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Comparison of the Vaginal Environment in Rhesus and Cynomolgus Macaques Pre and Post *Lactobacillus* Colonization

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Abstract

Background—Rhesus and cynomolgus macaques are valuable animal models for the study of Human Immunodeficiency Virus (HIV) prevention strategies. However, for such studies focused on the vaginal route of infection, differences in vaginal environment may have deterministic impact on the outcome of such prevention, providing the rationale for this study.

Methods—We tested the vaginal environment of rhesus and cynomolgus macaques longitudinally to characterize the normal microflora based on Nugent scores and pH. This evaluation was extended after colonization of the vaginal space with *Lactobacilli* in an effort to recreate NHP models representing the healthy human vaginal environment.

Results and Conclusion—Nugent scores and pH differed significantly between species, though data from both species were suggestive of stable bacterial vaginosis. Colonization with *Lactobacilli* was successful in both species leading to lower Nugent score and pH, though rhesus macaques appeared better able to sustain *Lactobacillus* spp over time.

Keywords

vagina; microbiota; lactobacillus; macaque

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Introduction

Worldwide, an estimated 38.8 million people currently live with HIV [1]. In the United States alone, approximately 1 million people are infected with HIV, and an estimated 14% of these individuals are undiagnosed [1, 2]. Vaginal acquisition represents the major portal of entry for HIV infection, and as such, is the focus of many studies seeking to prevent the sexual transmission of HIV [3–9]. Rhesus (*Macaca mulatta*) and Cynomolgus (*Macaca fascicularis*) macaques are valuable models for evaluating the safety and efficacy of HIV prevention strategies because of similarities to humans in their susceptibility to infection with simian immunodeficiency virus (SIV) or chimeric simian/human immunodeficiency virus (SHIV), and the gradual development of disease similar to that seen in human HIV infection [10]. However, susceptibility to vaginal HIV acquisition is heavily influenced by multiple factors, including mucosal host defenses, the presence of local infections, pH, inflammation, hormonal changes, and the resident microbiota [11–15].

In this study we conducted a comparative evaluation of the vaginal environment of rhesus and cynomolgus macaques via the evaluation of Nugent Scores and vaginal pH to determine if there is a significant species difference in these parameters. Nugent Scores represent a rapid test of the overall microbiota and are calculated on a scale of 0–10 with lower scores (0–3) indicating a healthy vagina, and higher scores (7–10) indicative of bacterial vaginosis, characterized by a reduced population of acid-producing *Lactobacillus* spp, and greater proportions of Gram negative and Gram variable bacteria such as *Mobiluncus*, *Bacteroides*, *Gardnerella* and *Mycoplasma* spp [16]. This condition has consistently been accompanied by an increased vaginal pH, and has been shown to increase susceptibility to vaginal acquisition of HIV [14, 17, 18]. We compared these known human risk factors in both species to better define the respective non-human primate models for prevention of vaginal acquisition of HIV in women. In addition, we sought to colonize rhesus and cynomolgus macaque vaginas with *Lactobacillus rhamnosus*, a facultative anaerobe commonly found in the healthy female genital tract, and used as a probiotic for the treatment of BV [19]. We monitored the longevity of *L. rhamnosus* and its effect on vaginal pH in each model. We submit that these data will allow for a better definition of vaginal challenge studies in the context of vaccine and microbicide evaluations using these nonhuman primate models.

Methods and Materials

Humane Care Guidelines

All animals used in this study were housed at the Yerkes National Primate Research Center (YNPRC) in Atlanta, GA. All protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Emory University (YER-2001013). All animal care procedures were compliant with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals.

Lactobacillus growth conditions and inoculations

Lactobacillus casei var *rhamnosus* GR-1 (*L. Rhamn.* ATCC 55826) was used in this study. The nucleotide sequence of HIV gp41-64 and anti-erythromycin were cloned into the *L.*

rhamn genome. *L. rhamn* was cultured using Man Rigosa Sharpe broth (Sigma Aldrich, Cat# 69966) containing 32µg/ml erythromycin. After seeding, the culture tubes were sealed and cultured without shaking at 30°C for 48 hrs. At the end of culture, the *L. rhamn* were washed three times with DPBS before being resuspended in 20mg/ml glycogen (Sigma Aldrich G0885) solution, and stored at 4°C overnight prior to inoculation. The colony forming units were determined on Bromo Cresol Purple agar (Himedia labs M1351) plated containing 30µg/ml erythromycin, and titrated generally around $1-2 \times 10^{10}$ per ml.

Study animals and experimental design

Fifteen normal cycling female adult specific-pathogen-free (SPF) cynomolgus macaques (CMs, age: 5–6.6 years old; weight: 3.9–10 kg) and twenty SPF Rhesus macaques (RMs, age: 3.25–6.58 years old; weight: 4.35–9.40 kg) were used in this study. Baseline blood, BactiSwab (Remel Cat# R12100; sent to Yerkes clinical pathology lab for Nugent Score), and cotton-tip swab (Fisher brand Cat# 23-400-110, for vaginal pH) samples were collected weekly from each monkey for a minimum of 3 baseline recordings. All collection procedures were performed under anesthesia.

L. Rhamn inoculation

For *L. rhamn* inoculation, five cynomolgus macaques and twenty rhesus macaques were used. The CMs were tested first with 3 consecutive strategies:

1. The monkey's perianal area was cleaned with gauze saturated with sterile saline solution and the vaginal mucus was removed with 1ml syringe. The monkey's vagina was then inoculated with 1ml DPBS containing $1-2 \times 10^{10}$ CFU/ml *L. rhamn* in 20mg/ml glycogen with a 1 ml needleless syringe. Animals were maintained in a supine position for 5 minutes before returning to their cage. This procedure was repeated once a week for 4 weeks, but the week of menses was skipped. Swabs were collected before inoculations for vaginal pH and Nugent Scores.
2. Ten weeks after the last inoculation, after perianal cleaning, the CM's vaginas were flushed with 10ml 1% lactic acid, pH=3.8 (prepared from Sigma Aldrich, Cat# 252476, ACS reagent, 85%) right before inoculation of 1ml of freshly prepared $1-2 \times 10^{10}$ CFU *L. rhamn* weekly for 5 weeks. Samples were collected as described above.
3. Ten weeks later, the five CM's vaginas were pretreated with 2ml of a suspension of 40mg/ml erythromycin one day before *L. rhamn* inoculation. The next day, the vaginal vaults were flushed with 10ml 1% lactic acid (pH3.8) right before inoculation of 1.5~ 2ml $1-2 \times 10^{10}$ CFU/ml *L. rhamn*. Swab samples were collected until 16 weeks after this inoculation.

Twenty SPF rhesus macaques were inoculated with *L. rhamn* according to the third strategy, preceded by vaginal erythromycin treatment followed one day later by a 10 ml 1% lactic acid flush as described above and inoculation of 1.5–2ml $1-2 \times 10^{10}$ CFU/ml *L. rhamn*. Swab samples were collected weekly for 7 weeks.

Determination of vaginal pH

The macaques' vaginal pH was determined by EMD Millipore MColor pHast™ pH Test Strips and Indicator Papers (pH 0–6 and 5–10, Cat# 1095310007 and 1095330001 respectively). In brief, a sterile cotton-tip applicator was inserted into the vagina, turned over 2 times, and then rolled onto pH test strips. The pH values were determined by two observers.

Nugent score determinations and calculations

Nugent scores have been used in evaluating the vaginal environment of women since 1991; scores of 7–10 indicate bacterial vaginosis whereas scores of 1–3 indicate lactobacillus-dominated microflora [20]. In order to evaluate bacterial vaginosis in the macaques, the Gram-stained vaginal BactiSwab smear slides were evaluated by a microbiologist in the clinical pathology lab at the Yerkes National Primate Research Center, blinded to the animal's experimental status. The evaluation scale included an Amsil test (fishy smell from the vaginal swabs after adding 1–2 drops of 10% fresh KOH), clue cells (the vaginal epithelia cells' border obscured by adherent small coccobacilli), and scores of three bacteria: Gardnerella, Curved Gram negative (G-) bacilli, and lactobacillus spp. See Supplemental Table 1.

Statistics

Differences between CM and RM vaginal pH and Nugent scores before and after *L. rhamn* inoculation were analyzed by unpaired two-tail t-test in GraphPad Prism.

Results

The average baseline Nugent score of the RMs was 8.36 ± 0.26 , while the average Nugent score of the CMs was 9.11 ± 0.12 ($p=0.0072$). These Nugent scores are indicative of bacterial vaginosis [21]. Figure 1A displays the tighter dispersion of Nugent scores amongst CMs as compared to RMs, some of which had lower Nugent scores comparable to those found in healthy humans. On the 0–10 scale, no individual CMs had a baseline Nugent score of less than 5. Baseline Nugent scores of RMs showed a higher level of variation, although vaginal cultures revealed that only 3 of 20 RMs had measurable levels of *Lactobacillus spp.* at baseline (data not shown). While the average Nugent scores of both species correlated with human bacterial vaginosis, the difference between the average Nugent scores of CM vs RMs was highly significant ($p=0.0072$) using an unpaired two-tailed t-test. The vaginal pH of both species was also evaluated and compared. The average vaginal pH of CMs was 7.14 ± 0.67 , while the average vaginal pH of RMs was 6.09 ± 0.07 ($p<0.0001$). As seen in figure 1B, vaginal pH correlated with the differences in Nugent scores and vaginal pH values in RM were stable over 2 reproductive seasons.

The initial inoculation protocol of CMs with *L. rhamn* consisted of a series of one inoculation per week without pre-conditioning and each preceded by the collection of samples to assess both the Nugent score and pH, to evaluate the colonization from the previous administration. As seen in Fig 2A five consecutive inoculations of $1-2 \times 10^{10}$ CFUs of *L. rhamn*, failed to alter the vaginal Nugent scores of CM, demonstrating failure of the *L.*

rhamn to durably colonize the vaginal vault. The repeated inoculations did however induce a lower vaginal pH (from 7.45 ± 0.32 to 6.5 ± 0.43) at week 2 (Fig 2B, $p=0.018$) with a similar but non-significant trend for the subsequent time points.

In our second attempt to inoculate CMs, the monkey vaginal vault was flushed with 10 mL of a 1% lactic acid solution (pH3.8) in an effort to reduce and inhibit the pre-existing microflora prior to inoculation of *L. rhamn*, at weekly intervals (Figure 3). This approach induced a slight but non-significant decrease in Nugent scores monitored weekly, but no evidence of colonization by *L. rhamn*. Somewhat surprisingly, the vaginal pH showed no appreciable change or perhaps even a slight increase (Fig 3B).

In a third attempt to colonize CMs, the animals were pre-conditioned by the vaginal administration of 1–2 ml of Erythromycin 24 hours prior to inoculation. The next day, each animal underwent vaginal flushing with 10mL 1% lactic acid followed by inoculation of $1-2\times 10^{10}$ CFUs of *L. rhamn*. Three animals received a single inoculation, while two animals received two inoculations 1 week apart. Nugent scores and pH were then monitored weekly for 12 weeks (Figure 4). Measurements starting at 1 week post inoculation revealed steep decreases in average Nugent score from 9.4 ± 0.24 to 3.33 ± 0.33 and pH from 6.65 ± 0.47 to 5.1 ± 0.08 . As reflected in figure 4, differences in Nugent scores reached statistical significance in weeks 1, 3, 9 ($p<0.0001$), 4 ($p<0.004$), and 5 ($p<0.01$) post inoculation. The trends seen for vaginal pH mirrored those noted in Nugent score, though the pH differences were not statistically significant. Overall, the *L. rhamn* colonization, while fluctuating over time, was durable in CMs.

For the vaginal colonization of RMs, *L. rhamn* was administered according to the same protocol as the final inoculation of CMs. RMs also received intravaginal erythromycin 24 hours prior to inoculation, and lactic acid flushing immediately prior to inoculation. All RMs received a single administration of *L. rhamn* ($1-2\times 10^{10}$ CFUs). Following inoculation, a sharp decrease in average Nugent scores was seen (6.2 to 5.6) that remained statistically significant despite small fluctuations in the average at each weekly time point for seven weeks as shown in in Figure 5A. Nugent scores were measured again at 42 weeks post inoculation, and were found to have remained significantly decreased compared to baseline (not shown) even at this late time point. Average pH was also followed weekly after inoculation, and showed statistically significant decreases in weeks 1, 2, 4, and 6 as seen in figure 5B.

To gain a better understanding of the kinetics of the microflora measured by Nugent score in the 2 species, relative values for *Gardnerella*, curved Gram⁻ bacilli and *Lactobacilli* were plotted over time and compared between the two macaque species (Figure 6). There was a marked difference between RMs and CMs in their ability to maintain lactobacilli over time and the inhibition of bacterial strains associated with bacterial vaginosis, despite relatively comparable initial representation of *Gardnerella*, curved Gram⁻ bacilli and *Lactobacilli*. While both species demonstrated successful colonization by *L. Rham*, the relative representation of this mucosal protective species was markedly higher in RMs than in CMS. Conversely, *Gardnerella*, after an initial decrease in CMs, regained prominence by week 5 post administration of lactobacilli, whereas RMs limited their rebound over time.

Discussion

A correlation between bacterial vaginosis and increased HIV acquisition in women has been well established [22, 23]. Since macaques are used as a major model of vaginal HIV infection for pathogenesis, vaccine and microbicide efficacy testing, we submit that the definition of the vaginal normal environment in macaque species is a critical parameter, and the ability to recreate various versions of the human microbial environment may represent further refinement of the model. Detailed analysis of the pigtail macaque vaginal microbiome has been reported resulting in preferred use of this species in microbicide studies [24]. RM and particularly CM vaginal environments have received comparatively less attention, even though both species are used extensively for the testing of vaccine efficacy, including protection from repeated vaginal challenges [25–28]. Moreover, the ability to colonize macaques with *Lactobacilli* to mimic a healthy human vaginal environment has not systematically been attempted. Additionally, there are differences in the reproductive cycle between tropical macaque species (CM and pigtailed macaques) which are cycling year round, and subtropical RMs which exhibit a mating and a reproductive season. Thus, we attempted to define differences in vaginal flora between RM and CM. Of note the data presented herein represents mating season recordings in attempts to match the CMs regular cycles. Indeed, differences were noted between the two species compared. While the average Nugent scores in both species were equivalent to bacterial vaginosis in humans, vaginal Nugent scores were significantly lower in RMs than CMs. Nugent scores are strongly correlated to the prevalence of various bacteria within the vagina, particularly gram negative and gram variable species that produce bacterial vaginosis in humans, relative to *Lactobacillus* spp [29]. While bacterial vaginosis is associated with increased acquisition of HIV in women, it remains difficult to assert whether a normal RM or CM vaginal flora renders them more susceptible to vaginal infection with SIV, although anecdotal observations suggest that this normal flora does not amount to the level of pro-inflammatory environment that accompanies human bacterial vaginosis [22, 23, 26]. However, it suggests that the benefits of a lactobacillus dominated environment (low pH, production of lactate, inhibition of gram negative bacteria and fungi) are largely absent and as such, the macaque model is more representative of women with bacterial vaginosis as opposed to those with healthy vaginal flora [30].

A low vaginal pH, primarily determined by *Lactobacillus* production of lactic acid, inactivates HIV [31]. In this study we confirmed that the average vaginal pH of both macaque species studied was higher than the average vaginal pH in women, likely due to the low number of acid producing *Lactobacillus* spp. However, there was an additional species specific difference with RMs able to maintain a lower vaginal pH than CMs, suggesting greater resistance to vaginal SIV infection in RMs as compared to CMs with a total absence of *Lactobacilli* spp. The low levels of *Lactobacilli* spp in pigtail and RMs has been correlated with low availability of glycogen in vaginal epithelium, suggesting that the vaginal environment may not be able to sustain *Lactobacilli* spp dominance over time[27]. However, in our attempts to recreate a model of healthy human vaginal environment, when the macaque vagina was pretreated with a short course of antibiotic treatment, inoculation of

both RMs and CMs with *L. rhamn* induced a lasting dominant albeit fluctuating presence of this beneficial flora more representative of a healthy human vagina.

Colonizing the RM vagina with *Lactobacillus* spp has been previously accomplished with strains that were either present in Chinese RMs or *L. jensenii* expressing cyanovirin-A, a lectin aimed at preventing HIV/SIV acquisition, though *Lactobacillus* spp are occasionally detected in RMs, and apparently more prevalent in Chinese RMs [32–34]. The initial decrease in pH while attempting to colonize the CM vagina by inoculation only, suggested limited and certainly subdominant colonization though there was no appreciable decrease in Nugent score, even after weekly inoculations of *L. rhamn*. Even flushing the vagina with 10mL 1% lactic acid immediately prior to inoculation did not appear to create an environment suitable for *L. rhamn* to prosper. As outlined above, upon successful colonization with *L. rhamn*, Nugent scores and pH dropped dramatically in CMs, but gradually climbed back up. Additionally, for reasons unknown, *L. rhamn* dominance transiently resumed at week 9 without external manipulation, indicating the *Lactobacillus* colonization was durable, but the extent of *Lactobacillus* dominance varied over time. In RMs, the *Lactobacillus* colonization and dominance appeared more durable, as it was still detectable several months after inoculation (not shown).

The results of our study outline differences between RM and CM vaginal environment, which may have to be taken into consideration when choosing a model for vaginal challenge with SIV or SHIV. While the reproductive season in RMs may be accompanied with differences in hormonal levels over the course of the year, the ability to colonize them with *Lactobacilli* to establish a vaginal microbiota resembling a healthy human vaginal microbiota seems readily achievable. CMs on the other hand provide a model of year round cycling similar to humans, but altering the microbiota may be more difficult than in RMs.

Acknowledgments

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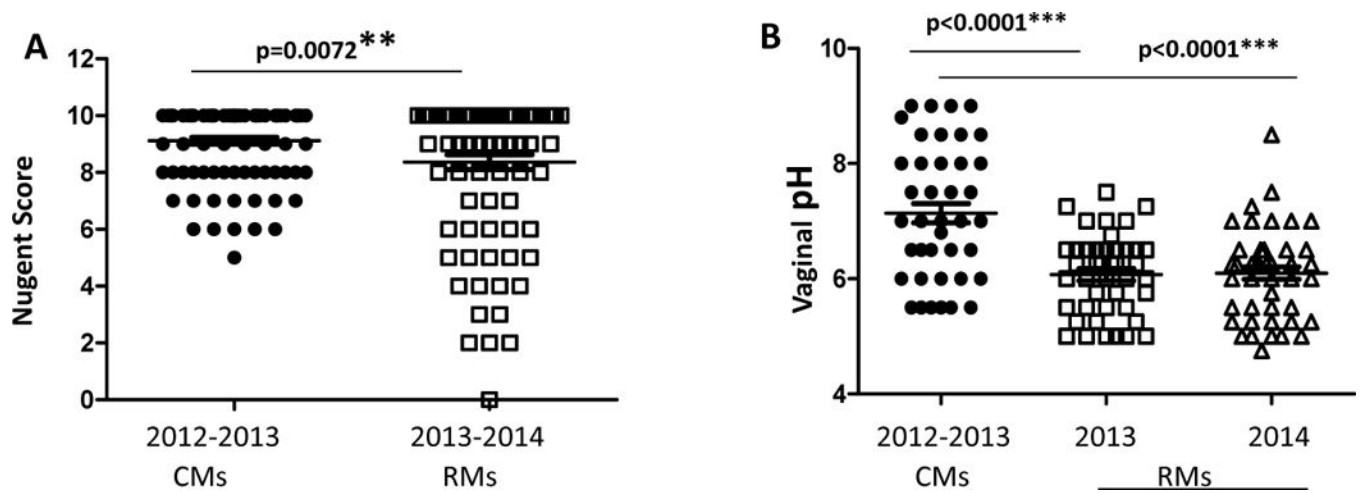


Figure 1.

(A) Comparison of Nugent scores between cynomolgus and rhesus macaques. Rhesus macaque Nugent Scores were collected during the reproductive period (Oct.–Dec.). (B) Comparison of vaginal pH between cynomolgus and rhesus macaques. Statistical values are presented as $**p=0.0072$; $***p<0.0001$

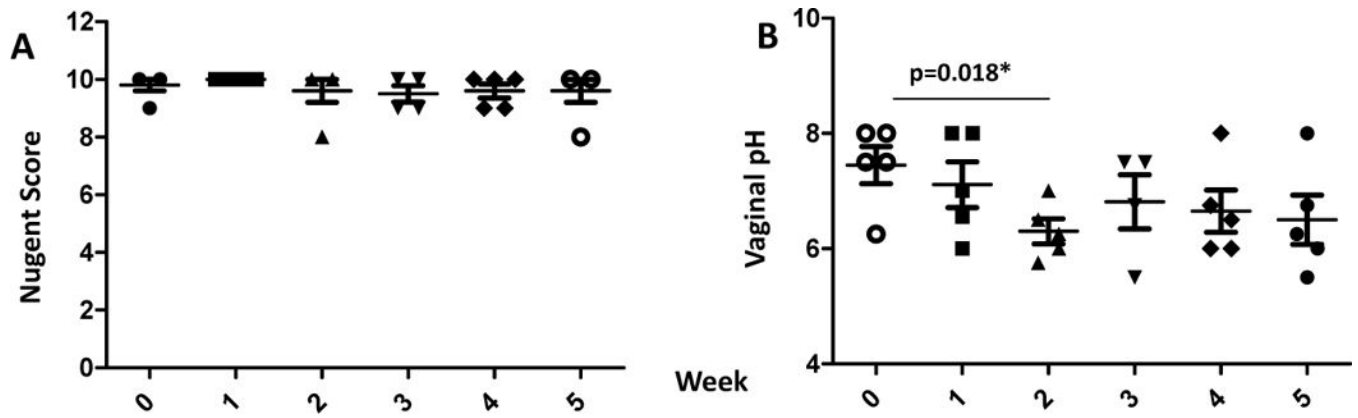


Figure 2. Nugent Scores (A) and vaginal pH (B) of cynomolgus macaques before and after weekly inoculation of $1\sim 2 \times 10^{10}$ CFU of *L. rhamn* without pre-conditioning. Statistical values are presented as * $p=0.018$.

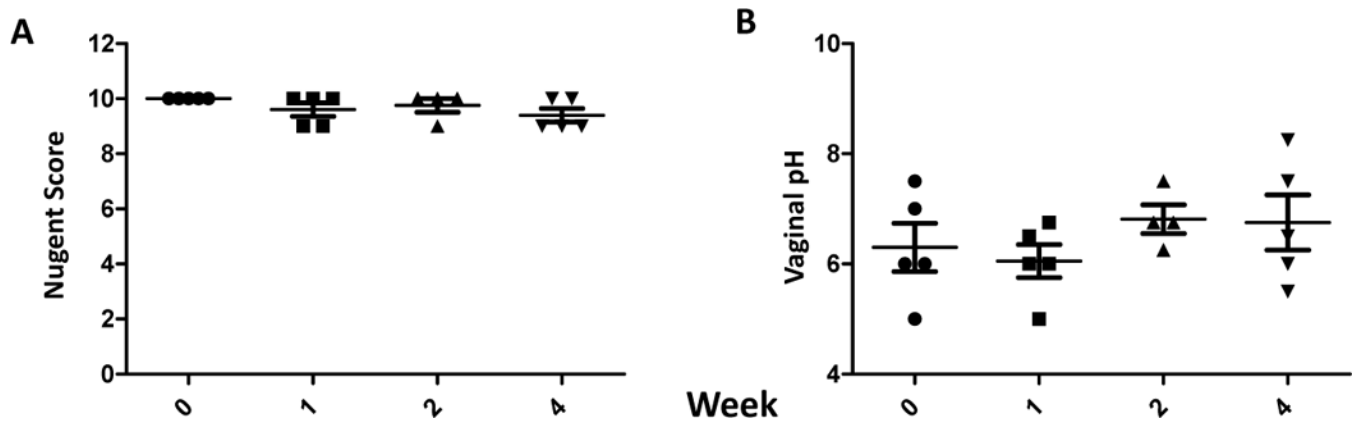


Figure 3. Nugent Scores (A) and vaginal pH (B) of 5 cynomolgus macaques before and after weekly inoculation of $1\sim 2 \times 10^{10}$ CFU of *L. rhamn* preceded by a 10ml 1% lactic acid flush immediately before *L. rhamn*. inoculation once a week. No statistically significant differences were observed.

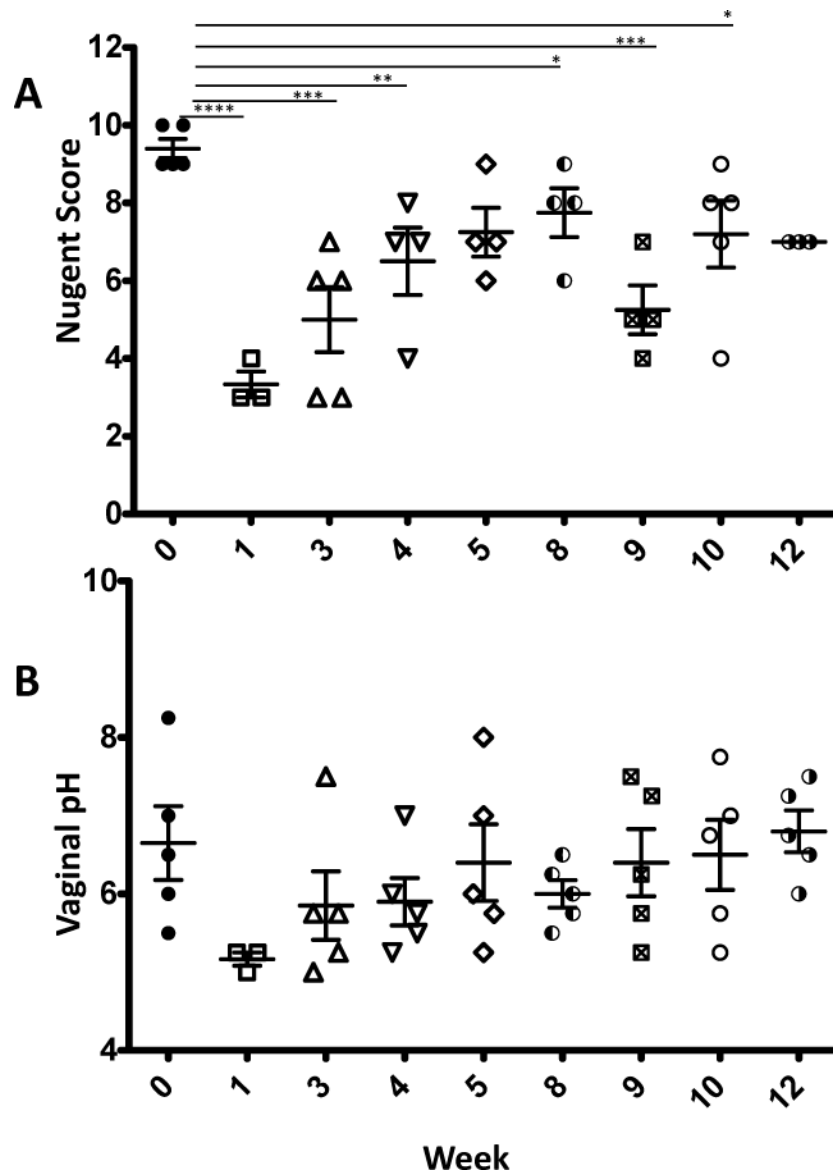


Figure 4. Nugent Scores (A) and vaginal pH (B) of 5 cynomolgus macaques before and after inoculation of $1\sim 2 \times 10^{10}$ CFU of *L. rhamn* preceded by the vaginal administration of 2 mls of erythromycin solution (40mg/ml) 24 hours before a 10ml 1% lactic acid flush followed by inoculation of *L. rhamn*. Each animal received 2 such administrations (on week 0 and 1) and were monitored weekly for up for 12 weeks. Statistical values are presented as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

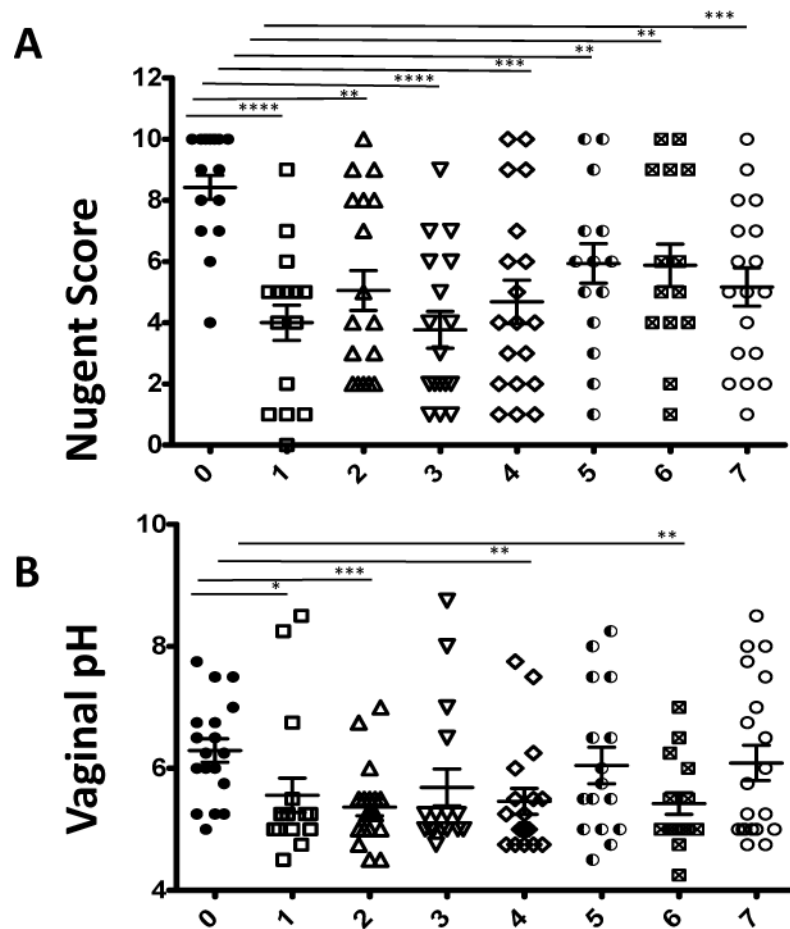


Figure 5. Nugent score and vaginal pH of rhesus macaques before and after *L. rhamn.* inoculation following erythromycin administration and 1% lactic acid flushing. **(A)** Nugent scores; after a single *L. rhamn.* inoculation, Nugent scores sharply decreased and remained low for 7 weeks and beyond (up to week 42, data not shown). **(B)** Vaginal pH after a single *L. Rhamn.* inoculation. Statistical values are presented as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

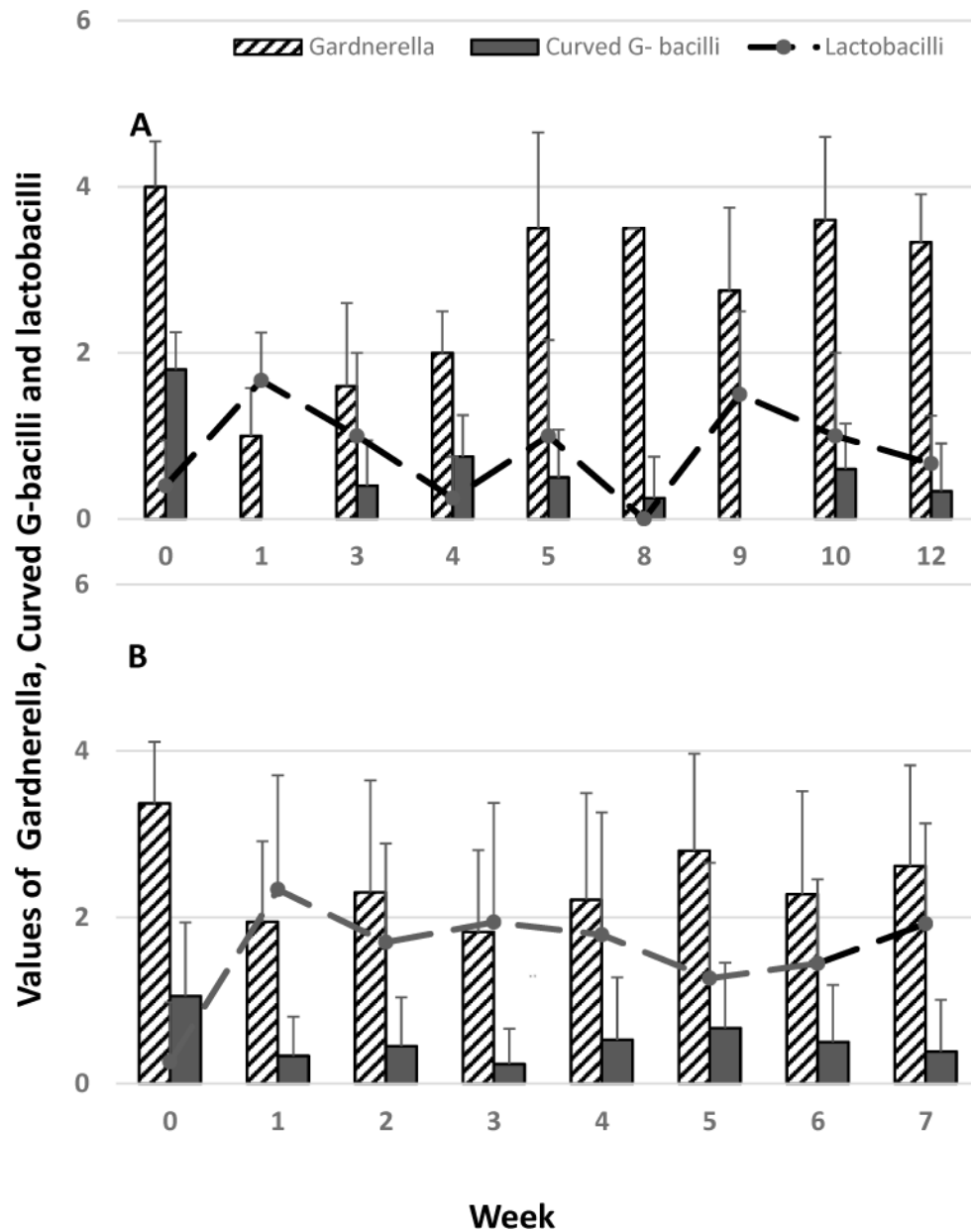


Figure 6. Relative abundance of *Gardnerella*, curved Gram⁻ bacilli and *Lactobacilli* in the vaginal environment of CMs (A) and RMs (B) before and after colonization with *L. Rhamn*. The data are representative of 5 CMs and 20 RMs. The values shown represent Nugent scoring values for *Gardnerella*, curved Gram⁻ bacilli and inverse scoring for *Lactobacilli* (1–4).