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Gut bacteria dysbiosis and necrotising enterocolitis in very low birthweight infants: a prospective case-control study

Prof Barbara B Warner, MD,

Department of Pediatrics, Washington University in St Louis School of Medicine, St Louis, MO, USA

Elena Deych, MS,

Department of Medicine, Washington University in St Louis School of Medicine, St Louis, MO, USA

Yanjiao Zhou, MD,

Department of Pediatrics, Washington University in St Louis School of Medicine, St Louis, MO, USA

Carla Hall-Moore, BS,

Department of Pediatrics, Washington University in St Louis School of Medicine, St Louis, MO, USA

Prof George M Weinstock, PhD,

McDonnell Genome Institute, Washington University in St Louis School of Medicine, St Louis, MO, USA

Erica Sodergren, PhD,

McDonnell Genome Institute, Washington University in St Louis School of Medicine, St Louis, MO, USA

Nurmohammad Shaikh, PhD,

Department of Pediatrics, Washington University in St Louis School of Medicine, St Louis, MO, USA

Julie A Hoffmann, RN,

Department of Pediatrics, Washington University in St Louis School of Medicine, St Louis, MO, USA

Laura A Linneman, RN,

Correspondence to Prof Phillip Tarr, Department of Pediatrics, Washington University in St Louis School of Medicine, St Louis, MO 63110, USA, tarr@wustl.edu or Prof William D Shannon, Department of Medicine, Washington University in St Louis School of Medicine, St Louis, MO 63110, USA, wshannon@dom.wustl.edu.

Contributors

BBW and PIT led the study design. ED, BAS, and WDS did the statistical analysis. GMW and ES led the sequencing effort. YZ analysed the resulting sequences. BBW, JAH, LAL, ME, JES, PGR, and AH supervised enrolment and managed specimen and data collection. GK read study radiographs. PIT supervised the laboratory efforts, assisted by CH-M, LCR-N, NS, and IMN. BBW and PIT wrote the first manuscript draft, and all authors contributed to manuscript revision.

Declaration of interests

We declare no competing interests.

Department of Pediatrics, Washington University in St Louis School of Medicine, St Louis, MO, USA

Aaron Hamvas, MD,

Department of Pediatrics, Washington University in St Louis School of Medicine, St Louis, MO, USA

Geetika Khanna, MD,

Department of Radiology, Washington University in St Louis School of Medicine, St Louis, MO, USA

Lucina C Rouggy-Nickless, BA,

Department of Pediatrics, Washington University in St Louis School of Medicine, St Louis, MO, USA

I Malick Ndao, MD,

Department of Pediatrics, Washington University in St Louis School of Medicine, St Louis, MO, USA

Berkley A Shands, BS,

Department of Medicine, Washington University in St Louis School of Medicine, St Louis, MO, USA

Prof Marilyn Escobedo, MD,

Department of Pediatrics, University of Oklahoma School of Medicine, Oklahoma City, OK, USA

Prof Janice E Sullivan, MD,

Department of Pediatrics, University of Louisville School of Medicine, Louisville, KY, USA

Paula G Radmacher, PhD,

Department of Pediatrics, University of Louisville School of Medicine, Louisville, KY, USA

Prof William D Shannon, PhD, and

Department of Medicine, Washington University in St Louis School of Medicine, St Louis, MO, USA

Prof Phillip I Tarr, MD

Department of Pediatrics, Washington University in St Louis School of Medicine, St Louis, MO, USA

Department of Molecular Microbiology, Washington University in St Louis School of Medicine, St Louis, MO, USA

Summary

Background—Gut bacteria might predispose to or protect from necrotising enterocolitis, a severe illness linked to prematurity. In this observational prospective study we aimed to assess whether one or more bacterial taxa in the gut differ between infants who subsequently develop necrotising enterocolitis (cases) and those who do not (controls).

Methods—We enrolled very low birthweight (1500 g and lower) infants in the primary cohort (St Louis Children's Hospital) between July 7, 2009, and Sept 16, 2013, and in the secondary cohorts

(Kosair Children's Hospital and Children's Hospital at Oklahoma University) between Sept 12, 2011 and May 25, 2013. We prospectively collected and then froze stool samples for all infants. Cases were defined as infants whose clinical courses were consistent with necrotising enterocolitis and whose radiographs fulfilled criteria for Bell's stage 2 or 3 necrotising enterocolitis. Control infants (one to four per case; not fixed ratios) with similar gestational ages, birthweight, and birth dates were selected from the population after cases were identified. Using primers specific for bacterial 16S rRNA genes, we amplified and then pyrosequenced faecal DNA from stool samples. With use of Dirichlet multinomial analysis and mixed models to account for repeated measures, we identified host factors, including development of necrotising enterocolitis, associated with gut bacterial populations.

Findings—We studied 2492 stool samples from 122 infants in the primary cohort, of whom 28 developed necrotising enterocolitis; 94 infants were used as controls. The microbial community structure in case stools differed significantly from those in control stools. These differences emerged only after the first month of age. In mixed models, the time-by-necrotising-enterocolitis interaction was positively associated with Gammaproteobacteria ($p=0.0010$) and negatively associated with strictly anaerobic bacteria, especially Negativicutes ($p=0.0019$). We studied 1094 stool samples from 44 infants in the secondary cohorts. 18 infants developed necrotising enterocolitis (cases) and 26 were controls. After combining data from all cohorts (166 infants, 3586 stools, 46 cases of necrotising enterocolitis), there were increased proportions of Gammaproteobacteria ($p=0.0011$) and lower proportions of both Negativicutes ($p=0.0013$) and the combined Clostridia–Negativicutes class ($p=0.0051$) in infants who went on to develop necrotising enterocolitis compared with controls. These associations were strongest in both the primary cohort and the overall cohort for infants born at less than 27 weeks' gestation.

Interpretation—A relative abundance of Gammaproteobacteria (ie, Gram-negative facultative bacilli) and relative paucity of strict anaerobic bacteria (especially Negativicutes) preceded necrotising enterocolitis in very low birthweight infants. These data offer candidate targets for interventions to prevent necrotising enterocolitis, at least among infants born at less than 27 weeks' gestation.

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Introduction

Necrotising enterocolitis is a catastrophic necro-inflammatory injury to the intestines in very low birthweight infants. Its incidence (5–10% of very low birthweight infants), mortality (about 25%), and treatment (bowel resection for severe cases) have changed little in more than three decades.¹ Necrotising enterocolitis does not occur in utero, and is positively associated with exposure to antibiotics^{2–4} and negatively associated with exposure to human milk⁵ and probiotics.⁶ These observations underlie the long-standing belief that necrotising enterocolitis and gut bacteria are causally associated.

Stool cultures cannot quantify individual fastidious bacteria and so culture-independent analyses have been used to study gut microbes in children at risk for necrotising enterocolitis. Improved technologies and databases have enabled direct sequencing and

interpretation of bacterial DNA from stools, but researchers using these methods have reported either no correlation between bacteria and necrotising enterocolitis,⁷⁻⁹ incriminated disparate organisms such as Gram-negative bacilli,¹⁰⁻¹³ Actinobacteria,¹³ Clostridia^{11,12} and other Firmicutes,¹⁰ or suggested age-dependent differences between cases and controls.¹¹ Additionally, certain taxa (Propionibacteria,¹⁰ Bifidobacteria, and Bacteroidetes¹³) have been proposed as preventers of development of necrotising enterocolitis. However, these studies analysed sequences from a median of only three stools (IQR two to seven) of infants before they developed necrotising enterocolitis.

There are many sampling challenges encountered by investigators attempting to define causative or protective bacterial communities in necrotising enterocolitis. Necrotising enterocolitis occurs unpredictably throughout the first 2 months of age,¹⁴ a period during which gut bacteria assemble into consensus communities in children who do not develop necrotising enterocolitis (rapidly rising then slowly declining proportions of Gammaproteobacteria and increasing proportions of anaerobic bacteria) at rates that vary according to duration of gestation at birth. Also, despite this non-random choreographed progression, sudden population shifts are common.¹⁵ Moreover, the variable frequency of stool production within and between infants of this age results in non-uniformly timed sample sets before the onset of necrotising enterocolitis. Hence, dynamic gut bacterial communities and the widely varying frequency with which stool can be obtained necessitate studying many samples from many very low birthweight infants to determine if dysbiosis precedes, and therefore might cause, necrotising enterocolitis. Finally, the resulting data must undergo statistical testing that accounts for both the broad span of gestational ages at birth among at-risk infants, the extended risk of necrotising enterocolitis over the first several months of age, diverse clinical data such as antibiotic use and feeds, and repeated measures from the same individuals. It is likely that these challenges contributed to differing conclusions in previous studies, which were drawn on relatively few pre-event samples.

In this three-site prospective case-control study, we describe an intensive culture-independent analysis of a large specimen set using a mixed model approach to control for confounding clinical variables from infants at risk for necrotising enterocolitis. We aimed to establish whether development of necrotising enterocolitis is associated with altered microbial content (dysbiosis).

Methods

Study design and participants

We used a prospective cohort design, within which we nested a case-control study. Our study population was formed of a primary cohort from St Louis Children's Hospital (St Louis, MO, USA) and secondary cohorts from Children's Hospital at Oklahoma University Medical Center (Oklahoma City, OK, USA) and Kosair Children's Hospital (Louisville, KY, USA), hereafter termed the St Louis, Oklahoma City, and Louisville cohorts, respectively. Secondary cohorts were included in the initial study design to increase case numbers and to serve as validation cohorts. Each site's institutional review board approved this study. Written informed consent was obtained from parents before enrolment.

Study structure, inclusion and exclusion criteria, and specimen handling protocols used in the St Louis cohort are as described in previous studies.^{15,16} Briefly, all infants of 1500 g birthweight or lower who were expected to survive beyond 1 week and who were hospitalised in a neonatal intensive-care unit at the study sites were considered eligible for enrolment. We excluded from analysis infants with complex congenital heart disease or spontaneous intestinal perforation without radiographic evidence of necrotising enterocolitis¹⁷ unless it subsequently ensued.

Procedures

All stools produced by these infants were collected and refrigerated at 4°C before freezing at -80°C. Stool collection began on admission to the neonatal intensive-care units, with parents approached for consent as soon as possible. If consent was not obtained, collected samples were destroyed. All stools were collected by nurses and frozen by study staff before knowing which infants would develop necrotising enterocolitis.

Cases were defined as infants whose clinical courses were consistent with necrotising enterocolitis and whose radiographs fulfilled criteria (confirmed by a study radiologist) for Bell's stage 2 or 3 necrotising enterocolitis.¹⁸ Controls (one to four per case; not fixed ratios) were selected after cases were identified and were chosen to correspond to cases from the same centre based on gestational age (plus and minus 1 week), birthweight (plus and minus 100 g), and birth date (details in appendix).

We analysed DNA extracted from all frozen stools from cases that had been collected up to and including the day before necrotising enterocolitis was diagnosed or 60 days of age, whichever occurred first. We analysed all frozen stools from controls up to age 30 days, and then every third stool thereafter, up to and including day of age 60 days. We sequenced V3 to V5 regions of the 16S rRNA gene on a Roche 454 platform, and classified reads using the Ribosomal Database Project Naive Bayesian Classifier version 2.5, training set 9. Bifidobacterial proportions were verified with quantitative PCR. Control stool sampling strategies and methods for sequencing all specimens and PCR quantification of Bifidobacteria are described in the appendix. Sequence and host data have been deposited in the NCBI dbGap (accession number phs000247.v5.p3) and BioProject (PRJNA46337).

Statistical analysis

For demographic and clinical features of case and control populations, we used the Student's *t* test or the Mann-Whitney test to identify statistically significant differences in continuous variables, and the χ^2 or Fisher's exact test to identify differences in categorical variables. We used the generalised Wald-type test for Dirichlet multinomial distribution to determine if composite microbial community structure in stools pre-event differed from that in stools of controls.¹⁹ The four 15 day analysis intervals are detailed in the appendix and include 1–15, 16–30, 31–45, and 46–60 days of age.

We then used mixed models accounting for repeated measures to determine if case or control status or other host variables (appendix) were associated with bacterial content in the serially collected stools. We compared Shannon diversity indices for bacterial content of stools in each analysis interval between cases and controls using mixed models, adjusting for

gestational age and the specific analysis interval in which specimens were produced.²⁰ For analyses we used SAS version 9.3 and R statistical software. We considered two-tailed *p* values of less than 0.05 as significant.

Role of funding sources

The funders had no role in study design, collection, analysis, or interpretation of data, or in writing this paper. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

We prospectively enrolled 972 infants (489, 276, and 207 infants from the three respective cohorts) over 4 years (primary cohort) or 2 years (secondary cohorts). The first participants were enrolled on July 7, 2009, in St Louis; Sept 12, 2011 in Oklahoma City; and Oct 3, 2011, in Louisville. The last participants were enrolled in this study on Sept 16, 2013, in St Louis, May 25, 2013, in Oklahoma City, and May 20, 2013, in Louisville. 58 (6%) of 972 infants developed necrotising enterocolitis; 21 (36%) of these infants died (appendix).

Infants born at less than 27 weeks' gestation (*n*=368) developed necrotising enterocolitis more frequently (37 [10%] vs 19 [3%]; *p*=0.0002) and later (median age of occurrence 31 days [IQR 23–49] vs 21 days [IQR 18–25]; *p*=0.003) than did those born after this gestational age (*n*=604).

We had sufficient pre-necrotising enterocolitis stools from 28, 13, and five infants (cases) from the St Louis, Oklahoma City, and Louisville cohorts, respectively, and compared these specimens to stools from 94, 20, and six infants (controls), from these respective cohorts. From the 166 infants in the three cohorts, we analysed DNA sequences from a total of 3586 stools, with a median of 6078 (IQR 4748–7600) reads per specimen, and a mean of 466 nucleotides per read.

The 28 cases in the primary (St Louis) cohort, compared with the 94 controls, were born after shorter gestations, weighed less at birth, had lower 5 min Apgar scores, were more frequently of black race, were more likely to die before discharge, and received more antibiotics and blood transfusions (table 1).

At the class level, Bacilli (largely Gram-positive cocci), Gammaproteobacteria (facultative Gram-negative bacilli), and Clostridia and Negativicutes (obligate anaerobes) accounted for 92% of reads from case stools and 93% of reads from control stools (figure 1). Except for Clostridia, within each class the same three genera (or family if a read could not be assigned to a genus) predominated independent of case or control status (figure 2; appendix). Dirichlet multinomial comparisons showed statistically significant differences in composite community structure at analysis intervals three (31–45 days of age; *p*=0.0035) and four (46–60 days of age; *p*=0.0001; figure 1; appendix), with samples from cases having greater proportions of Gammaproteobacteria and lower proportions of Clostridia and of Negativicutes than controls.

To account for postmenstrual age influence on the kinetics of gut microbial community assembly,¹⁵ microbial content was examined separately for infants born before 27 weeks' gestation or at or after 27 weeks' (this demarcation was chosen because the median duration of gestation for the subjects in this cohort was 26.7 weeks). After this stratification, Dirichlet multinomial distributions differed significantly between cases and controls in analysis intervals three (31–45 days; $p=0.0370$) and four (46–60 days; $p=0.0016$), but only for infants born at less than 27 weeks' gestation. Only one infant born at or after 27 weeks' gestation developed necrotising enterocolitis after day 30, thereby precluding Dirichlet multinomial comparison in analysis intervals three and four (figure 1; appendix).

We used mixed model analysis taking into account the repeated measures from the same individuals for the entire observation period to identify clinical factors associated with microbial differences. Greater gestational age at birth was associated with higher proportions of Negativicutes ($p=0.0079$) and the combined Clostridia–Negativicutes class ($p=0.0004$), and lower proportions of Bacilli ($p=0.0006$). Vaginal birth was associated with lower proportions of Bacilli ($p=0.0022$). Greater antibiotic exposure, which corresponds to higher mean proportion of days within an analysis interval that an infant received an antibiotic, was associated with higher proportions of Bacilli ($p=0.0212$), and lower proportions of Clostridia ($p=0.0142$), and the combined Clostridia–Negativicutes class ($p=0.0009$; table 2). Inotrope use, number of transfusions administered, and proportion of human milk feeds did not predict bacterial composition in the mixed models (table 2).

The mixed model also examined time-dependent factors associated with bacterial community progression. In both cases and controls, the fractions of Bacilli ($p<0.0001$) fell over time, whereas fractions increased of Gammaproteobacteria ($p=0.0001$), Clostridia ($p=0.0003$), and the combined Clostridia–Negativicutes class ($p<0.0001$). However, and most importantly, there were significant time-by-necrotising-enterocolitis interaction factors for the progression over time of Gammaproteobacteria, Negativicutes, and the combined Negativicutes–Clostridia class. Relative to control stools, case stools showed significant increases in Gammaproteobacteria ($p=0.0010$) over time, and decreases in Negativicutes ($p=0.0019$) and the combined Clostridia–Negativicutes class ($p=0.0010$). These time-by-necrotising enterocolitis interaction effects in the mixed models were largely limited to infants born at less than 27 weeks' gestation, with such cases being significantly associated with Gammaproteobacteria content ($p=0.0066$), and controls being associated with Negativicutes ($p=0.0300$), and the combined Clostridia–Negativicutes class ($p=0.0201$). For infants born at or after 27 weeks' gestation, the only significant interaction was for the combined Clostridia–Negativicutes class increasing over time among controls ($p=0.0279$; appendix).

Bifidobacteria, often used as probiotics, represented 1.2% of reads in case stools and 0.3% in control stools. PCR enumeration, done because of concerns that pyrosequencing might undercount Bifidobacteria,¹³ was in high agreement with pyrosequencing results (appendix).²¹

In a multivariate mixed model predicting the Shannon diversity index, adjusting for gestational age at birth, route of delivery, and birthweight, there was a significant time-by-

necrotising-enterocolitis interaction indicating discordant trends in bacterial diversity in stools from cases versus controls (figure 3; $p=0.0004$ for interaction). In a model stratified by occurrence of necrotising enterocolitis, diversity of bacterial content significantly increased in stools from controls ($p<0.0001$), but not from cases.

The bacterial classes and genera that predominated in St Louis cohort stools also predominated in the secondary cohorts (Louisville and Oklahoma City; appendix), and when all sites were amalgamated (appendix). In the secondary cohorts, stools were available for a median of only four cases per analysis interval (vs 14.5 cases per analysis interval in the primary cohort), preventing meaningful intrasite and intersite comparisons of case and control stools. However, when all cohorts were combined in a single mixed model, four associations became statistically significant: days on antibiotics versus diminishing Gammaproteobacteria content, gestational age at birth versus increasing Clostridia content, vaginal delivery versus increasing Clostridia content, and analysis interval during which samples were obtained versus increasing Negativicutes content. By contrast, only one association lost statistical significance: days on antibiotics versus diminishing proportion of Clostridia. Most importantly, the time-by-necrotising-enterocolitis interaction remained significantly associated with increased proportions of Gammaproteobacteria and decreased proportions of both Negativicutes and the combined Clostridia–Negativicutes class (table 2; appendix).

Discussion

The over-representation of Gram-negative facultative bacilli and potentially pathogenic organisms such as *Escherichia coli*, *Enterobacter*, and *Klebsiella* and the under-representation of obligate anaerobic bacteria, in particular Negativicutes and Clostridia, in infants' guts before necrotising enterocolitis develops are consistent with the hypothesis that dysbiosis precedes this severe event. The time-by-necrotising-enterocolitis interaction, reflecting the different rates of change in bacterial composition over time in serially collected stools from cases and controls, best portrays this Gammaproteobacteria–Clostridia–Negativicutes dysbiosis. This association is strongest in infants born at less than 27 weeks' gestation, and does not emerge until after the first month of age.

Our data show the need for exceptionally large patient and specimen sets to identify dysbiosis with confidence before a clinical event of interest. Indeed, the many ambiguities about microbial associations with necrotising enterocolitis might reflect the smaller scale of previous studies,⁷⁻¹³ a limitation that we designed our analysis of 1080 pre-event stools from 46 children who developed necrotising enterocolitis to overcome.

Even though we identified microbial class signatures associated with necrotising enterocolitis before it occurred, conclusions are necessarily imperfect because we cannot discount the alternative hypothesis that dysbiosis reflects, rather than causes, an infant's risk for this outcome. Nevertheless, several lines of evidence lend pathophysiological credence to the roles played by Gammaproteobacteria and by anaerobic bacteria. In animal models, Gram-negative bacteria elicit injury to the bowel that resembles necrotising enterocolitis,²² and antibiotics active against Gram-negative bacteria diminish mucosal injury.²³ Toll-like

receptor 4, which binds to bacterial lipopolysaccharide, is implicated in cellular processes that could underlie necrotising enterocolitis.^{24,25} Anaerobic bacteria, responding to microbiota-accessible carbohydrates,²⁶ produce anti-inflammatory short-chain fatty acids, especially acetate, propionate, and butyrate.²⁷ We hope our work stimulates mechanistic studies of necrotising enterocolitis using cellular and animal models, and moves the field beyond observational cohort studies and, ultimately, into microbiologically informed interventions. Relevant candidate bacterial drivers and attenuators of gut injury will be valuable reagents in these endeavours.

Human data indirectly corroborate our findings. Oral aminoglycosides, which reduce populations of Gram-negative gut bacteria²⁸ but do not inhibit anaerobic bacteria, have conferred protection against necrotising enterocolitis in clinical studies in populations similar to those reported here.²⁹ Bacterial gut community structure is intrinsically associated with degree of prematurity and age since birth,¹⁵ which are unequivocal epidemiological risk factors for necrotising enterocolitis. Clostridia become increasingly abundant as infants approach the equivalent of 36 weeks' post-menstrual age, but the rate of community assembly varies inversely according to the degree of prematurity at birth, and putatively harmful Gammaproteobacteria predominate longer in the most premature infants.¹⁵ Antibiotic use, a risk factor for necrotising enterocolitis, was significantly associated with lower proportions of potentially protective anaerobes, although unexpectedly also associated with lower proportions of Gammaproteobacteria. Our finding that case versus control status was associated with imbalanced proportions of Gammaproteobacteria and Clostridia–Negativicutes is, therefore, consistent with the worldwide epidemiology of necrotising enterocolitis, at least among the most premature infants.

Our data also support the hypothesis that necrotising enterocolitis might be related to lack of gut bacterial diversity.³⁰ However, because more than 90% of bacteria in the stool samples of cases and controls belonged to only four taxa, there are constraints on the degrees of freedom in which populations can differ at the class level. Hence, a change in fractional representation of a single taxon will produce a major change in bacterial diversity. Therefore, we cannot clearly attribute case or control status to changes in diversity in itself, versus changes in the ratios of the four taxa that define diversity in these non-complex communities. Also, we note that differences between cases and controls largely reflect maturation of diversity in control stool samples. That is, in controls, the decreasing proportions of Bacilli over time are counterbalanced by increasing proportions of anaerobes and stabilisation of Gammaproteobacteria but in infants that went on to develop necrotising enterocolitis bacterial populations do not show statistically convincing temporal changes in diversity. Our data also provide a microbiological explanation for the recent failure of the probiotic *Bifidobacterium breve* BBG-001 to prevent necrotising enterocolitis in a large and rigorous multicentre randomised controlled trial in the UK.³¹ Specifically, we found no evidence that Bifidobacteria were under-represented in the stools of infants who subsequently developed necrotising enterocolitis.

The associations between dysbiosis and necrotising enterocolitis among infants born at less than 27 weeks' gestation and divergence of bacterial content between cases and controls only after the first month of age warrant comment. Incidence and case fatality rates from

necrotising enterocolitis are highest in the most premature infants. Hence, the most apparent bacterial effect being identified in infants born at less than 27 weeks' gestation is potentially quite meaningful for the subset of premature infants in whom this disorder is most consequential. However, the demarcation point of 27 weeks' gestation, although providing a robust differentiation in microbial risk in this study, might not be applicable to all centres and should not be considered as a universally useful variable to assign risk. Notably, however, the delayed timing of these differences still permits a window to identify dysbiosis before necrotising enterocolitis occurs in the most premature infants, in whom this disorder has later onset than in less premature infants.¹⁴ Also, even though our data graphically present that microbial risk emerges after 30 days of age, we do not believe our findings yet support considering this day as a categorical boundary for seeking bacterial differences.

Our regression analysis should be considered as exploratory and not be used as a validated risk model. Nonetheless, our findings urge that future models of bacterial content and necrotising enterocolitis risk should address changes in proportions of Gammaproteobacteria and anaerobic bacterial populations, that children who are likeliest to benefit are those born most prematurely (<27 weeks), and that the interval of risk might not emerge until several weeks of age. Ultimately, microbiologically informed intervention studies will be needed to definitively establish the roles of specific bacterial populations in pre disposing to, or reducing the risk of, necrotising enterocolitis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research in context

Evidence before this study

We did PubMed and Medline searches before undertaking this study in 2009. We used the following keywords to identify papers of interest: “Gammaproteobacteria”, “microbiome”, “necrotising enterocolitis”, and “preterm birth”, with no date or language limitations. We identified only efforts to assess gut bacteria using gradient gel electrophoresis of amplified bacterial DNA and, occasionally, sequencing of selected bands excised from the gels. Our searches did not identify any studies that applied modern sequencing technology directly to stools to establish the role of bacterial populations in the development of necrotising enterocolitis.

During the assembly of our cohort, several groups reported data for gut microbial content before necrotising enterocolitis by sequencing bacterial DNA directly from stool. These studies produced conflicting data, probably because of the dynamic bacterial population content in this age group and the relatively few patients and samples in any single study. This problem is compounded by an inherently unstable gut bacterial population in this age group.

Added value of this study

We identified dysbiosis preceding necrotising enterocolitis through analysis of 10·7 gigabases of DNA from 22 945 218 reads of 3586 stool samples from 166 infants in three US hospitals. This dysbiosis consists of over-representation of Gammaproteobacteria in stools of cases before necrotising enterocolitis develops, and under-representation of anaerobic bacteria, in particular Negativicutes.

Implications of all the available evidence

Our data might form the basis for informed discussion about management strategies of gut microbes in very low birthweight infants to avoid this catastrophic complication of preterm birth.

Figure 1. Distribution of four major bacterial classes in composite community structure for each 15 day analysis interval in the St Louis cohort (A) and broken down into children born at less than 27 weeks' gestation (B) and at or greater than 27 weeks' gestation (C) in this cohort Bars show upper 95% CIs, which are symmetric around the means (lower bounds not shown). If a patient produced no samples within one of the 15 day intervals, no data were entered. If a patient produced more than one specimen within an interval, proportions were collapsed into composite values for that individual. For (C), one case was available for analysis intervals 3 and 4 so data for these intervals are not presented. Distributions are provided in 3D in the appendix. Dirichlet multinomial comparisons of class distribution (p values for case vs corresponding control comparisons) for each analysis interval for this cohort are provided in the appendix; significant p values are displayed in this figure. *p=0.0035. †p=0.0001. ‡p=0.0370. §p=0.0016.

Figure 2. Predominant genera or families within Gammaproteobacteria and Negativicutes
 Chart fractions represent the three most abundant genera (or families if a genus cannot be assigned) within Gammaproteobacteria and Negativicutes, among all reads generated by cohort, and by case or control status. Among these classes, Enterobacteriaceae is the only family identified. Colour code assignment for each pie fraction is provided beneath each class. Bacilli and Clostridia genera and families are presented in the appendix.

Figure 3. Shannon diversity indices in each 15 day analysis interval from the St Louis cohort Shows microbial diversity in stools from cases and controls. Horizontal line shows median, box boundaries show 25th and 75th percentiles, and whiskers show the differences between the 25th and 75th percentiles multiplied by 1.5. Values that exceed these boundaries are depicted as open circles. $p=0.0004$ for time-by-necrotising-enterocolitis interaction indicating significantly discordant trends in bacterial diversity in stools from cases versus controls.

Table 1

Baseline characteristics of St Louis cohort

	Cases (n=28)	Controls (n=94)	p value
Birthweight (g)	795 (720–980)	940 (800–150)	0.0181
Gestational age at birth (weeks)	26.0 (24.7–27.9)	27.0 (25.9–28.7)	0.024
Sex			0.190
Girls	10 (36%)	49 (52%)	
Boys	18 (64%)	45 (48%)	
Race			0.0081
Black	22 (79%)	45 (48%)	
White	6 (21%)	49 (52%)	
Number of singleton births	23 (84%)	68 (72%)	0.425
Number of births by caesarean delivery	20 (71%)	72 (77%)	0.759
Apgar scores			
1 min	2.0 (1–5)	4 (2–6)	0.131
5 min	5.5 (3.5–6)	7 (5–8)	0.0236
Age first stool analysed (days)	11.5 (6.0–17.4)	9.0 (5.4–13.4)	0.226
Number of stools analysed	12.5 (5.5–23.0)	21.5 (13.0–29.0)	0.0149
Age of necrotising enterocolitis (days)	24 (19.0–48.0)	NA	NA
Number who died before discharge	9 (32%)	1 (1%)	<0.0001
Day of age discharged alive	71 (55–80)	64 (52–72)	0.276
Exposure to human milk			0.0596
0	1 (4%)	2 (2%)	
<10%	1 (4%)	17 (18%)	
10–50%	5 (18%)	25 (27%)	
>50%	21 (75%)	50 (53%)	
Days of age on antibiotics (%)	43% (36–55)	28% (14–38)	<0.0001
First antibiotic-free age (days)	8 (4–8.5)	5 (4–9)	0.409
Number of bloodstream infections	4 (14)	11 (12)	0.746
Inotrope exposure	11 (39)	22 (23)	0.156
Blood transfusion exposure	4 (1–8)	1 (0–4)	0.0043

Data are median (IQR) or n (%), unless otherwise specified. The appendix defines antibiotic use, bloodstream infections, and exposures to human milk, inotropes, and blood transfusion. NA=not applicable.

Mixed-model analysis with bacterial content as outcome, from all infants whose stools were sequenced in the St Louis cohort and in all cohorts combined

Table 2

	Bacilli	Gamma proteobacteria	Clostridia	Negativicutes	Clostridia and Negativicutes
Gestational age at birth [*]					
Primary	-0.032 (p<0.0006)	0.002 (p=0.847)	0.008 (p=0.079)	0.013 (p=0.0079)	0.022 (p=0.0004)
All	-0.037 (p<0.0001)	0.003 (p=0.786)	0.015 (p=0.0002)	0.009 (p=0.0183)	0.024 (p<0.0001)
Vaginal delivery (yes)					
Primary	-0.132 (p=0.0022)	0.074 (p=0.174)	-0.033 (p=0.136)	-0.001 (p=0.961)	-0.034 (p=0.216)
All	-0.096 (p=0.0075)	0.055 (p=0.212)	-0.035 (p=0.0495)	0.006 (p=0.726)	-0.030 (p=0.186)
Proportion of days of antibiotics					
Primary	0.127 (p=0.0212)	-0.119 (p=0.0519)	-0.065 (p=0.0142)	-0.051 (p=0.067)	-0.119 (p=0.0009)
All	0.118 (p=0.0080)	-0.121 (p=0.0120)	-0.037 (p=0.077)	-0.035 (p=0.076)	-0.076 (p=0.0050)
Proportion of human milk feeds					
Primary	-0.057 (p=0.132)	0.072 (p=0.107)	0.013 (p=0.504)	-0.024 (p=0.2186)	-0.015 (p=0.546)
All	-0.046 (p=0.168)	0.065 (p=0.087)	0.000 (p=0.976)	-0.019 (p=0.2166)	-0.020 (p=0.337)
Time					
Primary	-0.112 (p<0.0001)	0.021 (p=0.0001)	0.047 (p=0.0003)	0.049 (p=0.0722)	0.096 (p<0.0001)
All	-0.118 (p<0.0001)	0.039 (p<0.0001)	0.045 (p<0.0001)	0.043 (p=0.0085)	0.087 (p<0.0001)
Necrotising enterocolitis (yes)					
Primary	0.097 (p=0.289)	-0.195 (p=0.074)	0.007 (p=0.880)	0.105 (p=0.0269)	0.111 (p=0.061)
All	0.035 (p=0.6397)	-0.134 (p=0.119)	0.020 (p=0.578)	0.070 (p=0.0435)	0.090 (p=0.0523)
Time-by-necrotising enterocolitis (yes; all gestational ages) [†]					
Primary	-0.057 (p=0.137)	0.146 (p=0.0010)	-0.021 (p=0.264)	-0.061 (p=0.0019)	-0.081 (p=0.0010)
All	-0.041 (p=0.185)	0.114 (p=0.0011)	-0.008 (p=0.587)	-0.046 (p=0.0013)	-0.053 (p=0.0051)

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Data are coefficient (p value) for the primary (St Louis) cohort and for all cohorts combined. The appendix defines proportion of days on antibiotics, human milk, and time (defined as the analysis interval during which samples were obtained). Positive coefficients represent positive associations between host or clinical factors and bacterial content, except for the time-by-necrotising enterocolitis interaction, for which positive values represent an association between bacterial content and case status. Transfusions and inotropic support were included and found to have no effect.

* Gestational age corresponds to weeks of gestation completed at time of birth.

[†] The time-by-necrotising enterocolitis interaction term indicates the difference in rate of change in bacteria between cases and controls over time; the appendix provides more information. The appendix provides 95% CIs.