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Abstract:	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 more severe NEC (NEC-2/3), we studied the gut microbiota and metabolome evolution of of children with suspected NEC (NEC-1). 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. 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-1

Evolution of Gut Microbiome and Metabolome during Suspected Necrotizing Enterocolitis (NEC-1): a Case-Control Study

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Short title: NEC-1 gut microbiome and metabolome

Abstract

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 more severe NEC (NEC-2/3), we studied the gut microbiota and metabolome evolution of of children with suspected NEC (NEC-1).

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.

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-1.

Keywords: necrotizing enterocolitis; intestinal microbiology; microbiome; infant gut; metabolomics.

Research in Context

Evidence before this study

Necrotizing Enterocolitis (NEC) is characterized by a change, named dysbiosis, in gut microbiota. However, the major attention has been paid on severe phenotypes of NEC such as NEC-2 and NEC-3. In this context, a microbial signature has not been found neither for NEC-2 nor for NEC-3. Thus, to date, there are no data about gut microbiota dysbiosis during suspected NEC, NEC-1.

Dysbiosis of gut microbiota is also accompanied by a change in metabolites from different biological samples. This may help identify biomarkers associated to a given disease. However, as for gut microbiota dysbiosis, there is also a lack of fecal metabolome studies during NEC-1.

Added value of this study

In NEC-1 children, we have analysed several clinical parameters and found a reduced enteral volume of nutrition at day 7 of life, when compared to healthy children.

Moreover, we provide the study of the evolution of the fecal microbial structure (microbiota) and microbial functions (microbiome) together with the analysis of fecal metabolome in NEC-1 preterm children, compared to healthy children.

We found that NEC-1 gut microbiota starts to diverge in both taxonomy and function by the third decade (10 days) of life, when compared to healthy children. Moreover, the fecal metabolome appears more divergent between healthy vs. NEC-1 children at the second month of life and amino-acids metabolism (serine and leucine) is the most affected metabolic pathway.

We also found NEC-1 microbiome more sensitive than healthy microbiome to factors such as childbirth, low birth weight and gestational age.

Implications of all the available evidence

Our study provides neonatal departments with immediate indications to blunt NEC-1 evolution by: i) increasing the enteral volume of nutrition, especially in the first days of life; ii) revising and reducing antibiotic therapy up to the first week of life in preterm infants.

Introduction

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 suspected NEC (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.

Materials and 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 necrotizing enterocolitis (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(15), 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(16). 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(17). Diseases and host genetic variation linked to NEC-1_21-30d associated gut microbiota were identified via MicrobiomeAnalyst(18), with the Taxon Set Enrichment Analysis module.

Fecal metabolome analysis

The metabolome (total metabolites) analysis of the feces was performed as previously described(19). Pathway-associated metabolite sets and SNP-associated metabolite sets (**Fig.S3C-D**, **Fig.S4B-C** and **Fig.S6G**) were analysed via MetaboAnalyst 4.0(20), 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.

Results

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

To understand the microbial and metabolomic evolution during the early onset of necrotizing enterocolitis (NEC), we studied clinical profile suspected 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.Fig.1A,B**) and a higher abundance of *Streptoccoccus* species (**Suppl.Fig.2A**) compared to healthy children. Both populations of children showed a high intragroup variance in terms of gut microbiota (**Suppl.Fig.2B**) and overall microbial diversity (**Suppl.Fig.2C**). NEC-1 microbiome showed increased activity for pathway related to transcription, glycosaminoglycan degradation and lysosome, compared to healthy children (**Suppl.Fig.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.Fig.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 (**Figure 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 (21), 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.Fig.3A**). The four NEC-1 groups also differed in terms of fecal metabolome, with regard to leucine, ethanol and serine amounts (**Suppl.Fig.3B**). Based on these results, we performed a metabolomic enrichment analysis on two levels: i) pathway-associated metabolite sets (**Suppl.Fig.3C**) and ii) single nucleotide polymorphism (SNP)-associated metabolite sets (**Suppl.Fig.3D**). NEC-1 metabolomic profile (increased ethanol and serine) was significantly associated to both homocysteine degradation and phosphatidylethanolamine biosynthesis (**Suppl.Fig.3C**), with serine being the metabolite the most linked to NEC-1-associated SNP (**Suppl.Fig.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.Fig.4A**). Healthy metabolomic profile (increased leucine, ethanol and dihydroxyacetone) was significantly associated to valine, leucine and isoleucine degradation and to ketone body metabolism (**Suppl.Fig.4B**), with leucine being the metabolite the most linked to healthy-associated SNP (**Suppl.Fig.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.Fig.5A**). By contrast, all the above factors, except the VLBW, affected the gut microbiome (**Suppl.Fig.5B-F**). Note that childbirth modality, ELBW and GA affected the gut microbiome only in NEC-1 children (**Suppl.Fig.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.Fig.6A-F**). Then, we performed again a metabolomic enrichment analysis on the pathway-associated metabolite sets, based on **Suppl.Fig.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.Fig.6G**).

Discussion

In this prospective study we focused on suspected necrotizing enterocolitis 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(22). 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(23). 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(24). Both this datum and the delayed intestinal colonization of preterm infants(25, 26) 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(27). 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(21). 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(28). Despite Anp32e-deficient mice display no sign of disease(29), 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(30), 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(31).

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.

Additional files.

Figure S1. Baseline plasma characteristics in healthy vs. NEC-1. **Figure S2.** Analysis of gut microbiota, microbiome and metabolome during NEC-1 over the first two months of life.

Figure S3. A specific microbiome and metabolome exist in healthy vs. NEC-1 children over the first two months of life. **Figure S4.** Fecal metabolome progression over the first two months of life in healthy children. **Figure S5.** Maternal and child factors shaping gut microbiota and microbiome in healthy vs. NEC-1 children. **Figure S6.** Maternal and child factors shaping fecal metabolome in healthy vs. NEC-1 children.

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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 and Legends

Fig. 1. Analysis of gut microbiota, microbiome and metabolome in the first decade of life in healthy vs. NEC-1 children. A) Gut microbiota analysis via Linear Discriminant Analysis (LDA) score between healthy (H) vs. NEC-1 children, 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.

Fig. 2. Analysis of gut microbiota, microbiome and metabolome in the second decade of life in healthy vs. NEC-1 children. A) Gut microbiota analysis via LDA score between healthy (H) vs. NEC-1 children, 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.

Fig. 3. A specific gut microbiota and microbiome exist in the third decade of life in healthy vs. NEC-1 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. NEC-1 children, 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.

Fig. 4. Diseases, host genetic variation and metabolome analysis in the third decade of life during NEC-1. 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. NEC-1 children. A) Gut microbiota analysis via LDA score between healthy (H) vs. NEC-1 children, 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.

Fig.S1. Baseline plasma characteristics in healthy vs. NEC-1 children. A) pH; B) enteral milk volume at day 7; C) hemoglobin (Hb); D) white blood cells (WBC); E) platelets; F) lactate; G) preterm premature rupture of the membranes. *P<0.05, ***P<0.001. Mann-Whitney. N=21 for H and N=11 for NEC-1.

Fig.S2. Analysis of gut microbiota, microbiome and metabolome during NEC-1 over the first two months of life. A) Gut microbiota analysis via LDA score between healthy (H) vs. NEC-1 children; B) 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<0.0001, 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=53 for H and N=27 for NEC-1.

Fig.S3. A specific microbiome and metabolome exist in healthy vs. NEC-1 children over the first two months of life. A) INTRA_NEC-1 LDA score for microbial pathways; B) histogram of the overall fecal metabolites and PCA as inset; C) INTRA_NEC-1 pathway-associated metabolite sets; D) INTRA_NEC-1 SNP-associated metabolite sets. **P<0.05, ***P<0.01, ****P<0.0001, 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).

Fig.S4. Fecal metabolome progression over the first two months of life in healthy children. A) histogram of the overall fecal metabolites and PCA as inset; B) INTRA_H pathway-associated metabolite sets; C) INTRA_H SNP-associated metabolite sets. ***P<0.01, ****P<0.0001, 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).

Fig.S5. Maternal and child factors shaping gut microbiota and microbiome in healthy vs. NEC-1 children. Six factors were examined: neonatal antibiotics (ABx), ABx treatment on mother, C-section (C-sec) vs. vaginal birth (VB), very low birth weight (VLBW), extremely low birth weight (ELBW) and gestational age (GA) > or ≤ 28 weeks (28w); A) Gut microbiota analysis via LDA score; B) to F) microbiome analysis via LDA score. P<0.05 or P<0.01 as indicated (B).

Fig.S6. Maternal and child factors shaping fecal metabolome in healthy vs. NEC-1 children. Six factors were examined: neonatal antibiotics (ABx), ABx treatment on mother, C-section (C-sec) vs. vaginal birth (VB), very low birth weight (VLBW), extremely low birth weight (ELBW) and gestational age (GA) > or ≤ 28 weeks (28w). A) to F) histogram of the overall fecal metabolites. **P<0.05, ***P<0.01, ****P<0.0001, 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). G) NEC-1_GA≤28w pathway-associated metabolite sets.

Figure 1

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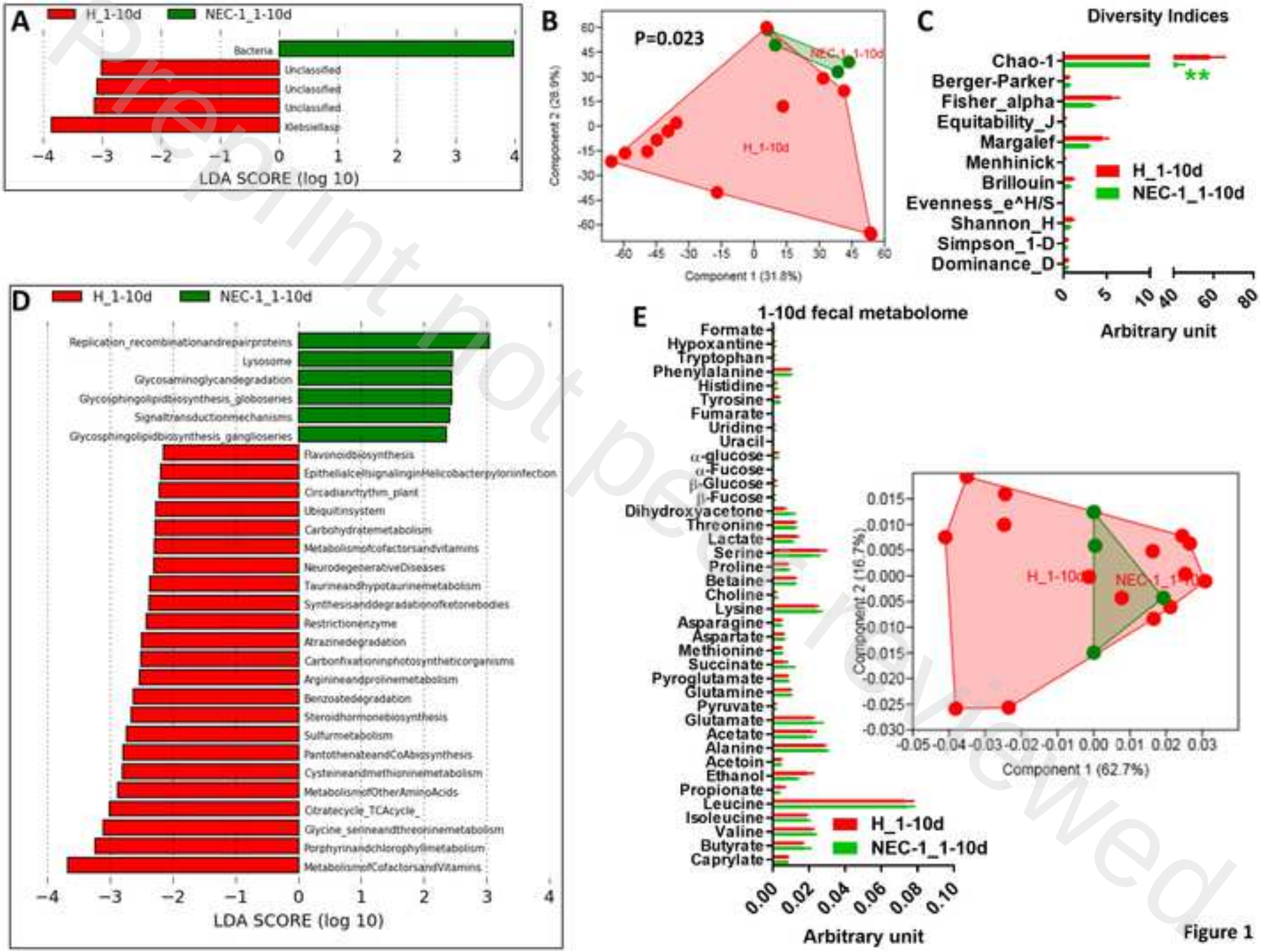

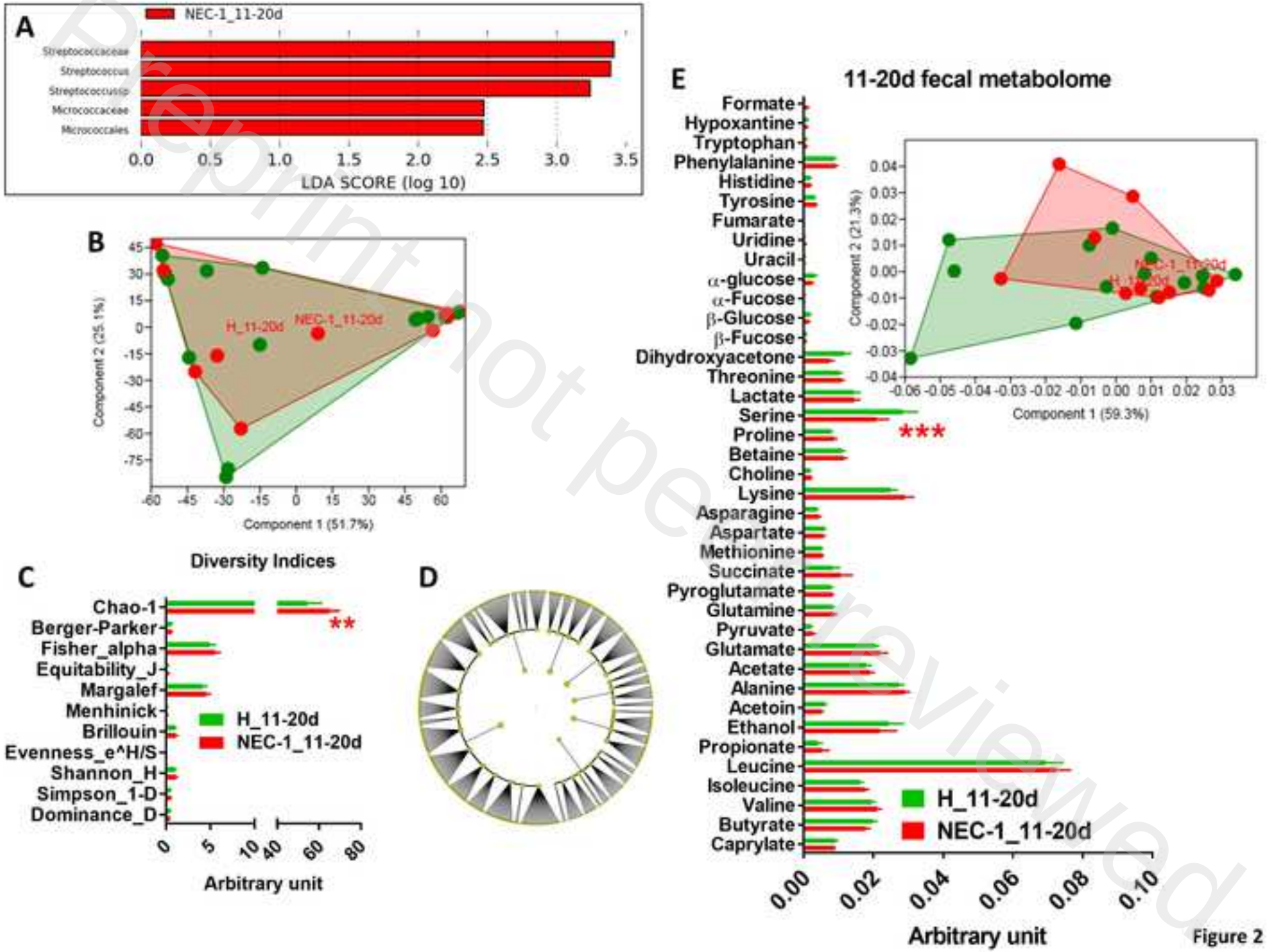

Figure 2

Figure 3

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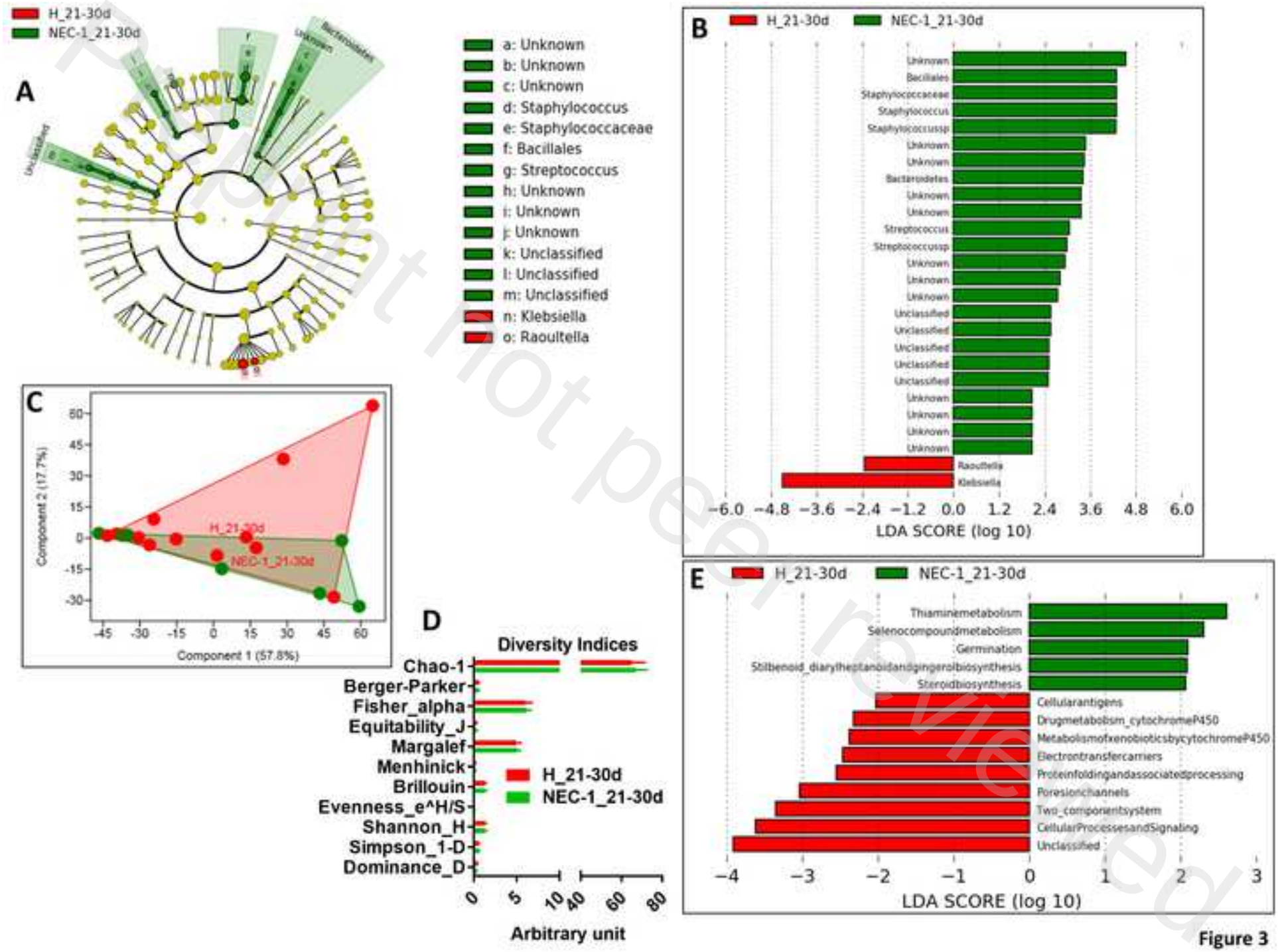
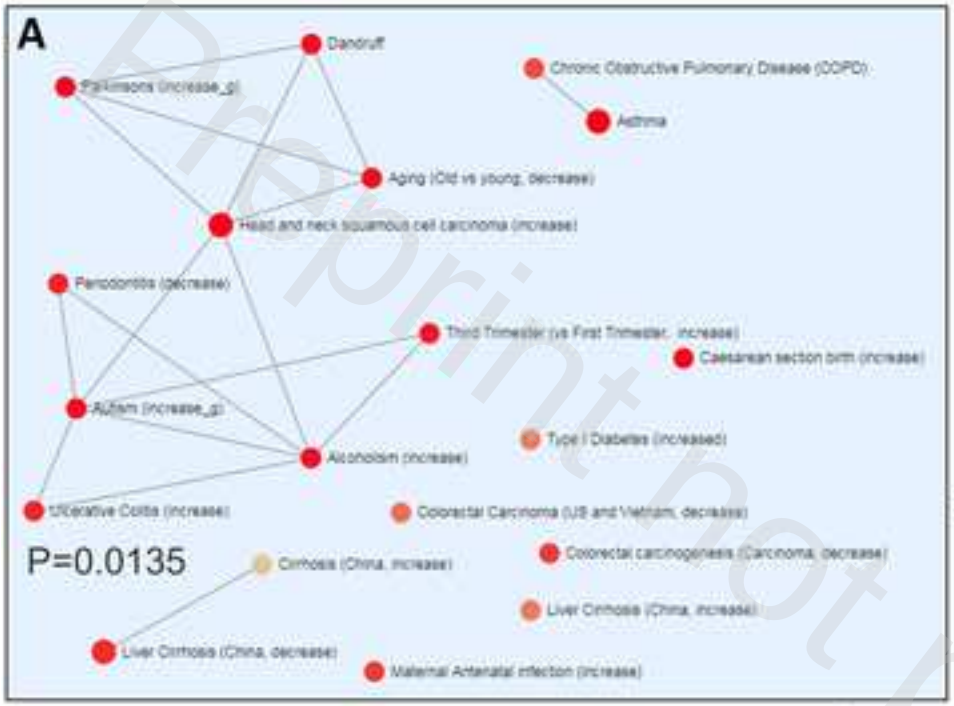
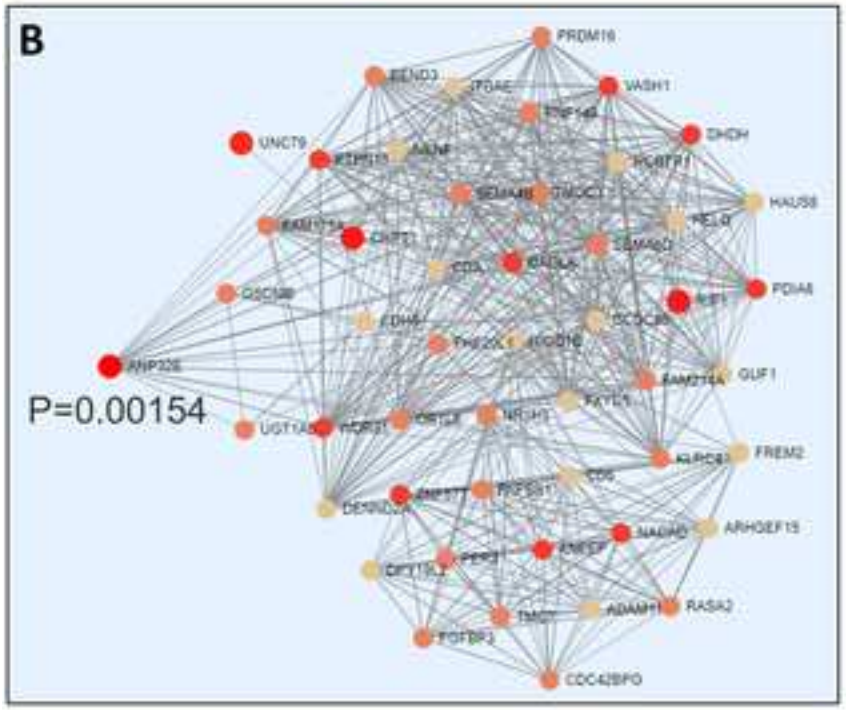


Figure 3

Figure 4





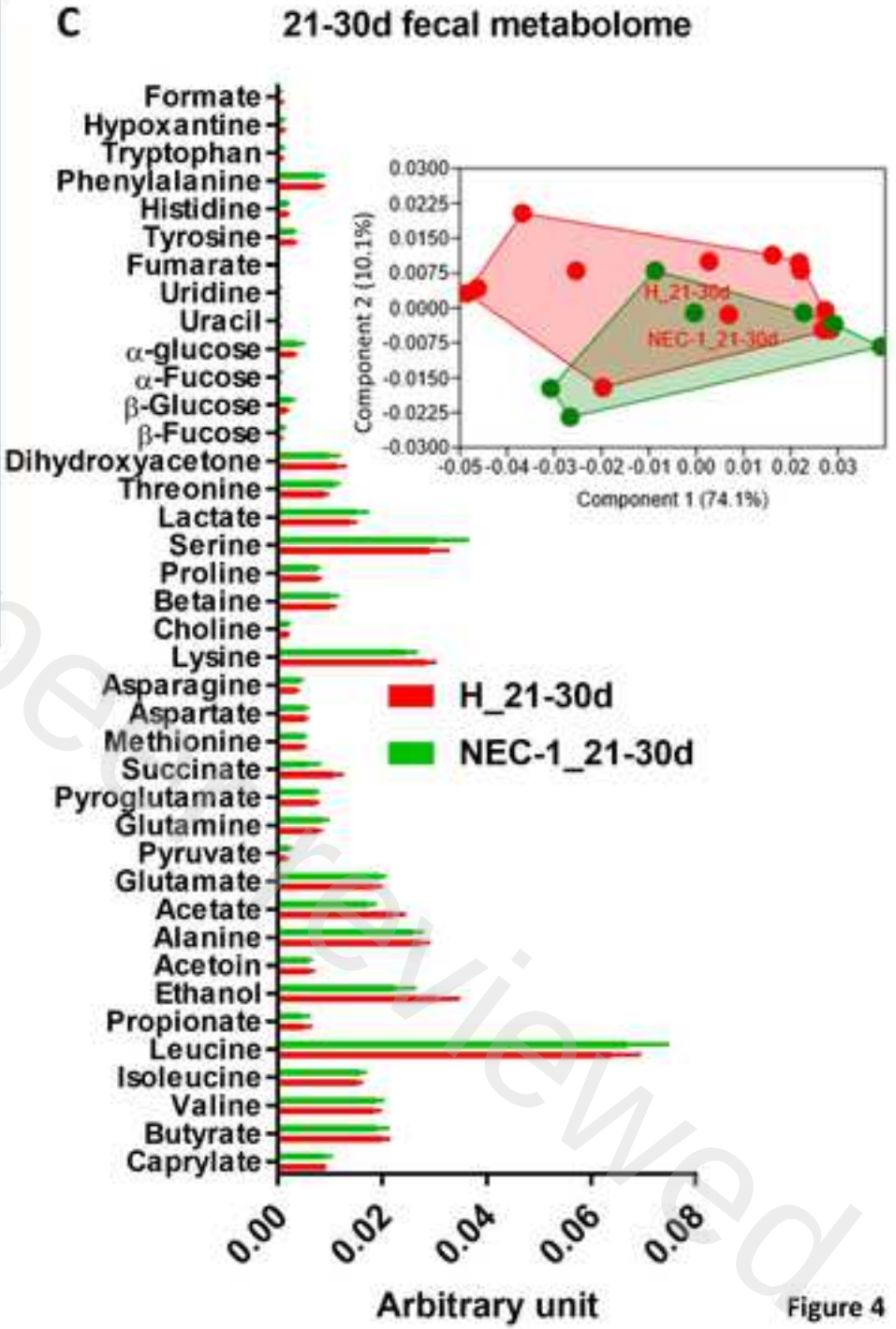


Figure 4

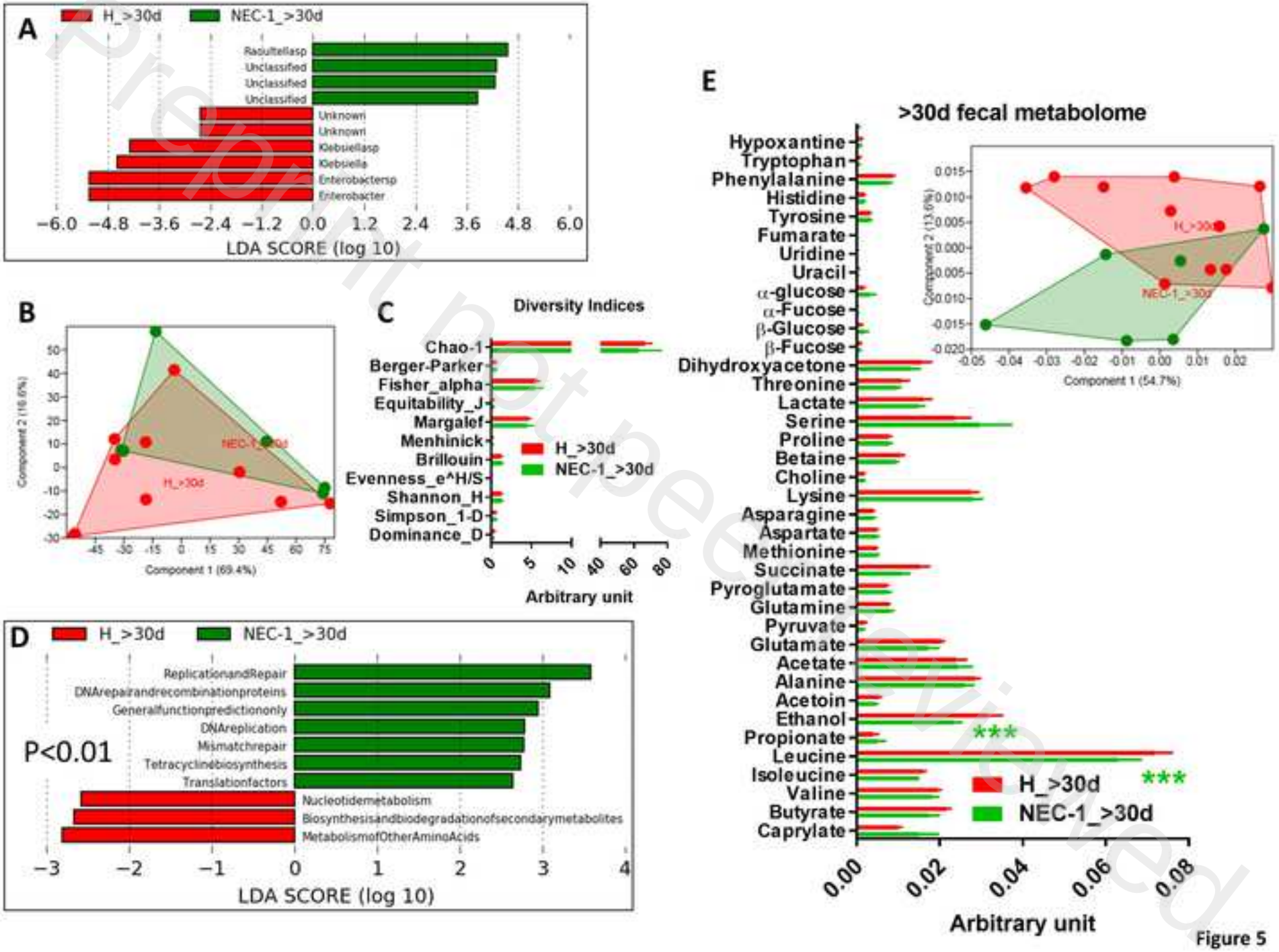


Figure 5

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

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Short title: NEC-1 gut microbiome and metabolome

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*****

16

17