

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

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***Lactobacillus rhamnosus* GR-1 and *L. fermentum* RC-14 are well-characterized probiotic strains with efficacy in the prevention and treatment of urogenital infections in women. The aim of the present study was to apply a molecular biology-based methodology for the detection of these strains and *L. rhamnosus* GG (a commercially available intestinal probiotic) in the human vagina in order to assess probiotic persistence at this site. Ten healthy women inserted vaginally a capsule containing either a combination of strains GR-1 and RC-14 or the GG strain for 3 consecutive nights. Vaginal swabs taken before and at various time points after probiotic insertion were analyzed, and the *Lactobacillus* flora was assessed by randomly amplified polymorphic DNA (RAPD) analysis. This method generated discrete DNA fingerprints for GR-1, RC-14, and GG and enabled successful detection of these strains in the vagina. Strain GR-1 and/or strain RC-14 was found to persist in the vaginal tract for up to 19 days after vaginal instillation, while *L. rhamnosus* GG was detectable for up to 5 days postadministration. In conclusion, the fates of probiotic *L. rhamnosus* and *L. fermentum* strains were successfully monitored in the human vagina by RAPD analysis. This technique provides molecular biology-based evidence that RC-14 and GR-1, strains selected as urogenital probiotics, persist in the human vagina and may be more suited to vaginal colonization than *L. rhamnosus* GG. This highlights the importance of proper selection of strains for urogenital probiotic applications.**

The vaginal ecosystem is a complex environment in which lactobacilli are the most predominant bacterial species in healthy women (30). A recent study showed that this microflora changes constantly, with only 22% of women having a normal *Lactobacillus*-dominated flora at any given time (27). As depletion of vaginal lactobacilli can have serious consequences, including increasing the risk of preterm labor and susceptibility to infections such as *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, human immunodeficiency virus, and urinary tract infections, bacterial vaginosis, and yeast vaginitis (2, 28, 30), restoration and maintenance of a normal microflora are important.

While antibiotics can be effective for eradication of urinary tract infections, they are often unable to cure bacterial vaginosis or prevent complications that arise from it (6). Furthermore, antibiotics have many side effects, multidrug-resistant microorganisms are common, and such chemotherapeutic agents ideally should not be used for prophylaxis or health maintenance.

The use of probiotics, defined as “microbial cell preparations or components of microbial cells that have a beneficial effect on health and well being of the host” (25), offers a potential alternative approach to health restoration and main-

tenance in the vaginal tract (2, 16, 29). The question becomes which probiotic strains will persist in and colonize the vagina, given that the vaginal microflora fluctuates daily in terms of both the types and the numbers of microorganisms present (27, 30).

Of the many probiotic microorganisms available commercially, the majority of which are used for the intestine (11), only *Lactobacillus acidophilus* NCFM has been tested for its potential to colonize the vagina, and it was found in a series of in vitro experiments not to have the optimal characteristics required of a urogenital probiotic (17). *L. rhamnosus* GG is one of the most studied intestinal probiotic microorganisms and has been used successfully to treat and prevent diarrhea (13). It adheres to intestinal epithelial cells and inhibits growth of enteropathogens (26), and one could expect that it would colonize the vagina and reduce the risk of infection; however, vaginal persistence has not been tested, until now.

On the other hand, *L. rhamnosus* GR-1 and *L. fermentum* RC-14 are extensively characterized urogenital isolates which possess a number of properties considered important for urogenital probiotics; both strains adhere to uroepithelial cells and inhibit the growth and adhesion of uropathogens, while GR-1 is resistant to the spermicide nonoxynol 9 and RC-14 produces hydrogen peroxide (19). Furthermore, studies with humans have shown that these strains are efficacious in the prevention and treatment of urogenital infections in women (4, 5, 21, 24).

In order to detect probiotic lactobacilli in the vagina, a specific diagnostic methodology must be used to differentiate

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exogenous from indigenous isolates, as the vaginal microflora of an individual can harbor five or more different strains of lactobacilli at any given time. Molecular biology-based techniques such as pulsed-field gel electrophoresis (PFGE), ribotyping, denaturing gradient gel electrophoresis, analysis with DNA probes, and randomly amplified polymorphic DNA (RAPD) analysis provide the means to do this (10, 15, 20, 33).

The objective of the present study was to use RAPD analysis to determine the ability of two products, one containing a combination of *L. fermentum* RC-14 and *L. rhamnosus* GR-1 and another containing *L. rhamnosus* GG, to colonize the vaginas of healthy women.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The probiotic strains used in this study, *L. fermentum* RC-14, *L. rhamnosus* GR-1, and *L. rhamnosus* GG (ATCC 53103), have been well characterized and were selected as a result of extensive *in vitro* experimentation and human studies (see references 13 and 18 for reviews). *Lactobacillus* strains obtained from culture collections (*L. fermentum* ATCC 9338, *L. rhamnosus* ATCC 7469, *L. reuteri* ATCC 23272, *L. plantarum* ATCC 14917, *L. casei* ATCC 393, *L. vaginalis* NCFB 2810, *L. acidophilus* ATCC 33199, and *L. jensenii* ATCC 25258) and some *Lactobacillus* strains previously isolated from the human urogenital tract (23) were included in the study for comparative purposes. All lactobacilli were routinely cultured at 37°C in MRS broth (Merck, Darmstadt, Germany) (9) under anaerobic conditions (anaerobic jars with BBL gas packs; Becton Dickinson and Co., Sparks, Md.).

Genetic fingerprinting by RAPD PCR analysis. RAPD PCR analysis (32) was performed with each *Lactobacillus* strain and with vaginal isolates recovered from participating subjects. Genomic DNA was first isolated from 1.5 ml of overnight MRS broth cultures by the method outlined by Coakley et al. (7), which uses shearing with glass beads to lyse the bacterial cells. The extracted DNA was then used as a template in subsequent PCR amplifications, which were performed in a total volume of 50 µl in a DNA thermal cycler (Eppendorf Scientific Inc., Westbury, N.Y.). PCR mixtures contained 1 µl of template DNA, 1× *Taq* polymerase buffer (Gibco BRL Life Technologies, Burlington, Ontario, Canada), 4 mM MgCl₂, each deoxynucleoside triphosphate (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada) at a concentration of 200 µM, 2.5 U of platinum *Taq* DNA polymerase (Gibco BRL), and each primer at a concentration of 1 µM. Two primers with arbitrary nucleotide sequences (5'ACGAGGCAC3' and 5'ACGCGCCT3') (31) were used, and these were synthesized by Gibco BRL. DNA was amplified for 40 cycles by using the following temperature profile: denaturation at 94°C for 30 s, annealing at 36°C for 30 s, and polymerization at 72°C for 2 min. The initial denaturation was performed at 94°C for 5 min, and a final extension step of 72°C for 10 min was also used. The PCR products (10 µl of each reaction mixture) were analyzed on a 1.5% (wt/vol) agarose (Fisher Biotech, Fair Lawn, N.J.) gel with 1× TAE buffer (40 mM Tris acetate, 1 mM EDTA [pH 8]) and ethidium bromide staining. A 100-bp ladder (Gibco BRL) was used as a molecular size standard. Gels were run for approximately 2 h at 100 V, and the DNA was visualized by UV transillumination.

Human study. Ten women between the ages of 21 and 51 (mean age, 35.5 years; standard deviation, 11 years) participated in the study, which was approved by the Review Board for Health Sciences Research Involving Human Subjects at the University of Western Ontario, London, Ontario, Canada. Although not specifically screened for urogenital disease, the women were considered healthy on entry into the study, as they did not present with any signs or symptoms of such diseases, and all women harbored vaginal lactobacilli at levels between 10⁵ and 10⁷ CFU/swab. Each subject signed an informed consent. Individuals were not included in the study if they were pregnant or breast-feeding, had a history of diabetes, or were on immunosuppressive or antibiotic therapy. The women were divided randomly into two groups, each of which contained five subjects. Immediately following menses, each member of one group inserted into the vagina a capsule containing a combination of *L. fermentum* RC-14 and *L. rhamnosus* GR-1 in freeze-dried form (a total of >10⁹ viable bacteria) at night for 3 consecutive days (days 0 to 2). Each member of the second group followed the same regimen with commercially available capsules containing >10¹⁰ CFU of *L. rhamnosus* GG (Con Agra Foods, Omaha, Nebr.). Vaginal swabs were obtained prior to capsule insertion (day 0); immediately after treatment (day 3); and 5 days (day 7), 12 days (day 14), and 19 days (day 21) following treatment.

Probiotic detection in *Lactobacillus* capsules and vaginal swabs. Each vaginal swab was placed in a tube containing modified Amies Clear transport medium,

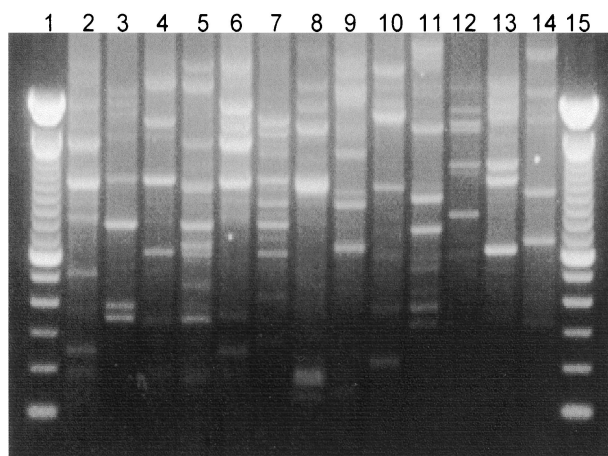


FIG. 1. RAPD fingerprints of probiotic *Lactobacillus* strains (*L. rhamnosus* GR-1 [lane 2], *L. fermentum* RC-14 [lane 3], and *L. rhamnosus* GG [lane 4]), culture collection *Lactobacillus* strains (*L. fermentum* ATCC 9338 [lane 5], *L. rhamnosus* ATCC 7469 [lane 6], *L. reuteri* ATCC 23272 [lane 7], *L. plantarum* ATCC 14917 [lane 8], *L. casei* ATCC 393 [lane 9], *L. vaginalis* NCFB 2810 [lane 10], *L. acidophilus* ATCC 33199 [lane 11], and *L. jensenii* ATCC 25258 [lane 12]), and vaginal isolates (*L. casei* 70 [lane 13] and *L. paracasei* 55 [lane 14]). Lanes 1 and 15, 100-bp ladders.

and the tubes were held at 4°C until analysis. Suspensions were made from each swab in 1 ml of 0.9% saline solution and were diluted 10-fold in the same medium. Portions (100 µl) of appropriate dilutions were spread plated onto MRS agar, and the plates were incubated anaerobically for 48 h at 37°C. The total number of colonies was counted in order to enumerate the total lactobacilli in the vaginal swabs. Then, on the basis of colony morphology, 4 to 20 representative *Lactobacillus* colonies from each swab (particularly those suspected of being GR-1, RC-14, or GG) were selected from the MRS agar plates for RAPD PCR analysis, performed as described above. The RAPD fingerprints of the probiotic strains were compared with those of the vaginal isolates and lactobacilli recovered from the probiotic capsules used for vaginal insertion. In addition, PFGE analysis was performed with selected vaginal isolates to confirm their identity as GR-1. PFGE was performed by the method of Brennan et al. (3), with the following modifications: genomic DNA was isolated from 1 ml of stationary-phase cultures, incubation with proteinase K was performed at 37°C, restriction digests were performed overnight at 25°C with *ApaI*, and gels were run for 18 h with the pulse time ramped from 1 to 15 s.

RESULTS AND DISCUSSION

DNA fingerprinting of probiotic strains by RAPD analysis.

RAPD analysis, a molecular biology-based method which uses PCR with arbitrary primers, was used to generate DNA fingerprints for cultures of *L. rhamnosus* GR-1, *L. fermentum* RC-14, and *L. rhamnosus* GG. The RC-14 and GG strains generated unique, reproducible RAPD profiles (Fig. 1) which were substantially different from the banding patterns of *Lactobacillus* species representative of those found in the human vagina (Fig. 1). While *L. rhamnosus* GR-1 could also be differentiated from most of the representative lactobacilli by RAPD analysis, it was difficult to distinguish GR-1 from some other *L. rhamnosus* strains (e.g., ATCC 7469) (Fig. 1). Therefore, PFGE was investigated and enabled differentiation of the GR-1 strain from these closely related *L. rhamnosus* strains (data not shown). However, RAPD analysis was chosen for subsequent tracking of probiotic lactobacilli in the human vagina, as it is faster and less laborious than the PFGE technique.

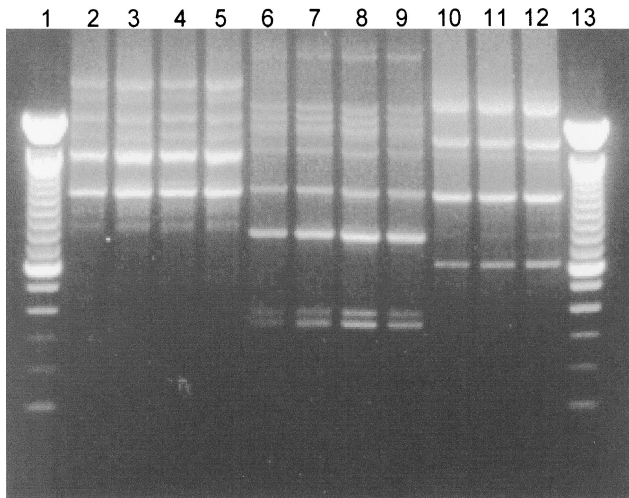


FIG. 2. RAPD fingerprints of *L. rhamnosus* GR-1 (lane 2), *L. rhamnosus* GR-1 isolates from a capsule containing the *L. rhamnosus* GR-1-*L. fermentum* RC-14 combination (lanes 3 to 5), *L. fermentum* RC-14 (lane 6), *L. fermentum* RC-14 isolates from the same *L. rhamnosus* GR-1-*L. fermentum* RC-14 capsule (lanes 7 to 9), *L. rhamnosus* GG (lane 10), and *Lactobacillus* isolates from a capsule containing the *L. rhamnosus* GG strain (lanes 11 and 12). Lanes 1 and 13, 100-bp ladders.

Human study. While previous studies in our laboratory have shown that vaginal insertion of GR-1 and B-54 (a strain almost identical to RC-14) leads to colonization of the vaginal tract and prevention of infection in women with acute or recurrent urinary tract infections (4, 5, 21, 24), probiotic identification was based on the Gram staining result and colony morphology only. Molecular biology-based methods have also been used for identification of these strains, with ribotyping used to identify GR-1 in the vagina of one patient following vaginal instil-

lation (22) and to detect RC-14 and GR-1 vaginally following oral administration (20). The RAPD method has also been used to monitor the survival of RC-14 and GR-1 in the human intestine (12) and to determine the survival of other probiotic lactobacilli in dairy products (10). The present data indicate that RAPD analysis can be used to identify constituents of the vaginal microflora.

As shown in Fig. 2, the RAPD fingerprints of *Lactobacillus* isolates from capsules containing the GR-1-RC-14 combination (lanes 3 to 5 and 7 to 9) or GG alone (lanes 11 to 12) were identical to those of the relevant probiotic strains isolated from the vaginal swabs (lanes 2, 6, and 10, respectively). The usefulness of RAPD analysis for the detection of GR-1, GG, and RC-14 in human vaginal swabs was further verified (Fig. 3A and 4). On examination of the vaginal microflora prior to probiotic treatment (day 0), it was found that all of the women were colonized vaginally by lactobacilli at levels of 10^5 to 10^7 CFU/swab and so were considered healthy, although no specific screening for urogenital disease was performed. Molecular biology-based analysis showed that no individual harbored strains with RAPD fingerprints corresponding to those for GR-1, RC-14, or GG (Fig. 3A, lane 3; Fig. 4, lane 2; Tables 1 and 2). Immediately following vaginal insertion (day 3) the *Lactobacillus* strains that had been applied were detected in the vaginas of all of the women except subject 209, in whom only one of the probiotic strains (*L. rhamnosus* GR-1) from the RC-14-GR-1 combination administered was detected (Tables 1 and 2). Thereafter, strain RC-14 or strain GR-1 or both strains were detectable as part of the dominant *Lactobacillus* microflora by RAPD analysis in the vaginas of four of the five women who inserted these strains 12 days following the cessation of treatment, and they were still present at 18 to 19 days posttreatment in three of these women (Fig. 3A; Table 1). Interestingly, *L. rhamnosus* GR-1 was recovered more frequently than *L. fermentum* RC-14 and so appears to have had

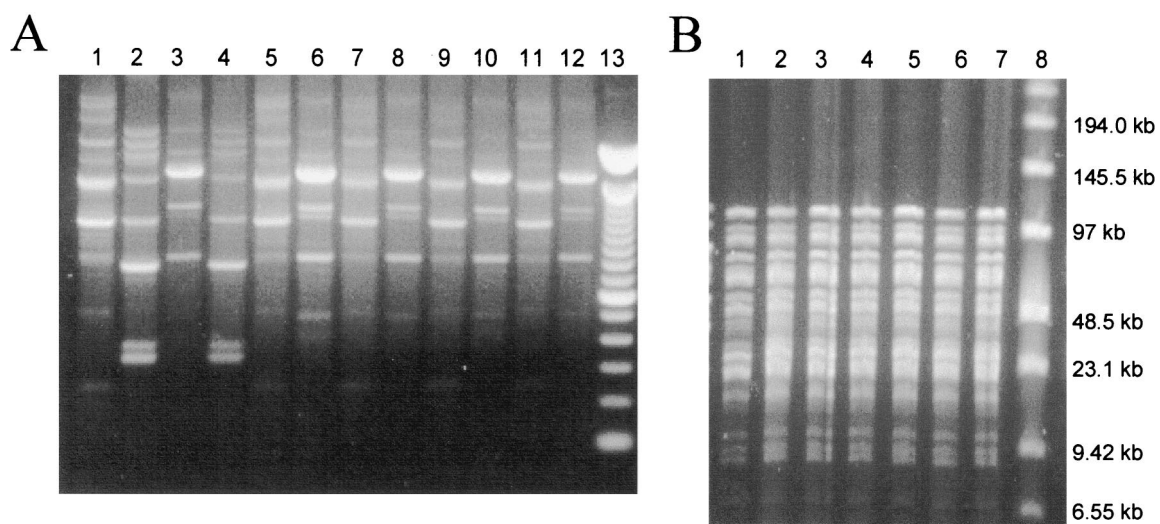


FIG. 3. (A) RAPD fingerprints of *L. rhamnosus* GR-1 (lane 1), *L. fermentum* RC-14 (lane 2), and representative lactobacilli isolated from vaginal swabs of subject 210 before (lane 3) and 1 (lanes 4 to 6), 5 (lanes 7 and 8), 12 (lanes 9 and 10), and 18 (lanes 11 and 12) days after vaginal insertion of an *L. rhamnosus* GR-1-*L. fermentum* RC-14 capsule for 3 consecutive nights. Lane 13, a 100-bp ladder. (B) PFGE analysis of *L. rhamnosus* GR-1 (lane 1) and selected vaginal *Lactobacillus* isolates from subject 207 at 1 day (lanes 2 to 4) and 5 days (lanes 5 to 7) after vaginal insertion of an *L. rhamnosus* GR-1-*L. fermentum* RC-14 capsule for 3 consecutive nights. Lane 8, low-range PFGE molecular size markers.

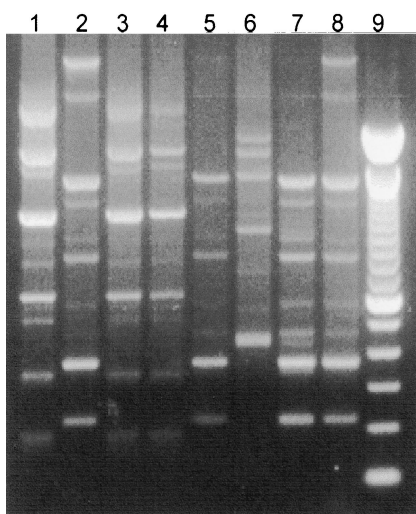


FIG. 4. RAPD fingerprints of *L. rhamnosus* GG (lane 1) and representative lactobacilli isolated from vaginal swabs of subject 222 before (lane 2) and 3 (lane 3), 7 (lanes 4 to 6), 14 (lane 7), and 21 (lane 8) days after vaginal insertion of an *L. rhamnosus* GG capsule for 3 consecutive nights. Lane 9, a 100-bp ladder.

a superior survival capability in all except one of the women (subject 206). Bearing in mind that GR-1 was difficult to distinguish from some other *L. rhamnosus* strains by RAPD analysis (Fig. 1), PFGE was also used in some cases to confirm the identity of GR-1 (Fig. 3B).

L. rhamnosus GG was detected in two of five women 5 days after the treatment ceased, and it was undetectable in the vaginas at all test points thereafter (Fig. 4; Table 2). A previous study had suggested that vaginal insertion of *L. rhamnosus* GG could improve symptoms of yeast vaginitis (14), but no data were reported on the recovery of GG from the vagina. The numbers of subjects evaluated in the present study are too small to conclude that GG is not effective in the vagina, but in vitro studies predicted this outcome (19) and oral administration of this strain did not prevent urinary tract infections (T. Kontiokari, M. Nuutinen, K. Sundqvist, T. Pokka, M. Koskela, and M. Uhari, Abstr. 38th Meet. Infect. Dis. Soc. Am., abstr. 179, 2000). Therefore, it does appear that GG is more suitable for intestinal applications (13, 26) than as a urogenital probiotic.

While a complete analysis of the vaginal microflora was not the aim of the present study, some interesting findings arose

TABLE 2. RAPD PCR detection of *L. rhamnosus* GG in the vaginal tract of women following vaginal instillation of this strain

Subject no.	Detection of GG on ^a :				
	Day 0	Day 3	Day 7	Day 14	Day 21
223	— ^a	+	—	—	—
221	—	+	—	— ^b	—
222	—	+	+	—	—
224	—	+	—	—	—
225	—	+	+	—	—

^a —, GG not detected; +, GG detected.
^b Swab taken at day 17.

regarding indigenous lactobacilli in the vagina. For example, a *Lactobacillus* strain present in the vagina prior to probiotic treatment in subject 210 (Fig. 3A, lane 3), who subsequently became colonized by GR-1, was still detectable immediately after treatment (Fig. 3A, lane 6) and remained for up to 18 days after discontinuation of treatment (Fig. 3A, lane 12). Similar results were obtained for subjects receiving the *L. rhamnosus* GG treatment (Fig. 4). This shows that endogenous lactobacilli can also be monitored by RAPD analysis, an advantage of using this method (i.e., PCR with random primers) over a PCR with strain-specific primers. Interestingly, in another subject (i.e., subject 230), all of the *Lactobacillus* colonies selected were identified as being of the exogenous strain (data not shown), suggesting that it could dominate the vaginal microflora of this individual. Previously, the predominant *Lactobacillus* species present in the vagina have been identified by molecular biology-based methods (1), and changes in the vaginal microflora over time have been examined by use of vaginal smears (27). Data from the present study suggest that further longitudinal molecular biology-based analysis of vaginal lactobacilli at the strain level would be worthy of pursuit.

In conclusion, to our knowledge, this is the first report of the application of RAPD analysis to the detection of probiotic strains in the vagina. Potentially clinically important probiotic strains *L. rhamnosus* GR-1 and GG and *L. fermentum* RC-14 as well as indigenous *Lactobacillus* microflora were differentiated by this method, which uses arbitrarily chosen PCR primers to amplify randomly sized DNA fragments, separation of which results in a characteristic DNA fingerprint (32). Recently, there has been a more rational approach to choosing probiotic strains both for urogenital and for intestinal use (8, 11, 18), and as a result, strains which have the best chance of survival in the host are selected. In this respect, RC-14 and GR-1 are well-characterized urogenital isolates selected on the basis of their probiotic properties at this site, while GG possesses characteristics considered more important for an intestinal probiotic. The present study provides a good example of how a molecular biology-based methodology can track properly selected probiotic lactobacilli as they colonize the vagina.

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TABLE 1. RAPD PCR detection of *L. rhamnosus* GR-1 and *L. fermentum* RC-14 in the vaginal tracts of women following vaginal instillation of a combination of these strains

Subject no.	Strain(s) detected on:				
	Day 0	Day 3	Day 7	Day 14	Day 21
210	— ^a	RC-14, GR-1	GR-1	GR-1	GR-1 ^b
207	—	RC-14, GR-1	GR-1	GR-1	—
206	—	RC-14, GR-1	RC-14, GR-1	RC-14	RC-14
209	—	GR-1	—	—	—
230	—	RC-14, GR-1	RC-14 GR-1	GR-1	GR-1

^a —, GR-1 or RC-14 was not detected
^b Swab taken at day 20.

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