Maternal Antenatal Treatments Influence Initial Oral Microbial Acquisition in Preterm Infants

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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 patterns 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

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been linked to later infant health and development of caries.[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.[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 colonization. For these reasons, we examined the impact of maternal 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.

Material and Methods

Patient Recruitment

The study was approved by the Human Research Review Board of New York University School of Medicine and Bellevue 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.

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°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 diagnoses and medical treatments were collected.

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°C until analysis.

16S rRNA Polymerase Chain Reaction

The bacterial specific primers used are listed in ►**Table 1**. Oligo nucleotide primers and the Power SYBR Green PCR Master Mix were purchased from Applied Biosystems (Carlsbad, CA) based on previous *Firmicutes* and *Bacteroidetes* phyla and *Bifidobacteria*, *Lactobacillus*, and *Bacteroides* species detection analysis.[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 samples were used for the determination of DNA by real-time

Table 1 Primer List for Targeted Bacterial Analysis

Target Organisms	Primer	Sequence (5′ to 3′)	Annealing Temp (°C)
Bacteroidetes[12] (126 bp)	Forward primer	GGARCATGTGGTTTAATTCGATGAT	60
	Reverse primer	AGCTGACGACAACCATGCAG	
Firmicutes[12] (126 bp)	Forward primer	GGAGYATGTGGTTTAATTCGAAGCA	60
	Reverse primer	AGCTGACGACAACCATGCAC	
All bacteria[13] (200 bp)	Forward primer	ACTCCTACGGGAGGCAGCAG	60
	Reverse primer	ATTACCGCGGCTGCTGG	
Lactobacillus[14] (90 bp)	Forward primer	TACATYCCAACHCCAGAACG	60
	Reverse primer	AAGCAACAGTACCACGACCA	
Lac-Probe		(FAM)AAGCCATTCTTRATGCCAGTTGAA(TAMRA)	
Bifidobacterium[15] (553 bp)	Forward primer	CTCCTGGAAACGGGTGG	55
	Reverse primer	GGTGTTCTTCCCGATATCTACA	
Bacteroides[16] (106 bp)	Forward primer	GAGAGGAAGGTCCCCCAC	60
	Reverse primer	CGCTACTTGGCTGGTTCAG	
AllBac-probe		(FAM)CCATTGACCAATATTCCTCACTGCTGCCT(TAMRA)	

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.[12] 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.[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 beats/min and fetal tachycardia > 160 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 2 Maternal Characteristics ($n = 65$)

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

PPROM, preterm premature rupture of membranes; NRFHT, nonreactive fetal heart tracing.
One-way ANOVA T- test.
*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

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

One-way analysis of variance *t* test. *n*, number of samples in each group ± SE; Δ, fold change; SE, standard error.
[a]Statistically significant ($p < 0.05$) compared with no medications group.
[b]Percent of total bacteria density.
[c]Percent of *Lactobacillus* of *Firmicutes* density.
[d]Percent of *Bacteroides* of *Bacteroidetes* density.
[e]Percent 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*

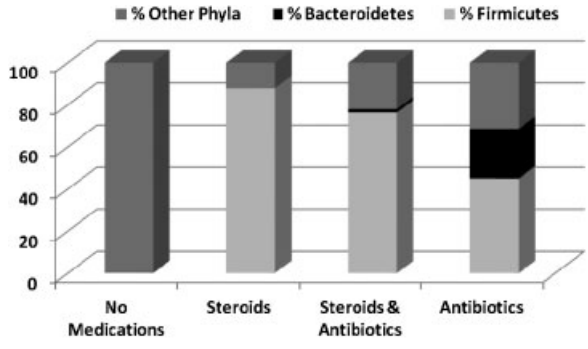

Figure 1 Antenatal treatment effects on infant oral total bacterial density patterns. The percent of *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* phyla in proportion to the total bacterial density in infant oral microflora. Total number of infants = 65, no medications ($n = 5$), steroid ($n = 26$), steroid and antibiotics ($n = 27$), antibiotics ($n = 7$).

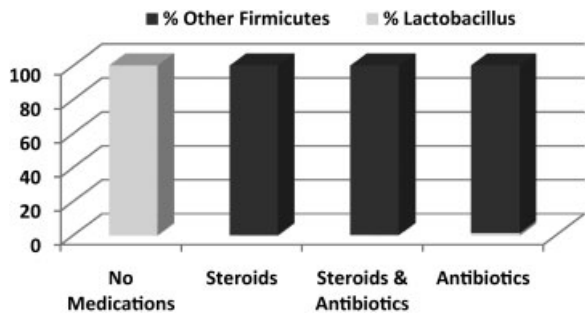

Figure 2 Antenatal treatment effects on infant oral *Lactobacillus* and other *Firmicutes* species. The percent of *Lactobacillus* and other *Firmicutes* species in proportion to the total *Firmicutes* bacterial density in infant oral microflora. Total number of infants = 65, no medications ($n = 5$), steroid ($n = 26$), steroid and antibiotics ($n = 27$), antibiotics ($n = 7$).

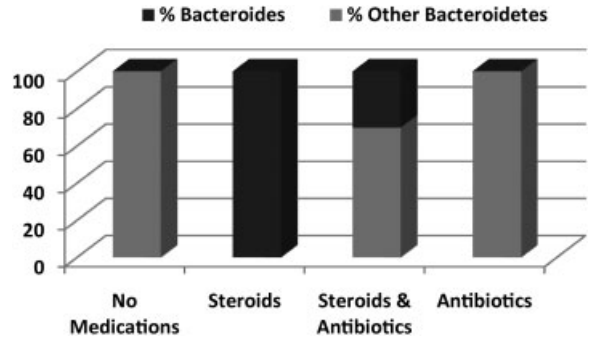

Figure 3 Antenatal treatment effects on infant oral *Bacteroides* species and other *Bacteroidetes* species. The percent of *Bacteroides* and other *Bacteroidetes* species in proportion to the total *Bacteroidetes* bacterial density in infant oral microflora. Total number of infants = 65, no medications ($n = 5$), steroid ($n = 26$), steroid and antibiotics ($n = 27$), antibiotics ($n = 7$).

increased almost 300-fold to the greatest levels of all groups reviewed. In this group, 30.3% of *Bacteroidetes* were of the *Bacteroides* species (►**Fig. 3**, ►**Table 3**). Of the total bacterial density in this group, 76.4% were of the metabolic repertoire (►**Fig. 1**, ►**Table 3**). The *Firmicutes* to *Bacteroidetes* ratio was notably increased to 49.3.

Oral Flora Characteristics of Antenatal Antibiotics

Infants exposed only to antenatal antibiotics demonstrated a suppressed total bacterial density fivefold lower than the unexposed group but a pattern of 20-fold increase in *Firmicutes* density (►**Fig. 1**, ►**Table 3)**. The prevalence of *Lactobacillus* species was depressed to 1.3%. Density levels of the *Bacteroidetes* increased by almost 250-fold compared with unexposed infant levels ($p < 0.02$), but *Bacteroides* species was only 0.01% of the total *Bacteroidetes* (►**Fig. 3**, ►**Table 3**).

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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