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***Lactobacillus* and *Pediococcus* species richness and relative abundance in the vagina of rhesus monkeys (*Macaca mulatta*)**

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Abstract

Background—The rhesus monkey is an important animal model to study human vaginal health to which lactic acid bacteria play a significant role. However, the vaginal lactic acid bacterial species richness and relative abundance in rhesus monkeys is largely unknown.

Methods—Vaginal swab samples were aseptically obtained from 200 reproductive aged female rhesus monkeys. Following Rogosa agar plating, single bacterial colonies representing different morphotypes were isolated and analyzed for whole-cell protein profile, species-specific PCR, and 16S rRNA gene sequence.

Results—A total of 510 *Lactobacillus* strains of 17 species and one *Pediococcus acidilactici* were identified. The most abundant species was *L. reuteri*, which colonized the vaginas of 86% monkeys. *L. johnsonii* was the second most abundant species, which colonized 36% of monkeys. The majority of monkeys were colonized by multiple *Lactobacillus* species.

Conclusions—The vaginas of rhesus monkeys are frequently colonized by multiple *Lactobacillus* species, dominated by *L. reuteri*.

Keywords

Vaginal microbiota; commensal bacteria; 16S rRNA; phylogenetic tree

Introduction

Lactobacilli are an important part of the human vaginal microbiota and play a significant role in maintaining vaginal health of women [1, 9]. Many species of *Lactobacillus* have been found in the vaginas of humans [1, 11, 13, 17, 25] and some of them have shown to possess various probiotic characteristics [5, 13, 30]. These probiotic bacteria inhibit the growth of many pathogens including bacteria, fungi and viruses by competitive exclusion and/or through the production of antimicrobial compounds, such as reuterin, organic acids and/or hydrogen peroxide [1, 3, 5, 8, 9, 13, 20, 30, 32, 36].

The rhesus monkey is a common nonhuman primate animal model for medical research due to its relatively close evolutionary relationship to humans. It provides a valuable animal model for investigating how vaginal microbiota may contribute to the health and disease of

the female reproductive tract [35]. If its vaginal lactobacilli show similar species composition and/or probiotic characteristics as those of humans, the rhesus monkey can be used as an appropriate animal model for investigating vaginal diseases such as bacterial vaginosis due to lack of lactobacilli and the instillation of *Lactobacillus*-based probiotics or therapeutics for disease prevention or treatment [16, 30, 40].

To date, information about vaginal lactobacilli of rhesus monkeys is limited. Only a few *Lactobacillus* species, mainly *L. johnsonii*, have been identified [35, 40]. Information on vaginal *Lactobacillus* species richness and relative abundance in this animal model is important for understanding its vaginal microbiota and innate immunity, and their role in promoting female reproductive health. Obtaining this information is a necessary first step in a broader microbial investigation that will elucidate the natural protective mechanisms of the vaginal lactobacilli and better design future related studies in this animal model.

Materials and methods

Subjects and vaginal sample collection

Study protocols were approved by the Institutional Animal Care and Utilization Committee and guidelines for humane care followed. Vaginal microbiological swab samples were aseptically collected from 200 healthy reproductive-aged, non-pregnant female rhesus macaques (*Macaca mulatta*). These were mixed Chinese and Indian macaques, but predominantly India in origin. Samples were taken from all phases of the menstrual cycle. Most female macaques in our colony cycle monthly, but cycles for individual animals were not tracked. All animals were housed in large social groups as outdoor colonies at the Oregon National Primate Research Center and were provided with humane care. Vaginal swab samples were stored in the RTF-glycerol buffer and kept at -70°C as previously described [13, 17, 25].

Bacterial sample preparation

The *Lactobacillus* selective Rogosa SL agar (Difco) was used for initial isolation of lactic acid bacterial single colonies. The *Lactobacillus* MRS broth (Difco) was used for subsequent culture of the new isolates. Single bacterial colonies were initially separated based on their morphological differences on Rogosa SL agar. The cell shape was observed under light microscope after Gram staining. Catalase activity, carbohydrate fermentations, thermo-resistance (growth at 45°C), acidogenecity and aciduricity (final pH), hydrogen peroxide (H_2O_2) and gas (CO_2) production were analyzed. All isolates were presumptively identified as *Lactobacillus* strains based on their ability to grow on the selective Rogosa SL agar, Gram-positive staining, catalase-negative phenotype and rod cell shape [14]. The *Pedococcus* strains were initially identified by the ability to grow at 50°C and sphere cell shape. Subsequently, the identity was confirmed by polymerase-chain reaction (PCR) with genus-specific primers and the 16S rRNA gene (rDNA) sequence analysis [13, 25].

Whole-cell protein profile analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell proteins of lactobacilli was performed to help group bacterial isolates of the same species. Each bacterial isolate was grown in 5 ml MRS broth for 16 h. The cells were harvested by centrifugation and washed twice with phosphate-buffered saline (PBS, pH 7.2). The gel system of Laemmli [18] was used with pre-made 10% SDS-PAGE gel from Bio-Rad. Proteins were visualized by staining with Commassie blue. Marker proteins were obtained from New England BioLabs.

Species-specific PCR assays—A group of *Lactobacillus* species-specific PCR primers were used to identify the species of these *Lactobacillus* isolates (Table 1). Two pairs of primers for detecting *L. plantarum* and *L. rhamnosus* did not yield positive results and thus were listed. These PCR primers were all derived from Song et al. [34] except for LJ1 [39] and 16S-R1 [25] for detecting *L. johnsonii*. Chromosomal DNA from these bacteria were isolated with a glass bead beating protocol [29]. The conditions of the PCR reactions were previously described [34, 39]. The PCR products were visualized after electrophoresis on 2% agarose gel.

16S rRNA gene sequence analysis

After the bacterial isolates were grouped based on their whole-cell protein profiles, one or more strains from each group were selected for 16S rRNA gene amplification and DNA sequence determination. Amplification of the 16S rRNA gene was performed by the PCR as described previously with universal primers based on the conserved regions of the *Escherichia coli* 16S rRNA gene [25]. The PCR products were purified with a QIAquick PCR DNA purification kit (Qiagen, Valencia, CA, USA). The sequence was determined by automated DNA sequencing at the University of Illinois at Chicago Research Resources Center.

The sequences of the 16S rRNA genes were initially analyzed by the BLAST program at the NCBI website [<http://blast.ncbi.nlm.nih.gov/Blast.cgi>] to search for the best matches among existing data in the GenBank. Subsequently, the DNA sequences were aligned along with existing sequences of closely related bacteria in the GenBank using the ClustalX program (Version 1.83). Two rounds of sequence analyses were performed. In the first round, only a fragment of 700 bp 16S rRNA gene was analyzed to identify strains with identical gene sequences. In the second round, one strain from each DNA homology group was selected for the complete 16S rDNA synthesis and analysis. A phylogenetic tree was constructed based on the full length of 16S rRNA gene with the TreeExplorer program (available from the web server http://evolgen.biol.metro-u.ac.jp/TE/TE_-man.html) by the Neighbor-Joining method in PHYLIP format [7].

Results

Bacterial isolation and grouping by whole-cell protein analysis

The bacteria were initially isolated on the Rogosa agar plates. As shown in Table 2, only 12 of the 200 vaginal swab samples did not show any *Lactobacillus* colonies on the Rogosa agar plates. All the rest of the samples showed one or more different colonial morphotypes representing varied lactic acid bacterial species and/or strains. Since our focus was on *Lactobacillus* and its related species only Gram-positive and catalase negative strains were isolated and further analyzed for species determination by species-specific PCR, whole-cell protein profiles and 16S rRNA gene analyses. Up to six bacterial colonies with different colonial morphologies on the Rogosa agar plates were isolated from each vaginal swab sample. For the whole-cell protein analysis, the samples were prepared with the glass bead method [29] and the released bacterial protein was electrophoresed on the SDS-PAGE [12, 17, 18]. By comparing the whole-cell protein profiles on the SDS-PAGE (Fig 1) and by species-specific PCR assays, we were able to group most of the isolated bacterial strains. From each group, one or more representative colonies were analyzed for the 16S rRNA gene (rDNA) sequence. For those isolates whose protein profiles and/or whose species-specific PCR results did not fit into any groups, their species were identified solely by the 16S rDNA sequence analysis.

Species identification

A total of 510 *Lactobacillus* strains were isolated from 200 vaginal swab samples of rhesus monkeys. Their chromosomal DNA was isolated and analyzed with 9 pairs of species-specific PCR primers. As shown in Table 3, a total of 304 isolates were detected positive with seven pairs of species-specific primers. These tentatively species-identified strains were further analyzed by protein profiles and 16rDNA analyses. Five of the initial PCR assay identified species, including *L. reuteri*, *L. johnsonii*, *L. fermentum*, *L. crispatus*, and *L. gasseri*, were confirmed. However, the species-specific PCR primers for *L. reuteri* and *L. fermentum* did not detect all strains in these two species. The primers for detecting *L. acidophilus* and *L. johnsonii* were found to yield some false positive results, which later were corrected by the 16S rDNA analysis (Table 3). For the 207 isolates that were not identified by the species-specific PCR assay, the 16S rDNA sequences of representative strains from each protein profile group were determined and analyzed with the BLAST program. The overall analyses of these results showed that the 510 *Lactobacillus* strains belonged to 17 species (Table 3). Only one strain belonged to *Pediococcus acidilactici*. A phylogenetic tree based on their 16S rDNA sequences was drawn by the Neighbor-Joining method (Fig. 2). It showed a wide range of *Lactobacillus* species in two major clusters. The *E. coli* 16S rRNA gene sequence was used as the root. Among the 17 *Lactobacillus* species, *L. reuteri* was the most abundant species, which colonized 86% of monkeys in the study population. A total of 306 different *L. reuteri* strains were isolated from 172 monkeys. This means that frequently more than one different *L. reuteri* strain (based on difference in colony morphologies) could be present in one monkey. *L. johnsonii* was the second most abundant species, which colonized 36% of monkeys. The remaining 15 *Lactobacillus* species colonized either individually or together with other *Lactobacillus* species in the vaginas of these monkeys.

Discussion

Our species identification included three methods: species-specific PCR assay, protein profiles, and 16S rDNA sequence analysis. The species-specific PCR assay was highly effective for identifying *L. johnsonii* but it missed about one half of the total *L. reuteri* strains, which were subsequently identified by protein profile and 16S rDNA sequence analysis. Nonetheless, the PCR assay is a highly efficient method for initial screening of a large number of *Lactobacillus* isolates. The protein profile analysis is more labor intensive but it is a good and reliable method for grouping these bacterial strains. Finally, the species of representative bacterial strains from each group or those that did not fit into any groups were determined or confirmed by the 16S rDNA sequence analysis. With these three methods, we have determined the species of 511 vaginal lactic acid bacterial strains isolated from the vaginas of 200 rhesus monkeys. These strains belong to 17 *Lactobacillus* species and one *Pediococcus* species. Although these species are genetically distinct, they are closely related in the phylogenetic tree (Fig. 2). To our knowledge, this is the first phylogenetic analysis of more than a dozen vaginal *Lactobacillus* species in rhesus monkeys with data on species richness and relative abundance.

The vaginal *Lactobacillus* species in the phylogenetic tree (Fig. 2) has two major clusters. The first cluster of eight species matched the *L. delbrueckii* group of Collins et al [4]. Because *L. johnsonii* is the most abundant species in this group we name it the *L. johnsonii/delbrueckii* group. The second cluster of nine *Lactobacillus* species and one *Pediococcus* species matched the *L. casei/Pediococcus* group of Collins et al [4]. Based on the phylogenetic distance, this group of nine *Lactobacillus* species is more closely related to *Pediococcus* than the *L. johnsonii/delbrueckii* group and *L. reuteri* is the most abundant species in this group. Therefore, we name it the *L. reuteri/Pediococcus* group.

Two previous studies [35, 40] reported that *L. johnsonii* was the most prevalent *Lactobacillus* species found in rhesus monkeys, but this information was based on limited number of monkeys. With a substantially enlarged animal population, we were able to identify that *L. reuteri* was actually the most abundant *Lactobacillus* species colonizing the vaginas of rhesus monkeys. *L. johnsonii* was a distant second abundant *Lactobacillus* species colonizing the vaginas of rhesus monkeys. The finding of these two dominant *Lactobacillus* species in rhesus monkeys is significant because these two species are not only phylogenetically distant (Fig. 2), but also phenotypically different [4, 14]. *L. reuteri* is a heterofermentative *Lactobacillus* species that metabolizes carbohydrates to lactic acid, acetic acid (ethanol) and carbon dioxide [14]. *L. johnsonii* is a homofermentative *Lactobacillus* species that metabolizes carbohydrates only to lactic acid [27]. Most importantly, *L. reuteri* produces a broad-spectrum antimicrobial substance, called “reuterin” by metabolizing glycerol [3, 36, 37].

Reuterin is a multi-compound dynamic equilibrium consisting of 3-hydroxypropionaldehyde, its hydrate, and its dimer [36]. Reuterin inhibits the growth of some harmful Gram-negative and Gram-positive bacteria, along with yeasts, fungi, and protozoa [37]. *L. reuteri* can secrete sufficient amounts of reuterin to cause the desired antimicrobial effects [3]. Furthermore, since about 4–5 times the amount of reuterin is needed to kill “good” bacteria (i.e. *L. reuteri* and other *Lactobacillus* species), this would allow *L. reuteri* to remove vaginal invaders while keeping normal vaginal flora intact.

Vaginal *Lactobacillus* species distribution in rhesus monkeys showed both similarities and differences from that of humans. *L. reuteri*, the most common vaginal *Lactobacillus* species in rhesus monkeys, is also the most common *Lactobacillus* species in women of Uganda [13], but is not common in women of other areas [1, 11, 13, 17, 25]. *L. johnsonii* is common in the vaginas of rhesus monkeys, but it is not very common in the vaginas of humans [1, 11, 13, 17, 25]. *Lactobacillus* species found in both humans and macaques include *L. salivarius*, *L. mucosae*, *L. fermentum*, *L. crispatus*, *L. gasseri* and *L. ruminis* [1, 11, 13, 17, 25]. However, the vaginal *Lactobacillus* species found only in rhesus monkeys included *L. amylovorus*, *L. animalis*, *L. kitasatonis*, *L. murinus*, *L. saerimneri*, and *L. sobrius*. Some common vaginal *Lactobacillus* species in humans, such as *L. vaginalis* and *L. jensenii*, were not found in the vaginas of rhesus monkeys. Moreover, the *Pediococcus* species found in the vaginas of humans [13] was also found in rhesus monkeys.

Glycogen stored in the vaginal epithelium is the major source of nutrients for vaginal lactobacilli. The metabolism of glycogen by lactobacilli produces lactic acid, which in turn creates an acidic environment in the vagina to inhibit the growth of other potentially harmful bacteria, such as those causing bacterial vaginosis [24]. It has been shown [22] that both women and macaques have vaginal glycogen but the glycogen level in women is much higher. Although *Lactobacillus* plays an important role in maintaining vaginal health of women [1, 13], the exact health-promoting mechanism is not clear. So far, several mechanisms have been proposed [1, 9, 13, 27, 30, 36]. These include competitive exclusion by their ability to adhere to the vaginal epithelium and productions of antimicrobial substances, lactic acid and/or hydrogen peroxide. Since hydrogen peroxide-producing lactobacilli were found to be associated with vaginal health [9], the production of hydrogen peroxide has been considered as an important vaginal health promoting factor. It was recently discovered [24] that the production of lactic acid, not hydrogen peroxide, is more critical for suppression of bacteria associated with bacterial vaginosis. Therefore, all lactic acid bacteria, including *Lactobacillus* and *Pediococcus*, regardless their ability to produce hydrogen peroxide, can play an important role in maintaining vaginal health.

L. reuteri and *L. johnsonii* were found in the vaginas of the rhesus monkey at 86% and 36% frequencies as opposed to 28% and 26% frequencies, respectively, in the milk of the same animal species [12]. Presumably *L. reuteri* and *L. johnsonii* in both the vagina and milk of rhesus monkeys are derived from the animal's gut, in which the two *Lactobacillus* species are common commensal bacteria [10]. As a probiotic, *L. reuteri* has been used to treat gut bacterial infections and rotavirus diarrhea [5, 16, 32] and *L. johnsonii* has been used to control *Helicobacter pylori* infection [8]. Additionally, *L. reuteri* has been used as a probiotic to promote urogenital health of women [30]. Due to similarities in vaginal microbiota and host physiology [22] between humans and rhesus monkeys, this animal model can be used to study vaginal infections and disease prevention with probiotic bacterial instillation. For example, a live recombinant *Lactobacillus* has been successfully tested in rhesus macaques to prevent vaginal SHIV transmission [19].

In summary, we have analyzed vaginal *Lactobacillus* and *Pediococcus* species from 200 rhesus monkeys in the Oregon National Primate Research Center. We have isolated 511 strains belonging to 17 *Lactobacillus* species and one *Pediococcus* species. We found that *L. reuteri* was the most abundant species and *L. johnsonii* was the second most abundant species. However, more studies are needed to evaluate whether the high presence of *L. reuteri* and *L. johnsonii* in the vaginas of rhesus monkeys play a major role in maintaining the animal's female reproductive health.

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Fig. 1.

SDS-PAGE analysis of *Lactobacillus* protein profiles. Line M indicates the protein molecular weight markers. Each letter in the gel indicates a bacterial species: A, *L. acidophilus*; J, *L. johnsonii*; M, *L. murinus*; R, *L. reuteri*; S, *L. saerimneri*; U, *L. ruminis*; Y, *L. amylovorus*. *, species determined by the 16S rDNA sequence analysis.

Fig. 2. Phylogenetic tree of 18 lactic acid bacterial species isolated from the vagina of rhesus monkeys. The *Escherichia coli* 16S DNA was used as the root as control. Alignment was carried out based on the first 1414 bases of the 16S rRNA genes. The tree was drawn with the Neighbor-Joining method. The bar indicates 2% sequence divergence. A. The *L. johnsonii/delbrueckii* group. B. The *L. reuteri/Pediococcus* group.

Table 1PCR primers used for *Lactobacillus* species identification

Species	Primer	(5' – 3')	Product (bp)
<i>L. acidophilus</i>	Laci-1	TGCAAAGTGGTAGCGTAAGC	210
	23-10C	CCTTTCCTCACGGTACTG	
<i>L. crispatus</i>	Lcri-3	AGGATATGGAGAGCAGGAAT	522
	Lcri-2	CAACTATCTCTTACACTGCC	
<i>L. gasseri</i>	Lgas-3	AGCGACCGAGAAGAGAGAGA	360
	Lgas-2	TGCTATCGCTTCAAGTGCTT	
<i>L. fermentum</i>	Lfer-3	ACTAACTTGACTGATCTACGA	192
	Lfer-4	TTCCTGCTCAAGTAATCATC	
<i>L. johnsonii</i>	LJ1	GATGATTTTAGTTCTTGCACTAA	600
	16S-R1	TCTACGCATTCCACCGCTAC	
<i>L. reuteri</i>	Lreu-1	CAGACAATCTTTGATTGTTTAG	303
	Lreu-4	GCTTGTTGGTTTGGGCTCTTC	
<i>L. salivarius</i>	Lsal-1	AATCGCTAAACTCATAACCT	410
	Lsal-2	CACTCTCTTTGGCTAATCTT	

Table 2

Bacterial species/strain distribution in the vagina of rhesus monkeys

Bacterial isolates per monkey	No. of monkeys (N=200)	No. of isolates (N=511)
0	12	0
1	23	23
2	48	96
3	86	258
4	22	88
5	8	40
6	1	6

Table 3

Lactobacillus species/strain distribution in the vagina of rhesus monkeys

Species (N=17)	No. of hosts (N=200)	No. of strains (N=510)	PCR+ (N=304)	16S rDNA (N=83)	Found also in other location
<i>L. reuteri</i>	172	306	159	25	Human vagina, milk, monkey milk [1, 12–14, 33]
<i>L. johnsonii</i>	72	85	85	7	Human gut, vagina, monkey milk [12–14, 27]
<i>L. ruminis</i>	23	25		9	Human gut, vagina [1, 10, 38]
<i>L. acidophilus</i>	21	22	33 ¹	4	Human gut, mouth, vagina [11, 14]
<i>L. salivarius</i>	17	17	22 ²	4	Human stomach, vagina, monkey milk [12–14, 31]
<i>L. kitasatonis</i>	16	16		6	Chicken gut [23]
<i>L. amylovorus</i>	9	9		7	Pig gut [15]
<i>L. mucosae</i>	7	7		3	Human vagina [25]
<i>L. saerimneri</i>	5	6		5	Human gut [38]
<i>L. bulgaricus</i>	3	4		3	Human vagina, yogurt [11, 14, 21]
<i>L. fermentum</i>	3	3	1	2	Human gut, milk, vagina [1, 13, 20, 25]
<i>L. murinus</i>	2	2		2	Mouse and dog gut [26]
<i>L. crispatus</i>	2	2	2	1	Human vagina [1, 13, 17, 20, 25]
<i>L. gasseri</i>	2	2	2	1	Human gut, vagina [1, 11, 12–14, 25]
<i>L. ingluviei</i>	2	2		2	Pigeon/chicken gut [6]
<i>L. animalis</i>	1	1		1	Dog gut, monkey milk [2, 12]
<i>L. sobrius</i>	1	1		1	Pig gut [15]

¹Includes 11 false positive strains which were further identified to be *L. amylovorus* or *L. johnsonii* by protein profiles and 16S rDNA sequences.

²Includes 4 false positive strains which were further identified to be *L. johnsonii*.