Unraveling the microbiome of necrotizing enterocolitis: insights in novel microbial and metabolomic biomarkers

Running title: Potential microbial biomarkers in necrotizing enterocolitis

Key words: necrotizing enterocolitis, NEC, microbiota, metagenomics, shotgun

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Supplementary Text

Composition and structure of the Pre-Term Community State Types (PT-CSTs).

Hierarchical Clustering Analysis (HCA) based on the species-level microbial profiling allowed the identification of five archetypal subgroups named Pre-Term Community State Types (PT-CSTs) (Table S4). In detail, PT-CST1 is composed mainly (75 %) by subjects classified as NEC. Specifically, PT-CST1 (n = 12) was characterized by a high abundance of *E. faecalis* (average of 59.44 %) with a prevalence of 100 %. Interestingly, the NEC subjects belonging to this subgroup showed higher average abundance of *Enterococcus faecalis* rather than Control samples (72.67 % vs 19.73 %, T-test p-value < 0.01) with concomitant presence of *Staphylococcus epidermidis* (Table S4, Figure 1b). In contrast, members of *Actinomyces*, *Corynebacterium*, *Schaalia*, and *Bifidobacterium*, which together constituted an average of about 52. 19 % of the healthy microbiome, were undetected in NEC samples (Table S5, Figure 2). While *St. epidermidis* commonly inhabits the human skin microbiota, in premature and very low bird weight infants this species can represent a bacterial agent causing neonatal sepsis and other neonatal morbidities, such as brain injury as well as pulmonary epithelial damage and was previously reported in NEC cases (1–4). Similarly, although *E. faecalis* was commonly found among early colonizers of the neonatal gut and, in certain circumstances, it can elicit biofilm-associated opportunistic infections, leading to clinical manifestations such as urinary tract infections, endocarditis, peritonitis, and NEC (5–8). Furthermore, acquired and natural antimicrobial resistance has been found among members of *Enterococcus* genus, likely providing *E. faecalis* with a biological advantage that allows it to defeat its niche competitors (5). Interestingly, the association of *S. epidermidis* and *E faecalis* appeared almost absent in preterm control newborns, thus emerging as possible NEC-associated biomarkers.

Moving the focus on other HCL-based clusters, PT-CST2, PT-CST4, and PT-CST5 were composed of both NEC and healthy subjects and were dominated by *Escherichia coli* (PT-

CST2), *S. epidermidis* (PT-CST4) or *Klebsiella pneumoniae* (PT-CST5) (Table S4, Figure 1b). Nevertheless, these three species showed statistically significant difference in intra-cluster abundance between NEC and healthy subjects. In particular, the 19 NEC cases of PT-CST2 showed a higher abundance of *E. coli* species (average of 86.51 %) in contrast to that found among the 14 healthy samples (67.44 %, T-test p-value < 0.05) (Table S4), accompanied by lower species richness than their healthy counterparts (average of 4 vs 7, T-test p-value < 0.05), with significantly reduction in *Bacteroides* genus members (T-test p-value < 0.05) (Table S4). In a similar fashion, the subjects with NEC of PT-CST5 exhibited a significantly higher abundance of *K. pneumoniae* compared to healthy subjects (66.54% vs 39.15 %, T-test p-value < 0.05) (Table S4) associated with a decreased biodiversity (9.6 vs 15.2 bacterial species per samples, T-test p-value < 0.05) and the lack of particular healthy infant gut-associated bacteria such as *Akkermansia* and *Bifidobacterium breve* taxa (9,10) (Table S5). Moreover, within PT-CST4 (n=18), showing *St. epidermidis* as dominant taxon with an average abundance of 78.33 %, NEC samples exhibited higher relative abundance of the abovementioned species (87.95 % compared to 72.22 % of the 11 Control samples, T-test p-value < 0.05) coupled with biodiversity loss (average of 5.4 vs 7.5 species per samples in healthy infants, T-test p-value < 0.05) (Table S4). Consistent with previous studies in preterm infants, these findings highlighted that although strains of *E. coli*, *K. pneumoniae*, and *St. epidermidis* were also detected in premature infants who do not develop NEC, there is a possible association between high intestinal levels of these taxa and NEC pathogenesis, specifically in a framework of biodiversity loss (11,12). In contrast, in PT-CST3 18 fecal samples from healthy subjects were combined with three NEC-affected. This subgroup was predominated by *S. agalactiae*, with an average abundance of 70.72 % and a prevalence of 66.6 % (Table S4, Figure 1b). Previous studies about *Streptococcus agalactiae* pathogenicity have reported that this microorganism can lead to neonatal infections with considerable variability in the degree of severity,

depending on the capsular serotype and strain-specific expression levels of other virulence factors. Thus, providing a possible explanation of the reasons, in this instance, supporting the identification of *Str. agalactiae* in the gut microbiota of healthy subjects (13,14)

Overall, these findings highlighted how NEC patients often experience a marked form of dysbiosis, where uncontrolled growth of a single bacterial species occurs with concomitant severe loss of biodiversity and/or particular microorganisms. This event eventually results in a permanent loss of competition between bacterial species that, in normal condition, leads to the early-stage development process of healthy human gut microbiota (15).

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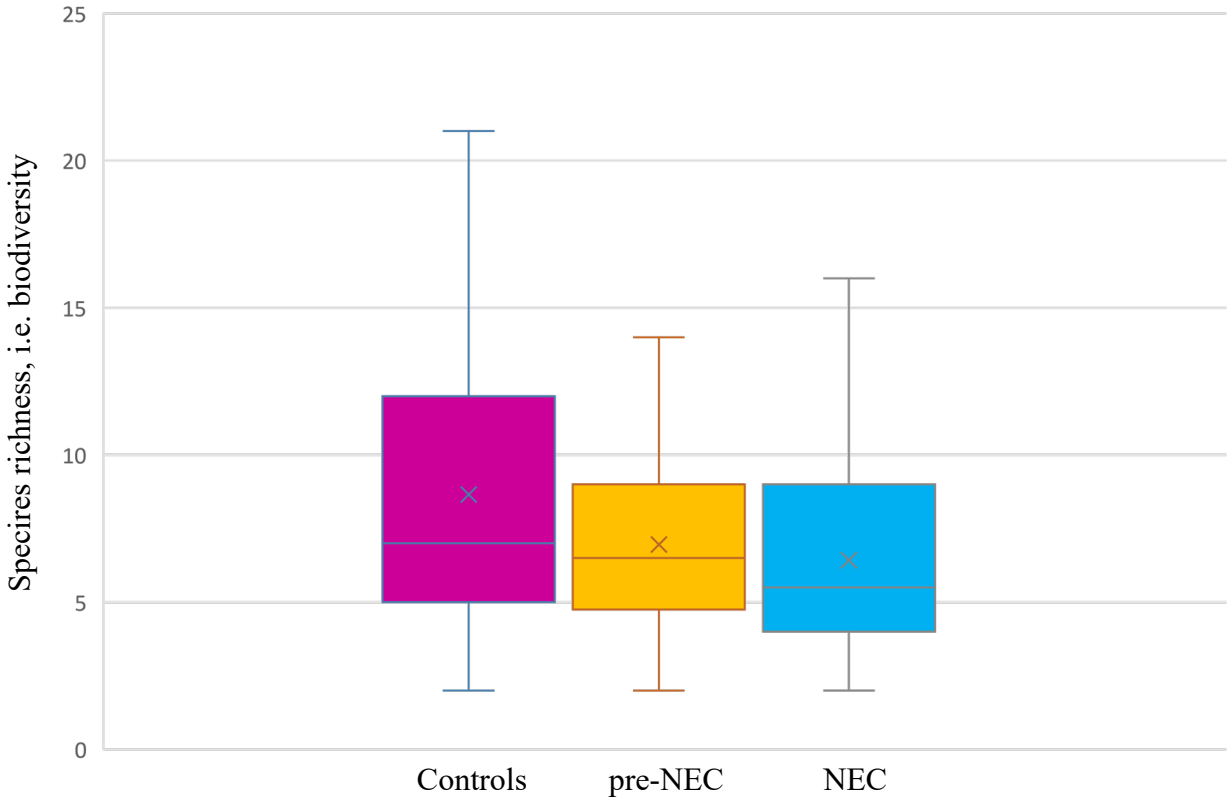
Supplementary Figure Legends

Figure S1. Exploration of the diversity and the gut microbiota composition of the pre-term infant. Panel a shows the box and whisker plot of the species richness observed in healthy, pre-NEC, and NEC infants. Panel b reports the principal coordinate analysis (PCoA) of the 171 collected pre-term infant samples.

a)


Specires richness, i.e. biodiversity
25
20
15
10
5
0
Controls
pre-NEC
NEC

b)

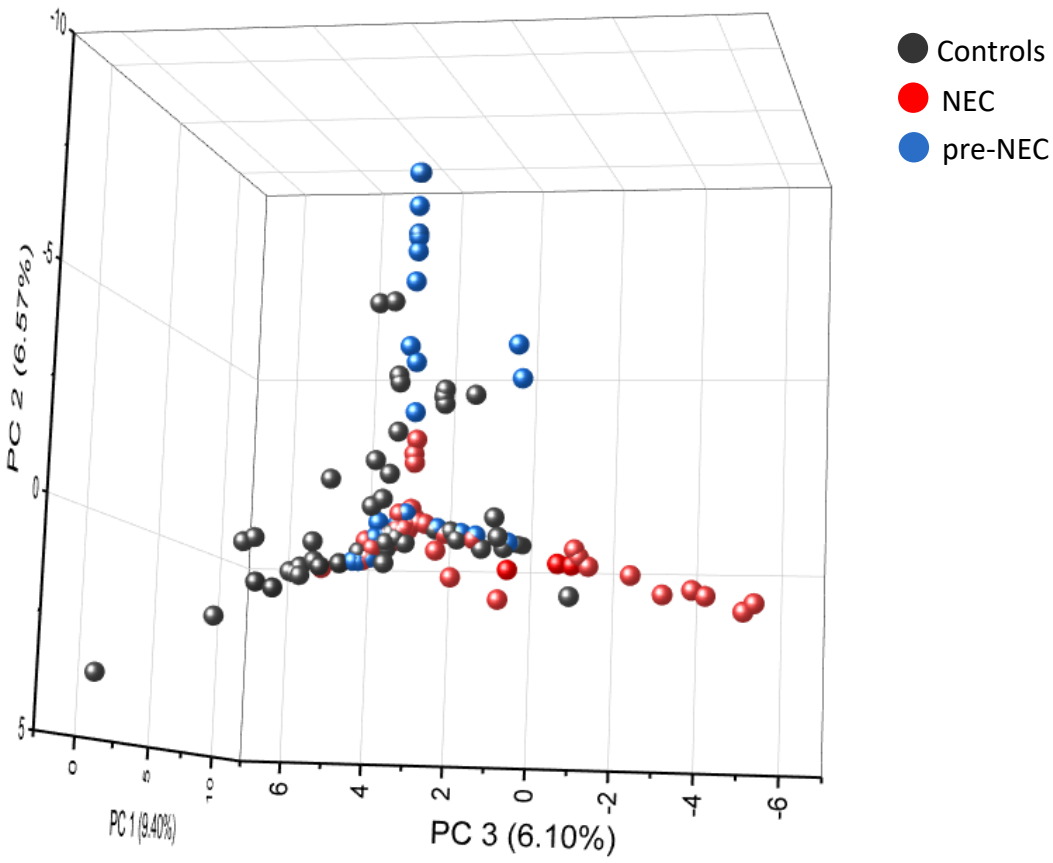

Controls
NEC
pre-NEC
PC 2 (6.57%)
PC 1 (9.40%)
PC 3 (6.10%)

Figure S1