Maternal Antenatal Treatments Influence Initial Oral Microbial Acquisition in Preterm Infants

Karen D. Hendricks-Muñoz, MD, MPH1 Guillermo Perez-Perez, DSc2 Jie Xu, PhD1 Yang Kim, MD3 Moi Louie, MD3

1 Division of Neonatal Medicine, Department of Pediatrics, Virginia Commonwealth University School of Medicine, Richmond, Virginia
2 Department of Medicine and Microbiology, New York University School of Medicine, New York, New York
3 Division of Neonatology, Department of Pediatrics, New York University School of Medicine, New York, New York


Abstract

Objective The purpose of this study was to analyze the association of maternal antenatal therapy on initial preterm infant oral microbial acquisition of gut metabolically important bacteria: Firmicutes, Bacteroidetes, Lactobacillus, Bifidobacterium, and Bacteroides species.

Study Design Infant oral samples were collected prefeeding at 24 hours and analyzed using group-specific primers by real-time 16S rRNA quantitative polymerase chain reaction with analysis of variance and logistic regression to evaluate effect of antenatal exposure.

Results Sixty-five infants <34 weeks’ gestational age (GA) were evaluated; mean GA was 28.6 ± 2.6 (standard deviation) weeks. Infants unexposed to antenatal treatment (n = 5) acquired <1% Firmicutes, which was composed of 100% Lactobacillus species with no detectable Bifidobacterium, Bacteroidetes, or Bacteroides species. Infants exposed to antibiotics (n = 7), acquired fivefold less total bacterial density (TBD) with 45% Firmicutes 1.3% Lactobacillus species, 23.5% Bacteroidetes and rare Bacteroides. Compared with unexposed infants, steroids (n = 26) or steroid and antibiotics (n = 27) exposure led to an eightfold increase in TBD with <1% Lactobacillus species and Bacteroides species 100% and 30%, respectively (p < 0.04). Bifidobacterium was undetectable in all groups.

Conclusion Preterm infant exposure to routine maternal antenatal treatments influence early oral microbial acquisition during the primary hours related to establishment of gut commensal bacteria.

Keywords
► antenatal therapy
► preterm infant
► oral microbiota
► Lactobacillus

In the naïve edentulous preterm infant, oral microbiota influence the initial pattern of bacteria exposure available for establishment of gut bacterial colonization.1–3 Despite their importance, factors that influence the pattern of these early oral bacterial colonizers have not been described. In the neonatal intensive care unit (NICU), preterm infants are indirectly exposed to antibiotics and steroids through antenatal maternal treatments. Recently, antibiotic treatment begun at birth and continued treatment have been associated with increased risk of necrotizing enterocolitis (NEC), implicating that initial establishment of microbial repertoires may be important for early mucosal protective properties or injury.4 Furthermore, oral early acquired commensal bacterial colonization patterns, altered by cesarean delivery, have
been linked to later infant health and development of
caries.\textsuperscript{5–7}

In most clinical settings, it is common to treat women who
present with risk for preterm delivery with antenatal steroids
to accelerate fetal lung maturity and antenatal antibiotics
to protect the preterm infant from infection risk.\textsuperscript{8–11} Although
antenatal treatments are beneficial for infant health, they
may indirectly influence the initial preterm oral bacterial
acquisition patterns important for later gut microbe coloni-
zation. For these reasons, we examined the impact of mater-
nal antenatal steroid and antibiotic treatment on preterm
infant initial oral bacterial acquisition patterns to identify
preliminary microbiota repertoire patterns as a basis to study
the impact of early acquired bacterial patterns on subsequent
preterm infant health.

\textbf{Material and Methods}

\textbf{Patient Recruitment}

The study was approved by the Human Research Review
Board of New York University School of Medicine and Belle-
vue Hospital Center. Mothers who delivered a preterm infant
at less than 34 weeks’ gestation signed informed written
consent as required by the Institutional Review Board for
their infant’s participation.

\textbf{Patient Sampling}

Infant samples were obtained at 24 hours of life using a sterile
dry soft swab that was rolled along the infant’s oral mucosal
surface of the mouth, inner cheeks, and tongue until saturated
with saliva, placed in 2.0 mL of phosphate-buffered saline,
centrifuged at 14,000 rpm for 6 minutes, and the pellets
stored at \(-80^\circ C\) prior to processing. Samples were analyzed
by the molecular methods described later to identify bacterial
DNA. All infants included were treated with antibiotics within
30 minutes of admission to the NICU consisting of ampicillin
and gentamicin, and no infant received any feedings prior to
oral sampling. Those mothers treated with antenatal steroids
received a complete 48-hour treatment of betamethasone or
dexamethasone. Antibiotics, when provided, consisted of
ampicillin or erythromycin. One mother received ampicillin
and gentamicin and one mother received ampicillin and
azithromycin. In our center, chorioamnionitis was defined
as maternal fever (>37.8°C) associated with two or more of
the following: maternal tachycardia >100 beats/min, fetal
tachycardia > 160 beats/min, maternal serum leukocytosis
>15,000/mm\(^3\), uterine tenderness, or malodorous vaginal
discharge. Infant demographic data such as birth weight,
gestational age, and race as well as maternal medical diagno-
ses and medical treatments were collected.

\textbf{Bacterial DNA Preparation}

Bacterial DNA was isolated with MasterPure DNA Purification
Kit (EPICENTRE Biotechnologies, Madison, WI), as described
by manufacturer. DNA was stored at \(-20^\circ C\) until analysis.

\textbf{16S rRNA Polymerase Chain Reaction}

The bacterial specific primers used are listed in \textit{\textbf{Table 1}}.
Oligo nucleotide primers and the Power SYBR Green PCR
Master Mix were purchased from Applied Biosystems
(Carlsbad, CA) based on previous \textit{Firmicutes} and \textit{Bacteroi-
detes} phyla and \textit{Bifidobacteria, Lactobacillus, and Bak-
eroides} species detection analysis.\textsuperscript{12–16} Detection of DNA
polymerase chain reaction (PCR) was performed with the
7900HT Fast Real-Time PCR System (Applied Biosystems)
using optical grade 384-well plates with control standards
determined by automatic analysis settings. Duplicate sam-
plexes were used for the determination of DNA by real-time

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Target Organisms} & \textbf{Primer} & \textbf{Sequence (5’ to 3’)} & \textbf{Annealing Temp (°C)} \\
\hline
\textit{Bacteroidetes}\textsuperscript{12} (126 bp) & Forward primer & GGARCATGTGGTTTAATTCGATGAT & 60 \\
& Reverse primer & AGCTGACGACAACCATGCG & \\
\hline
\textit{Firmicutes}\textsuperscript{12} (126 bp) & Forward primer & GGAGYATGTGGTTTAATTCGAAGCA & 60 \\
& Reverse primer & AGCTGACGACAACCATGAC & \\
\hline
\textit{All bacteria}\textsuperscript{13} (200 bp) & Forward primer & ACTCCTACGGGAGGCAGCAG & 60 \\
& Reverse primer & ATTACCGCGGCTGCTGG & \\
\hline
\textit{Lactobacillus}\textsuperscript{14} (90 bp) & Forward primer & TACATYCCAACHCACAGAAC & 60 \\
& Reverse primer & AAGCAACAGTACCACGACCA & \\
\hline
\textit{Lac-Probe} & & (FAM)AAACACATTCTRTATGCCCAGTG(TAMRA) & \\
\hline
\textit{Bifidobacterium}\textsuperscript{15} (553 bp) & Forward primer & CTCTGGAAACGGGTGG & 55 \\
& Reverse primer & GGTGTCTCTCCCGATATCATA & \\
\hline
\textit{Bacteroides}\textsuperscript{16} (106 bp) & Forward primer & GAGAGGAAGGTCCCCCAC & 60 \\
& Reverse primer & CCGTACTTGGCGTGTCAG & \\
\hline
\textit{AllBac-probe} & & (FAM)CCATTGACCAATATCCCTACTGCTGCTG(TAMRA) & \\
\hline
\end{tabular}
\caption{Primer List for Targeted Bacterial Analysis}
\end{table}
PCR, and mean values were calculated. The bacteria density for each target group was calculated using standard curves generated by 16S rRNA sequence containing plasmids. These primers amplify 90% of the rRNA coding sequence and minimize PCR bias. The PCR reaction was performed in a total volume of 10 μL. Bacteroidetes, Firmicutes, and total bacterial densities were detected using 100 nmol each of the forward and reverse primers and 1 ng of DNA for each reaction. PCR conditions for amplification were 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. A melting curve analysis was done after amplification.\textsuperscript{13-16}

**Statistical Methods**

Total bacterial density and specific bacteria phyla as well as the selected species in saliva were compared between exposed and nonexposed samples. Infants were grouped into four groups: no exposure, antibiotics, steroids, and steroids and antibiotics groups. Data were analyzed (SPSS 16 for Windows, SPSS, Inc., Chicago, IL) using descriptive, parametric, and nonparametric statistics according to the level of data obtained and the examination of the assumptions underlying the tests. All values were expressed as mean ± standard error of the mean unless otherwise indicated. Univariate analyses were performed to assess the distribution and variability of the data and to describe the sample. One-way analysis of variance and \( t \) test were used to assess differences in the quantity (density) of each bacterial phyla or species pattern within the defined groups. With adjustments for potential confounding factors, separate multiple logistic regression analyses were performed to assess impact of gender, race, and mode of delivery on microbial density and patterns. Statistical significant difference was defined as \( p < 0.05 \).

**Results**

**Baseline Characteristics**

Sixty-five preterm newborns born at <34 weeks’ gestation participated in this study. Demographic characteristics of the total population are described in Table 2. Five (7.6%) infants were not exposed to any medication, 7 (10.7%) infants were exposed to antibiotics only, 26 infants (40%) were exposed to only antenatal steroids, and 27 (41.5%) were exposed to both steroids and antibiotics. Of those mothers who received antibiotics, all demonstrated maternal fever \( >37.8°C \) associated with maternal tachycardia \( >100 \text{ beats/min} \) and fetal tachycardia \( >160 \text{ beats/min} \). No mother received antibiotics after birth. Fifty-three infants (81.5%) were delivered by cesarean section and 12 (18.5%) by vaginal delivery. No infant was diagnosed with early onset bacteremia diagnosed as a positive blood culture within the first week of life. Overall mean gestational age (±standard deviation) for the group was 28.6 ± 2.6 weeks with mean birth weight 1176 ± 357 g. Thirty-two infants were singletons and 23 were multiples. Thirty-four were males and 31 were females of a variety of racial and ethnic backgrounds (Table 2). Regression analyses did not show any likelihood of a microbial density or pattern significantly altered with infant mode of delivery ethnicity or infant gender.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No Medications ((n = 5))</th>
<th>Steroids ((n = 26))</th>
<th>Steroids and Antibiotics ((n = 27))</th>
<th>Antibiotics ((n = 7))</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPROM</td>
<td>0</td>
<td>9</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Hypertension/preeclampsia</td>
<td>3</td>
<td>8</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Incompetent cervix/NRFHT</td>
<td>1</td>
<td>4</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Chorioamnionitis</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Previa with bleeding</td>
<td>1</td>
<td>5</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Cesarean birth ((n = 53), n (%))</td>
<td>4 (80)</td>
<td>24 (92)</td>
<td>20 (74)</td>
<td>5 (71)</td>
</tr>
<tr>
<td>Multiples ((n = 23), n (%))</td>
<td>2 (40)</td>
<td>7 (27)</td>
<td>11 (41)</td>
<td>3 (43)</td>
</tr>
<tr>
<td>Males ((n = 34), n (%))</td>
<td>4 (80)</td>
<td>16 (61.5)</td>
<td>9 (33)\textsuperscript{*}</td>
<td>5 (71)</td>
</tr>
<tr>
<td>Race/Ethnicity, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Hispanic white ((n = 18))</td>
<td>0 (0)</td>
<td>7 (27)</td>
<td>10 (37)</td>
<td>1 (14)</td>
</tr>
<tr>
<td>Hispanic ((n = 11))</td>
<td>2 (40)</td>
<td>3 (12)</td>
<td>6 (22)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Asian ((n = 15))</td>
<td>1 (20)</td>
<td>9 (35)</td>
<td>4 (15)</td>
<td>1 (14)</td>
</tr>
<tr>
<td>Non-Hispanic black ((n = 21))</td>
<td>2 (40)</td>
<td>7 (27)</td>
<td>7 (26)</td>
<td>5 (71)</td>
</tr>
</tbody>
</table>

PPROM, preterm premature rupture of membranes; NRFHT, nonreactive fetal heart tracing.

One-way ANOVA T-test.

\textsuperscript{*}Statistically significant \((p < 0.05)\) from no medications group.

N represents the number of samples in each group ± SE (Standard Error).
Table 3 Infant Oral Microflora in Relation to Maternal Antenatal Treatment

<table>
<thead>
<tr>
<th>Bacterial DNA/mL</th>
<th>No Medications (n = 5)</th>
<th>Steroids (n = 26)</th>
<th>Steroids and Antibiotics (n = 27)</th>
<th>Antibiotics (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Category: Mean (±SE) ×10^6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Firmicutes</strong></td>
<td>0.145 ± 0.06</td>
<td>249.5 ± 41.1^a</td>
<td>96.6 ± 36.3^a</td>
<td>3.2 ± 1.8^a</td>
</tr>
<tr>
<td>% Firmicutes^b</td>
<td>0.4</td>
<td>88.0</td>
<td>76.4</td>
<td>45.0</td>
</tr>
<tr>
<td>% Lactobacillus^c</td>
<td>100</td>
<td>0.05</td>
<td>0.16</td>
<td>1.3</td>
</tr>
<tr>
<td>Fold Δ % Firmicutes</td>
<td>+220.0</td>
<td>+191.0</td>
<td>+112.5</td>
<td></td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>0.007 ± 0.0</td>
<td>0.034 ± 0.0^e</td>
<td>1.96 ± 0.00^e</td>
<td>1.67 ± 0.06^e</td>
</tr>
<tr>
<td>% Bacteroidetes^d</td>
<td>0.2</td>
<td>0.1</td>
<td>1.6</td>
<td>23.6</td>
</tr>
<tr>
<td>% Bacteroidetes^e</td>
<td>00.00</td>
<td>100%</td>
<td>30.3%</td>
<td>0.01%</td>
</tr>
<tr>
<td>Fold Δ % Bacteroidetes</td>
<td>-2.0</td>
<td>+8.0</td>
<td>+118.0</td>
<td></td>
</tr>
<tr>
<td>Total bacteria density</td>
<td>35.7 ± 10.4</td>
<td>283.4 ± 71.5^a</td>
<td>126.3 ± 55.3^a</td>
<td>7.09 ± 2.38^a</td>
</tr>
<tr>
<td>Fold Δ total density</td>
<td>+7.9</td>
<td>+3.5</td>
<td>-5.0</td>
<td></td>
</tr>
<tr>
<td>% other bacteria^a</td>
<td>99.4</td>
<td>11.9</td>
<td>22.0</td>
<td>31.4</td>
</tr>
</tbody>
</table>

One-way analysis of variance t test. n, number of samples in each group ± SE; Δ, fold change; SE, standard error.

^aStatistically significant (p < 0.05) compared with no medications group.

^bPercent of total bacteria density.

^cPercent of Lactobacillus of Firmicutes density.

^dPercent of Bacteroides of Bacteroidetes density.

^ePercent of total density that were other bacterial phylum.

Oral Flora Characteristics without Antenatal Medications

Bacterial counts for the unexposed infant group are summarized in Table 3. In untreated infants, the Firmicutes metabolic phylum, although not prominent at <1% of the total bacterial density, was composed completely of Lactobacillus species (Figs. 1 and 2). Organisms of the Bacteroidetes phylum were barely detectable, and species from Bifidobacterium or Bacteroides were absent.

Oral Flora Characteristics with Antenatal Steroids and Antenatal Steroids and Antibiotics

Total bacterial density was almost eightfold greater in infants exposed to antenatal steroids compared with the unexposed group with an increased composition of Firmicutes (88.0%) metabolic repertoire during this early period (Fig. 1, Table 3). Despite the greater density in Firmicutes, Lactobacillus prevalence was decreased to 0.05% (Fig. 2, Table 3). Density levels of the Bacteroidetes were significantly increased by 10-fold compared with unexposed infant levels composed completely of Bacteroides species (Fig. 3, Table 3). The addition of antibiotics with antenatal steroids also resulted in a 3.6-fold increase in total bacterial density compared with the unexposed group and an almost 1000-fold increase in Firmicutes density (Fig. 1, Table 3). Additionally, the prevalence of Lactobacillus species was minimal, decreased to 0.16% (Fig. 2, Table 3). Density levels of the Bacteroidetes...
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Discussion

The preterm infant’s oral cavity provides a gateway for first mucosal immune and gut colonizers for later overall intestinal function and nutritional health.17–20 Disruption of early colonizers can impact on later bacterial acquisition, such as occurs during cesarean delivery where colonization changes have been associated with later intestinal patterns and specific long-term health risks of infant caries.5,6 Recently, early antibiotic exposure in preterm infants has been associated with an increased risk of later NEC.4 Additionally, specific health-promoting bacteria, Lactobacillus and Bifidobacterium, provided after birth to the preterm infant appear to be important therapies that have been associated with decreased risk of later NEC development.21 Furthermore, the use of bacteria in therapeutic maternal antenatal probiotics has been associated with a decrease risk in later childhood atopic disease.22,23 These investigations support the important role of the establishment of bacterial colonization patterns in infant health and disease. Using quantitative 16S PCR technology, we provide additional data that demonstrate that routinely provided antenatal maternal treatment are also indirect mechanisms that alter preterm infant initial bacterial patterns and total bacterial density acquisition levels. Our infants were predominantly delivered by cesarean birth, and exposure to maternal antenatal common treatments influenced early preterm oral microbial acquisition.

Our results also detail patterns of specific bacteria during this acquisition period—specifically, that Firmicutes, Bacteroidetes, and Bifidobacterium of the Actinobacteria phyla oral acquisition is initially extremely limited in medication unexposed infants. Despite the small numbers, the results consistently demonstrate that the initial bacterial acquisition pattern is relatively devoid of these organisms. However, those Firmicutes present were completely of the Lactobacillus species. Furthermore, despite its prevalence in vaginal fluid, in all of our population samples Bifidobacterium was absent.

In contrast, infants exposed to any antenatal steroid treatments with or without antibiotics facilitated more oral bacterial density with a pattern composed primarily of Firmicutes with little Lactobacillus species but a greater prevalence of Bacteroides species. As expected, antenatal antibiotics suppressed preterm infant total bacterial acquisition but unexpectedly altered the acquisition pattern, favoring an increase in Bacteroidetes density to almost a quarter (23.5%) of the total bacterial density. Additionally, antibiotics limited Bacteroides species to <1%. Firmicutes density was increased compared with untreated infants with little Lactobacillus species. In these antibiotic-only infants the contribution of other phylum was decreased to 31% of the total oral density. The use and efficacy of antenatal steroids has been well established for preterm infant lung maturation.8,24,25 Additionally, steroids have additional benefits for infant survival including prevention of NEC.26 Further understanding of the impact of antenatal exposure in the full-term infant needs to be explored as our investigations were limited to the preterm infant and the depth of impact of antenatal treatment on acquisition of early commensal microbiota may be varied in the immature infant. It is known that antenatal amoxicillin for group B streptococcal prophylaxis in the term infant is associated with decreased Clostridium stool colonization at 3 days of life compared with untreated infants using culture-dependent methods.27 In our study, the small numbers of unexposed infants are a limitation in our observations; however, our current obstetric practice is to provide antenatal steroids to these high-risk infants. Those infants whose mothers received antenatal antibiotics did so due to perceived infection risk to the infant. Nevertheless, the bacterial density and acquisition patterns even in those infants whose mothers were treated for suspected chorioamnionitis or PPROM were strikingly similar within groups, providing support that maternal antenatal treatments indirectly impact on infant early microbial acquisition. Thus, the results of our study support an influence of altered bacterial acquisition patterns in those infants exposed to antenatal steroid with or without antibiotics. Furthermore, when used alone, antenatal antibiotics suppressed oral colonization density and bacterial diversity in support of previous studies that describe decreased microbial diversity in infants exposed to early antibiotics.28,29

Our results outline alteration of unique oral bacterial acquisition patterns acquired in preterm infants exposed to...
maternal antenatal steroids and antibiotics. Given the importance of the oral cavity in the acquisition of early gut microbes in the infant, our results further validate that maternal treatments alter infant bacterial acquisition, supporting antenatal therapy as an avenue of indirect infant therapy. Furthermore, our results identify the potential value of saliva as well as oral bacterial acquisition patterns as possible future biomarkers in the preterm infant. Finally, the results offer an initial step in investigating further potential mucosal changes that are important in the selection of specific host microbes to provide opportunities to follow bacterial and mucosal immune patterns to enhance our understanding of the role of early commensal bacteria acquisition important for immune and gut health during a pivotal period in these vulnerable preterm infants.

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