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ANTIPROLIFERATIVE EFFECT OF *LACTOBACILLUS PLANTARUM* L4 STRAIN ISOLATED FROM CERVICOVAGINAL MICROFLORA ON HELA CANCER CELL LINE

SERVİKOVAJİNAL MİKROFLORADAN İZOLE EDİLEN *LACTOBACİLLUS PLANTARUM* L4 SUŞUNUN HELA KANSER HÜCRE HATTI ÜZERİNDEKİ ANTİPROLİFERATİF ETKİSİ

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ABSTRACT

Objective: *Lactobacillus* has been shown to inhibit proliferation of various cancer cells, but the effects of vaginal *Lactobacillus* on cervical cancer cells have rarely been reported. The goal of this investigation was to assess the anti-proliferative effect on cancer cell line HeLa (Human Cervical Carcinoma Cell) and potential probiotic properties of *Lactobacillus plantarum* L4 isolated from cervicovaginal flora of healthy women in Turkey.

Materials and Methods: Molecular identification of the species was performed by 16S rDNA analysis. Probiotic properties of the L4 strain were investigated by conventional methods. Human Interleukin-10 (IL-10) and Tumor Necrosis Factor- alpha (TNF-alpha) ELISA kits were used in the evelation of the immune modulator effect of the L4 strain. The antiproliferative effect of the L4 strain on the HeLA cell line was performed using the XTT kit.

Results: *L. plantarum* L4 strain exhibited strong probiotic properties. The L4 strain showed an anti-inflammatory effect on HeLa by reducing the production of TNF-α and increased IL-10 production. The greatest antiproliferative effect of *L. plantarum* L4 strain on HeLa cells was observed at the highest dose of the metabolite 0.0006 gr/ml with a death rate of 90-95% while the number of living cells was found to be between 5-10%. The strain showed no anticancer effect on human umbilical vein endothelial cells (HUVEC).

Conclusion: *L. plantarum* L4 strain, with strong probiotic properties, can be considered a promising treatment candidate for HPV cancer due to its immunomodulatory effect and high antiproliferative effect, even in very small doses.

Keywords: *Lactobacillus plantarum*, antiproliferative effect, HeLa, IL-10, TNF-α

ÖZET

Amaç: *Lactobacillus* spp. bakterilerinin çeşitli kanser hücrelerinin proliferasyonunu inhibe ettiği gösterilmiştir. Ancak, vajinal *Lactobacillus*'ların rahim ağzı kanseri hücreleri üzerindeki etkileri nadiren bildirilmiştir. Bu çalışmada Türkiye'de yaşayan sağlıklı kadınların servikovajinal florasından izole edilen *L. plantarum* L4 suşunun çeşitli probiyotik karakterleri ve HeLa kanser hücre hattı üzerindeki antiproliferatif etkisi araştırılmıştır.

Gereç ve Yöntemler: Türün moleküler tanımlaması 16S rDNA analizi ile gerçekleştirilmiştir. L4 suşunun probiyotik özellikleri geleneksel yöntemlerle belirlenmiştir. L4 suşunun immun modilatör etkisini belirlemede insan Interleukin-10 (IL-10) ve Tümör Nekroz Factor-alfa (TNF-α) ELISA kitleri kullanılmıştır. HeLA hücre hattı üzerindeki antiproliferatif etkisi XTT kiti kullanılarak gerçekleştirilmiştir.

Bulgular: L4 suşu güçlü probiyotik özellikler sergilemiştir. L4 suşunun, TNF-α üretimini azaltarak HeLa hücreleri üzerinde anti-inflamatuar bir etki gösterdi ve IL-10 üretiminin artırılmasını sağladı. *L. plantarum* L4 suşunun HeLa hücreleri üzerinde en büyük antiproliferatif etkinin metabolitin 0,0006 gr/ml'lik en yüksek dozunda %90-95 düzeyinde ölüm oranı gözlemlenirken canlı hücre sayısının %5-10 arasında olduğu görülmüştür. İnsan göbek ven endotel hücreleri (HUVEC) üzerinde herhangi bir antikanser etkisi olmamıştır.

Sonuç: Güçlü probiyotik özelliklere sahip *L. plantarum* L4 suşu, immunmodilatör etkisi ve çok küçük dozlarda bile yüksek bir antiproliferatif etki göstermesi sebebiyle HPV kanseri için umut verici bir tedavi adayı olarak kabul edilebilir.

Anahtar Kelimeler: *Lactobacillus plantarum*, antiproliferatif etki, HeLa, IL-10, TNF-α

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INTRODUCTION

Cervical cancer (CVC) is the fourth most common cancer in women in terms of mortality and morbidity, with 570,000 new cases seen worldwide each year. The number of people who lost their lives in 2018 due to CVC is 311,000 (1). Human papilloma virus (HPV) is the biggest cause of CVC. More than 200 HPV genotypes have been identified, and there are low-risk and high-risk types of the virus (2). Low-risk HPV types can generally cause benign abnormalities and genital warts, while HPV types in the high-risk category can cause cervical, vulvar, penile, anal, and oropharyngeal cancer types (3).

Almost all women are infected with HPV types at least once during their lifetime. Only some individuals have the risk of persistence of oncogenic HPV types, premalignancy and progression to invasive cervical cancer (3, 4). The changing clinical picture depends on the type of virus, the localization of the lesion, the immunological status of the individual (more severe in pregnant women and those with immunodeficiency) and the nature of the epithelium (5). Several risk factors have also been identified, including smoking, sexual and reproductive factors, and human immunodeficiency virus HIV infection (6). In recent studies, there has been increasing evidence that cervicovaginal microbiota may be an important cofactor in the etiology of CVC (7-10).

The vaginal microbiota (VM) of healthy premenopausal women predominates in terms of the *Lactobacillus* species (11-13). Bacteria of the genus *Lactobacillus* protect the host against various genital infections by blocking the adhesion of pathogens in the VM, producing hydrogen peroxide (H_2O_2), diacetyl, acetaldehyde, reuterine, bacteriocin and bacteriocin-like substances, and antimicrobial substances, controlling the proliferation of pathogenic bacteria and ensuring that microflora is in balance (14). *Lactobacillus* and accompanying dysbiosis deficiency in VM increase the risk of genital infection (15, 16). In contrast, the presence of *Lactobacillus* spp. in the VM has been shown to decrease HPV infection intrauterine intraepithelial neoplasia and CVC development rate (3, 16-19). Therefore, the presence of *Lactobacillus* spp. in the VM is of great importance for prevention of HPV infection and prevention of CVC development after infection (20, 21). Studies have shown that different *Lactobacillus* species isolated from VM have the ability to modulate cancer cell proliferation and apoptosis with different roles and protective characters (16, 22).

In this study, we investigated the probiotic properties of the *Lactobacillus plantarum* L4 strain isolated from VM, its effect on the production of anti-inflammatory and pro-inflammatory cytokines released by HeLa cells, and its antiproliferative effect on the HeLa cancer cell line.

MATERIALS AND METHODS

Microorganism and culture conditions

The *Lactobacillus* spp. L4 strain was isolated from VM of healthy women who applied to the Kırşehir Ahi Evran University Education and Research Hospital. The vaginal swab was taken from voluntary patients whose age range was 18-45 years, who had no symptoms of menopause, who were not protected by any birth control method, and who had not used antibiotics within three months. In this study, a voluntary consent form was read and signed before the swab sample was taken from the patient. The study was approved by the Kırıkkale University Ethics Committee (Date: 27.10.2014, No: 25/02).

The isolated *Lactobacillus* spp. L4 strain was developed in Man Rogosa Sharpe (MRS) (Merck, UK) liquid and solid media under anaerobic conditions at 37°C for 24-48 hours (23). For the biochemical identification of gram-positive and catalase negative colonies, Analytical Profile Index (API) 50 CHL assay (BioMerieux, Inc., France) was used (24). The API50 CHL assay was carried out according to the manufacturer's recommendations and the results was evaluated via https://apiweb.biomerieux.com/. The bacterial culture was stored at -80°C in a Tryptic Soy Broth (TSB) medium containing 20% (v/v) glycerol (25).

Amplification of 16S rDNA genes

Genomic DNA isolation of the strain was performed with a Thermo Scientific GeneJET Genomic DNA Purification Kit (Kit No: #K0721). The 16S rDNA was amplified by using universal primers (27F forward, 5'-AGA GTT TGA TCM TGG CTC AG-3' and 1429R reverse, 3'-GGT TAC CTT GTT ACG ACT T-5') (26). Polymerase chain reaction (PCR) was performed with a Thermo Fisher Scientific Arctic Thermal Cycler 5020.

Resistance to low pH and tolerance to bile-salt condition

The survival rate of the identified *Lactobacillus* spp. L4 strain at low pH and high bile salt was measured by modification from previous studies (27). The culture, activated for 18 hours in MRS broth, was centrifuged at 3000 x g 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 in the phosphate buffer (pH 7.2) to 8.5±9.1 log CFU/ml. 100 µl from the prepared PBS buffer was injected into the 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. At hours 0 and 3 of the incubation, samples were taken from the L4 strain serial dilutions were made up to the 10^{-7} level, and cultured in triplicate on MRS solid media. After overnight incubation at 37°C, the L4 strain was counted as log cfu/mL by counting colonies in the control and test groups.

Survival rate (%) = (log cfu N1/log cfu N0)x100
N1: Number of live microorganisms in the test group
N0: The number of live microorganisms in the control group

Determination of antibiotic resistance

The antibiotic susceptibility of the isolated *Lactobacillus* spp. L4 strain was determined by the disk diffusion method against commonly used antibiotics (28). At the end of the incubation period, the diameters of the zones around the antibiotic discs were measured by caliper in millimeters. The antibiotic susceptibility levels of the strain were evaluated according to the 2007 criteria set by NCCLS (National Committee for Clinical Laboratory Standards) such as Resistant (R), Semi-sensitive (I) and Sensitive (S) (28).

Antagonistic activity

The antagonistic activity of the *Lactobacillus* spp. L4 strain against pathogenic microorganisms, which has clinical significance, was determined by the well diffusion method. Table 2 shows the development conditions and origins of indicator microorganisms and clinical preparations used in our study. Clinical isolates were obtained from the Kırşehir Ahi Evran University, Faculty of Medicine Microbiology Laboratory. The well diffusion method (Maldonado, 2012; Nami, 2014a) was carried out with minor modifications (29, 30).

Detection of hydrogen peroxide production

The H_2O_2 production capacity of the *Lactobacillus* spp. L4 strain was determined by a qualitative method. Plates of MRS agar that contained 5 mg/ml hemin, 1 mg/ml vitamin K, 0.01 mg/ml horseradish peroxidase (Sigma–Aldrich, USA) and 0.05 mg/ml 3,3′, 5,5′-tetramethylbenzidine (Sigma–Aldrich, USA) were spotted with a loop of the culture and incubated anaerobically at 37°C for 48 h. At the end of the incubation, Color changes in colonies were observed after subsequent incubation at room temperature. Colonies with H_2O_2 production capacity were classified as medium (brown), weak (light brown), or negative (white color) according to their density (blue) (28).

Auto-aggregation and co-aggregation assays

The method practiced by Juárez Tomás et al. was applied for determining Autoaggregation characteristics of *Lactobacillus* spp. L4 strain (31). Auto-aggregation percentage was calculated based on the formula 1- (A4/A0) x100. The same process was also prepared for use in the co-aggregation study for *Candida albicans* ATCC 10231, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 strains. The coaggregation percentage was calculated by the following formula:

$$\text{Co} - \text{aggregation } \% = \frac{(\text{AX} + \text{AY}/2) - \text{A}(\text{x} + \text{y})}{\text{AX} + \text{AY}/2}\ x\ 100$$

x & y: 2 genera in the control tubes
x+y: Mixture

In order to observe the co-aggregation study, 500 ul of the L4 strain was mixed with the suspension containing *Candida albicans* ATCC 10231, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 cell fluids (500 ul) at the same concentration, and after a short vortexing, the samples were mixed 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) (32, 33).

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 L4 strain 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.

HeLa cell lines and development conditions

HeLa cell lines were used to determine the anticancer activity of the secretory metabolites of the vaginal isolates on tumor cell. HeLa cells are routinely developed in the RPMI 1640 medium containing 10% fetal bovine serum and gentamicin antibiotics (34). All experiments were conducted at 37°C at 5% CO_2 atmosphere (Nüve EC 160) (34, 35).

Preparing culture supernatants

In this study, the L4 strain developed at 37°C for 18 hours was 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 performed at -58°C in the lyophilization device (Labfreez FD-10-R) (36).

Immunomodulatory effect of *Lactobacillus* spp. L4

HeLa cells prepared at a ratio of $1x10^6$ were treated with the L4 strain in 96-well plates. They were incubated for 24 hours at 37°C in 5% CO_2. The amount of cytokines released by HeLa cells was measured using human Interleukin-10 (IL-10) and Tumor Necrosis Factor-α (TNF-α) ELISA kits (Life Tech). Measurements were carried out in

an ELISA reader device (Biobase BIOBASE-EL10A, China) at 450 nm. Cytokine levels were expressed as pg/ml of each cytokine. Experiments were performed in triplicates (37).

XTT (Cell proliferation kit) test of L4 strain

An XTT (Biological Industries, Israel) kit was used to evaluate the anti-proliferative action of the vaginal L4 strain on the HeLa cell line. HeLa cells grown in CO_2 incubator for 24 hours were incubated in (36) a serial diluted supernatant medium of the L4 strain for 72 hours. Each run was carried out with the blank control column and the cell control column. After incubation, XTT reagent (BIOTEK) was added and measurement was performed at 482 nm with the reader (36).

Statistical analyses

A completely randomized experimental design was used with three replications in 10x2 and 6x4 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

Isolation and identification of *Lactobacillus* spp. L4 strain

The *Lactobacillus* spp. L4 strain we used in our study was isolated from the vaginal flora of healthy Turkish women aged 18-45 years. The L4 strain was obtained by a culture-based method in the MRS medium (26). According to morphological and biochemical properties, the L4 strain is gram positive, catalase negative, bacillus, and coke appearance. In our study, the 16S rDNA result was found to be compatible with the API 50 CHL test result (26). According to the 16S rDNA result, the NCBI (National Center for Biotechnology Information) gene bank number of strain L4 is MF155764 (26).

Resistance to low pH and tolerance to bile-salt condition

In our study, we investigated the resistance rate of the vaginal *Lactobacillus* spp. L4 strain in a low pH environment and it was found that the L4 strain maintained its viability at 63.5% at the end of the third hour in the pH 2.0 environment. The viability of L4 strains at pH 2.5 and 3.0 was 83.6% and 91.0%, respectively. The L4 strain was found to be highly resistant to different concentrations of bile salt simulating the small intestine system under in vitro conditions (Table 1).

Antibiotic resistance

The *Lactobacillus* spp. L4 strain was found to have high resistance to antibiotics commonly used in the treatment of various infectious diseases and was found to be resistant to ciprofloxacin gentamicin, tobramycin, amikacin aztreonam, and netilmicin antibiotics. The L4 strain was found to be susceptible to penicillin, ampicillin, cefazolin, tetracycline, rifampicin, chloramphenicol, erythromycin, clindamycin, cefoperazone, streptomycin, ceftazidime, and imipenem antibiotics. The L4 strain was also found to be resistant to vancomycin and teicoplanin antibiotics from the glycopeptide group, which is important for probiotics.

Antagonistic activity of L4 strain

In this study, the antagonistic activity of the *Lactobacillus* spp. L4 strain against pathogens that cause urogenital infections was observed to show antagonistic activity on all pathogen strains except *C. tropicalis* ATCC 13803 and *B. subtilis* W168 strains. In particular, the L4 strain was found to have a very good antimicrobial effect on *C. albicans*

Table 1: Survival rates of *Lactobacillus* spp. L4 strain in low pH and high bile environments

The survival count (log cfu/ml) and rate (%) of L4 strain in different ph value at the end of 3 hours			
	Start count**	**Count after application**	**Survival rate* (%)**
pH 2.0	8.64±1.1	5.49±2.1	63.5
pH 2.5	8.57±0.3	7.17±3.3	83.6
pH 3.0	8.91±3.5	8.11±3.1	91.0
The survival count (log cfu/ml) and rate (%) of L4 strain in different bile salt environments at the end of 3 hours			
0.3%	8.94±1.5	7.65±0.6	85.5
0.5%	8.85±0.9	6.42±4.1	72.5
1%	8.64±4.7	5.45±1.0	63.0

*Survival rate=Final (cfu/mL)/control (cfu/mL)x100.
**Viable count of vaginal strains determined at 0 h; the results are representative mean±SD of three independent experiments

ATCC 90028 and *K. pneumoniae* AEU5 strains isolated from the clinical samples (29, 30). Inhibition zone diameters of the L4 strain are given in Table 2.

Detection of hydrogen peroxide production

Lactobacillus spp. L4 was classified as a strong H_2O_2 producer because of the production of an intense blue color.

Auto-aggregation and co-aggregation assays

The auto-aggregation value of the *Lactobacillus* spp. L4 strain isolated from vaginal flora at the end of the 4th hour was 78.8%. In the co-aggregation study, the co-aggregation value of the L4 strain with *C. albicans* was found to be 54.4%, 42.7% with *E. coli*, and 27.6% with *P. aeruginosa*. Figure 1 shows the co-aggregation

Table 2: Antimicrobial activity against gastrointestinal and urogenital pathogens

Test organisms	Growth condition	Origin	L4 strain
E. coli	37°C, MacConkey agar	ATCC 25922	13±1.2
E. faecalis	37°C, MRS	ATCC29212	15±2.3
P. aeruginosa	37°C, MacConkey agar	ATCC 27853	14±1.6
S. aureus	37°C, blood agar	ATCC29213	12±3.4
B. subtilis	37°C, MPA	ATCC 6633	15±0.8
B. subtilis	37°C, MPA	W168	-
B. cereus	37°C, MPA	RSKK 709 ROMA	15±0.7
B. cereus	37°C, MPA	CU1065	17±2.4
C. tropicalis	28°C, MHA	ATCC 13803	-
C. albicans	28°C, MHA	ATCC 90028	18±1.8
C. albicans	28°C, MHA	ATCC 10098	15±0.7
C. albicans	28°C, MHA	Y-1200-NIH	17±1.9
C. glabrata AEU	28°C, MHA	Clinical isolate	14±2.3
E. coli AEU	37°C, MacConkey agar	Clinical isolate	13±0.5
E. coli AEU	37°C, MacConkey agar	Clinical isolate	16±1.6
E. faecalis AEU	37°C, MRS	Clinical isolate	12±2.7
K. pneumoniae AEU	37°C, MPA	Clinical isolate	18±2.1
P. mirabilis AEU	37°C, MacConkey agar	Clinical isolate	15±0.9
S. aureus AEU	37°C, blood agar	Clinical isolate	13±2.4

Values are the means±standard deviations of triplicate measurements. ATCC: American Type Culture Collection, Virginia, USA. RSKK: Refik Saydam National Type Culture Collection.

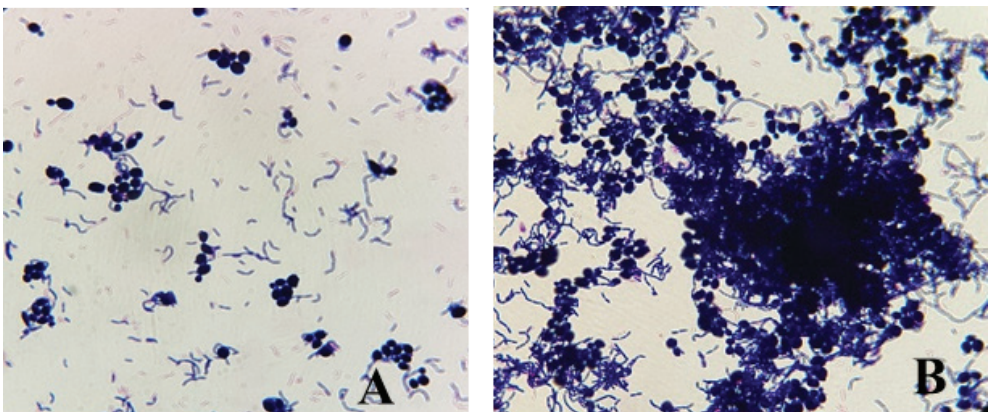

Figure 1: *Lactobacillus* spp. L4 strain *C. albicans* ATCC 10231 strain 1h (A) and 4h (B) co-aggregation at hour light microscope images (100x)

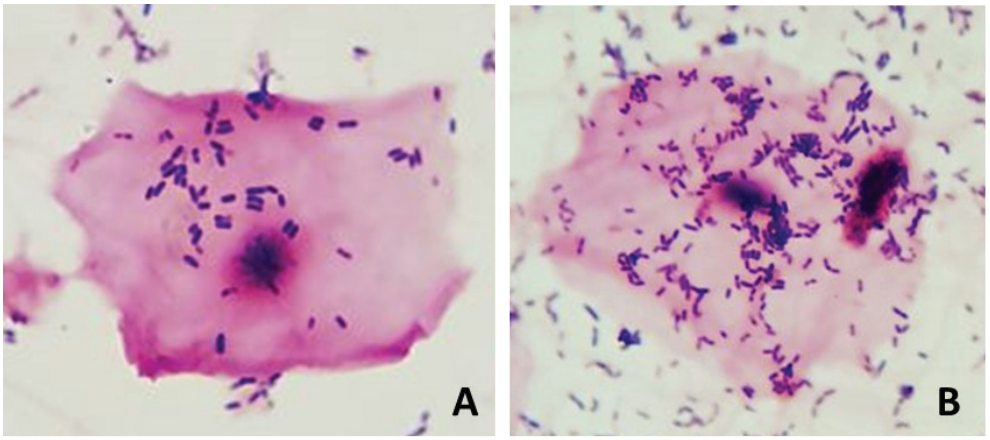

Figure 2: Binding of *Lactobacillus* spp. L4 strain to uropithelial cells, A: 1st hour, B: 4th hour 1h (A) and 4h (B) light microscope images (100x).

of the L4 strain with *C. albicans* ATCC 10231 at the end of the 4th hour.

Adhesion to uroepithelial cells

In our study, it was determined that the *Lactobacillus* spp. L4 strain had a high binding capacity to uroepithelial cells obtained from the urine of healthy women and the degree of binding was determined as (+4). The binding of the L4 strain to uroepithelial cells is given in Figure 2.

Immunomodulatory effect of L4 strain

Serving as an indicator of pro-inflammatory response, TNF-α was significantly decreased relative to control. In contrast, a significant increase in IL-10 production was observed in the sample treated with the L4 strain relative to the control (Figure 3).

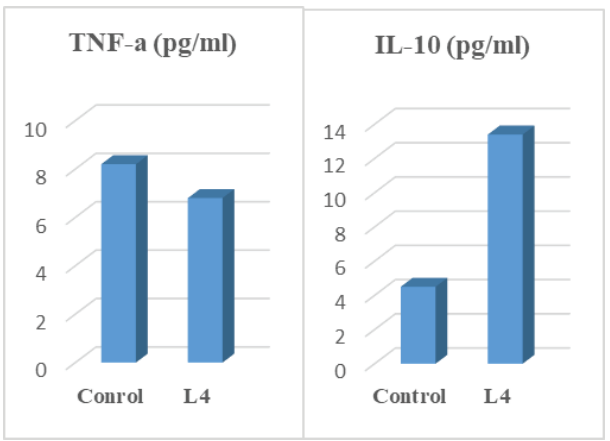

Figure 3: Effects of *Lactobacillus* spp. L4 strain on cytokine secretion by HeLa cells. The concentration of cytokine released by HeLa cells treated with 400 µg/ml of *L. plantaum* L4 strains was measured by ELISA to screen for changes in Tumor necrosis factor-α (TNF-α) and Interleukin-10 (IL-10) (n:5/group). *p<0.05: significant differences from the control.

The antiproliferative action of vaginal L4 strain on Hela cell line

The antiproliferative action of secretion metabolites of *Lactobacillus* spp. L4 isolated from vaginal microflora of healthy women on the HeLa cell line weas evaluated by the XTT method (72 h). With the LD50 (abbreviation of 50% lethal dose) program, in which the average lethal dose of a toxic substance is determined in toxicology, it was observed that L4 (LD_{50}: 0.0006 gr/ml) (36). As shown in Figure 2, deaths occurred at 90-95% at the highest metabolite dose of 0.0006 gr/ml, which showed the greatest antiproliferative action of the L4 strain on HeLa cells. When the antiproliferative effect of the L4 strain on HUVEC normal cells was also evaluated, it was seen that it had no effect on it. More than 94% of the HUVEC cells were found to grow well.

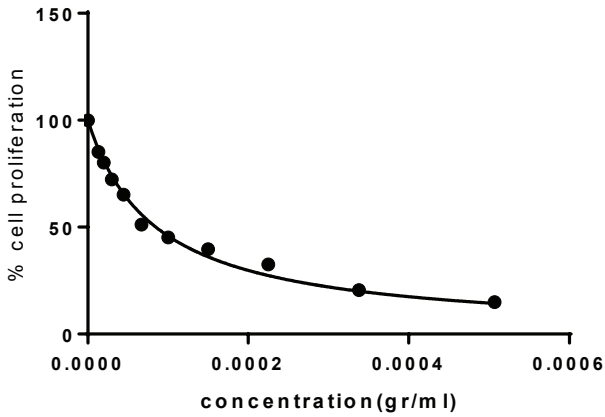

Figure 4: Antiproliferative effect of extracted metabolite products of *Lactobacillus* spp. L4 strain on HeLa cells using XTT cell proliferation kit.

DISCUSSION

Recent studies have also shown that probiotic vaginal *Lactobacillus* has an important protective role against urogenital infections and even has a protective and therapeutic role against various types of cancer, especially cervical cancer (9, 10, 16). Individual differences in *Lactobacillus* species are observed in the composition of women's vaginal flora in different geographical regions according to race and ethnicity (38). *L. crispatus*, *L. jensenii*, and *L. gasseri* are the predominant species in the vaginal microbiota that play a vital role in maintaining the balance of the vaginal microbiota, however *L. plantarum* is rare in vaginal flora (15). Each probiotic microorganism has its own biological effects and properties. Therefore, these organisms should be investigated in terms of various biological properties. The results show that this microorganism is susceptible to most antibiotics and can be regarded as a probiotic with optimal antagonistic and anticancer properties.

Probiotics were recently defined as live microorganisms which confer a health advantage on the host upon consumption in sufficient and suitable amounts (39). These two properties constitute the most important probiotic microorganism selection criteria. Several *Lactobacillus* strains lose their viability upon exposure to low pH for 3 h. (40). The L4 strain displayed high survival rates under low pH (91%) at pH 3.0 and high bile salt conditions (85.5%). Our outcomes on the viability of the L4 strain at pH 3.0 agree with the previous data (40, 41).

In our study, the *L. plantarum* L4 strain showed superior antagonistic activities against various pathogenic microorganisms (e.g. bacteria and fungi) causing gastrointestinal and urogenital infections. Previous studies have reported limited anti-pathogenic activity for this species (42-44). Vulvovaginal candidiasis (VVC) is one of the most common lower genital tract infections in women (45). In clinical studies, a variety of probiotic *Lactobacillus* spp. strains were found to be significantly effective in the treatment of VVC and in reducing recurrence. This activity is mainly attributed to the production of antimicrobial substances or organic acids (e.g. lactic acid) and metabolites such as hydrogen peroxide, which are toxic to *Candida* (46). VVC disease is likely to recur even in healthy individuals. Therefore, probiotic strains that have an antimicrobial effect on the *Candida* species are important in vaginal microflora. In our study, the *L. plantarum* L4 strain was found to be effective on the development of different *Candida* species.

Strains with a probiotic character should be sensitive to antibiotics administered in the clinic and should not have antibiotic resistance genes if they are resistant to antibiotics. In our study, the antibiotic susceptibility of the vaginal *Lactobacillus* spp. L4 strain was also tested. *Lactobacillus* has been shown to be resistant to aminoglycosides, beta-lactam antibiotics, cephalosporins, and glycopeptide antibiotics (46, 47). Similarly, in our study, it was found that the aminoglycosides were resistant to gentamicin, tobramycin, amikacin, and netilmicin antibiotics. Penicillin, ampicillin, cefazolin, tetracycline, rifampicin, chloramphenicol, erythromycin, clindamycin, cefoperazone, streptomycin, ceftazidime, and imipenem were also susceptible to antibiotics.

Vancomycin is one of the broad-spectrum antibiotics used in the treatment of multiple drug-resistant pathogens and various clinical infections. Therefore, resistance to vancomycin is important (29, 48). In our study, it was determined that the L4 strain showed high resistance to vancomycin and teicoplanin antibiotics from the glycopeptide group (49). This type of resistance doesn't cause concern for microorganisms with a probiotic character, as it differs from the inducible, transferable mechanism that may be in other bacteria, such as enterococci. Our results are similar to those reported previously (49, 50).

H_2O_2 is one of the important active compounds produced by vaginal *Lactobacillus*. Studies have shown that the *Lactobacillus* species with H_2O_2 production capacity can protect women against bacterial vaginosis infection (51, 52). In our study, the *L. plantarum* L4 strain was found to be a strong producer of H_2O_2. The L4 strain may be a suitable candidate for protection from genital infections.

Aggregation is an important feature of *Lactobacillus* because it can create a microenvironment around pathogens containing a high concentration of inhibiting agent and prevents the adherence of pathogens to the intestinal and/or vaginal epithelial cells. In this context, the *Lactobacillus* spp. L4 strain was determined to be a strain with high aggregation and co-aggregation ability. Cancer development and progression are thought to be associated with inflammation (53). In our study, it was observed that TNF-α value, which plays a role in proinflammatory response, significantly decreased compared to the control. In contrast, a significant increase in IL-10 production was observed in samples treated with the L4 strain relative to the control. IL-10 regulates the inflammatory response by suppressing the production of pro-inflammatory cytokines such as TNF-α (54).

This is a possible explanation for the decrease in TNF-αproduction observed in our study. It has been suggested that TNF-α is an inducer of proliferation, while IL-10 is an inhibitor of tumor growth. IL-10 plays an important role in the development of cervical cancer (55). In some IL-10 transfection studies, IL-10 has been shown to inhibit tumor growth and metastasis (56). Moreover, the decrease in IL-10 level is associated with the risk of cervical cancer (48). The results show that the L4 strain has an anti-inflammatory effect on HeLa cells and potentially

inhibits cell proliferation by reducing TNF-α production and increasing IL-10 production. This may explain the decrease in TNF-α production, which was also observed in our study. It has been suggested that TNF-α is a proliferation inducer and IL-10 is an inhibitor of tumor growth. IL-10 plays an important role in the development of CVC (55). The reduction in IL-10 level is associated with CVC (48). As a result, it shows that the L4 strain has an anti-inflammatory effect on HeLa cells and potentially inhibits cell proliferation by reducing TNF-α production and increasing IL-10 production.

The majority of anticancer studies of *Lactobacillus* are related to colorectal cancer (57, 58). Therefore, we decided to investigate the anticancer activity of the L4 strain, which has very good probiotic properties isolated from vaginal flora on HeLa cancer cells. In contrast, in this study the anticancer activity of the L4 strain on HeLa cancer cells was investigated. At the end of the study, it was found that even a very low dose of L4 strain metabolic products had a high antiproliferative effect on HeLa cells. In the present study, normal HUVEC cells were selected as controls. The results of the study showed that the L4 strain had no significant inhibitory or toxic effects on HUVEC normal cells.

The results of the meta-analysis conducted in 2019 show that there is a relationship between *Lactobacillus* species found in vaginal microbiota and HPV infection and HPV-related diseases, and it can be used as an aid in the treatment of these diseases (16, 20).

Studies investigating the anti-cancer activities of *L. plantarum* strains isolated from vaginal flora on cancer cell lines are very few. Nami et al. investigated the effect of the supernatant belonging to the *L. plantarum* 5BL strain on different cancer cell lines and observed the most important antiproliferative effect on HeLa cells (59). In the studies that different *Lactobacillus* species isolated from vaginal flora, the metabolites of the *L. acidophilus* 36YL strain have a reported cytotoxic effect on HT-29 and HeLa cells (29). Motevaselli et al. found that *L. gasseri* and *L. crispatus* strains showed a cytotoxic effect on HeLa cells (21). In another study, it was stated that the *L. fermentum* SK5 strain binds to HeLa, HT-29, and Caco-2 cells at rates of 92%, 93, and 93%, respectively and inhibits the growth of cells (28). Similarly, in our study, the greatest antiproliferative effect of the *Lactobacillus* spp. L4 strain on HeLa cells was observed as 90-95% mortality rate at the highest dose of metabolite 0.0006 g/ml, while the number of living cells was found to be between 5-10%.

CONCLUSION

Studies suggest that regular oral probiotic intake may play an effective role in gastrointestinal cancer treatment (20, 22). Vaginal probiotics may be effective in the development or prevention of gynecological cancers, as well as in vaginitis and HPV infection. The data we obtained from this study supported this result. In our study, the L4 strain with strong probiotic characters increased IL-10 cytokine production, proving that it plays an effective role on the system by inhibiting the production of TNF-α, whereas the metabolite of the *Lactobacillus* spp. L4 strain exhibited a high antiproliferative effect in bile in very small doses. The L4 strain is considered a promising treatment candidate for HPV cancer. However, additional research is needed to determine whether modulation of cervicovaginal microflora with probiotics is a preventative strategy or application to gynecological treatment.

Informed Consent: Written consent was obtained from the participants.

Ethics Committee Approval: This study was approved by the Institutional Ethics Committee of Kırıkkale University (Date: 27.10.2014, No: 25/02).

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