Novel Potential Probiotic Lactobacilli for Prevention and Treatment of Vulvovaginal Infections

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Abstract

Lactobacilli in the vaginal tract are essential to protect against microbial infections. We therefore focused on isolating vaginal lactobacilli from pregnant women and testing their functional properties. Lactobacilli were isolated from 50 vaginal swabs and the purified isolates were identified by MALDI-TOF MS. Functional properties (antimicrobial activity, organic acids and hydrogen peroxide production, antibiotic susceptibility, auto-aggregation, and hydrophobicity) of selected isolates were tested. Lactobacilli (41 strains) were identified in 58% of swabs with a predominance of *Lactobacillus crispatus* (48%) followed by *L. jensenii* (21%), *L. rhamnosus* (14%), *L. fermentum* (10%), and *L. gasseri* (7%). The highest antibacterial activity was determined for *L. fermentum* and *L. rhamnosus*. Strong anti-*Candida* activity was observed for strains *L. crispatus*, *L. fermentum*, and *L. rhamnosus*. Strain *L. jensenii* 58C possessed the highest production of hydrogen peroxide (6.32 ± 0.60 mg/l). The best lactic acid producer was strain *L. rhamnosus* 72A (11.6 ± 0.2 g/l). All strains were resistant to fluconazole and metronidazole. The highest auto-aggregation was observed for strain *L. crispatus* 51A ($98.8 \pm 0.1\%$ after 24 h). Strain *L. rhamnosus* 68A showed the highest hydrophobicity ($69.1 \pm 1.4\%$). Strains *L. fermentum* and *L. rhamnosus* showed high antibacterial activity and hydrophobicity, and strains *L. crispatus* possessed high auto-aggregation and anti-*Candida* activity. Thus, these strains alone or in a mix could be used for the preparation of probiotic products for treatment and prevention of vulvovaginal infections of pregnant and non-pregnant women.

Keywords Lactobacilli · Probiotics · Vulvovaginal infections · Vaginal microbiome

Introduction

The vaginal microbiome of healthy women is a dynamic ecosystem, and it is colonized by a variety of microorganisms. The composition of the vaginal microbiome is influenced by a number of factors such as age, hormonal levels, sexual activity, hygiene, phase of menstrual cycle, or diet [1, 2]. In healthy premenopausal women, bacteria of the genus *Lactobacillus* are dominant in the vaginal microbiome at 10^7–10^8 CFU/g of vaginal fluid [3]. The most frequently found are species of *L. crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii*, but this

depends on ethnic group or geographic location. There are also differences between pregnant and non-pregnant women; lower strain diversity was found in pregnant women. On the other hand, lactobacilli in pregnant women are more stable than in non-pregnant women [2, 4, 5].

The healthy urogenital tract plays a significant role in protecting against vaginal infections, and vaginal lactobacilli are important because of their protective functions (adhesion to the vaginal tissue and production of antimicrobial substances) [3]. An abnormal vaginal microbiome may lead to vaginal infections, and production of pro-inflammatory cytokines and prostaglandins, which can cause uterine contractions and weaken fetal membranes at pregnant women. Thus, the presence of abnormal microbiome in early pregnancy is recognized as a risk factor for preterm delivery and low birth weight. Preterm birth is defined as delivery before 37 completed weeks of gestation and it is responsible for neonatal morbidity and mortality almost in 80% [6–9]. Usually, preterm birth is caused by microbial attack in the amniotic cavity, especially by *Streptococcus agalactiae*. This pathogenic microorganism colonizes the lower vaginal tract and can also

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cause neonatal infections [4, 10, 11]. Preterm birth or amniotic fluid infection is also associated with bacterial vaginosis, which can be caused by a variety of microorganisms such as *Gardnerella vaginalis*, *Mycoplasma hominis*, or *Atopobium vaginae* [4, 12].

The protective role of vaginal lactobacilli is based on two main mechanisms. The first protective mechanism is specific adhesion of lactobacilli to the vaginal tissue, where formation of a biofilm can inhibit adhesion of pathogenic vaginal microorganisms by several mechanisms (e.g., competition for receptors, displacement of adhered pathogenic microorganisms, and avoiding their re-adhesion) [2, 13, 14]. The second protective mechanism is the production of antimicrobial substances (e.g., organic acids, hydrogen peroxide, bacteriocins) [15]. Organic acids, mainly lactic acid, are produced as the final product of carbohydrate fermentation and acidify the vaginal tract to pH 3.5–4.5. However, the pH is influenced by many factors (age, vaginal infection, and phase of menstrual cycle) [2, 9, 16, 17]. Some strains of vaginal lactobacilli, including *L. acidophilus*, *L. crispatus*, *L. fermentum*, and *L. jensenii*, are able to produce hydrogen peroxide. These lactobacilli are important because the hydrogen peroxide can reduce the development of bacterial vaginosis, as well as infections caused by HIV-1 and herpes simplex viruses [2, 18]. Other antimicrobial substances produced by vaginal lactobacilli are bacteriocins (proteins with antimicrobial activity), reuterin, and biosurfactants [2, 16, 18].

Lactobacilli, therefore, are able to inhibit the growth of pathogenic microorganisms through various mechanisms and may help in the reduction of vaginal infections. Knowledge of the biology and metabolic activities of vaginal lactobacilli is therefore important for the prevention and treatment of vaginal infections.

The aim of this study was to isolate vaginal lactobacilli from healthy pregnant women in the 36th week of pregnancy. We focused on functional properties testing, especially on antimicrobial activity testing, to find out strains suitable for the treatment of pregnant women's vaginal infections which induce preterm birth or neonatal infections.

Materials and Methods

Samples

Fifty swab samples were obtained from healthy pregnant women (36th week of pregnancy) from the Czech Republic. Samples were collected at the Department of Obstetrics and Gynecology of the First Faculty of Medicine, General University Hospital, Charles University, Prague, Czech Republic, and stored at 6 °C for 48–72 h. Sample collecting was approved by the Ethics Committee of the General University Hospital.

Isolation and Identification

Swabs were vortexed for 1 min at maximum speed (2800 min^{-1}) in de Man, Rogosa, and Sharpe (MRS) broth (Merck, Germany). The suspension was diluted 3 times with physiological saline. Each dilution (100 µl) was seeded on MRS agar (Merck, Germany) and blood agar (Columbia blood agar base with 5% defibrinated sheep blood) (Oxoid, UK) plates. The plates were incubated at 37 °C for 48 h, under a modified atmosphere containing 5% CO_2. After incubation, the colonies were identified by colony morphology, catalase test, and Gram staining. Selected colonies were identified by MALDI-TOF MS. Identified isolates were stored at -21 °C in MRS broth supplemented with glycerol (Penta, Czech Republic) (50% v/v).

Indicator Microorganisms

As indicator microorganisms for antimicrobial activity testing, we used the following pathogenic microorganisms. Five strains (*Escherichia coli* CCM 4517, *Gardnerella vaginalis* CCM 6221, *Staphylococcus aureus* CCM 4516, *Staphylococcus aureus* CCM 7719, *Candida albicans* CCM 8215) were obtained from the Czech collection of microorganisms (CCM, Czech Republic). *Gardnerella vaginalis* DSMZ 104275 was obtained from the German collection of microorganisms and cell cultures (DSMZ, Germany). Two strains (*Candida glabrata* ATCC 2001, *Candida parapsilosis* ATCC 22019) were obtained from the American type culture collection (ATCC, USA). Three strains (*Escherichia coli* MK 57B, *Streptococcus agalactiae* MK 14E, *Streptococcus agalactiae* MK 31B) were obtained from the Collection of microorganisms of the Department of Dairy, Fat and Cosmetics, both isolates originally from the vaginal tract.

Cultivation

Lactobacilli were cultivated in MRS broth at 37 °C, for 18 h, under a modified atmosphere containing 5% CO_2 (overnight grown culture). Pathogenic bacteria were cultivated aerobically in Brain heart infusion (BHI) broth (Himedia, India) at 37 °C, for 24 h. Pathogenic yeasts were cultivated aerobically in Malt extract broth (Oxoid, UK) at 30 °C, for 24 h.

Antibacterial Activity

Antibacterial activity of selected *Lactobacillus* spp. isolates was tested by agar spot-diffusion method against eight pathogenic microorganisms (*E. coli* CCM 4517, *E. coli* MK 57B, *G. vaginalis* DSMZ 104275, *G. vaginalis* CCM 6221, *S. aureus* CCM 4516, *S. aureus* CCM 7719, *St. agalactiae* MK 14E, *St. agalactiae* MK 31B). The BHI soft agar (pH 5.6 before sterilization) was inoculated with the indicator

pathogenic microorganisms at a density of 10^5 CFU/ml. The inoculated BHI soft agar was poured into a Petri dish and left to solidify. Live cells of each *Lactobacillus* strain (10 μl) were injected into the inoculated BHI soft agar. These plates were incubated aerobically at 37 °C for 24 h. Antibacterial activity was evaluated by the diameter of the zones of inhibition ($\leq$ 5 mm, low inhibition; 5–10 mm, medium inhibition, $\geq$ 10 mm, high inhibition).

Anti-*Candida* Activity

Anti-*Candida* activity of lactobacilli isolates was tested against three pathogenic yeasts (*C. albicans* CCM 8215, *C. glabrata* ATCC 2001, and *C. parapsilosis* ATCC 22019). Bacterial cells of an overnight grown culture were harvested by centrifugation at 8600g, for 5 min, at 4 °C. Cells were washed twice in physiological saline under the same conditions. Washed cells were adjusted to McFarland 2 in physiological saline. This suspension (1 ml) was inoculated onto a Petri dish and overlaid with MRS agar. Plates were incubated at 37 °C, for 24 h, under a modified atmosphere containing 5% CO_2. After 24 h, Malt extract agar was poured onto the inoculated plates. Yeast cells from an overnight grown culture were harvested by centrifugation at 8600g, for 5 min, at 4 °C. Cells were washed twice in physiological saline and then adjusted to McFarland 0.5 with the same solution. This suspension (100 μl) was inoculated onto the Malt extract agar layer. Plates were incubated at 37 °C, for 24 h, under a modified atmosphere containing 5% CO_2. These plates were subsequently incubated aerobically at 25 °C, for 24 h. The anti-*Candida* activity was assessed by yeast growth and inhibition activity was evaluated as follows: +++, high anti-*Candida* activity (total inhibition of *Candida* spp.); ++. medium anti-*Candida* activity; +, low anti-*Candida* activity; 0, no anti-*Candida* activity (high growth of *Candida* spp.).

Production of Organic Acids

Production of organic acids was detected by HPLC (Agilent 1260 Infinity, USA). Bacterial cells from an overnight grown culture were centrifuged at 17,000g, for 10 min, at 4 °C. The supernatant (250 μl) was transferred to an Eppendorf tube and 1600 μl of ethanol (96%) were added and mixed. This mixture was incubated for 30 min at room temperature and then centrifuged at 17,000g, for 10 min, at 4 °C. After centrifugation, the mixture was filtered through a 0.22 μm PVDF filter. HPLC was performed using a Polymer IEX H column (Watrex, Czech Republic) (350 × 8 mm) with a pre-column (50 × 8 mm). The column and pre-column were preheated to 60 °C. The mobile phase was H_2SO_4 (9 mmol/l) with a flow of 0.6 ml/min. Detection was by UV detector (210 nm; 55 °C; 500 mV). The concentration of lactic acid was evaluated using a calibration curve.

Hydrogen Peroxide Production—Plate Method

Determination of hydrogen peroxide produced by lactobacilli was tested on MRS agar supplemented with 3,3′,5,5′-tetramethylbenzidine (250 μl/ml) (Sigma-Aldrich, USA) and horseradish peroxidase (0.01 mg/ml) (Sigma-Aldrich, USA). Plates were inoculated with selected *Lactobacillus* spp. and incubated anaerobically at 37 °C, for 48 h, using an anaerobic jar containing AnaeroGen™ (Oxoid, UK). After incubation, plates were exposed to aerobic conditions for 30 min. Colonies of hydrogen peroxide producing strains form a blue pigment.

Detection of Hydrogen Peroxide Concentration—Spectrophotometric Method

Bacterial cells from an overnight grown culture were centrifuged at 8600 g, for 5 min, at 4 °C. Supernatant was removed and the cells were washed twice by phosphate buffer (1 mol/l; pH 6.8). Cooled phosphate buffer (20 ml) was added to washed cells and mixed. This suspension was cultivated at 5 °C, for 48 h. After cultivation, the suspension was centrifugated at 8600g, for 5 min, at 4 °C. Obtained supernatant was used for the quantification of H_2O_2. The supernatant (5 ml) was mixed with water solution of horse radish peroxidase (1 ml; 0.001% w/v) and methanolic solution of o-dianisidine (0.1 ml; 1% w/v) (Sigma-Aldrich, USA), and this mixture was cultivated at 37 °C, for 10 min. Then HCl solution (0.2 ml; 4 mol/l) was added and the absorbance of samples was measured at 400 nm. Concentration of H_2O_2 produced by lactobacilli was evaluated according to the calibration curve of H_2O_2, which was measured by the same procedure with H_2O_2 solutions in phosphate buffer instead of supernatant.

Hemolytic Activity

Lactobacilli were streaked onto blood agar with 5% of defibrinated sheep blood. Inoculated plates were incubated at 37 °C, for 48 h, under a modified atmosphere with 5% of CO_2. The result was evaluated visually according to zones of hemolysis. The *Staphylococcus aureus* CCM 3953 was used as a positive control, which showed β-hemolysis.

Antibiotic Resistance

Antibiotic resistance was tested by disk diffusion method with 15 antibiotics (Oxoid, UK): ampicillin (AMP; 10 μg), cefotaxime (CTX; 30 μg), chloramphenicol (C; 30 μg), ciprofloxacin (CIP; 5 μg), clindamycin (DA; 2 μg), erythromycin (E; 15 μg), fluconazole (FCA; 25 μg), gentamicin (CN; 10 μg), kanamycin (K; 30 μg), metronidazole (MTZ;

5 μg), nitrofurantoin (F; 300 μg), ofloxacin (OFX; 5 μg), streptomycin (S; 10 μg), tetracycline (TE; 30 μg), and vancomycin (VA; 30 μg). Bacterial cells of an overnight grown culture were centrifuged at 8600g, for 5 min, at 4 °C. Cells were washed in physiological saline under the same conditions. Washed cells were adjusted to McFarland 1 in physiological saline. This suspension (100 μl) was inoculated onto Mueller-Hinton agar (Oxoid, UK) plates and MRS agar plates. Antibiotic discs were placed on the surface of the agar and the plates were incubated at 37 °C, for 48 h, under a modified atmosphere containing 5% of CO_2. Diameters of inhibition zones were measured after incubation.

Auto-Aggregation

Bacterial cells from an overnight grown culture were harvested by centrifugation at 8600g, for 5 min, at 4 °C. Cells were washed twice in phosphate buffered saline (PBS) (pH 7.2 before sterilization) under the same conditions. Cells were adjusted to an absorbance (650 nm) of 0.45–0.55. Samples were incubated aerobically at 37 °C, for 24 h. At times 0, 6, and 24 h, 1 ml of the upper layer was transferred to a cuvette and the A_{650} was measured. The percentage of auto-aggregation was calculated using the following equation. As a blank, pure PBS was used.

$$\text{Auto}-\text{aggregtion }(\%) = \left[1 - \left(\frac{A_0}{A_x}\right)\right] 100$$

$A_0 \ldots A_{650}$ at 0 h
$A_x \ldots A_{650}$ at times 6 or 24 h

Hydrophobicity

Bacterial cells from an overnight grown culture were harvested by centrifugation at 8600g, for 5 min, at 4 °C. Cells were washed twice in PBS (pH 7.2 before sterilization) under the same conditions. The suspension was adjusted to an A_{650} of 0.45–0.55 and 3 ml were added to a glass tube. Hexane (0.5 ml) was added, vortexed for 1 min and incubated for 20 min at room temperature. The water layer was then removed and the A_{650} was measured. The percentage of hydrophobicity was calculated using the following equation. As a blank, pure PBS was used.

$$\text{Hydrophobicity }(\%) = \left[\frac{(A_{\text{before}} - A_{\text{after}})}{A_{\text{before}}}\right] 100$$

$A_{\text{before}} \ldots A_{650}$ adjusted before extraction
$A_{\text{after}} \ldots A_{650}$ after extraction

Results

Identification of Vaginal Lactobacilli

Bacteria from the genus *Lactobacillus* occurred in 29 (58%) out of 50 samples of vaginal swabs of pregnant women and 41 strains of lactobacilli were isolated. The most frequent species was *L. crispatus* (48%). The next most frequent were *L. jensenii* (21%), *L. rhamnosus* (14%), *L. fermentum* (10%), and *L. gasseri* (7%). Pre-screening of all isolates was focused especially on growth ability and afterwards 21 isolates were selected for further testing of functional properties.

Antibacterial Activity

Antibacterial activity of lactobacilli was tested by the agar spot-diffusion method against five indicator pathogenic microorganisms. The antibacterial activity was measured with live lactobacilli. Results are summarized in Table 1. The major proportion of isolates was inhibitory against all pathogenic bacteria tested. All isolates inhibited growth of *G. vaginalis* DSMZ 104275 and *Str. agalactiae* MK 14E. Strains *L. rhamnosus* 10A, *L. fermentum* 74A, and *L. fermentum* 74B were strongly inhibitory against all pathogenic bacteria tested.

Anti-*Candida* Activity

Lactobacillus spp. isolates were tested against three pathogenic yeasts and results are presented in Table 2. The highest anti-*Candida* activity was observed against *C. albicans* CCM 8215 by all lactobacilli tested. Isolates of *L. crispatus*, *L. fermentum*, and *L. rhamnosus* showed very good inhibitory activity against *Candida* spp. whereas most strains of *L. gasseri* and *L. jensenii* showed poor or no anti-*Candida* activity.

Production of Hydrogen Peroxide and Organic Acids

Lactobacilli are able to produce antimicrobially active substances. The majority are organic acids (mainly lactic acid) and hydrogen peroxide. All isolates were tested for hydrogen peroxide production and this was determined by pigment changes in colonies. It was found that 71% (Table 3) of isolates were able to produce hydrogen peroxide. Production was divided into two groups, low production of H_2O_2 (light blue pigmented colonies) and high production of H_2O_2 (dark blue pigmented colonies). Color changes of colonies were detected for all strains of *L. crispatus*, *L. gasseri*, and *L. jensenii*. On the other hand, detection of hydrogen peroxide by spectrophotometry method showed all strains are able to produce hydrogen peroxide in different concentrations. The highest concentration of H_2O_2 was detected for *L. jensenii* 58C (6.32 ±

Table 1 Antibacterial activity of *Lactobacillus* spp. (live cells) against pathogenic bacteria

Lactobacillus spp.		Vaginal strains			Commercial culture strains				
		St. agalactiae MK[a] 14E	*St. agalactiae* MK[a] 31B	*E. coli* MK[a] 57B	*E. coli* CCM[b] 4517	*G. vaginalis* DSMZ[c] 104,275	*G. vaginalis* CCM[b] 6221	*S. aureus* CCM[b] 4516	*S. aureus* CCM[b] 7719
L. crispatus	2A	+++	+++	++	++	+++	+++	++	++
	46B	+++	+++	++	++	+++	+++	++	++
	49C	+++	++	++	++	++	++	++	++
	51A	+++	+++	++	++	+++	+++	++	++
	55B	++	++	++	+	+++	+++	+	++
	69E	++	++	++	++	+++	+++	++	++
L. gasseri	8D	+++	+++	++	++	+++	+++	+	++
	71A	++	+	+	+	++	++	0	+
	71B	++	+	+	++	++	+	0	0
	71C	++	++	0	0	++	++	0	+
	71D	+++	++	+	++	+++	++	++	0
L. jensenii	49E	+++	++	++	+	++	++	0	0
	51E	++	++	++	++	++	++	++	+
	58C	++	++	+	+	++	++	++	++
	62A	+++	++	++	+	+++	+++	+	+
L. rhamnosus	10A	+++	+++	+++	+++	+++	+++	+++	+++
	68A	++	++	++	+++	+++	+++	++	++
	72A	+++	+++	+++	+++	+++	+++	++	++
L. fermentum	43B	+++	+++	+++	++	+++	+++	+++	+++
	74A	+++	+++	+++	+++	+++	+++	+++	+++
	74B	+++	+++	+++	+++	+++	+++	+++	+++

0, without inhibitory activity; +, low inhibitory activity (diameter of zone ≤ 5 mm); ++, medium inhibitory activity (diameter of zone 5–10 mm); +++, high inhibitory activity (diameter of zone ≥ 10 mm)

[a] Strains isolated from swabs of non-pregnant women with vaginal infection (MK)

[b] Strains obtained from the Czech collection of microorganisms (CCM)

[c] Strain obtained from the German collection of microorganisms and cell cultures (DSMZ)

0.60 mg/l). The lowest concentration of H_2O_2 was detected for strain *L. rhamnosus* 72A (0.33 ± 0.05 mg/l).

Production of organic acids was tested by HPLC and the concentration of lactic acid was calculated (Table 3). The concentration of lactic acid ranged from 4.6 ± 0.1 g/l to 11.6 ± 0.2 g/l and was strain specific. The best lactic acid producer was strain *L. rhamnosus* 72A (11.6 ± 0.2 g/l), followed by strains *L. crispatus* 2A and *L. crispatus* 69E (10.4 ± 0.0 g/l for both of them). The lowest concentration of lactic acid was detected for strain *L. crispatus* 51A (4.6 ± 0.1 g/l).

Hemolytic Activity

No strain showed the β-hemolysis (complete lysis of erythrocytes) on blood agar with defibrinated sheep blood. Strains *L. gasseri* (71 B, 71C) showed α-hemolysis (partial decomposition of hemoglobin). No hemolysis was detected for other tested lactobacilli.

Antibiotic Resistance

Antibiotic resistance was tested for all 21 *Lactobacillus* spp. isolates on Mueller-Hinton agar and MRS agar plates. Differences between each tested media were detected in 28% cases without significant variance. Table 4 shows the sensitivity of each strain to antibiotics on Mueller-Hinton agar. All isolates were resistant to fluconazole and metronidazole and sensitive to ampicillin, erythromycin, chloramphenicol, and tetracycline. When antibiotic susceptibility was tested on MRS agar, lactobacilli possessed higher resistance to gentamicin, kanamycin, and streptomycin. Sensitivity to other antibiotics tested was strain specific.

Auto-Aggregation and Hydrophobicity

The results of auto-aggregation and hydrophobicity testing are summarized in Table 3. These results represent strain diversity between isolates. Our isolates showed auto-aggregation

Table 2 Anti-*Candida* activity of *Lactobacillus* spp. isolates

Lactobacillus spp.		*C. albicans* CCM[a] 8215	*C. glabrata* ATCC[b]2001	*C. parapsilosis* ATCC[b] 22,019
L. crispatus	2A	++	++	+
	46B	+++	++	++
	49C	++	+	+
	51A	++	+	+
	55B	+++	+++	++
	69E	++	++	++
L. gasseri	8D	++	+	+
	71A	+	+	0
	71B	+	0	0
	71C	0	0	0
	71D	0	0	0
L. jensenii	49E	0	0	0
	51E	0	0	0
	58C	+	+	+
	62A	+	+	0
L. rhamnosus	10A	+++	++	++
	68A	+++	++	+
	72A	+++	++	++
L. fermentum	43B	+++	++	+
	74A	+++	+++	++
	74B	+++	+++	++

+++, high anti-*Candida* activity (total inhibition of *Candida* spp.); ++, medium anti-*Candida* activity; +, low anti-*Candida* activity; 0, no anti-*Candida* activity (high growth of *Candida* spp.)

[a] Strain obtained from the Czech collection of microorganisms (CCM)

[b] Strains obtained from American type culture collection (ATCC)

between 12.1–89.9% after 6 h and 32.5–98.8% after 24 h. A significant proportion of *L. crispatus* strains tested showed very strong auto-aggregation, more than 90% after 24 h. The highest auto-aggregation was determined for strain *L. crispatus* 51A (98.8 ± 0.1% after 24 h) and the lowest was observed for strain *L. rhamnosus* 10A (32.5 ± 3.8% after 24 h).

Hydrophobicity of our isolates was in the range 19.1–69.1%. The highest hydrophobicity was exhibited by strain *L. rhamnosus* 68A, which achieved 69.1 ± 1.4% and the lowest was shown by strain *L. jensenii* 51E (19.4 ± 2.4%). In this study, no correlation between auto-aggregation and hydrophobicity was observed.

Discussion

Lactobacilli were isolated from vaginal swabs obtained from healthy pregnant women in the third trimester. All swabs were collected in the same time in the 36th week of pregnancy to obtain relevant results. During this period, estrogen levels are high, as well as lactobacilli abundance. Romero et al. [19] found out that bacterial microbiota is more stable during normal pregnancy than in the non-pregnant state. Also, they identified four lactobacilli (*L. crispatus*, *L. gasseri*, *L. jensenii*, *L. vaginalis*) as species with higher relative abundance in pregnant women. In our study, the most frequent species were *L. crispatus*, followed by *L. jensenii*, *L. rhamnosus*, *L. fermentum*, and *L. gasseri*. These results are typical for European woman and they correspond with several studies. For example, Kiss et al. [20] observed lactobacilli in the vaginal microbiome of pregnant women of Caucasian origin, with *L. crispatus* and *L. gasseri* being the most frequently found species, followed by *L. jensenii* and *L. rhamnosus*. Petricevic et al. [6] identified five leading *Lactobacillus* spp. in the vaginal microbiome of pregnant women: *L. crispatus*, *L. iners*, *L. gasseri*, *L. johnsonii*, and *L. jensenii*. In our study *L. iners* was not identified. *Lactobacillus iners* grows on blood agar and is uncultivatable on MRS agar, in contrast with other lactobacilli that grow readily on MRS agar. In our study, lactobacilli were isolated only by cultivation, in contrast with other studies where genetic methods, independent of cultivation, were used for identification purposes. This may explain why *L. iners* was not detected in our study.

Antimicrobial activity of isolates is considered as a significant criterion for probiotic strain selection. The majority of

Table 3 Functional properties of vaginal lactobacilli

Lactobacillus spp.		H₂O₂		Lactic acid [g/l]	Auto-aggregation [%]		Hydrophobicity [%]
		Colony pigment	[mg/l]		6 h	24 h	
L. crispatus	2A	++	1.32 ± 0.06	10.4 ± 0.0	56.3 ± 0.2	72.2 ± 4.5	25.7 ± 0.9
	46B	+	1.86 ± 0.33	9.1 ± 0.2	35.9 ± 1.3	96.5 ± 2.4	39.5 ± 0.6
	49C	+	0.97 ± 0.01	6.7 ± 0.0	89.9 ± 4.9	96.1 ± 1.6	24.8 ± 0.2
	51A	+	2.27 ± 0.01	4.6 ± 0.1	73.2 ± 3.6	98.8 ± 0.1	62.8 ± 1.7
	55B	+	0.71 ± 0.08	7.6 ± 0.0	29.0 ± 2.7	87.3 ± 2.5	19.1 ± 0.6
	69E	++	1.69 ± 0.05	10.4 ± 0.0	82.6 ± 2.1	93.2 ± 8.7	49.2 ± 0.6
L. gasseri	8D	++	0.29 ± 0.09	10.2 ± 0.2	61.2 ± 1.0	80.2 ± 3.4	62.1 ± 0.4
	71A	++	1.88 ± 0.23	6.5 ± 0.0	30.3 ± 3.2	70.4 ± 1.9	40.9 ± 0.9
	71B	++	4.22 ± 0.2	7.9 ± 0.0	36.3 ± 3.2	40.6 ± 1.4	24.6 ± 0.2
	71C	++	1.95 ± 0.12	6.5 ± 0.1	37.9 ± 0.6	53.6 ± 2.6	35.1 ± 1.8
	71D	++	4.17 ± 0.10	6.9 ± 0.1	41.2 ± 2.7	52.7 ± 1.8	29.1 ± 0.5
L. jensenii	49E	+	5.05 ± 0.17	6.5 ± 0.1	26.1 ± 0.1	45.5 ± 3.6	25.4 ± 0.2
	51E	+	1.58 ± 0.37	7.7 ± 0.2	27.4 ± 1.9	58.9 ± 1.9	19.4 ± 2.4
	58C	+	6.32 ± 0.60	8.3 ± 0.2	41.2 ± 3.9	73.3 ± 1.9	42.0 ± 0.1
	62A	++	0.67 ± 0.00	5.9 ± 0.1	39.7 ± 1.0	64.2 ± 0.8	64.8 ± 4.2
L. rhamnosus	10A	–	0.90 ± 0.08	8.5 ± 0.3	16.1 ± 2.2	32.5 ± 3.8	50.4 ± 0.8
	68A	–	0.53 ± 0.03	10.3 ± 0.1	36.8 ± 0.1	51.0 ± 0.2	69.1 ± 1.4
	72A	–	0.33 ± 0.05	11.6 ± 0.2	36.4 ± 0.5	53.5 ± 0.1	23.8 ± 0.2
L. fermentum	43B	–	1.13 ± 0.07	7.2 ± 0.1	12.1 ± 2.1	42.3 ± 0.6	53.4 ± 0.9
	74A	–	0.55 ± 0.06	6.9 ± 0.0	23.5 ± 1.2	47.0 ± 0.1	36.3 ± 1.0
	74B	–	0.74 ± 0.07	6.5 ± 0.1	28.8 ± 1.4	51.9 ± 1.9	50.9 ± 0.5

–, without production of H₂O₂; +, production of H₂O₂ (light blue pigment); ++, high production of H₂O₂ (dark blue pigment)

isolates showed medium or high inhibitory activity against the pathogenic bacteria tested. All strains inhibited growth of *Str. agalactiae* MK 14E. *Streptococcus agalactiae* can colonize newborns during delivery and can cause fetal sepsis [6]. Our vaginal isolates therefore have the potential to decrease the number of *Str. agalactiae* in pregnant women and to decrease the risk of fetal sepsis. All isolates were able to inhibit *G. vaginalis* DSMZ 104275, the bacteria associated with bacterial vaginosis, and almost all isolates also showed inhibitory activity against bacteria that are usually associated with aerobic vaginitis, such as *E. coli* and *S. aureus*. Aerobic vaginitis and bacterial vaginosis appear to be linked with spontaneous miscarriage, premature rupture of membranes, and increased risk of preterm birth [21, 22].

Some strains of vaginal lactobacilli inhibit the growth of *Candida* spp. [23]. Our results show that the highest inhibitory activity occurred with strains of *L. crispatus*, *L. fermentum*, and *L. rhamnosus*. These results are in accordance with the study of Strus et al. [23], where strains of *L. fermentum* and *L. rhamnosus* showed partial or total inhibition of *Candida* growth. Hütt et al. [24] tested antagonistic activity of *L. crispatus*, *L. gasseri*, and *L. jensenii* against *C. albicans* and *C. glabrata*. In that study, *L. crispatus* showed substantially higher antagonistic activity than *L. gasseri* and

L. jensenii. In general, inhibitory activity of lactobacilli decreased in the order *C. albicans*, *C. glabrata*, and *C. parapsilosis*.

Hydrogen peroxide is an antimicrobial substance produced by certain strains of vaginal lactobacilli. These lactobacilli play a significant role, especially in the vaginal tract of pregnant women. Hydrogen peroxide has a protective effect against catalase-negative microorganisms such as *G. vaginalis*, which cause bacterial vaginosis [4, 25]. Hydrogen peroxide producing strains are responsible for maintaining microbial balance and their absence is associated with the development of bacterial vaginosis [2, 18]. In this study, we found that 71% of isolates were able to produce hydrogen peroxide, especially strains of *L. crispatus*, *L. gasseri*, and *L. jensenii*. Rabe and Hillier [26] also observed production of hydrogen peroxide by the same vaginal lactobacilli. Bouridane et al. [25] tested 70 isolates and 57 of them (81.42%) were able to produce hydrogen peroxide.

Lactic acid is an antimicrobial compound produced by lactobacilli as the main product of carbohydrate fermentation. Lactic acid maintains vaginal pH at values 3.5–4.5 and inhibits growth of pathogenic microorganisms [17]. Production of lactic acid is strain specific. In a previous study, the best lactic acid producers were strains of *L. gasseri* [24]. In

Table 4 Antibiotic susceptibility of vaginal isolates on Mueller-Hinton agar

Lactobacillus spp.		AMP	CTX	CN	E	C	TE	OFX	S	DA	FCA	K	MTZ	CIP	VA	F
L. crispatus	2A	S	S	R/S	S	S	S	R	R/S	S	R	R/S	R	R	S	S
	46B	S	S	S	S	S	S	S	S	S	R	R/S	R	R	S	S
	49C	S	S	S	S	S	S	R	R/S	S	R	S	R	R/S	S	S
	51A	S	S	S	S	S	S	R	S	S	R	R	R	R	S	R
	55B	S	S	S	S	S	S	R	R/S	S	R	R/S	R	R	S	R/S
	69E	S	S	R/S	S	S	S	R	R	S	R	R	R	R	S	R
L. gasseri	8D	S	S	S	S	S	S	R	S	R	R	R/S	R	R	S	R
	71A	S	S	S	S	S	S	R	S	S	R	R/S	R	S	R	R
	71B	S	S	S	S	S	S	R	S	R/S	R	S	R	R	S	S
	71C	S	S	S	S	S	S	R	R/S	S	R	R/S	R	R	S	S
	71D	S	S	S	S	S	S	R	S	R	R	R/S	R	R	S	S
L. jensenii	49E	S	S	R/S	S	S	S	R	S	R/S	R	R/S	R	R	S	R
	51E	S	S	R	S	S	S	S	S	S	R	S	R	R	S	R
	58C	S	S	S	S	S	S	R	R/S	S	R	S	R	R	S	R
	62A	S	S	S	S	S	S	R	S	S	R	S	R	R	S	S
L. rhamnosus	10A	S	S	S	S	S	S	R/S	R/S	S	R	R/S	R	R/S	R	S
	68A	S	S	S	S	S	S	R	R/S	S	R	R/S	R	R/S	R	S
	72A	S	S	S	S	S	S	S	R/S	S	R	R/S	R	S	R	S
L. fermentum	43B	S	S	R	S	S	S	S	R/S	S	R	R	R	R	R	S
	74A	S	R	S	S	S	S	R	R/S	S	R	S	R	R	R	S
	74B	S	R	S	S	S	S	R	R/S	S	R	S	R	R	R	S

R. resistant (diameter of zone < 12 mm); R/S, intermediate (diameter of zone 12–16 mm); S, sensitive (diameter of zone > 16 mm)

our study, the highest concentration of lactic acid was detected for strain *L. rhamnosus* 72A, which was followed by strains of *L. crispatus*.

It is very important to know the antibiotic susceptibility of vaginal lactobacilli, because resistance to an antibiotic can be considered as advantageous. A resistant strain can be administered together with a vaginal drug and the strain can start colonizing the urogenital mucosa [25, 27]. In our study, it was determined that all strains were resistant to metronidazole and fluconazole. Usually, metronidazole is used to treat bacterial vaginosis [16, 28]. Thus, these strains could be co-administrated with these antibiotics during treatment of bacterial vaginosis.

Adherence to vaginal cells is one antimicrobial mechanism. A previous study showed that there was a correlation between adhesion to the vaginal tissue and auto-aggregation [29]. Auto-aggregation can correlate with hydrophobicity [27, 30], but in our study, this correlation was not proven. Generally, strain diversity was detected for each property tested.

For future testing, six strains with appropriate functional properties were selected. Strains *L. crispatus* 46B and *L. crispatus* 69E were capable of substantial production of hydrogen peroxide, strong auto-aggregation, and high or medium anti-*Candida* activity. High antibacterial activity against all indicator pathogenic microorganisms was proven for strains *L. rhamnosus* 10A, *L. fermentum* 74A, and *L. fermentum* 74B. Relatively satisfactory properties were determined for strain *L. gasseri* 8D, which showed high hydrogen peroxide production, strong auto-aggregation, and high inhibitory activity against *G. vaginalis* and *Str. agalactiae*. Thus, these lactobacilli isolates could be appropriate candidates on the base of their stability and properties according to Romero et al. [19], where higher stability of lactobacilli from pregnant women was detected. On the other hand, Romero et al. [19] compared isolates from pregnant women with isolates from non-pregnant women. This comparison has not been done in our study, so it could be little limitation of our research. Also, weaknesses of this study may be that all tested properties were determine only in vitro and lactobacilli may show a little bit different property in the real system of vaginal tract. Therefore, it is necessary to subject selected strains for clinical trials.

In conclusion, lactobacilli are important for maintaining the normal environment of the vaginal tract. Lactobacilli isolated in our study have a promising potential for future use and six selected strains (*L. crispatus* 46B and 69E, *L. fermentum* 74A and 74B, *L. gasseri* 8D, *L. rhamnosus* 10A) could be potential probiotic candidates for clinical trials. They could be used for preparation of probiotic products for treatment and prevention of vulvovaginal infections, especially for pregnant women with abnormal microbiome to reduce risk of preterm birth or

neonatal infection causes by *Streptococcus agalactiae*. The advantage of lactobacilli isolated from the vaginal tract of pregnant women is the increased chance of their later adhesion and colonization of the tract since this represents a transfer back to their natural environment. They can also be potentially used in non-pregnant women as a replacement for antibiotic therapy of vulvovaginal infections.

Compliance with Ethical Standards

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Sample collecting was approved by the Ethics Committee of the General University Hospital, Prague.

Conflict of Interest The authors declare that they have no conflict of interest.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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