Persistence of Lactobacilli in Postmenopausal Women – A Double-Blind, Randomized, Pilot Study

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Key Words

Lactobacillus spp. · Colonization · Postmenopausal women · Probiotic ointment · Intimate area

Abstract

Aim: To investigate the ability of lactobacilli to persist in the genital area (vagina and labia) of women after the topical application of an ointment containing *Lactobacillus gasseri* LN40, *L. fermentum* LN99 and *L. rhamnosus* LN113. Secondary objectives were to study the presence of *Escherichia coli* and other contaminants, as well as subjective symptoms in the genital tract. **Methods:** Eighteen healthy postmenopausal women were randomized to use either the study product or placebo for 10 days. Gynecological examinations, labial and vaginal samplings for bacterial cultivation were performed at baseline (visit 1), after treatment (visit 2), and at a 10-day follow-up (visit 3). LN strains were identified by specific cultivation methods. Subjective symptoms were evaluated by a self-administered question-

naire. **Results:** The presence of LN99 was shown in 7 out of 8 women in the investigational group at visit 2 ($p < 0.001$ compared to placebo) and in 5 out of 8 at visit 3 ($p < 0.05$), whereas the presence of LN113 was shown in 2 out of 8 at visit 2 and in 1 out of 8 at visit 3. Subjective symptoms were significantly reduced ($p < 0.01$) at visits 2 and 3 for both products. **Conclusion:** Topical application of a probiotic ointment is feasible to achieve persistence of lactobacilli for at least 10 days.

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Introduction

The complex microbial ecology of the genital tract of healthy fertile women is predominated by bacterial species belonging to the genus *Lactobacillus* [1–3], which is considered to prevent the occurrence of vaginal infections [4]. The lactobacilli colonize not only the vagina but also the labia [5].

During menopause, the level of estrogen declines and the level of lactobacilli in the genitals dramatically decreases, thereby resulting in increased incidents of urogenital infections in postmenopausal women [8, 9]. The decreased estrogen level also results in thinner, drier and less elastic genital tissues [8, 10, 11]. Local estrogen treatment is conventionally used for relief of this condition and leads to natural lubrication of the mucosa. However, women who do not want to use estrogen and women having breast cancer, who cannot use estrogen products, are in need of an alternative method for lubrication of the genital mucosa and for improvement of the microbiological ecology in their urogenital tract.

Probiotics are empirically selected strains of microorganisms, often lactobacilli, which when administered in adequate amounts are considered to confer a health benefit to the host (FAO/WHO 2001) [12] and to improve the natural bacterial microbiota when it becomes disturbed. The lactobacilli are considered to protect the genital health by competitive exclusion of genitourinary pathogens, co-aggregation with uropathogenic bacteria, formation of a biofilm and production of inhibitory compounds such as lactic acid and other metabolites [6, 7, 9, 13, 14].

The lactobacilli used in this study were originally isolated from a healthy woman. They were partly chosen due to their ability to be used in large-scale production. Their colonization ability in the vagina has been studied previously by use of vaginal capsules [15]. *L. gasseri* LN40, *L. fermentum* LN99 and *L. rhamnosus* LN113 are used in hygienic tampons and intimate care products (Ellen Probiotic Tampon and Probiotic Intimate Crème, Ellen AB, Sweden).

We hypothesize that lactobacilli can persist in the genital area of postmenopausal women after being added to an ointment. We designed a randomized, placebo product controlled double-blind pilot study to evaluate the ability of LN40, LN99 and LN113 to persist in the external and internal genital areas (labia and vagina) of postmenopausal women after the topical application of a probiotic ointment containing freeze-dried powder of the LN strains, vegetable fat and oil. The primary objective of this study was to show the presence of at least one LN bacterial strain in the internal and external genital area (vagina and labia) of women in the investigational group at the end of a 10-day period of use of the investigational product. Secondary objectives were to obtain information about the presence of coliform bacteria and subjective symptoms in the genital area before and after the treatment.

Materials and Methods

Study Design

The study was conducted as a randomized, double-blind, placebo-controlled study. It was approved (2013/873-31/2) by the regional Ethical Review Board at the Karolinska Institutet in Stockholm, and performed on the basis of Good Clinical Practice Guidelines, the Declaration of Helsinki and applicable regulatory requirements. The study was registered at ClinicalTrials.gov with registration number NCT02162160 prior to the recruitment of women.

Study Population

Healthy women were recruited between September 1, 2013 and November 30, 2013, by advertising at the Danderyd Hospital as well as in a newsletter of a member club for women over 40 years.

Inclusion criteria were age in the range 45–65, having reached menopause, latest menstruation at least 3 years prior to inclusion in the study, signed informed consent, having a feeling of dryness in the intimate area and being able to abstain from intercourse and the use of other creams and natural remedies in the intimate area during the study period. Exclusion criteria were clinical symptoms of Candida vaginitis (diagnosed by ocular examination) or bacterial vaginosis (diagnosed by Amsel's criteria), concomitant medication with any antibiotics or antifungals during the last 2 months, concomitant estrogen therapy or participation in another study.

The Investigational Product

The investigational product in this study was an intimate care ointment provided by Ellen AB, consisting of vegetable fat (hydrogenated coco-glycerides), oil (caprylic/capric triglyceride), cetyl alcohol, as well as freeze-dried powder of the LN strains *L. gasseri* LN40, *L. rhamnosus* LN113 and *L. fermentum* LN99. The freeze-dried bacterial powder in the investigational product constituted 3% (w/w), and the proportion of the viable cells were 10:45:45 of the 3 *Lactobacillus* strains in the order as mentioned above, totally $6\text{--}8 \times 10^8$ cfu/g ointment.

Purity and Safety Checks

Online supplementary information S1 (for all online suppl. material, see www.karger.com/doi/10.1159/000446946).

The Control Product

The control product was a commercially available intimate care product, available on the market in pharmacies and in health food stores in Sweden. The ingredients of the product were aqua, paraffinum liquidum, butylene glycol, glycerine, canola oil, petrolatum, cetyl PEG/PPG-10/1 dimethicone, cera microcristallina, polyglyceryl-3 oleate, hydrogenated castor oil, sodium chloride, citric acid and tetradibutyl pentaerithrityl hydroxyhydrocinnamate (INCI names). The product did not contain lactobacilli. The product was provided in 15-ml white aluminum tubes, identical to the investigational product.

Randomization Procedure

The investigational and control products were stored in identical white metallic tubes marked with numbers 1–18. The tubes were prepared prior to the study by a representative from Ellen AB who randomly assigned the numbers to the tubes according to a treatment-randomization list generated in Microsoft Excel. The randomization list was kept at Ellen AB with a copy stored and

locked away at the study center at Danderyd Hospital. The tubes were given out to patients in a consecutive order. The instructions on how to use the study product were given to the women by a study coordinator nurse midwife. The representative from Ellen AB did not take an active part in the study.

Study Objectives

The primary objective of this study was to show the presence of at least one LN bacterial strain in the internal and external genital area (vagina and labia) of women in the investigational group at the end of a 10-day period of use of the investigational product (days 8–10), as well as 9–11 days after the 10-day period of use using bacterial cell culture.

Secondary objectives were (1) to obtain information about the coliform bacterial microbiota in the internal and external genital area before and at the same sampling occasions as above; (2) to obtain information about the subjective feeling of dryness, malodor, discharge and irritation in the vulva and/or vagina at the sampling occasions.

Study Outline

Clinical examinations and sampling were carried out at the Department of Obstetrics and Gynecology at Danderyd Hospital, and microbiological analysis of samples at the Department of Microbiology, Tumor and Cell biology, Karolinska Institutet.

After receiving written and oral information about the study, women, fulfilling inclusion criteria and willing to participate in the study, signed an informed consent in the presence of the principal investigator.

The women were screened for the inclusion and exclusion criteria by clinical examinations and randomized to investigational or control product. They were instructed to use the ointment, quantity equal to that of the size of a pea, on the mucus membranes of the vulva, 10 days, twice daily, every morning and evening at bed time, and to carefully smear their labia majora and minora as well as around the vaginal opening and the perineum. A pea size amount corresponded to ≈ 0.5 – 0.7 ml (≈ 0.4 – 0.6 g) product. The daily dose thus corresponded to ≈ 1.0 – 1.4 ml (≈ 0.8 – 1.2 g), which corresponded to ≈ 6 – 8×10^8 cfu of lactobacilli in the investigational product. The women were asked to return the empty tubes, as well as any unused study products for accountability and compliance checks.

Follow-up visits were carried out at the end of the 10 days usage of the study product (8th–10th day), as well as 9–11 days after this 10-day period with recording of adverse events and concomitant medication. In addition, the dryness of the mucus membranes in the urogenital area of women was carefully examined by a gynecologist and subjectively rated on a scale 1–5 (not dry, dry, medium dry, very dry and excessively dry, respectively). The vaginal pH was measured using a pH-indicator paper. Symptoms regarding dryness, malodor, discharge, and irritation in the internal and external genitalia were subjectively graded by the women from zero to 10 in a 10-point scale self-administered questionnaire (zero represented no inconvenience; 10 represented great inconvenience).

At each visit, vaginal and labial swabs were collected. For vaginal sampling, the labia were pulled apart and a speculum was used to visualize the upper third part of the lateral vaginal wall. The swab was performed beyond the tip of the speculum to avoid contamination and rolled across the vaginal surface to obtain the sample. For labial sampling, the labia majora and minora were held apart and a sterile swab gently rubbed back and forth 5 times dur-

ing rotation on the left side of the vulva near the vaginal orifice. The labial examination area was approximately 2 cm^2 . The swabs were placed in a tube containing 1.8 ml cold sterile anaerobic transport medium, that is, reducing agents (cysteine 1.0 g/l and sodium thioglycollate, 1.0 g/l) in sodium phosphate buffer, pH 7.5 (PBS) (Anaerobe Systems, Morgan Hill, Calif., USA), and sent to the laboratory for microbiological analysis within 4 h, kept at 4°C .

Microbiological Assessment

Online supplementary information S2.

Data Handling and Statistics

Online supplementary information S3.

Results

Clinical Data

Eighteen women aged 49–65, having given informed consent, were included in the study. Seventeen (8 in the investigational and 9 in the control group) conducted the study as outlined by the protocol. The flow of women is described in figure 1 (consort flow chart). There were no significant differences between the investigational and control groups regarding demographic characteristics and baseline symptoms such as perceived dryness, malodor, discharge or irritation (table 1). In addition, there were no significant differences between the groups concerning pH (table 1), the presence of cracks or dryness of the mucosa examined and rated by the physician (results not shown).

The women reported reduced subjective symptoms in the self-administered questionnaire at visits 2 and 3 compared to visit 1 (table 1). Statistically significant improvements were noted for dryness, malodor and irritation at the second visit after using any of the ointments for 10 days.

Microbiological Assessment

A total of 52 vaginal and 52 labial samples (18 from visit 1, 17 from visit 2 and 17 from visit 3 for each kind of sample) were analyzed. All samples were received and analyzed blindly. Before the treatment (visit 1), lactobacilli were found in all (8/8) women in the investigational group and in 7 out of 9 women in the control group. None of the lactobacilli found at visit 1 was identical to any of the LN strains used.

Overall, LN strains were recovered from 7 out of 8 women in the investigational group and from 1 out of 9 women in the control group (table 2). LN99 was found in 7 women at visit 2 and in 5 women at visit 3 (table 2; fig. 2), while LN113 was found in 2 women at visit 2 and in 1 at visit 3. However, LN99 and LN113 were also present at visit 3 in

Fig. 1. Consort flow diagram.

1 woman in the control group. LN40 was not recovered either from labia or vagina in any woman in any group.

Lactobacilli, other than the LN strains, were found in about 70% of the samples. The total cfu of lactobacilli, however, did not change because of colonization with LN strains. The occurrence of lactobacilli was not related to whether the LN strains colonized the mucosa or not.

Escherichia coli, as well as *Klebsiella* spp., *Enterococcus* spp. and staphylococci were rarely detected in the pre-treatment samples, but at end of the treatment period (visit 2), at least one of these contaminants were detected in 12 out of the 17 sampling occasions in both groups, a figure that decreased to 7/18 at the final visit.

Adverse Events

Vulvar itching was reported at visit 3 by 1 woman in each group. One woman in the control group reported both vulvar itching and irritation at both visits 2 and 3. One woman in the investigational group had a small crack on the mucus membranes. One woman in the control group reported abdominal pain at visit 3.

The occurrence of adverse events did not differ between the groups. The correlation of the vulvar itching and the test products was graded as likely. No serious adverse events were reported.

Discussion

In this randomized, double blind pilot study, we could show that lactobacilli can colonize the genital area of postmenopausal women for at least up to 10 days after application of a topical ointment containing *L. gasseri* LN40, *L. fermentum* LN99 and *L. rhamnosus* LN113.

The strengths of this study include the strict randomized double-blind design, the short time (maximum 4 h) between sampling at the clinic and cultivation of bacteria in the laboratory, as well as the cultivation-dependent method for detection of lactobacilli. The short transportation time from the clinic to the laboratory might contribute to a high recovery rate of lactobacilli, while the bacterial cultivation methods allowed identification of

Table 1. Mean symptoms according to a 10-point scale self-administered questionnaire and pH

Means*												
dryness			malodor			discharge			irritation			pH
visit 1	visit 2	visit 3	visit 1	visit 2	visit 3	visit 1	visit 2	visit 3	visit 1	visit 2	visit 3	visit 1
Control (n = 9)	6.67	3.00**	4.44	2.33	0.89	1.56	1.89	1.22	1.22	1.44	2.67	4.3
Investigational (n = 8)	5.13	2.14	4.25	2.25	0.63	1.38	1.00	0.50	0.75	1.63	2.25	4.7
All (n = 17)	5.94	2.63	4.35	2.29	0.76	1.47	1.47	0.88	1.00	1.53	2.47	4.5**
p values <0.05 between visits***												
dryness			malodor			discharge			irritation			pH
visits	visits	visits	visits	visits	visits	visits	visits	visits	visits	visits	visits	visits
1 vs. 2	1 vs. 3	2 vs. 3	1 vs. 2	1 vs. 3	2 vs. 3	1 vs. 2	1 vs. 3	2 vs. 3	1 vs. 2	1 vs. 3	2 vs. 3	1 vs. 2
0.0088	ns	0.0498	0.0311	ns	ns	ns	ns	ns	0.0403	ns	ns	ns
Investigational (n = 8)	0.0335	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
All (n = 17)	0.0007	0.008	0.006	0.0341	ns	ns	ns	ns	0.0036	ns	ns	ns

ns = Not significant.

* 0 = No inconvenience (minimum); 10 = great inconvenience (maximum).

** n = 7 (missing data from 1 woman).

*** Wilcoxon non-parametric paired test.

Table 2. Individuals colonized by LN lactobacilli, strains LN99 or LN113, after use of the study products (visit 2) and 10 days thereafter (visit 3). Strain LN40 was never recovered during the study period. Fisher's exact test was used

LN99			LN113 ^a			LN99 or LN113 ^b		
investigational group	control group	significance between groups, p value	investigational	control	significance between groups, p value	investigational group	control group	significance between groups, p value
<i>Visit 2^c</i>								
Vagina	6	0	2	0	0	6	0	
Labia	7	0	2	0	<0.001	7	0	<0.001
Vagina or labia	7	0	2	0		7	0	
<i>Visit 3</i>								
Vagina	3	0	0	1		3	1	
Labia	5	1	1	0	<0.05	5	1	<0.05
Vagina or labia	5	1	1	1		5	1	

^a No significant differences.

^b Primary endpoint.

^c LN strains were not found in any of the samples from visit 1, that is, pre-treatment.

Fig. 2. Colonization of *L. fermentum* and *L. rhamnosus* in control (n = 36) and investigational (n = 32) groups. Each point represents log₁₀ cfu/ml either from labial or vaginal samples from either visits 2 or 3. Horizontal lines represent geometric mean values.

lactobacilli at the strain level. The frequencies of colonization of the vagina by lactobacilli decrease after menopause and different results have been reported with different methods [9, 14].

The main weakness of the study is the low number of women, but this study was designed as a pilot study. The results, however, are robust with clear significant differences regarding the presence of lactobacilli 10 days after treatment between the investigational and control groups. Strangely, lactobacilli from the studied strains were found at visit 3 in 1 woman from the placebo group. She did not harbor any LN strains at visits 1 or 2. We suspect, but have no evidence that she may have purchased the investigational product, which was available for purchase online at the time of the study and used it despite strict instructions not to do so.

The placebo product differed from the investigational product. The main reason for this was that the investigational product without the bacterial powder is in the near-liquid form and as a placebo control would have thus physically differed significantly from the investigational product. Although the fact that the investigational product differs from the control product may affect the subjective symptoms of women, there were no significant differences between the groups (table 1). The pH of women in this study was close to normal and did not change in spite of colonization of lactobacilli. It may be that the women in our sample did not have severe symptoms of vulvovaginal atrophy as is usually the case in studies of specific treatment of this condition. The colonization ability of lactobacilli may be influenced if the mucosa is more severely affected. This should be evaluated in a larger study.

Fig. 3. Colonization levels of *E. coli* vs. levels of lactobacilli. Each point represents log₁₀ cfu/ml of *E. coli* (y-axis) vs. lactobacilli (x-axis) in labial or vaginal samples.

The fact that LN40 was not recovered from any women in this study differs from previous findings in a study by Ehrström et al. [15], in which freeze-dried bacteria of LN strains were administered in vaginal capsules. However, in the previous study, the women studied were premenopausal. LN40 is more nutrition sensitive (Ellen AB, data on file) and the environment in postmenopausal women may be too demanding for this strain compared to LN99 and LN113. In addition, the cell count of LN40 in the study ointment was low compared to that of the other strains. Furthermore, the colonies formed by LN40

are smaller, and although great efforts were directed toward pre-study experiments to avoid missing them upon cultivation, during the study there still might have been some difficulties to detect their presence.

In this study, lactobacilli were found in almost all the women at baseline. However, the levels of lactobacilli were lower than those in normal healthy premenopausal women as estimated by the semi-quantitative analyses [14]. Interestingly, the colonization of the LN strains did not increase the total numbers of lactobacilli in the colonized women, a fact that may indicate a limited niche for these bacteria in the genital area. It might be speculated that women with even lower or zero levels of lactobacilli (and possibly higher pH) before treatment might benefit more from active colonization by the selected lactobacilli strains, a question that merits further investigations.

At the end of the treatment period (visit 2), there was an increased presence of contaminating bacteria such as *E. coli*, *Klebsiella* spp., *Enterococcus* spp. and staphylococci. This may be due to contaminating bacteria being unintentionally added to the genital tract during the application of the ointment. However, no infections occurred during the study period and these species may occasionally be found in the vagina of healthy women [2]. Furthermore, clear negative correlations between levels of lactobacilli and levels of contaminating bacteria were seen (fig. 3).

Subjective symptoms such as feeling of dryness, malodor and irritation were reduced during the study for

both groups. In order to investigate the specific effect of lactobacilli on these symptoms, a larger study with a specifically designed identical placebo product is needed.

Thus, in this study, we could show that topical application of a probiotic ointment is a feasible method for achieving the colonization of lactobacilli in the external and internal genital area of postmenopausal women for at least 10 days after cessation of treatment.

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