Evolution of Gut Microbiome and Metabolome during Stage 1 Necrotizing Enterocolitis: a Case-Control Study

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Research

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Abstract

Background : Necrotizing enterocolitis (NEC) is a devastating condition of the preterm new-born due to multiple factors, including gut microbiota dysbiosis. Since NEC development is poorly understood due to main focus on late stages 2 and 3, we studied the gut microbiota and metabolome evolution of NEC at a very early onset. Results NEC-1 gut microbiota had a higher abundance of Streptococcus (second decade of life) and Staphylococcus (third decade of life) species. NEC-1 children showed a microbiome evolution in the third decade of life being the most divergent and associated to a different metabolomic signature than in healthy children. NEC-1 microbiome had increased glycosaminoglycan degradation and lysosome activity by the first decade of life and was more sensitive to factors such as childbirth, low birth weight and gestational age, than healthy microbiome. NEC-1 fecal metabolome was more divergent by the second month of life.

Conclusions: The modifications of gut microbiota and microbiome during NEC-1 development appear more distinguishable by the third decade of life, when compared to healthy children. These data identify a precise window of time (i.e. third decade of life) and provide microbial targets to fight/blunt the progression of NEC by stage 1.

Background

Necrotizing enterocolitis (NEC), defined by the Bell classification[1–3], is the most severe intestinal disease in preterm infants, with a mortality score of 25% and long-term neurological morbidity[4]. Yet, a precise initiating factor of this pathology is missing. In the last decade gut microbiota was identified and recognized as a specific organ with functions widely beyond digestion[5]. Both its taxonomic (relative abundance) and functional (microbial pathway) alterations, named dysbiosis, were described in several pathologies, in particular metabolic diseases such as type 2 diabetes and obesity[6–8], and intestinal inflammatory diseases[9]. Importantly, a dysbiotic gut microbiota associated with a very high inflammatory status of the gut[10, 11] may trigger NEC development, since germ-free mice do not develop NEC[12].

From a clinical and microbiological point of view, studies of NEC were focused only on established and severe phenotypes such as NEC-2 and NEC-3. Based on the French study EPIPAGE 2, the incidence of proved NEC-2 and NEC-3 is 1–5% in preterm infants born at less than 32 weeks of gestation[13].

By contrast, NEC suspicions such as lethargy, bradycardia, thermic instability associated to biliary gastric residues, vomiting, abdominal distension with or without rectal bleeding, with a normal abdominal x-ray image or a simple dilatation, which identifies stage 1 (NEC-1), have not been studied yet. In fact, enteropathies are frequent in the first weeks of life in preterm infants, though no data are available about NEC-1 incidence. This induces the end of alimentation, a prolonged (sometime life-lasting) parenteral nutrition, with a delayed gut maturation and failure to thrive[14]. Therefore, to study the evolution of gut microbiota and microbiome during the early onset of NEC, we focused on NEC-1 children within the first

two months of life. We studied fecal metabolome to understand how a change in gut microbiota may drive alterations in intestinal metabolites. To further understand which factor of mother and child may affect the evolution of gut microbiota, microbiome and fecal metabolome during NEC-1, we analysed: presence of neonatal antibiotherapy (ABx), ABx treatment on the mother, childbirth (Cesarean-section [C-sec] vs. vaginal birth [VB]), very low birth weight (VLBW), extreme low birth weight (ELBW) and gestational age (GA) > or ≤ 28 weeks.

Results

Analysis of gut microbiota, microbiome and fecal metabolome during stage 1 NEC (NEC-1).

To understand the microbial and metabolomic evolution during the early onset of necrotizing enterocolitis (NEC), we studied clinical profile suspected (stage-1) NEC (NEC-1) preterm infants. NEC-1 children underwent more glycopeptides treatment, showed significantly higher cordon lactates, bacteremia and a longer full enteral feeding, when compared to age-matched healthy children (Table 1). NEC-1 children also displayed a lower plasma pH and enteral milk volume at day 7 (Suppl. Figure 1A,B) and a higher abundance of Streptoccoccus species (Suppl. Figure 2A) compared to healthy children. Both populations of children showed a high intragroup variance in terms of gut microbiota (Suppl. Figure 2B) and overall microbial diversity (Suppl. Figure 2C). NEC-1 microbiome showed increased activity for pathway related to transcription, glycosaminoglycan degradation and lysosome, compared to healthy children (Suppl. Figure 2D). Then, we analysed the fecal metabolome to appreciate NEC-1-induced changes in gut microbial metabolic activity. NEC-1 children displayed a reduced intragroup variation and significantly lower levels of ethanol (Suppl. Figure 2E). Overall, these data show that NEC-1 is characterized by a precise gut microbiota, microbiome and gut microbial metabolites profile.

Analysis of gut microbiota, microbiome and fecal metabolome during the evolution of NEC-1 over decades up to the second month of life.

Given the presence of a NEC-1-specific gut microbiota and microbiome profile, we aimed at identifying at what time these profiles establish. We divided both NEC-1 and healthy children populations in subgroups according to decades (period of ten days of life) as it follows: 1-10d (d stands for “days”), 11-20d, 21-30d for the first month of life and > 30d for the second one. In the first decade, NEC-1 children displayed a divergent and more homogenous gut microbiota compared to healthy children, with the latters characterized by a higher abundance of Klebsiella species (Fig. 1A-B). At this stage of life, gut microbiota in NEC-1 had a lower diversity based on Chao-1 index (Fig. 1C) and a different microbial activity related to replication, recombination and repair proteins, lysosome and glycosaminoglycan degradation (Fig. 1D). No significant changes were observed in fecal metabolites (Fig. 1E). Overall, these data show that gut microbiome starts to diverge at the early onset of NEC-1.

In the second decade, NEC-1 gut microbiota was characterized again by a higher abundance of Streptococcus species and bacteria from the Micrococcales order (Fig. 2A), with a high intragroup variance (Fig. 2B). At this stage of life, NEC-1 gut microbiota also showed a higher diversity based on

Chao-1 index (Fig. 2C), but no microbial pathway differently regulated (Fig. 2D). As for the fecal metabolome, NEC-1 children displayed significant lower levels of serine (Fig. 2E). Overall, these data show a stronger evolution of gut microbiota than gut microbiome in the second decade, between NEC-1 and healthy children.

In the third decade of life, changes in NEC-1 gut microbiota compared to healthy children occurred to a bigger extent and were related to increased Staphylococcus and Streptococcus species (Fig. 3A-B), together with a high intragroup variance (Fig. 3C) and no change in the overall diversity indices (Fig. 3D). We also observed a NEC-1 microbiome profile mainly based on thiamine and seleno-compound metabolism (Fig. 3E). The NEC-1 gut microbiota profile of the third decade of life was associated with: i) multiple diseases and found significantly increased in ulcerative colitis (Fig. 4A); ii) host genetic variation and significantly related to ANP32E, a gene involved in ulcerative colitis [15], in line with previous reports. In terms of fecal metabolome, we observed no significant changes in NEC-1 vs. healthy children (Fig. 4C). Then, we studied feces collected in the second month of life. In this period of life, the taxonomical differences in the gut microbiota of NEC-1 vs. healthy children were related to the increase in Raoultella species in NEC-1 gut microbiota (Fig. 5A), with a still high intragroup variance (Fig. 5B) and no change in the overall microbial diversity indices (Fig. 5C). We also observed microbial functions related to DNA repair increased in the NEC-1 gut microbiome (Fig. 5D). This period of life was characterized by the highest separation in terms of fecal metabolome, with significant lower levels of ethanol and leucine in NEC-1 children.

Specific impact of NEC-1 on the evolution of gut microbiota. microbiome and fecal metabolome over the first two months of life, compared to healthy children.

To investigate the evolution of gut microbiota, microbiome and fecal metabolome over the first two months of life, we conducted an intra-group study in both NEC-1 and healthy children, according to the four groups reported above: 1-10d, 11-20d, 21-30d and > 30d. We did not observe any taxonomic significant change in the gut microbiota of NEC-1 children. However, the group NEC-1_21-30d had a specific gut microbiome with an increased restriction enzyme activity, among others (Suppl. Figure 3A). The four NEC-1 groups also differed in terms of fecal metabolome, with regard to leucine, ethanol and serine amounts (Suppl. Figure 3B). Based on these results, we performed a metabolomic enrichment analysis on two levels: i) pathway-associated metabolite sets (Suppl. Figure 3C) and ii) single nucleotide polymorphism (SNP)-associated metabolite sets (Suppl. Figure 3D). NEC-1 metabolomic profile (increased ethanol and serine) was significantly associated to both homocysteine degradation and phosphatidylethanolamine biosynthesis (Suppl. Figure 3C), with serine being the metabolite the most linked to NEC-1-associated SNP (Suppl. Figure 3D). By contrast, in healthy children the four groups reported above did not differ in terms of both gut microbiota and microbiome, but only with regard to fecal metabolome (Suppl. Figure 4A). Healthy metabolomic profile (increased leucine, ethanol and dihydroxyacetone) was significantly associated to valine, leucine and isoleucine degradation and to ketone body metabolism (Suppl. Figure 4B), with leucine being the metabolite the most linked to healthy-associated SNP (Suppl. Figure 4C). Overall, these data suggest that: i) a different intragroup evolution

exist between NEC-1 and healthy children with regard to gut microbiota and microbiome and ii) NEC-1 microbiome appears to be more sensitive to mother-related factors.

Maternal and child Factors influencing the gut microbiota, microbiome and fecal metabolome during NEC-1.

Next, we asked which factor related to both mother and child may affect the most the above reported parameters. We analysed six conditions: neonatal antibiotherapy (ABx), ABx treatment on mother, childbirth (C-section (C-sec) vs. vaginal birth (VB)), very low birth weight (VLBW), extreme low birth weight (ELBW) and the gestational age (GA) > or ≤ 28 weeks.

Only neonatal ABx treatment affected the gut microbiota in both NEC-1 and healthy children (Suppl. Figure 5A). By contrast, all the above factors, except the VLBW, affected the gut microbiome (Suppl. Figure 5B-F). Note that childbirth modality, ELBW and GA affected the gut microbiome only in NEC-1 children (Suppl. Figure 5D-F). Moreover, all the above factors, except the neonatal ABx treatment and ELBW, affected the fecal metabolome between NEC-1 and healthy children (Suppl. Figure 6A-F). Then, we performed again a metabolomic enrichment analysis on the pathway-associated metabolite sets, based on Suppl. Figure 6F, in which there is an increase in ethanol and succinate within in the NEC-1_GA ≤ 28w. Ketone body and butyrate metabolism were the most significantly associated with this metabolomic set (Suppl. Figure 6G).

Discussion

In this prospective study we focused on suspected (stage-1) necrotizing enterocolitis NEC (NEC-1) preterm infants. NEC-1 phenotype has been poorly clinically investigated, with no data available on gut microbiota, microbiome and fecal metabolome. By contrast, NEC-2 and NEC-3, more severe and established phenotypes, have been more characterized. As for clinical parameters, the increased cordon lactate levels we found in NEC-1 has been recently positively correlated to the development of enteropathy[16]. Hence, the hypothesis of hypoxic lesions in utero or during birth may not be excluded and be even predictive of neonatal morbidity. Importantly, the observed reduced enteral nutrition volume in NEC-1 is not a protective factor during NEC but rather it may lengthen hospitalization and infections risk[17]. NEC-1 children showed a high general variance for gut microbiota and fecal metabolome which is in line with a personalized microbiota and fecal metabolome profiles of preterm infant[18]. Both this datum and the delayed intestinal colonization of preterm infants[19, 20] may explain the lack of NEC-1-specific microbial group in the first decade of life. The analysis by decades of life revealed a divergence for both gut microbiota and microbiome in NEC-1 by the third decade of life. In particular, the higher abundance of Staphylococcus in NEC-1 is in accordance with the early colonization by Staphylococcus bacteria of the intestine of preterm infants[21]. This datum suggests the third decade as an optimal time window to be targeted by antibiotics directed against bacterial species higher in NEC-1 such as Staphylococcus. However, in our study NEC-1 children that underwent glycopeptide and aminoglycoside therapy were more numerous than healthy children. Therefore, this evidence suggests that NEC-1 may be

associated to glycopeptide and/or aminoglycoside-resistance, since NEC-1 gut microbiota was characterized by an increase, and not a decrease, of Staphylococcus. Since aminoglycosides are active antibiotics against Enterobacteria, their administration could delay intestinal colonization by Proteobacteria and thus promote the implantation of resistant genera such as Staphylococcus and Streptococcus. Based on this evidence, our data suggest not to prolong antibiotic therapy beyond the first week of life in preterm infants. Furthermore, NEC-1 gut microbiota profile was associated to ulcerative colitis and host genetic variation in the ANP32E gene, encoding a protein implicated in cortico-resistance during ulcerative colitis[15]. NEC-1 children showed increased exposition to antenatal corticosteroids compared to healthy children, even though a study has not identified antenatal corticosteroids as a NEC-inducing factor[22]. Despite Anp32e-deficient mice display no sign of disease[23], it has not to be excluded the role of Anp32e in a model of gut inflammation mimicking ulcerative colitis. Hence, further studies are warranted on genetic factors of NEC. In terms of microbial functions, the intragroup analysis showed in the third decade of life a higher restriction enzyme activity in the NEC-1 gut microbiome. This bacterial activity, directed against bacteriophages and enriched in the new-born intestine[24], suggests an increased virus activity and, hence, a virome dysbiosis, beyond a microbiota dysbiosis, during NEC-1 evolution. All these microbial data are associated with our observation about a change in fecal amino-acids, such as leucine and serine, confirming the association between gut microbiota dysbiosis and a change in amino-acids metabolism[25].

Conclusions

Our study may provide neonatal departments with immediate indications to blunt NEC-1 evolution such as: i) increase the enteral volume of nutrition, especially in the first days of life; ii) revise and reducie antibiotic therapy up to the first week of life in preterm infants.

Methods

Study design

Cohort constitution. We conducted a prospective monocentric case-control cohort study. This study was approved (number of the approval: DC 2016-2804) by Neonatal and Pediatric Intensive Care Unit and Neonatology Department of Purpan Hospital in Toulouse, France. The parents of the children involved in this study gave their approval by written consensus. The inclusion criteria regarding all of the children hospitalised into the Neonatal and Pediatric Intensive Care Unit or Neonatology Departments of the Purpan Hospital, were:

- newborn of gestational age under 34 weeks of gestation

- diagnosis of suspected (stage 1) necrotizing enterocolitis (NEC, NEC-1) made by a neonatalogist

- obtainment of the non-opposition from parents of their legal representative

Following the inclusion of every case, we conducted in parallel a search for two controls, according to the following matching criteria, listed in decreasing priority:

- gestational age (±1 week of gestation, priority to matched age)

- body weight

- neonatal antibiotherapy

- childbirth (C-section vs. vaginal)

- maternal antibiotherapy

Inclusion criteria for controls were:

- newborn of gestational age under 34 weeks of gestation

- respect of the matching according to the priority order of the established criteria

- obtainment of the oral non-opposition from parents of their legal representative.

Children with complex congenital cardiopathy or with spontaneous intestinal perforation without a radiological evidence of NEC were excluded from the study.

Based on these criteria, we included 11 NEC-1 children, with 27 feces collection and 21 healthy children, with 53 feces collection. A total of 80 fecal samples was analysed in our study. The period of collection was day 1 to day 68 of life of the new-born.

Taxonomic and functional analysis of gut microbiota

Feces analysed in this study were collected by nurses in the related department in the first week of life and once a week till the end of the hospitalization. Feces were firstly kept at 4 °C in a 5 ml Eppendorf tube with 20% glycerol/Lysogeny Broth and then stored at -80 °C. Total DNA was extracted from feces as previously described[26], with a modification: a thermic shock of 30 seconds was performed between each bead-shaking step (3 bead-shaking steps of 30 seconds each at maximum speed). The 16S bacterial DNA V3-V4 regions were targeted by 357wf-785R primers and analysed by MiSeq (RTLGenomics, http://rtlgenomics.com/, Texas, USA). An average of 68,669 sequences was generated per sample. A complete description of the bioinformatic filters applied is available at http://www.rtlgenomics.com/docs/Data_Analysis_Methodology.pdf. Cladogram and LDA scores were drawn using the Huttenhower Galaxy web application (http://huttenhower.sph.harvard.edu/galaxy/) *via* the LEfSe algorithm[27]. Diversity indices were calculated using the software Past 3.23 (Hammer, Ø., Harper, D.A.T., and P. D. Ryan, 2001. PAST: Paleontological Statistics Software Package for Education and Data Analysis. Palaeontologia Electronica 4(1): 9pp). The predictive functional analysis of the gut

microbiota was performed *via* PICRUSt[28]. Diseases and host genetic variation linked to NEC-1_21-30d associated gut microbiota were identified via MicrobiomeAnalyst[29], with the Taxon Set Enrichment Analysis module.

Fecal metabolome analysis

The metabolome (total metabolites) analysis of the feces was performed as previously described[30]. Pathway-associated metabolite sets and SNP-associated metabolite sets (**Fig.S3C-D**, **Fig.S4B-C** and **Fig.S6G**) were analysed via MetaboAnalyst 4.0[31], with the Enrichment Analysis module.

Statistical analysis

The results are presented as mean±SEM for histograms and box and whiskers graphs. Statistical analyses were performed by two-way ANOVA followed by a two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli to correct for multiple comparisons by controlling the False Discovery Rate (<0.05) (for histograms) or Mann-Whitney test (for box and whiskers), as indicated in the figure legend, by using GraphPad Prism version 7.05 for Windows Vista (GraphPad Software, San Diego, CA). For Table 1, results are presented as median or as indicated and P value was calculated using Fisher's exact test. Significant values were considered starting at $P<0.05$. For the taxonomical and predictive functional analysis of gut microbiota significant values were considered starting at $P<0.05$ or $P<0.01$ when indicated. PCA graphs were drawn by using Past 3.23.

Table 1: Cohorts characteristics

Variables, description	NEC-1 n=11	Healthy n= 21	P (Fisher's exact test)
Birth weight, median (g)	1150	1360	0.09
Gestational age, median (weeks)	28.4	30	0.2
Gender			0.43
Girls, number (%)	2 (18)	8 (38)	
Boys, number	9	13	
Patent Ductus arteriosus, number (%)	3 (27)	6 (28)	>0.9999
Parity, number (%)	3 (27)	3 (10)	0.39
Antenatal corticosteroids, number (%)	11 (100)	19 (90)	0.53
Hypertension, eclampsia, number (%)	2 (18)	3 (14)	>0.9999
Multiple births, number (%)	2 (18)	4 (19)	>0.9999
Antenatal antibiotics, number (%)	4 (36)	5 (24)	0.68
Chorioamniotitis, number (%)	2 (18)	1 (5)	0.27
Apgar Score			
1 min	8	7	0.07
5 min	10	8	0.3
Cordon pH	7.23	7.31	0.27
Cordon lactates	5.7	3	**0.04***
Mean arterial pressure at hospital admission	29	29.5	0.7
Hospital Admission T (°C)	36.5	36.8	0.23
Antibiotics in the first week of life (%)	10 (90)	18 (85)	>0.9999
Days under antibiotics	7,5	3	0.052
Days under antibiotics (3GC ± Penicillin A, ± aminoglycoside) in the first week of life	3	3	0.39
Children under glycopeptides number (%)	8 (72)	3 (14)	**0.0018****
Bacteremia	5	1	**0.01***
Exposition to mother milk	11	21	>0.9999
Age of enteropathy (days)	12 (4-	-	-

	60)		
Exposition to inotropes	1	0	0.34
Blood transfusion	2	4	>0.9999
Full enteral feeding (days)	23	11	**0.0002*** **

Declarations

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Authors' contributions C.B., made substantial contribution to study concept, contributed to acquisition of fecal samples and clinical data, writing and critical review of the manuscript for important intellectual content; D.D. made substantial contributions to acquisition of fecal samples; O.D. and S.B. made substantial contributions to constitution of the H vs. NEC-1 cohorts; E.O. reviewed the manuscript; M.S. made substantial contributions to concept and design of the overall study, acquisition, analysis and interpretation of data, prepared the figures and wrote the manuscript. All authors gave final approval of the version to be published.

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Competing interests The authors declare no competing interests.

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Availability of data and materials All data are available in the main text or the supplementary materials and *via* the following repositories: Sequence Read Archive (SRA) database https://submit.ncbi.nlm.nih.gov/subs/sra/ with the assigned identifier PRJNA579480.

Ethics approval and consent to participate

This study was approved (number of the approval: DC 2016-2804) by Neonatal and Pediatric Intensive Care Unit and Neonatology Department of Purpan Hospital in Toulouse, France. The parents of the children involved in this study gave their approval by written consensus.

Consent for publication

Not applicable

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Figures

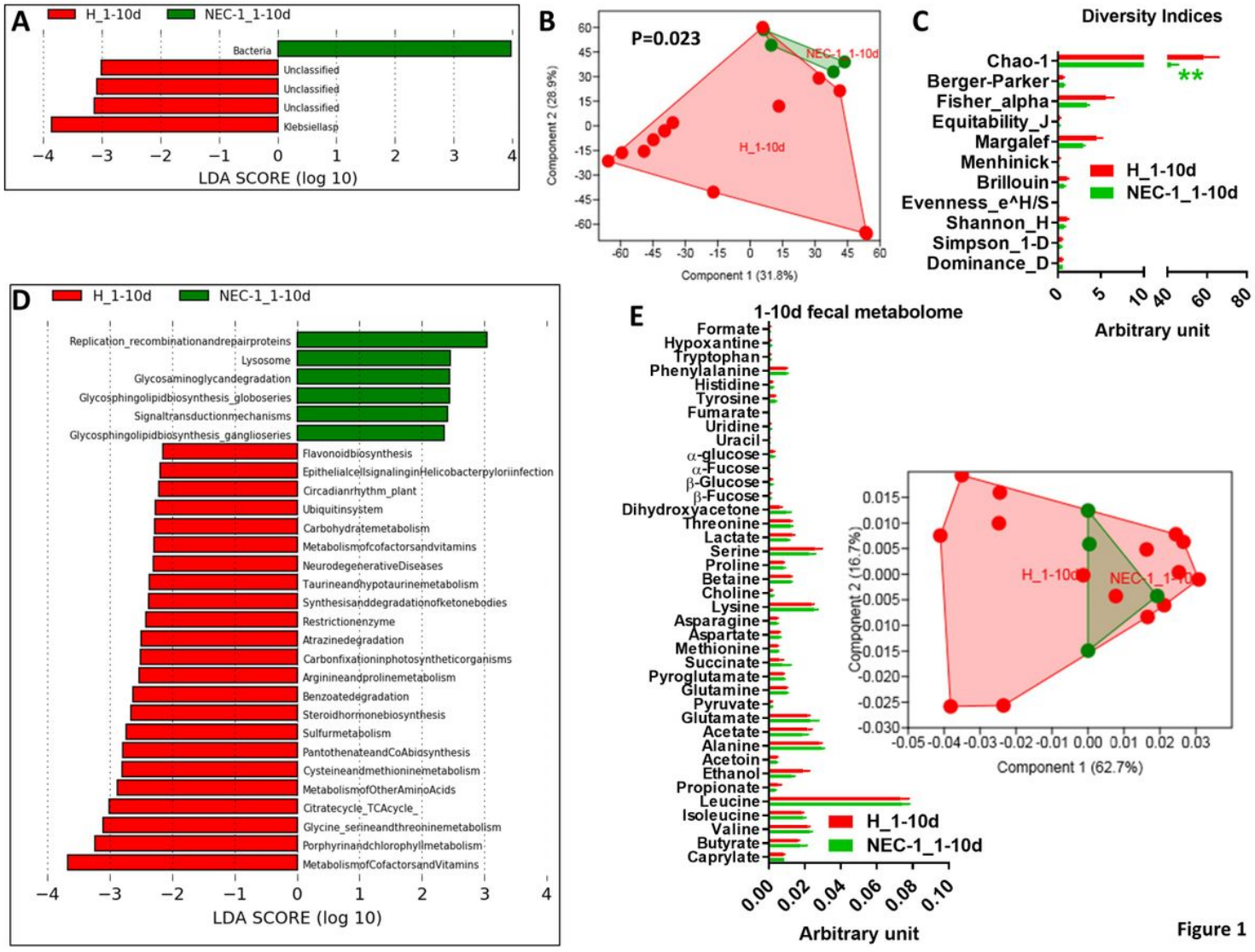

Figure 1

Figure 1

Analysis of gut microbiota, microbiome and metabolome in the first decade of life in healthy vs. stage 1 necrotizing enterocolitis children. A) Gut microbiota analysis via Linear Discriminant Analysis (LDA) score between healthy (H) vs. stage 1 necrotizing enterocolitis children (NEC-1), in the first decade of life 1 to 10 days (d); B) Principal Component Analysis (PCA) of the gut microbiota; C) Indices of gut microbiota diversity; D) LDA score for microbial pathways; E) histogram of the overall fecal metabolites and PCA as inset. **P<0.01. two-way ANOVA followed by a two-stage linear step-up procedure of Benjamini. Krieger and Yekutieli to correct for multiple comparisons by controlling the False Discovery Rate (<0.05); N=15 for H and N=4 for NEC-1.

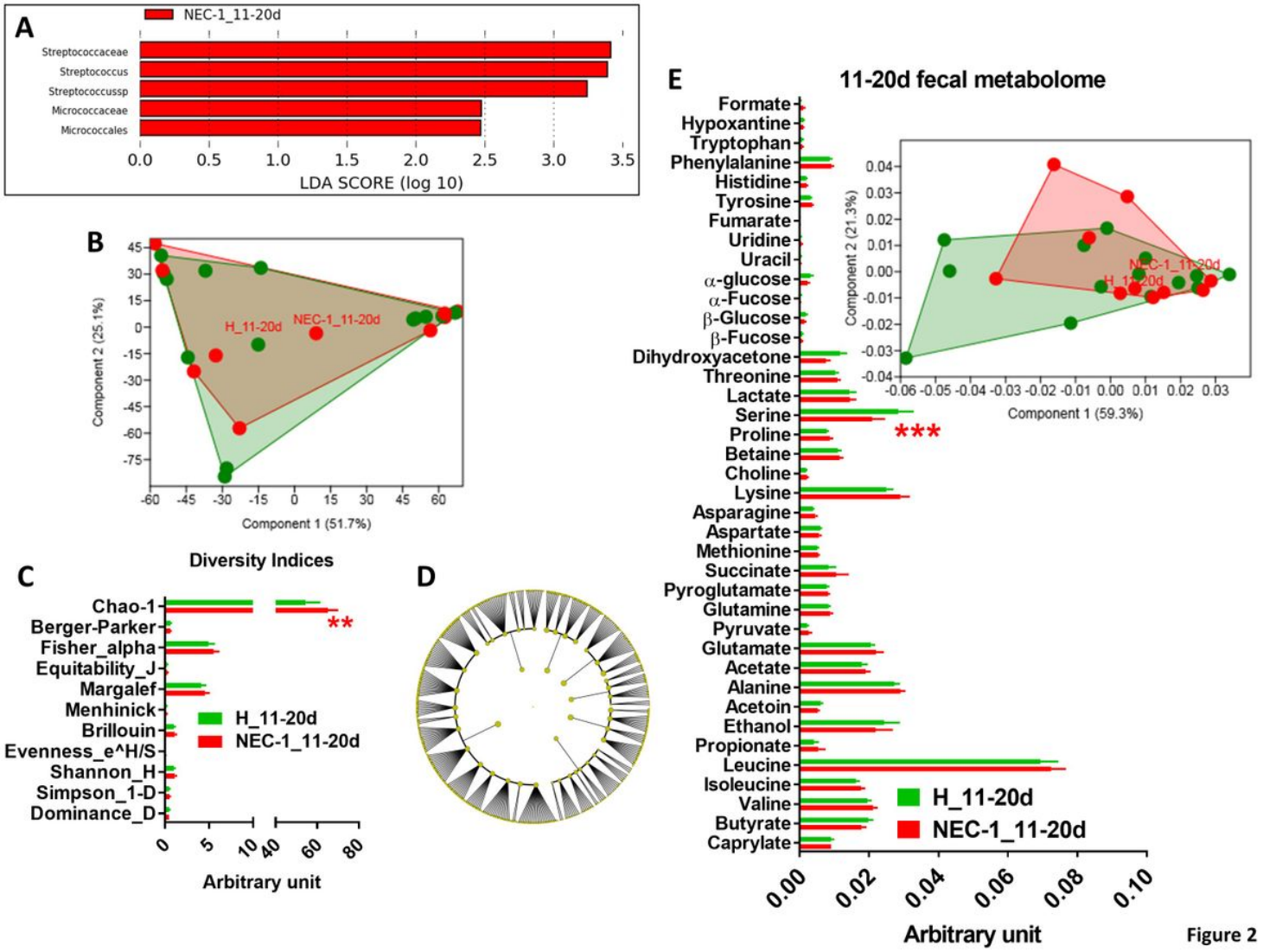

Figure 2

Analysis of gut microbiota, microbiome and metabolome in the second decade of life in healthy vs. stage 1 necrotizing enterocolitis children. A) Gut microbiota analysis via LDA score between healthy (H) vs. stage 1 necrotizing enterocolitis children (NEC-1), in the second decade of life 11 to 20 days (d) (the score is only shown for NEC-1 children meaning that no bacteria are significantly higher in the H group vs. NEC-1); B) PCA of the gut microbiota; C) Indices of gut microbiota diversity; D) Null cladogram for microbial pathways; E) histogram of the overall fecal metabolites and PCA as inset. **P<0.01. ***P<0.001. two-way ANOVA followed by a two-stage linear step-up procedure of Benjamini. Krieger and Yekutieli to correct for multiple comparisons by controlling the False Discovery Rate (<0.05); N=14 for H and N=10 for NEC-1.

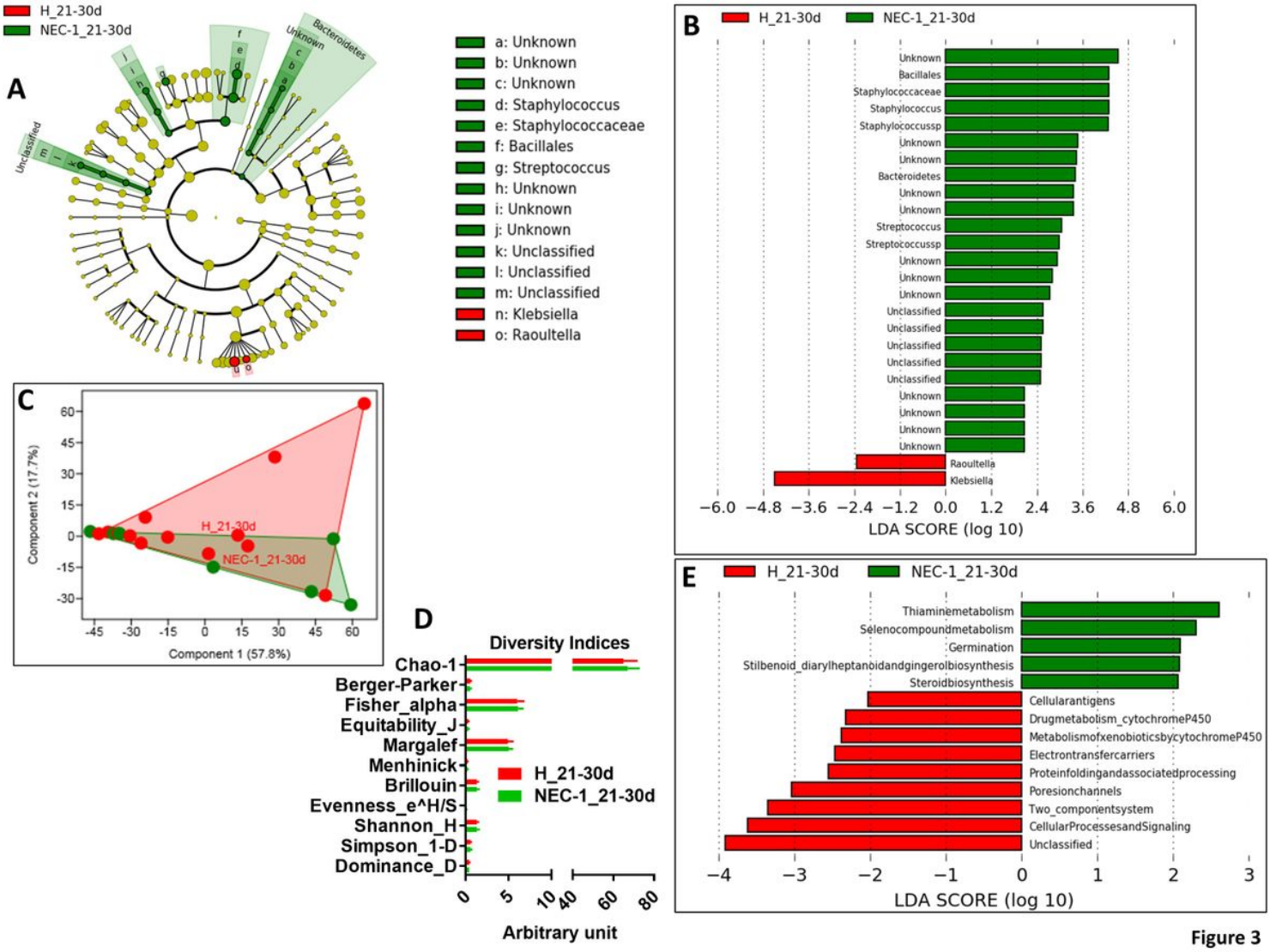

Figure 3

Figure 3

A specific gut microbiota and microbiome exist in the third decade of life in healthy vs. stage 1 necrotizing enterocolitis children. A) Comparative analysis of the gut microbiota by LDA Effect Size (LEfSe): the cladogram shows bacterial taxa significantly higher in the group of children of the same color, in the fecal microbiota between healthy (H) vs. stage 1 necrotizing enterocolitis children (NEC-1), in the third decade of life 21 to 30 days (d) (the cladogram shows the taxonomic levels represented by rings with phyla at the innermost and genera at the outermost ring and each circle is a bacterial member within that level); B) LDA score used to build the cladogram in (A); C) PCA of the gut microbiota; D) Indices of gut microbiota diversity; E) LDA score for microbial pathways. N=13 for H and N=7 for NEC-1.

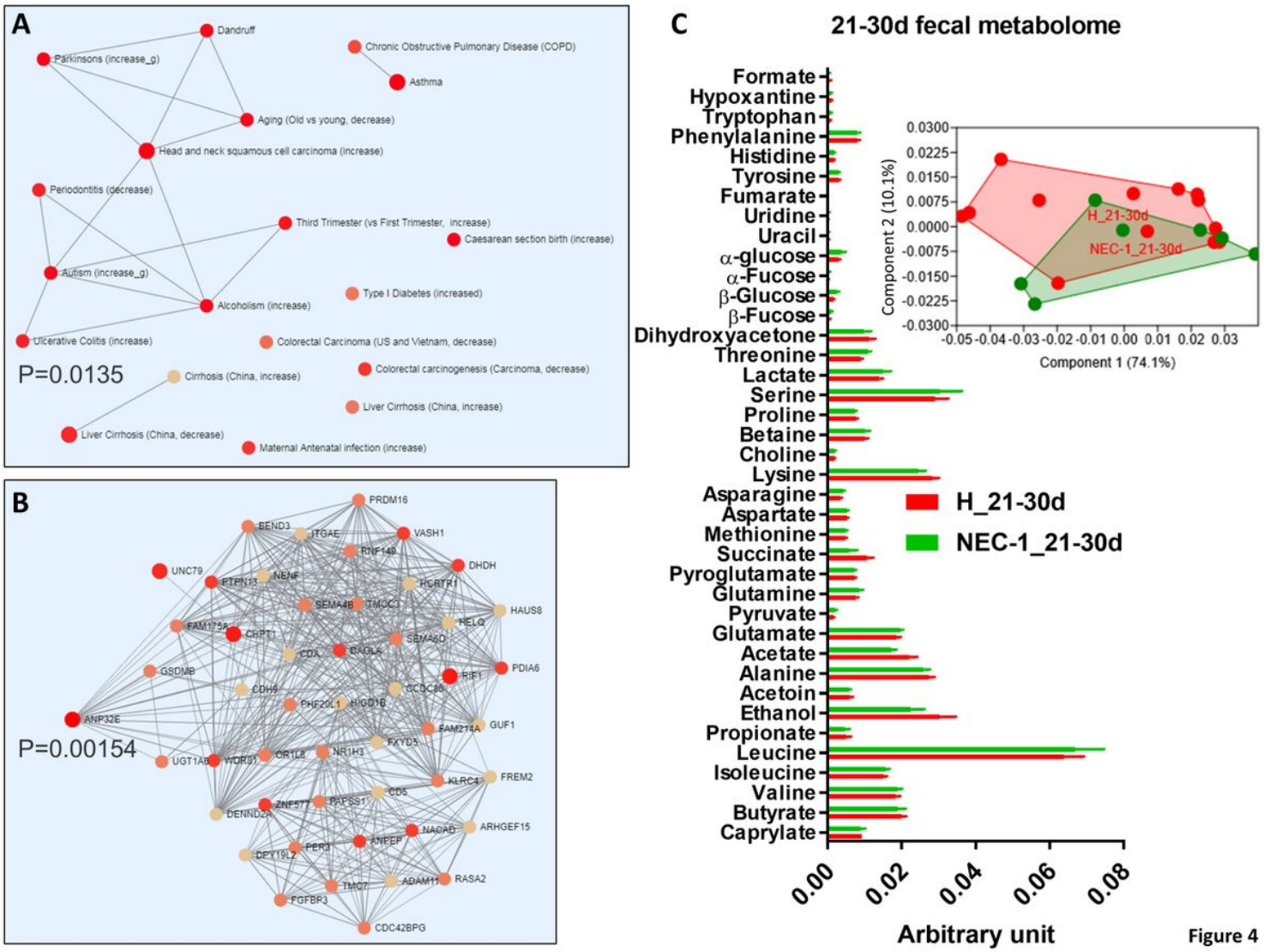

Figure 4

Diseases, host genetic variation and metabolome analysis in the third decade of life during stage 1 necrotizing enterocolitis. A) Diseases and B) host genetic variation linked to NEC-1_21-30d associated gut microbiota; C) histogram of the overall fecal metabolites and PCA as inset. N=13 for H and N=7 for NEC-1.

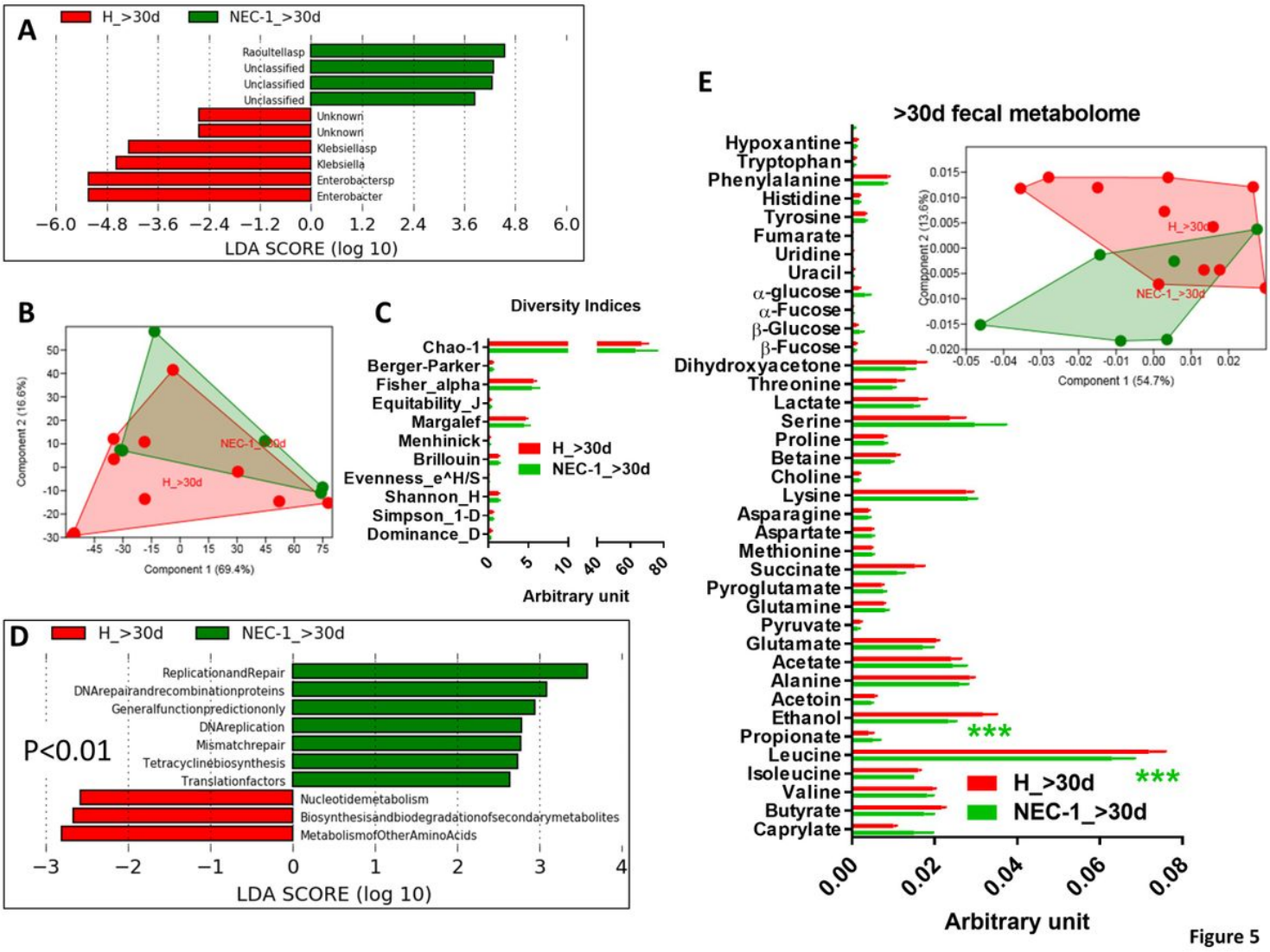

Figure 5

Analysis of gut microbiota, predicted microbiome and metabolome in the second month of life in healthy vs. stage 1 necrotizing enterocolitis children. A) Gut microbiota analysis via LDA score between healthy (H) vs. stage 1 enterocolitis children (NEC-1), in the second month of life >30 days (d); B) Principal Component Analysis (PCA) of the gut microbiota; C) Indices of gut microbiota diversity; D) LDA score for predictive microbial pathways (P<0.01); E) histogram of the overall fecal metabolites and PCA as inset. ***P<0.001. two-way ANOVA followed by a two-stage linear step-up procedure of Benjamini. Krieger and Yekutieli to correct for multiple comparisons by controlling the False Discovery Rate (<0.05); N=11 for H and N=6 for NEC-1.

Supplementary Files

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- SupFigureLegends.docx
- BrehinetalSuppl.Fig.1.JPG
- BrehinetalSuppl.Fig.4.JPG
- BrehinetalSuppl.Fig.3AB.JPG
- BrehinetalSuppl.Fig.6G.JPG