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Species diversity and relative abundance of lactic acid bacteria in the milk of rhesus monkeys (*Macaca mulatta*)

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Abstract

Background—Mother's milk is a source of bacteria that influences the development of the infant commensal gut microbiota. To date, the species diversity and relative abundance of lactic acid bacteria in the milk of non-human primates have not been described.

Methods—Milk samples were aseptically obtained from 54 female rhesus monkeys (*Macaca mulatta*) at peak lactation. Following GM17 and MRS agar plating, single bacterial colonies were isolated based on difference in morphotypes, then grouped based on whole-cell protein profiles on SDS–PAGE. Bacterial DNA was isolated and the sequence the 16S rRNA gene was analyzed.

Results—A total of 106 strains of 19 distinct bacterial species, belonging to five genera, *Bacillus*, *Enterococcus*, *Lactobacillus*, *Pediococcus*, and *Streptococcus*, were identified.

Conclusions—Maternal gut and oral commensal bacteria may be translocated to the mammary gland during lactation and present in milk. This pathway can be an important source of commensal bacteria to the infant gut and oral cavity.

Keywords

16S rRNA; breastfeeding; commensal bacteria; *Lactobacillus reuteri*; milk microbiota; *Streptococcus gallolyticus*

Introduction

Mother's milk provides not only all the required nutrients for infants but also defensive factors, including immunoglobulin (Ig) A, immunocompetent cells, fatty acids, oligosaccharides, lysozyme, or lactoferrin [26], to protect infants against infectious diseases [9, 25]. Recently, various commensal bacteria have been found in the breast milk of healthy human mothers, including *Lactobacillus gasseri*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, or *Enterococcus faecium* [5, 10, 20–23]. Food bacteria, such as nisin-producing lactococcus strains, have also been isolated from human milk [1, 22]. These bacteria are presumably transported from the maternal intestine to the mammary by mononuclear cells in peripheral circulation via mesenteric lymph nodes [30]. Therefore,

milk is considered a potential source of probiotic bacteria that may provide additional benefits to the infant's digestive and immunological health [10, 20]. In fact, some of these bacteria, mostly lactic acid bacteria, inhibit the growth of many pathogenic bacteria by competitive exclusion and/or through the production of antimicrobial compounds, such as bacteriocins, organic acids, or hydrogen peroxide [1, 15, 20, 21, 28]. In this way, commensal bacteria in milk, particularly lactic acid bacteria, may be a natural maternal protective mechanism to promote the health of infants by improving the development of their gut [7] and reducing infection [9, 25].

The rhesus monkey is a common non-human primate animal model for medical research owing to their relatively close evolutionary relationship to humans. Although to date commensal bacteria in rhesus monkey milk have not been studied, this species provides a valuable animal model for investigating how mother's milk constituents may contribute to infant developmental trajectories [14]. The species diversity and relative abundance of lactic acid bacteria present in the milk of rhesus monkeys may be important to the milk innate immunity. Describing the bacteria present in mother's milk is a necessary first step in a broader immunological investigation that will elucidate the natural maternal protective mechanisms of the milk provided to infants and inform study design for related future research in this animal model.

Materials and methods

Subjects and milk sample collection

Milk samples were aseptically collected from 54 healthy lactating rhesus macaques (*Macaca mulatta*) at peak lactation between three and four months after parturition. All animals were housed in large social groups in ½ acre outdoor enclosures at the California National Primate Research Center. All subjects received a standard commercial diet (Purina LabDiet Monkey Diet 5038) twice daily, supplemented semi-weekly with fresh fruit and vegetables, and water was available *ad libitum*. After 3.5–4 hours of milk accumulation, mothers were sedated with ketamine hydrochloride (5–10 mg/kg), nipple areas were cleaned with 97% isopropyl rubbing alcohol, mothers were administered exogenous oxytocin (0.1 mg/kg) intramuscularly for milk letdown, and samples were collected from each mammary separately by hand into sterile tubes on wet ice and then subsampled and stored at –80°C until further processing. The samples used in this study represent one mammary from each mother. These methods were approved by the Institutional Animal Care and Use Committee at the University of California Davis and have been described in further detail elsewhere [12–14].

Bacterial sample preparation

The agar plates made with GM17 medium (Difco) (M17 supplemented with 0.5% glucose) suitable for growing lactococci and the MRS broth (Difco) suitable for growing lactobacilli were used for initial isolation of lactic acid bacterial single colonies. Single bacterial colonies were initially separated based on their morphological differences on these agar plates. Cell morphology was observed under light microscope after Gram staining. Catalase activity, carbohydrate fermentations, acidogenicity and aciduricity (final pH), and gas (CO₂) production were analyzed. All isolates were presumptively identified as lactic acid bacterial strains based on their ability to grow on the MRS agar, Gram-positive staining, and catalase-negative phenotype [16]. Subsequently, the identities of these strains were confirmed by polymerase chain reaction (PCR) with genus-specific primers and the 16S rRNA gene (rDNA) sequence analysis [29]. Sugar fermentation assays were performed in purple broth (Difco) supplemented with 0.5% testing sugar.

Whole-cell protein profile analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of whole-cell proteins of lactic acid bacteria was performed to help group bacterial isolates of the same species. Each bacterial isolate was grown in 5 ml MRS broth for 16 hours. The cells were harvested by centrifugation and washed twice with phosphate-buffered saline (PBS, pH 7.2). To optimize resolution of the protein bands, three methods were tested for sample preparation: direct boiling of the bacteria with the 6× sample buffer [10% SDS, 4.2% Tris base, 30% glycerol; 9.3% dithiothreitol, 0.0012% bromophenol blue (w/v)], pre-treating the bacteria with lysozyme to weaken the cell wall, or pre-treating the bacteria with glass bead beating to break the bacterial cells. For the glass bead beating method, the sample was resuspended in 300 μ l PBS plus 10 mg 0.1- mm glass beads. The cells were then cooled on ice and shaken for 3 $\times$ 5 min with a Turbomix™ bead-beater (Scientific Industries, Inc., Bohemia, NY, USA) with maximal speed. The supernatant was harvested after centrifugation at 10,000 $\times$ g at 4°C for 30 min and boiled in the sample buffer for 5 min and then centrifuged at 10,000 $\times$ g for 1 min to remove any precipitates. The gel system of Laemmli [18] was used. The pre-made 10% SDS–PAGE gel from Bio-Rad was used. Proteins were visualized by staining with Coomassie blue. Marker proteins were obtained from New England BioLabs.

16S rRNA gene sequence analysis

After the bacterial isolates were grouped based on comparison of their whole-cell protein profiles by visual inspection, several strains from each group were selected for 16S rRNA gene amplification and DNA sequence determination. The isolation of chromosomal DNA from these lactic acid bacteria was carried out with a glass bead beating protocol [32]. 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 [29]. 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 rDNAs 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 Gen- Bank 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 rDNA was analyzed to identify strains with identical gene sequences. In the second round, one strain from each DNA homology group was selected for 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 [8].

Carbohydrate fermentation assay

For bacterial species that could not be readily separated with the 16S rRNA gene analysis, their carbohydrate fermentation patterns were analyzed. Purple broth supplemented with various carbohydrates at 0.5% was used for growing these bacteria. The tested carbohydrates included arabinose, inulin, lactose, mannitol, melibiose, raffinose, sucrose, and starch.

Results

Bacterial isolation and whole-cell protein analysis

The bacteria were initially isolated on the GM17 and MRS agar. Nearly all 54 milk samples showed some colonies that are Gram-positive and catalase-positive cocci resembling *Staphylococcus aureus* on the GM17 agar. As our focus was on lactic acid bacteria, only Gram-positive and catalase-negative strains were isolated and further analyzed for the whole-cell protein profiles and 16S rDNA analyses. Up to seven bacterial colonies with different colonial morphologies were isolated from each milk sample. None of the milk samples was sterile; all showed one or more types of bacterial colonies on these agar plates. For the whole-cell protein analysis, three sample preparation methods were tested, but only the pre-treatment with glass beads showed significantly increased protein bands on the SDS-PAGE. By comparing the whole-cell protein profiles on the SDS-PAGE, we were able to group nearly all of the isolated bacterial strains (Table 1 and Fig. 1).

Species identification

By visual inspection of colonial morphological differences and comparison of the bacterial whole-cell protein profiles on SDS-PAGE, a total of 106 unique bacterial strains were identified and isolated from 54 milk samples of rhesus monkeys. Among strains that showed identical or similar protein profiles, two to five representative strains were selected for one round of 16S rDNA sequence determination of about 700 bp. The BLAST analysis of these DNA sequences showed that these strains belonged to 19 bacterial species of five genera: *Bacillus*, *Enterococcus*, *Lactobacillus*, *Pediococcus*, and *Streptococcus* (Table 1). From each of these 19 species, one strain was selected to obtain the full length of the 16S rDNA sequence of about 1.5 kb. These sequences were first aligned with the ClustalX program. Protruding sequences at both ends were trimmed. A phylogenetic tree based on the aligned and trimmed full-length 16S rDNA sequences was drawn as shown in Fig. 2 by the neighbor-joining method. The phylogenetic tree showed a wide range of bacterial species and genetic similarities and distances among these bacteria.

In addition to DNA analysis, carbohydrate fermentations were used to help differentiate certain bacterial species that share similar 16S rRNA gene sequences. For example, *Streptococcus bovis*, *Streptococcus equinus*, and *Streptococcus gallolyticus* cannot be separated by their 16S rRNA gene sequences, but can be separated by their sugar fermentation patterns. Among 41 isolates sharing the same short 700-bp 16S rDNA sequences and/or the whole-cell protein profiles, 36 were identified to be *S. gallolyticus* owing to their ability to ferment mannitol and inability to ferment melibiose [36]; five strains were identified to be *S. bovis* owing to their ability to ferment melibiose and inability to ferment mannitol. None of the 41 strains were *S. equinus*, which could not metabolize any of these tested carbohydrates.

Enterococcus faecium cannot be readily separable from *Enterococcus durans* and *Enterococcus faecalis* based on 16S rRNA gene sequences. Carbohydrate fermentation pattern was used to differentiate them. The five enterococcal strains were identified to be *E. faecium* owing to their positive fermentation of sucrose, mannitol, and arabinose [2].

Discussion

Nineteen species belonging to five Gram-positive bacterial genera, *Bacillus*, *Enterococcus*, *Lactobacillus*, *Pediococcus*, and *Streptococcus*, were isolated from the rhesus monkey milk. It is possible that some of these bacteria were contamination from skin microbiota; however, this is unlikely given the aseptic method of sample collection. Although these bacteria are genetically distinct and distant based on their positions in the phylogenetic tree (Fig. 2), they

belong to the group of bacteria that produce lactic acid as a common end product of carbohydrate fermentation. To our knowledge, this is the first report on lactic acid bacteria species distribution in the milk of rhesus monkeys.

The majority of bacterial species isolated from the monkey milk were gut commensal bacteria (Table 1), suggesting a possible gut origin. Previous studies on milk microbiota have showed that gut-derived bacteria are present in the milk of humans and mice [20, 30]. In mice, the bacterial translocation from the gut to the mammary is presumably mediated by the blood mononuclear cells and is thought to program appropriate immune responses to pathogenic vs. commensal gut bacteria in the developing pup [30]. All subjects were fed commercial monkey chow, suggesting that a more diverse diet may lead to greater variance in the bacterial species present in milk and their relative abundances. Anaerobic bacterial species might also be present in the rhesus milk, but could not be identified in the present study owing to the selective plating method used for lactic acid bacteria that does not promote anaerobic bacterial growth.

Macaque milk showed both similarities and differences from human milk. *Enterococcus faecium* was isolated from the milk of rhesus monkeys, which has similarly been isolated from human milk [33]. Unlike in humans [1], however, macaque milk showed no evidence of *Lactococcus* bacteria. Even though we used the GM17 agar that promotes the growth of *Lactococcus*, we failed to find bacteria belonging to this genus. A possible reason is that women that consume fermented dairy products made with *Lactococcus*, such as cheese, may be colonized by *Lactococcus* in their milk [1, 22]. The rhesus monkeys at the CNPRC did not consume cheese as a part of their diet and might not be exposed to *Lactococcus* and therefore are not colonized by this bacterium.

We found that *Streptococcus* and *Lactobacillus* are major lactic acid bacterial genera in the milk of rhesus monkeys. At the genus level, this finding is similar to previous report on human milk [5, 20–23]. However, *S. gallolyticus*, the most dominant species found in rhesus monkeys in our study population, and two other streptococcal species, *S. alactolyticus* and *S. bovis*, have not been found in human milk. Likewise, the four *Lactobacillus* species, *L. animalis*, *L. brevis*, and *L. johnsonii*, and two *Bacillus* species, found in the milk of this rhesus monkey population, have not been reported in human milk by multiple studies including culture-independent studies [5, 20–23]. This may be a unique microbiological characteristic for rhesus monkeys. *L. reuteri* was found in the milk of the rhesus monkeys at a 28% frequency as opposed to 15% in the breast milk of humans [38]. As a probiotic, *L. reuteri* has been used to treat rotavirus diarrhea in young children [37]. Likewise, the rhesus monkey gut-derived probiotic *L. reuteri* has been used as a supplement to the infant formula to promote the digestive health of infant monkeys [17]. More studies are needed to evaluate whether the natural presence of *L. reuteri* in the mother's milk benefits the infant's digestive health.

Finding a wide range of milk lactic acid bacteria indicates that mother's milk itself may serve as a source of naturally occurring probiotics for the infant. It is of particular note that even though all gut micro-organisms might have an equal opportunity to enter the milk through a putative entero-mammary gland translocation route [30], the underlying mechanisms appear to selectively enrich the number of beneficial lactic acid bacteria and suppress pathogenic gut bacteria. Presumptive host factors in the milk involved in such a microbial selection may include the high concentration of lactose and immune factors inhibiting gut pathogenic bacteria. Also, the presence of some lactic acid bacteria that have probiotic potential in the milk may inhibit the growth of some pathogenic bacteria.

Several bacteria, such as *L. salivarius*, *S. mitis*, *S. oralis*, *S. parasanguinis*, and *S. salivarius*, found in the milk of rhesus monkeys are common commensal species of the oral cavity. A similar group of oral streptococci were also identified by Martin et al. [22] in human milk. It is not clear as to how these oral bacteria become a part of the milk microbiota. We speculate that there might be three possible sources. First, these bacteria might be from the oral cavity of infants. During breastfeeding, oral bacteria of the infants might enter the mother's mammary gland via possible regurgitation. Second, oral bacterial translocation to the mammary gland might occur in the gut because some oral bacteria may transiently pass through the gut after swallowing or even colonize the gut. Third, these oral bacteria might translocate to the mammary gland directly from the maternal oral cavity. They might transiently enter the peripheral circulation from various salivary glands and/or from the lymphatic system of tonsils and then be relocated to mammary gland.

Further studies will be needed for identification of possible oral and gut translocation routes of maternal commensal bacteria to the milk that is provided to the infant. Moreover, many questions remain regarding the magnitude of inter-individual variation among mothers in the presence and abundance of milk microbiota and how milk microbiota profiles may change as infant age. Although this report of the presence and abundance of milk lactic acid bacteria in rhesus monkey mothers represents the necessary first step in understanding this complex system, future directions should directly address consequences of milk microbiota for infant gut colonization, digestive function, and immunological development.

Acknowledgments

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Fig. 1.

Electrophoretic protein profiles of a selection of bacteria isolated from the rhesus monkey milk samples. Bacteria that showed the identical protein profiles were grouped, and their species were determined by analysis of 16S rRNA gene sequences. A, *Lactobacillus animalis*; B, *Streptococcus gallolyticus*; C, *Pediococcus pentosaceus*; D, *Enterococcus faecium*; E, *Lactococcus johnsonii*.

Fig. 2. Phylogenetic tree of five bacteria genera isolated from the milk of rhesus monkeys. 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.

Table 1

Distribution of lactic acid bacteria in the milk of rhesus monkeys

Genus	Species	No. of strains	Found also in other location
<i>Bacillus</i>	<i>cereus</i>	1	Soil and animal gut [39]
	<i>panaciterrae</i>	1	Soil [40]
<i>Enterococcus</i>	<i>faecium</i>	6	Human gut and milk [20]
	<i>hirae</i>	1	Human and animal guts [19]
<i>Lactobacillus</i>	<i>animalis</i>	13	Dog gut [3]
	<i>brevis</i>	2	Human gut [42]
	<i>johnsonii</i>	14	Human gut [31]
	<i>reuteri</i>	15	Human and monkey gut [17, 35]
	<i>salivarius</i>	1	Human gut, saliva, and stomach [11, 35]
<i>Pediococcus</i>	<i>pentosaceus</i>	1	Pork meat [43]
<i>Streptococcus</i>	<i>alactolyticus</i>	1	Dog gut [6]
	<i>anginosus</i>	1	Human saliva [24]
	<i>bovis</i>	5	Human and animal guts [41]
	<i>gallolyticus</i>	36	Human and animal guts [34]
	<i>mitis</i>	2	Human saliva [27]
	<i>oralis</i>	1	Human saliva [27]
	<i>parasanguinis</i>	2	Human saliva [27]
	<i>salivarius</i>	1	Human saliva [27]
	<i>thoraltensis</i>	2	Pig and rabbit guts [4]