Probiotic assessment of *Enterococcus durans* 6HL and *Lactococcus lactis* 2HL isolated from vaginal microflora

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Forty-five lactic acid bacteria (LAB) were isolated from the vaginal specimens of healthy fertile women, and the identities of the bacteria were confirmed by sequencing of their 16S rDNA genes. Among these bacteria, only four isolates were able to resist and survive in low pH, bile salts and simulated *in vitro* digestion conditions. *Lactococcus lactis* 2HL, *Enterococcus durans* 6HL, *Lactobacillus acidophilus* 36YL and *Lactobacillus plantarum* 5BL showed the best resistance to these conditions. These strains were evaluated further to assess their ability to adhere to human intestinal Caco-2 cells. *Lactococcus lactis* 2HL and *E. durans* 6HL were the most adherent strains. *In vitro* tests under neutralized pH proved the antimicrobial activity of both strains. Results revealed that the growth of *Escherichia coli* O26, *Staphylococcus aureus* and *Shigella flexneri* was suppressed by both LAB strains. The antibiotic susceptibility tests showed that these strains were sensitive to all nine antibiotics: vancomycin, tetracycline, ampicillin, penicillin, gentamicin, erythromycin, clindamycin, sulfamethoxazole and chloramphenicol. These data suggest that *E. durans* 6HL and *Lactococcus lactis* 2HL could be examined further for their useful properties and could be developed as new probiotics.

INTRODUCTION

The concept of eating or using live bacteria for health benefits goes back a hundred years (Metchnikoff, 1907). Lactic acid bacteria (LAB) are generally regarded as safe and constitute part of the probiotic concept, which has received significant attention over the past years (Metchnikoff, 1907; Salminen *et al.*, 1998). Probiotics are live micro-organisms in the form of single or mixed cultures; when consumed at sufficient amounts, these probiotics provide beneficial health effects for hosts (Guenner *et al.*, 2008; Lilly & Stillwell, 1965; Surawicz, 2003). Probiotics significantly affect the bioavailability of nutrients in the human body (Young & Huffman, 2003). These micro-organisms stimulate immune mechanisms and maintain normal human microbiota compositions; as a result, these micro-organisms reduce the risk of diarrhoea (Binns & Lee, 2010), antibiotic-related diarrhoea (Friedman, 2012), irritable bowel syndrome (Barouei *et al.*, 2009; Parkes, 2010), inflammatory bowel disease (Geier *et al.*, 2007; Gionchetti *et al.*, 2002), vaginal infections (Reid & Bocking, 2003), atopic eczema (Bunselmeier & Buddendick, 2010; Wickens *et al.*, 2008), rheumatoid arthritis (Guenner *et al.*, 2008) and cancers (Serban, 2014; Zhu *et al.*, 2011). They are believed to be the dominant members of normal postpubertal and premenopausal vaginal microbiota (Nam *et al.*, 2007). Clinical proof shows that the vaginal and urogenital floras play a dominant role in maintaining both protection from illnesses and the well-being of women (Burton *et al.*, 2003). The vaginal microbiota generate a balanced ecosystem because of the interaction of factors of the native bacterial biota, in which the bacteria generally play a central role in inhibiting colonization by pathogenic organisms, including those responsible for yeast infections, bacterial vaginosis, urinary tract infections and other sexually transmitted diseases (Shi *et al.*, 2009).

**Abbreviation:** LAB, lactic acid bacteria.
Many recognized probiotics belong to the LAB genera, such as *Lactobacillus*, *Lactococcus*, *Enterococcus* and bifidobacteria, but the most important genera commonly used as probiotics are *Lactobacillus* and bifidobacteria. Probiotics are defined as ‘living micro-organisms which, upon ingestion in certain numbers, employ health advantage beyond inherent general nutrition’ (Gorbach, 2002) and play a role in the deterrence or treatment of infectious diseases, namely, vaginal infections (Reid et al., 2009), allergies (Kuitunen et al., 2012), irritable bowel syndrome (Hoveyda et al., 2009), inflammatory bowel diseases (Damaskos & Kolios, 2008), chronically high cholesterol levels (Huang & Zheng, 2010), colitis (Resta-Lenert & Barrett, 2009) and colon cancer (Andersson et al., 2001; Liong, 2008).

To obtain the ability to exert their probiotic effects, potential strains are expected to have desirable properties (Ouwehand et al., 1999; Zavisic et al., 2011). Probiotics have many selection criteria: human origin for human usage, acid and bile tolerance, ability to adhere to the mucosal surface, safe for food and clinical use and gastric resistance (Dunne et al., 2001; Saarela et al., 2000).

Another of the most important selection criteria for probiotics is antimicrobial activity, which targets pathogens and the undesirable enteric elements (Klaenhammer & Kullen, 1999). The antimicrobial effects of LAB are caused by their production of some substances such as organic acids (i.e. lactic, acetic and propionic acids), hydrogen peroxide, carbon dioxide, bacteriocins, diacetyl and low molecular mass antimicrobial substances (Ouwehand & Vesterlund, 2004). The aim of this study was to isolate and characterize select LAB of the human vaginal tract as new potential probiotics.

**METHODS**

**Isolation of vaginal LAB.** Vaginal samples used in this study were isolated from 40 healthy fertile women of childbearing age (17–36 years). LAB were isolated by inoculating the samples onto de Man Rogosa agar (MRS agar; Merck) and M17 medium (Merck) supplemented with 0.3% oxgall at pH 3.0 using a streak plate technique. The inoculated plates were incubated at 37 °C for 24 h under anaerobic conditions using anaerobic jars (Mitsubishi) equipped with anaerobic gas generating kits. Individual colonies were selected and subcultured in a sterile MRS medium broth. Gram-positive and catalase-negative cocci and bacilli colonies were considered as LAB and stored at −70 °C in MRS broth supplemented with glycerol (25 % v/v).

**Identification, PCR amplification and gene sequencing.** The universal primers used for LAB were those primarily used by Mirzaei & Brazgari (2012). The primers amplify a region of the 16S rDNA gene of approximately 1500 bp. The primer sequences were: Hal6F 5′-AGATTTTGATCMTGCCAG-3′ and Hal6R 5′-TACCTGTGTA-GACCTCACCC-3′. All amplification reactions were performed in an MWG Biotech thermal cycler (Gallito) with the following temperature profile: an initial denaturation at 94 °C for 4 min, followed by 32 cycles involving denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 1 min, with a 5 min final extension step at 72 °C (Dubernet et al., 2002). The total reaction mixture (25 μl) contained 13 pmol of each primer, a 0.2 mM concentration of each deoxynucleobnucleotide triphosphate (Invitrogen), 1 x PCR buffer without MgCl₂, 2.0 mM MgCl₂, 100 ng of bacterial DNA and 2.0 U of *Taq* DNA polymerase (MP Biomedicals). The PCR products were resolved through electrophoresis in a 1.0 % (w/v) agarose gel and were visualized by ethidium bromide staining. PCR products were purified and sequenced by the Macrogen DNA Sequencing Service (Korea). Sequence similarity was determined using the BLAST (Basic Local Alignment Search Tool) program of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul et al., 1997).

**Resistance to low pH.** To determine the acid permanency of isolated LAB, the method described previously by Vernazza et al. (2006) was performed with some modifications. The pH of MRS broth was adjusted to 3.0 with 1 M HCl before autoclaving; pH 7.0 served as a control. Survival was evaluated using exponential phase cultures (8 log₁₀ c.f.u. ml⁻¹) by plating on MRS agar after 0, 1, 2 and 3 h of incubation of bacterial cells in acidic MRS broth (pH 3.0) at 37 °C by a pour plate technique.

**Survival in bile salts.** Tolerance to bile salts was determined using the method of Pereira & Gibson (2002) with some modifications. MRS broth supplemented with 0.3 % (w/v) oxgall, and MRS broth without supplement (as a control) were inoculated with actively growing bacteria. Samples were incubated for 4 h at 37 °C and the bacterial maintenance was evaluated using exponential phase cultures (8 log₁₀ c.f.u. ml⁻¹) by a plate count on MRS agar at time points of 0, 1, 3 and 4 h of incubation in MRS broth containing bile salts at 37 °C. During the incubation for 4 h, viable colonies were enumerated every hour using a pour plate technique in MRS agar medium.

**Survival in simulated in vitro digestion.** To determine in vitro digestion, the method described previously by Seiquer et al. (2001) was performed with some modifications. To recreate gastric digestion, pepsin to a final concentration of 5 % (w/v) was added to the samples which were then adjusted to pH 3.0. The samples were incubated for 120 min at 37 °C with gentle agitation (110 r.p.m.). To simulate intestinal digestion, the samples were adjusted to pH 6.0 and solutions of bile salts and pancreatin were added at a final concentration of 0.3 % and 0.1 % (w/v), respectively. The samples were incubated at 37 °C for 180 min with gentle agitation at 110 r.p.m. To determine the cell count, the samples were removed before and after gastric and intestinal digestion; the aliquots were serially diluted and plated in triplicate on MRS agar. The plates were incubated for 48 h under anaerobic conditions.

**Adhesion of LAB to Caco-2 cells.** Bacteria were evaluated for their adhesion ability to the human colon carcinoma cell line, Caco-2. The cells were cultures in RPMI medium (1640; Sigma) supplemented with 10 % heat-inactivated fetal bovine serum and 1 % penicillin/ streptomycin mixture. Cells were cultured on 24-well tissue culture plates and incubated at 37 °C in 5 % CO₂ in a relatively humid atmosphere until a confluent monolayer was achieved.

Before the adhesion assay, the medium in the wells containing a Caco-2 cell monolayer was removed and replaced once with fresh antibiotic-free RPMI. Then, 1 × 10⁷ c.f.u. ml⁻¹ of bacteria were added to each well with a total volume of 1 ml and were incubated for 3 h at 37 °C in an atmosphere of 5 % (v/v) CO₂. To remove non-attached bacterial cells, the wells were washed three times with a sterile, pre-warmed PBS solution. To detach the cells from the wells, 1 ml of trypsin/EDTA solution (0.5 % porcine trypsin and 0.2 % EDTA in PBS; Sigma) was added to each well and the mixture was gently stirred for 5 min. To measure the viable Caco-2 cell count, the cells were counted in a Bürker haematocytometer chamber (Merck). Afterwards, this cell suspension (Caco-2 and bacteria cells) was subjected to c.f.u. determination by the
pure plate method onto MRS agar and incubated at 37 °C under anaerobic conditions. Bacterial adhesion was expressed as the total number of bacteria attached to viable Caco-2 cells.

**Antimicrobial activity.** The well diffusion technique was used to detect the production of inhibitory substances in the supernatant fluid of the isolates (Schillinger & Lücke, 1987). In the agar well diffusion assay, overnight cultures of the indicator strains were used to inoculate appropriate agar growth media at 37 °C (Dimitonova et al., 2007). Wells of 5 mm diameter were cut into agar plates and 50 μl of filtered, cell-free supernatant fluid obtained from the third subculture of the micro-organisms grown in MRS broth was added to each well. Supernatant fluid was obtained by growing the inhibitory producer strains overnight in MRS broth at 37 °C. Then, cells were removed by centrifugation, the supernatant fluid was placed in the wells, and the fluid was allowed to diffuse into the agar for 2 h at room temperature. Consequently, the plates were incubated at the optimum growth temperature of the indicator strains and examined after 24 h for the inhibition zone. At the end of the incubation, inhibition zone diameters (surrounding the wells) were measured (Maldonado et al., 2012; Nowroozi et al., 2004).

**Antibiotic susceptibility.** The patterns of resistance/sensitivity to antibiotics of the LAB isolates were determined using the disc diffusion method (Maldonado et al., 2012; Rahmati et al., 2011). LAB strains were incubated overnight under anaerobic conditions at 37 °C and 5% CO₂. Exactly 100 μl of the diluted cultures (approximately 10⁶–10⁷ viable cells) were diffused onto Mueller–Hinton agar and antibiotic discs were applied on the surface using an antibiotic disc dispenser. Plates were incubated at 37 °C under anaerobic conditions and assessed after 24 h of incubation. Inhibition zones around the discs were calculated using a digital caliper. The results were expressed in terms of resistance, moderate susceptibility, or susceptibility by comparing with the interpretative zone diameters given by the performance standards for antimicrobial disc susceptibility tests (Hoesl & Altwein, 2005).

**RESULTS**

**Isolation and identification of human vaginal LAB**

Forty-five strains were isolated from MRS and M17 agars. Among these strains were four Enterococcus faecium, five Enterococcus malodoratus, eight Enterococcus faecalis, three Enterococcus durans, four Enterococcus avium, two Enterococcus gilvus, four Enterococcus hirae, two Enterococcus pseudoavium, four Enterococcus lactis, two Lactobacillus casei, one Lactobacillus plantarum, three Lactobacillus acidophilus and three Lactococcus lactis (Table 1). The fact that the primers used were capable of amplifying enterococci, lactobacilli and lactococci genes shows that these primers are as specific as expected for amplifying genes of the LAB.

**Resistance to acidic condition**

All 45 LAB strains were incubated at pH 3.0 for 3 h. After 3 h incubation, 30 strains were found to be resistant to low pH (pH 3.0) with survivability higher than 50%. Only five strains demonstrated low pH resistance with a survival rate greater than 80% (Table 1). Strains such as Lactobacillus plantarum 5BL, E. durans 6HL and Lactococcus lactis 2HL were the species most resistant to low pH.

**Survival in bile salts**

All bacteria were incubated for 4 h in the presence of 0.3% bile salts. Among the bacteria, only 39 strains were resistant to 0.3% bile salts after 3 h incubation, with a survival rate higher than 50%. Only 14 strains showed bile salt resistance with survivability greater than 80% (Table 1). The most resistant species to bile salt conditions were E. durans 6HL and Lactococcus lactis 2HL, with 93% survivability.

**Survival in simulated in vitro digestion**

One of the most desirable features required for probiotics is their capability to stay alive in the gastrointestinal tract. To further examine the strains through a simulated digestion test, nine of the best strains that were resistant to low pH and bile salts were tested. Four strains showed considerable digestion survivability. The resistant strains were E. durans 6HL, Lactobacillus plantarum 5BL, Lactobacillus acidophilus 36VL and Lactococcus lactis 2HL. The highest percentage of survivability was observed for E. durans 6HL and Lactococcus lactis 2HL, with survivability values of 46% and 49%, respectively (Table 2). Based on our findings, the resistance to acid/bile conditions is strain-specific because of a wide range of diversity in survivability even among the same species.

**Adhesion assay to Caco-2 cells**

The four digestion-resistant LAB strains were examined for their capability to adhere to Caco-2 cells. The results showed that E. durans 6HL and Lactococcus lactis 2HL were the most adherent strains, with adhesion values of 4.8 × 10⁴ c.f.u. ml⁻¹ and 2.8 × 10⁵ c.f.u. ml⁻¹, respectively.

**Activity against pathogenic micro-organisms**

The results of the inhibitory action of two selected vaginal strains against pathogenic micro-organisms are shown in Table 3. In vitro tests under neutralized pH were performed. The results demonstrated antimicrobial activity for both examined strains. These strains were recognized as active because the capability to inhibit the growth of one or more target pathogenic strains was observed. The growth of Escherichia coli O26, Staphylococcus aureus and Shigella flexneri was suppressed by both LAB strains. The results showed that the inhibitory action of Lactococcus lactis 2HL was better than that of E. durans 6HL.

**Antibiotic susceptibility**

E. durans 6HL and Lactococcus lactis 2HL indicated good sensitivity to all nine antibiotics tested in this study, such as vancomycin, tetracycline, ampicillin, penicillin, erythromycin, clindamycin, gentamicin, chloramphenicol and sulfamethoxazole. The aureolys around the antibiotic discs in Fig. 1 illustrate the good susceptibility of E. durans 6HL for all nine examined antibiotics.
The majority of bacteria commonly recognized as probiotic are LAB, which are commensal micro-organisms in the human genital and gastrointestinal tracts. These bacteria have demonstrable useful properties and have a long history of being safe (Nueno-Palop & Narbad, 2011; Petricevic et al., 2012). Among the genital microflora, vaginal microbiota from healthy women can be considered as a good reservoir of probiotics for isolation. The presence of these probiotics in vaginal microbiota plays a significant role in the prevention of vaginal infections and in the maintenance of health (Strus et al., 2012). This study was undertaken to assess the potential probiotic properties of vaginal LAB isolated from Iranian women.

**Table 1.** Screening of LAB strains for their resistance to low pH (pH 3.0) and bile salts (0.3 % v/v)

The low pH survivability was measured after 3 h and bile salt survivability was determined after 4 h.

<table>
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<th>Strain code</th>
<th>Species</th>
<th>Low pH survival (%)</th>
<th>Bile salt survival (%)</th>
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Increasing interest has been given to the use of LAB isolated from human genitalia and gastrointestinal tract as probiotics to treat urogenital tract infections (Okkers et al., 1999). The potential application of vaginal LAB isolated from healthy women as probiotics has not been investigated in detail. In this study, vaginal swabs were collected from the lateral vaginal wall of 40 healthy Iranian women. As a result of the identification tests, 45 LAB isolates were identified as four Lactobacillus casei, one E. faecalis, E. durans, E. hirae, Lactobacillus plantarum, three Enterococcus faecalis, E. avium, E. pseudoavium, E. malodoratus, two Lactobacillus casei, one Lactobacillus casei, three Lactobacillus acidophilus and three Lactococcus lactis (Table 1). Enterococci were predominant among the LAB genera; this genus represented 80 % of the isolates, followed by lactobacilli (13 %) and lactococci (7 %). Similar results have been reported by other researchers. Redondo-Lopez et al. (1990) reported that Lactobacillus acidophilus was the dominant species among lactobacilli of vaginal microflora. A requirement for bacteria to be recognized as a probiotic is their capability to stay alive while passing through the upper digestive tract to reach the large intestine, where their useful actions are expected (Bezkorovainy, 2001; Tuomola et al., 2001). To be colonized in the intestine, probiotic bacteria have to adhere to the intestinal mucosa to avoid being removed from the colon by peristalsis. Nine vaginal isolates were examined for survival in simulated in vitro digestion and, among them, only four strains were able to survive exposure to the simulated digestion conditions of the stomach. Their adhesion ability to Caco-2 cells was evaluated by further analysis. In conclusion, we found that the strains Lactococcus lactis 2HL and E. durans 6HL were the strains most resistant to digestion conditions and were the best strains to adhere to Caco-2 cells. This screening showed that these strains could be candidates as new probiotics.

The growth of pathogens is inhibited by antagonistic substances produced during the growth of LAB which decrease the involvement of Escherichia coli via a reduction of the vaginal pH to 4.5 (Tomás et al., 2003). In vitro growth of many enteric pathogens has been demonstrated to be inhibited by LAB. LAB can treat a wide range of gastrointestinal disorders and can be used in both humans and animals (Aslim & Kılıç, 2006). Lactococcus lactis 2HL has been demonstrated to strongly inhibit the in vitro growth of Streptococcus mutans, Shigella flexneri, Gardnerella vaginalis, Bacillus cereus, Escherichia coli O26 and Salmonella typhimurium. The growth of other enteropathogenic bacteria such as Escherichia coli O157, Staphylococcus aureus, Listeria monocytogenes, Klebsiella pneumoniae, Pseudomonas
Aeruginosa, Candida albicans, Serratia marcescens and Staphylococcus saprophyticus was inhibited by Lactococcus lactis 2HL. The growth of five other strains, namely, Shigella flexneri, Listeria monocytogenes, Staphylococcus aureus, Escherichia coli O26 and Salmonella typhimurium, was inhibited by E. durans 6HL.

Annually, one million women are affected by urogenital tract infections worldwide (MacPhee et al., 2010). Such infections are responsible for high healthcare costs as well as high morbidity and mortality rates in females (Donders et al., 2009). Although Iranian women are not an exception, limited official epidemiological data are available on the subject. The most common pathogen associated with desquamative inflammatory vaginitis is Escherichia coli (Donders et al., 2009). According to Barzegari & Saei (2012), probiotics must be isolated with respect to native microbiomes and be used in the same population in the interest of efficacy. The efficiency of a probiotic can be found in the target population before usage to guarantee the health and therapeutic benefits. Therefore, two native (Escherichia coli O26) and non-native (Escherichia coli O157) pathogenic organisms were assessed in this study. The growth of the native strain was inhibited by both LAB strains, but the growth of the non-native strain was only inhibited by Lactococcus lactis 2HL.

Vancomycin resistance is one of the most important concerns in antibiotic resistance because vancomycin is one of the last antibiotics largely effective against clinical infections caused by multidrug-resistant pathogens (Johnson et al., 1990; Woodford et al., 1995). We determined the susceptibility of strains 2HL and 6HL to several antibiotics. Both strains were sensitive to tetracycline, ampicillin, penicillin, erythromycin, clindamycin, gentamicin, chloramphenicol and sulfamethoxazole. In conclusion, we identified two strains (E. durans and Lactococcus lactis) from the human vaginal tract that can survive the digestive system and potentially colonize the intestinal epithelial cells. We also show that these strains have good ability to inhibit the growth of many pathogenic micro-organisms and are sensitive to a broad range of antibiotics. These strains have good potential to be used as probiotics.

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**References**


