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Miscellaneous

Preterm infants have distinct microbiomes not explained by mode of delivery, breastfeeding duration or antibiotic exposure

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Abstract

Background: Preterm infants have low gut microbial diversity and few anaerobes. It is unclear whether the low diversity pertains to prematurity itself or is due to differences in delivery mode, feeding mode or exposure to antibiotics.

Methods: The Norwegian Microbiota Study (NoMIC) was established to examine the colonization of the infant gut and health outcomes. 16S rRNA gene Illumina amplicon-sequenced samples from 519 children (160 preterms), collected at 10 days, 4 months and 1 year postnatally, were used to calculate alpha diversity. Short-chain fatty acids (SCFA) were analysed with gas chromatography and quantified using flame ionization detection. We regressed alpha diversity on gestational age, taking into account possible confounding and mediating factors, such as breastfeeding and antibiotics. Taxonomic differences were tested using Analysis of Composition of Microbiomes (ANCOM) and SCFA profile (as a functional indicator of the microbiota) was tested by Wilcoxon rank-sum.

Results: Preterm infants had 0.45 Shannon units lower bacterial diversity at 10 days postnatally compared with infants born at term (95% confidence interval: –0.60, –0.32). Breastfeeding status and antibiotic exposure were not significant mediators of the gestational age–diversity association, although time spent in the neonatal intensive care unit was. Vaginally born, exclusively breastfed preterm infantss not exposed to antibiotics at 10 days postnatally had fewer Firmicutes and more Proteobacteria than children born at term and an SCFA profile indicating lower saccharolytic fermentation.

Conclusions: Preterm infants had distinct gut microbiome composition and function in the early postnatal period, not explained by factors more common in preterms, such as shorter breastfeeding duration, more antibiotics or caesarean delivery.

Key words: gut microbiome, preterm birth, microbial diversity, breastfeeding duration, antibiotic use, NICU, SCFAs, mediation analysis, ANCOM

Key Messages

- Infants born preterm are at high risk of infections and devastating conditions such as necrotizing enterocolitis (NEC), which may be related to their gut microbiome.
- Preterm infants were found to have low bacterial diversity and a different bacterial composition, with more Proteobacteria and *Enterococcus* in their gut compared with full-term infants.
- Preterm infants also had higher levels of many short-chain fatty acids compared with full-term infants.
- Preterm-related factors such as caesarean delivery, formula feeding and exposure to antibiotics were not primarily responsible for the differences found, although a longer stay in the neonatal intensive care unit did matter to some degree.

Background

Preterm birth (<37 weeks of gestation) is a common but serious event, even in high-income countries. It occurs in 5–13% of births and has increased in recent years, in part due to increases in *in vitro* fertilization and multiple pregnancies, but also due to reasons not identified.[1] Preterm infants have an immature gut barrier and immune system, which puts them at high risk for infections and diseases such as necrotizing enterocolitis (NEC),[2,3] associated with high morbidity and mortality rates. In conjunction with their immaturity, factors such as caesarean delivery, prolonged exposure to hospital environments, less breastfeeding and frequent antibiotic use increase the risk of adverse outcomes.

From birth, the gut microbiota is important for the development of gut function and in the normal maturation of the immune system.[4] It contributes to the regulation and maintenance of intestinal barrier function, provides protection against pathogens and promotes tolerance of foods.[4] Preterm infants typically have a very sparse microbiota, with low diversity and few anaerobes.[2,5] Bacterial colonization in the preterm gut also differs from that in the healthy, full-term infant gut over time.[2,6] However, it is not known whether the low-diversity microbiome is a consequence of the immaturity per se or whether differences in external factors are responsible. Preterm infants are commonly delivered by caesarean section and caesarean delivery has been shown to delay the gut establishment of specific taxa such as *Lactobacillus*, commonly found in the maternal vaginal microbiota, and instead favor the colonization of common skin bacteria such as *Staphylococcus*, *Streptococcus* or *Propionibacterium*.[7,8] Preterm infants often receive a higher proportion of formula feed compared with breastmilk than full-term infants, which will also affect the gut microbiota, as the oligosaccharides contained in human milk selectively support growth of specific microbes.[9,10] Several studies have demonstrated a dose–response relationship between the amount of breast milk received in the early period of life (14–28 days after birth) and the reduced risk of short- and long-term morbidities such as NEC, chronic lung disease, developmental delay and other conditions in very-low-birthweight and extremely-low-birthweight infants.[11,12] The higher incidence of opportunistic infections also increases exposure to antibiotics, which has a profound effect on gut microbiota.[13] The degree of microbiota disturbance depends on the type and dose of antibiotics used, along with the timing. Disruption of the colonization in early life may have permanent effects on the colonization due to potentially critical windows in which the immune system is being 'educated' by microbial signals.[14]

Currently, the influence of gestational age on the microbiome, independently of factors such as delivery mode, feeding mode and antibiotic use, is not clear. In this study, we investigated the association between gestational age and gut microbiome characteristics in children at 10 days, 4 months and 1 year postnatally, taking into account preterm-related factors.

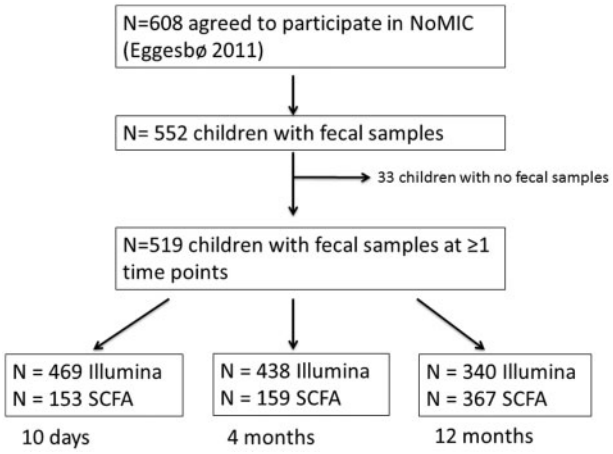

Figure 1. Flowchart of samples included in the study. SCFA, short-chain fatty acids.

Methods

Study cohort

The Norwegian Microbiota Study (NoMIC) was established to study the development of the gut microbiota and subsequent health outcomes. Participating mothers were recruited from the maternity ward at a county hospital (Sykehuset Østfold) from 2002 to 2005. For every preterm-birth mother, two control mothers of consecutively born term infants were enrolled. Mothers who were fluent in Norwegian and resided in the Østfold area (south-eastern Norway) were eligible to participate. Questionnaires and containers for faecal samples were distributed to the participants at the maternity ward. Details on the study population have been reported by Eggesbø *et al.*[15] and details on the faecal sample storage and analyses are found in the Supplementary Material, available as Supplementary data at IJE online. Briefly, samples were retrieved from the mothers and kept frozen at –20°C until DNA was extracted by an automated procedure and amplified by PCR reactions using 16S rRNA specific primers. The V4 region of the 16S rRNA gene was then sequenced using the Illumina HiSeq instrument. In this study, we use information from 519 infants with data on gut microbiota composition and SCFA concentrations on at least one of three time points: 10 days, 4 months and 12 months (Figure 1). The three time points were selected to examine the gestational age–gut microbiota relationship across patterns of breastfeeding (exclusive, weaning, weaned).

Exposure: gestational age

Information on gestational age, obtained from the Medical Birth Registry (MBR), was calculated based on the last menstrual period (LMP). Ultrasound estimates were routinely performed in the second trimester, but only used if the discrepancy between LMP and ultrasound exceeded 14 days. We entered gestational age continuously in the regression models, but also in a piecewise linear regression (splines) with breakpoints at 259 days (37 weeks, i.e. preterm/early term limit) and 280 days (40 weeks, i.e. the length of a full-term pregnancy).

Outcomes

We focused on three alpha diversity measures (i.e. bacterial diversity within samples): Shannon diversity, phylogenetic diversity (PD) and observed operational taxonomic units (observed OTUs). Shannon diversity takes into account the total number of species (species richness) and their relative abundances (species evenness). PD is a measure based on the amount or proportion of branch length in a phylogenetic tree that leads to different organisms (species richness), whereas observed OTUs is the count of unique OTUs found within the sample. Beta diversity (i.e. bacterial diversity across the entire community) was calculated using weighted UniFrac.[16] Clustering by preterm and term groups was visualized with principal coordinate analysis (PCoA) and tested using permutational analysis of variance (PERMANOVA). To detect possible differences in phylum and OTU abundance between preterm and term infants, we applied analysis of composition of microbiomes (ANCOM), which considers the compositional structure of the microbiome data, reduces false discovery rate and increases power compared with conventional statistical methods.[17] The following SCFAs were analysed: acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, caproate and isocaproate. Additionally, we used two fermentation indices to describe the saccharolytic and proteolytic properties of the gut microbiota, respectively. These indices were calculated according to Tjellström *et al.*[18] as follows: Index A (saccharolytic fermentation), i.e. the concentration of acetic minus propionate minus butyrate divided by the total amount of SCFAs; and Index B (proteolytic fermentation), i.e. the sum of concentrations of isobutyrate and isovalerate.

In the ANCOM and the SCFA analyses, we restricted analyses to comparing preterm and term groups of vaginally born infants, not exposed to antibiotics and with the following breastfeeding status at the sampling time: exclusive at Day 10, exclusive or partial at 4 months and not breastfed at 12 months.

Confounders

Confounders identified by daggity, version 2.3 (http://www.dagitty.net/)[19] for estimating the total effect were: delivery mode, nationality, siblings, pets, maternal smoking, maternal

body mass index (BMI), maternal antibiotics and maternal gut alpha diversity. We obtained the following variables from the questionnaires: delivery mode (vaginally/acute caesarean/elective caesarean, validated through the MBR), ethnicity (proxy based on whether the mother and her parents were born in Norway), number of siblings (categorized into two groups: none and one/several), household pets (yes/no) and antibiotic use during pregnancy (yes/no). BMI (kg/m^2, continuous) at the beginning of pregnancy was calculated from reported weight and height in the pregnancy journal (filled out by the doctor or midwife). The MBR provided data on smoking at the beginning of pregnancy (daily or occasionally vs no). Maternal gut microbiome was characterized by measured Shannon diversity (continuous) in the mother at 4 days postnatally.

Mediators

The questionnaires (at 1 month and 6 months postpartum) gave information on introduction to formula (yes/no) before 10 days postnatally. Numbers of daily breast feedings, formula and other meals were reported on the questionnaires at 6 and 12 months and were calculated into proportion of breastfeeding (0–1) of total daily meals relative to formula and other food given to the child. Antibiotic exposure (retrospectively from questionnaires at 1 month, 6 months and 12 months) was reported in days and weeks during the first month and monthly thereafter. Time in the neonatal intensive care unit (NICU) is often highly correlated with gestational age. NICU time was reported by the mothers in the 1-month and 6-months questionnaires. For the 10th-day sampling point, it was converted into NICU-free time (i.e. the number of days since birth the child had been outside the NICU), as some of the children were still in the NICU at that time. NICU time at 4 and 12 months was used as recorded on the 6- and 12-months questionnaires (in days).

Ethical approvals

The NoMIC study was approved by the Regional Ethics Committee for Medical Research in Norway (S-02216) and the Norwegian Data Inspectorate (2002/1934). Written, informed consent was obtained from each participant before enrollment.

Statistics

Linear regression models were used to estimate the association between gestational age (exposure) and bacterial alpha diversity (outcome), considering the occurrence of twins and other siblings in the sample (10%) by using the robust cluster variance estimator in STATA. Missing covariates (0.2–67.8%) were imputed using multiple imputation by chained equations[20,21] with predictive mean matching including all the variables in the full model, in addition to birthweight and maternal variables such as smoking, marital status, education and income. Fifteen sets were imputed and variances were combined using Rubin's rule.[20] The clustering by twins and siblings had to be considered in the imputation; therefore, the data were imputed in two rounds for each time point, including only one of the twins/siblings from each pair in each round. Supplementary Table 1, available as Supplementary data at *IJE* online, compares Complete Case (observed values) vs Multiple Imputed (MI) values across covariates.

In a mediation analysis, we estimated the effects of the mediators: formula introduction (by 10 days), breastfeeding proportion (at 4 and 12 months), antibiotics and time spent in the NICU, one by one and all together. The 'seemingly unrelated regression' approach (sureg in STATA) was used to estimate confidence intervals for the controlled indirect effects (CID) in each imputed dataset, and estimates were combined using Rubin's rule.[20] The CID assume no interactions between gestational age and mediators; however, as a sensitivity analysis, we tested for interaction in the first imputed set using the paramed command in STATA. Interaction between gestational age and delivery mode was also checked. All confounders were included in the mediation analysis, along with any confounders of the exposure–mediator and the mediator–outcome association.

All diversity measures were calculated in the Quantitative Insights Into Microbial Ecology (QIIME) pipeline version 1.7.0.[22] STATA 14 was used for imputation and regression analysis. PCoA plots were visualized using Emperor[23] and ANCOM was applied in R(version 3.0) to formally test for taxonomic differences.

Results

Characteristics of the participants

Gestational age ranged from 23 weeks (163 days) to 44 weeks (312 days); 31% of the children were preterm (7% <223 gestational days) and 13% were twins. Mothers of preterm infants did not differ in education (>12 years) or ethnicity (Norwegian) from mothers of term infants but were younger and more likely to report smoking at the beginning of pregnancy (Table 1). Preterm infants were more often born by caesarean section and exposed to antibiotics, and their duration of both any and exclusive breastfeeding was shorter (Table 1). Fewer preterm infants were introduced to formula before 10 days postnatally (Table 1) and they were generally introduced to solid foods later than

Table 1. Characteristics of the study participants

Characteristic[a]	Source of data	Total population ($N = 519$)	Term (≥37 weeks' gestation) ($N = 359$)	Preterm (<37 weeks' gestation) ($N = 160$)	*P*-value[b]
Maternal factors					
Maternal alpha diversity at 4 days postpartum	Measured				
Shannon diversity		5.8 (5.3–6.1)	5.8 (5.3–6.2)	5.7 (5.4–6.1)	0.45
Phylogenetic diversity		13.9 (11.6–15.9)	14.2 (11.6–16.1)	13.7 (11.6–15.4)	0.37
Observed OTUs		900 (766–992)	913 (770–913)	864.5 (751–964)	0.30
Missing (%)		67.8	67.4	68.8	
Maternal age	From personal identification number	30 (27–33)	30 (28–33)	30 (26–32)	0.03
Missing (%)		0	0	0	
Maternal pre-pregnancy BMI	Pregnancy journal	24.0 (21.2–27.1)	24.0 (21.5–26.6)	23.7 (20.4–27.5)	0.80
Missing (%)		4.8	2.8	9.4	
Maternal smoking at the start of pregnancy (%)	Medical Birth Registry				0.001
Yes (including occasional)		10.2	10.5	22.7	
Missing (%)		27.6	25.9	31.3	
Education (%)	Questionnaire				0.24
<12 years		11.2	10.1	13.9	
12 years		19.4	18.4	21.9	
>12 years		69.3	71.6	64.2	
Missing (%)		3.9	3.1	5.6	
Ethnicity[c] (%)					0.70
Non-Norwegian		11.6	11.9	10.7	
Missing (%)		10.2	6.7	18.1	
Maternal antibiotics during pregnancy (% yes)	Questionnaire at 1 month postpartum	26.6	26.7	26.2	0.92
Missing (%)		16.6	13.4	23.8	
Infant factors					
Birth weight (gram)	Medical Birth Registry	3320 (2470–3800)	3610 (3275–3980)	2085 (1524–2525)	<0.001
Missing (%)		0	0	0	
Sex (% female)	Medical Birth Registry	46.6	50.1	38.8	0.016
Missing (%)		0	0	0	
Household pets (% yes)	Questionnaire	47.2	41.5	63.4	<0.001
Missing (%)		24.9	19.6	36.9	
Days in neonatal intensive care unit (NICU)	Questionnaire	0 (0–6)	0 (0–0)	17 (5–28)	<0.001
Missing (%)		0.7	0.5	0.6	
Antibiotic exposure at 10 days (% yes)	Questionnaire at 1 month postpartum	18.8	11.6	37.2	<0.001
Missing (%)		16.8	13.4	24.4	
Antibiotic exposure at 4 months (% yes)	Questionnaire at 6 months postpartum	20.1	14.6	35.9	<0.001
Missing (%)		30.8	25.6	42.5	
Antibiotic exposure at 12 months (% yes)	Questionnaire at 12 months postpartum	43.5	37.5	59.5	0.001
Missing (%)		40.7	37.6	47.5	

(Continued)

Table 1. Continued

Characteristic[a]	Source of data	Total population ($N = 519$)	Term (≥37 weeks' gestation) ($N = 359$)	Preterm (<37 weeks' gestation) ($N = 160$)	*P*-value[b]
Mother–child shared factors					
Delivery mode (%)	Questionnaire, and Medical Birth Registry if missing				<0.001
Vaginal		68.5	76.1	50.3	
Acute caesarean		23.0	16.5	38.3	
Elective caesarean		8.6	7.4	11.4	
Missing (%)		3.5	2.0	6.9	
Months of exclusive breastfeeding	Questionnaire	4 (0–5)	4 (1–5)	3 (0–5)	0.03
Missing (%)		0	0	0	
Months of total breastfeeding	Questionnaire	10 (4–14)	10 (5–14)	8 (4–13)	0.04
Missing (%)		0	0	0	0
Formula introduced before 10 days (% yes)	Questionnaire	16.8	19.7	9.2	0.009
Missing (%)		18.3	15.3	25.0	
Age solid food introduced (month)	Questionnaire	5 (4–6)	5 (4–6)	5 (4–7)	<0.001
Missing (%)		20.2	15.3	31.8	
Number of siblings (%)	Questionnaire				<0.001
0		44.4	36.3	62.5	
1		36.1	42.2	22.5	
≥2		19.5	21.5	15.0	
Missing (%)		0.2	0.3	0.0	
Twins (N=62) or siblings (N=6) (% years)	Questionnaire	13.1	5.0	31.3	<0.001
Missing (%)		0	0	0	

[a]Continuous measures described by median (25th–75th percentile); categorical measures described by frequencies (%).
[b]Wilcoxon-ranksum or *t*-tests (if normally distributed) used for comparing continuous variables and Chi-squared for categorical variables.
[c]Proxy of ethnicity based on whether both parents were born in Norway.

term infants (Table 1). Correlation between gestational age and NICU-free time was $r_{spearman} = 0.69$.

Beta diversity

There was clustering of beta diversity by preterm/term status at 10 days ($p = 0.01$, PERMANOVA) and 4 months ($p = 0.01$, PERMANOVA) when including all infants and also in the subset of only vaginally born infants without antibiotic exposure (Supplementary Figures 2 and 3, available as Supplementary data at *IJE* online). No clustering was found at 12 months ($p = 0.65$, PERMANOVA, Supplementary Figure 4, available as Supplementary data at *IJE* online).

Regression analysis with alpha diversity

Median (IQR) Shannon diversity at 10 days was 2.83 (2.39–3.25) and increased to 3.11 (2.71–3.56) and 4.32 (3.85–4.78) at 4 and 12 months, respectively. Preterm infants had 0.45 Shannon units lower bacterial diversity at 10 days postnatally compared with infants born at term [95% confidence interval (CI): –0.60, –0.32]. Table 2 gives total and direct effects of gestational age (continuous, per 30 days) on Shannon diversity at 10 days. A positive association between gestational age and Shannon diversity was found in infants at 10 days, also when considering confounders and mediators such as early formula introduction, antibiotic exposure and time spent in the NICU (Table 2). A piecewise regression of gestational age on Shannon diversity at 10 days showed that the preterm period up to 259 days seemed to account for most of this association (Supplementary Tables 2 and 3 and Supplementary Figure 5, available as Supplementary data at *IJE* online). No associations were found between gestational age and Shannon diversity at 4 months and 12 months (Supplementary Table 2, available as Supplementary data at *IJE* online).

Table 2. Association [β, 95% confidence interval (CI)] between gestational age (per 30 days), and Shannon diversity at 10 days postpartum, total and direct effects. NoMIC cohort, $N = 469$

	β (95% CI)	
Total effect	0.24 (0.18, 0.3)***	
Adjusted total effect[a]	0.26 (0.18, 0.33)***	
Direct effect[a] of gestational age controlling for *mediators*:		Proportion mediated β(indirect effect)/β(total effect)
Formula introduction (FI)	0.26 (0.18, 0.30)***	0.027
Antibiotic exposure (AB)	0.24 (0.16, 0.31)***	0.085
FI & AB	0.24 (0.16, 0.31)***	0.095
NICU-free[b]	0.17 (0.072, 0.26)**	0.31

P-value: **<0.001; ***<0.0001.

[a]Adjusted for: delivery mode, ethnicity, maternal pre-pregnancy BMI, maternal gut microbiota (Shannon diversity), maternal antibiotics in pregnancy, maternal smoking before pregnancy, pets and number of siblings in the household.

[b]Number of days spent outside the NICU at sampling time 10 days postnatally.

After adjusting for covariates (gestational age, delivery mode and NICU-free time), early formula introduction was associated with higher Shannon diversity at 10 days postnatally (β = 0.19, 95% CI: 0.025, 0.36), although antibiotic exposure between the time of birth and sampling was not. Early formula introduction and antibiotic exposure did not mediate the association between gestational age and Shannon diversity at 10 days (Table 2). NICU-free time was associated with Shannon diversity at 10 days (β = 0.024, 95% CI: 0.002, 0.045) and found to be a partial mediator of the gestational age–Shannon diversity association (Table 2).

Taxonomic composition in vaginally born infants not exposed to antibiotics

At 10 days, exclusively breastfed preterm infants had a lower proportion of Firmicutes and higher proportion of Proteobacteria compared with exclusively breastfed children born at term (Figure 2). However, these differences did not persist to later time points.

Five genera were found to be differently abundant between the preterm and term infants at 10 days: two were from the genus *Bifidobacterium*, one from *Streptococcus* and one from *Enterococcus* (Table 3). There was also one unknown genus from the class Bacilli (Table 3). At 4 and 12 months, these genera were no longer differentially abundant; however, there were differences in the family Enterobacteriaceae (4 months), unclassified genera within Ruminococcaceae and Clostridiaceae (12 months), and Eubacterium (12 months) (Supplementary Table 4, available as Supplementary data at *IJE* online).

SCFA composition in vaginally born infants not exposed to antibiotics

Table 4 shows the amount and proportion of SCFAs in preterm and term infants at different time points postnatally. Total SCFA amount was lower in preterm compared with term infants at 10 days, both groups exclusively breastfed. This was mainly due to a somewhat lower proportion of acetate. However, preterms had a higher proportion of propionate and butyrate. At 4 months, the preterm infants had higher proportions of several SCFAs (acetate, propionate, isobutyrate, butyrate and isovalerate) compared with children born at term, although there were only five preterm children in the group at this time point. Preterm infants had a lower saccharolytic fermentation index (Index A) compared with full-term infants at 10 days and at 4 months. No significant differences were found at 12 months of age.

Discussion

The present study looked at the effect of gestational age on bacterial diversity after birth and on how much of this effect was mediated through formula introduction, antibiotic exposure and time in the NICU. Gestational age showed a strong positive association with neonatal gut microbiota diversity of 0.26 (0.18–0.33) Shannon units increase per 30 days of gestational age. The proportions mediated through formula introduction, antibiotic exposure and NICU-free days were 3%, 5% and 31%, respectively. Even under optimal microbiota-promoting conditions (vaginal birth, exclusive breastfeeding and no antibiotic exposure), preterm infants had a different bacterial composition in their gut (e.g. more Proteobacteria and more *Enterococci* at 10 days) and a different SCFA profile.

Primary colonization of the gut is important for the development of intestinal function and maturation of the immune system.[14,24] The first colonizing bacteria are mainly aerobic (e.g. *Staphylococci*, *Enteroccoci* and Enterobacteriaceae). When aerobic bacteria have consumed much of the oxygen, the anaerobic bacteria (e.g. *Bacteroides*, *Bifidobacterium*

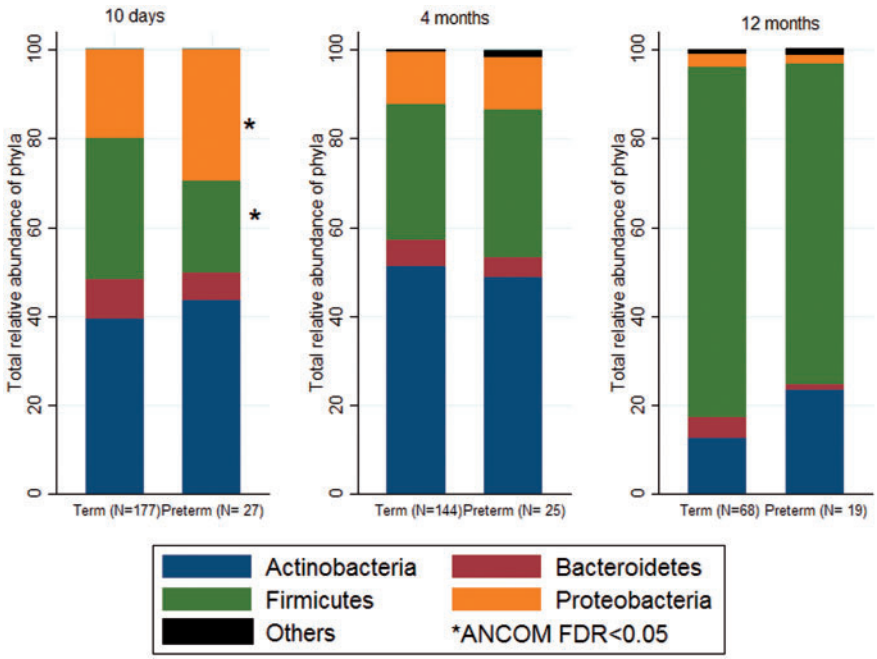

Figure 2. Phyla level gut microbial relative abundance at 10 days, 4 months and 12 months postnatally, according to term/preterm status at birth in the NoMIC cohort. Only vaginally born infants with no antibiotic exposure (exclusively breastfed at Day 10, partially breastfed at 4 months and not breastfed at 12 months).

and *Clostridium*) can colonize the gut.[2,25,26] The progress of bacterial colonization has been shown to be different in preterm infants compared with healthy infants born at term.[2,24] Preterm infants have an underdeveloped immune system and a slower progression of colonization,[2] possibly resulting in low diversity, which was supported in our study. However, we found that the diversity did not differ by gestational age at 4 months of age, indicating that the microbiome of preterms may move towards the term microbiome over time. To study the function of the microbiota, we included data on SCFAs. SCFAs in faecal samples constitute the unabsorbed fraction of SCFAs produced within the gastrointestinal tract, mainly the proximal colon.[18] In this study, we found higher levels of many SCFAs in preterms (both relative to total SCFAs and in absolute values). However, the amount of acetate (which constitutes the largest proportion of SCFAs[27]) was somewhat lower; therefore, the total SCFA amount was lower in preterm infants. In addition, Index A, proposed by Tjellström *et al.*[18] to reflect the saccharolytic properties of the gut microbiota, was lower in preterm infants, suggesting a reduced capability of the gut microbiota to degrade luminal carbohydrates. Intriguingly, disturbances of gut microbiota carbohydrate degradation have previously been proposed to play a major role in the pathophysiology of NEC[28] and our findings of altered SCFA levels may thus be of clinical significance.

Gut microbiota in preterm infants has been extensively studied,[2,5,26,29–55] but studies have often been small, performed only in preterm infants without comparison to infants born at term, and with little opportunity to separate the effect of gestational age from preterm-related factors. Penders *et al.*[52] did compare preterm with term infants by including 1032 infants at 1 month of age from the KOALA birth cohort. Full-term infants born at home and those exclusively breastfed were found to have the most 'beneficial' gut microbiota, with the highest number of *Bifidobacteria* and lowest number of *Clostridium difficile* and *Escherichia coli*. Still, they did not take into account the compositional nature of the microbiome, nor did they consider mediation by preterm-related factors. Ardissone *et al.*[40] also compared preterm with term infants and found gestational age to have the largest influence on the bacterial community of meconium, but they did not study this over time. La Rosa *et al.*[2] studied the colonization of bacteria in the gut of 58 preterm infants residing in the NICU. Results from their study correspond with our study, with more *Enterococci* (aerobic) and fewer of certain *Bifidobacteria* (anaerobic) in the preterm infants. La Rosa *et al.*[2] also observed that host biology (gestational age) was the most important factor in microbiota establishment, although recent re-analysis of the La Rosa data using ANCOM suggests that delivery mode, feeding type and antibiotic use also may play a role.[40] Arboleya *et al.*[46] found no differences in relative concentrations of SCFAs between the preterm and full-term infants,[46] which is contrary to our results. The inconsistency is probably due to the small sample size (only 21 preterm infants) in Arboleya *et al.*, which made it impossible to separately study vaginally born infants not exposed to antibiotics. A recent study by Forsgren *et al.*[29] also found

Table 3. Genera that were found to be differently abundant (FDR < 0.05, ANCOM procedure) in preterm vs term infants at 10 days postnatally. Only vaginally born, exclusively breastfed children not exposed to antibiotics included. NoMIC cohort

Green genes ID	Genbank accession number	Phylum	Class	Family	Genus	BLAST result[a]	Relative abundance (%) preterms	Relative abundance (%) full terms
365385	FJ369662.1	Actinobacteria	Actinobacteria	Bifidobacteriaceae	*Bifidobacterium*	*Bifidobacterium bifidum* strain	0.3	3.9
4347159	JQ187021.1	Actinobacteria	Actinobacteria	Bifidobacteriaceae	*Bifidobacterium*	*Bifidobacterium stercoris* strain	39.9	27.6
4425214	JQ471213.1	Firmicutes	Bacilli	Streptococcaceae	*Streptococcus*	*Streptococcus vestibularis* strain	3.5	7.8
224813	EF510486.1	Firmicutes	Bacilli	Enterococcaceae	*Enterococcus*	*Enterococcus faecalis* strain	1.8	1.0
225919	EF454749.2	Firmicutes	Bacilli	Enterococcaceae	*Enterococcus*	*Enterococcus saccharolyticus* strain	0.3	0.2

[a]Max score on accession number in NCBI database. Also other strains with equal query cover were found, but are not listed here.

gestational age to be the main predictor of bacterial composition, although they included only late preterm infants and chose to analyse only a few clinically relevant bacteria.

The most influential mediator of the gestational age–microbiota association in our study was the number of days since the infant had been discharged from the NICU. The NICU environment has the potential to impact microbial colonization, not only through feeding practices and antibiotic exposure, but also through other factors such as parental skin-to-skin contact, contact with nurses, environmental surfaces, pacifiers and equipment,[56] which are difficult to quantify. In addition, many infants in the NICU require total parenteral nutrition during their first days of life, possibly slowing down the gut-maturation process. Together with reduced microbe exposure, it could explain why we observed lower diversity in infants with longer stays in the NICU and a significant mediation of the gestational age effect on gut bacterial diversity through length of time outside the NICU.

Strengths and limitations

The present study included a relatively large number of preterm infants, giving the opportunity to examine the effect of gestational age on bacterial diversity, while considering external factors. We were also able to investigate this association over time. The inclusion of both preterm and term infants permitted comparisons across the entire gestational age spectrum. Additionally, we used ANCOM for bacterial composition comparisons, reducing the false discovery of microbial taxa.

Limitations included the use of questionnaire data for information on infant feeding and antibiotic use, which may be misclassified due to mothers' recall. For example, it is possible that the mothers did not know or remember whether their child had received antibiotics, particularly if the child had a prolonged stay in the NICU. Unfortunately, we did not have data on whether the infant received parenteral feeding and/or donor milk in the NICU. It is likely that infants who received donor milk would transfer to formula feeding once discharged, but we do not know how this could affect our results. Every NICU has its own microflora; data from the present study were collected in one NICU and in one hospital, and our results may therefore not be generalizable to other geographic areas. Although we attempted to make infant groups comparable by adjusting for known factors associated with gestational age and bacterial diversity, there may still be factors associated with preterm status and diversity (e.g. maternal intrauterine/vaginal infections or fortification of breast milk and formula given in the NICU) that we were not able to take into account. It is possible that the difference in preterm/term gut microbiota in infants is simply a reflection

Table 4. Short-chain fatty acid (SCFA) concentrations (mmol/kg) and proportions (percentage of total SCFA) in preterm and full-term infants, at 10 days, 4 months and 12 months postnatally. Only vaginally born infants, not exposed to antibiotics. NoMIC cohort

SCFA concentrations in mmol/kg	Preterms 10 days, exclusively breastfed ($N = 15$)		Fullterms 10 days, exclusively breastfed ($N = 59$)		*P*-value	Preterms 4 months, breastfed ($N = 6$)		Fullterms 4 months, exclusively breastfed ($N = 48$)		*P*-value	Preterms 12 months, not breastfed ($N = 20$)		Fullterms 12 months, not breastfed ($N = 71$)		*P*-value
	Median	IQR	Median	IQR		Median	IQR	Median	IQR		Median	IQR	Median	IQR	
Acetate	33.8	25.6–63.2	48.4	30.3–91.8	0.22	105.3	72.7–134.3	87.8	47.0–116.9	0.36	79.6	48.6–115.8	72.5	53.3–108.1	0.98
% of total	92.3	88.0–96.5	95.3	91.0–98.4	0.059	68.8	61.3–73.5	84.9	74.7–93.3	0.02	68.2	58.4–78.3	65.9	60.0–73.4	0.90
Propionate	2.3	1.1–3.4	1.3	0.67–3.0	0.18	27.5	16.3–36.8	9.9	3.0–17.6	0.006	14.5	10.8–19.6	15.4	11.1–23.7	0.56
% of total	6.4	2.5–8.3	2.8	1.03–6.4	0.04	19.9	11.4–27.3	10.9	4.7–16.6	0.04	13.9	10.5–16.7	15.7	9.4–21.0	0.47
Isobutyrate	0.0	0.0–0.07	0.0	0.0–0.14	0.34	1.6	1.2–3.0	0.7	0.3–1.4	0.02	1.8	0.9–2.3	1.55	0.82–2.42	0.65
% of total	0.0	0.0–0.14	0.0	0.0–0.2	0.37	1.3	0.76–1.8	0.8	0.36–1.4	0.1	1.7	1.1–2.3	1.43	0.77–2.03	0.30
Butyrate	0.66	0.33–1.17	0.41	0.2–0.8	0.11	11.7	1.2–20.1	1.05	0.23–2.7	0.008	11.2	8.1–25.7	13.4	8.9–21.0	0.73
% of total	1.3	1.09–2.4	0.70	0.2–1.8	0.04	8.6	0.8–12.4	1.23	0.46–2.7	0.02	14.2	8.6–20.3	12.9	8.8–17.5	0.80
Isovalerate	0.1	0.0–0.26	0.04	0.0–0.12	0.20	1.7	1.5–3.5	0.7	0.09–1.9	0.03	2.3	0.6–3.2	1.96	0.92–2.99	0.47
% of total	0.19	0.0–0.45	0.02	0.0–0.2	0.17	1.5	0.9–1.8	0.8	0.12–1.7	0.11	2.4	1.2–3.5	1.8	0.86–2.69	0.27
Valerate	0.0	0.0–0.08	0.0	0.0–0.04	0.48	0.08	0.0–0.5	0.05	0.0–0.11	0.55	0.18	0.03–0.27	0.25	0.07–0.82	0.14
% of total	0.0	0.0–0.25	0.0	0.0–0.04	0.47	0.06	0.0–0.3	0.04	0.0–0.14	0.78	0.17	0.02–0.32	0.21	0.07–0.42	0.27
Isocaproate	0.0	0.0–0.0	0.0	0.0–0.0	0.50	0.06	0.0–0.4	0.0	0.0–0.0	0.10	0.0	0.0–0.0	0.0	0.0–0.0	0.94
% of total	0.0	0.0–0.0	0.0	0.0–0.0	0.47	0.04	0.0–0.3	0.0	0.0–0.0	0.17	0.0	0.0–0.0	0.0	0.0–0.0	0.95
Caproate	0.0	0.0–0.0	0.0	0.0–0.1	0.98	0.0	0.0–0.0	0.0	0.0–0.06	0.12	0.0	0.0–0.0	0.0	0.0–0.0	0.49
% of total	0.0	0.0–0.0	0.0	0.0–0.0	0.92	0.0	0.0–0.0	0.0	0.0–0.07	0.12	0.0	0.0–0.0	0.0	0.0–0.0	0.45
Total SCFA	35.0	28.2–91.2	53.8	33.8–94.4	0.29	154.5	140.7–158.5	106.5	53.3–131.8	0.06	116.0	75.6–146.9	114.3	88.4–150.6	0.67
Fermentation Index A	0.85	0.77–0.93	0.91	0.8–0.96	0.04	0.44	0.27–0.49	0.73	0.52–0.87	0.03	0.32	0.21–0.59	0.37	0.24–0.50	0.97
Fermentation Index B	0.14	0.0–0.35	0.13	0.0–0.25	0.81	3.3	2.5–6.5	1.63	0.37–3.24	0.02	4.1	1.4–5.5	3.57	1.68–4.99	0.47

of a different vaginal microbiota in mothers and not due to the gestational age per se. We chose to focus on bacterial diversity as the main outcome measure, because low diversity has been found to be associated with many diseases, such as NEC[32,57] and *C. difficile* infection.[57] However, it is a crude summary statistic and important differences may be missed. Therefore, we also applied ANCOM for comparison of preterm and term infants. This may, however, be limited by the 16S rRNA method, which cannot detect differences in species and strains. Faecal SCFA profile may give important information about the function of the microflora, but SCFAs produced within the colon are readily absorbed, and only small amounts are excreted within the feces. Faecal SCFA levels are therefore an uncertain estimate of colonic SCFA production.

Conclusion

We found gestational age to be an important factor for bacterial diversity in infants 10 days postnatally and some of this association is mediated through the length of stay in the intensive care unit. Future research should aim at understanding why gestational age has such a large influence on infant gut microbiota, possibly leading to effective preventive measures for devastating diseases such as NEC.

Supplementary Data

Supplementary data are available at *IJE* online.

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