INSTITUT PASTEUR

Original article

Lactic acid bacteria colonization and clinical outcome after probiotic supplementation in conventionally treated bacterial vaginosis and vulvovaginal candidiasis

Sophia Ehrström [a,*], Katalin Daroczy [b], Eva Rylander [a], Carolina Samuelsson [c], Ulrika Johannesson [a], Bo Anzén [a], Carl Påhlson [b]

[a] Karolinska Institutet, Department of Clinical Sciences, Division of Obstetrics and Gynecology, Danderyd Hospital, Stockholm, PA 182 88, Sweden
[b] Mälardalen University, School of Sustainable Development of Society and Technology, Department of Chemistry, Eskilstuna, Sweden
[c] Department of Anaesthesia and Intensive Care, Halmstad County Hospital, Halmstad, Sweden

Abstract

This randomized double-blind placebo controlled study assessed the vaginal colonization of lactic acid bacteria and clinical outcome. Vaginal capsules containing *L gasseri* LN40, *Lactobacillus fermentum* LN99, L. *casei subsp. rhamnosus* LN113 and *P. acidilactici* LN23, or placebos were administered for five days to 95 women after conventional treatment of bacterial vaginosis and/or vulvovaginal candidiasis. Vulvovaginal examinations and vaginal samplings were performed before and after administration, after the first and second menstruation, and after six months. Presence of LN strains was assessed using RAPD analysis. LN strains were present 2–3 days after administration in 89% of the women receiving LN strains (placebo: 0%, $p < 0.0001$). After one menstruation 53% were colonized by at least one LN strain. Nine percent were still colonized six months after administration. Ninety-three percent of the women receiving LN strains were cured 2–3 days after administration (placebo: 83%), and 78% after one menstruation (placebo: 71%) (ns). The intervention group experienced less malodorous discharge 2–3 days after administration ($p = 0.03$) and after the second menstruation ($p = 0.04$), compared with placebo. In summary, five days of vaginal administration of LN strains after conventional treatment of bacterial vaginosis and/or vulvovaginal candidiasis lead to vaginal colonization, somewhat fewer recurrences and less malodorous discharge.
© 2010 Elsevier Masson SAS. All rights reserved.

Keywords: Bacterial vaginosis; Vulvovaginal candidiasis; Lactobacilli; Probiotics; RAPD analysis

1. Introduction

The healthy vaginal flora of women of childbearing age is characterized by a complex ecology of microorganisms [1–3] where lactobacilli are the predominant species. By producing lactic acid, lactobacilli protect the vagina against ascending microbes from the GI-tract, thus conserving a physiological pH below 4.5 [4,5]. Lactobacilli may also produce hydrogen peroxide and bacteriocins, thereby inhibiting the adhesion of pathogenic microorganisms to the vaginal epithelial cells [6–10].

Bacterial vaginosis (BV) is a polymicrobial syndrome where the lactobacilli-dominated vaginal microflora is replaced by a mix of other, mainly anaerobic bacteria species [11,12]. BV is characterized by a thin, greyish-white, malodorous discharge, a vaginal pH > 4.5, and presence of clue cells on microscopy (Amsel criteria) [13]. Metronidazole, orally or vaginally, or vaginal clindamycin are effective treatments for BV on the short term. In a meta-analysis, the expected cure rate after one month was 70–80% for metronidazole [14], and 82% for clindamycin [15]. However, one and three months following the antibiotic treatment period, the

* Corresponding author. Tel.: + 46 8 655 50 00; fax: + 46 8 655 77 87.
 E-mail address: sophia.ehrstrom@ds.se (S. Ehrström).

recurrence rate has been reported to be as high as 15–30% and 50–60%, respectively [16,17], possibly due to an inability of the host to restore the lactobacilli-dominated vaginal flora [18,19]. Consequently, there is a need for a treatment with long lasting effect. Untreated, BV may lead to premature birth [20], late miscarriage, intrauterine fetal death [21], postoperative infections and pelvic inflammatory disease [22].

Vulvovaginal candidiasis (VVC) occurs when there is an overgrowth of yeast, most frequently *Candida albicans*, which normally inhabits the vaginal ecology in low numbers [23–25], the vaginal lactobacilli levels are not altered in women with VVC [26]. Typical symptoms include itching, discharge, and sometimes a burning sensation of the vulvar skin. Approximately 75% of all women experience at least one yeast infection in their lifetime. VVC may be treated with local or oral antifungals [27]. However, recurrences are frequent, and 5–8% of all women report more than 3–4 episodes of yeast infection every year (recurrent vulvovaginal candidiasis; RVVC) [24]. This disorder causes great health care costs and suffering, and may require long-time oral antifungal treatment [28].

BV and VVC are the two most common causes of vulvovaginal discharge, itching and irritation in fertile women, responsible for 80–90% of all cases of vaginitis [16,29].

Since the disturbed vaginal flora in BV is associated with a reduced number of lactobacilli, the idea of obtaining better long-term cure by administrating exogenous lactobacilli is appealing. Exogenous probiotic lactobacilli have been considered as supplementation therapy to conventional treatment of BV and VVC since lactobacilli have shown antimicrobial effects *in vitro* on both a range of BV associated microorganism and *C. albicans* [6,30–32]. However, these results do not necessarily apply *in vivo*. In addition to *in vitro* properties of the lactobacilli, it may be critical that the probiotic strains have the ability to colonize the vagina. Previous studies have shown that some strains of lactobacilli may colonize the vagina of women up to one month after administration [33–36] and certain investigated lactobacilli have been shown to reduce the recurrence rate of BV [37,38]. However, other studies have failed to repeat these results [39]. Clinical trials in women with VVC have so far showed conflicting results regarding the benefit of probiotics, but there are methodological problems with several studies, including too small sample size, lack of control groups, and means of bacteria administration [38,40,41]. Further studies are needed to evaluate these issues.

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [42]. Probiotics are principally lactic acid bacteria (LAB) strains. The lactic acid bacteria strains that were used in this study were originally isolated from the vaginal flora of healthy women and belong to the most common *Lactobacillus* species found in the healthy vagina [12,43–46].

This randomized, placebo controlled, double-blind trial evaluated *L. gasseri* LN40, *Lactobacillus fermentum* LN99, *Lactobacilli casei subsp. rhamnosus* LN113 and *P. acidilactici* LN23 as supplementation to conventional treatment of BV and VVC. The aim of the study was to assess the colonization ability of the LN strains after vaginal administration. Clinical symptoms, vaginal pH, and recurrence rate of BV or VVC related to the probiotic supplementation were also evaluated.

2. Materials and methods

2.1. Study population

Between March 2006 and December 2007, 168 women, recruited by advertising in regular newspapers, were examined at the Department of Obstetrics and Gynecology at Danderyd hospital, Stockholm, Sweden, due to symptoms of malodorous discharge. They were screened for BV and VVC.

One hundred women fulfilled the inclusion criteria, which were: women aged between 18 and 45, diagnosed and treated for VVC and/or BV, signed informed consent, examined at least eight days before the first day of next menstruation. Exclusion criteria were: pregnancy, breastfeeding, pelvic inflammatory disease, current sexually transmitted disease, severe medical conditions, allergy to clindamycin or clotrimazole, concomitant medication with antibiotics/antimycotics for other diagnoses, or use of natural remedies in the urogenital area. Five women were excluded from the study: one patient due to a positive *Chlamydia* PCR, three women due to recent use of tampons containing the same bacteria strains as in this study, and one woman due to antibiotic treatment of urinary tract infection between inclusion and randomization.

The study population consisted of 95 women. At screening (visit 0), 39 were diagnosed and treated for BV, and 45 for VVC. Eleven women were diagnosed with both BV and VVC, and treated for both conditions. Sixty-three percent (60/95) of the women were randomized to the intervention group, and 37% (35/95) of the women to the placebo group. BV was present in 53% (32/60) of the women in the intervention group and 51% (18/35) in the placebo group. Fifty eight percent (35/60) in the intervention group and 60% (21/35) in the placebo group had VVC. Twelve percent (7/60) in the intervention group and eleven percent (4/35) in the placebo group fulfilled the criteria for both BV and VVC. Mean age was 31.4 years (SD 7.6) in the intervention group, and 29.3 years (SD 8.3) in the placebo group (ns). There were no differences regarding weight, height, nicotine use or use of oral contraceptives between the two groups.

2.2. The study product

The lactic acid bacteria strains investigated in this study were provided by Ellen AB in form of vaginal capsules. The hydroxypropyl methylcellulose capsules (Capsugel, Belgium) contained an inert carrying matrix (maltodextrin and magnesium stearate) and between 10^8 and 10^{10} viable cells of a probiotic substance, which was a mixture of freeze-dried *L. gasseri* LN40 (36 weight %), *L. fermentum* LN99 (27 weight %), *L. casei subsp. rhamnosus* LN113 (27 weight %), and *P. acidilactici* LN23 (10 weight %). The blend of lactobacilli that

was chosen was aimed at resembling the normal lactobacilli in a healthy vagina. The *Pediococcus* strain was present in a lower amount (10%) than the Lactobacillus strains (32% LN40, 27% LN99 and LN113), because it was not meant to colonize the vagina but to serve as a starter culture for the lactobacilli. The viability and the concentration of the LAB were checked at several checkpoints during the production of the vaginal capsules:

The freeze-dried powder of all lactic acid bacteria strains was checked separately for viability, concentration and purity before and after being blended to a bacteria pool and used for production of the vaginal capsules. The viability and the concentration of lactic acid bacteria were also checked in the vaginal capsules after production. Previous studies have shown stability of the freeze-dried bacteria in the produced vaginal capsules. The CFU of the lactic acid bacteria strains were measured by serial dilutions from the LN bacteria capsules (and also from the freeze-dried powder), culturing equal amounts from the dilution on agar plates selective for lactic acid bacteria (MRS) (pour plate method).

Safety and toxicology controls have been performed and documented on all LN strains. According to *in vitro* tests, all LN strains used in the vaginal capsules inhibit the growth of Candida albicans to some degree. *L. fermentum* LN99 is a powerful yeast inhibitor *in vitro* (data not published) and might thereby affect overgrowth of yeast in the vaginal cavity. LN23, LN99 and LN113 have shown clear inhibitory activity against some urogenital pathogens such as strains of *Escherichia coli*, *Streptococcus agalactiae*, *Enterococcus faecalis*, *Staphylococcus aureus* and *Enterobacter cloacae* (data not published). The antimicrobial activity of the LN strains against common bacteria species associated with BV is during evaluation.

Identical placebo capsules contained only the carrying matrix.

2.3. Study outline

At the screening visit (visit 0), all women underwent a careful vulvovaginal examination. A wet mount was examined microscopically, and a fungal culture was obtained. Presence of three out of four Amsel criteria (malodorous greyish discharge, a vaginal pH of > 4.5, positive whiff-test on addition of 10% potassium hydroxide, and clue cells on microscopy) was diagnostic for BV. Growth of Candida species on fungal culture or presence of fungal hyphae on microscopy, in combination with clinical symptoms of vaginitis or vulvitis was diagnostic for VVC. The women were treated for BV with local clindamycin 100 mg ovules, while the women having VVC were treated with clotrimazol 200 mg vaginal tablets for 3 consecutive nights. The women diagnosed with both BV and VVC were treated with both clindamycin and clotrimazol. According to pharmacokinetical studies on clindamycine, it's half-life plasma elimination (t1/2) ranges between 0.6 h and 3.6 h, depending on liver function. Since there were 2–3 days between the end of the antibiotic administration and start of the probiotic administration, only trace amounts of clindamycine should theoretically be left at start of the LN bacteria administration. According to our knowledge, clotrimazol does not affect the growth of lactobacilli.

At randomization (visit 1), vaginal sampling for wet smear and fungal culture were performed. Vaginal swabs for detection of LN strains were collected. Participants were randomly allotted to vaginal administration of either capsules containing LN bacteria strains (the intervention group) or identical placebo capsules twice daily, for five consecutive days. The intervention group consisted of 60 women. Thirty-five women received placebo capsules. The women, who were not cured from BV or VVC after the clindamycin or clotrimazole treatment received a new treatment before use of the vaginal capsules.

Follow-up visits including vulvovaginal examinations and vaginal sampling for wet smear and fungal culture, as well as sampling of vaginal swabs for detection of LN bacteria strains were performed 2–3 days after administration of LN bacteria strains/placebo (visit 2), after the first and second menstrual period (visit 3 and visit 4, respectively) and finally six months following treatment (visit 5).

Clinical signs and symptoms were registered and a self-reported rating form regarding vulvovaginal symptoms such as bad smell, discharge and itching, graded from zero to 10, was filled out at each visit (zero represented no discomfort, while 10 represented great discomfort).

A relapse was defined as three out of four Amsel criteria fulfilled in the BV patients, and a positive fungal culture in combination with clinical symptoms such as foul smelling discharge, itching and sometimes vulvitis or vaginitis in the VVC patients. Clinical cure was defined as absence of the above mentioned criteria. Any adverse events were recorded.

At each visit 1–5, the vaginal swabs, rolled over the upper third part of the lateral vaginal wall and then placed in modified Stuart medium (Copan transport medium), were sent to the Department of Chemistry at the Mälardalen University, Eskilstuna, Sweden for detection of the LN strains.

2.4. Cultivation

The vaginal fluid samples were cultured for isolation of lactic acid bacteria within 24 h from collection. Each vaginal swab was vortexed in 1 ml sterile Phosphate Buffered Saline (PBS), pH 7.4. The suspensions were diluted 10-fold in PBS (three dilutions for each suspension). Aliquots (100 μl) of each dilution were spread pleated onto MRS (Difco, BD) and Rogosa (Difco, BD) agar. The plates were incubated anaerobically at 37 °C, in 7–10% CO_2 (Gas generating kit, Anaerobic system, Oxoid) for a minimum of 48 h. Up to six representative colonies, with colony morphology identical to the reference LN40, LN99, LN113 and LN23 strains, were selected from the plate with the highest non-confluent growth. The selected colonies were pure cultured on MRS plates. A maximum of 12 colonies were selected from each original swab. Isolates were identified to genus *Lactobacillus* or *Pediococcus* by Gram staining, colony morphology and negative catalase test. Cells from each pure cultured isolate were frozen at −80 °C in 0.5 ml MRS broth (Difco) containing 30%

Glycerol. The reference strains were also cultured on MRS agar plates under the same conditions as the patient-isolates.

2.5. Species identification of isolates

Selected *Lactobacillus* and *Pediococcus* isolates were identified to species level by amplifications of 16S rDNA regions using species specific primer sets (Thermo Electron Corporation, Germany): 5′-TACCAGGTCTTGACATCCAGT-3′ together with 5′-TCCTACCTTAGACGGCTGAC-3′ giving 496 bp fragment (*L. gasseri*) 5′-CACCTGATTGATTTTGGTCGCC-3′ together with 5′-CCTATCTCTAGGGTTGGCGCA-3′ giving 957 bp fragment (*L. fermentum*), 5′-GGCAATGATACGTAGCCGAACT-3′ together with 5′-AACAGTTACTCTGCCGACCA-3′ giving 196 bp fragment (*L. casei subsp. rhamnosus*) and 5′-GATTATGACGTGCTTGCACT GAAT-3′ together with 5′-GAAACCCTCCAACACTTAGTAAT-3′ giving 794 bp fragment (*P. acidilactici*). Cells of the pure cultured isolates suspended in dH$_2$O were used as a template. The PCR was performed on PuReTaq Ready-To-Go PCR Beads (Amersham Biosciences) and the PCR cycling was carried out in a DNA Thermalcycler (Biometra). The 5 min initial denaturation at 94 °C was followed by 35 cycles amplification when annealing was performed at 60 °C (*L. gasseri* and *L. casei subsp. rhamnosus*) or 55 °C (*L. fermentum* and *P. acidilactici*) for 30 s, and extension was performed at 72 °C for 45 s (*L. gasseri* and *L. casei subsp. rhamnosus*) or for 60 s (*L. fermentum* and *P. acidilactici*). The final extension was performed at 72 °C for 10 min. Aliquots of the amplified products were subjected to gel electrophoresis in 1.5% agarose gel (Amersham Bioscience) with 1× TBE buffer, and visualized by ethidium bromide staining and UV transillumination. A 100-bp ladder (Amersham Biosiences) was used as a molecular size standard.

2.6. Genomic fingerprinting by RAPD analysis

The vaginal lactic acid bacteria isolates were identified down to strain level using molecular biology based Random Amplification of Polymorphic DNA (RAPD) technique. The RAPD analysis was performed on genomic bacterial DNA, purified from the vaginal isolates and from the reference LN strains. The DNA was isolated using a modified cell lysis method [33] as well as the "QIAamp DNA Mini Kit Protocol for Isolation of genomic DNA from Gram-positive bacteria and Tissue"-protocol (Qiagen). A loopful of bacteria, removed from the culture plates, was washed in 1 ml sterile dH$_2$O. The pellets were resuspended in 200 µl lysis buffer (25% ultrapure sucrose, 5 mM Tris, 1 mM EDTA, pH 8.0) containing 20 mg ml^{-1} lysozyme (Lysozyme from Chicken Egg White, Sigma). The samples were stored at −20 °C until DNA extraction. For genomic DNA isolation the cells in the lyses buffer were incubated at 37 °C over night. Five µl (10 mg ml^{-1}) RNase A (Sigma) and 80 µl 10% SDS were added, and the samples were incubated at 60 °C for 1 h. The DNA extraction was continued by addition of 20 µl Proteinase K, using the QIAamp DNA Mini Kit in accordance to the instructions of the manufacturer. The concentration of the purified DNA was carefully measured by spectrophotometer, and then stored at −20 °C until needed. The genomic DNA served as a template in RAPD PCR amplifications using Ready-To-Go RAPD Analysis Beads (Amersham Biosciences). The PCR mixture contained 10 ng template DNA, 25 pmol of single Cy5 end-labelled RAPD primer with short arbitrary sequence (5′-Cy-AACGCGCAAC-3′) and sterile water in a total volume of 25 µl. DNA was amplified in a DNA thermal cycler (Biometra) using the following profile: one cycle at 95 °C for 5 min, then 45 cycles at 95 °C for 60 s and 36 °C for 60 s. The RAPD PCR product (25 µl) was mixed with 25 µl Deionised Formamide − Dextran Blue 2000 solution (5%), and heat incubated at 95 °C for 2 min. An aliquot (1−1.5 µl) randomly amplified DNA were visualized and analysed by automated electrophoresis with ALFexpress Genetic analyser (Amersham Pharmacia Biotech) using ReproGel High Resolution polyacrylamide gel (Amersham Pharmacia Biotech) and the software ALFwin Fragment Analyser 1.0.0.1 (Amersham Pharmacia Biotech). The runtime for the gels was 750 min at 55 °C and 25 W. The genetic fingerprints of the isolates were visually compared with the fingerprints of the reference LN strains. A vaginal isolate was identified as an LN strain if the band pattern of its genetic fingerprinting was identical with the fingerprinting of a reference LN strain. Molecular size marker was not used in the electrophoresis.

2.7. Data handling and statistics

The study was conducted with a double-blind, placebo controlled, parallel design, and was performed in accordance with the International Conference on Harmonization Good Clinical Practice Guidelines, the Declaration of Helsinki and applicable regulatory requirements. The local Ethics Committee of the Karolinska hospital approved the study. Participation was voluntary and anonymous. An informed consent was signed before randomization. Probiotic or placebo supplementation was randomly assigned to the patients in the proportion of 2:1, in order to obtain a better description of the larger intervention group with only a marginal reduction in statistical power. The BV and VVC groups were separately randomized. Women who had been treated for both BV and VVC were randomized in the BV group. The randomization code was provided in sealed envelopes to each of the investigators. Based on frequencies of colonization of 0.05 (placebo) and 0.5 (intervention group), alpha = 0.05 and beta = 0.2 (power = 0.8), 18 patients per group were required.

Chi-square test was used for analyses between the intervention group and the placebo group. The efficacy variables were not expected to be normally distributed; therefore non-parametric statistical analyses were performed. Continuous variables were described with mean, SD, median and range and categorical variables with n and %. For comparison between two groups Fisher's exact test was used for dichotomous variables and Mann−Whitney *U*-test for continuous and ordered variables. A complementary sensitivity analysis was done regarding the primary efficacy variable, colonization, in the two groups, adjusting for diagnosis at baseline using chi-square test with Mantel−Haenszel pooling technique. In calculation of accumulated relapse both relapses between visits and at visits were

included, and compared with the result at visit 1. All significance tests were two-tailed and conducted at the 0.05 significance level.

All included women and all samples from all visits were investigated and included in the statistical analyses, although all women did not attend all visits, or did not comply fully with the protocol. Vaginal isolates with lactobacilli morphology, but not identified as an LN strain, were regarded as endogenous *Lactobacillus* species.

The primary objective of the study was to determine whether LN lactic acid bacteria strains could colonize the vagina. Colonization ability was defined as survival of the LN strains in the vaginal cavity over one or more menstrual cycles.

3. Results

Out of the 95 participants, 92 women demonstrated full compliance by administering all the 10 vaginal capsules. Two women in the intervention group used only eight of the 10 capsules, while one woman in the placebo group used the capsules orally. Of the 95 women, 88% returned at visit 2, 82% at visit 3, 77% at visit 4, and 74% at visit 5. Three women did not return after randomization (drop-outs). There were no significant differences between the placebo group and the intervention group with respect to incomplete follow-ups or drop-outs.

3.1. Colonization

A total of 399 vaginal samples (95 from visit 1, 83 from visit 2, 78 from visit 3, 73 from visit 4, and 70 from visit 5) were analysed for presence of the *L. gasseri* LN40, *L. fermentum* LN99, *L. casei subsp. rhamnosus* LN113 and *P. acidilactici* LN23 strains. The RAPD analysis of the reference LN strains, as well as of the vaginal isolates, generated unique, reproducible band patterns of the bacterial DNA, thereby any of the LN strains were easily distinguishable from any other strains. Representative gels are shown in Figs. 1 and 2.

Before the probiotic supplementation (visit 1), lactobacilli was found in 47% (28/60), of the women in the intervention group, and in 54% (19/35) of the women in the placebo group. None of the lactobacilli found at visit 1 was identical with any of the LN strains used for the supplementation.

At visit 2, 1—3 days after the probiotic supplementation, one or more LN strains were present in the vagina in 89% (47/53) of the women in the intervention group, compared to 0% in the placebo group ($p < 0.0001$). *L. gasseri* LN40 was present in 79% (42/53) of the women, *L. fermentum* LN99 in 49% (26/53) of the women, *L. casei subsp. rhamnosus* LN113 in 28% (15/53) of the women, while *P. acidilactici* LN23 was present in 42% (22/53) of the women (Table 1). In only six percent (3/53) of the women all four LN strains were found.

The presence of the LN strains in the vagina decreased over time after visit 2 (Table 1; Fig. 3). After the first menstrual period (visit 3), at least one of the fourLN bacteria strains colonized the vagina in 53% (27/51) of the women in the intervention group. *L. gasseri* LN40 colonized 39% (20/51) of the women, *L. fermentum* LN99 colonized 18% (9/51) of the women, *L. casei subsp. rhamnosus* LN113 colonized 20% (10/51) of the women, while the *P. acidilactici* LN23 strain was present in only two percent (1/51) of the women. All women were colonized by one or two LN strains.

Almost nine percent of the women (one woman with BV and three women with VVC) were still colonized after six months when *L. gasseri* LN40 was found in six percent (3/47) of the women, while *L. casei ssp rhamnosus* LN113 was found in one percent (1/47) of the women in the intervention group (Fig. 2).

During all visits, *L. gasseri* LN40 was found most frequently followed *by L. fermentum* LN99, and then *L. casei subsp. rhamnosus* LN113. The distribution of the LN strains in the BV and the VVC group within the intervention group is presented in Table 1. None of the women in the placebo group was colonized with LN bacteria strains at any time.

3.2. Clinical outcome

Demographics and baseline symptoms were similar in the intervention group and in the placebo group.
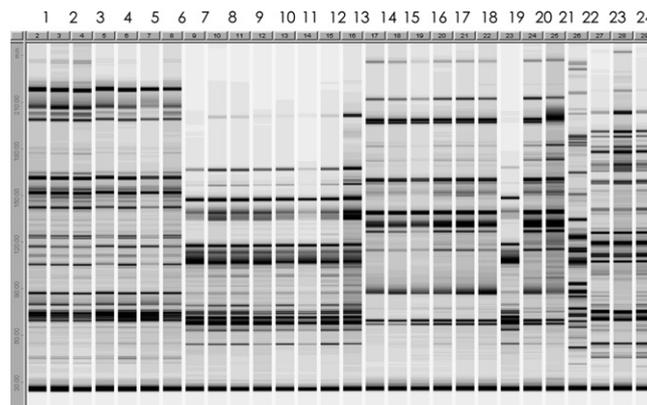

Fig. 1. Genetic RAPD fingerprints of vaginal isolates, previously identified as *P. acidilactici* [lane 1—6], *L. fermentum* [8—14], *L. gasseri* [lane 16—22] and *L. casei subsp. rhamnosus* [lane 25—27], and RAPD fingerprints of the reference *P. acidilactici* LN23 [lane 7], *L. fermentum* LN99 [lane 15], *L. gasseri* LN40 [lane 23 and 24] and *L. casei subsp. rhamnosus* LN113 [lane 28] strains. The *P. acidilactici* isolates in the lane 1—6, the *L. fermentum* isolates in the lane 8—14, the *L. gasseri* isolates in the lane 16—21, and *L. casei subsp. rhamnosus* isolates in the lane 26—27 had identical fingerprints with the fingerprints of the reference LN strains in lane 7, 15, 23—24, and 28, respectively. The fingerprints of the vaginal isolates in lane 22 and 25 were not identical with the fingerprints of any of the LN strains.
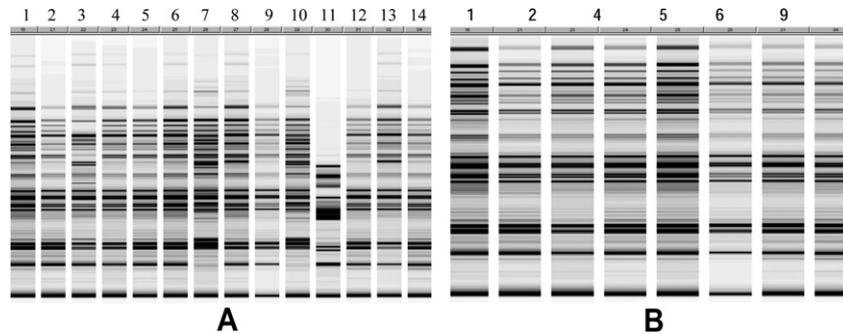
Fig. 2. (A) RAPD fingerprints of 12 vaginal lactobacilli isolates previously identified as *L. casei subsp. rhamnosus* [lane 1−11 and 13], and RAPD fingerprints of the reference *L. casei subsp. rhamnosus* LN113 strain [lane 12 and 14]. The RAPD fingerprints of the *L. casei subsp. rhamnosus* isolates in the lane 1, 2, 4, 5, 6 and 9 were identical with the fingerprints of the reference LN113 strain, while the fingerprints in line 3, 7, 8, 10 and 11 were different. (B) The vaginal *L. casei subsp. rhamnosus* isolates in lane 1, 2, 4, 5, 6 and 9 with identical fingerprints with the LN113 strain were identified as LN113 strains. The lanes in (B) and (A) are processed from the same electrophoresis.

At randomization (visit 1), immediately after treatment with clindamycin and/or clotrimazole, fungal culture was positive in seven percent (4/60) of the women in the intervention group, and in nine percent (3/35) in the placebo group, Out of the women in the intervention group.; one woman was treated for BV and one woman was treated for VVC (3%). In the placebo group, two women treated for VVC were not cured clinically (5.7%). After initial treatment with local antibiotics and/or antifungals, pH, irritation and itching, as well as experience of malodorous discharge were lowered in both the intervention group and the placebo group ($p = 0.0010$, $p < 0.0001$, and $p < 0.0001$, respectively).

Eleven percent (9/84) of the women in the intervention group were diagnosed as having clinical relapse 2−3 days after the probiotic supplementation. In fact, the recurrence rate was 17% (5/30) in the placebo group, and seven percent (4/54) in the intervention group (ns). The cumulative cure rate after the first menstrual period was 78% in the intervention group, and 71% in the placebo group (ns). After the second menstrual period, as well as six months after the probiotic supplementation (visits 4 and 5), there was no difference in clinical cure rate between the intervention group and the placebo group (Fig. 4). As expected, there were no appreciable differences in the vaginal pH values between the intervention group and the placebo group at any of the visits. The vaginal pH was normal at all visits in the women who were diagnosed with VVC, while the antibiotic treatment lowered the pH level in the vagina until the next relapse in all women who were treated for BV.

According to the self-reported rating of vulvovaginal symptoms in the protocol, women in the intervention group reported less malodorous discharge after the probiotic supplementation ($p = 0.03$) and after the second menstruation

Table 1
Vaginal colonization of the LN strains after probiotic supplementation.

	Intervention group	n[f]	Any LN strain	LN40	LN99	LN113	LN23
Visit 1[a]	All patient	60	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	BV	32	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	VVC	28	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Visit 2[b]	All patient	53	47 (89%)	42 (79%)	26 (49%)	15 (28%)	22 (42%)
	BV	27	24 (89%)	22 (82%)	14 (52%)	9 (33%)	12 (44%)
	VVC	26	23 (88%)	20 (77%)	12 (46%)	6 (23%)	10 (38%)
Visit 3[c]	All patient	51	27 (53%)	20 (39%)	9 (18%)	10 (20%)	1 (2%)
	BV	26	16 (62%)	12 (46%)	3 (12%)	9 (35%)	0 (0%)
	VVC	25	11 (44%)	8 (32%)	6 (24%)	1 (4%)	1 (0%)
Visit 4[d]	All patient	47	12 (26%)	9 (19%)	2 (4%)	3 (6%)	1 (2%)
	BV	22	5 (23%)	3 (14%)	0 (0%)	3 (14%)	0 (0%)
	VVC	25	7 (28%)	6 (24%)	2 (8%)	0 (0%)	1 (4%)
Visit 5[e]	All patient	47	4 (9%)	3 (6%)	0 (0%)	1 (2%)	0 (0%)
	BV	25	1 (4%)	0 (0%)	0 (0%)	1 (4%)	0 (0%)
	VVC	22	3 (14%)	3 (14%)	0 (0%)	0 (0%)	0 (0%)

[a] After conventional treatment of BV or/and VVC and before probiotic supplementation.

[b] Two−three days after probiotic supplementation with *L gasseri* LN40, *L. fermentum* LN99, *L. casei subsp. rhamnosus* LN113 and *P. acidilactici* LN23.

[c] After the first menstruation.

[d] After the second menstruation.

[e] Six months after the probiotic supplementation.

[f] Numbers of vaginal samples assessed for presence of LN strains (equal with the numbers of women who participated at the visits apart from visit 2 when one sample did not arrive to the laboratory).
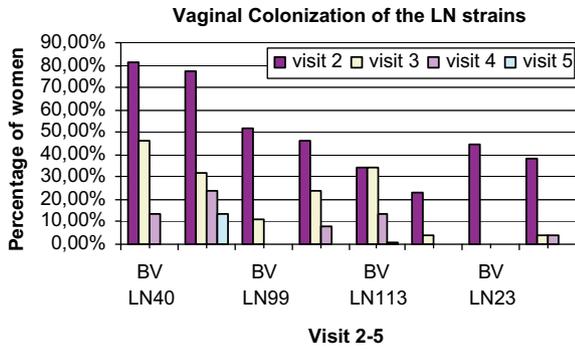
Vaginal Colonization of the LN strains

Fig. 3. The colonization ability of *L gasseri* LN40, *L. fermentum* LN99, *L. casei subsp. rhamnosus* LN113 and *P. acidilactici* LN23 in the intervention group.

($p = 0.04$), compared with the placebo group (Fig. 5). The values of reported irritation/itching after application of the vaginal capsules was somewhat higher in the intervention group, compared with the placebo group (ns).

There were no differences between the colonized women and non-colonized women with respect to vaginal pH, patient ratings of vulvovaginal symptoms, or clinical cure rate. Subgroup analysis of the clinical outcome was not performed in the BV and the VVC group due to lack of statistical power (e.g. small sample size).

3.3. Adverse events (AE)

Two (3.2%) women in the intervention group and two women (5.3%) in the placebo group with BV were diagnosed with VVC infection during the study, while two (3.2%) patients in the intervention group and one (2.6%) patient in the placebo group with VVC fulfilled the criteria for BV later in the study. These patients received treatment with clotrimazol or clindamycine, respectively. Vulvovaginal pruritus was reported by one (1.6%) woman in the intervention group, and by five (13.2%) women in the placebo group. One woman (1.6%) in the intervention group experienced a small vaginal bleeding, while one (2.6%) woman had a swollen and red vulva after use of the placebo capsules. One (2.6%) woman in the placebo group
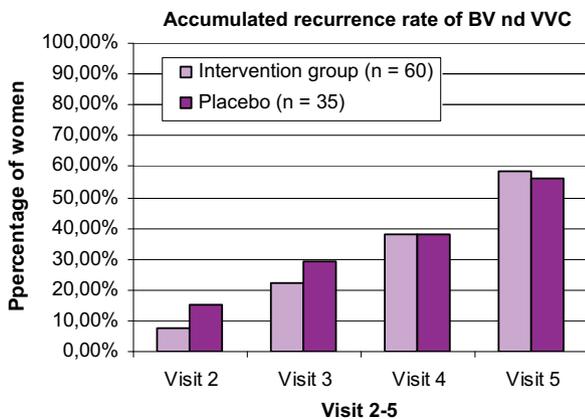
Accumulated recurrence rate of BV nd VVC

Fig. 4. Cumulative recurrence of BV and VVC after probiotic/placebo supplementation.

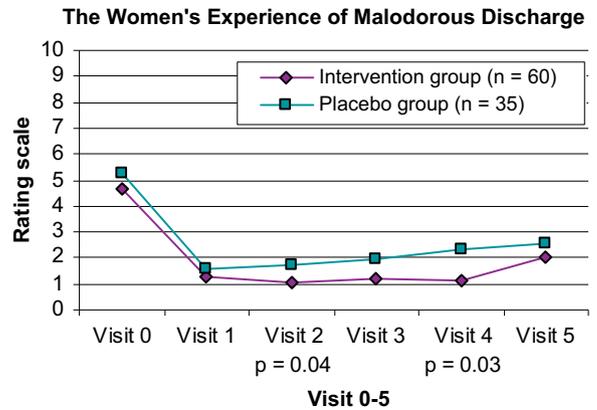**The Women's Experience of Malodorous Discharge**

Fig. 5. The women's own experience of malodorous discharge, graded from zero to 10, was lower at each visit after the probiotic supplementation with the LN strains (intervention group) compared with the women's who received placebos ($p = 0.04$ at visit 2 and $p = 0.03$ visit 4). 0 = no discomfort; 10 = great discomfort.

reported headache. Most adverse events were reported at visit 2, while one was reported at visit 3 (vulvar itching/placebo). No serious adverse events were reported.

4. Discussion

This double-blind, placebo controlled clinical study showed the first evidence that a short (five days) period of probiotic supplementation can lead to vaginal colonization of the exogenous lactobacilli for up to six months. Women in the intervention group experienced less malodorous discharge after administration of LN bacteria and after the second menstruation, compared with placebo.

L. gasseri LN40, *L. fermentum* LN99, *L. casei subsp. rhamnosus* LN113 and *P. acidilactici* LN23 colonized the vagina of almost all women who received probiotic supplementation .The LN strains colonized the vagina of women with VVC and BV to the same degree. This is interesting since we had expected that in VVC patients, who per definition have normal endogenous lactobacilli flora level, the instilled LN strains would not colonize. *P. acidilactici* LN23 was found in the vagina of some women 2−3 days after administration but it colonized poorly. The rationale for including *P. acidilactici* LN23 in the study product was that pediococci have a shorter generation time than lactobacilli, and may thereby serve as a starter culture by quickly lowering the vaginal pH, making the milieu less favourable for acid sensitive pathogens. Over time, the lactobacilli, which are more resilient, will eventually out-compete the pediococci. This hypothesis was supported by our results.

It has been discussed whether administration of probiotics may alter the vaginal flora. It has been shown that probiotics (*Lactobacillus rhamnosus* GR-1 and *L. fermentum* RC-14), administered orally for 60 consecutive days, can increase the total number of vaginal lactobacilli over time in healthy women [38]. In some studies, vaginal colonization of probiotic bacteria has been shown for up to one month [33−36]. However, few colonization studies have been performed using

molecular biology based methods. In our study, identification of the vaginal isolates down to the strain level was employed using RAPD analysis. For optimal resolution of the RAPD fragments, automated gel electrophoresis on high resolution polyacrylamide gels were used. This method, using ALF express, is very sensitive, and can detect single bas pair differences between two DNA fragments. Consequently, the method is excellent for distinguishing between strains belonging to the same species.

Since recurrences of BV and VVC are common, there is a need for long-time prophylaxis. During the past 25 years, several *in vitro* studies and some clinical studies using different probiotic lactobacilli candidates have been performed. Previous *in vitro* studies have shown that some probiotic lactobacilli inhibit the growth of microorganisms related to BV [6,8]. *In vitro* inhibition of yeast growth has also been documented [32,47]. Most of the clinical studies have focused on BV, showing promising results. In one study, *L. fermentum* RC-14 and *L. rhamnosus* GR-1 were used as oral supplementation for 30 days after conventional treatment with metronidazole. At the 30-day follow-up, the recurrence of BV, diagnosed by Nugent score and negative sialidase test, was reduced from 60% to 12% in the intervention group, compared with the placebos group [48]. Recently, vaginal capsules with *L. casei subsp. rhamnosus* Lcr-35, used for seven days after treatment with clindamycin, showed reduction of Nugent score in 83% (89/83) of the women with BV, while the reduction in the placebo group was detected in 35% (31/88) of the women [37]. Larsson et al. also reported that the recurrence rate of BV was reduced at 6 months in women using vaginal capsules with *L. rhamnosus* Lbp PB01-DSM 14870 and *L. gasseri* Lba EB01-DSM14869 for 10 days during three menstrual cycle following clindamycin treatment [49]. However, after one month, the cure rate was only 64% in the lactobacilli group compared with 78% in the placebo group (ns). The few clinical studies performed regarding supplementation of lactobacilli after conventional antifungal treatment in women with VVC show conflicting results [38,40,41]. Women who suffer from recurrent vulvovaginal candidiasis do not recover until treated with oral antimycotics for several months [28]. It would be interesting and urgent to determine whether longer or repeated treatment periods with probiotic strains might lower the recurrence rate of VVC.

Our days' global increase in resistance to antimicrobial drugs strengthens the interest in alternatives to antibiotic/antimycotic treatments. The preventive efficacy of probiotics, especially in women with BV and VVC with documented high relapse rates, is therefore an interesting field of research.

The probiotic supplementation in our study resulted in numerically higher overall clinical cure rate 1−3 days after the vaginal administration, as well as after the first menstrual period, compared with the placebo group. Although not significant, this result is worth mentioning. According to the self-reported rating form, 1−3 days after the probiotic supplementation and after the second menstrual period, the women in the intervention group experienced significantly less malodorous discharge compared with placebo. In view of the

fact that the treatment period was as short as five days, these results seem rather promising.

The limitations in our study are the small sample size of the BV and the VVC group, as well as the relatively short treatment period. Moreover, the questionnaire did not include information whether the women had changed sex partner during the study period. Changing sex partner is a risk factor for acquiring BV. However, this error is systematic.

In conclusion, vaginal administration of *L. gasseri* LN40, *L. fermentum* LN99, and *L. casei subsp. rhamnosus* LN113 and *P. acidilactici* LN23 for five consecutive days lead to vaginal colonization. The probiotic supplementation of the conventional treatment of BV and/or VVC resulted in less malodorous discharge, and a trend towards higher clinical cure rate, compared with the placebo group. Further randomized clinical studies with extended and/or repetitive treatment with probiotic LN bacteria strains in combination with conventional treatment are needed to evaluate the promising colonization results of this study.

Acknowledgements

References

[1] K.C. Anukam, E.O. Osazuwa, I. Ahonkhai, R. Gregor, *Lactobacillus* vaginal microbiota of women attending a reproductive health care service in Benin City, Nigeria. Sex. Transm. Dis. 33 (2006) 59−62.

[2] R.W. Hyman, M. Fukushima, L. Diamond, J. Kumm, L.C. Giudice, R.W. Davis, Microbes on the human vaginal epithelium. Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 7952−7957.

[3] A.N. Masfaril, B.I. Duerden, G.R. Kinghorn, Quantitative studies of vaginal bacteria. Genitourin. Med. 62 (1986) 256−263.

[4] E.R. Boskey, K.M. Telsch, K.J. Whaley, T.R. Moench, R.A. Cone, Acid production by vaginal flora in vitro is consistent with the rate and extent of vaginal acidification. Infect. Immun. 67 (1999) 5170−5175.

[5] E.R. Boskey, R.A. Cone, K.J. Whaley, T.R. Moench, Origins of vaginal acidity: high D/L lactate ratio is consistent with bacteria being the primary source. Hum. Reprod. 16 (2001) 1809−1813.

[6] S. Boris, J.E. Suarez, F. Vasquez, C. Barbes, Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens. Infect. Immun. 66 (1998) 1985−1989.

[7] D.A. Eschenbach, P.R. Davick, B.L. Williams, S.J. Klebanoff, K. Young-Smith, C.M. Critchhlow, K.K. Holmes, Prevalence of hydrogen peroxide-producing *Lactobacillus* species in normal women and women with bacterial vaginosis. J. Clin. Microbiol. 27 (1989) 251−256.

[8] J.K. Kaewsrichan, Peeyananjarassri, J. Kongprasertkit, Selection and identification of anaerobic lactobacilli producing inhibitory compounds against vaginal pathogens. FEMS Immunol. Med. Microbiol. 48 (2006) 75−83.

[9] M.L. Harold Jr., B.A. Richardson, P.M. Nyange, L. Lavreys, S.L. Hillier, B. Chohan, K. Mandaliya, J.O. Ndinya-Achola, J. Bwayo, J. Kreiss, Vaginal lactobacilli, microbial flora, and risk of human

Immunodeficiency Virus type 1 and sexually transmitted disease Acquisition. Infect. Dis. 180 (1999) 1863–1868.

[10] D.J. Okkers, L.M.T. Dicks, M. Silvester, J.J. Joubert, H.J. Odendaal, Characterization of pentocin TV35b, a bacteriocin-like peptide isolated from *Lactobacillus pentosus* with a fungistatic effect on *Candida albicans*. J. Appl. Microbiol. 87 (1999) 726–734.

[11] D.A. Eshenbach, History and review of bacterial vaginosis. Am. J. Obstet. Gynecol. 169 (1993) 441–445.

[12] A. Vásquez, T. Jakobsson, S. Ahrné, U. Forsum, G. Molin, Vaginal *Lactobacillus* flora of healthy Swedish women. J. Clin. Microbiol. 40 (2002) 2746–2749.

[13] R. Amsel, P.A. Totten, C.A. Spiegel, K.C. Chen, D. Eschenbach, K.K. Holmes, Non-specific vaginitis. Diagnostic criteria and microbial and epidemiologic associations. Am. J. Med. 74 (1983) 14–22.

[14] K.Y. Kane, R. Pierce, What are the most effective treatments for bacterial vaginosis in non-pregnant women? J. Fam. Pract. 5 (2001) 399–400.

[15] M.R. Joesoef, G. Schmid, Bacterial vaginosis. Clin. Evid. 11 (2004) 2054–2063.

[16] C.S. Bradshaw, A.N. Morton, J. Hocking, S.M. Garland, M.B. Morris, L.M. Moss, L.B. Horvath, I. Kuzevska, C.K. Fairley, High recurrence rates of bacterial vaginosis over the course of 12 Months after oral metronidazole therapy and factors associated with recurrence. J. Infect. Dis. 193 (2006) 1478–1486.

[17] J.D. Sobel, C. Schmitt, C. Meriwether, Long-term follow-up of patients with bacterial vaginosis treated with oral metronidazole and topical clindamycin. J. Infect. Dis. 167 (1993) 783–784.

[18] R.H. Beigi, M.N. Austin, L.A. Meyn, M.A. Krohn, S.L. Hillier, Antimicrobial resistance associated with the treatment of bacterial vaginosis. Am. J. Obstet. Gynecol. 191 (2004) 1124–1129.

[19] P. Nyirjesy, M.J. McIntosh, D.J. Gattermeir, R. Scumacher, J.I. Steinmetz, J.L. Joffrion, The effects of intravaginal clindamycin and metronidazole therapy on vaginal lactobacilli in patients with bacterial vaginosis, Am. J. Obstet. Gynecol. 194 (2006) 1277–1282.

[20] S.L. Hillier, R.P. Nugent, D.A. Escenbach, M.A. Krohn, R.S. Gibbs, D.H. Martin, M.F. Cotch, R. Edelman, J.G. Pastorek, A.V. Rao, D. McNellis, J.A. Regan, J.C. Carey, M.A. Klebanoff, Association between bacterial vaginosis and preterm delivery of a low-birthweight infant, the vaginal infections and prematurity study group. N. Engl. J. Med. 333 (1995) 1737–1742.

[21] P.G. Larsson, M. Bergström, U. Forsum, B. Jacobsson, A. Strand, P. Wolner-Hanssen, Bacterial vaginosis. Transmission, role in genital tract infection and pregnancy outcome: an enigma. APMIS 113 (2005) 233–245.

[22] R.B. Ness, K.E. Kip, S.L. Hillier, D.E. Soper, C.A. Stamm, R.L. Sweet, P. Rice, H.E. Richter, A cluster analysis of bacterial vaginosis-associated microflora and pelvic inflammatory disease. Am Epidemiol. 162 (2005) 585–589.

[23] E.M. de Leon, S.J. Jacober, J.D. Sobel, B. Foxman, Prevalence and risk factors for vaginal *Candida* colonization in women with type 1 and type 2 diabetes. BMC Infect. Dis. 2 (2002) 1.

[24] P.L. Fidel Jr., J.D. Sobel, Immunopathogenesis of recurrent vulvovaginal candidiasis. Clin. Microbiol. Rev. 9 (1996) 335–348.

[25] P.L. Fidel Jr., J. Vazquez, J.D. Sobel, Candida glabrata: review of epidemiology, pathogenesis, and clinical disease with comparison to *C. albicans*. Clin. Microbiol. Rev. 12 (1999) 80–96.

[26] J.D. Sobel, W. Chaim, Vaginal microbiology of women with acute recurrent vulvovaginal candidiasis. J. Clin. Microbiol. 34 (1996) 2497–2499.

[27] J.D. Sobel, Vulvovaginal candidosis. Lancet 9577 (2007) 1961–1971.

[28] J.D. Sobel, Vaginitis. N. Engl. J. Med. 337 (1997) 1896–1903.

[29] H.L. Kent, Epidemiology of vaginitis. Am. J. Obstet. Gynecol. 165 (1991) 1168–1176.

[30] J. Osset, R.M. Bartolome, E. Garcia, A. Andreu, Assessment of the capacity of *Lactobacillus* to inhibit the growth of uropathogens and block their adhesion to vaginal epithelial cells. J. Infect. Dis. 183 (2001) 485–491.

[31] J. Osset, E. Garcia, R.M. Bartolome, A. Andreau, Role of *Lactobacillus* as protector against vaginal candidiasis. Med. Clin. 117 (2001) 285–288.

[32] D. Rönnqvist, U. Forsgren-Brusk, E. Husmark, E. Grahn-Håkansson, J. Med. Microbiol. 56 (2007) 1500–1504.

[33] M.A.D. Antonio, S.L. Hillier, DNA fingerprinting of *Lactobacillus crispatus* strain CTV-05 by repetitive element sequence-based PCR analysis in a pilot study of vaginal colonization. J. Clin. Microbiol. 41 (2003) 1881–1887.

[34] G. Gardiner, C. Heinemann, A.W. Bruce, D. Beuerman, G. Reid, Persistence of *Lactobacillus fermentum* RC-14 and *Lactobacillus rhamnosus* GR-1 but not *L. rhamnosus* GG in the human vagina as demonstrated by randomly amplified polymorphic DNA. Clin. Diagn. Lab. Immunol 9 (2002) 92–96.

[35] G. Reid, A.W. Bruce, N. Fraser, C. Heinemann, J. Owen, B. Henning, Oral probiotics can resolve urogenital infections. FEMS Immunol. Med. Microbiol. 30 (2001) 49–52.

[36] P.D. Rönnqvist, U.B. Forsgren-Brusk, E.E. Grahn-Håkansson, Lactobacilli in the female genital tract in relation to other genital microbes and vaginal pH. Acta. Obstet. Gynecol. Scand. 85 (2006) 726–735.

[37] L. Petricevic, A. Witt, The role of *Lactobacilli casei rhamnosus* Lcr35 in restoring the normal vaginal flora after antibiotic treatment of bacterial vaginosis. BJOG 115 (2008) 1369–1374.

[38] G. Reid, D. Charbonneau, J. Erb, B. Kochanowski, D. Beuerman, R. Poehner, A.W. Bruce, Oral use of *Lactobacillus rhamnosus* GR-1 and *L. fermentum* RC-14 significantly alters vaginal flora: randomized, placebo-controlled trial in 64 healthy women. FEMS Immunol. Med. Microbiol. 35 (2003) 131–134.

[39] K. Eriksson, B. Carlsson, U. Forsum, P.G. Larsson, A double-blind treatment study of bacterial vaginosis with normal vaginal lactobacilli after an open treatment with vaginal clindamycin ovules. Acta. Derm. Venereol. 85 (2005) 42–46.

[40] E. Hilton, P. Rindos, H. Isenberg, *Lactobacillus* GG vaginal suppositories and vaginitis. J. Clin. Microbiol. 33 (1995) 1433.

[41] M.E. Falagas, G.I. Betsi, S. Athanasiou, Probiotics for prevention of recurrent vulvovaginal candidiasis: a review. J. Antimicrob. Chemother. 58 (2006) 266–272.

[42] Food and Agriculture Organization of the United Nations and World Health Organization, 2001 posting date. Regulatory and clinical aspects of dairy probiotics, Food and Agriculture Organization of the United Nations and World health Organization Expert Consultation report. Food and Agriculture Organization of the United Nations and World health Organization Working group report (online).

[43] M.A.D. Antonio, S.E. Hawes, S.L. Hillier, The identification of vaginal *Lactobacillus* species and the demographic and microbiologic characteristics of women colonized by these species. J. Infect. Dis. 180 (1999) 1950–1956.

[44] S.I. Pavlova, A.O. Kilic, S.S. Kilic, J.-S. So, M.E. Nader-Macias, J.A. Simoes, L. Tao, Genetic diversity of vaginal lactobacilli from women in different countries based on 16S rRNA gene sequences. J. Appl. Microbiol. 92 (2002) 451–459.

[45] G.D. Stoyancheva, S.T. Danova, I.Y. Boudakov, Molecular identification of vaginal lactobacilli isolated from Bulgarian women. Antonie Van Leeuwenhoek 90 (2006) 201–210.

[46] W. Zhong, K. Millsap, H. Bialkowska-Hobrzanska, G. Reid, Differentiation of *Lactobacillus* species by molecular typing. Appl. Environ. Microbiol. 64 (1998) 2418–2423.

[47] M. Strus, A. Kucharska, G. Kukla, M. Brzychczy-Włoch, K. Maresz, P.B. Heczko, The in vitro activity of vaginal *Lactobacillus* with probiotic properties against *Candida*. Infect. Dis. Obstet. Gynecol. 13 (2005) 69–75.

[48] K. Anukam, E. Osazuwa, I. Ahonkhaia, M. Ngwue, G. Osemene, A.W. Bruceb, G. Reid, Augmentation of antimicrobial metronidazole therapy of bacterial vaginosis with oral probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14: randomized, double-blind, placebo controlled trial. Microbes Infect. 6 (2006) 1450–1454.

[49] P.G. Larsson, B. Stray-Pedersen, K.R. Ryttig, S. Larsen, Human lactobacilli as supplementation of clindamycin to patients with bacterial vaginosis reduce the recurrence rate; a 6-month, double-blind, randomized, placebo-controlled study. BMC Woman's Health 8 (2008) 3.