lose their viability after exposure to low pH for 3 hours (Nami *et al*, 2014a). In our study, it was observed that none of the strains were able to maintain their vitality at the end of the hour 3 in the pH 2.0. However, the viability of the strains was 67.6% at pH 2.5 and this ratio increased to 89.3% at pH 3.0. It was found that

especially L15, L16, L18 and L20 strains were able to maintain high viability in gastric conditions and thus maintain sufficient numbers for competition and attachment with pathogenic bacteria when they reached the digestive tract (Nami *et al.*, 2014a; Nami *et al.*, 2014b). Bile tolerence is another essential criterion for the selection of a probiotic strains. The resistance to different salt concentrations in vaginal lactobacilli is strain-specific, because the behavior is different in strains identified into the same species. In the studies, L16, L18 L19 and L21 strains were found to be highly resistant to high bile salt environment as seen in Table 1, and all strains were found to be resistant to different concentrations of bile salts.

Probiotic strains should be sensitive to antibiotics. If they do not contain infectious antibiotic resistance genes, they are resistant to antibiotics (Zhou *et al.*, 2005; Argyri *et al.*, 2013). Resistance to vancomycin is the most common source of concern. Vancomycin is one of the broad-spectrum antibiotics that are effective against clinical infections caused by multidrug-resistant pathogens (Johnson *et al.*, 1990; Woodford *et al.*, 1995). In addition, antibiotic resistant probiotics are an advantage for patients and can be given to patients with antibiotics (Kaewnopparat *et al.*, 2013). In our study, all strains were found to be resistant to ciprofloxacin, gentamicin, tobramycin amikacin, netilmicin and cefoperazone antibiotics. In addition, except for two strains (L14 and L21), all other strains were found to have vancomycin and teicoplanin resistance. The strains were found to be susceptible to other tested antibiotics. The data obtained from this study are similar to the previously reported findings (Katla *et al.*, 2001; Ammor *et al.*, 2007; Kassaa *et al.*, 2014).

In our study, we investigated the antimicrobial activity of vaginal probiotics and the antagonistic effect of strains on pathogen microorganisms causing urogenital infections and vaginal candidiasis was found to be high (Van de Wijgert and Verwijs, 2019). Recent meta-analysis results show that vaginal lactobacilli are promising for the treatment and prevention of BV, but have poor efficacy for VVC. As shown in Table S3, most of the strains except *C. tropicalis* ATCC 13803 were found to be effective on other *Candida* strains. These strains appear promising in the development of well-focused probiotics based on new evidence targeting female urogenital system diseases.

Hydrogen peroxide is an active compound produced by vaginal lactobacilli, and has been proven by several studies that have been shown to protect the vaginal microbiota against recurrent urinary tract infection and various vaginal infections (BV, gonorrhea, HIV) by H_2O_2-producing lactobacilli (Knezević et al, 2005). In this study, we investigated the H_2O_2 production capabilities of vaginal strains with a semiquantitative method, the strain with the highest production ability was identified as *L. plantarum* L19. This result differs from the results of the other researchers who used semiquantitative methods (Wilks *et al.*, 2004).

The autoaggregation and coaggregation capabilities of lactobacilli are one of the most important probiotic properties, allowing the pathogen microorganisms to serve as a barrier against colonization of mucosal surfaces (Guan *et al.*, 2017). The strains isolated in our study have different autoaggregation capacities. *L. plantarum* L16 (85.2%) constitutes the highest percentage of autoaggregation followed by L18 and L20.

The presence of the highest autoaggregation strain as *L. plantarum* L16 may be that *L. plantarum* strains contain LysM regions capable of binding to cell surfaces and polymers containing N-acetylglucosamine (Sánchez *et al.*, 2010). The presence of strains having different autoaggregation capacity in the present study can be explained by the effect of such specific compounds.

In the vaginal coaggregation study, *C. albicans* ATCC 10231, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 strains were used to

investigate the relationship of lactobacilli with pathogenic bacteria. Only some lactobacilli gather in clusters and prevent the growth of pathogens (Kassaa *et al.*, 2014). The coaggregation of lactobacilli species with *Candida* strains is very significant in protecting against vaginal yeast infections because lactobacilli can create a small environment around pathogens via coaggregation and can inhibit the spread of pathogens by synthesizing high amount of inhibitory agents (Verdenelli *et al.*, 2014). It was observed that all strains except L14, L17 and L20 had high coaggregation ability with *C. albicans* in the present study. In addition, vaginal lactobacilli were found to be well coaggregation with *E.coli*, but they had low coaggregation ability with *P. aeruginosa*.

FAO (Food and Agriculture Organization) reported that one of the most important tests in potential probiotic probiotics is the adhesion of probiotic microorganism to mucin and human epithelial cells (WHO, 2002). The binding of lactobacilli to vaginal epithelial cells is defined as the first step in the formation of a barrier to prevent colonization of opportunistic pathogenic microorganisms (Claudia Otero *et al.*, 2007). The binding of lactobacilli to epithelial cells is due to their biofilm characteristics, as well as autoaggregation and surface hydrophobicity (Dunne *et al.*, 2001). Several lactobacilli cell surface proteins have been characterized as putative competitive exclusion factors responsible for the suppression of pathogen invasion (Nishiyama *et al.*, 2015). In our study, uroepithelial cells obtained from urine of healthy women were used to determine the binding capacity of lactobacilli isolated from vaginal microbiota to epithelial cells. The strains showing the best binding to uroepithelial cells were L16, L19 and L21.

According to API-ZYM test kit, most of the strains have high activity with leucine aramidase, valine aramidase, acid phosphatase, naphthol - AS-BI-phosphohydrolase and α-glucosidase enzymes in our study. None of the strains showed any reaction with trypsin, α-mannosidase and β-glucuronidase enzymes. When compared with other studies (Herreros *et al.*, 2003). it was determined that the enzyme activities of the strains were high.

Probiotics are microorganisms with beneficial health effects and different therapeutic activities and anti-carcinogenic characteristics. Studies have shown that probiotics have high apoptic and antiproliferative effects on different cancer cell lines such as breast, bladder, colon, cervical, liver, and stomach (Ouyang *et al.*, 2019). In the literature studies, it was observed that the studies conducted by the researchers mainly focused on the efficacy of probiotics on colorectal cancer (Hendler and Zhang, 2018). In our study, after the antiproliferative effect of the lactobacilli on the cervical cancer cell line, which was primarily isolated from vaginal microbiota, was determined, strains with antitumor activity on HeLa cells were investigated for antiproliferative action on the Caco-2 cell line.

Regarding the antiproliferative action of the metabolite products obtained as a result of lyophilization of the lactobacilli supernatant isolated from vaginal microbiota on the HeLa and Caco-2 cancer cell lines, it was observed that as the dose of the metabolites tested increased, the cytotoxic action on the cells and the rate of viability in the cell lines decreased. Based on the study, it was observed that all strains had a significant antiproliferative action on HeLa cell line and especially L12 (LD50: 0.009 g/ml), L13 (LD50: 0.009 g/ml) and L19 (LD50: 0.007 g/ml) strains resulted in a signification action. The highest antiproliferative action on the HeLa and Caco-2 cells was found to vary between 75-90% at the highest doses of the metabolite products of the L12, L13 and L19 strains.

CONCLUSION

In our study which investigated effective probiotic strains in maintaining vaginal health and protection from vaginal and urogenital

infections, it was observed that different probiotic characteristics of different strains were prominent. In particular, strains L16, L19 and L21 show strong probiotic activity and are likely to contribute to the development of new probiotic products that may be effective in the prevention and treatment of vaginal and urogenital infections. Each probiotic strain is specific and its efficacy should be investigated. Each probiotic strain is specific and the strains need to be clinically well characterized by in vivo and in vitro studies.

In our study, it was determined that various metabolite supernatants belonging to L12, L13 and L19 strains were found to have antiproliferative effect on cervical and colorectal cancer. The data obtained indicate that the metabolites of these strains are possible candidates that can be used to kill cancerous cells or to prevent cancer. It is of great importance that the strains alone or in combination with other probiotic microorganisms can contribute to the development of new probiotic formulations as oral, vaginal, dry powder or suspension.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

The decision of the ethics committee of the study was taken from Kırıkkale University Ethics Committee with the decision no 25/02 on 27.10.2014.

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