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Original Article

Identifying probiotic characteristics of *Lactobacillus crispatus* isolated from the vagina

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Lactobacilli prevent overproduction of pathogenic microorganisms and contribute protecting vaginal microbiota. Many probiotic microorganisms are categorized as Lactic Acid Bacteria. In this study, it was aimed identifying probiotic characteristics of *Lactobacillus crispatus* isolated from the vagina of a healthy woman. For this purpose, lactic acid, hydrogen peroxide and proteolytic activity quantities and auto-aggregation, co-aggregation and hydrophobicity abilities of *Lactobacillus crispatus*, which has been isolated and identified by 16s rRNA sequence analysis, were determined. Additionally, bile salt and acid resistance, along with antibiotic susceptibility of *Lactobacillus crispatus* were analyzed by the end of 3 hours. Lactic acid, hydrogen peroxide and proteolytic activity quantities of *Lactobacillus crispatus* were measured 2.275%, 0.334±0.075 µg/mL and 2.131±0,000 mg/mL respectively. The findings include existence of co-aggregation and auto-aggregation ability, but not hydrophobicity. By the end of 3 hours, the viability was preserved in 0.1% and 0.3% bile salt medium and, at pH 3. *L. crispatus* exhibited resistance to methicillin, metronidazole, oxacillin, and sulfamethoxazole + trimethoprim, but the bacteria exhibited susceptibility to tested the other antibiotics. This study will make an important contribution to the literature about probiotic characteristics of *L. crispatus* and our strain isolated from the vagina might be considered as a candidate probiotic.

Keywords: *Lactobacillus crispatus*/probiotic. Vaginal flora. Lactic acid bacteria.

INTRODUCTION

Lactobacilli are normal inhabitants of vaginal of healthy women. Vaginal fluid contains 10^{10} *Lactobacillus* spp. per mL (Redondo-Lopez, Cook, Sobel, 1990). Albert S. Döderlein, a German physician, described bacterium isolated from a pregnant woman's vagina as Döderlein bacillus (Lepargneur, Rousseau, 2002).

Vaginal microbiota has a critical role in preserving urogenital health (Jespers *et al.,* 2012). Lactobacilli prevent overproduction of pathogenic microorganisms and contribute protecting vaginal microbiota. Lactobacilli in the vagina compete with the pathogens in order to prevent them adhere to epithelial cells (Kaewnopparat *et al.,* 2013). Previous studies suggested that *Lactobacillus* species bind to host epithelial cell receptors to block attachment of various urogenital pathogens including Group B *Streptococcus* species, *Staphylococcus aureus*, *Gardnerella vaginalis*, *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* to epithelial cells (Fettweis *et al.,* 2012).

FAO/WHO described probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit to the host" in 2001 (FAO/WHO, 2001). Many probiotic microorganisms including *Lactobacillus* sp., *Bifidobacterium* sp., and *Enterococcus* sp. are categorized as Lactic Acid Bacteria (LAB) (Bergonzelli *et al.,* 2005). Probiotic microorganisms have been suggested as candidate microorganisms to be included in probiotics for vaginal use (Shah, 2007). Probiotic effect is strain-specific (Paolillo *et al.,* 2009). Therefore, it is important to establish the probiotic properties of lactic acid bacteria before the bacteria is developed as a

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probiotic product. In this study, it was aimed identifying probiotic characteristics of *Lactobacillus crispatus* isolated from the vagina of a healthy woman. For this purpose, lactic acid, hydrogen peroxide and proteolytic activity quantities and auto-aggregation, co-aggregation and hydrophobicity abilities of *Lactobacillus crispatus*, which has been isolated and identified by 16s rRNA sequence analysis, were determined. Additionally, bile salt and acid resistance, along with antibiotic susceptibility of *Lactobacillus crispatus* were analyzed by the end of 3 hours. There are not many studies in the literature regarding the investigation of the probiotic properties of *L. crispatus* isolated from the vagina, so the work we have done will be a guide to *L. crispatus* and will greatly contribute to the literature.

MATERIAL AND METHODS

Bacteria isolation

In this study, bacteria isolated from the vagina of a healthy woman was inoculated with de Man, Rogosa, and Sharpe (MRS) agar) and incubated for 48 hours at 37°C 5% CO_2. Following incubation, gram staining and catalase test were performed. The bacteria isolate was stored in 25% glycerol, at -80°C.

This study was approved by İstanbul Medipol University Non-Interventional Clinical Researches Ethics Board on 11/04/2013 with decision number 38. This work was supported by a grant from the Anadolu of University and Research, within the research project 1305F089 / 2016. In this project, samples were taken from vaginal smear of 30 pre-menopausal healthy women between 20-40 years of age. It was also taken into consideration that women did not use antibiotics until the last 2 months.

Genotypic identification of bacteria with 16s rRNA sequence analysis

16s rRNA sequence analysis was performed genotypic identification of the bacteria isolate. The genomic DNA of the isolate was purified using GeneJET genomic DNA purification kit (ThermoFischer Scientific). Obtained genomic DNA was used as template DNA and PCR reaction was performed for 16s rRNA gene locus. 27F 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R 5'TACGGYTACCTTGTTACGACTT-3' universal primers were used. PCR reaction components include 2.5 µL of 10X TaqBuffer (+ KCl-$MgCl_2$), 2.5 µL of 25 mM $MgCl_2$, 2.5 µL of 2.5 mM dNTP mix, 2.5 µL of 2.5 mM 27F primer, 2.5 µL of 2.5 mM 1492R primer, 0.25 µL of Taq polymerase (5 u/ µL), 11.75 µL nuclease-free ddH_2O and 1 µL of template DNA. PCR products obtained from the reaction were screened in 1% agarose gel. 1492R and 907R (5'-CCGTCAATTCMTTTRAGTTT-3') primers were used for the sequence analysis of nearly 1400 base pair region (Doi *et al.,* 2013). The sequence analysis of isolate was performed by MedSanTek Laboratory Supplies Trade & Industry Ltd.

Lactic acid production assay

The LAB isolate was incubated in the MRS broth medium at 37°C 5% CO_2 for 48 hours. Following incubation, 1 mL of fresh LAB culture was transferred to a clean flask and filled up to 100 mL by sterile dH_2O. 2-3 drops of phenolphthalein indicator were added and titrated with 0.1 M NaOH solution. The amount of NaOH was recorded. Acid produced by the culture was calculated as percent titrable acidity. Lactic acid amount that the bacteria produced was calculated by the formula below. The study was conducted in duplicate (Demirci, Gündüz, 1994).

Acidity %: 0.1 N NaOH (mL) amount used × 0.9/mL

Hydrogen peroxide production assay

The LAB isolate was incubated in the MRS broth medium at 37°C 5% CO_2 for 48 hours. Following incubation, 5 mL of dH_2O was added to LAB culture and centrifuged at 5000 x rpm for 15 minutes. The supernatant phase following centrifugation was filtered through Whatman no 42 filter paper. 4 mL of filtrate obtained from filtering process was transferred to a tube. 0.5 mL sulfuric acid, 0.5 mL ammonium molybdate and 0.5 mL potassium iodide were added to the filtrate respectively and mixed thoroughly after each chemical addition. Optical density (OD) was measured by 350 nm wavelength spectrophotometer for 3 times (Shimadzu, UV-1800). OD values were converted into µg/mL formulation based on standard curve adjusted previously. The study was conducted in duplicate (Patrick, Wagner, 1949).

Proteolytic activity assay

The LAB isolate was incubated in 5 mL non-fat milk liquid medium at 37°C 5% CO_2 for 48 hours. Following incubation, 1 mL of dH_2O and 10 mL of trichloroacetic acid (TCA) were added to the medium respectively, mixed gently, incubated at room temperature for 10 minutes and filtered through Whatman no 1 filter paper. 2.5 mL of the filtrate was transferred to a separate tube, in which 5 mL of $Na_2CO_3.Na_4P_2O_7$ solution was added and

mixed thoroughly. 1.5 mL of phenol indicator was added and shaken until dark blue color appears. The sample was centrifuged at 8000 x rpm for 15 minutes. OD of the supernatant was measured by 650 nm wavelength spectrophotometer for 3 times and the values were recorded, which were converted into µg/mL formulation based on standard curve adjusted for proteolytic activity. The study was conducted in duplicate (Kıvanç, Yapıcı, 2015).

Auto-aggregation

The LAB isolate was incubated in the MRS broth medium at 37°C 5% CO_2 for 48 hours. Following incubation, the final concentration was adjusted to 10^8 colony forming unit (cfu)/mL with McFarland Densitometer (DEN-1, Biosan) using phosphate buffer saline (PBS) with pH 6.2. Microbial suspension was spread on a microscope slide and observed by light microscope (Leica DM500) with 100x magnification. Auto-aggregation test is considered positive if the cells aggregate within 2 minutes. The results were recorded (Pascual *et al.,* 2008).

Co-aggregation

In this assay, *Escherichia coli* ATCC 25922 strain was used as test microorganism. The incubation conditions for the LAB isolate was in the MRS broth medium at 37°C 5% CO2 for 48 hours and for *E. coli* ATCC 25922 in brain-heart infusion broth (BHI) medium at 37°C for 24 hours. Following incubation, the LAB isolate and *E. coli* ATCC 25922 adjusted to 10^8 cfu/mL with McFarland Densitometer (DEN-1, Biosan) in the PBS were transferred to a separate tube and mixed for 15 seconds. Samples from both bacteria were seeded in a 24-well plate and incubated at 37°C for 48 hours while shaking gently. Following incubation, samples were taken from each well, Gram stained and samples were examined under light microscope (Leica DM500). Co-aggregation condition of the LAB isolate with *E. coli* ATCC 25922 was recorded (Pascual *et al.,* 2008).

Hydrophobicity

The LAB isolate was incubated in the MRS broth medium at 37°C 5% CO_2 for 48 hours. Following incubation, the LAB culture were centrifuged at 12000 x g, 5°C for 5 minutes, washed twice in 0.05 M K_2HPO_4 (pH: 6.5) buffer and finally solved in the same buffer. The cell suspension was adjusted to an A560 nm value (Shimadzu, UV-1800) of 1.0 with the buffer and 3 mL of the bacterial suspension is transferred to a separate tube. 0.6 mL of n-hexadecane was added in the tube and mixed for 120 seconds. The two phases were allowed to separate for 0 h at 37°C. The aqueous phase was carefully removed and spectrophotometric measurement was performed on A560 nm (Vinderola, Reinheimer, 2003).

Hydrophobicity was calculated by the formula below:

$$\%= (A_0 - A)A_0 \times 100$$

A_0: Initial absorbance value; A: Final absorbance value

Identifying acid resistance

The LAB isolate was incubated in the MRS broth medium at 37°C 5% CO_2 for 48 hours. Following incubation, the LAB culture were inoculated with the MRS broth, in which pH was adjusted to 2 and 3 by 1 mol/L HCl. The MRS broth with pH 7.2 was used as control group. Following incubation, samples were collected at 0, 1, 2 and 3-hour time points, diluted serially and 10 µL from each sample was inoculated on the petri dishes containing the MRS agar. Petri dishes were incubated at 37°C 5% CO_2 for 48 hours. Following incubation, colonies in the petri dishes were counted and results were recorded as cfu/mL (Claire *et al.,* 2006).

Identifying bile salt resistance

The LAB isolate was incubated in the MRS broth medium at 37°C 5% CO_2 for 48 hours. Following incubation, the LAB culture was inoculated with the MRS broth medium containing 0.1% and 0.3% bile salt. Bile salt free-MRS broth medium was used as control. Following incubation, samples were collected at 0, 1, 2 and 3-hour time points, diluted serially and 10 µL from each sample was inoculated on the petri dishes containing the MRS agar. Petri dishes were incubated at 37°C 5% CO_2 for 48 hours. Following incubation, colonies in the petri dishes were counted and results were recorded as cfu/mL (Pereira, Gibson, 2002).

Antibiotic susceptibility

Disc Diffusion Susceptibility Method was applied in order to clarify antibiotic susceptibility of the LAB isolate to various antibiotics. In this study, total of 12 antibiotics including ampicillin, erythromycin, gentamycin, clindamycin, methicillin, metronidazole, oxacillin,

penicillin, cephalothin, sulfamethoxazole + trimethoprim, tetracycline and vancomycin were tested. The LAB isolate incubated on the MRS agar medium at 37°C 5% CO_2 for 48 hours was adjusted to 0.5 McFarland Turbidity Standard (10^8 cfu/mL) in the 0.85% physiological saline. Microbial suspension was inoculated on Mueller Hinton Agar medium using a swab. Cultured petri dishes were dried for 15 minutes. Antibiotic discs were taken by forceps and placed on surface of petri dish under aseptic conditions. 15 minutes later, they were incubated at 37°C 5% CO_2 for 24 to 48 hours. Zone diameters around the discs were measured, recorded and the results were evaluated. The study was conducted in duplicate (Kaewnopparat *et al.,* 2013).

RESULTS

Gram staining and catalase test were performed on the isolate obtained from the vagina of a healthy woman. The isolate Gram-positive had catalase-negative activity. Genotypic identification through 16s rRNA sequence analysis suggested that the isolate was *Lactobacillus crispatus* (Accession number: LC065039.1) with 99% similarity.

Lactic acid production, hydrogen peroxide production and proteolytic activity of the isolate were recorded as 2.275%, 0.334±0.075 µg/mL and 2.131±0,000 mg/mL respectively (Table I). Although the isolate had auto-aggregation and co-aggregation activity, it had not hydrophobicity activity (Table II). By the end of 3-hour period, the bacteria protected its viability on the 0.1% and 0.3% bile salt conditions and, at pH 3 (Figure I).

The acid and bile salt resistance of *L. crispatus* were determined by colony counting at the end of 0th, 1st, 2nd and 3rd hours. h:hour.

The zone diameters around the antibiotic discs (ampicillin, erythromycin, gentamycin, clindamycin, penicillin, cephalothin, tetracycline and vancomycin discs) were recorded as 36mm, 36 mm, 24 mm, 38 mm, 38 mm, 38 mm, 30 mm and 24 mm, respectively. Zone formation around methicillin, metronidazole, oxacillin and sulfamethoxazole + trimethoprim discs was not observed.

DISCUSSION

Lactobacillus species have beneficial effects predominate in the vaginal microbiota of healthy women. These bacteria are very important in terms of vaginal ecosystem health protection (Aroutcheva *et al.,* 2001). *Lactobacillus* species are catalase negative,

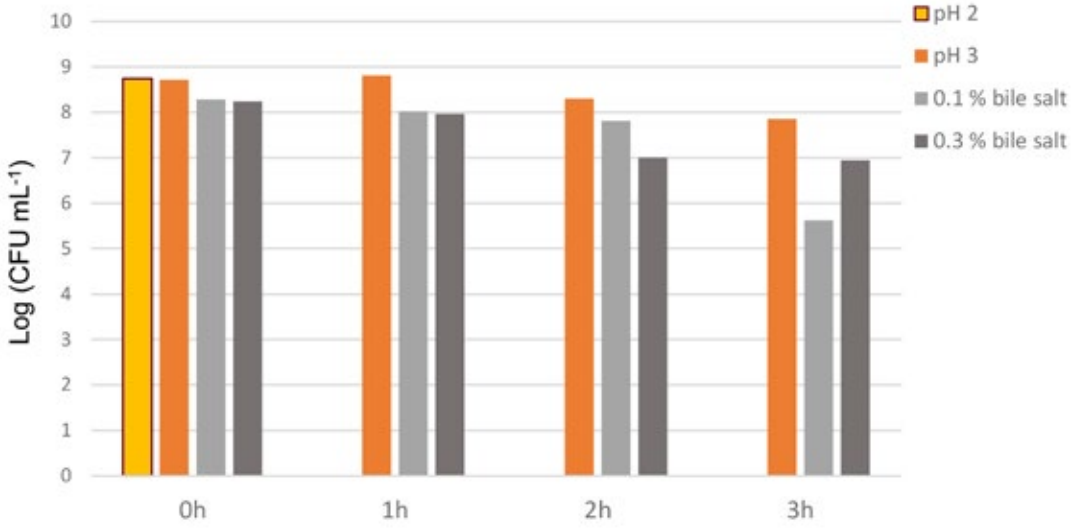

FIGURE 1 - Acid and bile salt resistances of *L. crispatus.*

TABLE I - The amounts of lactic acid, hydrogen peroxide and proteolytic activity of the *L. crispatus*

% of Acidity	The amount of hydrogen peroxide (µg / mL)	Proteolytic activity (mg/mL)
2.275%	0.334±0.075	2.131±0.000

L. crispatus was incubated in the MRS broth medium at 37°C 5% CO_2 for 48 hours. Acid produced by the isolate was calculated as percent titrable acidity. The amount of hydrogen peroxide and proteolytic activity of the isolate were determined by spectrophotometrically. The tests were conducted in duplicate.

TABLE II - The auto-aggregation, co-aggregation and hydrophobicity activities of the *L. crispatus*

Auto-aggregation	Co-aggregation	Hydrophobicity
(+)	(+)	(-)

(+): positive (-): negative. Auto-aggregation of *L. crispatus* was examined microscopically (Leica DM500). Auto-aggregation test is considered positive if the cells aggregate within 2 minutes. In the co-aggregation assay, *E. coli* ATCC 25922 strain was used as test microorganism. The coaggregation of *L. crispatus* with *E. coli* ATCC 25922 was examined microscopically. The amount of hydrophobicity activity of *L. crispatus* were determined by spectrophotometrically.

oxidase negative, gram positive, bacil shaped, facultative aerotolerant, immobilized and non-spore forming bacteria (Hanghshenas *et al.,* 2014).

Previous studies reported that lactic acid bacteria including *Lactobacillus acidophilus, L. plantarum, L. casei, L. cellobiotus, L. oris, L. reuteri, L. ruminis, L. salivarius, L. brevis, L. delbrueckii, L. vaginalis, L. gasseri,* and *L. crispatus* are isolated from the vagina of healthy women (Atassi *et al.,* 2006; Kıvanç, Yazıcıoğlu, Dinçer, 2011). In this study, we had isolated *L. crispatus* from a healthy woman's vagina.

Lactic acid bacteria protect the vagina from pathogens by producing antimicrobials such as lactic acid and bacteriocin (Redondo-Lopez, Cook, Sobel, 1990). The vaginal homeostasis is preserved by lactic acid production of the LABs in the vagina. By the production of organic acids such as lactic acid, vaginal pH is kept below 4.5. By the acidic nature of the vaginal, the development of pathogen microorganisms is prevented. (Cheeti, Warrier, Lee, 2006). In a study conducted by Aslim and Kilic in 2006, it was revealed that the amount of acidity in *L. crispatus* strains isolated from healthy women vaginal was 0.48-0.85% (Aslim, Kilic, 2006). In our study, we monitored that lactic acid production rate of isolated *L. crispatus* was 2.275%.

In vitro experiments indicated that H_2O_2 suppress development of endogenous pathogens and various pathogen microorganisms including *G. vaginalis* and HIV (Jespers *et al.,* 2012). In a study conducted by Aslim and Kilic in 2006, it was revealed that although some of *L. crispatus* strains isolated from healthy women vagina did not produced H_2O_2, although some of the *L. crispatus* strains produced 1.24±0.02 µg/mL - 3.84±0.04 µg/mL of H_2O_2 (Aslim, Kilic, 2006). Abramov et al. reported that *L. crispatus* 2029 isolated from the healthy women vagina produced 0.120±0.05 µg/mL of H_2O_2 (Abramov *et al*., 2014). We showed that *L. crispatus* isolated from the vagina produced 0.334±0.075 µg/mL of H_2O_2. Additionally, *L. crispatus'* proteolytic activity amount was found 2.131±0.000 mg/mL. There hasn't been found any study about proteolytic activity of *L. crispatus* isolated from the vagina.

Autoaggregation and coaggregation activities of lactic acid bacteria have great importance (Jankovic *et al.,* 2003). Kmet and Lucchini have shown that 12 of the 60 lactic acid bacteria isolated from vagina have auto-aggregation activity (Kmet, Lucchini, 1997). In our study, we found that *L. crispatus* has auto-aggregation activity. It has been reported that *Lactobacillus* species also have co-aggregation activity with pathogenic bacteria (Reniero *et al.,* 1991). It is clear that they are able to aggregate and co-aggregate with uropathogenic *E. coli.* Kmet and Lucchini have shown that only 3 of 60 lactic acid bacteria isolated from vagina have co-aggregation activity with *E. coli* strains (Kmet, Lucchini, 1997). Our results also indicated that *L. crispatus* did not co-aggregate with *E. coli* ATCC 25922.

One of the most important features of probiotics is that microorganisms are resistant to low pH and high bile salts (Brink *et al.,* 2006). Probiotics should be able to tolerate certain conditions such as high bile salt (0.3%) and low pH (2 - 3) for a minimum of 90 minutes (Hanghshenas *et al.,* 2014). It has been demonstrated that *L. crispatus* preserved its viability by the end of 3-hour period at pH 3 in our study. Previous studies showed that *Lactobacillus crispatus* ATCC 33820 strain does not survive more than 2 hours at pH 2 (Pereira, Gibson, 2002).

Bile salts have been reported to be toxic to bacterial cells because of disrupting organization of the cell membrane. Lactic acid has to produce bile salt to survive in the small intestine (Succi *et al.,* 2005). In a study conducted, it was revealed that *Lactobacillus lactis* ATCC 11454, *L. paracasei* ATCC 27092, *L. casei* ATCC 393 ve *L. rhamnosus* ATCC 53103 strains were able to maintain their viability at the end of the 3rd hour in 0.4% bile salt solution (Kaewnopparat *et al*., 2013). Moreover, *L. fermentum* SK5 strain can survive in 0.1% and 0.2% bile salt conditions (Succi *et al.,* 2005). In our study, we revealed that *L. crispatus* could maintain its viability in 0.1% and 0.3% bile salt conditions at the end of the 3rd hour. Besides, our results indicate that *L. crispatus* did not exhibit hydrophobicity.

In this study, antibiotic susceptibility of *L. crispatus* investigated with disc diffusion method. Total of 12 antibiotics, namely ampicillin, erythromycin, gentamycin, clindamycin, methicillin, metronidazole, oxacillin, penicillin, cephalothin, sulfamethoxazole + trimethoprim, tetracycline and vancomycin were tested. Test results

revealed that *L. crispatus* showed resistance to methicillin, metronidazole, oxacillin, and sulfamethoxazole + trimethoprim, yet showed susceptibility to ampicillin, erythromycin, gentamycin, clindamycin, penicillin, cephalothin, tetracycline and vancomycin. In a study conducted by Hütt et al. in 2016, it was revealed that all bacteria from 26 *L. crispatus* strains isolated from vagina showed resistance to metronidazole, kanamycin ve trimethoprim+sulfamethoxazole, although they showed susceptibility to ampicillin, chloramphenicol, eriythromycin, gentamycin, nitrofurantoin, tetracycline and vancomycin. Metronidazole is one of the most commonly used antibiotics in bacterial vaginosis (Ocana, Silva, Nader-Mac´ıas, 2006). In our study, it was observed that our isolate showed metronidazole resistance in agreement with these results.

It has been reported that strains develop resistance to a specific antibiotic may be given with antibiotics during antibiotic treatment (Cebeci, Gürakan, 2003). It has been reported that antibiotic along with LAB strains that are not susceptible to the antibiotic may be given for the protection of microbiota during bacterial vaginosis treatment (Ocana, Silva, Nader-Mac´ıas, 2006). Thus, it was observed that the microbiota could regenerate in a shorter time (Cebeci, Gürakan, 2003).

The normal vaginal microbiota has been found to consist primarily of one or more of merely four distinct species, in particular *L. crispatus, L. jensenii, L. gasseri*, and *L. iners* (Verstraelen *et al*., 2009). *Lactobacillus crispatus* was the most frequent cultured species in vaginal samples. Presence of *L. crispatus* is considered a major determinant to the stability of the normal vaginal microbiota in women of reproductive age (Hütt *et al*., 2016). Besides, *L. crispatus* appears to be substantially prevailing over the other hydrogen peroxide producing *Lactobacillus* species (Abramov *et al*., 2014). In addition, *L. crispatus* is a lactic acid bacteria that produces the highest lactic acid when compared to *L. iners, L. jensenii,* and *L. gasseri* (Ravel *et al.,* 2011). Lactic acid bacteria in the vagina protects vaginal health by producing antimicrobial agents such as lactic acid and hydrogen peroxide against bacterial pathogens (Hütt *et al.,* 2016).

Lactic acid bacteria that are used as probiotic are beneficial microorganisms (Fuller, 1989). Recently, probiotics are widely used in therapeutic preparations (Shah, 2007). *L. crispatus* are potential probiotic (Roy *et al*., 2000). *L. crispatus* CTV-05 was used as a vaginal suppository for healthy women with a history of recurrent urinary tract infections (Abramov *et al*., 2014). This study did produce findings that contribute to the probiotic characterization of a *L. crispatus* strain but it is not yet a readily marketable probiotic product. Our strain promises hope and so further studies are needed to be able to use this strain as a probiotic.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ETHICAL APPROVAL

This study was approved by Istanbul Medipol University Non-Interventional Clinical Researches Ethics Board on 11/04/2013 with decision number 38. All applicable international, national, and/ or institutional guidelines for the care and use of human were followed.

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