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Gut Dysbiosis With Bacilli Dominance and Accumulation of Fermentation Products Precedes Late-onset Sepsis in Preterm Infants

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Background. Gut dysbiosis has been suggested as a major risk factor for the development of late-onset sepsis (LOS), a main cause of mortality and morbidity in preterm infants. We aimed to assess specific signatures of the gut microbiome, including metabolic profiles, in preterm infants <34 weeks of gestation preceding LOS.

Methods. In a single-center cohort, fecal samples from preterm infants were prospectively collected during the period of highest vulnerability for LOS (days 7, 14, and 21 of life). Following 16S rRNA gene profiling, we assessed microbial community function using microbial metabolic network modeling. Data were adjusted for gestational age and use of probiotics.

Results. We studied stool samples from 71 preterm infants with LOS and 164 unaffected controls (no LOS/necrotizing enterocolitis). In most cases, the bacteria isolated in diagnostic blood culture corresponded to the genera in the gut microbiome. LOS cases had a decelerated development of microbial diversity. Before onset of disease, LOS cases had specific gut microbiome signatures with higher abundance of Bacilli (specifically coagulase-negative Staphylococci) and a lack of anaerobic bacteria. In silico modeling of bacterial community metabolism suggested accumulation of the fermentation products ethanol and formic acid in LOS cases before the onset of disease.

Conclusions. Intestinal dysbiosis preceding LOS is characterized by an accumulation of Bacilli and their fermentation products and a paucity of anaerobic bacteria. Early microbiome and metabolic patterns may become a valuable biomarker to guide individualized prevention strategies of LOS in highly vulnerable populations.

Keywords. gut; microbiome; preterm infant; dysbiosis; bacterial metabolism.

Preterm infants are at high risk for late-onset sepsis (LOS; occurrence after 72 hours of life), a leading cause of mortality and long-term morbidity including brain injury and chronic lung disease [1–4]. Hence, there is an urgent need to evaluate new diagnostic markers and individualized strategies for prevention of LOS.

The hypothesis that gut dysbiosis precedes the development of LOS is supported by several facts. First, previous reports noted alterations in intestinal microbiome composition and lower bacterial diversity prior to the onset of LOS in preterm infants [5–9]. These data are, however, inconsistent due to variable study designs and small cohort sizes. Second, the most vulnerable period for LOS is between days 7 and 21 of life [10]. The first weeks of life are associated with a high rate of exposure to microbiome-disturbing influences such as antibiotic treatment or invasive measures [5, 11]. Third, host factors such as gestational age and immaturity of mucosal barriers contribute to the sepsis risk in mutual interaction with colonizing bacteria known to modulate immune responses, tight junction integrity, and metabolic function [12, 13]. Conventional culture studies have demonstrated concordance between bloodstream isolates and bacteria resident in the infants' gut [5, 11, 14–16]. Causative organisms are *Staphylococcus aureus*, coagulase-negative Staphylococci (CoNS), group B streptococci, and Enterococci, as well as gram-negative bacteria, that is, Enterobacteriaceae [10, 17].

Recent advances in molecular microbiology and bioinformatics have enabled direct sequencing of bacterial DNA from stool in preterm infants to determine gut dysbiosis. Here, we also investigate the yet unexplored function of the microbiota ecosystem and its impact on LOS risk. Functional community traits such as the release of metabolic by-products emerge from complex interactions between gut-inhabiting bacteria and their nutritional environment [9, 18]. These data might improve the predictive value

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of microbiome patterns for LOS risk as they reflect important determinants of intestinal homeostasis [19, 20].

To confirm our hypothesis, we performed a prospective longitudinal study in a large cohort of preterm infants <34 weeks of gestational age at significant LOS risk during the neonatal period. Major readouts were microbiome signatures obtained from 16S rRNA sequencing and profiles of metabolic by-products released by intestinal bacteria using a systems biology approach.

METHODS

Study Cohort

Between January 2012 and January 2017, we collected fecal samples from preterm infants with a gestational age 23 0/7 to 33 6/7 weeks within our single-center prospective study (Immunoregulation of Neonates) [21]. Fresh samples were collected on day 7 ± 3, 14 ± 3, and 21 ± 3 of life and stored at −80°C in a freezer. Infants without LOS or necrotizing enterocolitis (NEC) were used as controls. Infants with lethal malformations or congenital anomalies of the gastrointestinal tract were excluded from our study cohort [22]. Detailed information and definitions are outlined in the Supplementary Methods.

Ethics

Written informed consent was obtained from parents or legal representatives on behalf of the infants enrolled in our study. The study was approved by the local committee on research in human subjects at the University of Lübeck, Germany.

Bacterial DNA Isolation

Fecal samples (approximately 200 mg) were processed using the PowerSoil DNA Isolation Kit (MOBIO, Carlsbad, Canada). Details are outlined in the Supplementary Methods.

Polymerase Chain Reaction Amplification and Sequencing

We amplified V3/V4 16S gene sequences from each DNA sample. The primer design, polymerase chain reaction, quantification of amplicons, and library preparation were performed as described elsewhere [23]. Sequencing by synthesis runs was carried out on a MiSeq platform (Illumina, San Diego, CA) using the MiSeq Reagent Kit v3 (600 cycles). Negative extraction controls were included to verify that reagents were uncontaminated.

Bioinformatics and Statistics

Bioinformatic processing [24, 26–28], modeling of bacterial community metabolism [25], and statistical analysis are outlined in the Supplementary Methods.

RESULTS

Clinical Characteristics

We recruited a large cohort of preterm infants and prospectively collected fecal samples during the first weeks of life (n >600 infants/>2000 fecal samples). We included only infants with thorough longitudinal collection of fecal samples in the first weeks of life, that is, 3 stool samples in the first 24 days of life and additionally infants with LOS with at least 1 sample before diagnosis (n = 71 infants with LOS between 72 hours and 35 days after birth, n = 164 control infants without LOS or NEC) in order to match the fecal sample of controls with the corresponding time point of the pre-event sample of LOS cases. Hence, a total of 607 fecal samples were analyzed; 574 had a positive sequencing result. A total of 31 infants had blood culture–proven LOS with exclusively single pathogen detection (CoNS in 26 cases [*Staphylococcus epidermidis*, 12; *Staphylococcus haemolyticus*, 10; *Staphylococcus hominis*, 2; undefined species, 2); *Escherichia coli*, 4; and *S. aureus*, 1; Figure 1, Supplementary Tables 1 and 2].

The clinical characteristics of the study cohort are provided in Table 1, specifically the lower gestational age in the LOS group compared with the control group (median [interquartile range, IQR], 25.9 [24.8–28.5] vs 30.7 [28.3–32.6] gestational weeks). Infants with LOS and controls had no differences in exposure to antibiotics on day 1 of life (Table 1). LOS occurred

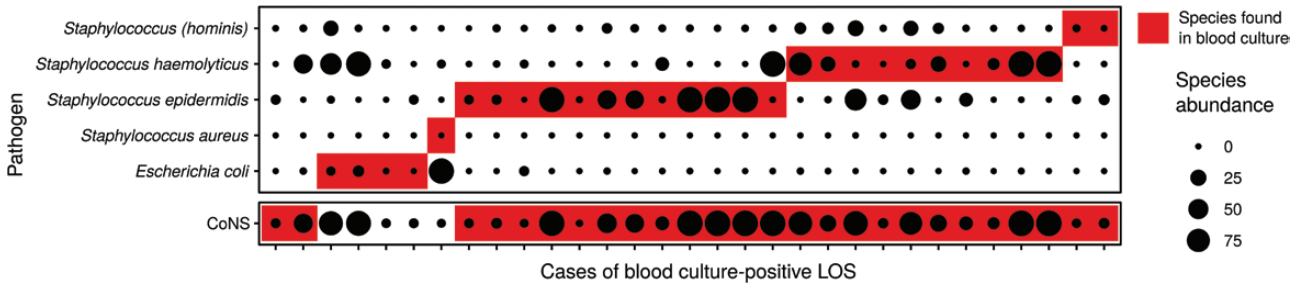

Figure 1. The dominance of opportunistic pathogens in fecal samples frequently explains the identity of bacterial species found in blood cultures. The figure shows the relative abundance (point size) of 5 bacterial species in fecal samples (rows), which were isolated in at least 1 case from blood cultures from the study cohort. Each column corresponds to a blood culture–positive late-onset sepsis case. The identity of the isolated pathogen is highlighted in red. If a pre-event stool sample was available, the abundances are shown for the last fecal sample before the onset of sepsis in each respective case. Otherwise, the sample closest to sepsis onset was chosen. The group CoNS (coagulase-negative Staphylococci) comprise the abundances of the species *Staphylococcus hominis*, *Staphylococcus haemolyticus*, and *Staphylococcus epidermidis*. In 2 blood cultures (first 2 columns), the exact CoNS species could not be further defined. *Staphylococcus hominis* was not identified in our sequencing data from stool samples. The abundances shown correspond to sequences that were annotated with the genus *Staphylococcus* without species classification. Abbreviations: CoNS, coagulase-negative Staphylococci; LOS, late-onset sepsis..

Table 1. Summary of Patient Demographics

	No LOS	LOS		Total
	n = 164	n = 71	P^a	n = 235
Gestational age (weeks)	30.7 (28.3–32.6)	25.9 (24.8–28.5)	<.001	29.4 (26.6–32.0)
Birth weight (g)	1400.0 (1018.8–1688.8)	755.0 (630.0–1057.5)	<.001	1220.0 (816.5–1490.0)
Male gender	87 (53.05)	40 (56.34)	.747	127 (54.04)
Multiple birth	60 (37.04)	22 (30.99)	.459	82 (35.19)
Caesarean section	145 (89.51)	65 (91.55)	.627	210 (90.13)
Probiotics	65 (39.63)	37 (52.11)	.103	102 (43.4)
Apgar score, 5 min	8 (7–9)	7 (6–8)	<.001	8 (7–8)
Apgar score, 10 min	9 (8–9)	8 (8–9)	<.001	9 (8–9)
Early-onset sepsis	21 (12.8)	15 (21.13)	.153	36 (15.32)
Amnion infection syndrome	16 (9.82)	10 (14.08)	.466	26 (11.11)
Necrotizing enterocolitis[b]	0 (0)	7 (9.86)	<.001	7 (3.00)
Focal intestinal perforation	1 (0.62)	11 (15.49)	<.001	12 (5.15)
Intraventricular hemorrhage	9 (5.56)	16 (22.54)	<.001	25 (10.73)
Small for gestational age	18 (10.98)	14 (19.72)	.112	32 (13.62)
LOS–day	…	12 (9–16)	…	…
Antenatal antibiotics	44 (26.83)	27 (38.03)	.149	71 (30.21)
Exposure to antibiotics from day of life 1	144 (85.21)	60 (90.91)	.344	204 (86.81)
Duration (days)	5 (3–6)	5 (4–7)	<.001	5 (3–7)
Ampicillin	143 (99.31)	60 (100)	…	…
Gentamicin	142 (98.61)	60 (100)	…	…
Teicoplanin	1 (0.69)	2 (3.33)	…	…
Meropenem	2 (1.39)	1 (1.67)	…	…
Cefotaxime	6 (4.17)	3 (5.00)	…	…
Tobramycin	2 (1.39)	0 (0)	…	…
Postnatal antibiotics Day of life 1–35	149 (90.85)	71 (100)	.034	220 (93.62)
Days	5 (4–12)	15 (7–26)	<.001	8 (5–20.75)
Ampicillin	144 (96.64)	66 (85.92)	…	…
Gentamicin	147 (98.66)	71 (100)	…	…
Teicoplanin	44 (29.53)	70 (98.59)	…	…
Meropenem	16 (10.74)	46 (64.79)	…	…
Vancomycin	1 (0.67)	1 (1.41)	…	…
Tazobactam	1 (0.67)	5 (7.04)	…	…
Cefuroxime	1 (0.67)	1 (1.41)	…	…
Erythromycin	3 (2.01)	5 (7.04)	…	…
Cefotaxime	7 (4.70)	11 (15.49)	…	…
Tobramycin	3 (2.01)	7 (9.86)	…	…
Linezolid	1 (0.67)	6 (8.45)	…	…
Other	2 (1.34)	5 (7.04)	…	…

Data are described as median (interquartile range) or n (%).

Abbreviation: LOS, late-onset sepsis.

[a]Kruskal-Wallis rank sum test and χ^2 test or Fisher's exact test for count data.

[b]Clinical necrotizing enterocolitis (NEC) classified as Bell stage II or Bell stage III with the need for laparotomy with or without resection of necrotic gut and the macroscopic diagnosis of NEC.

at a median (IQR) day of life 12 (9–16), and total duration of antibiotic treatment was longer in LOS cases compared with controls (median [IQR] 15 [7–26] vs 5 [4–12] days).

Development of Microbial Diversity

The analysis of the bacterial DNA sequences from all 574 stool samples resulted in a median of 11 392 (IQR, 6873–28 764) reads per specimen. We compared mean relative abundances of bacterial taxa for different time points of stool samples from all preterm infants. In detail, Bacilli, Actinobacteria, Gammaproteobacteria, and Clostridia accounted for 97% of reads on days 7, 14, and 21 of life. The analysis revealed a decrease of Bacilli ($P < .0001$) and an increase of Gammaproteobacteria ($P = .0032$), Clostridia ($P = .0003$), Alphaproteobacteria ($P = .0001$), Betaproteobacteria ($P = .0133$), and Fusobacteria ($P = .0183$) within the first weeks of life (Figure 2A). Thus, the microbiota structure substantially changed over time. At the species level, Bacilli were mostly identified gram-positive cocci such as *S. epidermidis*, *S. haemolyticus*, *Enterococci*, and *Streptococci*; Gammaproteobacteria were mainly *E. coli*, *Enterobacter hormaechei*, *Klebsiella oxytoca*, and other *Enterobacteriaceae*; Clostridia included *Veillonella* (it is debated

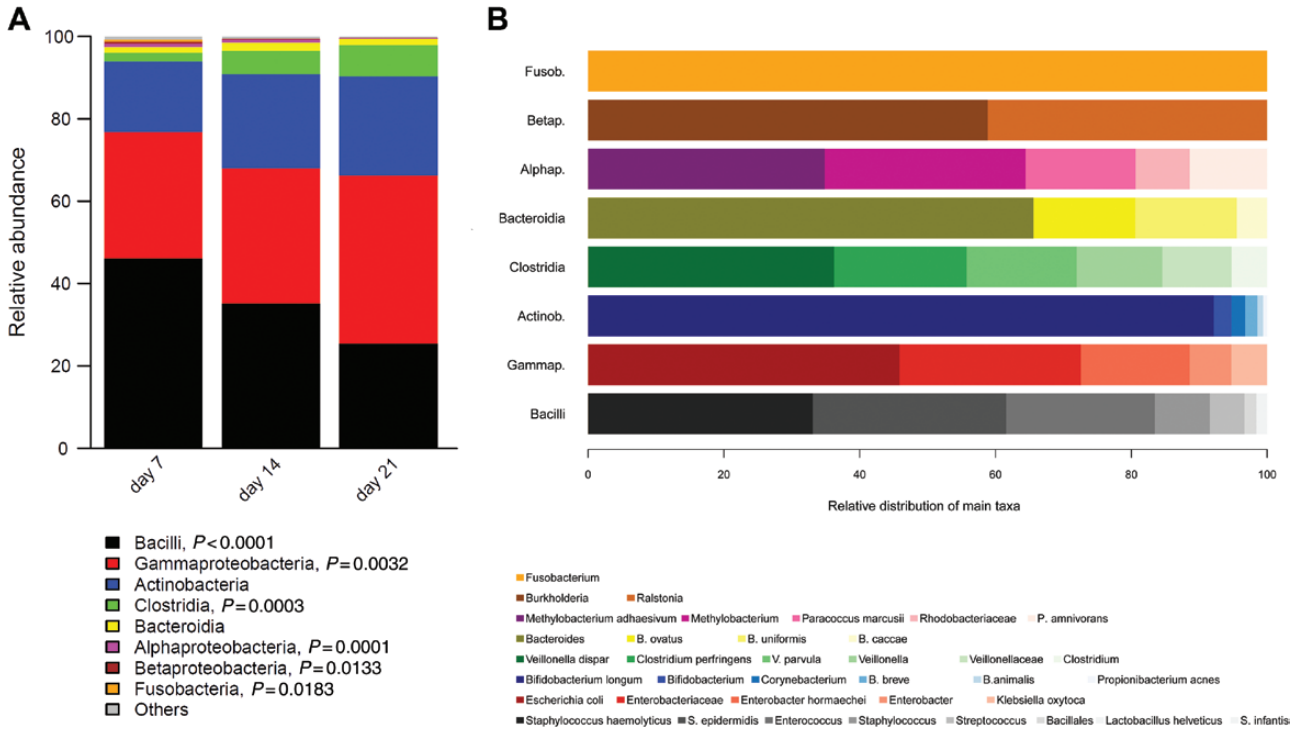

Figure 2. Intestinal microbiota development of preterm infants in the first weeks of life. *A*, Mean relative abundances of bacterial taxa on class level and species level for different time points in stool samples from preterm infants using nonparametric analysis of covariances with gestational age and probiotic use as covariates. The comparison of relative abundances of bacterial taxa of different time points revealed a significant decrease of Bacilli and a significant increase of Gammaproteobacteria, Clostridia, Alphaproteobacteria, Betaproteobacteria, and Fusobacteria within the first month of life (day 7, n = 204; day 14, n = 202; day 21, n = 164). *B*,The most abundant taxa within each represented class. Abbreviations: Actinob., Actinobacteria; Alphap., Alphaproteobacteria; Betap., Betaproteobacteria; Fusob., Fusobacteria; Gammap., Gammaproteobacteria.

whether *Veillonella* belongs to the Clostridia taxa or is in its own group); and Actinobacteria were dominated by *Bifidobacteria* (Figure 2B).

The overall branch length–weighted phylogenetic diversity in all infants increased over time (linear mixed-effect model fit, $\chi^2(1) = 11.857$; $P < .0001$; Figure 3).

Gut Dysbiosis With Bacilli Dominance Precedes LOS

To characterize the intestinal bacterial composition preceding LOS, we compared stool samples from infants before onset of clinical or blood culture–proven LOS (pre-LOS, median interval 3 days from sample to event) with stools from unaffected infants at corresponding time points of sampling. Only the first episode of LOS was included. The pathogen identified by blood culture was concordant with the operational taxonomic units (OTUs) detected in the gut microbiota before diagnosis in 29 of 31 blood culture–positive LOS cases and in all cases of CoNS sepsis (Figure 1 and Supplemental Tables 1 and 2).

A maturational increase in phylogenetic diversity was only observed in infants who did not develop LOS ($P < .0001$; Figure 3). The diversity did not significantly change over time in infants with blood culture–confirmed LOS ($P = .2678$) or in infants with blood culture–negative LOS ($P = .4806$; Figure 3).

The pre-event microbiome pattern of LOS patients was dominated by Bacilli ($P = .0330$), which were largely gram-positive bacteria such as *S. epidermidis*, other *Staphylococci*, and

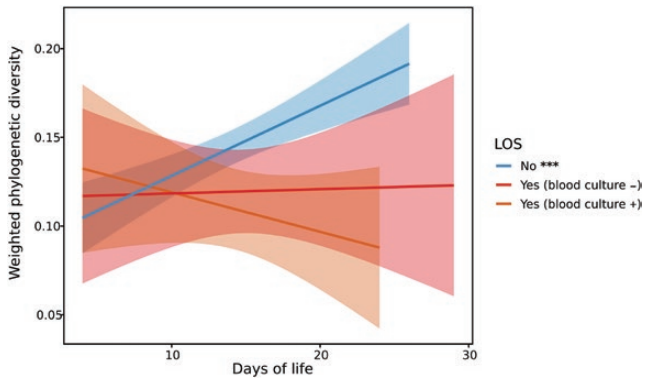

Figure 3. Succession of bacterial phylogenetic diversity in the first 24 days of life of preterm infants without late-onset sepsis (LOS; blue, n = 164 infants), with clinical but blood culture–negative LOS (red, n = 32 infants), and with culture-confirmed LOS (orange, n = 31 infants). Intercepts and slopes of lines and standard error intervals (shaded areas) were calculated using linear regression models. Blood culture–confirmed LOS significantly affected the development of the intestinal bacterial diversity (linear mixed-effect model fit, $P = .007$). While infants without LOS displayed an increase in bacterial diversity with time (linear mixed-effect model fit, $P < .0001$), the diversity in infants who developed LOS did not significantly change over time in both blood culture–positive ($P = .2678$) and blood culture–negative LOS ($P = .4806$). Abbreviation: LOS, late-onset sepsis.

Bacillales (Figure 4, Supplementary Figure 1). This was true for the group of infants with blood culture–positive as well as blood culture–negative LOS. A comparable high level of Bacilli relative abundance could be observed at earlier sampling time points prior to onset of LOS. We further analyzed the taxa within the Bacilli class at the species level and normalized the reads of each taxon to the total Bacilli reads for each sample. At the species level, the LOS cases (blood culture–positive and –negative group) were associated with a higher abundance of *S. haemolyticus* (Mann-Whitney *U* test, $P = .0200$) in comparison with controls (Supplementary Figure 2).

Furthermore, we analyzed the colonization of the preterm infant gut over time in the first 24 days of life (Figure 5). We incorporated only those control stool samples in our analysis that had been collected within the same time span as the available pre-LOS samples. We also checked that there was no significant difference between the groups regarding the variance of day of life of the stool samples (Levene's test, $P = .1074$). LOS status significantly affected the colonization with Bacilli ($P < .0001$). Control infants had a significantly reduced abundance of Bacilli over time ($P = .0019$), whereas the infants with blood culture–positive LOS showed an increase in their Bacilli colonization ($P = .0141$) before onset of disease (Figure 5). In contrast, LOS was associated with decreased colonization of anaerobic bacterial communities. Clostridia abundance increased over time in infants without LOS ($P = .0232$), while there was no significant increase in Clostridia abundance over time in infants before onset of blood culture–positive LOS (Figure 5).

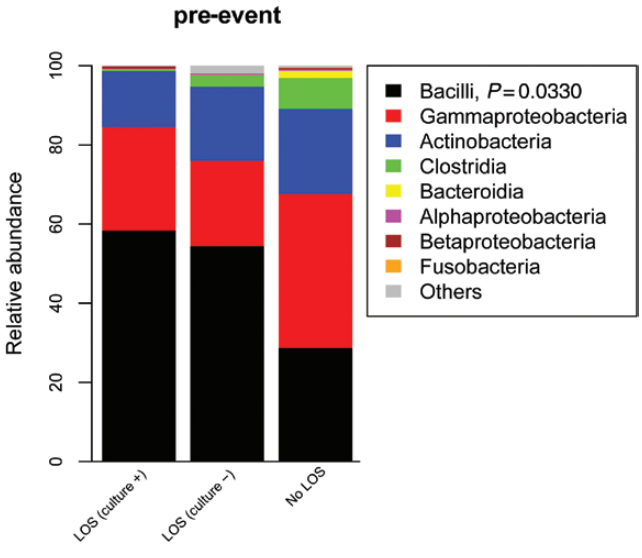

Figure 4. Mean relative abundances of bacterial taxa on class level according to late-onset sepsis (LOS) status before the onset of sepsis using nonparametric analysis of covariances with gestational age and probiotic use as covariates. Infants who developed LOS and non-LOS infants differed significantly in their abundance of Bacilli prior to the onset of disease (blood culture–positive LOS, n = 20; blood culture–negative LOS, n = 24; no LOS, n = 164). Abbreviation: LOS, late-onset sepsis.

Profiles of Metabolites Released by Bacteria Preceding LOS

Based on 16S rRNA gene sequencing data from the gut microbiota communities, we analyzed bacterial metabolism within the preterm infant gut bacterial communities using in silico metabolic models and assuming the nutrients from the human milk diet as a resource for bacterial growth (Supplementary Table 3). Based on the computer simulations, formic acid, acetic acid, ethanol, DL-lactic acid, ammonium, L-alanine, propionic acid, and carbon dioxide were predicted to be the most prominent bacterial metabolites produced (Figure 6, Supplementary Figure 3). The release of the fermentation products ethanol and formic acid were increased in infants with blood culture–confirmed LOS before disease onset compared with unaffected infants (Figure 6). The simulations suggested further that the elevated production of ethanol and formic acid could be assigned to Bacilli ($P < .0001$; Figure 7), while the frequencies of Actinobacteria and Gammaproteobacteria were inversely associated with ethanol production (Figure 7).

When we considered only those samples that are dominated by Bacilli (more than 75% of 16S reads), our model predicted still higher amounts of ethanol in blood culture–positive LOS cases before the onset of sepsis compared with controls (Mann-Whitney *U* test, $P = .029$; Supplementary Figure 4). The production of formic acid in Bacilli-dominated samples displayed a similar trend, although not significant ($P = .067$). This could potentially be explained by the different taxonomic distribution of Bacilli between both groups described above (Supplementary Figure 2), because the different taxa might also express different metabolic phenotypes.

DISCUSSION

In this prospective longitudinal study of preterm infants, we demonstrated that the pattern of gut dysbiosis preceding LOS is characterized by a high abundance of Bacilli and an accumulation of their fermentation products such as ethanol and formic acid. The strengths of our comprehensive study include the number of infants and specimens available for analysis and the novelty of evaluating the preterm gut for the pre-LOS habitat of the pathogens. The main limitation is lack of sequencing data on the genetic identity between blood culture isolates and pathogens in fecal samples.

The colonization of the preterm infant gut in the first month of life is a highly dynamic process. The gut microbial community generally matures along with a substantial increase in microbial diversity over many weeks at rates that are proportional to gestational age at birth (the communities in the more preterm infants mature at a slower rate). In our cohort, the gut was colonized with typical skin-associated bacteria in the first week of life, which were dominated by Bacilli, primarily CoNS. Within the second and third week of life, proportions of Bacilli rapidly decreased and there was a switch from these rather aerobic and facultative anaerobic bacterial communities

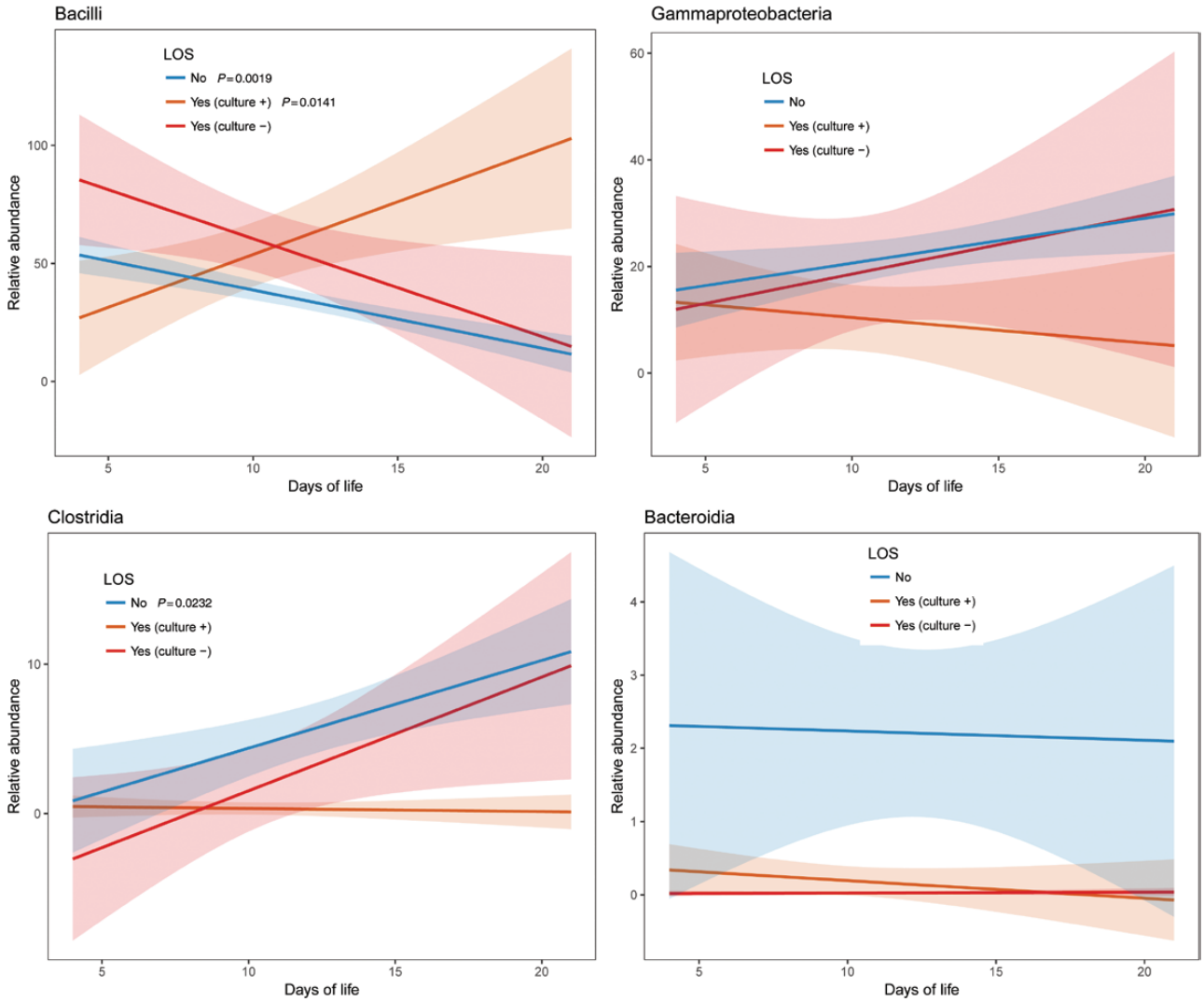

Figure 5. Relative abundances of Bacilli, Gammaproteobacteria, Clostridia, and Bacteroidia in the first 24 days of life in infants before late-onset sepsis (LOS) onset (blood culture negative = red: n = 24; blood culture positive = orange: n = 20) and infants without LOS (blue: n = 162) using linear mixed effect models while controlling for probiotic administration and gestational age. Blood culture–proven LOS significantly affected the development of Bacilli colonization (*P* value < .0001). While Bacilli abundance increased over time in blood culture–proven LOS (linear mixed-effect model fit, *P* = .0141), it decreased in control infants (linear mixed-effect model fit, *P* = .0019). In addition, the relative abundance of Clostridia increased over time in control infants (linear mixed-effect model fit, *P* = .0232), while this increase was absent in infants who developed blood culture–proven LOS.

toward increasingly anaerobic bacterial communities including Gammaproteobacteria (ie, largely *E. coli*, *Enterobacteriaceae*, and *K. oxytoca*), Clostridia (including *Veillonella,* based on Greengenes database, although the taxonomy is still a matter of debate), Alphaproteobacteria, Betaproteobacteria, and Fusobacteria. Similar patterns of bacterial colonization have been shown by other groups in smaller cohorts of preterm infants [29–31].

For LOS patients, however, we noted different microbiome patterns. The first hallmark was a decelerated phylogenetic diversity of LOS patients over time, which is in line with previous studies [6, 7]. The second distinct phenomenon was overrepresentation of aerobic bacteria and a lack of anaerobic bacterial populations prior to the onset of disease. Hence, LOS-affected infants showed significantly increased abundances of Bacilli, which were largely gram-positive Staphylococci such as *S. epidermidis*, *S. haemolyticus*, and *Bacillales*. On the species level, the LOS cases were associated with a higher ratio of *S. haemolyticus* in comparison with controls. These high proportions of Bacilli in the microbiome of LOS-affected infants did not decrease over time. A Bacilli-dominated microbiome after birth is known for infants delivered by Caesarean section. In our cohort, LOS cases and unaffected infants had a similar Caesarean section rate, but apparently unaffected infants display a gut microbiome with decreasing Bacilli abundance during the critical period of sepsis vulnerability. A critical microbiome-disrupting factor is exposure to antibiotics, which was similar in LOS patients

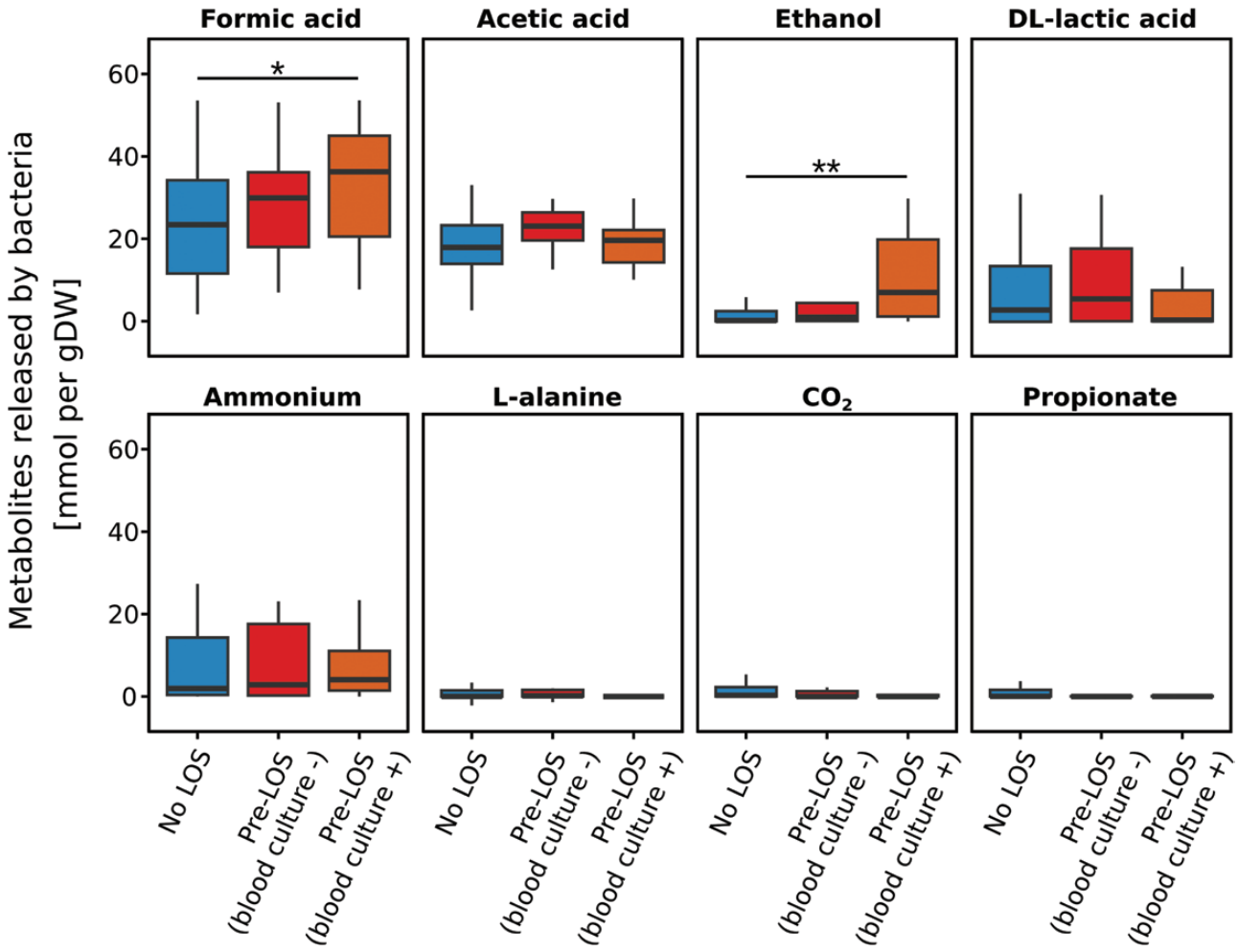

Figure 6. In silico estimates for the production of individual metabolites by bacterial communities depending on late-onset sepsis (LOS). The simulations suggested an increased production of the fermentation products ethanol and formic acid in infants who later developed LOS (red: blood culture–negative LOS, n = 24; blue: blood culture–confirmed LOS, n = 20) in comparison with nonaffected infants (n = 164). One-way nonparametric analysis of covariances was used to calculate *P* values while controlling for probiotic administration and gestational age. Shown are only metabolites with a median production rate above 0.1 mmol/gDW (gram bacterial dry weight) in at least 1 of the 3 groups. Abbreviations: gDW, gram bacterial dry weight; LOS, late-onset sepsis.

and controls immediately after birth but significantly different with regard to total days of therapy. The third hallmark of the pre-event microbiome of LOS infants is a diminished colonization with anaerobic bacterial communities such as Clostridia. These distinct aspects of gut dysbiosis are supported by previous studies that showed that Bacilli such as *S. aureus* caused perturbations in the microbiota composition of the human adult colon, reducing the proportion of anaerobic species such as *Bifidobacterium* [32]. The lack of anaerobic bacteria, which have a role in prevention of bacterial translocation and stabilizing epithelial integrity, has been previously proposed to increase sepsis risk originating from the gut [33–36].

In our cohort, 31/71 LOS infants had blood culture–proven sepsis. We identified a concordance of 93.5% between the causative LOS organism and its identification in fecal samples prior to LOS, which is consistent with previous studies (from 64% to 95%) [11, 14–16]. In LOS cases caused by CoNS, concordance with the corresponding gut taxa was noted in all cases. Hence, our data support the concept that apart from catheter-associated bloodstream infections, CoNS sepsis may evolve from gut translocation. Noteworthy, limitations in the interpretation of blood culture results need to be considered. In preterm infants, blood culture has a sensitivity of 10%–20% due to low bacteremia, low sample volumes, and previous antibiotic treatment [37].

Using a systems biology approach, we determined whether the pre-event gut dysbiosis in LOS is also characterized by distinct patterns of microbiota metabolic processes. Modeling functional community traits such as the release of metabolic by-products revealed that LOS-affected infants showed an accumulation of the fermentation products ethanol and formic acid prior to onset of sepsis. The increased release of formic acid and ethanol could be assigned to the abundance of Bacilli. The accumulation of those fermentation products is

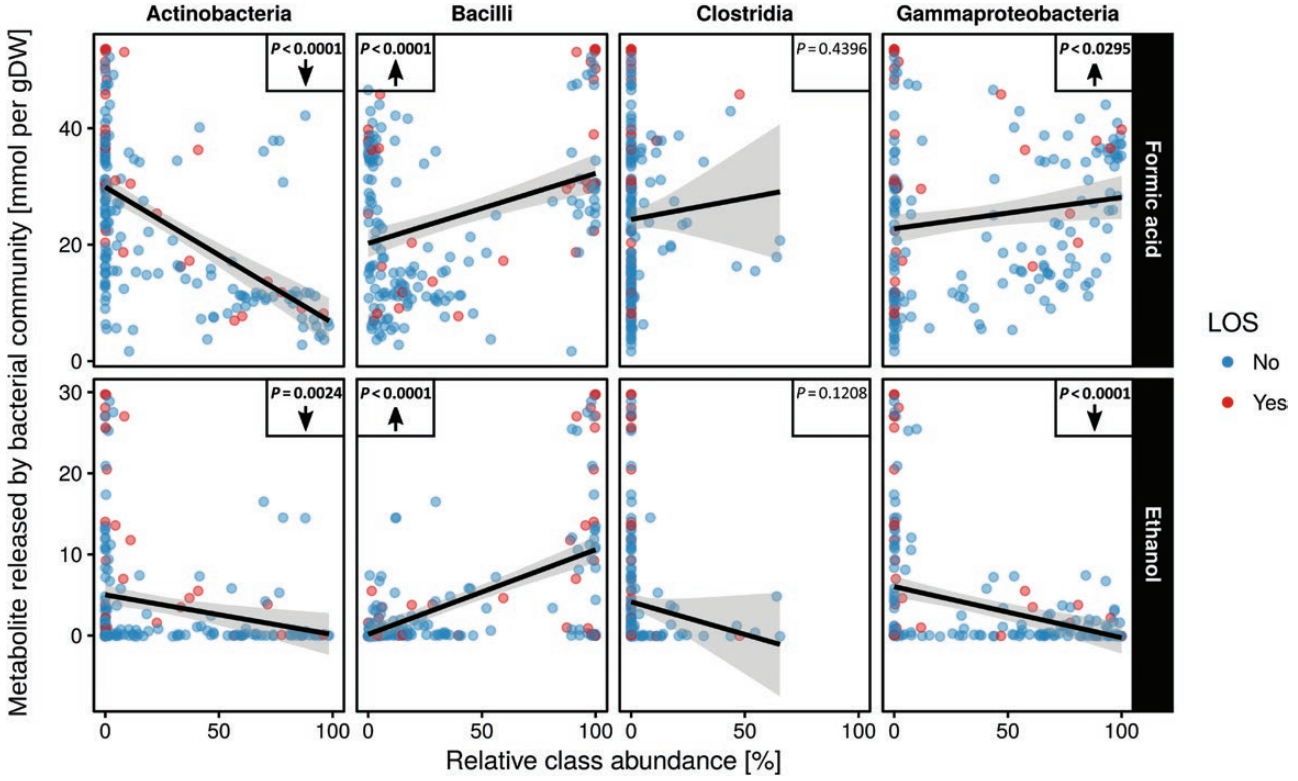

Figure 7. Correlations between predicted rates of formic acid and ethanol production by the bacterial community and the relative abundance of phylogenetic bacterial classes. Pearson product-moment correlation tests were used to obtain *P* values. Arrows denote the sign of the correlation coefficient in case of a *P* value < .05. Abbreviation: gDW, gram bacterial dry weight.

probably compounded by an immaturity of the intestinal lactase activity in preterm neonates. Animal studies suggested that excessive production and accumulation of bacterial fermentation products contribute to the pathogenesis of NEC [38]. Thus, fermentation products can directly cause damage to the intestinal mucosa and have indirect impact due to the induction of inflammation, which might lead to disruption of the mucosal barrier and translocation of luminal contents [38, 39]. Moreover, the distinct functional community traits that precede sepsis might impede further colonization with beneficial commensal bacteria and thereby inhibit the diversification of the microbiota.

Thus, we propose that distinct patterns of gut dysbiosis lead to intestinal barrier defects in preterm infants and thereby increase their risk for bacterial translocation. We suggest that a high Bacilli abundance may increase the potential for translocation through a damaged intestinal barrier. In addition, the high ratio of *S. haemolyticus* in the LOS cases might indicate that the colonization of virulent species increases the LOS risk [40].

Our novel findings on the structure of the gut microbiome in preterm infants encourage the concept that functional community traits can improve prediction models based on 16S rRNA sequencing data for LOS risk. The combination of intestinal dysbiosis and distinct metabolic interaction in a complex ecosystem may identify preterm infants at high risk for sepsis before onset.

We made a comprehensive single-center attempt to identify microbiome signatures in stool samples from infants before LOS; however, our approach has several limitations. The single-center study is of advantage for thorough sampling, rapid storage, and standardized analysis of fecal samples; however, our data need to be confirmed in a multicenter study. Noteworthy, daily instead of weekly sampling would have been ideal to study the dynamics of microbiome establishment; however, this approach is less feasible in the neonatal intensive care unit setting. Second, we did not sequence bloodstream isolates in order to compare the genotype with gut-colonizing strains. Due to this limitation, we cannot provide data on genetic identity of invasive isolates and pre-event colonizing species. In addition, we did not culture tips of removed central venous lines as this is not part of standardized clinical practice in our unit. Third, we noted an overwhelming role of gram-positive cocci as causes of bloodstream infections. In the experience of the German Neonatal Network, gram-positive cocci are the most dominant bacteria found in blood cultures of preterm infants with LOS (75%–80%) [41]. To overcome the risk of contamination, we only counted CoNS infection as "true" when infants additionally had clinical signs of infection and an elevated laboratory marker [42].

In conclusion, we demonstrated a distinct intestinal microbiome pattern of gut dysbiosis preceding LOS, which is dominated by Bacilli and a lack of anaerobic bacteria. The pre-event microbiome ecosystem of LOS infants is characterized by an accumulation of the fermentation products formic acid and ethanol. Future studies are needed to evaluate this distinct "sepsis signature" as a diagnostic biomarker and a prognostic tool for individualized strategies. Systems biology approaches can help to simulate specific supplementation strategies and their impact on the developing microbiome. In addition to administration of specific probiotic compositions, selective decontamination with antibiotic treatment might be an option for a high-risk microbiota pattern. Mechanistic studies of sepsis in preterm neonates using cellular and animal models with relevant candidate bacterial drivers and attenuators of gut injury will have to complement this important translational approach.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. Experimental concept and design: J. P., C. H., J. R., S. G., S. W. Performance/realization of experiments: J. P., S. G., K. C., S. K. Contribution of reagents/materials/analysis tools: J. P., S. G., S. W., C. K., J. R., S. K., J. F. B., N. T., W. G., E. H., J. Z. Data analysis: J. P., C. H., J. R., S. G., S. W., T. R., D. V. Writing of the paper: J. P., C. H., S. W.

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