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Demonstration of vaginal colonization with GusA-expressing *Lactobacillus jensenii* following oral delivery in rhesus macaques

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Abstract

The vaginal microbiome, which harbors beneficial *Lactobacillus* strains, is believed to be a major host defense mechanism for preventing infections of the urogenital tract. It has been suggested that the gastrointestinal tract serves as a reservoir for lactobacilli that colonize the vagina. Using rhesus macaques, we examined whether oral delivery of human vaginal *Lactobacillus jensenii*-1153-1646, a GusA-producing strain, would result in colonization of the rectum and the vagina. Lactobacilli were identified from the vagina tracts of three macaques on the basis of β -glucuronidase enzyme production, 16S rRNA gene sequence and DNA homology using a repetitive sequence-based polymerase chain reaction.

Keywords

Lactobacillus; Colonization; Oral; Vaginal; Rectal

1. Introduction

The bacteria that colonize the vagina are collectively referred to as the vaginal microbiome. The amount and species of bacteria present have significant implications on a woman's overall health. The vaginal microbiome consists primarily of lactobacilli (Ravel et al., 2010), which may serve to protect the mucosa from the entry of pathogens (Klebanoff et al., 1991). Lactobacilli also acidify the vagina through lactic acid production, which may contribute to vaginal health (Boskey et al., 2001).

The normal rectal microbiome includes members of the *Lactobacillus* genus such as *L. plantarum*, *L. rhamnosus* and *L. paracasei* (Ahrne et al., 1998). Recent papers have suggested that the vagina and rectum may share common species of *Lactobacillus*, with *L.*

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crispatus, *L. gasseri* and *L. jensenii* being those vaginal strains most commonly found rectally (Antonio et al., 2005; El Aila et al., 2009; Marrazzo et al., 2009). Using a repetitive element sequence-based PCR (repPCR) method, Marrazzo et al. showed that the same *Lactobacillus* strain could be detected from both the vagina and rectum of women (Marrazzo et al., 2009). Another group described the same *Lactobacillus* genotype in paired vaginal/rectal samples from women using randomly amplified DNA polymorphism analysis (RAPD) (El Aila et al., 2009; El Aila et al., 2011).

In an important predicate study, Reid et al. were the first to show that oral delivery of *Lactobacillus rhamnosus* GR-1 and *Lactobacillus fermentum* RC14 could result in increased vaginal colonization by *Lactobacillus* (Reid et al., 2001). While this increase was likely comprised of the administered strains, the authors acknowledge that the ribotyping technique they used “lacks the precision that is necessary for accurate strain discrimination” (Zhong et al., 1998). In another study, the probiotic strains, *L. rhamnosus* GR-1 and *L. fermentum* RC14, were administered orally to healthy women for 60 days (Reid et al., 2003). Compared to placebo, women given *Lactobacillus* had a 0.9 log increase in *Lactobacillus* counts at day 28 and a 0.5 log increase at day 60 (Reid et al., 2003). However, the authors did not perform strain typing and thus could not definitively conclude that the increase was due solely to the administered strain; notably, the authors also found minor, but significant, increases in non-administered coliforms and yeast strains.

To determine whether the rectum can serve as a possible bacterial reservoir for colonization of the vagina, a GusA tagged-*L. jensenii* strain was delivered orally via gavage or feeding to macaques and subsequently cultured from the rectum, feces and vagina. Using molecular techniques and DNA sequence, we definitively establish that the rectal, fecal and vaginal colonizing lactobacilli were the orally administered strain. Our experiments verify that some strains of lactobacilli can traverse the gastrointestinal (GI) tract, and that the rectum may serve as a reservoir for vaginal colonization with *Lactobacillus* species.

2. Materials and methods

2.1. Lactobacillus preparation

Lactobacillus jensenii 1153-1646 used in the study was created at Osel, Inc. (Mountain View, CA) as an indicator strain. The DNA corresponding to the *L. gasseri* *gusA* gene encoding β -glucuronidase A and promoter was cloned. The *gusA* gene cassette was stably integrated by homologous recombination as a single copy gene into the *poxI* site of the *L. jensenii* 1153 genome as described in Liu et al. for the integration of the cyanovirin-N gene (Liu et al., 2006). The resolved strain, *L. jensenii* 1153-1646, secretes β glucuronidase enzyme, which acts on the (5-bromo-4-chloro-3 indoxyl-beta-D-glucuronic acid, cyclohexylammonium salt) substrate turning the colonies blue on Mann Rogosa Sharp (MRS) agar plates (a selective agar for *Lactobacillus* sp.).

2.2. Lactobacillus growth conditions, culture and identification

L. jensenii 1153-1646 was cultured using BD Difco™ Lactobacilli MRS broth or agar (Becton Dickinson, Franklin Lakes, NJ). For inoculation of animals, lactobacilli were grown in 200 ml of MRS broth and incubated for about 20 h at 37°C, 5% CO₂. Lactobacilli were pelleted by centrifugation at 1942 × g for 10 min, then the supernatant decanted, and the pellet resuspended in 6 ml of PBS. The cell suspension was pelleted again at 1942 × g for 10 min, and the supernatant decanted. The pellet was resuspended in 2 ml of PBS (1 ml per animal), containing approximately 1×10⁹ colony-forming units (CFU) of *Lactobacillus*.

The vaginal and rectal/fecal microflora of each animal was sampled using the Port-A-Cul™ swab/tube collection system (Becton Dickinson, Cockeysville, MD, USA). Swabs were first

plated on MRS plates containing X-Gluc substrate (Gold Biotechnology, St Louis, MO), and incubated anaerobically using the Anaerobe Gas Generating Pouch System (Becton Dickinson). *Lactobacilli* were identified by selection of blue colonies and Gram stain for Gram-positive rods. Single colonies were grown on MRS and harvested with a sterile swab into API 50 CHL medium, which was used to inoculate an API 50-CH Microorganism Identification Strip (Biomerieux, Inc, St. Louis, MO). After 48 h of anaerobic incubation, results were recorded. *Lactobacillus* DNA was extracted using a Mo Bio™ UltraClean Microbial DNA Isolation Kit (Mo Bio, Carlsbad, CA). The ~900 bp 16S rRNA gene was amplified using gene-specific primers, 8f (5′ AGA GTT TGA TCC TGG CTC AG-3′) and 926r (5′ -CCG TCA ATT CCT TTR AGT TT-3′), and a unique sequence of 214 bp spanning the *gusA* gene and *pox1* cassette was amplified using primers F*gusA* (5′-GGA ATT TTT ACT CGC GAT CGT G-3′) and R cassette (5′ -TGG CTC GCT ACA ATA TGC AC-3′). The 16S ribosomal DNA (rDNA) and the unique *gusA* cassette were cloned using a Topo TA kit (Invitrogen, Carlsbad, CA) and sequenced. The 16S rRNA gene sequence was analyzed using the Nucleotide Basic Local Alignment Search Tool (BLAST) and compared to the *L. jensenii* 1153 parental strain (Accession: NZ_GL545251.1), which has been fully sequenced. The *gusA* gene sequence was compared to the known sequence within the *Lactobacillus* cassette. Finally, strains were typed using a Diversilab® DNA Fingerprinting Kit for *Lactobacillus* and Diversilab® Analysis System (Biomerieux Inc.).

Due to poor recovery of *Lactobacillus* isolates from the rectum, selected rectal/fecal swabs were pre-incubated 12 h in MRS broth containing clindamycin 20 µg/ml as a selection broth to enhance *Lactobacillus* growth.

2.3. Rhesus macaque studies

Four female Chinese-origin rhesus macaques (*Macaca mulatta*), ages 4–6 yrs, were used in this study. Animals were housed at BIOQUAL, Inc. in Rockville, MD. Non-human primate housing, care and treatments were performed in accordance with the standards of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), the Animal Welfare Act as amended, the Public Health Service Policy on Humane Care and Use of Laboratory Animals, 2002 and the NIH guidelines for Research Involving Recombinant DNA Molecules. For all procedures, animals were sedated with 10 mg/kg ketamine and 1 mg/kg acepromazine when required.

Prior to the start of the study vaginal and rectal cultures were taken from M4698 and M4701, and no *L. jensenii* 1153-1646 strain was isolated. In the first study, 5 ml sodium bicarbonate solution (130mg/ml) followed 5 min later by 1×10^9 CFU *L. jensenii* 1153-1646 were administered to two macaques by intragastric gavage on days 0–3 and 6–8. Vaginal and rectal swabs (Port-A-Cul™ swab/tube collection system, Becton Dickinson, Cockeysville, MD) were taken on days 2, 3, 7, 8, 10, 15 and 28. Macaques M4698 and M4701 were treated with vaginal suppositories of azithromycin (200 mg in a micronized silica gel and MBK-base gel for 5 days) to clear their *Lactobacillus* flora and were recycled into the second study. Prior to the second study, vaginal, rectal and fecal cultures were taken from macaques M4698, M4701, M4700, and M4703, and no *L. jensenii* 1153-1646 strain was isolated.

Once vaginal colonization had been established in the first study, we tried a less invasive technique to deliver the *Lactobacillus*. In the second study, 1 ml of sodium bicarbonate solution (650 mg/ml) in a food treat followed 30 min later by 2×10^8 CFU *L. jensenii* 1153-1646 in a different food treat were administered orally for 3–4 consecutive weeks. This procedure did not require anesthesia. Swabs from vaginal, rectal and feces were taken on days 7, 14, 21, 28, 35, 42, 49 and 56. We collected samples for a longer period of time to follow the duration of colonization in animals that became positive.

3. Results and discussion

3.1. Colonization of recombinant *L. jensenii* 1153-1646 in macaques

Lactobacilli were readily isolated from the vaginal tract of three macaques. However rectal isolation was more cumbersome due to the high load of diverse numbers of microbial species; therefore, we used a *Lactobacillus* strain that has been genetically tagged with a *gusA* gene and we used clindamycin for improved selection of this strain. The *gusA* gene, which encodes the enzyme β -glucuronidase, was integrated into the *L. jensenii* 1153 chromosome and thus *Lactobacillus* colonies are blue when cultured in the presence of the chromogenic X-gluc substrate (Fig. 1A). In addition, *L. jensenii* 1153-1646 has a characteristic fermentation profile when examined biochemically using API 50-CH (Fig. 1B). In the first study, two macaques were gavaged with sodium bicarbonate solution and *L. jensenii* 1153-1646. The dosing and distribution of *L. jensenii* 1153-1646 by anatomic sites is depicted in Fig. 2. Both M4698 and M4701 became positive for *L. jensenii* 1153-1646 vaginally at day 28, approximately three weeks post-oral dosing. In addition, we also detected *L. jensenii* 1153-1646 in the rectum of macaque 4701. We treated these two macaques vaginally with azithromycin suppositories to clear the *Lactobacillus*. Vaginal cultures were taken from these macaques to ensure clearance of the *L. jensenii* 1153-1646 strain; then they were given sodium bicarbonate and the *L. jensenii* 1153-1646 mixture in two different food treats for a total of 18 days over a 4-week period. *L. jensenii* 1153-1646 was detected vaginally at days 7, 14, 21 and 56 from M4701. *L. jensenii* 1153-1646 was detected rectally on day 56 from M4698. In the second study, two different macaques were fed sodium bicarbonate and the *L. jensenii* 1153-1646 mixture in two different food treats for a total of 15 days over a 3-week period. *L. jensenii* 1153-1646 was detected both rectally and vaginally from M4700, and was detected rectally at day 35 from M4703, the last day the swabs were taken. Taking both experiments together, 3 out of 4 macaques were colonized vaginally after oral delivery.

The delay in rectal and vaginal colonization following oral dosing was unexpected and suggested that the bacteria survived transit through the upper GI tract and were capable of persisting in the lower GI tract and possibly needed to achieve a threshold before the vagina was colonized. In a recent study, another group found that using quantitative PCR to detect species of *Lactobacillus* yielded much higher numbers of positive cultures rectally and vaginally, in contrast to culture-based methods (El Aila et al., 2011). Thus, culture-based detection levels may not be as sensitive in recovery of *Lactobacillus* in rectal and fecal cultures. However, it was important to show that live, culturable *L. jensenii* 1153-1646 could be isolated in this study.

3.2. Identification of *L. jensenii* 1153-1646 in macaques

The strains were identified first by culture of blue colonies on MRS agar (as shown in Fig. 1A) and by Gram stain for Gram-positive rods. Pure cultures were analyzed biochemically using API 50 CH carbohydrate fermentation strips (as shown in Fig. 1B). Colonies were isolated and DNA was extracted for 16S rRNA gene sequencing analysis and for analysis of the unique sequence corresponding to the insertion of the *gusA* cassette into the *pox1* gene. All strains were identified as *L. jensenii* by 16S rRNA gene homology of 98% or greater, and each isolate contained the unique *gusA* sequence cassette. In addition, the strains were typed using the DiversiLab *Lactobacillus* DNA fingerprinting kit based on rep-PCR. The results of the fingerprinting are shown in Fig. 3. All *GusA*-positive strains isolated from the rectum, feces or vaginal tract of the macaques were shown to be the *L. jensenii* 1153-1646 strain. Two endogenous *L. johnsonii* strains are shown for comparison.

Several papers have suggested that probiotic strains of *Lactobacillus* can be delivered orally and have a benefit on the outcome of women with bacterial vaginosis or urogenital infections. However, it was unclear in these studies whether the orally delivered strains exerted their effects by vaginally colonizing the subjects. It is difficult to distinguish native lactobacilli strains without molecular markers and these papers did not demonstrate that the strains isolated vaginally were indeed the same strains administered orally (Anukam et al., 2006a; Anukam et al., 2006b; Reid et al., 2004; Reid et al., 2003).

In a previous paper, we isolated endogenous *Lactobacillus johnsonii* in the vagina and rectum of rhesus macaques (Yu et al., 2009). Although *L. johnsonii* was found in both anatomic sites, the strains isolated from the rectum were different from the vaginal strains by either rep-PCR DNA fingerprinting or by biochemical analysis (Yu et al., 2009). Therefore, we decided to undertake this oral to vaginal colonization study using the *gusA*-tagged human *L. jensenii* 1153-1646 strain to definitively determine vaginal colonization of our strain. Although *L. jensenii* is a human vaginal isolate not naturally found in the macaque, it is capable of colonizing macaques when administered vaginally (Yu et al., 2009).

The mechanism of vaginal colonization by rectal *Lactobacillus* spp. is not well understood and the concordance of species at both sites has only been sporadically documented (El Aila et al., 2009; El Aila et al., 2011; Marrazzo et al., 2009). In a study of college-age women, lactobacilli were the predominant organism at multiple sites including the perineum, periurethral and introitus (Elkins and Cox, 1974); thus, there is some evidence that lactobacilli may traverse between sites. In our study, we found that oral delivery of a vaginal strain of *Lactobacillus* resulted in vaginal colonization of the strain. In one animal, the strain persisted for several weeks, whereas in the other animals, the strain was isolated on the last day of the experiment, so persistence could not be evaluated. These experiments provide evidence that vaginal colonization may be achieved in humans by oral delivery methods, which are easier to administer and more acceptable in some cultural settings than vaginal delivery methods.

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Fig. 1.

(A) Recovery of blue (GusA-expressing) *L. jensenii* 1153-1646 colonies from a vaginal culture on an MRS agar plate containing X-Gluc. (B) Biochemical analysis of *L. jensenii* 1153-1646 using API Microorganism Identification Strips (Biomerieux, Inc, St. Louis, MO). Fermentation is designated by a color change of blue to yellow detected by a pH indicator present in the medium. *L. jensenii* 1153-1646 ferments d-glucose, d-fructose, d-mannose, wells 11–13, *N*-acetylglucosamine, amygdalin, arbutin, wells 22–24, salicin, d-cellobiose, d-maltose, wells 26–28, d-saccharose, amidon (starch) and gentiobiose wells 31, 36, 39 and hydrolyzes esculin ferric citrate (well 25). Hydrolysis of esculin produces insoluble iron salts and turns the medium black.

Fig. 2.

Study design and results of vaginal, rectal and fecal *Lactobacillus* isolation. Black arrows indicate days of dosing. Empty triangles indicate negative culture. Filled triangles indicate positive cultures. The quadrant of the plate that was positive is indicated by 1+ (one quadrant), 2+ (two quadrants) or 3+ (3 quadrants). Vaginal azithromycin treatment is noted. Study 1: Both macaques were negative for *L. jensenii* 1153-1646 at the beginning of the study. M4698 was positive vaginally for *L. jensenii* 1153-1646 and M4701 was positive both vaginally and rectally at day 28. Following antibiotic treatment, both macaques were negative for *L. jensenii* 1153-1646, but after repeat of oral dosing, M4701 was positive at day 7, day 14 and day 21 and was still positive at day 56. M4698 was positive rectally at day 56.

Study 2: Both macaques were negative for *L. jensenii* 1153-1646 at the beginning of the study. M4700 became positive vaginally at day 28 and was positive vaginally and rectally at day 35. M4703 was positive rectally and in fecal culture at day 35.

Fig. 3.

Lactobacillus DNA fingerprinting kit results based on rep-PCR. The profile of the individual isolates from each macaque (M4698, M4701, M4703 and M4700) was compared to the parental *L. jensenii* 1153-1646. The day of positive sample is noted. Two endogenous *L. johnsonii* strains were typed for comparison.