

## Clinical Study

# Vaginal Impact of the Oral Administration of Total Freeze-Dried Culture of LCR 35 in Healthy Women

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The use of probiotics in the prevention or treatment of some vaginal infections has been the subject of numerous studies. To assess the presence of *Lactobacillus casei rhamnosus* (LCR35) in the vagina after an oral administration, an open randomised pilot study was conducted on 20 healthy women of child-bearing age. *Materials and Methods.* 2 groups of 10 women were given a 28-day oral course, that is, at least 108 CFU/day (group 1) or  $2 \times 108$  CFU/day (group 2) of LCR35. Nugent score and vaginal screening for LCR35 were undertaken before and after 28 days of treatment. *Results.* The mean Nugent score decreased in group 1 (-0,2) as well as in group 2 (-0,3). 10% of women in group 1 versus 40% of women in group 2 were carrying LCR35 at the end of the trial. *Conclusion.* LCR35, at the minimal dose of  $2 \times 108$  CFU/day, can return the Nugent score to normal in healthy women of child-bearing age, by means of a well-tolerated vaginal temporary presence. Phase III clinical trials will specify the preventive or curative impact of this orally administered strain on a range of vaginal disorders such as bacterial vaginosis or vulvovaginal candidiasis.

## 1. Introduction

Lactobacilli play a fundamental role in the ecological balance of the vagina. Many vaginal infections result from the disappearance or the quantitative or qualitative decrease in lactobacilli naturally present in the vagina. This is particularly true with bacterial vaginosis (BV), one of the most common vaginal infections estimated to have a prevalence between 15% and 30% [1].

Over the last few years, studies have been conducted to assess the usefulness of probiotics to treat or prevent vaginal infections such as BV or vaginal candidiasis. The clinical results differ widely because of the range of probiotics used, the routes of administration of probiotics (oral or vaginal), the duration of treatment and the cohorts studied. Among the points requiring clarification are the type of lactobacilli used in the probiotic preparations and the route of administration (oral or vaginal).

*Lactobacillus casei rhamnosus* LCR35 (LCR35) has shown *in vitro* that it has the required characteristics for vaginal colonization [2]:

- (i) proven fast adherence (one hour) to the vaginal wall, and
- (ii) prevention of growth of potential pathogens such as *Prevotella bivia*, *Gardnerella vaginalis*, and *Candida albicans* from the 4th hour after incubation [2].

About the route of administration, an oral preparation seems to be interesting since studies have shown that the rectum was the natural reservoir for commensal vaginal lactobacilli [3, 4].

An open pilot study was conducted in healthy women to assess the efficacy and safety of this route of administration for LCR35.

## 2. Material and Methods

An open randomised trial was conducted on 20 healthy women to assess the vaginal impact of the daily oral administration of two different doses of LCR35 for 28 days.

The protocol was submitted to the Agence Française de Sécurité Sanitaire des Produits de Santé (AFSSaPS) and the

Ethics Committee (Comité de Protection des Personnes, CPP Ile de France VII) who, respectively, issued an authorisation and a favourable opinion.

**2.1. Study Cohort and Trial Design.** This single-centre trial (Institute Alfred Fournier, Paris) included 20 healthy women who were randomized into two groups of ten women each.

At the screening visit, inclusion criteria were women between 18 and 45 years old, premenopausal, nonpregnant, using effective contraception (i.e., all kind of contraception (hormonal, IUD, chemical, condoms) except “natural contraception” like coitus interruptus), not taking any anti-infectious treatment and not immunodeficient.

All patients were asked for genital symptoms of infections (abnormal discharge, pruritus, burning, or dyspareunia etc.). Symptomatic patients were excluded.

The simple practical technique of self-sampling was chosen because studies demonstrated that self-collected samples had the same microbiological diversity as physician-collected samples [5, 6]. All the women took a sample from their own vagina at the inclusion visit using two sterile cotton swabs mounted on a wooden handle (Deltalab) and introduced over a distance of 2 to 3 cms into the vagina. One swab was used to determine the Nugent score [7]. The other swab was placed in a survival medium (FT-MRS) and frozen for *LCR35* screening.

During the screening visit, all the women received an information leaflet on the study and all signed an informed consent form.

Three to seven days later, women were included, randomized in two groups, and given the treatment.

- (i) Group 1 (10 women): 1 gel capsule of *LCR35 per os* per day (i.e., a minimum of  $10^8$  CFU) for 28 days.
- (ii) Group 2 (10 women): 2 gel capsules of *LCR35 per os* per day (i.e., a minimum of  $2 \times 10^8$  CFU) for 28 days.

The period of 28 days for the control visit has been chosen to sample vaginal secretions at the same time of the menstrual cycle.

## 2.2. Microbiological Methods

**2.2.1. Nugent Score.** After spreading the vaginal secretions on a slide and colouring with the Gram technique, the score was used to classify the vaginal microbiota in three categories: normal (0 to 3), intermediate (4 to 6), and bacterial vaginosis (7 to 10).

**2.2.2. *LCR35* Identification.** At days D7 and D30, immediately after sampling, vaginal swabs were homogenised in FT-MRS, then the solution was divided into 6 cryotubes (containing 33% final glycerol). The divided fractions were then stored at  $-80^\circ\text{C}$  before analysis [(1) to (3)].

(1) *Culture.* After thawing at  $37^\circ\text{C}$ , a fraction was used to establish a diluted series at 1/10th (up to a dilution of  $-7$ ). Cultures were done on MRS, Rogosa and DP media.

The Rogosa, and DP media were incubated in anaerobic conditions for 72 h at  $37^\circ\text{C}$  and the MRS medium was incubated in both aerobic and anaerobic conditions for 72 h at  $37^\circ\text{C}$ . The cultures were done in duplicate for the purposes of statistical analysis.

(2) *REP-PCR.* After culture on MRS for 72 h at  $37^\circ\text{C}$  in aerobic conditions, 10 colonies per sample were taken and the REP-PCR profile of each one was performed. The colony was lysed by heat shock directly in the thermocycler on the basis of previous tests carried out in our laboratory (data not shown). The DNA of each of the 10 colonies was amplified in a reagent medium containing MgCl buffer<sub>2</sub> 1X (MPbiomedicals), 0.2 mM of dNTP (MPbiomedicals), 0.5  $\mu\text{M}$  of primer (GTG)<sub>5</sub>, 1.5 mM of MgCl<sub>2</sub> (MPbiomedicals), 2.5 units of Taq polymerase (MPbiomedicals) with sterile water added to make 50  $\mu\text{L}$ . The amplification cycle is as follows: 5 min/ $94^\circ\text{C}$ ; (30 s/ $94^\circ\text{C}$ ; 1 min/ $45^\circ\text{C}$ ; 2 min/ $72^\circ\text{C}$ )  $\times$  29 cycles; 7 min/ $72^\circ\text{C}$ ; Hold  $10^\circ\text{C}$ . The result of amplification is visible on 1.5% agarose gel, supplemented with 10% B.E.T, following migration for 52 min at 75 volts.

(3) *Specific PCR.* The total DNA of a fraction from a sample is extracted according to supplier recommendations with the QIAamp MiniKit for stool (QIAGEN). Amplification of a specific region of strain *LCR35* was done using hyb 21 primers [8]. The adjustment of this technique enabled us to obtain an amplification of *LCR35* at a hybridization temperature of  $56^\circ\text{C}$ , in a mix made up of 3 mM of MgCl<sub>2</sub>, 2U of Taq polymerase (QBiogen), 0.2 mM of each dNTP, 0.5 mM of primers and 1X of Taq buffer (Qbiogen). The DNA is amplified using successive cycles of: ( $94^\circ\text{C}$ , 5 min,  $94^\circ\text{C}$ , 30 s;  $56^\circ\text{C}$ , 30 s;  $72^\circ\text{C}$ , 1 min/kb)  $\times$  25–35,  $72^\circ\text{C}$ , 7 min). The amplified fragments were then deposited on 2% agarose gel before migration at 100 V for 30 min.

## 2.3. Efficacy and Safety Criteria

**2.3.1. Vaginal Criteria.** The vaginal impact of the oral treatment was evaluated on the following.

- (i) Assessment of the Nugent scores on inclusion and at the end of the trial.
- (ii) PCR screening for *LCR35* in the vagina at the end of treatment.

**2.3.2. Safety.** Patients were told to note any adverse event (local or general) during the study and to consult their physician if necessary. At the control visit, the physician asked the patients about genital or general symptoms and taken medications during the study.

## 3. Results

The mean age of women included was  $27.2 \pm 6.8$  years. No significant difference was noted as regards demographic characteristics (age, weight, height, BMI, urinary pregnancy test) between groups 1 and 2.

TABLE 1: Changes in Nugent scores.

Nugent score	Group 1 ( $n = 10$ )		Group 2 ( $n = 10$ )	
	V1	V3	V1	V3
Normal (0 to 3)	8 (80%)	9 (90%)	10 (100%)	10 (100%)
Intermediate (4 to 6)	1 (10%)	1 (10%)	—	—
Bacterial vaginosis (7 to 10)	1 (10%)	—	—	—

All the women in group 1 took their treatment in accordance with the protocol. 1 patient in group 2 missed 1 day of treatment but nevertheless took all the capsules.

### 3.1. Vaginal Criteria (Table 1)

**3.1.1. Group 1 (at Least  $10^8$  CFU of LCR35 per Os per Day).** Before treatment, the mean Nugent score for group 1 was 1.8: 8 women had normal microbiota, 1 had intermediate microbiota, and 1 had BV (asymptomatic patient).

After treatment, the mean score for group 1 was 1.6: 9 women had normal microbiota, 1 had intermediate microbiota. Note that the patient with microbiological BV on inclusion had normal microbiota by the end of the trial. At the end of the trial, LCR35 had been revealed in the vaginal microbiota of one out of 10 women (10%) in this group.

**3.1.2. Group 2 (at Least  $2 \times 10^8$  CFU of LCR35 per os per Day).** Before treatment, the mean Nugent score was 1 (10 women with normal microbiota).

After treatment, the mean Nugent score was 0.7 (10 women with normal microbiota).

At the end of the trial, LCR35 had been revealed in the vaginal microbiota of four out of 10 women (40%) in this group.

Nugent score decreased in both groups but slightly more significantly in group 2 despite a lower score at inclusion (resp.,  $-0.3$  versus  $-0.2$ ).

At the end of the trial, LCR35 was revealed in the vaginal microbiota of 5 out of 20 women (25%).

**3.2. Safety.** No side effects and no withdrawal of treatment were reported either by patients or by physician during the trial involving 20 women.

## 4. Discussion

One of the exclusion criteria was postmenopausal women, which led to an age group between 18 and 45 years old. After menopause, the lack of oestrogen causes a change in the vaginal ecosystem, with a significant decrease in the number of lactobacilli [9, 10]. The inclusion of postmenopausal women would therefore have introduced bias into the trial.

The exclusion of women with clinical signs of vaginal infection is justified for ethical reasons. It would have been controversial not to start specific treatment of the infection and this, again, would have introduced a bias into the trial.

On the other hand, the patient with asymptomatic BV was not excluded because there was no risk of complications (as this patient was not pregnant). Treatment of the infection could therefore be postponed, especially in the absence of any clinical symptoms.

The two bacteriological samples were taken at an interval of four weeks to ensure that the women were at the same point in their cycle or in the taking of contraception, that is, under the same conditions of hormonal impregnation as studies [11, 12] demonstrated slight changes in lactobacillus microbiota during the menstrual cycle.

The Nugent score has been used for this trial because, even if it is an imperfect test, it stays the benchmark score [5] used to evaluate the quality of the vaginal microbiota and the load of lactobacilli in a semiquantitative manner. It is based on a direct examination after colouring of the vaginal secretions. The examination has been standardised but remains observer-dependent. During this trial, all the Nugent tests were conducted in the same microbiology laboratory (Institute Fournier, Paris) by the same observer.

At the end of treatment, LCR35 was identified, by REP-PCR, four times more often (resp., 40% versus 10% of women) among women in group 2 (at least 2 capsules dosed at  $10^8$  CFU of LCR35/day) than among the women in group 1 (at least 1 capsule dosed at  $10^8$  CFU of LCR35/day). The daily dose of LCR35 therefore played a significant role in the vaginal uptake of the probiotic.

19 of the 20 treated women had a normal Nugent score at the end of the study (Table 1); one woman had an intermediate score. Note that the patient with asymptomatic BV at inclusion (Nugent score  $\geq 7$ ) returned to a normal Nugent score at the end of the study. Thus overall, the treatment improved the Nugent scores, particularly at the dose of  $2 \times 10^8$  CFU of LCR35/day.

Few studies have been conducted on the vaginal uptake of lactobacilli following an oral dose of probiotics. Reid et al. [13] randomised 42 healthy women (mean age 31 years) into 3 groups:  $8 \cdot 10^8$  or  $6 \cdot 10^9$  or  $2 \times 8 \cdot 10^8$  of a combination of *L. rhamnosus* GR-1 + *L. fermentum* RC-14 for 28 days. A change to normal vaginal microbiota (appreciated semiquantitatively by the Nugent score) was significant only at a dose of  $1.6 \cdot 10^9$  GR-1/RC-14, showing the potential ability of oral probiotics to restore or maintain a normal vaginal ecosystem.

The same author published in 2003 [14] an other study about 64 healthy women who received either an oral dose of *L. rhamnosus* + *L. reuteri* or an oral dose of placebo for 60 days. A microbiological examination showed a significant rise in the level of vaginal lactobacilli at D28 and D60 in the probiotic group compared to the placebo group. However, in that study, there was no PCR identification of the strains of vaginal lactobacilli before or after treatment.

Morelli [15] administrated *L. rhamnosus* GR-1 + *L. fermentum* RC-14 for 14 days per os to a group of 10 women. A vaginal colonisation was identified by REP-PCR between 0% and 60% depending on the observation period after the oral administration.

The results of our study of LCR35 therefore agree with the results of other studies using other strains of lactobacilli.

Finally, the literature on probiotics reports an improvement in general immunity [16–18] when taking probiotics, mainly resulting from indirect effects:

- (i) increased migration of immune cells (macrophages, lymphocytes, etc.);
- (ii) increased phagocytosis;
- (iii) Production of proinflammatory cytokines, and so forth.

The same type of result was shown in children with *LCR35* [19].

## 5. Conclusion

This randomised trial is the first formal demonstration of the positive impact of the oral administration of *LCR35* on vaginal microbiota. Treatment with a daily dose of 2 gel capsules *LCR35 per os* produced an overall decrease in the Nugent score in healthy women and, therefore, the maintenance of the quality of their vaginal microbiota. A return to normal was even observed in one case of asymptomatic BV. Our work also demonstrated, by using REP-PCR, *LCR35*'s ability to be temporarily present in the vagina after 28 days oral treatment without generating any adverse events. This means that the probiotic is able to survive through the gastric tract before being temporarily present in the vaginal area. Note that the vaginal impact of *LCR35* appears to be dose-dependent since, after treatment, we observed that 40% of women who received at least 2 capsules dosed at  $10^8$  CFU/day of *LCR35* were carrying *LCR35*, compared to only 10% of women in the group that received at least one capsule dosed at  $10^8$  CFU/day.

This shows that, at a dose of  $2 \times 10^8$  CFU/day, *LCR35* is able to restore or maintain a normal vaginal microbiota in healthy premenopausal women, with a high level of safety and compliance with treatment.

New clinical trials are now required to specify the preventive or curative impact of this strain, administered orally, in the treatment of various vaginal diseases such as BV or vulvovaginal candidiasis.

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