Necrotizing enterocolitis and preterm infant gut bacteria

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Summary

Necrotizing enterocolitis remains an intractable consequence of preterm birth. Gut microbial communities, especially bacterial communities, have long been suspected to play a role in the development of necrotizing enterocolitis. Direct-from-stool nucleic acid sequencing technology now offers insights into the make-up of these communities. Data are now converging on the roles of Gram-negative bacteria as causative agents, despite the dynamic nature of bacterial populations, the varying technologies and sampling strategies, and the overall small sample sizes in these case–control studies. Bacteria that confer protection from necrotizing enterocolitis have not been identified across studies. The beneficial effect of probiotics is not apparent in infants with birth weights <1000 g (these infants are at highest risk of, and have the highest case fatality rate from, necrotizing enterocolitis). Further work should be directed to the modulating gut microbes, or the products they produce, to prevent this devastating complication of preterm birth.

Keywords

Necrotizing enterocolitis; Gammaproteobacteria

1. Introduction

The newborn gut microbiome is an area of intense and growing interest in perinatology. There is emerging appreciation of the roles played by gut microbes in intestinal health, and, indeed, in lifelong health. Most relevant to preterm infants in neonatal intensive care units (NICUs), several very important disorders are likely to originate from either abnormal proportions of microbial content (dysbiosis), or when a vulnerable host encounters a specific pathogen. In this review, we focus on early-in-life bacterial population assembly in the preterm infant gut, recent data on the biology and ecology of the bacterial community, and
the role these microbes play in the development of necrotizing enterocolitis (NEC).
Experimental data are mentioned as examples that corroborate or extend observations from
the human host.

2. The in-population of the human infant gut by microbes

Classic teaching holds that the human gut (i.e., the meconium) contains no bacteria, or at
least no viable bacteria, at birth. However, recent data prompt reconsideration of this dogma.
Mshvidnadze et al. [1] identified bacterial sequences in freshly produced meconium, as have
Heida et al. [2], and Ardissone et al. [3]. Stout et al. [4] have identified bacterial bodies on
electron microscopy in the basal plate of placentas delivered via cesarean section. Aagaard
et al. [5] reported bacterial 16S and metagenomic sequences in placentas, finding similarities
between these sequences and those of bacteria resident in the mouth. Additional studies have
identified bacterial sequences in amniotic fluid from term pregnancies delivered by elective
cesarean section [6]. Notably, these papers rarely present evidence of viable bacteria in
specimens putatively colonized based on nucleic acid sequencing (Table 1).

The possibility that the fetal gut is colonized by bacteria before maternal membranes rupture
is intriguing. However, low grade bacteremia occurs independent of pregnancy in healthy
adults after brushing or flossing teeth, or defecation [11]. The finding of bacterial sequences
in or on a newborn infant immediately after rupture of membranes may reflect colonization
of the newborn while passing through the birth canal or being delivered through the skin.
These sequences may also reflect nucleic acid remnants of viable bacteria that circulated in
the mother's blood, but which have no replicative potential or relevance to the assembly of
the earliest-in-life members of the bacterial community of the infant gut. An additional
argument against prenatal colonization of the gut with bacteria acquired in utero is the
observation that germ-free animals, which generally require immersion in iodine solution
during the derivation process, are generated from mothers (usually mice) who are not free of
germs. Iodine immersion would not sterilize the colonized gut, so if the gut is an
intergenerational habitat for viable bacteria, we would expect that it would be impossible to
derive germ-free animals.

Recent publications illuminate rules of assembly for the human infant gut microbiome.
Among healthy term and near-term infants, early gut colonization patterns are driven largely
by delivery route and feeding patterns [12,13], with emerging data suggesting a role for the
gut virome [10]. Infants born very preterm (<32 weeks), however, have a distinct set of
exposures compared to those born near term. Parenteral antibiotic use is nearly universal
during the first several days of life in preterm infants, feeding tubes are placed early, and
enteral nutrition is commenced cautiously. In the days and weeks after birth, preterm infants
reside almost exclusively in NICUs. These environments are designed to limit microbial
transmission, and contact with bacteria is controlled to the extent possible. Visitors are
restricted and often only parents and professional staff are permitted to touch the infant.
Hand hygiene is stressed, line care is protocolized, and nutrition is either human breast milk
(mother of infant or pasteurized donor pool), or sterilized liquid formula. There is no
exposure to pets, or physical contact with other relatives. This microbiologically constrained
biosphere offers a rich opportunity to study the transition of the neonatal gut from sterility or
near sterility at birth to an organ that houses, for the rest of the life of the host, the greatest
density of microbes in the human body. Not unexpectedly, bacterial communities assemble
differently in the preterm gut than in the term infant gut.

Whereas the in-population of the infant gut with bacteria is a fascinating ecologic event
worthy of study in its own right, accumulating experimental data suggest that the earliest-in-
life gut bacteria affect the future well-being of their hosts [14]. Hence, the study of these
communities in infants is justifiable in order to determine whether the animal data are
relevant for humans. However, pertinent to infants born very prematurely, there are
additional compelling reasons to study the gut microbiome because of the high frequency
with which these infants experience complications of premature birth that are plausibly
associated with this biomass. The two most dire consequences in which gut bacteria could
play major roles in outcome are NEC and late-onset neonatal bloodstream infections (P.I.
Tarr and B.B. Warner, Chapter 4, this issue).

The “normal” preterm infant gut microbiome has been characterized among infants born
very preterm who were at risk of developing NEC, but who did not experience this event,
i.e., controls. Until recently, these analyses used culture-based technology, or polymerase
chain reaction amplification of DNA extracted from stool and testing for mobility in a gel.
Most recently, advances in sequencing technology, expansion of ribosomal RNA gene
databases, and metagenomic capabilities (DNA sequencing not confined to 16S rRNA gene
regions) have made feasible the direct-from-stool amplification of extracted bacterial DNA.
These approaches provide a less circuitous, and deeper and more economical, portrayal of
bacterial populations in polymicrobial substances. In the targeted approach, conserved
regions of the 16S ribosomal RNA gene of bacteria are primed and amplified, a technique
employed in the NIH-sponsored Human Microbiome Project [15]. This targeted approach
enables deep “censusing” of bacterial populations, as all such mass readouts are confined to
the regions of the bacterial chromosome that identify the organism from which they are
derived.

We are aware of six publications from NICUs in eight different centers in which bacterial
community assembly in “normal” preterm infants has been interrogated in depth using
direct-from-stool sequencing. For the purposes of this review, our criteria for including such
studies are those that included at least 100 stools from at least 25 subjects who did not
develop NEC, and that the enumeration technology employed 16S rRNA gene or
metagenomic sequencing (Table 2).

Even though only one of the papers in Table 2 exclusively focused on defining the pattern of
progression in children without NEC [20], data supplied in the others [16,18,21] were
sufficient to confirm the findings of La Rosa et al. [20]. In that comprehensive study of
preterm infants, 16S rRNA gene sequencing demonstrated a remarkably choreographed
pattern: namely, the early-in-life gut bacterial content is predominated by Bacilli (despite
their name, Bacilli are Gram-positive cocci such as staphylococci, streptococci, and
enterococci). Bacilli are soon overtaken by Gram-negative facultative organisms (a diversity
of genera and species within the Gammaproteobacteria class). This surge in
Gammaproteobacteria is counterbalanced by a gradually increasing abundance of Clostridia

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(many genera and species) and Negativicutes (predominantly *Veillonella*). Overall, four bacterial classes (Bacilli, Gammaproteobacteria, Clostridia, and Negativicutes) account for >90% of the taxa present. Compared to the gut content of older children and adults, these preterm infant gut bacterial populations have much higher content of Gammaproteobacteria (one to two orders of magnitude difference), and approximately half the density of obligate anaerobes.

There is a convergence on a consensus community structure by the equivalence of 33–36 weeks postmenstrual age (the sum of gestational age at birth, and day of life on which the sample was obtained). The content of this consensus community at this postmenstrual age (but not earlier) is independent of gestational age at birth. In particular, anaerobic bacteria gain abundance more rapidly in the gastrointestinal tracts of infants born least prematurely. This choreographed progression is punctuated unpredictably and substantially by short-lived changes in composition, before the communities self-revert to the choreographed progression. Such abrupt changes have been noted in older children and adults [12,13,15,22]. Unexpectedly, the factors believed to be influential in microbial community assembly (at least in children born after full-term gestation), namely mode of delivery (vaginal vs cesarean section), antibiotic administration in the aggregate, and feeds (breast milk), were either not determinative of bacterial content, or had only minimal or temporary influence on this progression.

These non-associations between diverse exposures, each of which could logically be considered to influence bacterial community structure in the gut, prompts us to interpret that in the preterm human infant, the major driver of bacterial population assembly is intrinsic host biology or succession ecology rather than exogenous factors. However, we offer two caveats. First, as described above, the microbial exposures of very preterm infants differ considerably from those of infants born at term, in whom mode of delivery and breast-milk feeding appear to influence gut bacterial population assembly. Second, we wish to note that in a subsequent study of the St Louis Children’s Hospital cohort [19], specific antibiotics (meropenem, cefotaxime, and ticarcillin–clavulanate), which were used in few subjects in La Rosa et al. [20], were associated with substantial directional changes in microbial content. It is also noteworthy that when metagenomic sequencing technology was applied [19], bacterial community population characteristics as previously defined by 16S rRNA gene sequencing were recapitulated [20].

### 3. The gut microbiome and NEC

Multiple lines of circumstantial evidence suggest that NEC is influenced by bacteria in the very preterm infant gut. Most notably, NEC does not occur in utero, and, in fact, rarely occurs before approximately day of life 10, after bacterial populations start to assemble in the newborn gut. Also, NEC is statistically and independently associated with increased antibiotic use, especially prolongation of antibiotics during the first week of life [23–25]. Moreover, H2 blockers, which could affect gut microbial populations by reducing the gastric acid line of defence against bacterial colonization, are associated with increased NEC risk [26].
Multiple studies suggest that probiotics prevent NEC (reviewed in [27]), but this benefit accrues chiefly to infants who weigh <1000 g at birth, and who are at lesser risk of experiencing NEC, and of dying from NEC, than those whose birthweights are <1000 g. Indeed, a recent large and well-conducted multi-center randomized control trial of *Bifidobacterium breve* BBG-001 failed to demonstrate any protective effect against NEC in a population in which most of the children weighed ≤1000 g at birth [28]. Whereas this failure might represent the use of a single probiotic instead of a combination of probiotics, and though the choice of the probiotic intervention could be subject to debate, it seems unlikely that we will soon identify viable microbes that can exert a profoundly protective effect against NEC, especially among infants whose birth weights are ≤1000 g.

A review of the many taxa that have been associated with NEC is beyond the scope of this article. However, the diversity of incriminated species, the overall small numbers of subjects in these studies, and small effect sizes reported (often only in subgroup analysis), cast doubt on the existence of a specific mono-microbial driver of NEC [29]. Nonetheless, as reviewed above, direct-from-stool sequencing of DNA now offers new opportunities to compare cases with NEC to controls, to determine whether microbial populations are associated with this outcome. In the past decade, multiple groups have attempted to apply direct-from-stool sequencing to identify bacteria that might cause NEC. Some such attempts are summarized in Table 3, focusing on studies that utilized 16S rRNA amplification methods rather than culture or gel electrophoresis-based methods.

These studies suggest that diverse bacterial taxa are associated with either risk of, or protection from, developing necrotizing enterocolitis among preterm infants. One interpretation is that there are center-specific differences in microbial drivers of NEC, as described for variability in gut microbial populations before the onset of bloodstream infections [35]. An alternative explanation is that the population biology of bacteria in the gut is exceptionally dynamic in the interval during which NEC occurs, which obligates the assembly of exceptionally large cohorts to study this disorder, and the need to interrogate an abundance of specimens prior to the event. Indeed, only one of the studies in Table 3 reported the analysis of >100 pre-NEC specimens.

The dynamism of bacterial populations poses immense challenges. In the first 60 days of life, as described above, there is a week-by-week aggregate progression from Bacilli to Gammaproteobacteria predominance, while Clostridia slowly rise in abundance. In reality, the Clostridia class described by La Rosa et al. contains Clostridia and Negativicutes, because Negativicutes (Gram-negative obligate anaerobic bacteria) have recently been assigned their own class [20]. NEC generally does not present until after the second week of life, and risk extends to approximately day of life 60, with infants born most prematurely developing NEC later in this period of vulnerability [29]. To illustrate this challenge, stools from a case occurring on day of life 25 would ideally be compared to stools from a control group of infants produced on day of life 25, these controls having been born after the same gestational duration. However, control specimens for a case of NEC that occurs on day of life 45 would greatly differ in content from controls chosen on day of life 25, even if controlling for gestational age at birth. That is to say, the norm changes throughout the interval of risk, during which NEC can occur at any time. When one also takes into account
the additional abrupt changes in populations, it is clear that a substantial number of subjects and specimens must be assembled to characterize the microbial population in children at risk in a case–control study. Notably, the larger studies tend to lean towards a predominance of Gram-negative bacteria as being drivers of NEC. Consensus protective organisms have generally not emerged from these large studies. A final complicating note is that specimens obtained immediately before NEC is apparent may reflect changes of NEC that are already under way before infants become visibly affected. It therefore seems prudent to “censor” sequence data from the hours preceding the onset of clinical NEC if trying to identify signatures well in advance of NEC that could be associated with this disorder. Interestingly, in one study in which specimens were analyzed late (tissue at resection) \[30\] or early (meconium) \[2\], anaerobic bacteria were associated with NEC. In the largest study (in terms of numbers of cases and numbers of pre-NEC stools analyzed) reported to date, an overrepresentation of Gammaproteobacteria was associated with NEC, whereas anaerobic bacteria, especially Negativicutes and secondarily Clostridia, were associated with control status (i.e., protection). Gammaproteobacteria risk has been suggested in several smaller studies \[16,18,34\]. In contrast, however, several publications employing direct-from-stool sequencing have not identified overabundant Gram-negative bacteria as a prelude to NEC \[2,33\].

Indirect data support that Gram-negative bacteria are causal in NEC pathogenesis. In animal models, toll-like receptor 4, the ligand for lipopolysaccharide, is believed to play a central role in mucosal injury \[36\], and antibiotics active against Gram-negative bacteria confer protection \[37\]. Moreover, anaerobic bacteria, in response to microbiota-accessible carbohydrates, generate anti-inflammatory short-chain fatty acids, notably acetate, propionate, and butyrate \[38\]. Several literature reviews \[39,40\] have evaluated studies in which infants were administered oral aminoglycosides in attempts to prevent NEC. Aminoglycosides would be active against Gammaproteobacteria in the gut, but not suppress anaerobic bacterial populations. In the aggregate, these studies \[41–44\] support the use of oral aminoglycosides to prevent NEC. However, because of concerns about selecting for aminoglycoside resistance \[45\] and of absorption of the oral aminoglycosides from the gut (albeit confined to very early in life before the incidence of NEC increases \[46\]), enteral antibiotics to prevent NEC are not widely used. It is interesting to note that the oral aminoglycosides were often discontinued in these studies before the time of life at which the most premature infants develop NEC. This timing raises the possibility that the beneficial effects of antibiotics in these studies might have been understated.

Bacterial diversity – defined as the number of different taxa present, weighed according to their proportionality – is considered to reflect a healthy luminal microbial community in inflammatory bowel disease and \textit{C. difficile} infections \[47,48\]. Even before these associations between lack of diversity and gut inflammation were reported, Claud and Walker proposed the hypothesis that diminished diversity of the premature infant gut could result in NEC \[49\]. Subsequent studies failed to find an association between lack of bacterial diversity and development of NEC \[16–18,31,33,34,50\], though again, as for NEC microbial associations, the numbers were limited. However, in a recent study \[21\], an association between subsequent development of NEC and comparatively lower gut bacterial diversity
was noted. The difference appeared to be related to delayed or suppressed maturation of microbial diversity in infants who subsequently developed NEC, compared to those who did not. In other words, diversity slowly increased over the first 60 days of life in the controls but not the cases. However, this association is not straightforward: gut bacterial communities are exceptionally non-complex in preterm infants. Therefore, a change in the proportionality of one taxon is necessarily counterbalanced by a change in one or more of the few other taxa present. With only four dominant taxonomic “degrees of freedom,” it is difficult to attribute NEC to lack of gut bacterial diversity per se, versus an increase or a decrease in one or another taxon. In other words, it cannot be stated that lack of diversity is the driver of risk for NEC, versus an overrepresentation of Gammaproteobacteria, which directly ordains the lack of diversity in these sample sets. The role of bacterial diversity in protecting from NEC remains an intriguing hypothesis, however.

4. Conclusion

NEC remains a catastrophic disorder. It is concerning that we have not had meaningful and durable improvements in incidence or outcomes of NEC in the nearly four decades since widespread recognition of this entity permeated neonatology. The finding of a microbial signature prior to development of NEC, and/or a protective signature in the form of obligate anaerobic bacteria, now offers new opportunities to prevent this devastating consequence of preterm birth.

Acknowledgments

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References


Practice points

- The causes of NEC are unknown.
- Judicious use of antibiotics and promotion of human milk use might lower the risk of NEC, but these interventions are unlikely to categorically reduce disease incidence, and are justifiable for multiple additional reasons.
### Research directions

- How can we anticipate the microbial community changes that lead to NEC?
- How can we modulate the gut microbial community to reduce bacteria-associated processes that might lead to NEC?
### Table 1
Data in support of prenatal colonization of the new-born gut with microbes.

<table>
<thead>
<tr>
<th>Study</th>
<th>Subject of study</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jimenez et al. [7]</td>
<td>Cord blood cultures of term neonates born by elective cesarean section</td>
<td>Enterococci, streptococci, staphylococci, and propionibacterium recovered from cord blood</td>
</tr>
<tr>
<td>Mshvidladze et al. [1]</td>
<td>Meconium by 16S rRNA gene sequencing</td>
<td>Viable bacteria not sought</td>
</tr>
<tr>
<td>DiGiulio et al. [8]</td>
<td>Amniotic fluid cultures and 16S rRNA gene sequencing in preterm deliveries</td>
<td>16S rRNA gene sequences identified in, and <em>Mycoplasma hominis</em>, <em>Ureaplasma</em> sp., <em>Streptococcus agalactiae</em>, <em>Lactobacillus</em> sp., <em>Prevotella</em> sp., <em>Fusobacterium nucleatum</em>, coagulase-negative <em>Staphylococcus</em> sp., <em>Bacillus</em> sp. (not anthrax), <em>Peptostreptococcus</em> sp., and <em>Gardnerella vaginalis</em> recovered from the amniotic fluid</td>
</tr>
<tr>
<td>Rautava et al. [9]</td>
<td>Bacterial DNA detected in amniotic fluid at time of elective cesarean section by 16S rRNA gene sequencing</td>
<td>Viable bacteria not sought</td>
</tr>
<tr>
<td>Stout et al. [4]</td>
<td>Electron microscopy of placenta</td>
<td>Bacteria identified in basal plate, no attempt to culture</td>
</tr>
<tr>
<td>Aagaard et al. [5]</td>
<td>16S rRNA gene sequences and metagenomic sequences</td>
<td>Bacterial sequences identified and reflected periodontal microbes, no attempt to culture</td>
</tr>
<tr>
<td>Lim et al. [10]</td>
<td>First in life stool (days 1–4) subjected to 16S rRNA gene sequencing and virome analysis</td>
<td>Few bacterial species, many bacteriophages, based on sequence analysis</td>
</tr>
</tbody>
</table>
Table 2

Studies of gut bacterial assembly in preterm infants without necrotizing enterocolitis (NEC).

<table>
<thead>
<tr>
<th>Study and location</th>
<th>Sequencing technology</th>
<th>No. Of subjects without NEC</th>
<th>No. of specimens</th>
<th>Conclusions about pattern of bacterial community assembly</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zhou et al. [16], Brigham and Women's Hospital, Boston, MA, USA</td>
<td>16S rRNA gene sequencing</td>
<td>26</td>
<td>111</td>
<td>Increasing proportion of Clostridia over time, balanced by slowly diminishing proportion of Gram-negative genera, with little effect of antibiotics on this trend.</td>
</tr>
<tr>
<td>Ward et al. [17], Cincinnati, OH, and Birmingham, AL, USA</td>
<td>Metagenomic sequencing</td>
<td>89</td>
<td>185</td>
<td>Clostridia class increases over time (specifically veillonella and C. freundii), with consistently high Proteobacteria (specifically E. coli).</td>
</tr>
<tr>
<td>Shaw et al. [18], St Mary's Hospital, Queen Charlotte's and Chelsea Hospital, London, UK</td>
<td>16S rRNA gene sequencing</td>
<td>44</td>
<td>369</td>
<td>Bifidobacteria and klebsiella increased in proportionality, and Gram-positive bacteria decreased in proportionality, over time.</td>
</tr>
<tr>
<td>Gibson et al. [19], St Louis Children's Hospital, St Louis, MO, USA</td>
<td>Metagenomic sequencing</td>
<td>84</td>
<td>401</td>
<td>Some of these subjects and specimens were also analyzed in La Rosa et al. [21]. Notably, metagenomic sequencing recapitulated the 16S sequence analysis of this cohort in these two companion publications.</td>
</tr>
<tr>
<td>La Rosa et al. [20], St Louis Children's Hospital, St Louis, MO, USA</td>
<td>16S rRNA Gene sequencing</td>
<td>58</td>
<td>922</td>
<td>Bacterial classes proceed from Bacilli to Gammaproteobacteria to Clostridia in these infants, but these populations are prone to changes in content. When infants near 33–36 weeks postconceptional age (i.e., an interval that is equivalent to the 3rd to the 12th week of age, in view of the wide range of gestational ages in this cohort), the populations converge on a consensus community, with ~40% of the bacteria being obligate anaerobes (especially Clostridia and Negativicutes), and an equal percentage being Gammaproteobacteria. There was little or no effect of use of postnatal antibiotics, mode of delivery, or breast milk, and the community composition at this point.</td>
</tr>
<tr>
<td>Warner et al. [21], St Louis Children's Hospital, St Louis, MO; Children's Hospital at Oklahoma University, Oklahoma City, OK; Kosair Children's Hospital, Louisville, KY, USA</td>
<td>16S rRNA gene sequencing</td>
<td>120</td>
<td>2720</td>
<td>Includes the 58 subjects without NEC and their 922 stools in La Rosa et al. [21]. Patterns in NICUs in Oklahoma City and in Louisville recapitulate those in St Louis cohort.</td>
</tr>
</tbody>
</table>

NICU, neonatal intensive care unit.

*Based on data from Supplemental Table 1 in [18]*.
Table 3
Summary of studies in which DNA sequences obtained directly from stools have been used to associate bacterial risk and development of necrotizing enterocolitis (NEC), listed in ascending order according to number of pre-NEC stools sequenced.

<table>
<thead>
<tr>
<th>Study</th>
<th>Sequencing technology</th>
<th>No. of subjects without NEC</th>
<th>No. of specimens from subjects without NEC</th>
<th>No. of subjects with NEC</th>
<th>No. of pre-NEC specimens from subjects who subsequently developed NEC</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brower-Sinning et al. [30], Pittsburgh, PA, USA</td>
<td>16S rRNA gene sequencing</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>9</td>
<td>Tissue analysis only, no pre-NEC samples; Proteobacteria, Clostridia associated with risk, as was diminished bacterial diversity</td>
</tr>
<tr>
<td>Mai et al. [31], three University of Florida-affiliated NICUs, FL, USA</td>
<td>16S rRNA gene sequencing</td>
<td>9</td>
<td>18</td>
<td>9</td>
<td>18</td>
<td>Case stools demonstrated an increase in Proteobacteria, and a decrease in Firmicutes in the second of the paired samples (i.e., week before NEC)</td>
</tr>
<tr>
<td>McMurtry et al. [32], Louisiana State University Health Sciences Center, Touro Infirmary, East Jefferson General Hospital and Children's Hospital of New Orleans, LA, USA</td>
<td>16S rRNA gene sequencing</td>
<td>74</td>
<td>74</td>
<td>21</td>
<td>21</td>
<td>Bacterial diversity and relative abundance of Clostridia was significantly lower in NEC specimens compared to controls</td>
</tr>
<tr>
<td>Raveh-Sadka et al. [33], Pittsburgh, PA, USA</td>
<td>Metagenomic sequencing</td>
<td>5</td>
<td>34</td>
<td>5</td>
<td>21</td>
<td>No clear association between bacterial content as identified by metagenomics and outcome; no microbiologic evidence of time-space clustering</td>
</tr>
<tr>
<td>Heida et al. [2], Groningen, The Netherlands</td>
<td>16S rRNA gene sequencing</td>
<td>22</td>
<td>57</td>
<td>11</td>
<td>30</td>
<td>Clostridium perfringens and Bacteroides dorei associated with risk, and staphylococci associated with protection.</td>
</tr>
<tr>
<td>Torrazza et al. [34], Gainesville, FL, USA</td>
<td>16S rRNA gene sequencing</td>
<td>35</td>
<td>77</td>
<td>18</td>
<td>40</td>
<td>Novel sequence matching closest to Klebsiella pneumoniae during week 1 associated with subsequent development of NEC</td>
</tr>
<tr>
<td>Ward et al. [17], Cincinnati, OH, and Birmingham, AL, USA</td>
<td>Metagenomic sequencing</td>
<td>89</td>
<td>185</td>
<td>27</td>
<td>60</td>
<td>Specific sequence types of E. coli associated with NEC</td>
</tr>
<tr>
<td>Sim et al. [18], St Mary's Hospital, Queen Charlotte's and Chelsea Hospital, London, UK</td>
<td>16S rRNA gene sequencing</td>
<td>44</td>
<td>369</td>
<td>22</td>
<td>88</td>
<td>Klebsiella, clostridium associated with risk; no microbiologic evidence of time-space clustering</td>
</tr>
<tr>
<td>Zhou et al. [16], Brigham and Women's Hospital, Boston, MA, USA</td>
<td>16S rRNA gene sequencing</td>
<td>26</td>
<td>111</td>
<td>10</td>
<td>88</td>
<td>Age-specific differences identified, with early- and late-onset NEC having an association with Clostridia and Gammaproteobacteria, respectively</td>
</tr>
<tr>
<td>Warner et al. [23], St Louis Children's Hospital, St Louis, MO; Kosair Children's Hospital, Louisville, KY; Children's Hospital at Oklahoma University, Oklahoma City, OK, USA</td>
<td>16S rRNA gene sequencing</td>
<td>120</td>
<td>2720</td>
<td>46</td>
<td>866</td>
<td>Gammaproteobacteria associated with risk, and Negativicutes associated with protection; lack of diversity is associated with risk</td>
</tr>
</tbody>
</table>

NICU, neonatal intensive care unit.