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# Clostridium butyricum Strains and Dysbiosis Linked to Necrotizing Enterocolitis in Preterm Neonates

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**Background.** Necrotizing enterocolitis (NEC) is the most common and serious gastrointestinal disorder among preterm neonates. We aimed to assess a specific gut microbiota profile associated with NEC.

**Methods.** Stool samples and clinical data were collected from 4 geographically independent neonatal intensive care units, over a 48-month period. Thirty stool samples from preterm neonates with NEC (n = 15) and controls (n = 15) were analyzed by 16S ribosomal RNA pyrosequencing and culture-based methods. The results led us to develop a specific quantitative polymerase chain reaction (qPCR) assay for *Clostridium butyricum* and we tested stool samples from preterm neonates with NEC (n = 93) and controls (n = 270). We sequenced the whole genome of 16 *C. butyricum* strains, analyzed their phylogenetic relatedness, tested their culture supernatants for cytotoxic activity, and searched for secreted toxins.

**Results.** *Clostridium butyricum* was specifically associated with NEC using molecular and culture-based methods (15/15 vs 2/15; P < .0001) or qPCR (odds ratio, 45.4 [95% confidence interval, 26.2–78.6]; P < .0001). Culture supernatants of *C. butyricum* strains from preterm neonates with NEC (n = 14) exhibited significant cytotoxic activity (P = .008), and we identified in all a homologue of the  $\beta$ -hemolysin toxin gene shared by *Brachyspira hyodysenteriae*, the etiologic agent of swine dysentery. The corresponding protein was secreted by a NEC-associated *C. butyricum* strain.

**Conclusions.** NEC was associated with *C. butyricum* strains and dysbiosis with an oxidized, acid, and poorly diversified gut microbiota. Our findings highlight the plausible toxigenic mechanism involved in the pathogenesis of NEC.

**Keywords.** necrotizing enterocolitis; *Clostridium butyricum*; gut microbiota; dysbiosis; toxins.

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Necrotizing enterocolitis (NEC) is the most common and serious acquired gastrointestinal disorder among preterm neonates. Its incidence rises to 12% in infants with very low birth weight (ie, 500–1000 g) [1]. The mortality associated with NEC ranges from 20% to 50% [2]. A high morbidity rate has also been reported; in particular, neurodevelopmental impairment is observed [1]. Low birth weight and prematurity have been identified

as critical risk factors for NEC [3]. Although intestinal immaturity and highly immunoreactive intestinal mucosa appear to be important elements of the disease, it could not be reproduced in germ-free animals, suggesting a critical role of microbial colonization [4]. Interestingly, NEC does not occur until at least 8–10 days postpartum in preterm neonates, the period when gut microbiota diversification and anaerobic bacteria colonization begins [3, 5]. To date, multiple causative organisms have been proposed, including viruses, *Staphylococcus* species, various gram-negative bacilli, and *Clostridium* species [6]. However, no etiologic organism has been definitively established.

The gut microbiota from preterm neonates both prior to and during NEC has recently been studied using molecular methods [6–8]. These studies suggested an association between the pattern of gut microbiota and the occurrence of NEC. An interindividual variation and overall reduction in the diversity of microbiota, especially when there has been a prolonged previous antibiotic therapy, was retrieved. However, using genomic tools induces several biases. Microbial diversities measured from the same sample as well as relative taxa abundances may differ depending on the choice of 16S ribosomal RNA (rRNA) gene variable region(s) studied [9]. There is also a depth bias in that clinically relevant bacteria such as *Salmonella Typhi* can be missed by sequence-based methods in concentrations  $<10^5$ /mL [10].

As microbial culturing is the only method to characterize the physiological properties of bacteria and evaluate their virulence expression, there has been a renewed interest in culture methods allowing the identification of species hitherto considered nonculturable [11]. A previous comprehensive approach has allowed the culture of 341 different bacterial species from 3 different stool samples with only 15% of the species overlapping, with the bacteria detected by concomitant pyrosequencing [10]. This work has facilitated the selection of optimal conditions and media for bacterial cultivation from stool samples.

In the present study, we compared the gut microbiota between preterm neonates with NEC and controls using complementary 16S rRNA pyrosequencing and culture-based methods. We then analyzed the potential role of *Clostridium butyricum*, our etiologic candidate, in the pathogenesis of NEC.

## METHODS

### Patients

Preterm neonates were recruited from 4 neonatal intensive care units (NICUs) in southern France between February 2009 and March 2013. All data were prospectively recorded. The ethics committee at the IFR48 approved the study protocol, and informed consent was obtained from the parents of all patients.

In the first part of the study, we included the first 15 case patients (Figure 1) with definite and advanced NEC, corresponding to Bell stages II (n = 10) and III (n = 5) [12]. Stool samples

were collected the day of the symptom's onset. We matched 15 control patients by sex, gestational age, birth weight, days of life, type of feeding, mode of delivery, and duration of previous antibiotic therapy (Supplementary Table 1). All controls were present in the same NICU at the same time as the cases of NEC. None of the selected patients had received probiotics or been included in therapeutic protocols before the stool sampling. All of them were negative for routine microbial investigation. Bacteria and viruses as well as *Clostridium difficile* toxins were screened on stool samples using standard methods as previously described [13].

In the second part of the study, we tested for the presence of *C. butyricum* 363 stool samples from preterm neonates with NEC (n = 93), preterm control neonates (n = 70), infants with gastroenteritis (n = 100), and control infants with no symptoms of gastroenteritis (n = 100). Patients with NEC included over a period of 48 months and sampled at the onset of the disease (Figure 1) were also matched with the same criteria as for the first part of the study to control preterm neonates (Supplementary Table 2). Stool samples from infants with and without gastroenteritis were collected independently at the pediatric ward, corresponding to external controls.

### Study Design and Procedures

#### Bacterial Composition of Stool Samples

We compared the bacterial composition of stool samples from 30 preterm neonates with and without NEC. Samples were tested by pyrosequencing of a 16S rRNA amplicon targeting the V6 region. This region was used because of its considerable sequence variability that distinguishes among most bacterial species [9]. The mothur program and the recommended procedure for trimming the 16S rRNA gene amplicon data were used [14]. This program was also used to determine Shannon diversity indices at the 0.02 level, based on operational taxonomic unit (OTU) sequences. The Ribosomal Database Project classifier was used for taxonomic assignments from phylum to genus [15]. Assignment at the species level from 16S rRNA pyrosequences was attempted using basic local alignment search tool (BLAST) similarity searches against the database from the National Center for Biotechnology Information (NCBI). The species level was defined with a minimum sequence identity of 98.7% as the best BLAST hit [16]. Raw data are available on the public databases NCBI/European Bioinformatics Institute (EBI) under BioProject ERP004064. In parallel, the same samples were analyzed by an original culture-based method. We inoculated stool samples into 54 preselected culture conditions. Anaerobic cultures were performed in an anaerobic chamber (AES Chemunex, Combourg, France). Identification of bacterial colonies was first performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) as previously described [17]. Isolates that were not identified by MALDI-TOF MS were submitted to molecular identification using 16S rRNA gene amplification and sequencing.

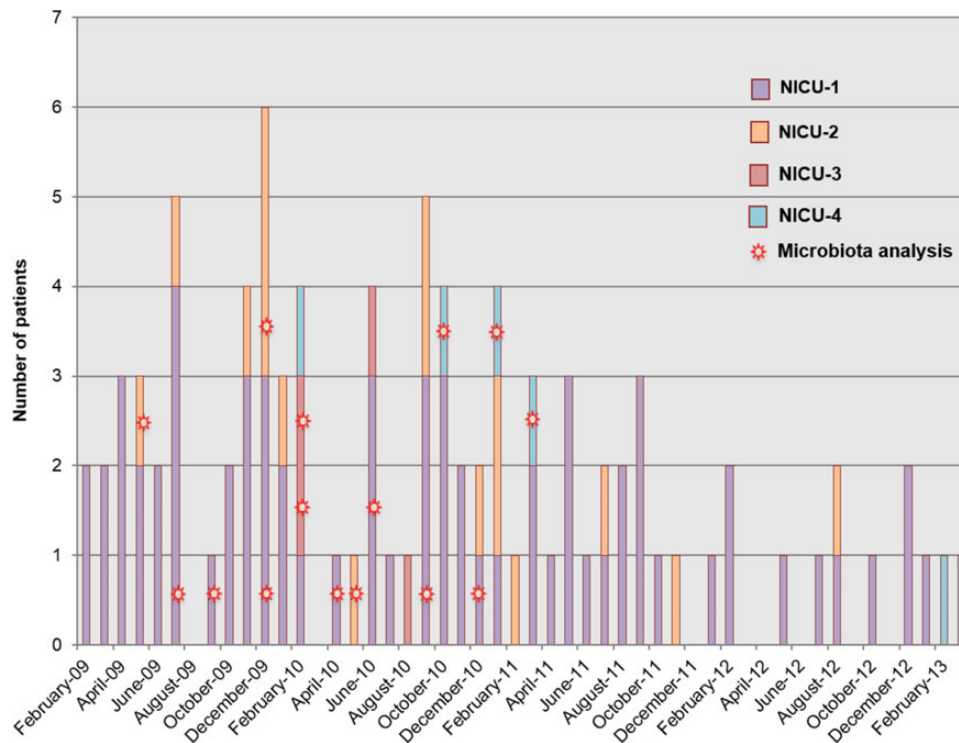


Figure 1. Time course of patients with definite necrotizing enterocolitis (Bell stage I, II, or III) from 4 geographically independent neonatal intensive care units (NICUs) during a period of 48 months. We highlighted patients for whom microbiota analysis was performed (by culture-based method using a developed technique [“microbial culturomic”] and a culture-independent method [16S ribosomal RNA gene-based pyrosequencing]) and *Clostridium butyricum* identified from their stool samples ( $n = 15$ ) in the first part of the study.

#### Analysis of Stool Samples for *C. butyricum*

We tested 363 stool samples, including the 30 samples studied before, for the presence of *C. butyricum* DNA with a specific quantitative polymerase chain reaction (qPCR) assay. For this, we developed and validated a specific qPCR assay for *C. butyricum* (Supplementary Figure 1 and Supplementary Table 4). In brief, all *rpoB* gene sequences from clostridia strains available in the NCBI GenBank database were collected and aligned using the ClustalW program [18]. One region of about 150 nucleotides with high divergence between *C. butyricum* and all other clostridia was selected. This sequence was analyzed with Primer3 software [19] to obtain primers and probes specific for *C. butyricum*.

#### Whole-Genome Sequencing of *C. butyricum* and Genotyping

We sequenced the whole genomes of all of our *C. butyricum* strains ( $n = 16$ ). We used Roche 454 pyrosequencing with a library of 3-kb paired-end fragments. Shotgun pyrosequencing was also performed using the Ion Torrent Personal Genome Machine sequencer. Reads were assembled with Newbler 2.7 and manually improved by using CLC genomics software. The whole genomes are available on the public databases NCBI/EBI (Supplementary Material). To distinguish different strains of *C. butyricum* at the molecular level, we performed

in silico multilocus sequence typing (MLST) as previously described [20]. For that, we analyzed the 11 whole genomes of *C. butyricum* strains accessible in the NCBI database together with our 16 strains. We selected 6 housekeeping genes present in all strains that exhibited the most variation between each strain (Supplementary Material). The relatedness between each MLST was shown as a dendrogram constructed by the unweighted pair group method with arithmetic mean. To retrieve potential toxins within the genome, we compared the sequences of all coding sequences (CDS) with an in-house database of 11 633 toxic proteins (Supplementary Figure 2). This database includes all existing proteins that are described as toxins in the Uniprot database [21]. Functional domains were also searched using the Pfam database [22] for each CDS related to a potential toxin. In parallel, we performed the proteomic analysis by nanoliquid chromatography of 1 randomly chosen *C. butyricum* strain from a patient with NEC. Tryptic peptides were separated on a nano-liquid chromatography system (Ultimate 3000, Thermo Fisher Scientific, Illkirch, France) and detected using a linear ion trap mass spectrometer (LTQ XL, Thermo Fisher Scientific). The MS spectra were computed using an In-Chorus search (Peaks 6.0, BSI) against *C. butyricum* sequences from internal annotation (4264 sequences, April 2013).

**Cytotoxic Activity of *C. butyricum* Strains Supernatant**  
 We evaluated the mean cytotoxic activity (experiment performed in triplicate) of the *C. butyricum* supernatants by flow cytometry. Supernatants were tested for cytotoxicity using a

commercial kit (SYTOX Green Dead Cell Stain, Invitrogen) and the Jurkat leukemia cell line. Analyses were performed on a flow cytometer (LSRFortessa Cell Analyzer; BD, Franklin Lakes, New Jersey). We adjusted the logarithmic amplification

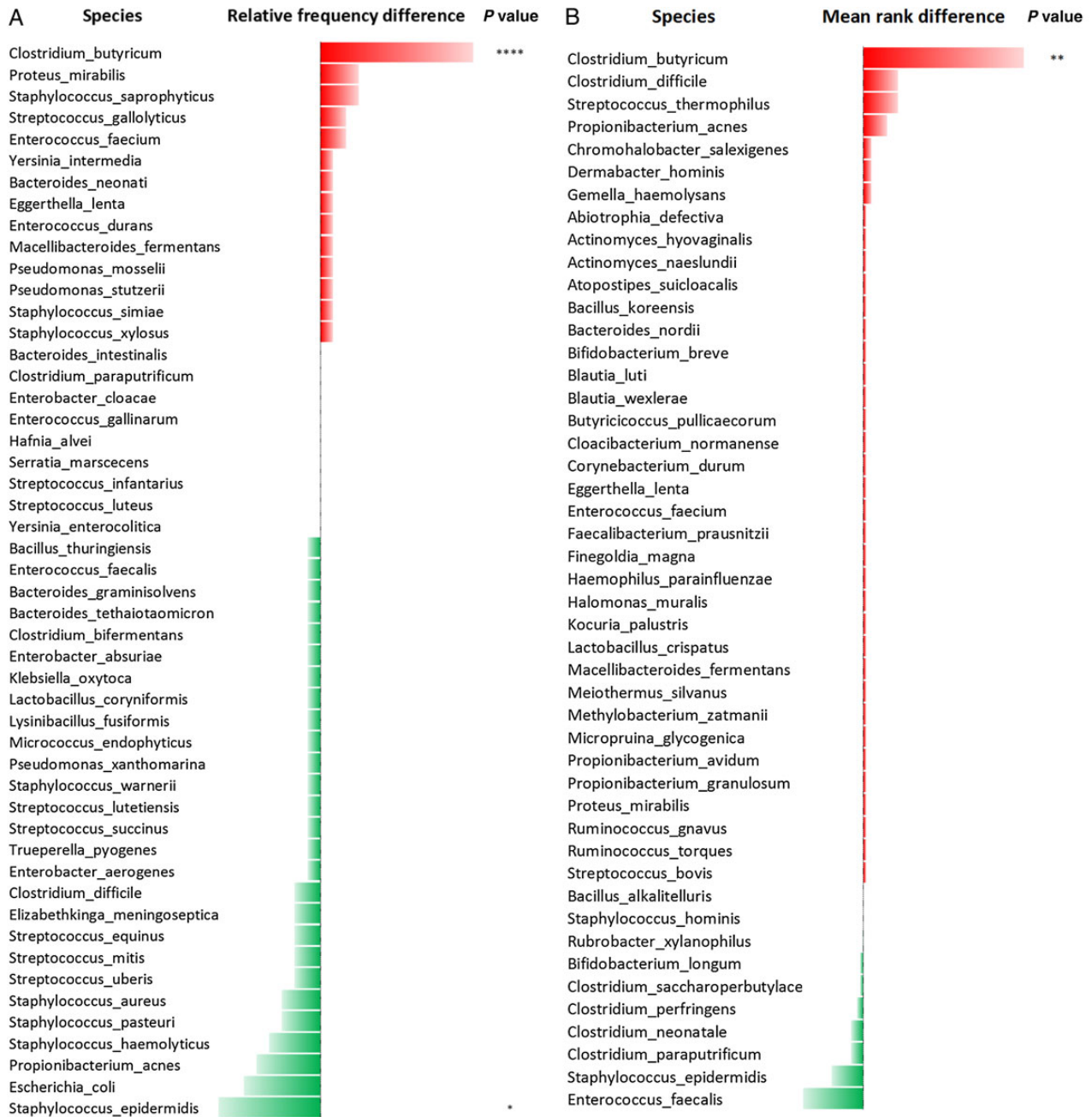


Figure 2. Species identified by culturomic and 16S ribosomal RNA (rRNA) pyrosequencing. A, The relative frequency of the species identified by culture was compared between patients with necrotizing enterocolitis (NEC) and controls. We used the Fisher exact test, as appropriate. *Clostridium butyricum* was significantly associated with NEC patients ( $P < .00001$ ), whereas *Staphylococcus epidermidis* was significantly associated with control patients ( $P < .01$ ). B, Mean rank values of the species identified by 16S rRNA pyrosequencing were compared between patients with NEC and controls. By this method, we took into account the frequency and the relative abundance of the reads. We used the nonparametric Mann–Whitney U test, as appropriate. *Clostridium butyricum* was significantly associated with NEC patients ( $P < .001$ ). \* $P < .05$ ; \*\* $P < .001$ ; \*\*\*\* $P < .00001$ .

scale to distinguish between viable cells from late apoptotic and/or necrotic cells with compromised plasma membranes as previously described [23]. We tested the culture supernatants of our 16 different *C. butyricum* strains by comparing them with a toxigenic *C. difficile* strain (positive control) and a nontoxigenic *C. difficile* strain (negative control) that were produced under the same conditions.

#### pH Values and Redox Potential of Stool Samples

We measured the pH values and the redox potential of all stool samples. One gram of stool was diluted in 10 mL of distilled water and centrifuged at 8000 rpm for 10 minutes. A pH and redox meter (PCE-228-R, PCE Instruments, Southampton, United Kingdom) was used according to the manufacturer's instructions.

#### Statistical Analysis

Continuous variables were summarized as the mean and standard deviation. For categorical variables, the percentages of patients in each category were calculated. Comparisons were made using the  $\chi^2$  test or Fisher exact test for categorical variables and Student t test or the Mann-Whitney U test for continuous variables, according to their distribution. A P value of <.05 was considered statistically significant. All analyses were performed with the R statistical package (version 3.1.2, Vienna, Austria). We used the Shannon index to compare diversity.

## RESULTS

### Patients

Ninety-three patients with NEC were included over a period of 48 months (Figure 1). Among the 15 patients for whom we analyzed the gut microbiota, 11 (73%) presented with specific radiological signs (pneumatosis intestinalis or portal venous air); all received antibiotic therapy, while 5 of them were treated by additional surgery, 4 (26.6%) had at least 1 episode of NEC recurrence, and 3 (20%) died.

### Bacterial Community Analysis Using 16S rRNA Pyrosequencing

Overall, we analyzed a total of 356 503 sequences from the 30 stool samples that clustered at 97% similarity levels to obtain 961 OTUs. A lower diversity was retrieved in samples from case patients (Supplementary Figure 3). At the phylum level, we observed a nonsignificant lower relative abundance of Proteobacteria in samples from case patients (35% vs 41%;  $P = .38$ ). At the genus level, we highlighted an interindividual variability with no hierarchical clustering between the samples from case patients and controls (Supplementary Figure 4). At the species level, this method yielded 47 different species. The species most frequently identified in stool samples from case patients was *Clostridium butyricum* (11/15 vs 2/15;  $P = .001$ ). By mean rank difference, taking into

account the relative abundance, we identified *C. butyricum* as the bacteria with the strongest association with the occurrence of NEC ( $P < .0001$ ). No other species were significantly enriched or depleted in case patients or controls (Figure 2). In terms of richness, we identified a higher mean number of OTUs in the 2 control samples positive with *C. butyricum* compared with those from case patients ( $236.5 \pm 51.6$  vs  $55.4 \pm 69.5$ ;  $P = .001$ ). We also distinguished case patients positive with *C. butyricum* from control patients negative with *C. butyricum*, based on their stool bacterial composition (Figure 3).

### Bacterial Species Identified by Culture

Overall, we obtained 3000 colonies by culture from the 30 stool samples, which yielded 51 different bacterial species including 9 (11%) that overlapped with the species identified by molecular method (Supplementary Figure 5). The richness corresponding to the number of species identified per sample was lower in case patients ( $5.4 \pm 1.2$  vs  $7.2 \pm 1.9$ ;  $P = .004$ ). The species most frequently isolated in stool samples from case patients was *C. butyricum* (14/15 vs 2/15;  $P < .0001$ ). By comparing the relative frequency of the species identified by culture, *C. butyricum* was significantly associated with the occurrence of NEC ( $P < .0001$ ), whereas *Staphylococcus epidermidis* was found to be associated with controls ( $P < .01$ ) (Figure 2). Combining

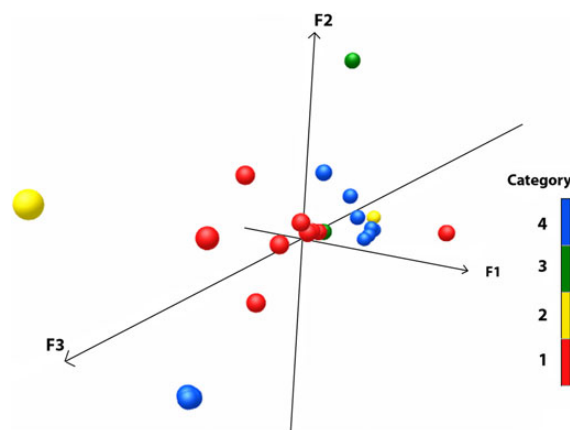


Figure 3. Link between the overall bacterial composition (excluding *Clostridium butyricum*), the presence of *C. butyricum* in stool samples, and the occurrence of necrotizing enterocolitis (NEC). Principal component analysis (Pearson [n]) using XLSTAT-3DPlot (Addinsoft, Paris, France) was performed on species raw data (number of reads) obtained by 16S ribosomal RNA pyrosequencing. Case patients with NEC and *C. butyricum* positive are shown in red (category 1); case patients with NEC and *C. butyricum* negative are shown in yellow (category 2); *C. butyricum*-positive controls are shown in green (category 3); *C. butyricum*-negative controls are shown in blue (category 4). *Clostridium butyricum*-positive case patients were grouped together and differed from *C. butyricum*-negative controls according to their stool bacterial composition: the first (F1), second (F2), and third (F3) components accounted for 32%, 14%, and 10%, respectively, of the overall variability.

Table 1. Clostridium butyricum–Specific Quantitative Real-time Polymerase Chain Reaction Testing Comparison Between Necrotizing Enterocolitis Samples and Overall Controls

Sample	Positive	Negative
Patients with NEC (n = 93)	74 (79.6)	19 (20.4)
Controls (n = 70)	9 (12.8)	61 (87.2)
Infants with gastroenteritis (n = 100)	7 (7)	93 (93)
Infants without symptoms (n = 100)	4 (4)	96 (96)

Data are presented as No. (%). Comparison showed a strong association between the occurrence of NEC and the presence of *C. butyricum* in the stool (odds ratio, 45.4 [95% confidence interval, 26.2–78.6];  $P < .0001$ , Pearson  $\chi^2$  test).

Abbreviation: NEC, necrotizing enterocolitis.

the results from 16S rRNA gene–based pyrosequencing and culture-based method, *C. butyricum* was present in all stool samples from patients with NEC.

Clostridium butyricum Prevalence and Loads Assessed by qPCR Of the 363 secondarily collected samples, 74 neonates with NEC (79.5%), 9 preterm control neonates (12.8%), 7 infants with gastroenteritis (7%), and 4 asymptomatic infants (4%) were positive for *C. butyricum* by qPCR (odds ratio, 45.4 [95% confidence interval, 26.2–78.6];  $P < .0001$ ; Table 1). Among positive samples, significantly higher loads of *C. butyricum* were found in stools from patients with NEC ( $P = .015$ ; Supplementary Figure 6). From the first part of the study, all but 1 case patient and the same 2 controls yielded a positive for *C. butyricum* via qPCR (14/15 vs 2/15;  $P < .0001$ ).

#### Clostridium butyricum Toxin Identification Using Whole-Genome and Proteomic Analysis

The comparison of the predicted proteins of *C. butyricum* from all our strains ( $n = 16$ ) against our dedicated database highlighted 4 proteins that were strikingly similar to already described toxins

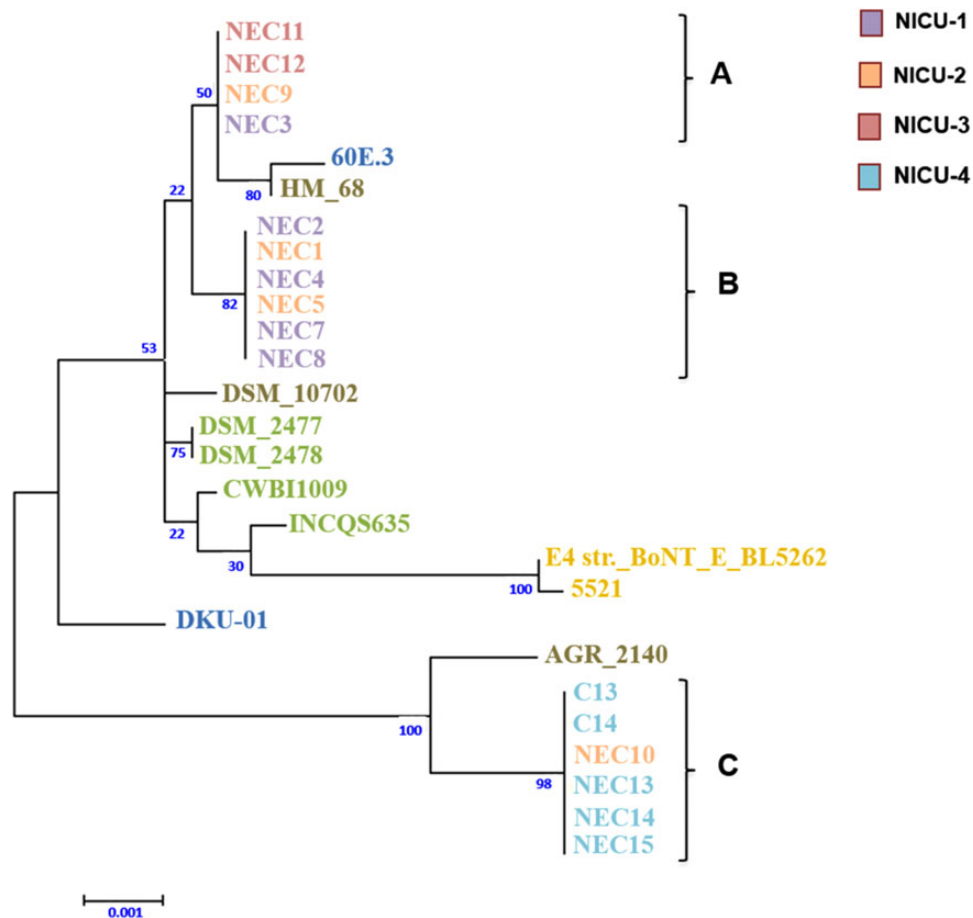


Figure 4. Genotyping by in silico multilocus sequence typing of 27 *Clostridium butyricum* strains. We distinguished 3 clades (A, B, and C) regrouping all our *C. butyricum* strains from patients with necrotizing enterocolitis (NEC). Represented in blue are strains from asymptomatic infant feces (60E.3) and adult gut (DKU-01); in green, strains from soil (INCQS635), cottonwood tree (DSM\_2477), lake sediment (DSM\_2478), and anaerobic sludge (CWBI1009); in brown, strains from chicken intestine (HM\_68), pig intestine (DSM\_10702), and cow rumen (AGR\_2140); in yellow, strains from infants with botulism (E4 str. BoNT\_E\_BL5262 and 5521). Abbreviation: NICU, neonatal intensive care unit.

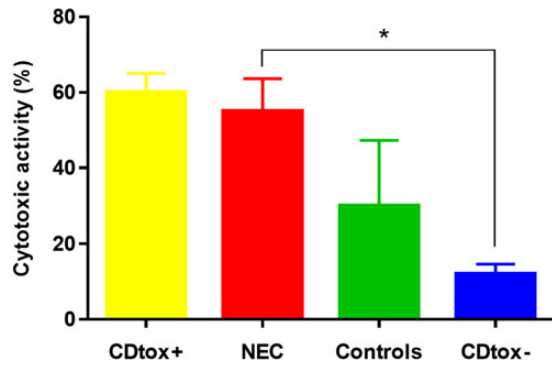


Figure 5. Cytotoxic activity on Jurkat cells tested by flow cytometry. Clostridium butyricum strains from patients with necrotizing enterocolitis (NEC; n = 14) and controls (n = 2) were tested. Clostridium butyricum strains from patients with NEC had a significantly higher cytotoxic activity compared with the nontoxic Clostridium difficile strain (P = .008). We used the unpaired t test with Welch correction. Analyses were performed on a flow cytometer (LSRFortessa CellAnalyzer), and we used a commercial kit for staining (SYTOX Green Dead Cell Stain, Invitrogen). \*P < .05. Abbreviations: CDtox+, toxigenic Clostridium difficile (positive control); CDtox-, nontoxic Clostridium difficile (negative control).

in *Brachyspira hyodysenteriae* [24]: hemolysin A (tlyA gene, Uniprot: HLYA\_TREHY), hemolysin B (tlyB gene, Uniprot: HLYB\_TREHY), hemolysin C (tlyC gene, Uniprot: HLYC\_TREHY) and  $\beta$ -hemolysin (hlyA gene, Uniprot: ACP\_TREHY) (Supplementary Table 5). The functional domains were highly conserved.

We then performed the proteomic analysis of 1 randomly chosen *C. butyricum* strain from a patient with NEC. The nano-liquid chromatography–tandem mass spectrometry (nano-LC-MS/MS) analysis of the secretome permitted identification of 1 of the target *C. butyricum* putative toxins. Two

unique peptides corresponded to 24% sequence coverage with the hlyA  $\beta$ -hemolysin shared by *B. hyodysenteriae*. Each peptide was detected with 3 and 7 MS/MS spectra, respectively. This was consistent with the presence of a hlyA-like protein secreted by *C. butyricum*.

#### Genotyping by In Silico MLST

Phylogenetic analysis distinguished 3 clades of *C. butyricum* strains from preterm neonates with NEC (Figure 4).

#### Cytotoxic Activity

The mean cytotoxic activity of the 14 *C. butyricum* strains from patients with NEC was significantly higher than that of a nontoxic *C. difficile* strain (P = .008; Figure 5).

#### Oxidation Reduction Potential and pH Level Testing

There was a higher redox potential (P < .0001) and lower pH levels (P < .0001) in stools from patients with NEC (n = 93) compared with controls (n = 70). Oxidative stress and acidity were then associated with the occurrence of NEC (Figure 6).

## DISCUSSION

Our results showed strong evidence of an association between the presence of cytotoxic *C. butyricum* strains in stools from preterm neonates, dysbiosis, and the occurrence of NEC. Multiplication of culture conditions and use of optimized anaerobic conditions (anaerobic chamber) led us to identify a large number of bacterial species by culture. The use of the V6 region for 16S rRNA pyrosequencing, which has been recognized to be the best for detection of clostridia [9], allowed us to identify 6 different clostridial species (*C. butyricum*, *C. difficile*, *C. paraputrificum*, *C. bifermentans*, *C. perfringens*, and *C. neonatale*). Moreover, the low diversity within the neonatal intestine

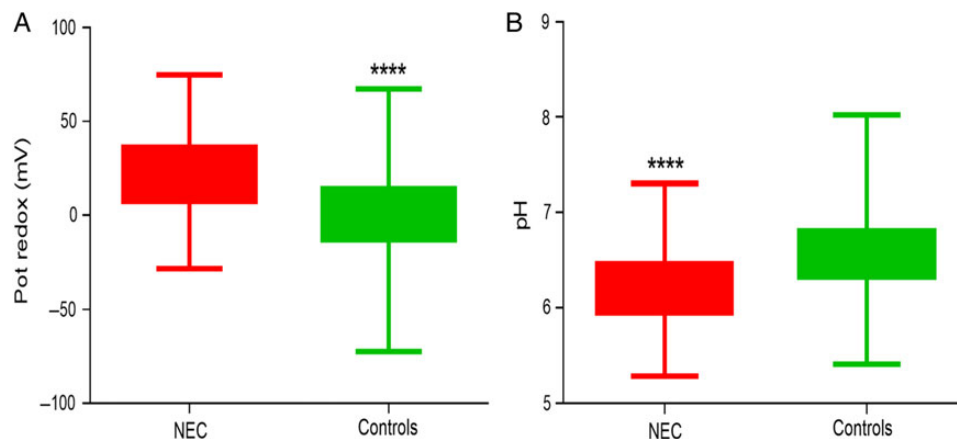


Figure 6. Oxidized and acid gut environment associated with necrotizing enterocolitis (NEC) There was a significantly higher redox potential (A) and lower pH levels (B) in stools from patients with NEC. We used the nonparametric Mann–Whitney U test. \*\*\*\*P < .00001.



made it possible to study gut microbial communities at a high level of resolution [5]. We finally identified a specific presence of *C. butyricum* in stool samples from patients with NEC. We confirmed our results by testing a larger population of preterm neonates with NEC (n = 93), among which 79.5% were positive with a qPCR assay specific for *C. butyricum*. The population matching led us to strengthen the role of *C. butyricum* in the occurrence of NEC independent of intrinsic factors. However, other clostridial species besides *C. butyricum* have been significantly associated with the occurrence of NEC (ie, *C. perfringens*, *C. neonatale*) [6, 25].

The first description of the association between *C. butyricum* and NEC was described in 1977. Howard et al identified *C. butyricum* in blood cultures of 9 of 10 preterm neonates during an outbreak of NEC [26]. Recently, Smith et al detected the presence of *C. butyricum* with high density in 2 surgical samples from preterm neonates with NEC. Both specimens were characterized by histological pneumatosis intestinalis [27]. Several experimental studies showed in animal models the capacity of *C. butyricum* to reproduce NEC-like lesions [28–32]. We demonstrated a significant cytotoxic activity of the *C. butyricum* strain's supernatants from case patients (Figure 5) and identified 4 genes encoding for polypeptides that are highly similar to hemolysins shared by *B. hyodysenteriae*, the etiologic agent of swine dysentery [24], one being described as the main virulence factor [33]. Further studies are currently in progress with Caco-2 cells that are likely more relevant to study bacterial adherence and enteropathogenicity. The fact that we also isolated *C. butyricum* with significant cytotoxic activity from stool samples of 2 asymptomatic preterm neonates and also identified our putative toxin gene in the genome of those strains suggests a similar pathogenesis to that of toxigenic *C. difficile* that associates asymptomatic carriage and multiple clinical presentations ranging from asymptomatic carriage to life-threatening disease [34]. Studies on factors influencing toxin expression in *C. difficile* revealed complex mechanisms involving the microbiota ecosystem, host immune, nutritional and metabolic status, and particular virulent strain proliferation [35]. Similarly, important conditions must be met to cause NEC that could explain variable susceptibility in preterm neonates and different stages of severity [3]. A recent study showed that preterm neonates' *C. butyricum* colonization incidence increased during hospitalization, suggesting an essential role of the environment and nursing conditions [36]. This is consistent with the occurrence of outbreaks of NEC cases and the effectiveness of implemented infection control measures [37]. Perinatal exposure to antibiotics has been identified as an independent risk factor for NEC, this risk being proportional to the duration of exposure and related to substantial microbiota modifications [38]. In this study, we described that *C. butyricum* loads in stools from case patients were significantly higher than those of controls, and this was linked with dysbiosis associated

with reduced bacterial richness and diversity in stool samples. Finally, accumulating data suggest that host immature immunity and oxidative stress play a major role in NEC pathogenesis [3]. We highlighted here the association between oxidative stress and the occurrence of NEC (Figure 6). Notably, it has been demonstrated that spores of *C. butyricum* were able to germinate and outgrow in highly oxidized and acid environments [39].

In conclusion, our findings show a strong association between NEC and the presence of cytotoxic *C. butyricum* strains in an oxidized, acid, and abnormally poorly diversified gut microbiota. Whether this result is part of a geographically restricted outbreak or could be generalized to other parts of the world warrants further investigation. Moreover, the plausible toxigenic mechanism involved in the pathogenesis of NEC needs further confirmation. Similarly, with *C. difficile* and pseudomembranous colitis, the identification of toxins in patients' stool or intestinal tissue and demonstration of its effects will be essential for determining the causal relationship between toxigenic *C. butyricum* strains and NEC.

#### Supplementary Data

Supplementary materials are available at Clinical Infectious Diseases online (<http://cid.oxfordjournals.org>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

#### Notes

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