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Profound Pathogen-Specific Alterations in Intestinal Microbiota Composition Precede Late-Onset Sepsis in Preterm Infants: A Longitudinal, Multicenter, Case-Control Study

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Background. The role of intestinal microbiota in the pathogenesis of late-onset sepsis (LOS) in preterm infants is largely unexplored but could provide opportunities for microbiota-targeted preventive and therapeutic strategies. We hypothesized that microbiota composition changes before the onset of sepsis, with causative bacteria that are isolated later in blood culture.

Methods. This multicenter case-control study included preterm infants born under 30 weeks of gestation. Fecal samples collected from the 5 days preceding LOS diagnosis were analyzed using a molecular microbiota detection technique. LOS cases were subdivided into 3 groups: gram-negative, gram-positive, and coagulase-negative *Staphylococci* (CoNS).

Results. Forty LOS cases and 40 matched controls were included. In gram-negative LOS, the causative pathogen could be identified in at least 1 of the fecal samples collected 3 days prior to LOS onset in all cases, whereas in all matched controls, this pathogen was absent ($P = .015$). The abundance of these pathogens increased from 3 days before clinical onset. In gram-negative and gram-positive LOS (except CoNS) combined, the causative pathogen could be identified in at least 1 fecal sample collected 3 days prior to LOS onset in 92% of the fecal samples, whereas these pathogens were present in 33% of the control samples ($P = .004$). Overall, LOS (expect CoNS) could be predicted 1 day prior to clinical onset with an area under the curve of 0.78.

Conclusions. Profound preclinical microbial alterations underline that gut microbiota is involved in the pathogenesis of LOS and has the potential as an early noninvasive biomarker.

Keywords. gram-negative sepsis; gut-derived sepsis; microbiota; dysbiosis.

Late-onset sepsis (LOS), defined as sepsis occurring ≥72 hours post-natally, contributes substantially to neonatal morbidity and mortality at neonatal intensive care units (NICUs) [1]. Approximately 20% of very low-birth-weight infants (<1500 g) admitted to NICUs develop at least 1 LOS episode [2]. Infants suspected of LOS often present with nonspecific symptoms. This may lead to, in retrospect following negative blood cultures, unnecessary exposure to broad-spectrum antibiotics. Excessive antibiotics administration in NICUs has been associated with adverse outcomes, including increased risk for infections with antibiotic-resistant bacteria, bronchopulmonary dysplasia, necrotizing enterocolitis (NEC), and death [3–5]. To date, the gold standard for LOS diagnosis is a positive blood culture, but its diagnostic accuracy in preterm infants is limited due to low bacteremia, low sample volumes, and previous antibiotic treatment [6].

Classically, it has been hypothesized that LOS was predominantly caused by the presence of intravenous catheters, especially in cases with a prolonged dwell time or with frequent replacements of catheters [7]. A more recent hypothesis suggests that LOS may also be caused by pathogens that originate from the gut [8, 9]. The preterm intestine has an increased permeability,

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facilitating bacterial translocation to the bloodstream, which may lead to gut-derived sepsis [10]. Previous conventional culture studies have demonstrated concordance between bloodstream isolates and bacteria resident in the infant gut, suggesting bacterial translocation from the gut to the bloodstream [11–13]. Little is known about whether specific microbial alterations could be linked to the development of certain types of LOS. Identification of LOS-specific microbiota signatures could lead to early detection and targeted treatment initiation or even prevention of LOS, improving outcomes. We hypothesized that preclinical microbiota composition and dynamics of patients who develop LOS differ from those of matched controls. Thus, the gut microbiota could potentially serve as a noninvasive predictive biomarker for LOS in preterm infants.

METHODS

Participants

In this case-control study, infants born at less than 30 weeks' gestation at 1 of 7 participating NICUs between May 2014 and December 2016 were included (Supplementary Table 1). Fecal samples and clinical data were prospectively collected on a daily basis from birth to 28 days post-natally. In case of transfer to a referral hospital or death during the first 28 days of life, data and sample collection stopped. None of the participating centers administered probiotics. Only the first episode of LOS was included for analysis. Infants diagnosed with LOS (Table 1) were strictly matched in a 1:1 ratio to controls without LOS based on center of birth, gestational age (±2 days), and post-natal age at onset. The exclusion criteria for both cases and controls were early-onset sepsis (positive blood cultures <72 hour post-natally), NEC (≥Bell's stage 2A), congenital gastrointestinal malformation, spontaneous intestinal perforation, fewer than 2 fecal samples available with a minimum weight of 100 mg, and an incomplete or missing medical file. Study approval was provided by the local institutional review boards of all participating centers. Written informed consent was obtained from parents or legal caretakers.

Sample Collection

Fecal samples were collected from the diaper into a stool container (Stuhlgefäß 10 mL, Frickenhausen, Germany) and immediately stored at −20°C until further handling. In case of multiple stool productions per day, only the first fecal sample was stored. Samples collected up to 5 days prior to clinical onset were included for analyses. Samples were always transported on dry ice (−80°C).

Microbiota Analyses

Microbiota analysis was performed using the IS-pro microbiota assay (inBiome, Amsterdam, the Netherlands), a molecular microbiota profiling technique developed for clinical diagnostics. Microbiota analyses were performed according to a previously published protocol [14, 15]. In short, 100 mg of feces was used as input for DNA isolation with the easyMAG machine (Biomérieux, Marcy l'Etoile, France) with the Specific A protocol, as described by the manufacturer, and eluted in 110 µL. Eluted DNA was diluted 1:10 and amplified with the IS-pro microbiota assay. IS-pro uses 2 polymerase chain reactions (PCRs). The first PCR has 2 fluorescently labeled PCR primers, one that amplifies the phylum *Bacteroidetes* and the other that amplifies the phyla *Firmicutes*, *Actinobacteria*, *Fusobacteria*, and *Verrucomicrobia* (*FAFV*) [14]. A second PCR has a primer specific for the phylum *Proteobacteria* [14]. After the amplification step, 5 µL of PCR product was added to 20 µL eMix (inBiome, Amsterdam, the Netherlands). Then, DNA fragments were separated based on their length in an ABI Prism 3500 Genetic Analyzer (Thermo Fisher, California). In essence, the technique identifies bacteria based on specific length polymorphisms in the 16S-23 rDNA interspace region combined with phylum-specific sequence polymorphisms in the 16S rDNA. Resulting data consist of peak profiles with different colors that relate to the different phylum groups *FAFV*, *Bacteroidetes*, and *Proteobacteria* and length signatures that correspond to the specific species. A true peak is considered to be an individual operational taxonomic unit. The data were preprocessed and analyzed using the IS-pro software suite [14, 15]. To control for potential contamination, negative control samples were taken along with each DNA isolation run. Negative control samples were taken following the entire identical IS-pro process as patient samples, and data were analyzed accordingly. In order to determine whether the cultured pathogen from the blood was present in the fecal samples,

Table 1. Definitions Used in the Current Study

LOS diagnosis	Established based on the Vermont Oxford criteria [31]
CoNS-LOS	Combination of a positive blood culture for CoNS, presence of LOS-like symptoms, and a C-reactive protein value of >10 mg/L
t_{0}	First day of LOS was defined as the day on which the blood culture that yielded the qualifying pathogen was obtained
t_{-1}	One day prior to clinical onset
t_{-2}	Two days prior to clinical onset
t_{-3}	Three days prior to clinical onset
t_{-4}	Four days prior to clinical onset
t_{-5}	Five days prior to clinical onset

Abbreviations: CoNS, coagulase negative S*taphylococci*; LOS, late-onset sepsis.

the species analyzed in feces had to match the blood-cultured pathogen on the species level.

Data and Statistical Analyses

Clinical and demographical characteristics were analyzed using Statistical Package for the Social Science (SPSS) version 26.0 (IBM, Armonk, NY). When considered appropriate, clinical and demographical data were analyzed using an independent *t* test, Mann-Whitney *U* test, or χ^2 test. Distribution of the data was assessed using histogram shape; upon indication, the Shapiro-Wilk test was performed.

A log2 transformation was performed on peak heights to improve the estimated correlation coefficient consistency. The absolute and relative abundances at predefined time points were analyzed using the Mann-Whitney *U* test. The alpha diversity was calculated as the Shannon diversity index for each phylum and all phyla combined. Second, the presence of the causative LOS pathogen was assessed in samples of both cases and matched controls.

All analyses were performed for the subgroups gram-negative, gram-positive (excepting CoNS), and CoNS-LOS. Analyses were performed by comparing cases and controls based on the causative pathogen. Furthermore, all different comparisons were performed per predefined time point (ie, t_{-5}, t_{-4}, t_{-3}, t_{-2}, and t_{-1}).

A boosting approach for classification was applied, combining weak learners in order to create more accurate classifiers [16]. A logistic boost algorithm with 1-node decision trees was used as the weak learners. The model was assessed based on repeated 5-fold cross-validation with 100 repeats. The tuning parameter was the number of boosting steps needed, and the optimal value was determined by grid search. For this classification model, feeding type and the different species identified in the fecal samples collected 1 day prior to clinical onset were included.

The results were corrected for multiple testing by applying the Bonferroni correction where appropriate. The corrected *P* value is mentioned in the footnote of the tables.

RESULTS

Clinical and Demographical Characteristics

In total, 753 preterm infants were included during the study period, of which 187 (25%) developed at least 1 LOS episode (Figure 1). Based on sample availability at predefined time points, 40 cases and 40 matched controls were included in the current study, providing 186 samples. Table 2 depicts the general characteristics of both groups in which no statistically significant differences were identified. The median post-natal age at clinical onset was 9 days (interquartile range, 6–11). Most cultured pathogens were CoNS (67.5%; eg, *Staphylococcus epidermidis*), followed by other gram-positive bacteria (17.5%; ie, *Staphylococcus aureus and Enterococcus faecalis*) and gram-negative bacteria (15.0%; ie, *Klebsiella pneumonia* and *Escherichia coli*) (Table 3).

Shannon Diversity Analyses and Overall Microbiota Composition

The Shannon diversity indices of the 3 phyla were not discriminative between the different subgroups of LOS compared with controls (Figure 2). When all phyla were combined, the Shannon diversity indices did not differ between LOS and controls and between the LOS subgroups and controls (Supplementary Table 2). On the phylum level, no significant differences in absolute and relative abundances were identified between the different subgroups. In all subgroups and time points, species within the phyla *FAFV* were most abundant, followed by *Proteobacteria* spp. (Supplementary Tables 3–6). *Bacteroidetes* were absent from almost all of the fecal samples analyzed, both in cases (17.5%) and controls (32.5%; *P* = .196) (Supplementary Tables 3–6). All negative control samples were found to be negative.

Presence of Causative Pathogen

Gram-Negative LOS

In gram-negative LOS (n = 6), the causative agent was detectable in 1 or more of the fecal samples collected in the 3 days preceding onset of sepsis in all cases, whereas in all matched controls, this pathogen was absent (*P* = .015) (Table 4). The abundance of these pathogens increased from 3 days before clinical onset in the cases (Figure 3).

When assessing the presence of the causative gram-negative pathogens in the cohort of all available controls (n = 40), we found that at t_{-3}, t_{-2}, and t_{-1}, the causative pathogen was present in 11%, 10%, and 13% of the fecal samples, respectively (Supplementary Table 7).

Gram-Positive LOS

In gram-positive LOS (n = 7, excluding CoNS), the causative agent was always detectable in 1 or more of the fecal samples collected in the 3 days preceding onset of sepsis in all cases, whereas this pathogen was present in 66% of the fecal samples from all matched controls (*P* = .19) (Table 4 and Figure 4). The causative gram-positive pathogen was variably present in the fecal samples of the matched controls (50%–67% of samples at all time points) (Table 4).

When assessing the presence of the causative pathogen in all control samples (n = 40), we found the causative pathogen was detectable in less than 50% of the fecal samples at all predefined time points (Supplementary Table 7).

CoNS-LOS

In contrast to the increased abundance of causative pathogens before gram-negative LOS onset, we found that CoNS presence

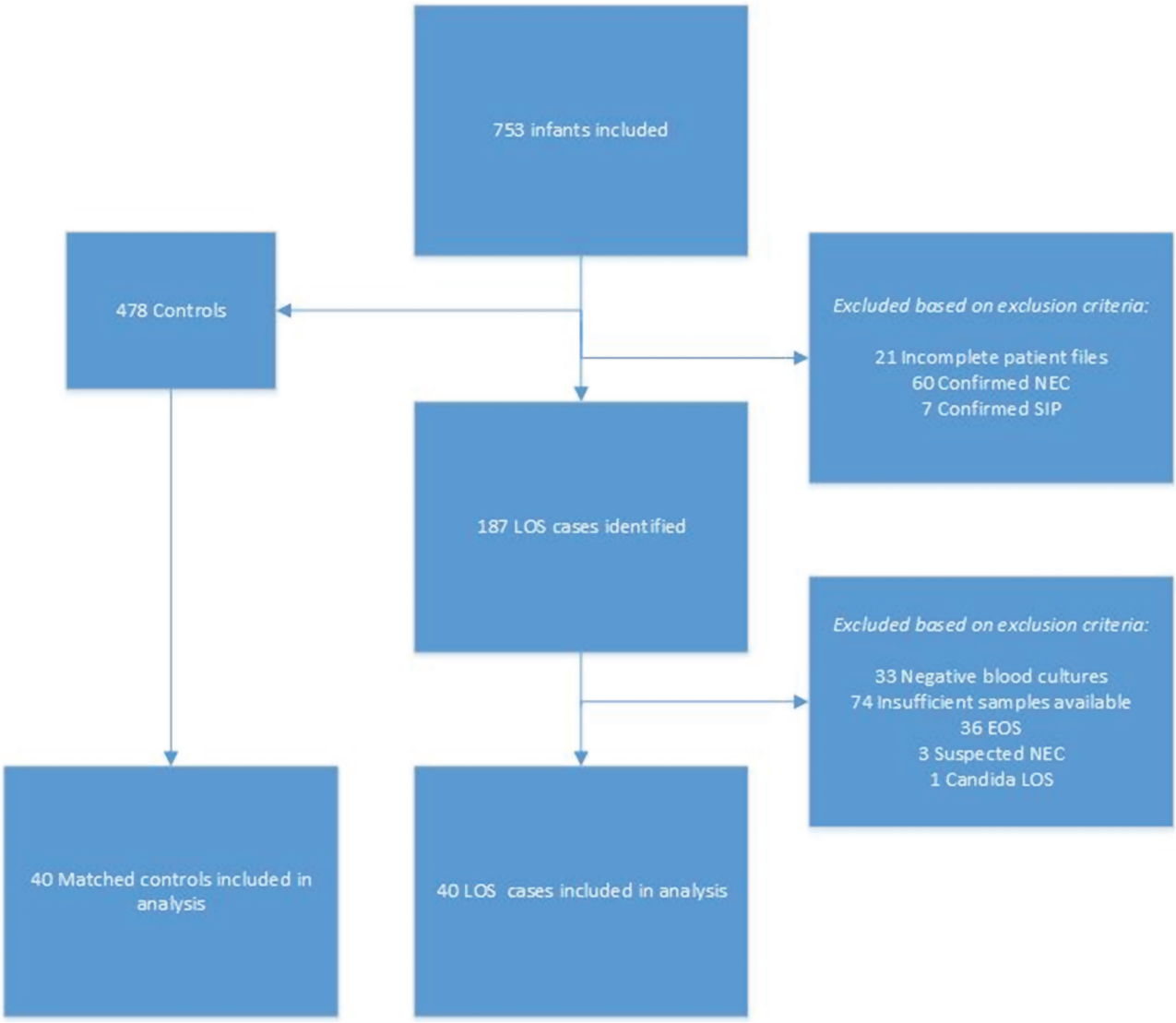

Figure 1. Flow diagram for inclusion process. In total, 40 LOS cases were included for analysis and matched to 40 controls. Abbreviations: EOS, early-onset sepsis; LOS, late-onset sepsis; NEC, necrotizing enterocolitis; SIP, spontaneous intestinal perforation.

was variable over time in the cases (n = 27) and consistently present in matched controls. These differences were not statistically significant.

All LOS Cases Combined Excluding CoNS

When combining the samples of all LOS cases except CoNS, the causative pathogen could be identified in at least 1 of the fecal samples collected 3 days before LOS onset in, on average, 92% of all fecal samples of cases, while these pathogens were present in, on average, 33% of the samples of the matched controls ($P = .004$).

Prediction Model

With the logistic Boosting model, LOS, irrespective of causative pathogen, could be predicted with an area under the curve (AUC) of 0.71 one day prior to clinical onset. Gram-negative and gram-positive (expect CoNS) could be predicted with an AUC of 0.78 one day prior to clinical onset. For both prediction models, feeding type and individual microbial species were included, resulting in 81 and 37 features, respectively.

DISCUSSION

In this case-control study, we observed profound microbial alterations before LOS onset that were pathogen-specific and most evident in gram-negative cases. In gram-negative LOS, the causative pathogen could be identified in at least 1 of the fecal samples collected 3 days prior to onset in all cases, whereas in all matched controls, this pathogen was absent. One day prior to clinical onset of gram-negative and gram-positive (except CoNS) LOS, cases could be discriminated from controls with a diagnostic accuracy of 0.78.

Table 2. Demographic and Clinical Characteristics

Variables	Late Onset-Sepsis Cases (n = 40)	Matched Controls (n = 40)	*P* Value[a]
Gestational age, mean [SD], weeks + days	27 + 6 [12]	28 + 1 [9]	.432
Birth weight, mean [SD], g	1053.5 [309.3]	1124.5 [274.6]	.281
Gender male, n [%]	20 [50]	23 [57.5]	.501
Delivery mode vaginal, n [%]	20 [50]	20 [50]	1.000
Multiple births, n [%]	18 [45]	11 [27.5]	.104
Enteral feeding type, n [%]			.291
Breast milk	27 [67.5]	19 [50]	
Formula milk	6 [15.0]	9 [23.7]	
Combination	7 [17.5]	10 [26.3]	
Antibiotics exposure prior to clinical onset, n [%]	35 [88]	33 [83]	.531
Antibiotic exposure during sampling, n [%]	2 [5.0]	4 [10.0]	.378
Antibiotics days prior to clinical onset, median [interquartile range]	3 [3, 4]	3 [2–4]	.326
Type of antibiotics during sampling, n [%]			.428
Amikacin	1 [2.5]	1 [2.5]	
Amoxicillin	1 [2.5]	3 [7.5]	
Flucloxacillin	1 [2.5]	0 [.0]	
Gentamicin	0 [.0]	2 [5.0]	
Catheter exposure at time of sampling, n [%]			.187
None	4 [10.0]	11 [27.5]	
Peripheral catheter	21 [52.5]	14 [35.0]	
Central catheter	11 [27.5]	11 [27.5]	
Peripheral and central catheter	4 [10.0]	3 [7.5]	
Fecal samples analyzed (n)			
t_{-1}	25	24	NA
t_{-2}	22	21	
t_{-3}	20	21	
t_{-4}	17	18	
t_{-5}	8	10	
Mortality, n [%]	2 [5.0]	0 [.0]	.152
Age at death, mean [SD], days	15 [1]	NA	NA

Abbreviations: NA, not applicable; SD, standard deviation.

[a]Results were considered statistically significant at $P = <.05$.

Several studies have investigated the microbiota in preterm infants affected by LOS. CoNS (eg, *S. epidermidis)* was the most commonly cultured causative pathogen in these studies, followed by *S. aureus* and gram-negative pathogens (eg, *E. coli)* [12, 17–19]. In one study with 31 LOS cases, it was demonstrated that the pathogen isolated in blood culture corresponded to intestinal genera that were found preceding LOS onset [17]. A comparable observation was done in 2 smaller studies (7 and 10 LOS cases, respectively) [18, 19]. We found preclinical microbiota differences to be strongly associated with the causative pathogen of LOS. These microbial differences were present up to 3 days before LOS onset, dependent on LOS subtype, and were driven by differences in the presence of the causative pathogens, rather than preclinical differences in, for example, α diversity. This phenomenon was most evident in gram-negative LOS. In this study, the diagnostic accuracy for detection of gram-negative LOS outperformed detection of other pathogens. These findings suggest that differences in microbiota composition before LOS onset are specific for different LOS pathogens and that gram-negative LOS, in particular, may originate from the gut.

Table 3. Distribution of Blood-Cultured Pathogens

Blood-Cultured Pathogens	Number of Cases
Coagulase-negative *Staphylococci*	27
Staphylococcus epidermidis	14
Staphylococcus capitis	7
Staphylococcus haemolyticus	1
Staphylococcus lugdunis	1
Staphylococcus warneri	1
Staphylococcus epidermidis and *S. hominis*	1
S. haemolyticus, *S. capitis*, and *S. warneri*	1
Coagulase-negative *Staphylococcus* unspecified	1
Gram-positive bacteria	*7*
Enterococcus faecalis	1
Staphylococcus aureus	6
Gram-negative bacteria	*6*
Escherichia coli	5
Klebsiella pneumonia	1

Previously, it has been described that a decreased microbial diversity is linked to the development of LOS [17, 20]. In a study by Mai et al, there was no difference in α diversity between LOS

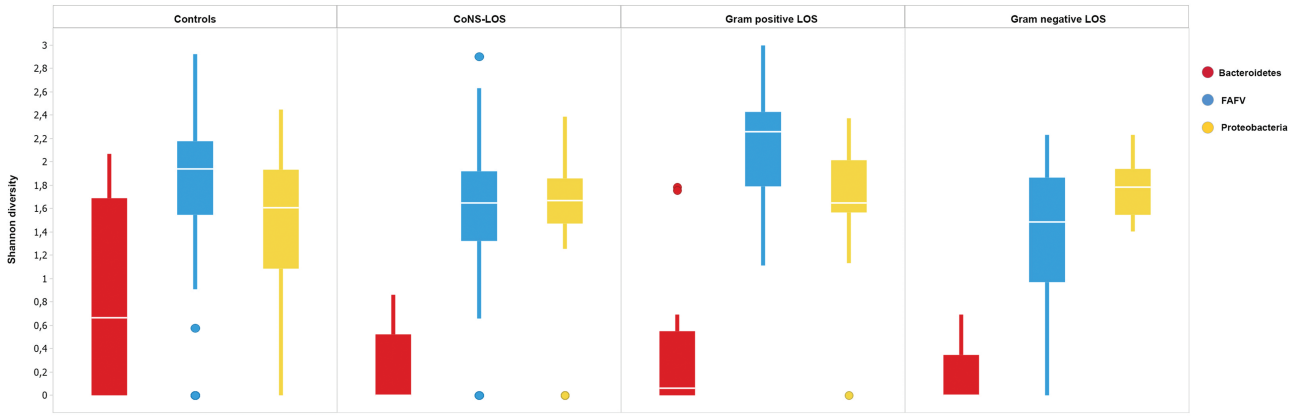

Figure 2. Shannon diversity indices per phylum per subgroup LOS vs controls. The Shannon diversity indices are displayed per phylum and subgroup LOS vs controls. *Bacteroidetes* are displayed in red, *FAFV* in blue, and *Proteobacteria* in yellow. No significant differences were identified between the different subgroups of LOS vs controls. Abbreviations: CoNS, coagulase negative *Staphylococcus*; *FAFV*, *Firmicutes*, *Actinobacteria*, *Fusobacteria*, and *Verrucomicrobia*; LOS, late-onset sepsis.

cases and controls identified [19]. Here, no significant differences in the Shannon diversity indices were identified between controls and the different subtypes of LOS.

We demonstrated that in gram-negative LOS, the causative agent was always detectable in 1 or more of the fecal samples collected in the 3 days preceding onset of sepsis and in increasing abundance toward the day of diagnosis. Furthermore, *E. coli* and *K. pneumoniae* were absent in all samples from matched controls at all predefined time points. Based on this observation, it could be hypothesized that colonization by *E. coli*, and possibly *K. pneumonia*, was crucial in the pathogenesis of gram-negative LOS. In a study by Smith et al in which fecal samples of very low-birth-weight infants were weekly sampled, it was also observed that the causative gram-negative pathogen was present in the rectal swabs of 51 LOS cases prior to clinical onset [12]. In the current study, fecal samples were collected on a daily basis, allowing for the comparison of longitudinal microbiota profile between cases and controls in the days preceding LOS. Our data show that the window between initial colonization and onset of LOS is small (3 days). Therefore, potential future applications to analyze gut microbiota composition in order to predict LOS should report results quickly.

In gram-positive LOS, *S. aureus* and *Enterococcus faecalis* were always detectable in at least 1 fecal sample collected in the 3 days preceding LOS onset, whereas this pathogen was present in 66% of the matched controls in this sampling period. In an in vitro colonic model, it has been demonstrated that the presence of *S. aureus* resulted in decreased abundances of *Bifidobacterium* and *Bacteroidetes* [21], which are characterized by health-promoting properties by antimicrobial activities [22, 23].

The traditional hypothesis of LOS by CoNS has considered skin abrasions as the main port of entry of pathogens [24]. More recently, several studies have suggested the gut as an alternative port of entry [11]. Here, we observed that although the gut is certainly a common reservoir of CoNS in preterm infants, it seems to be unlikely as an important source of infection in our population. In this case-control approach, we found that CoNS was variably present at all predefined time points in the cases and consistently present in controls. This counterintuitive finding is supported by previous work showing that patients with previous mucosal exposure to *Staphylococci* had less-severe infections [25]. Likewise, it could be hypothesized that the presence of CoNS in the preterm gut might provide protective properties against development of CoNS-LOS. However,

Table 4. Presence of Causative Pathogen in Fecal Samples

	All Causative Pathogens (n = 40)			All Causative Pathogens Except for CoNS (n = 13)			Gram-Negative LOS (n = 6)			Gram-Positive LOS (n = 7)			CoNS-LOS (n = 27)		
	Cases, n [%]	Controls, n [%]	*P* Value	Cases, n [%]	Controls, n [%]	*P* Value	Cases, n [%]	Controls, n [%]	*P* Value	Cases, n [%]	Controls, n [%]	*P* Value	Cases, n [%]	Controls, n [%]	*P* Value
t_{-1}	14 [58.3]	21 [91.3]	.609	5 [71.4]	2 [33.3]	.29	2 [66.7]	0 [.0]	.40	3 [75.0]	2 [50.0]	1.00	9 [52.9]	11 [73.3]	.29
t_{-2}	12 [57.1]	16 [80.0]	.410	7 [70.0]	4 [44.4]	.37	3 [100.0]	0 [.0]	.10	4 [57.1]	4 [66.7]	1.00	5 [45.5]	8 [88.9]	.07
t_{-3}	13 [65.0]	18 [94.7]	.605	10 [90.9]	4 [40.0]	.02	4 [80.0]	0 [.0]	.048	6 [100.0]	4 [66.7]	.46	3 [33.3]	7 [77.8]	.15
t_{-4}	9 [56.3]	14 [82.3]	.601	5 [71.4]	2 [33.3]	.29	0 [.0]	0 [.0]	NA	5 [100.0]	2 [50.0]	.17	4 [44.4]	6 [66.7]	.63
t_{-5}	2 [25.0]	9 [90.0]	1.000	2 [50.0]	1 [25.0]	1.00	0 [.0]	0 [.0]	NA	2 [100.0]	1 [50.0]	1.00	0 [.0]	3 [75.0]	.14

The number of fecal samples in which the causative pathogen was identified in a case and matched control setting is displayed with corresponding percentage. Abbreviations: CoNS, coagulase negative *Staphylococci*; LOS, late-onset sepsis; NA, not applicable.

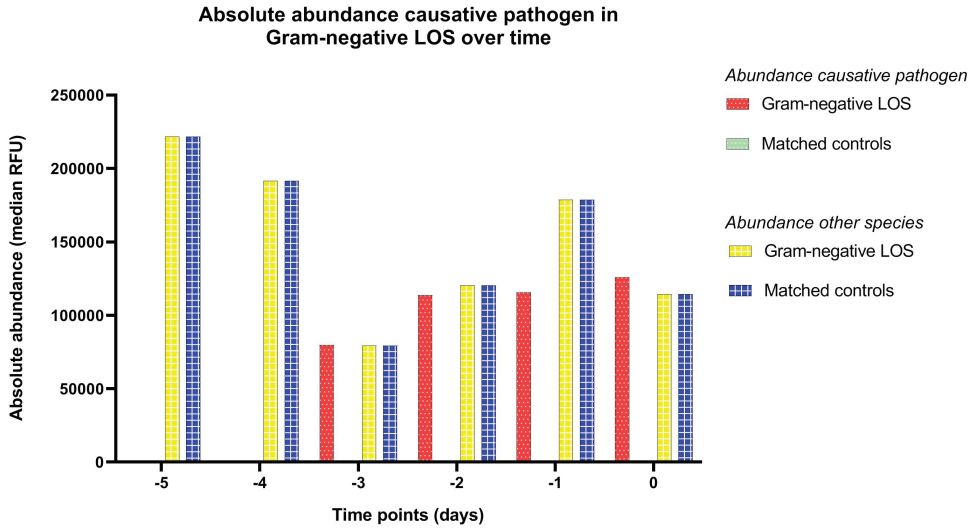

Figure 3. Absolute abundance of causative pathogen over time in gram-negative LOS and matched controls. The absolute abundance of the causative pathogen is displayed per time point for both cases and controls. Abundance of both *Escherichia coli* and *Klebsiella pneumonia* in the cases is indicated in red and in green for controls. The abundance of other species analyzed are indicated in yellow for cases and blue for controls. At 5 and 4 days prior to clinical onset, *E. coli* and *K. pneumoniae* are absent in the fecal samples analyzed. From 3 days prior to clinical onset, *E. coli* and *K. pneumoniae* are increasingly abundant until clinical onset. *Escherichia coli* and *K. pneumoniae* are both absent at all time points in the matched controls. Abbreviations: LOS, late-onset sepsis; RFU, relative fluorescent unit.

a difficulty in the analyses of CoNS-LOS is the possibility of contamination during blood culture withdrawal, which might influence results. Therefore, we only considered those infants with elevated CRP levels as LOS cases.

In our study population, bacteria belonging to the phylum *Bacteroidetes* were rarely identified and almost absent in cases. This suggests that the presence of *Bacteroidetes* may protect against LOS development. It has been previously proposed that a lack of anaerobic bacteria, which have a role in prevention of bacterial translocation and stabilizing epithelial integrity, increases the risk of gut-derived LOS [26]. Also, colonization with *Bacteroidetes* has been considered an essential factor in priming the immune system [27]. Interestingly, in a recent mouse study, it was demonstrated that gut-derived sepsis could be diverted by fecal microbiota transplantation due to an expansion of butyrate-producing *Bacteroidetes* [28]. These findings emphasize the importance of the presence of *Bacteroidetes* for a healthy and balanced gut microbiota composition and illustrate the potential of butyrate-producing *Bacteroidetes* in preventing LOS.

A microbiota-based prediction model for development of LOS showed promise, with a diagnostic accuracy of 71% that

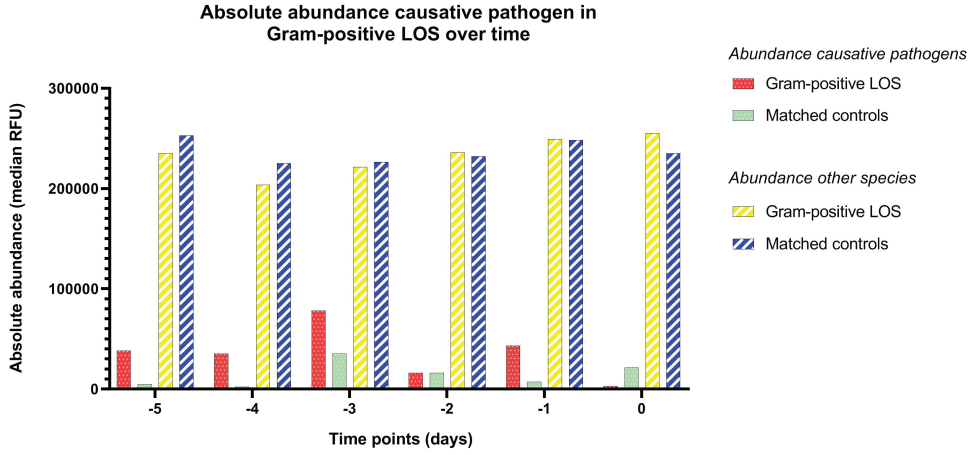

Figure 4. Absolute abundance of causative pathogen over time in gram-positive LOS and matched controls. The absolute abundance of the causative pathogen is displayed per time point for both cases and controls. Abundance of both *Staphylococcus aureus* and *Enterococcus faecalis* in the cases is indicated in red and in green for controls. The abundance of other species analyzed are indicated in yellow for cases and blue for controls. Both *S. aureus* and *E. faecalis,* were present in the fecal samples up to 5 days prior to clinical onset. Abbreviations: LOS, late-onset sepsis; RFU, relative fluorescent unit.

increased to 78% when only gram-negative and gram-positive (except CoNS) LOS were included. Thus, the current study demonstrates that a more stratified approach that focuses on specific pathogens, rather than a one-size-fits-all model, could likely increase diagnostic accuracy. A combination of diagnostic tools, including clinical, metabolic, and microbial features, integrated in a more sophisticated algorithm based on a larger dataset could be of great value to clinical practice, allowing for early LOS detection with high diagnostic accuracy and providing a time window for early intervention. Based on this algorithm, infants can be stratified based on their microbiota profile in a preclinical state, even predicting which pathogen is most likely to cause LOS. This allows for more targeted and personalized treatments and allows for the manipulation of microbiota composition, for example, by selective gut decontamination, probiotics administration, and a novel more experimental approach of administration of fecal microbiota transplantation (FMT). Recently, the potential of FMT was demonstrated as a novel treatment option for patients at increased risk for the development of gut-derived sepsis [28]. Although the study was performed in mice, it demonstrated the deteriorating effect of 2 days administration of FMT in mice infected by 4 member pathogen communities isolated from a patient with lethal sepsis. Future studies should assess the effect of early diagnosis using a clinical-, metabolic-, and microbial-based algorithm and prompt treatment by means of FMT, ultimately increasing LOS survival.

One strength of our study is its prospective multicenter design in which fecal samples and clinical data were collected daily in a standardized way. This allowed for strict matching to reduce the risk of bias by potential confounders influencing the microbiota composition. Since the overall number of cases was reasonable, we were able to focus on different subtypes of LOS. Last, a molecular-based microbial analytical technique was used that allows for high-throughput analyses to assess the microbial composition on the species level within hours after sampling. This is in contrast with other sequencing-based techniques that take days to provide results. This technique has previously been validated in neonates and children [29, 30]. Furthermore, in our study, an experimental approach was used that resulted in a high-risk gut microbiota profile that could be translated into clinical practice.

There are also some limitations. A relatively small number of gram-negative and gram-positive (excluding CoNS-LOS) LOS cases were included. Larger numbers are needed in future studies to validate our findings. Furthermore, preterm infants do not always produce stool on a daily basis, hampering longitudinal assessment due to missing time points. Also, we were unable to determine whether the blood-cultured pathogen was genetically similar to those bacteria isolated from the fecal samples preceding clinical onset. However, the bacteria isolated from the fecal sample had to match the blood-cultured pathogen on the species level.

CONCLUSIONS

We demonstrated that in both gram-negative and gram-positive pathogens, the causative pathogen of LOS could be detected up to several days before LOS onset. Our results illustrate the potential of longitudinal microbiota profiling as an early biomarker for LOS detection. Future studies should focus on mechanisms of how gut pathogens are involved in LOS pathophysiology and explore whether targeted microbial manipulation could decrease LOS incidence.

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

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Potential conflicts of interest. N. d. B. has served as a speaker for AbbVie and MSD and has served as consultant and principal investigator for TEVA Pharma BV and Takeda. He has received a (unrestricted) research grant from Dr Falk, TEVA Pharma BV, MLDS, and Takeda. A. E. B. has proprietary rights on the IS-pro platform technology and is a cofounder of a spinoff company developing this technique. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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