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CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF VAGINAL *LACTOBACILLUS* ISOLATE

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Abstract - The aim of this study was to investigate the probiotic potential of bacteriocin-producing lactobacilli strain *Lactobacillus plantarum* G2 isolated from the vaginal mucus of healthy women. The antimicrobial effect of G2 was confirmed in the mixed culture with pathogenic *Escherichia coli, Staphylococcus aureus, Salmonella abony and Pseudomonas aeruginosa,* while bacteriocine activity was detected against *S. aureus* and *S. abony* only. The strain showed an excellent survival rate in low pH and in the presence of bile salts. The percentage of adhered cells of *L. plantarum* G2 to hexadecane was 63.85±2.0 indicating the intermediate hydrophobicity.

Key words: *Lactobacillus*, vaginal isolate, antimicrobial activity, probiotics

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INTRODUCTION

Among lactic acid bacteria members, lactobacilli present a diverse group of homofermentative and heterofermentative species. Lactobacilli are gram-positive rods, primarily facultative or rarely strictly anaerobic bacteria (Kandller and Weiss, 1986; Hammes and Hertel, 2009) that can produce a variety of antimicrobial substances such as lactic acid, ethanol, formic acid, acetone, hydrogen peroxide, diacetyl and bacteriocins. These compounds can serve as a natural competitive means for overcoming other microorganisms sharing the same niche (Oliveira et al., 2008). Lactobacilli are the most important bacteria of the normal vaginal flora of healthy pre-menopausal women and are present at 10^7-10^8 CFU/ml of vaginal fluids (Fazeli et al., 2006). Lactobacilli have been used in the prevention of genital and urinary tract infections (Reid, 2005). The purpose of this study was to investigate several important probiotic features of one vaginal *Lactobacillus* strain, including its antimicrobial potential, gastric and bile tolerance and cell surface hydrophobicity.

MATERIALS AND METHODS

Subjects and sampling

Seven different vaginal swab samples were collected from healthy women of 18 to 50 years of age. The vaginal samples were taken aseptically with a cotton swab, placed in MRS broth (De Man et al., 1960), and taken to the laboratory where they were transferred to MRS plates. The inoculated plates were incubated at 37°C in anaerobic and microaerophilic environment (Gas pack vessel, BioMerieux, France) for 72 h. The obtained individual colonies were restrickled on fresh MRS plates and used as the starting material for bacterial assessment.

Bacterial strains and media conditions

The selected G2 isolate was grown on MRS medium at 37°C. To detect the antimicrobial activity of G2, the following indicator strains were used: *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538-P, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella abony* NTCC 6017 and *Candida albicans* ATCC 10231. The indicator strains were grown on the following selective media: *E. coli* on MacConcay medium, *S. aureus* on Baird Parker medium, *S. abony* on Deoxycholate lactose medium, *P. aeruginosa on* Cetrimide agar and *C. albicans* on Saburo dextrose medium. *C. albicans* was incubated at 25°C for 72 h. The other listed strains were incubated at 37°C for 48 h. Tryptone soy broth (TSB) was used for the cultivation of lactobacilli in mixed culture with pathogenic bacteria. All media were obtained from Torlak, Belgrade, Serbia. To determine the number of viable cells, a set of ten-fold dilutions was made in the sterile phosphate buffer, pH 7.2 (Taylor, 1962). Subsequently, 100 µl of each dilution was smeared on the surface of agar, depending on the strain.

Preliminary identification of the selected lactobacilli isolate

A homofermentative lactobacilli isolate, G2, was preliminarily characterized to the genus level by standard morphological and physiological tests according to the criteria in Bergey's Manual (Kandler and Weiss, 1986; Hammes and Hertel, 2009). The biochemical identification of the isolate was carried out by a standard API 50CH test containing 49 different substrates, according to the manufacturer's instructions. The results were interpreted by comparing the test results with the biochemical profile of the species in the identification table *(*API Analitical index profile). All tests mentioned above were performed in triplicate.

DNA isolation and manipulations

The total DNA from G2 was isolated using the QIA DNA Mini Kit (Qiagen GmbH, Hilden, Germany). PCR assay with genus-specific primers UNI16SF (5'-GAG AGT TTG ATC CTG GC-3) and UNI16SR (5'-AGG AGG TGA TCC AGC CG-3'), which amplify the 16S rRNA gene, was conducted for species determination (Jovcic et al., 2009). PCR amplifications were carried out in tubes containing 25 µl reaction mixture composed of 1xTaq buffer, 1 U Taq polymerase (Pharmacia, Vienna, Austria), 1.5 mmol $MgCl_2$, 200 µmol dNTPs each and 1.5 µmol primer each. PCR amplification conditions were as follows: 5 min at 96°C; 30 cycles of 96°C for 30 s, 55°C for 30 s and 72°C for 30 s, and an additional extension step of 5 min at 72°C. The resulting PCR product was purified using the QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany) and sequenced at Macrogen in Seoul, South Korea. The BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST; RID: 1138633900-27581-131272740575. BLASTQ4) was used to determine the most related sequence relatives in the NCBI nucleotide sequence database. A multiplex PCR assay with the *recA* gene-based specific primers plantF, paraF, pentF and pREV (Torriani et al., 2001) was performed for the final species determination.

Microbial adhesion to hexadecane - MATH

For the assessment of the degree of hydrophobicity, the microbial adhesion to hydrocarbon (MATH) test was employed. The *in vitro* test in which hexadecane, an apolar *n*-alkane is used as a solvent, was performed in order to assay the adhesion potential of the G2 strain. The strain was incubated until the stationary phase of growth was reached (18 h) and then centrifuged (5000 x g, 10 min). The pellet was subsequently washed twice in 0. 1 M KNO_3 (pH 6.2) and resuspended in the same buffer to obtained the optical density 0.4 at 600 nm (A_0). Next, 0.2 ml of the solvent was added to the resuspended cells (1.2 ml) after 10 min of incubation at room temperature. The obtained two-phase system was vortexed for 2 min and after 15 min of incubation at room temperature, the water phase was separated and the optical density was measured at 600 nm (A_1). The percentage of adhered cells was calculated using the formula: $(1 - A_1/A_0) \times 100$. The values θ from 0-35

indicate low hydrophobicity, 36-70 intermediate hydrophobicity and 71-100 high hydrophobicity. In other words, the numbers indicate the percentages of bacteria adhering to hexadecane (Ocaña et al., 1999). The adhesion of microorganisms to hexadecane is a criterion of the hydrophobicity or hydrophilia of a bacterial surface due to the absence of electrostatic interaction (caused by a large quantity of electrolytes in 0.1M KNO_3).

In vitro testing – resistance to artificial gastric and intestinal fluids

The test of bacterial survival in artificial gastric juice (AGJ) was performed as follows: 10 ml of MRS medium was inoculated at 1% with lactobacilli and incubated at 37°C for 18 h. After washing the bacterial cells, 10 ml of the cell suspension (10^8 CFU/ml) was added to 90 ml of AGJ (0.03 M NaCl, 0.32% pepsin at pH 2.0 adjusted with 0.58 ml 10M HCl) and incubated with gently agitation (58 rpm) to simulate peristalsis. Bacterial aliquots were taken for the enumeration of viable cells at 0, 60 and 120 min. Bacterial survival was expressed with reference to the initial bacteria count. Pepsin and HCl were obtained from Sigma (Sigma-Aldrich, Sent Louis, MO, USA).

The effect of bile salts solution on bacterial survival was studied by resuspending the harvested cells (grown in MRS medium at 37°C for 18 h) in PBS buffer (0.01 M K_2HPO_4, 0.01M KH_2PO_4 and 0.15M NaCl) containing 0.5% bile salts and adjusting it to pH 8.0 with 1M NaOH. The suspensions were incubated at 37°C for up to 2 h with gently agitation (58 rpm). The samples for total viable counts were taken at 0, 30, 60, 90 and 120 min and expressed with reference to the initial bacteria count.

Detection of antimicrobial activity

An agar-well diffusion assay was prepared for the detection of antimicrobial activity. The overnight cultures of the indicator strains (10^8 CFU/ml) were mixed at 1% with melted nutrient agar and poured into sterile Petri dishes. A 6-mm-wide well was cut in the agar across the centre of the dish. The cell-free filtrate was obtained after centrifuging the overnight culture of strain G2 (18 h), at 5000 rpm for 10 min and filtration through a 0.45μm membrane (Sarstedt, Numbrecht, Germany). The aliquots (100 μl) of cell-free filtrate were poured into the 6-mm wells. The plates were first incubated at 4°C for 2 h to allow the test material to diffuse in the agar and then incubated for 24 h for bacteria and 48 h for *C. albicans* ATCC 10231, at appropriate temperatures. After the incubation, a clear zone of inhibition around the well was measured. The result was positive if a clear inhibition zone ≥ 8mm in diameter was found. Control tests of the culture medium (pH 4 and pH 6) were performed. All the experiments were carried out in triplicate. For bacteriocin activity detection, the cell-free filtrate was previously buffered to pH 6.5 and/or treated with 1 mg/ml Proteinase-K (Merck, GmbH Darmstadt, Germany), incubated at 37°C for 60 min and placed into each well. The MRS medium buffered to 6.5 was used as the control. The proteinaceous nature of the antimicrobial substances was assessed as the absence of clear inhibition zones around the wells, which is the result of bacteriocin degradation by the added Proteinase-K (Oh et al., 2000).

Analytical method: HPLC assay of lactic acid in the cell-free filtrate

The content of lactic acid was determined by HPLC (HP1100, Hewlett Packard, Palo Alto, CA, USA) with an ion exchange column (Supelcogel C-610H, Supelco, USA) using 0.1% H_3PO_4 as the mobile phase. The flow rate of the mobile phase was 0.5 ml/min and absorbance was measured at 210 nm by a Diode Array Detector (DAD 1100, Hewlett Packard). LA verification was determined by HPLC (LC-6A, Shimadzu, Kyoto, Japan), with the same column, mobile phase, flow rate and Refraction Index Detector (RID, 9100 Varian, Inc., Palo Alto, CA, USA). Reproducibility was assessed by triplicate tests. The system suitability and linearity for the concentration of lactic acid was estimated using a standard organic acid kit (cat. No. 47264, Supelco Inc., Bellefonte, PA, USA) (Huh et al., 2006).

Table 1. Antimicrobial and bacteriocin activity of the cell-free filtrate obtained from *L. plantarum* G2.

Indicator strain	Inhibition zone (mm) (unbuffered CFF)	Inhibition zone (mm) (buffered CFF)	Inhibition zone (mm) (buffered CFF +Proteinase K)
S. aureus ATCC 6538-P	18	12	-
E. coli ATCC 8739	14	-	-
P. aeruginosa ATCC 9027	14	-	
S. abony NTCC 6017	20	10	
C. albicans ATCC 10231	-	-	

CFF-cell-free filtrate

The effect of L. plantarum G2 on E. coli ATCC 8739, S. aureus ATCC 6538-P, S. abony NTCC 6017 and P. aeruginosa ATCC 9027 co-cultivated in mixed cultures

The method was used for studying the antimicrobial activity of G2 on the growth of pathogenic bacteria co-cultivated in mixed cultures. A 9-ml TSB was inoculated with 1 ml of the overnight culture of G2 (10^9 CFU/ml) and 0.1ml of each pathogenic bacteria: *E. coli* ATCC 8739 (10^7CFU/ml), *S. aureus* ATCC 6538-P (10^8 CFU/ml), *S. abony* NTCC 6017 (10^7CFU/ml) or *P. aeruginosa* ATCC 9027 (10^7CFU/ml). The inoculated media were mixed immediately and incubated at 37°C for the next 24 h. To determine the number of pathogenic cells that survived after 24 h, the following media were used: MacConcay agar for *E. coli*, Baird Parker agar for *S. aureus*, Deoxycholate Lactose agar for *S. abony*, Cetrimide agar for *P. aeruginosa* and MRS agar for lactobacilli. The plates were incubated at 37°C for 48 h and each strain count (CFU/ml) was determined. All the experiments were carried out in triplicate.

RESULTS

Ten different vaginal lactobacilli strains were isolated on MRS plates from the vaginal smears of seven healthy women. Only one isolate (named G2) that showed antimicrobial potential, was selected for further characterization. According to the results obtained after morphological and physiological testing, the isolate was classified as a member of the *Lactobacillus* genus. The testing was further resumed by assessing the ability of the isolate to ferment carbohydrates. The obtained results showed that the match of the properties with bacterial species was not sufficient for their identification to the species level. Therefore, the unidentified isolate was subjected to 16S rDNA sequencing. The 16S rDNA analysis revealed that the isolate G2 belongs to the *L. plantarum*/*paraplantarum*/*pentosus* group. The isolate was finally identified as *L. plantarum* by using multiple PCR assays with primers used for the differentiation of *L. plantarum* sp (data not shown).

Preliminary tests have shown that the indigenous isolate of *L. plantarum,* G2, was able to strongly inhibit the growth of *E. coli* ATCC 8739, *S. aureus* ATCC 6538-P, *S. abony* NTCC 6017 and *P. aeruginosa* ATCC 9027. The inhibition zones, determined by the agar-well diffusion assay, were 14, 18, 20 and 14 mm, respectively (Table 1). Nevertheless, it failed to inhibit *C. albicans* ATCC 10231. In order to investigate bacteriocin or bacteriocin-like substance production, the cell-free filtrate from an overnight culture was buffered to pH 6.5 and the enzyme Proteinase-K was added. No inhibition zones were detected against the indicator *S. aureus* ATCC 6538-P and *S. abony* NTCC 6027. Additionally, the final pH value of cell-free filtrate was 3.8 - 4.0 and the content of lactic acid in the filtrate was 11.75 g/l.

The effect of G2 on growth inhibition of *E. coli* ATCC 8739, *S. aureus* ATCC 6538-P, *S.abony* NTCC 6017 and *P. aeruginosa* ATCC 9027 co-cultured in TSB, after 24-hour incubation, was also investigated. When G2 was mixed with the above-mentioned

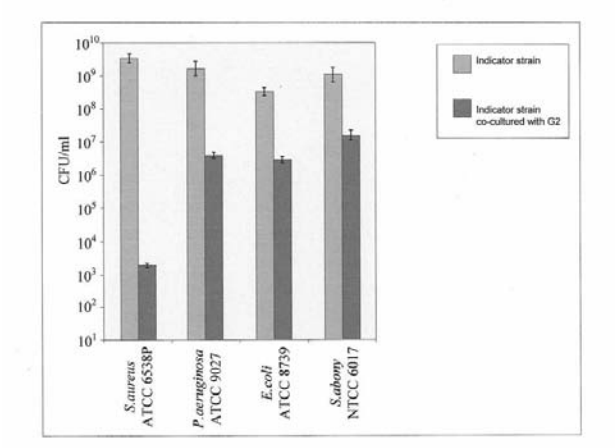

Fig. 1. The antimicrobial activity of *L. plantarum* G2 on the growth of *E. coli* ATCC 8739, *S. aureus* ATCC 6538-P, *S. abony* NTCC 6017 and *P. aeruginosa* ATCC 9027 co-cultivated in mixed cultures.

strains, growth of the pathogen was lowered by 2.0 $\log_{10}$ for *E. coli* ATCC 8739, 6.7 $\log_{10}$ for *S. aureus* ATCC 6538-P, 1.9 $\log_{10}$ for *S. abony* NTCC 6017 and 2.6 $\log_{10}$ for *P. aeruginosa* ATCC 9027 when compared to the growth of the pure culture (control) of the pathogen (Figure 1). Also, the final pH value of the supernatants was between 3.6 – 4.0, depending on the mixture.

Survival of the vaginal strain *L. plantarum* G2 in AGJ and in bile salts was tested under conditions similar to those found in the gastrointestinal tract, at time intervals corresponding to the actual presence of lactobacilli in the intestines. After a 120-min exposure of G2 to AGJ, a decreased count of viable cells by 1.54 log CFU/ml was detected. Following a 120-min exposure to the bile salts solution, the decrease of viable G2 cells was 1.00 log CFU/ml (Figure 2).

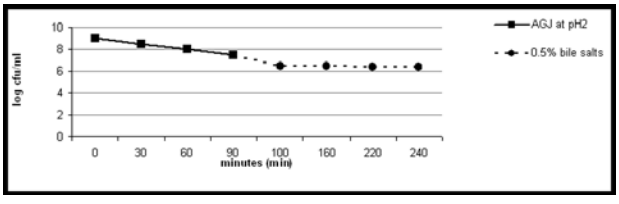

Fig. 2. Survival of *L. plantarum* G2 in simulated artificial gastric juice (AGJ) at pH 2.0 (■) and in the solution containing 0.5% of bile salts (•).

The percentage of adhered cells of *L. plantarum* G2 to hexadecane was 63.85±2.0. According to the results from this study, the MATH values indicate the intermediate hydrophobicity of the cells.

DISCUSSION

The *Lactobacillus plantarum* strain is found to be a normal inhabitant of healthy women (Anukam and Reid, 2007). Commensal lactobacilli in the genital tract reduce the risk of infection in women through the production of different antimicrobial compounds (Barrons and Tassone, 2008). In the present study it was found that among ten vaginal *Lactobacillus* isolates, one possessed an antimicrobial potential against different human pathogenic bacterial strains. According to results it could be speculated that the inhibition of *E. coli* ATCC 8739 and *P. aeruginosa* ATCC 9027 is solely a consequence of lactic acid activity. On the other hand, the antimicrobial activity of *S. aureus* ATCC 6538-P and *S. abony* NTCC 6017 is due to the synergistic activity of lactic acid and bacteriocin or bacteriocin-like substance/s. Under optimal growth conditions for *L. plantarum* G2, the final pH value of the culture reached 3.8-4.0, which is comparable to the pH of the vaginal environment of healthy women (Baskey et al., 1999). The acid production by vaginal flora *in vitro* is consistent with the rate and extent of vaginal acidification. Reduction of pathogenic viable cells in mixed cultures showed that the isolated G2 was able to inhibit the growth of *E. coli, S. aureus*, *S. abony* and *P. aeruginosa*. The predominant vaginal microflora of healthy women can be maintained at pH of < 4.5 (Renoldo-Lopez et al., 1990). Bacterial vaginosis is characterized by a vaginal pH> 4.5 and by an overgrowth of anaerobic bacteria (Eschenbach, 1993). In our study, the final pH of mixed cultures measured after 24 h of co-cultivation was lower than 4.0. The obtained acidic environment prevents the growth of pathogens, including potential uropathogens *S. aureus* ATCC 6538-P and *E. coli* ATCC 8739.

Research on the survival of ingested bacteria in the GIT is important for the selection of probiotic

strains and the development of probiotics (Vasiljevic and Shah, 2008). Tolerance of the low pH of the stomach and the bile content and the ability to colonize the intestinal tract appears to be very important (Jacobsen et al., 1999). Until now, results have shown that strains of human origin exhibit better survival rates than isolates from other sources. Compared to the literature data, the strain G2 showed excellent viability at the low pH in simulated gastric juice and in bile salts. Moreover, after passage through the simulated proximal part of a simulated GIT the number of cells decreases only by 2.54 log CFU/ml.

The capacity of lactobacilli to adhere to and colonize the epithelium of the gastrointestinal and urogenital tracts is one of the features that may be beneficial to the host (Ocaña et al., 1999). The test of microbial adhesion to hexadecane (MATH) is very important for examining lactobacilli hydrophobicity by its correlation with adherence. Adhesion to the intestinal epithelium is an property affecting the selection of probiotic lactic acid bacterial strains (Ocaña et al., 1999). The analysis of the surface characteristics of G2 performed by measuring strain adhesion to hexadecane showed that the strain has a basic and hydrophobic surface.

According to the presented results, the vaginal *L. plantarum* G2 strain is capable of inhibiting the growth of *E. coli* and other pathogenic bacteria such as *P. aeruginosa*, *S. aureus* and *S. abony*. The obtained results indicate a probiotic potential of G2, although more detailed analyses, including human clinical trials, are required before the *L. plantarum* G2 strain can be considered for use for biotherapeutic purposes.

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