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1 **Antibiotics may influence gut microbiome signaling to the brain in preterm neonates**

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15 **Abstract**

16 Antibiotic use in neonates can have detrimental effects on the developing gut microbiome,
17 increasing the risk of morbidity. A majority of preterm neonates receive antibiotics after birth
18 without clear evidence to guide this practice. Increasing evidence suggests associations between
19 early-life antibiotic use, intestinal dysbiosis, and morbidity that challenge the efficacy of this
20 common neonatal practice. Here we present microbiome and metabolomic results from the
21 Routine Early Antibiotic use in Symptomatic preterm Neonates (REASON) study, which is the
22 first trial to randomize symptomatic preterm neonates to receive or not receive antibiotics in the
23 first 48 hours after birth. Using 16S rRNA sequencing of stool samples collected longitudinally
24 for 91 neonates, we show that antibiotics alter trends in microbiome diversity and development
25 between randomized neonates and illustrate that type of nutrition helps shape the early infant gut
26 microbiome. Integrating multi-omic data for the gut microbiome, stool immune markers, stool
27 metabolites, and inferred metabolic pathways, we identified an association between *Veillonella*
28 and the neurotransmitter gamma-aminobutyric acid (GABA). Our results suggest early antibiotic
29 use may impact the gut-brain axis with the potential for consequences in early life development.

30 **Main**

31 Premature infants are particularly susceptible to infections secondary to increased need for
32 invasive procedures and immaturity of the immune system, skin, and gastrointestinal tract[1–3].
33 Increasingly, there is growing concern that risk factors for mortality may originate from
34 underlying pathologies that could also be responsible for premature birth[4]. Symptoms of
35 prematurity are difficult to discern from symptoms of infection which, compounded by the

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36 increased risk of infection, have led to most premature infants being exposed to antibiotics early
37 in life[5–7]. Despite high mortality rates, the incidence of culture positive early onset sepsis (EOS)
38 is relatively low, between 0.2-0.6%[8]. In the absence of a positive culture, a majority of preterm
39 infants receive antibiotics immediately after birth based on maternal risk factors (e.g. intra-
40 amniotic infection) or laboratory abnormalities (e.g. elevated serum C-reactive protein (CRP)
41 because of the risk of mortality[8]. Given the low incidence of culture positive EOS in this
42 population, it is possible that such high rates of antibiotic use are unnecessary and may increase
43 morbidity in these infants[9]. Other morbidities in the neonatal intensive care unit (NICU) such as
44 necrotizing enterocolitis (NEC) also have high mortality rates and have been associated with
45 prolonged antibiotic exposure.[10,11]. Nevertheless, antibiotics remain the most commonly
46 prescribed medication in the NICU[12,13].

47 The gut microbiome comprises a highly volatile community structure early in life[14]. Microbial
48 colonization is influenced as early as birth by mode of delivery, and perhaps even in the uterine
49 environment by maternal factors[15,16]. Not surprisingly, antibiotic use has been shown to also
50 change the composition of the preterm gut community[17–20]. Furthermore, antibiotic use early in
51 life has increasingly been associated with adverse outcomes both short- and long-term[21,22]. One
52 possible consequence is the disruption of the gut-brain axis (GBA), which involves bi-directional
53 transmission of bio-molecular signals between the gut microbiota and the nervous system[23].
54 Aberrations in the GBA have been associated with altered immune homeostasis, as well as
55 psychiatric, behavioral and metabolic conditions in adulthood[24]. It is therefore imperative to
56 determine if such high rates of antibiotic use in preterm infants is necessary, as it could have
57 lifelong consequences on future health.

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58 Randomized clinical trials have the advantage of controlling for the numerous covariates that
59 could interfere with answering whether preemptive antibiotic use in preterm infants affects
60 outcomes. The Routine Early Antibiotic use in Symptomatic preterm Neonates (REASON) study
61 is the first to randomize symptomatic premature infants to either receive or not receive
62 antibiotics soon after birth. Previously reported results from this study demonstrate the feasibility
63 of such a trial and that withholding antibiotics did not lead to a significant increase in neonatal
64 mortality or morbidity. By employing a multi-omic approach, this randomized trial also provides
65 the unique opportunity to understand how antibiotic intervention perturbs the early life gut
66 microbiome, metabolome, and inflammatory environment in ways that may be consequential to
67 health and development.

68 **Results**

69 **Cohort and study description**

70 Ninety-eight infants were enrolled in the study over the two-year period: group A – antibiotics
71 indicated (n=32), group B – antibiotics not indicated (n=11), and group C – eligible for
72 randomization (n=55). Twenty-eight infants were randomized to C1 (antibiotics x 48 hours) and
73 27 infants were randomized to group C2 (no antibiotics). For 13 infants (48%) in group C2,
74 antibiotics were prescribed in the first 48 hours after birth. Neither sex nor mode of delivery
75 were significantly different between all groups and were not significantly different between the
76 two randomized subgroups using the chi-square test. Both weight (p-value=0.0001) and
77 gestational age (p-value=0.0002) were significantly different between groups overall, with group
78 A infants on average with lower gestational age (GA) and at lower birth weights. Neither birth
79 weight nor GA were significantly different between the randomized subgroups by ANOVA. A
80 summary of the infants in this analysis is provided in Extended Data Fig. 1.

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81 Six hundred ninety-three stool samples were collected longitudinally for 91 of the total 98 infants
82 enrolled. Stool data was not available for 7 infants due to early mortality. Sequencing data for
83 16S rRNA was able to be obtained for 656 of those samples. After rarefying to an even
84 sequencing depth of 10,000 reads per sample, 642 samples remained for further analysis. The
85 breakdown of samples by group is provided in Extended Data Fig. 1. The aim for this analysis is
86 to test the effects of randomization to antibiotics vs. no antibiotics has on the developing gut
87 microbiome, metabolome and inflammatory environment using high-throughput 16S rRNA
88 sequencing, quantitative PCR (qPCR), metabolomics, pathway inference, immune marker
89 analysis and open-source statistical tools.

90 **Antibiotic use affects trends in early gut microbiome development over time**

91 The within-sample diversity differed by enrollment groups A, B, C1, and C2. Using amplicon
92 sequencing variants (ASVs), the richness, and the alpha diversity were not significantly different
93 between enrollment groups overall (Kruskal-Wallis, Richness: p = 0.31, Shannon: p = 0.90) and
94 did not differ across time using a linear mixed effects model (LME) through Qiime2[25–27]
95 (Shannon p-values: group A = 0.98, group B = 0.70, group C1 = 0.83, group C2 = 0.926).
96 Though not statistically significant, scatter plots of each sample's alpha diversity, including
97 regression lines for each group, indicate trends in diversity development over time between the
98 groups (Fig. 1a, Shannon diversity). These trends are expected given the variation in antibiotic
99 levels used by each group. For example, group B (antibiotics not indicated) tended to have the
100 highest trend in diversity over time and is the group with the lowest antibiotic use. In contrast,
101 group A (antibiotics indicated) infants saw very little diversity development over time while
102 receiving the most antibiotics. Certain considerations need to be made when comparing infants

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103 by enrollment group. For example, group A infants had significantly lower GA and birth
104 weights, particularly compared to group B infants who on average had the highest GAs and birth
105 weights, and the shortest stays in the NICU. Furthermore, infants in randomized group C2 who
106 were changed to receive antibiotics in the first 48 hours after birth can no longer be considered as
107 receiving no antibiotics and would be more similar to group C1. To account for this in testing the
108 effect of antibiotic use, we focus only on the randomization group, taking into consideration
109 those infants who were changed to receive antibiotics by instead separating infants based on any
110 antibiotic use within 48 hours after birth (any_abx).

111 Samples from group C infants that were collected during antibiotic use (abx_during_sample =
112 TRUE) were compared with those during periods of no treatment (abx_during_sample =
113 FALSE). As anticipated, samples taken during periods of antibiotic use had lower richness and
114 lower bacterial load, as determined by quantitative PCR (qPCR) of 16S rRNA copies per gram of
115 stool (Figs. 1b,c). As this result may be driven by samples collected early in life, just those
116 samples collected in the first week of life were also compared and were not significantly
117 different in richness (Wilcoxon, $p = 0.70$), alpha diversity (Wilcoxon, $p = 0.43$) and bacterial
118 load (Wilcoxon, $p = 0.50$).

119 Taking into consideration those infants in group C2 who were changed to antibiotic use, bacterial
120 richness was plotted as a function of time (Collection_week) considering whether the infant
121 received antibiotics (any_abx) (Fig. 1d). Pairwise comparisons of the antibiotic and no antibiotic
122 infants at each collection week were not statistically significant (Wilcoxon test, $p > 0.05$).
123 Initially within the first few weeks of life, bacterial richness is similar regardless of antibiotic

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124 use. However, beginning at the fifth collection week (30 days after birth on average) infants who
125 never received antibiotics trended towards higher bacterial richness compared to infants that
126 were treated. Average bacterial richness remained higher among the no antibiotic infants through
127 collection week 8 (53 days after birth on average). Again using linear mixed effects modelling,
128 we found that both bacterial richness and Shannon diversity trended higher in infants who never
129 received antibiotics compared with those that did, though neither diversity metric was significant
130 as a function of time (Richness: $p = 0.35$, Shannon: $p = 0.26$) (Figs. 1e,f). As with the infants in
131 group C, infants who were randomized to not receive antibiotics and were not changed to receive
132 antibiotics (any_abx = FALSE) had shorter stays in the NICU. Furthermore, although the
133 randomized groups are almost evenly powered without considering change in antibiotic use
134 status, removing C2 infants who received antibiotics underpowers the group of infants who never
135 received antibiotics, limiting the strength of comparisons.

136 Changes in overall bacterial community structure were apparent between groups when assessing
137 beta-diversity. Both the Bray-Curtis and Jaccard distance indices were used to assess community
138 structure taking into account quantitative ASV abundance and qualitative presence/absence
139 information, respectively. Beta dispersion was not significantly different between groups using
140 the Bray-Curtis metric (ANOVA; Df = 3, Sum of Squares = 0.01589, Mean Squares =
141 0.0052974, $F = 2.547$, $p = 0.055$) but was significant using Jaccard (ANOVA: Df = 3, Sum of
142 Squares = 0.0315, Mean Squares = 0.01050, $F = 8.4013$, $p = 1.75E-05$), specifically group A
143 versus all other groups (TukeyHSD, B-A $p = 0.0153$, C1-A p-value = 0.0001, C2-A $p = 0.0028$).
144 With the test assumption met for the Bray-Curtis index, PERMANOVA also showed distance

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145 dissimilarity to be significant between groups (PERMANOVA; Df = 3, Sum of Squares = 4.642,
146 R_2 = 0.01632, F = 3.544, p = 0.001). Principle coordinates analysis (PCoA) suggests an effect of
147 antibiotic use by enrollment group. The infants exposed to the least amount of antibiotics
148 through the NICU course (group B and group C2 that were not changed to receive antibiotics)
149 had tighter clustering using both Bray-Curtis and Jaccard, whereas groups A and C1 had the
150 highest variability, suggesting less antibiotic use led to greater microbiome stability (Figs. 2a,b).
151 Similarly, group C1 had greater spread by PCoA using Bray-Curtis but not Jaccard (Figs. 2c,d).
152 Infants in group C2 who were changed to receive antibiotics in the first 48 hours but did not
153 receive antibiotics again had much tighter clustering, indicating the outcome of early antibiotics
154 in the randomized group and the effect antibiotics use has on microbiome stability (Figs. 2e,f).

155 **Feeding patterns drive changes in gut diversity and bacterial load**

156 For preterm infants, diet generally consists of mother's breast milk, pasteurized donor breast
157 milk, and/or formula. To control for any effect of antibiotics, only samples from group C2
158 infants that were not changed to receive antibiotics in the first 48 hours were used. Using the
159 calculated alpha diversity metrics used previously, feeding type was found to significantly
160 associated with bacterial community richness (Kruskal-Wallis, p = 0.0016) and Shannon
161 diversity (Kruskal-Wallis, p = 6.2E-05) (Figs. 3a,b). In addition, feeding type had an effect on
162 total bacterial load measured by the number of 16S rRNA copies per gram of stool for each
163 sample (Kruskal-Wallis, p = 0.003) (Fig. 3c). As expected, stool collected during times of no
164 enteral feeding (NPO) had the lowest richness and total bacterial load. Samples collected during
165 formula feeding had just as high of richness and diversity as samples during feeding with

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166 mother's milk. However, samples collected during feeding with mother's milk tended to have the
167 higher bacterial load (Wilcoxon, $p = 0.0057$). Donor breast milk (DBM), which is pasteurized
168 prior to use, tended to have lower richness, diversity, and bacterial load compared to other
169 feeding types except NPO. Interestingly however, combining formula with DBM trended
170 towards increased diversity and bacterial load, though not significantly, most likely due to the
171 limited number of samples collected during feeding with DBM + formula from group C (n= 2).

172 Clustering analysis by PCoA also showed trends in gut composition based on feeding type (Figs.
173 3d,e). Stool collected while feeding with mother's milk had the widest variability in overall
174 composition by both Bray-Curtis and Jaccard, whereas donor milk had the tightest clustering.
175 Notably, samples taken during formula or formula mixed with mother's milk had similar
176 clustering by Bray-Curtis, but formula feeding alone led to more variability with Jaccard,
177 suggesting addition of mother's milk had little effect on the abundance of bacteria but formula
178 feeding did contribute more variability in the presence or absence of certain bacteria. Not
179 considering samples from infants in group C2 who were changed to antibiotics, feeding type had
180 a significant effect on community structure by both Bray-Curtis (PERMANOVA, Df = 7, Sum of
181 Squares = 5.527, $R_2 = 0.133$, $F = 1.98$, $p = 0.001$) and Jaccard (PERMANOVA, Df = 7, Sum of
182 Squares = 4.692, $R_2 = 0.118$, $F = 1.720$, $p = 0.001$). Along the same lines, pairwise comparisons
183 identified significant differences in beta diversity between mother's milk vs. formula, donor milk
184 vs. formula, mother's milk vs. donor milk, and mother's milk vs. donor milk mixed with formula
185 (PERMANOVA, $p < 0.05$). These results indicate feeding type as a significant source of
186 variation in the composition of the preterm infant gut in addition to antibiotic use.

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187 **Gut microbial community development is highly variable and unique to each infant**

188 Although the infants in this study were enrolled into 4 groups, each infant's stay in the NICU is
189 highly personalized, by type, frequency, number or length of antibiotic use, type and length of
190 feeding patterns, and adverse clinical events over time. To aid in visual identification of patterns
191 throughout the NICU course, detailed charts were created for each infant that depict both clinical
192 and laboratory data over time (Days Post Birth) integrated into a single graphic per infant (Fig.
193 4). This includes results from the 16S rRNA analysis as pie charts for each stool sample, color
194 coded by bacterial taxonomy and sized based on the $\log_{10}$-transformed number of 16S rRNA
195 copies per gram of stool, as well as adverse clinical events coded by a single letter code
196 (Extended Data Fig. 2 and Supplementary Figure 1). With these visualizations, patterns are more
197 easily observed between antibiotic treatments, feeding types, and the gut microbiome. For
198 example, anti-fungal fluconazole administration resulted in lower bacterial loads and diversity.
199 Also, the effects of antibiotic use and feeding patterns become visually apparent. To illustrate,
200 infant 5 was exclusively fed mother's milk from day 14 through day 71 post birth. At day 31,
201 antibiotics vancomycin and piperacillin were administered (Supplementary Figure 1). During
202 treatment, *Veillonella* was entirely removed from the stool microbiome, falling from 3.23E+06
203 cells per gram (73%) from the pre-treatment time point to undetected while antibiotics were
204 used. At day 47, 9 days after antibiotic treatment ceased, *Veillonella* again dominated the stool at
205 3.83E+06 cells per gram (80%), almost a complete replacement of levels before treatment. Also,
206 the proportions of the other 2 genera found in the stool, *Escherichia* and an unclassified
207 Enterobacteriaceae spp., were nearly identical after treatment and continuation of mother's milk

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208 as before treatment (pre-treatment: *Escherichia* – 21%, Enterobacteriaceae spp. – 4.7%; post-
209 treatment: *Escherichia* – 15%, Enterobacteriaceae spp. – 3.3%), demonstrating that mother's
210 milk effectively restored the stool microbiome to its pre-treatment state.

211 **Bacterial genera correlate with stool metabolites and inferred metabolic pathways**

212 In addition to 16S rRNA profiling, 90 stool samples from 10 infants were sent for metabolomic
213 profiling. Each of the four enrollment groups were included in these samples for comparison.
214 Peak height responses were recorded for 454 identifiable metabolites. To determine if gut
215 bacteria were associated with relative concentrations of metabolites in stool, the top 10 most
216 abundant bacterial genera associated with identified metabolites were determined. Spearman
217 correlation values were plotted using a heatmap, which indicated numerous significant, positive
218 and negative, associations between bacteria and metabolites (Fig. 5a). Next, 6 of the top 10
219 bacterial genera were correlated between infant groups (Fig. 5b). Interestingly, *Veillonella* was
220 positively associated with the neurotransmitter 4-aminobutanoate (GABA) (Spearman R = 0.48,
221 p = 0.003) and *Veillonella* counts were significantly different between groups A and C2
222 (Wilcoxon, p = 0.0475). *Veillonella* counts were plotted using a boxplot, along with the
223 normalized concentrations of GABA between groups (Figs. 5c,d). Furthermore, using
224 PICRUSt2, functional pathway abundances were inferred based on the rarefied 16S rRNA data[28].
225 The *Veillonella* counts of predicted pathways were strongly correlated with biosynthesis of the
226 GABA precursor L-glutamate and L-glutamine (Spearman R = 0.88, p = 3.02E-27) (Extended
227 Data Fig. 3). Thus, *Veillonella* may be at least partially responsible for GABA neurotransmitter
228 production and that this function is negatively impacted by antibiotic use early in life.

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229 Alternatively, a negative correlation between *Bifidobacterium* counts and glycocholic acid was
230 observed (Spearman R = -0.39, p = 0.0098), which was impacted by antibiotic use between
231 groups (Extended Data Fig. 4). Furthermore, bifidobacteria were negatively associated with other
232 conjugated bile acids including taurocholic (Spearman R = -0.45, p = 0.0021) and glycocholic
233 acids (Spearman R = -0.39, p = 0.0098), but positively associated with deconjugated cholic
234 (Spearman R = 0.36, p = 0.0203). Thus, gut microbiota affected by antibiotic use may be
235 responsible for modification of neuroactive metabolites (i.e. deconjugated bile salts) in addition
236 to production of neurotransmitters.

237 **Immune markers in stool correlate with bacterial abundance**

238 Antibiotic use was examined for its correlation with inflammatory marker level. These levels
239 were also correlated with gut bacterial abundances. Twelve immune markers were measured in
240 110 samples across 18 of the first enrolled infants. Twelve bacterial genera had at least one
241 significant correlation with an immune marker ($p < 0.05$) (Fig. 6a). Significant correlations
242 between the bacterial genera and stool immune markers were classified as either inflammatory or
243 anti-inflammatory based on the known function of the marker (Fig. 6b). Interestingly,
244 *Enterococcus* was consistently negatively correlated with most of the immune markers
245 measured. *Enterococcus* counts were lower in group A compared to the randomized groups
246 (Wilcoxon, A vs. C1 p = 1.6E-06, A vs. C2 p = 9.4E-07) (Fig. 6c). *Enterococcus* counts did not
247 differ significantly between infants who received or did not receive antibiotics (Wilcoxon, p =
248 0.2) (Fig. 6d). *Enterococcus* had a significant negative correlation with IL8:IL10 ratios, whereas
249 this genus was positively correlated with IL1a:IL1ra ratios, suggesting that mechanisms of

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250 controlling inflammation can be unique depending on the microbe. Notably, MIP1a was the only
251 marker that differed significantly depending on antibiotic use (Wilcoxon, p = 0.039). *Citrobacter*
252 was positively correlated with MIP1a (Spearman R = 0.47, p = 3.74E-05), and was significantly
253 higher in group C1 compared to group C2 (Wilcoxon, p = 7.2E-08). *Citrobacter* was higher in
254 those infants that received antibiotics compared to those that did not (Wilcoxon, p = 0.037).

255 **Discussion**

256 There is an urgent need for evidence supporting or refuting the widespread practice of routine
257 antibiotic use after birth in symptomatic preterm neonates. The REASON study represents a
258 significant step as it is the first randomized controlled trial to test the feasibility of randomizing
259 symptomatic preterm infants to antibiotics versus no antibiotics, evaluating the effect of
260 antibiotic treatment on the developing gut microbiome, metabolome, and inflammatory
261 environment. Our results expand upon previous reports with new evidence that early routine
262 antibiotic use leads to greater beta-diversity dissimilarity and lowered trends in richness and
263 Shannon diversity development over time, even after discontinuation of antibiotics[17,29,30]. The
264 power to detect significant associations was decreased mainly because many of the infants
265 randomized to not receive antibiotics were changed to antibiotic administration. A larger multi-
266 center randomized study is needed to validate and expand upon the extended effect of antibiotics
267 on the developing gut microbiome.

268 Our results confirm that feeding type also have a significant influence on gut microbiome
269 richness, diversity and bacterial load[31–33]. Exclusive or partial feeding with mother's own milk
270 tended to associate with high bacterial load, which is supported by evidence that breast milk
271 harbors maternal-originating bacteria, as well as nutritional components (prebiotics) that support

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272 bacterial proliferation in the intestinal tract[34,35]. Interestingly, formula-fed infants had
273 comparable levels of richness, diversity and bacterial load as mother's milk. This supports the
274 notion that mother's milk drives early colonization of a limited set of dominant microbes through
275 nutrient and antimicrobial-mediated selection[36–38].

276 Integrating detailed and personalized records of clinical and laboratory data led us to identify
277 overlooked patterns in the data. One such peculiar pattern was that stool samples taken during
278 administration of the anti-fungal fluconazole had lowered copies of 16S rRNA, suggesting lower
279 bacterial load. A previous study reported that fluconazole, though not inherently bactericidal,
280 increased the bactericidal activity of neutrophils[39]. Immune marker data were collected for 18 of
281 the first enrolled infants, and one or more of those markers, such as calprotectin which is
282 secreted by neutrophils, may help explain this pattern[40]. However, unfortunately only 3 of the 18
283 infants that had immune marker data received fluconazole, and therefore more data is needed to
284 test this hypothesis. Interestingly, counts of *Enterococcus* were often negatively correlated with
285 levels of many of the immune markers tested, both pro- and anti-inflammatory, an odd finding
286 considering Enterococci have been associated with risk for infection in preterm neonates[41,42]. On
287 the other hand, *Citrobacter* counts were associated with increased levels of the macrophage
288 chemokine MIP1α and counts were significantly higher in infants who received antibiotics.
289 Increased levels of MIP1α are likely related to recruitment of intestinal macrophages leading to a
290 heightened inflammatory environment, suggesting that antibiotic use may select for bacteria
291 which lead to intestinal inflammation[43]. Further work including a larger sample size will be
292 needed to understand how changes in the preterm infant gut caused by routine antibiotics impacts
293 the gut inflammatory environment.

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294 Neurological development can be impaired in infants born very prematurely compared to their
295 full-term counterparts; a trend that extends into delayed cognitive and behavioral development
296 through childhood[44–46]. Could routine antibiotic use in preterm neonates play a role in this
297 association? Intestinal microbes produce a plethora of metabolites and bio-active compounds that
298 can be absorbed by the host[47]. Some of these compounds have direct neurologic implications
299 including neurotransmitters such as GABA, which is reduced in preterm infants, is critical for
300 early brain development, and possesses immunomodulatory properties[48,49]. Our results indicate
301 that antibiotic use negatively affected the abundance of *Veillonella* and that *Veillonella* were
302 positively correlated with GABA concentrations in the gut. Furthermore, *Veillonella* correlated
303 strongly with the L-glutamine biosynthesis pathway, the precursor to GABA production. Aside
304 from production of neurotransmitters, we identified negative correlations between
305 *Bifidobacterium* abundance and concentrations of conjugated bile acids, particularly glyco- and
306 taurocholic acid, which were significantly different based on antibiotic use. Bifidobacteria,
307 which were more abundant in infants that did not receive antibiotics, are known to deconjugate
308 bile acids to primary forms including cholic acid, which was positively correlated with
309 bifidobacteria abundance[50,51]. Cholic acid can passively diffuse into the brain where it blocks
310 signaling in the $GABA_A$ receptor[52]. Bifidobacteria may therefore be essential in regulating
311 GABA signaling in the developing brain. These are significant findings, for they suggest routine
312 antibiotic use could be disrupting processes involved in the gut-brain axis and
313 immunomodulatory pathways critical for neonatal and future childhood development.

314 Evidence-based antibiotic use to prevent infection in preterm neonates is critical in preventing
315 unnecessary treatment that may be doing more harm than good. Routine overuse of antibiotics
316 can change the developmental trajectory of the infant gut microbiome during a time of critical

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317 establishment and interaction. Given the potential for extensive crosstalk between gut microbiota
318 and the host, changes in microbiome composition could have both short- and long-term effects
319 on outcomes and overall health and development. Future randomized studies with greater infant
320 enrollment will be crucial in our understanding of the effects current neonatal practice has on
321 health which will allow for the reevaluation of practices. Such trials will need to expand on the
322 findings from this pilot study from a multi-omic standpoint to identify direct links between
323 antibiotic-induced dysbiosis and health outcomes.

324 Materials and Methods

325 Experimental design, enrollment, and clinical sample and data collection

326 The REASON study was conducted from January 2017 - January 2019 at the University of
327 Florida and was approved by the institutional review board (IRB201501045). This study is
328 funded by the NIH (R21HD088005). A detailed description of the study design including
329 enrollment, group selection, randomization, and collection of clinical samples and data including
330 outcomes has been previously described. Briefly, 98 premature infants were enrolled in the study
331 and placed into one of three groups according to previously described criteria: group A with
332 indication for antibiotic use, group B without indication for antibiotic use, and group C eligible
333 for randomization to antibiotics (C1) or no antibiotics (C2) in the first 48 hours after birth.
334 Infants not receiving antibiotics in the first 48 hours after birth (group B, C2) could be changed
335 to receive antibiotics at any time at the medical team's discretion. Clinical samples relevant to
336 this analysis include weekly fecal collection starting with meconium when possible (all stored at
337 -80°C) and results of bacterial and fungal cultures (blood, urine, sputum, and cerebrospinal fluid
338 - when available) and laboratory measurements of CRP, white blood cell count and immature to
339 neutrophil ratio. Clinical metadata from the mothers such as antepartum antibiotic use, type,

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340 duration, and proximity to delivery were recorded. Pertinent clinical metadata from the infants
341 include group placement, antibiotic use status, antibiotics and antifungal use including type and
342 duration throughout NICU course, feeding type and duration, GA at birth, sex, mode of delivery
343 and any serious adverse events (SAEs) including NEC, late onset sepsis, spontaneous intestinal
344 perforations, bronchopulmonary dysplasia, and death.

345 **Stool DNA extraction, 16S rRNA PCR and Sequencing Analysis**

346 DNA extraction and 16S rRNA barcoded PCR was carried out exactly as described previously[53].
347 Approximately 60 gigabases of nucleotide sequencing data was generated across 5 Illumina
348 Miseq flowcells for stool samples collected from 91 (of the 98 total) study participants where
349 samples were collected (ICBR, Gainesville, FL, USA). The resulting sequencing reads were
350 merged, demultiplexed, trimmed, filtered for quality and processed into amplicon sequencing
351 variants (ASVs) as previously described with no alterations in method[53]. Briefly, sequences were
352 processed to ASVs using the DADA2 package in R (https://www.R-project.org) and assigned
353 taxonomy using the SILVA_v132 training dataset[54–58]. Samples were rarefied to 10,000 reads per
354 sample, leaving 642 of the total 656 individual longitudinal stool samples for analysis.

355 **Total bacterial quantification by universal 16S qPCR**

356 Total bacterial load per gram of stool was determined by universal 16S rRNA qPCR using the
357 same primer set used for amplicon sequencing (341F and 806R). QPCR assays were performed
358 on a QuantStudio 3 system (Applied Biosystems, Life Technologies, USA). The reaction mixture
359 contained 12.5 µl PowerUp SYBR Green 2X Master Mix (Applied Biosystems), 1 µl each of
360 forward (341F) and reverse (806R) primer (10 µM), 1 µl of DNA template, 0.1 µg/µl BSA and
361 brought to a final volume of 25 µl with nuclease free water. Cycling conditions were identical to

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362 those of the endpoint PCR used for sequencing, however with a total of 40 cycles and replacing
363 the final elongation step with a melt curve. Each sample reaction was performed in triplicate and
364 these values were averaged for each sample copy calculation. A standard curve was generated
365 for copy quantification using known concentrations of the expected PCR product amplified from
366 a similar stool sample. Copies of 16S rRNA per gram of stool was calculated by multiplying the
367 average copy number per replicate reaction (i.e. 1 µl DNA template) by the total DNA extraction
368 volume (75 µl) and dividing this value by the mass of stool extracted in grams.

369 **Absolute bacterial abundance by copy number correction**

370 Absolute bacterial abundance was calculated on a per gram of stool basis by correcting the
371 relative sequencing abundance by the variable number of copies of the 16S rRNA gene in each
372 observed organism. This correction was done using the “Estimate” tool provided as part of the
373 rrnDB copy number database[59]. Briefly, after rarefying each sample to an even sequencing depth,
374 the ASV sequences were submitted through the rrnDB online portal where they were classified
375 down to the genus level using the RDP classifier version 2.12 and copy number adjusted using
376 rrnDB copy number data version 5.6[59,60]. The copy number adjusted relative abundance for each
377 observed taxon was multiplied by the total number of 16S rRNA copies obtained by qPCR,
378 resulting in the absolute abundance of each taxon per gram of stool.

379 **Fecal inflammatory markers**

380 Inflammatory markers were analyzed using a combination of multiplex technologies using the
381 Bio-Rad Bio-Plex platform (Bio-Rad, California, USA). The markers evaluated include common
382 markers of intestinal inflammation including calprotectin and S100A12, in addition to other
383 markers such as IL-6, TNF, IL-10 and other cytokines and chemokines that may play a role in

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384 inflammatory or anti-inflammatory processes. The data were analyzed using direct comparisons
385 of all infant groups using analysis of variance (ANOVA) and subsequent individual
386 comparisons. Fecal calprotectin and S100A12 levels were measured using the fCal ELISA kit
387 from BUHLMANN Laboratories AG (Schonenbuch, Switzerland) and the Inflamark S100A12
388 kit from Cisbio Bioassays (Codolet, France), respectively, according to the manufacturer's
389 instructions. Samples were then analyzed for the presence of both pro-inflammatory and anti-
390 inflammatory cytokines/chemokines using Multiplex Human Cytokine Magnetic kit, Milliplex
391 MAP Kit (Millipore, Billerica, MA, USA). Twelve cytokines/chemokines, including EGF, IL-
392 10, IL-1RA, IL-B, IL-4, IL-6, IL-8, IP-10, MCP-1, MIP-1a, TNFα, and VEGF were analyzed
393 according to the manufacturer's instructions. Plates were read using the MILLIPLEX Analyzer
394 3.1 xPONENT™ System (Luminex 200). Cytokine concentrations were determined using
395 BeadView software (Millipore, Billerica, MA, USA).

396 **Metabolomics**

397 The infant stool samples were suspended in 400 µl 5 mM ammonium acetate. Homogenization
398 was done 3 times for 30 s each time using a cell disruptor. Protein concentrations of the
399 homogenates were measured using Qubit Protein Assay. Samples were normalized to 500 µg/ml
400 protein at 25 µl for extraction. Each normalized sample was spiked with 5 µl of internal
401 standards solution. Extraction of metabolites was performed by protein precipitation by adding
402 200 µl of extraction solution consisting of 8:1:1 acetonitrile: methanol: acetone to each sample.
403 Samples were mixed thoroughly, incubated at 4°C to allow protein precipitation, and centrifuged
404 at 20,000 x g to pellet the protein. 190 µl supernatant was transferred into clean tube and dried
405 using nitrogen. Samples were reconstituted with 25 µl of reconstitution solution consisting of

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406 injection standards, mixed, and incubated at 4° C for 10-15 min. Samples were centrifuged at
407 20000 x g. Supernatants were transferred into LC-vials.
408 Global metabolomics profiling was performed as previously described using a Thermo Q-
409 Exactive Orbitrap mass spectrometer with Dionex UHPLC and autosampler[61]. Briefly, samples
410 were analyzed in positive and negative heated electrospray ionization with a mass resolution of
411 35,000 at m/z 200 as separate injections. Separation was achieved on an ACE 18-pfp 100 x 2.1
412 mm, 2 µm column with mobile phase A as 0.1% formic acid in water and mobile phase B as
413 acetonitrile. The flow rate was 350 µl/min with a column temperature of 25°C. 4 µl was injected
414 for negative ions and 2 µl for positive ions.
415 Data from positive and negative ions modes were processed separately. LC-MS files were first
416 converted to open-format files (i.e. mzXML) using MSConvert from Proteowizard[62]. Mzmine
417 was used to identify features, deisotope, align features and perform gap filling to fill in any
418 features that may have been missed in the first alignment algorithm[63]. Features were matched
419 with SECIM internal compound database to identify metabolites. All adducts and complexes
420 were identified and removed from the data set prior to statistical analysis.

421 **Statistical Analysis**

422 The ASV and taxonomy tables resulting from DADA2 were manipulated using the phyloseq R
423 package v1.30.0[64]. Inferred metabolic pathway abundances were determined from the rarefied
424 16S rRNA data using PICRUSt2[28]. Alpha diversity measures, including the observed number of
425 ASVs and the Shannon diversity index, as well as Spearman correlations and associated
426 heatmaps, were calculated and drawn using the microbiome R package v1.8.0
427 (https://bioconductor.org/packages/devel/bioc/html/microbiome.html). Violin and box plots
428 (including statistical testing where applicable) were generated using the ggpubr R package v0.2.4

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429 (https://github.com/kassambara/ggpubr), which serves as a wrapper for ggplot2[65]. The linear
430 mixed effects modeling, volatility analysis and associated plots were done using the longitudinal
431 plugin "q2-longitudinal" offered in Qiime2 v2019.4[25–27]. The biomformat R package
432 (https://biom-format.org) was used to convert data in phyloseq format to BIOM format for
433 import into Qiime2[66]. Bray-Curtis and Jaccard distance dissimilarities were calculated using the
434 vegan R package v2.5.6 (https://github.com/vegandevs/vegan) and PCoA plots were made using
435 ggplot2 v3.3.0[65]. Individual infant charts were also generated using ggplot2. Non-parametric
436 statistical tests including the Wilcoxon and Kruskal-Wallis tests were used for pairwise and
437 overall comparisons of 3 or more factors, respectively[67,68]. The permutational analysis of
438 variance (PERMANOVA) test was used in the vegan package to compare overall microbiome
439 dissimilarities between antibiotic use, feeding type, and enrollment groups. P-values were
440 adjusted for false discovery rate (FDR) via the Benjamin-Hochberg method[69].

441 **Data availability**

442 The demultiplexed 16S rRNA sequencing data generated in this study is deposited in the NCBI
443 Sequence Read Archive (SRA) under BioProject PRJNA515272.

444 **Trial Registration**

445 This project is registered at clinicaltrials.gov under the name "Antibiotic 'Dysbiosis' in Preterm
446 Infants" with trial number NCT02784821.

447 **References**

448 1. Israel, E. J. Neonatal necrotizing enterocolitis, a disease of the immature intestinal mucosal
449 barrier. *Acta. Paediatr. Suppl.* **396**, 27–32 (1994).

bioRxiv preprint doi: https://doi.org/10.1101/2020.04.20.052142; this version posted April 21, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

450 2. Sadeghi, K. *et al.* Immaturity of Infection Control in Preterm and Term Newborns Is
451 Associated with Impaired Toll-Like Receptor Signaling. *J. Infect. Dis.* **195**, 296–302 (2007).
452 3. Hunter, C. J., Upperman, J. S., Ford, H. R. & Camerini, V. Understanding the Susceptibility of
453 the Premature Infant to Necrotizing Enterocolitis (NEC). *Pediatr. Res.* **63**, 117–123 (2008).
454 4. Basso, O. & Wilcox, A. Mortality risk among preterm babies: Immaturity vs. underlying
455 pathology. *Epidemiology* **21**, 521–527 (2010).
456 5. Tripathi, N., Cotten, C. M. & Smith, P. B. Antibiotic use and misuse in the neonatal intensive
457 care unit. *Clin. Perinatol.* **39**, 61–68 (2012).
458 6. Schulman, J. *et al.* Neonatal intensive care unit antibiotic use. *Pediatrics* **135**, 826–833
459 (2015).
460 7. Greenberg, R. G. *et al.* Prolonged duration of early antibiotic therapy in extremely
461 premature infants. *Pediatr. Res.* **85**, 994–1000 (2019).
462 8. Puopolo, K. M., Benitz, W. E., Zaoutis, T. E., Newborn, C. on F. A. & Diseases, C. on I.
463 Management of Neonates Born at ≤34 6/7 Weeks' Gestation With Suspected or Proven
464 Early-Onset Bacterial Sepsis. *Pediatrics* **142**, 1006 (2018).
465 9. Fajardo, C., Alshaikh, B. & Harabor, A. Prolonged use of antibiotics after birth is associated
466 with increased morbidity in preterm infants with negative cultures. *J. Matern.-Fetal*
467 *Neonatal Med.* **32**, 4060–4066 (2019).
468 10. Samuels, N., van de Graaf, R. A., de Jonge, R. C. J., Reiss, I. K. M. & Vermeulen, M. J. Risk
469 factors for necrotizing enterocolitis in neonates: a systematic review of prognostic studies.
470 *BMC Pediatr.* **17**, 105 (2017).

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471 11. Alganabi, M., Lee, C., Bindi, E., Li, B. & Pierro, A. Recent advances in understanding
472 necrotizing enterocolitis. *F1000Res.* **8**, 107 (2019).
473 12. Clark, R. H., Bloom, B. T., Spitzer, A. R. & Gerstmann, D. R. Reported Medication Use in the
474 Neonatal Intensive Care Unit: Data From a Large National Data Set. *Pediatrics* **117**, 1979–
475 1987 (2006).
476 13. Hsieh, E. M. *et al.* Medication use in the neonatal intensive care unit. *Am. J. Perinatol.* **31**,
477 811–821 (2014).
478 14. Derrien, M., Alvarez, A.-S. & Vos, W. M. de. The Gut Microbiota in the First Decade of Life.
479 *Trends Microbiol.* **27**, 997–1010 (2019).
480 15. Shao, Y. *et al.* Stunted microbiota and opportunistic pathogen colonization in caesarean-
481 section birth. *Nature* **574**, 117–121 (2019).
482 16. Stinson, L. F., Boyce, M. C., Payne, M. S. & Keelan, J. A. The Not-so-Sterile Womb: Evidence
483 That the Human Fetus Is Exposed to Bacteria Prior to Birth. *Front. Microbiol.* **10**, 1124
484 (2019).
485 17. Dardas, M. *et al.* The impact of postnatal antibiotics on the preterm intestinal microbiome.
486 *Pediatr. Res.* **76**, 150–158 (2014).
487 18. Gasparrini, A. J. *et al.* Antibiotic perturbation of the preterm infant gut microbiome and
488 resistome. *Gut Microbes* **7**, 443–449 (2016).
489 19. Sun, X., Zhuan, C., Xiao, J., Yao, E. & Chen, L. Impact of postnatal exposure to antibiotics on
490 intestinal microbiome in preterm infants. *Chinese Journal of Perinatal Medicine* **21**, 458–464
491 (2018).

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492 20. Zou, Z.-H. *et al.* Prenatal and postnatal antibiotic exposure influences the gut microbiota of
493 preterm infants in neonatal intensive care units. *Ann. Clin. Microbiol. Antimicrob.* **17**, 9
494 (2018).
495 21. Cantey, J. B., Pyle, A. K., Wozniak, P. S., Hynan, L. S. & Sánchez, P. J. Early Antibiotic
496 Exposure and Adverse Outcomes in Preterm, Very Low Birth Weight Infants. *J. Pediatr.* **203**,
497 62–67 (2018).
498 22. Langdon, A., Crook, N. & Dantas, G. The effects of antibiotics on the microbiome
499 throughout development and alternative approaches for therapeutic modulation. *Genome*
500 *Med.* **8**, 39 (2016).
501 23. Sherman, M. P., Zaghouani, H. & Niklas, V. Gut microbiota, the immune system, and diet
502 influence the neonatal gut–brain axis. *Pediatr. Res.* **77**, 127–135 (2015).
503 24. Collins, S. M., Surette, M. & Bercik, P. The interplay between the intestinal microbiota and
504 the brain. *Nat. Rev. Microbiol.* **10**, 735–742 (2012).
505 25. Lindstrom, M. J. & Bates, D. M. Newton—Raphson and EM Algorithms for Linear Mixed-
506 Effects Models for Repeated-Measures Data. *J. Am. Stat. Assoc.* **83**, 1014–1022 (1988).
507 26. Bokulich, N. A. *et al.* q2-longitudinal: Longitudinal and Paired-Sample Analyses of
508 Microbiome Data. *mSystems* **3**, e00219-18 (2018).
509 27. Bolyen, E. *et al.* Reproducible, interactive, scalable and extensible microbiome data science
510 using QIIME 2. *Nat. Biotechnol.* **37**, 852–857 (2019).
511 28. Douglas, G. M. *et al.* PICRUSt2: An improved and customizable approach for metagenome
512 inference. *bioRxiv* 672295 (2020) doi:10.1101/672295.

bioRxiv preprint doi: https://doi.org/10.1101/2020.04.20.052142; this version posted April 21, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

513 29. Tanaka, S. *et al.* Influence of antibiotic exposure in the early postnatal period on the
514 development of intestinal microbiota. *FEMS Immunol. Med. Mic.* **56**, 80–87 (2009).
515 30. Greenwood, C. *et al.* Early Empiric Antibiotic Use in Preterm Infants Is Associated with
516 Lower Bacterial Diversity and Higher Relative Abundance of Enterobacter. *J. Pediatr.* **165**,
517 23–29 (2014).
518 31. Cong, X. *et al.* Gut Microbiome Developmental Patterns in Early Life of Preterm Infants:
519 Impacts of Feeding and Gender. *PLoS One* **11**, e0152751 (2016).
520 32. Cong, X. *et al.* Influence of Infant Feeding Type on Gut Microbiome Development in
521 Hospitalized Preterm Infants. *Nurs. Res.* **66**, 123–133 (2017).
522 33. Parra-Llorca, A. *et al.* Preterm Gut Microbiome Depending on Feeding Type: Significance of
523 Donor Human Milk. *Front. Microbiol.* **9**, 1376 (2018).
524 34. Gregory, K. E. *et al.* Influence of maternal breast milk ingestion on acquisition of the
525 intestinal microbiome in preterm infants. *Microbiome* **4**, 68 (2016).
526 35. Kordy, K. *et al.* Contributions to human breast milk microbiome and enteromammary
527 transfer of Bifidobacterium breve. *PLoS ONE* **15**, e0219633 (2020).
528 36. Pannaraj, P. S. *et al.* Association Between Breast Milk Bacterial Communities and
529 Establishment and Development of the Infant Gut Microbiome. *JAMA Pediatr.* **171**, 647–
530 654 (2017).
531 37. Forbes, J. D. *et al.* Association of Exposure to Formula in the Hospital and Subsequent Infant
532 Feeding Practices With Gut Microbiota and Risk of Overweight in the First Year of Life.
533 *JAMA Pediatr.* **172**, e181161 (2018).

bioRxiv preprint doi: https://doi.org/10.1101/2020.04.20.052142; this version posted April 21, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

534 38. Mueller, E. & Blaser, M. Breast milk, formula, the microbiome and overweight. *Nat. Rev.*
535 *Endocrinol.* **14**, 510–511 (2018).
536 39. Zervos, E. E., Bass, S. S., Robson, M. C. & Rosemurgy, A. S. Fluconazole increases bactericidal
537 activity of neutrophils. *J. Trauma* **41**, 10–14 (1996).
538 40. Rougé, C. *et al.* Fecal Calprotectin Excretion in Preterm Infants during the Neonatal Period.
539 *PLoS One* **5**, e11083 (2010).
540 41. Coggins, S. A., Wynn, J. L. & Weitkamp, J.-H. Infectious Causes of Necrotizing Enterocolitis.
541 *Clin. Perinatol.* **42**, 133–154 (2015).
542 42. Dobson, S. R. M. & Baker, C. J. Enterococcal Sepsis in Neonates: Features by Age at Onset
543 and Occurrence of Focal Infection. *Pediatrics* **85**, 165–171 (1990).
544 43. O'Grady, N. P. *et al.* Detection of Macrophage Inflammatory Protein (MIP)-1α and MIP-β
545 during Experimental Endotoxemia and Human Sepsis. *J. Infect.* **179**, 136-141 (1999).
546 44. Bhutta, A. T., Cleves, M. A., Casey, P. H., Cradock, M. M. & Anand, K. J. S. Cognitive and
547 Behavioral Outcomes of School-Aged Children Who Were Born Preterm: A Meta-analysis.
548 *JAMA* **288**, 728–737 (2002).
549 45. Counsell, S. J. & Boardman, J. P. Differential brain growth in the infant born preterm:
550 Current knowledge and future developments from brain imaging. *Semin. Fetal. Neonat. M.*
551 **10**, 403–410 (2005).
552 46. Marlow, N. & Samara, M. Neurologic and Developmental Disability at Six Years of Age after
553 Extremely Preterm Birth. *N. Engl. J. Med.* **352**, 9-19 (2005).
554 47. Sharon, G. *et al.* Specialized Metabolites from the Microbiome in Health and Disease. *Cell*
555 *Metab.* **20**, 719–730 (2014).

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556 48. Kwon, S. H. *et al.* GABA, Resting State Connectivity and the Developing Brain. *Neonatology*
557 **106**, 149–155 (2014).
558 49. Jin, Z., Mendu, S. K. & Birnir, B. GABA is an effective immunomodulatory molecule. *Amino*
559 *Acids* **45**, 87–94 (2013).
560 50. Grill, J. P., Manginot-Dürr, C., Schneider, F. & Ballongue, J. Bifidobacteria and probiotic
561 effects: Action of Bifidobacterium species on conjugated bile salts. *Curr. Microbiol.* **31**, 23–
562 27 (1995).
563 51. Jia, W., Xie, G. & Jia, W. Bile acid–microbiota crosstalk in gastrointestinal inflammation and
564 carcinogenesis. *Nat. Rev. Gastro. Hepat.* **15**, 111–128 (2018).
565 52. Kiriyama, Y. & Nochi, H. The Biosynthesis, Signaling, and Neurological Functions of Bile
566 Acids. *Biomolecules* **9**, 232 (2019).
567 53. Russell, J. T. *et al.* Genetic risk for autoimmunity is associated with distinct changes in the
568 human gut microbiome. *Nat. Commun.* **10**, 1–12 (2019).
569 54. Callahan, B. J. *et al.* DADA2: High resolution sample inference from Illumina amplicon data.
570 *Nat. Methods.* **13**, 581–583 (2016).
571 55. Pruesse, E. *et al.* SILVA: a comprehensive online resource for quality checked and aligned
572 ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res.* **35**, 7188–7196
573 (2007).
574 56. Glöckner, F. O. *et al.* 25 years of serving the community with ribosomal RNA gene reference
575 databases and tools. *J. Biotechnol.* **261**, 169–176 (2017).
576 57. Yilmaz, P. *et al.* The SILVA and “All-species Living Tree Project (LTP)” taxonomic frameworks.
577 *Nucleic Acids Res.* **42**, D643–D648 (2014).

bioRxiv preprint doi: https://doi.org/10.1101/2020.04.20.052142; this version posted April 21, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

578 58. Quast, C. *et al.* The SILVA ribosomal RNA gene database project: improved data processing
579 and web-based tools. *Nucleic Acids Res.* **41**, D590–D596 (2013).
580 59. Stoddard, S. F., Smith, B. J., Hein, R., Roller, B. R. K. & Schmidt, T. M. rrnDB: improved tools
581 for interpreting rRNA gene abundance in bacteria and archaea and a new foundation for
582 future development. *Nucleic Acids Res.* **43**, D593–D598 (2015).
583 60. Wang, Q., Garrity, G. M., Tiedje, J. M. & Cole, J. R. Naive Bayesian classifier for rapid
584 assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.*
585 **73**, 5261–5267 (2007).
586 61. Chamberlain, C. A., Hatch, M. & Garrett, T. J. Metabolomic and lipidomic characterization of
587 Oxalobacter formigenes strains HC1 and OxWR by UHPLC-HRMS. *Anal. Bioanal. Chem.* **411**,
588 4807–4818 (2019).
589 62. Chambers, M. C. *et al.* A cross-platform toolkit for mass spectrometry and proteomics. *Nat.*
590 *Biotechnol.* **30**, 918–920 (2012).
591 63. Pluskal, T., Castillo, S., Villar-Briones, A. & Orešič, M. MZmine 2: Modular framework for
592 processing, visualizing, and analyzing mass spectrometry-based molecular profile data. *BMC*
593 *Bioinform.* **11**, 395 (2010).
594 64. McMurdie, P. J. & Holmes, S. phyloseq: An R Package for Reproducible Interactive Analysis
595 and Graphics of Microbiome Census Data. *PLOS ONE* **8**, e61217 (2013).
596 65. Wickham, H. *ggplot2: Elegant Graphics for Data Analysis*. (Springer-Verlag, New York,
597 2009).
598 66. McDonald, D. *et al.* The Biological Observation Matrix (BIOM) Format Or: How I Learned to
599 Stop Worrying and Love the Ome-Ome. *Gigascience* **1**, 1-7 (2012).

bioRxiv preprint doi: https://doi.org/10.1101/2020.04.20.052142; this version posted April 21, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

600 67. Wilcoxon, F. Individual Comparisons by Ranking Methods. *Biometrics Bulletin* **1**, 80–83
601 (1945).
602 68. Kruskal, W. H. & Wallis, W. A. Use of Ranks in One-Criterion Variance Analysis. *J. Am. Stat.*
603 *Assoc.* **47**, 583–621 (1952).
604 69. Benjamini, Y. & Hochberg, Y. Controlling the False Discovery Rate: A Practical and Powerful
605 Approach to Multiple Testing. *J. R. Statist. Soc.* **57**, 289–300 (1995).
606

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631 J.N. designed and oversaw the study. J.L.R. aided in implementation in the NICU, review of

632 clinical events for the cohort, preparation for the DSMB and IRB reviews, and analyzed clinical

633 data. J.N, J.L.R., D.C., and C.B. carried out patient enrollment/consent/group allocation,

634 provided care for the infants in the NICU during the study. J.L.R, C.B. L.P. recorded clinical

635 data. N.L. maintained/distributed the samples and performed the stool immune marker assays.

636 T.J.G performed the stool metabolomics assays and analysis. K.L.M. assisted with stool DNA

637 extraction, 16S PCR/qPCR, and figure generation. J.T.R. performed the stool microbiome

638 analysis, data integration, figure generation and wrote the manuscript. R.A.P. and E.W.T.

639 assisted with data interpretation. All authors reviewed the manuscript before submission.

640

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643 **Ethics Declarations**

644 Dr. Josef Neu is the principal investigator of a study with Infant Bacterial Therapeutics and
645 on the Scientific Advisory Boards of Medela and Astarte. No other authors have conflicts of
646 interest to disclose.
647

648 **Figures**

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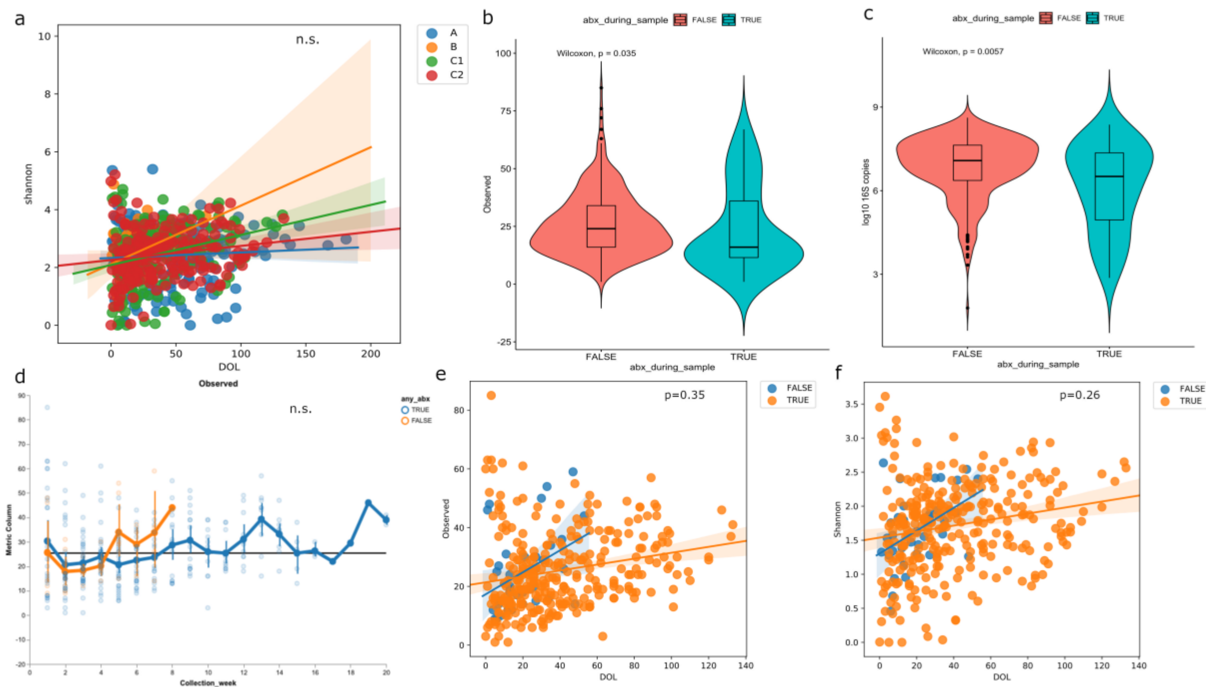
649



650

651 **Figure 1 – Antibiotic use affects early gut richness, bacterial load and trends in diversity**

652 **a**, Regression scatter plot applying linear mixed effects (LME) modeling to test the relationship
653 between enrollment group and Shannon diversity longitudinally. (group A n=232, group B n=42,
654 group C1 n=171, group C2 n=197). Time scale on the x-axis is day after birth (DOL). Greyed
655 area represents a 95% confidence interval. **b**, **c**, Violin boxplots showing the **(b)** observed
656 number of ASVs (richness) and **(c)** the bacterial load (log_{10} 16S rRNA copies) of samples which
657 were taken when antibiotics either were (TRUE, n=83) or were not (FALSE, n=562)
658 administered (abx_during_sample). Comparisons performed using the Wilcoxon test. **d**,
659 Volatility plot of the observed number of ASVs by whether the infants in the randomized group
660 received antibiotics (any_abx, TRUE, n=323 vs. FALSE, n=45). Pairwise comparisons at each
661 timepoint done using the Wilcoxon test. **e**, **f**, Regression scatter plots applying LME to the **(e)**
662 observed number of ASVs and **(f)** Shannon diversity longitudinally for infants in group C that

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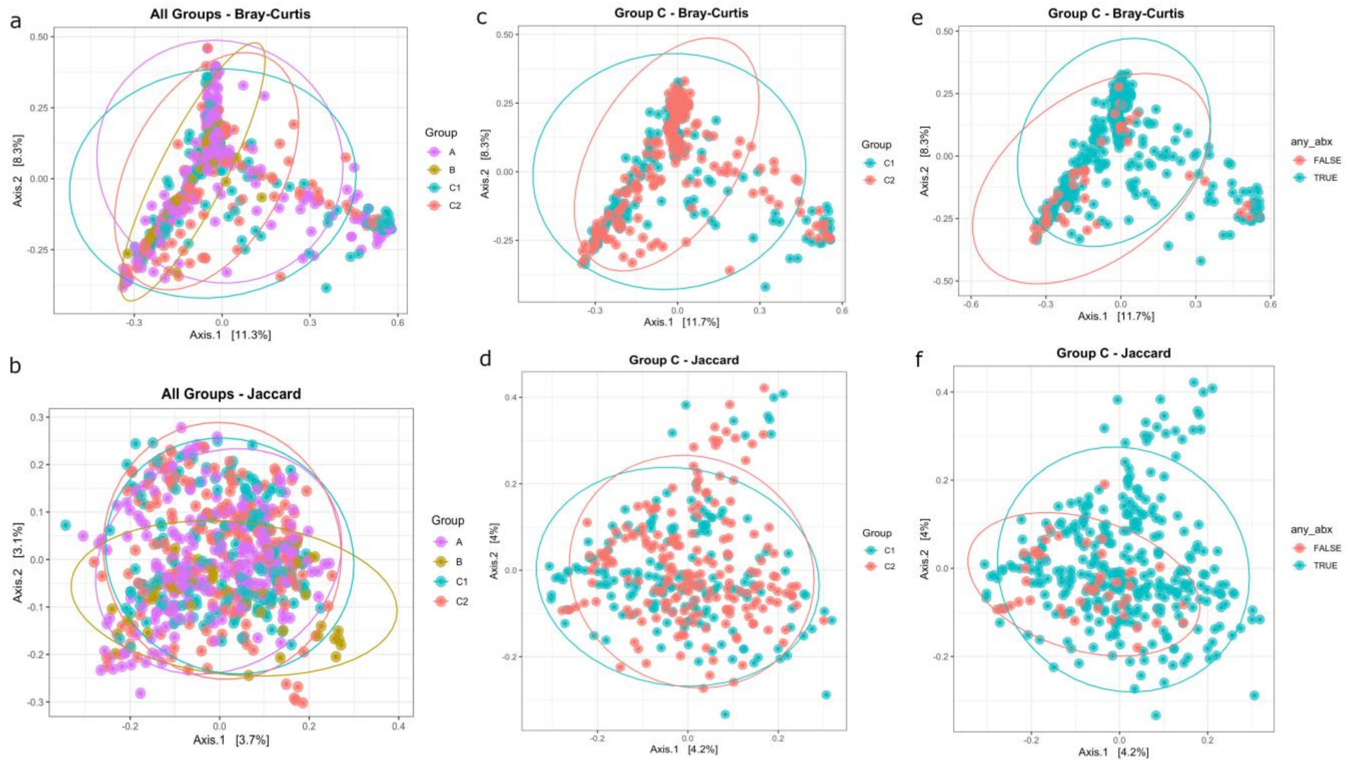
663 either received (TRUE, n=323) or did not receive (FALSE, n=45) antibiotics (any_abx). Greyed

664 area represents a 95% confidence interval.

665



666

667 **Figure 2 – Beta-diversity shows effects of antibiotics on the infant gut communities**

668 **a**, **b**, **c**, **d**, **e**, **f**, PCoA ordination of stool samples by **(a, b)** enrollment group, **(c, d)** group C1 or

669 C2, and **(e, f)** whether they received antibiotics (C1 plus C2 changed: any_abx = TRUE, n=323)

670 or not (C2 not changed: any_abx = FALSE, n=45) using the quantitative Bray-Curtis distance

671 index **(a, c, e)** and the qualitative Jaccard distance index **(b, d, f)**. (group A n=232, group B

672 n=42, group C1 n=171, group C2 n=197). Ellipses are calculated based on a 95% confidence

673 interval.

674

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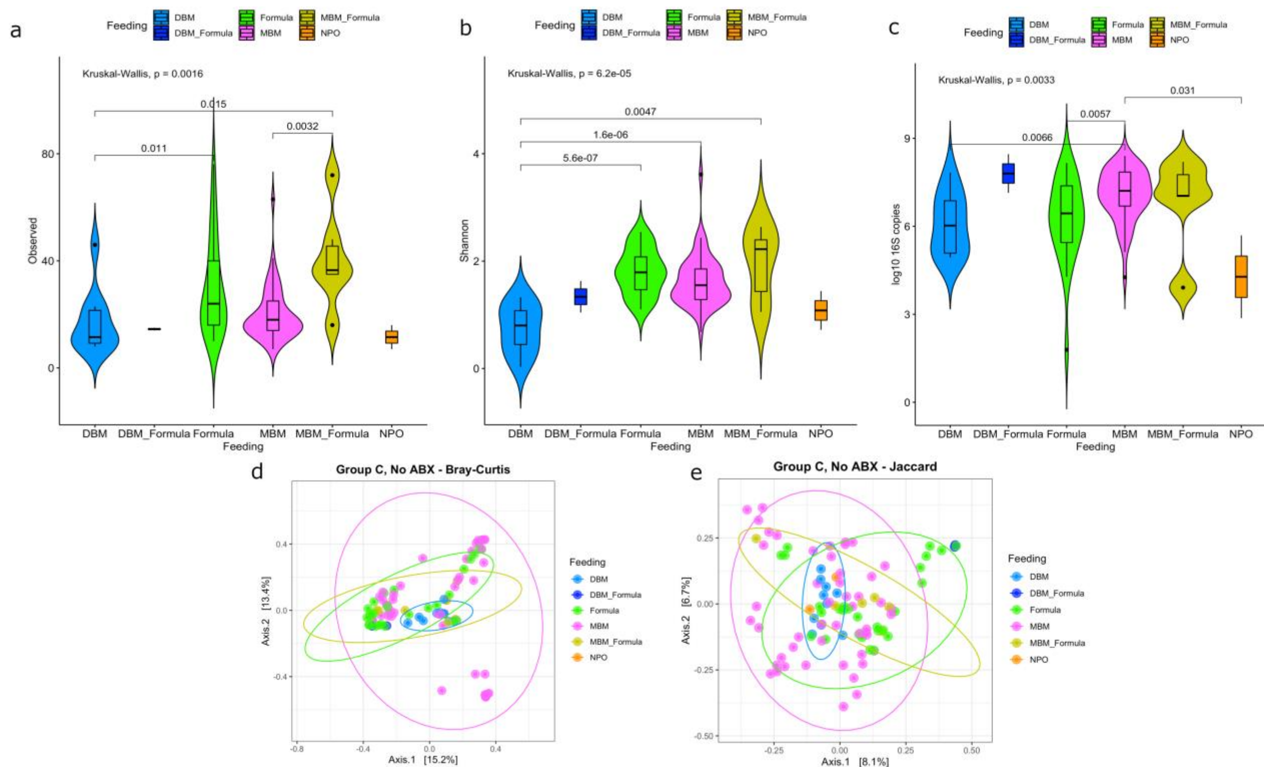

675

676 **Figure 3 – Feeding patterns influence bacterial community richness, diversity, size and**

677 **structure**

678 **a**, **b**, **c**, Violin boxplots comparing the **(a)** observed number of ASVs (richness), **(b)** Shannon

679 diversity, and **(c)** log_{10}-transformed number of 16S copies (bacterial load) of samples across

680 different feeding types. Overall comparisons between feeding types was performed using the

681 Kruskal-Wallis test and pairwise comparisons by the Wilcoxon test. Only significant pairwise

682 comparisons are shown for clarity ($p < 0.05$). **d, e**, PCoA ordination of stool samples collected

683 during different feeding types using the **(d)** Bray-Curtis and **(e)** Jaccard distance indices. Ellipses

684 are calculated based on a 95% confidence interval. DBM: donor breast milk, MBM: mother's

685 breast milk, NPO: no enteral nutrition, ABX: antibiotics. (MBM n=49, DBM n=10, Formula

686 n=27, MBM_Formula n=6, DBM_Formula n=2, NPO n=2).

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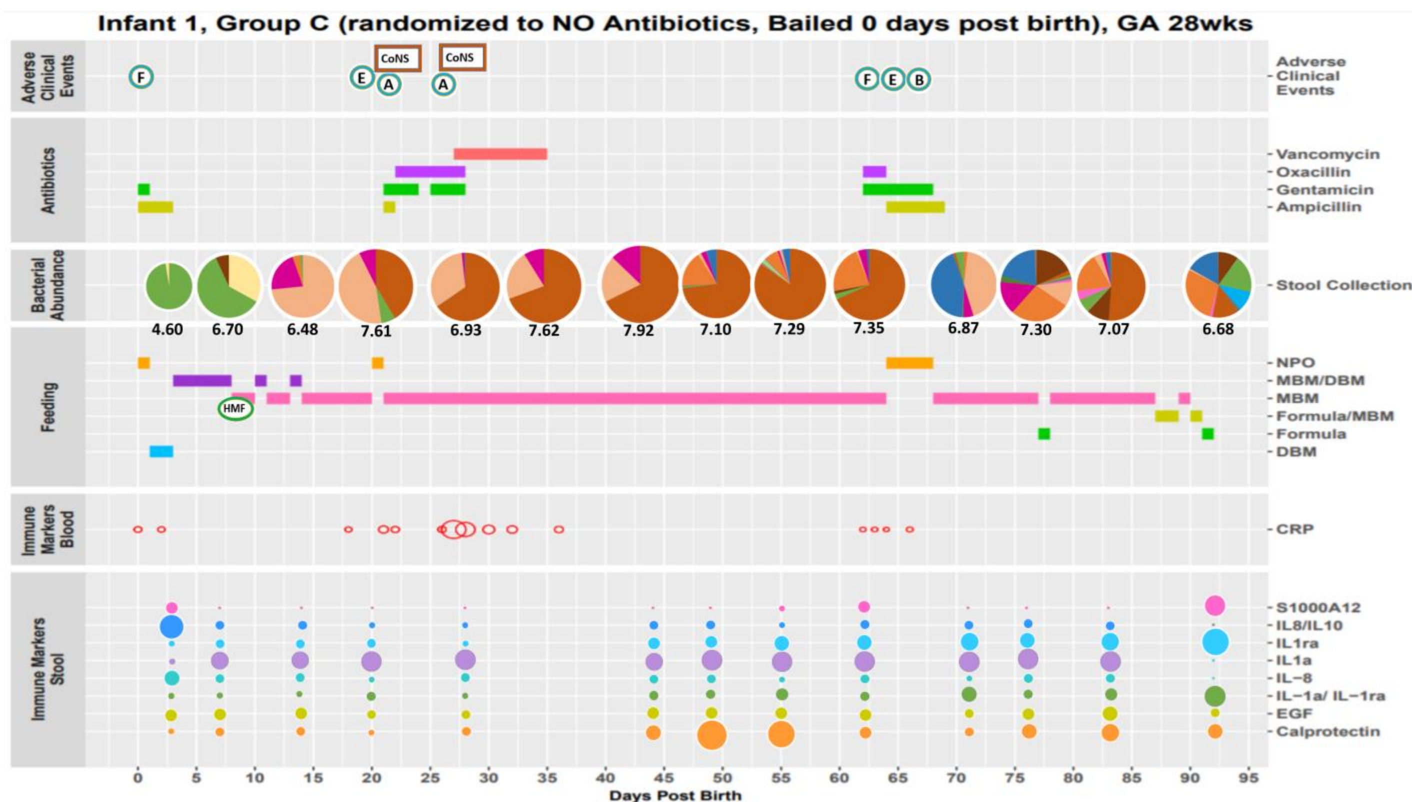

687

688 **Figure 4 – Integration of clinical and laboratory data gives detailed view of infant stay in**

689 **NICU**

690 Extensive clinical and laboratory data, when combined, provide a detailed summary of each
691 infant's stay in the NICU. Data included in each chart from top to bottom include: the infant ID,
692 group assignment, antibiotic change status (bail), gestational age, any adverse clinical events
693 (which are further described in Supplementary Figure 1), the type and duration of antibiotic use
694 (if any), the copy-number corrected absolute composition of each weekly stool sample and it's
695 $\log_{10}$-scale number of bacterial 16S rRNA copies, the type and duration of each feeding
696 including administration of human milk fortifier, the relative levels of C-reactive protein
697 measured from blood, and relative concentrations of measured stool immune markers (for infants
698 where these measurements were performed). DBM: donor breast milk, MBM: mother's breast
699 milk, NPO: no enteral nutrition, CRP: C-reactive protein, EGF: epidermal growth factor.

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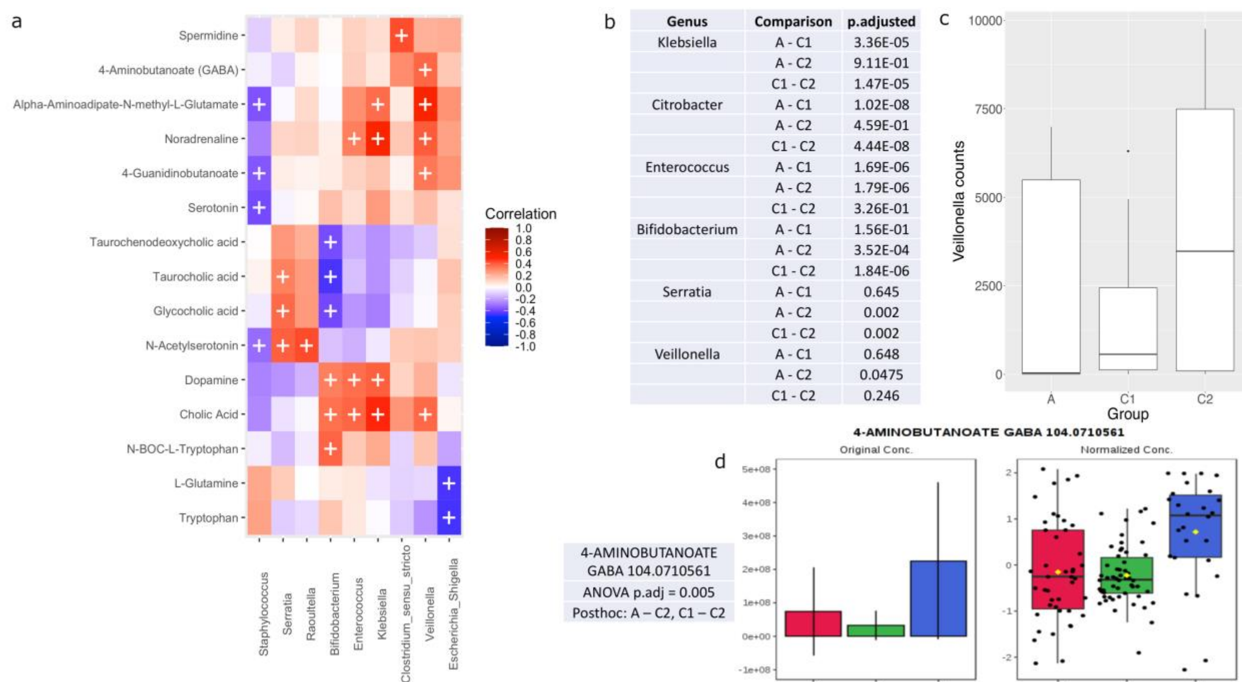
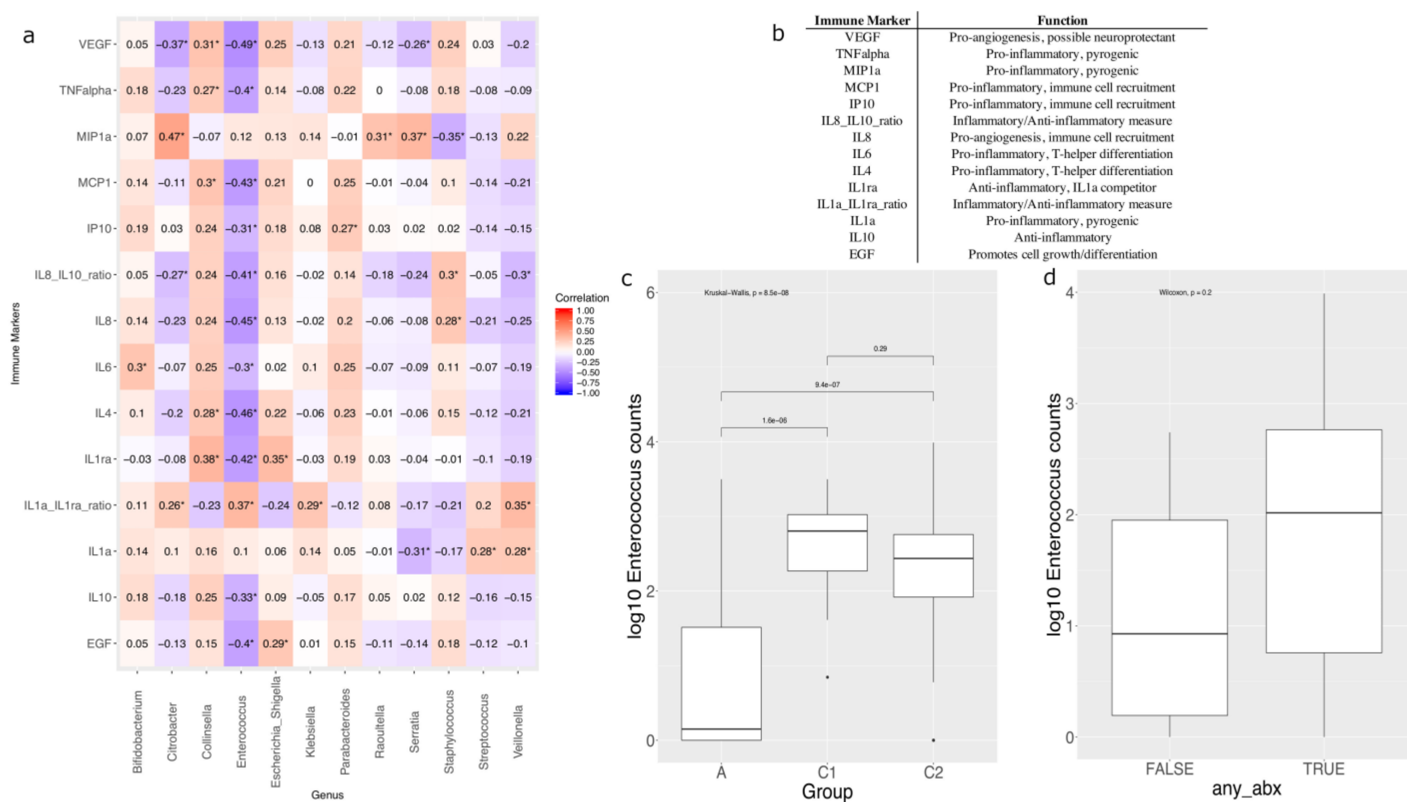


Genus	Comparison	p.adjusted
Klebsiella	A - C1	3.36E-05
	A - C2	9.11E-01
	C1 - C2	1.47E-05
Citrobacter	A - C1	1.02E-08
	A - C2	4.59E-01
	C1 - C2	4.44E-08
Enterococcus	A - C1	1.69E-06
	A - C2	1.79E-06
	C1 - C2	3.26E-01
Bifidobacterium	A - C1	1.56E-01
	A - C2	3.52E-04
	C1 - C2	1.84E-06
Serratia	A - C1	0.645
	A - C2	0.002
	C1 - C2	0.002
Veillonella	A - C1	0.648
	A - C2	0.0475
	C1 - C2	0.246

700

701 **Figure 5 – Metabolites in stool correlate with abundance of bacterial genera**

702 **a**, Heatmap of Spearman correlation coefficients between peak response heights of identified
703 metabolites in stool and the top 10 bacterial genera from the same samples (n=90 stool samples).
704 Only genera with at least one significant correlation with an identified metabolite are displayed
705 (9 of 10). Significant correlations are indicated by a '+' with FDR-corrected p-values < 0.05. **b**,
706 Bacterial genera which had significantly different counts when comparing at least 2 enrollment
707 groups (at least 1 pairwise comparison). P-values are FDR adjusted. **c**, Boxplot comparing the
708 number of rarefied *Veillonella* counts between the enrollment groups. **d**, Original and normalized
709 concentrations of the neurotransmitter 4-aminobutanoate (GABA) by enrollment group and
710 significant comparisons of normalized concentrations between groups by ANOVA.



b

Immune Marker	Function
VEGF	Pro-angiogenesis, possible neuroprotectant
TNFalpha	Pro-inflammatory, pyrogenic
MIP1a	Pro-inflammatory, pyrogenic
MCP1	Pro-inflammatory, immune cell recruitment
IP10	Pro-inflammatory, immune cell recruitment
IL8_IL10_ratio	Inflammatory/Anti-inflammatory measure
IL8	Pro-angiogenesis, immune cell recruitment
IL6	Pro-inflammatory, T-helper differentiation
IL4	Pro-inflammatory, T-helper differentiation
IL1ra	Anti-inflammatory, IL1a competitor
IL1a_IL1ra_ratio	Inflammatory/Anti-inflammatory measure
IL1a	Pro-inflammatory, pyrogenic
IL10	Anti-inflammatory
EGF	Promotes cell growth/differentiation

711

712 **Figure 6 – Stool immune marker levels show modest correlation with gut microbiota**

713 **a**, Heatmap of Spearman correlation coefficients between immune markers measured from stool
714 and the most abundant bacterial genera from the same samples (n=110 stool samples). Only the
715 bacterial genera with at least one significant correlation with an immune marker are displayed
716 (n=12). Significant correlations are marked with an '*' by the coefficient, with FDR-adjusted p-
717 values < 0.05. **b**, Table listing the immune markers used for correlation analysis and their role in
718 inflammatory processes. **c**, Comparison of $\log_{10}$-transformed number of *Enterococcus* counts by
719 enrollment group and their significance by Kruskal-Wallis. **d**, Comparison of $\log_{10}$-transformed
720 number of *Enterococcus* counts by whether the infant in received any antibiotics (any_abx) and
721 their significance by the Wilcoxon test. VEGF: vascular endothelial growth factor, TNFalpha:
722 tumor necrosis factor alpha, MIP1a: macrophage inflammatory protein 1a, MCP1: monocyte
723 chemoattractant protein 1, IP10: interferon inducible protein 10, EGF: epidermal growth factor.

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724 **Extended Data**

	Group A	Group B	Group C1	Group C2	Changed from C2
Total	32	11	28	27	13
Changed from no antibiotics		1		13	
Sex (M::F)	14::14	4::7	13::15	17::10	10::3
Delivery Mode (V::C)	13::19	8::3	11::17	8::19	11::2
Gestational Age Range (median)	23 - 32 (28)	29 - 32 (32)	23 - 32 (29)	23 - 32 (29)	24 - 32 (28)
Maternal antibiotic exposure (Y::N)	23::9	6::5	21::7	19::8	12::1
Number of samples post-rarefying	232	42	171	197	97

725

726 **Extended Data Fig. 1 – Summary of infant enrollment and covariates**

727 Summary of the number of enrolled infants per group used in this analysis and the number of
728 infants changed from groups C2 and B (no antibiotics). Enrollment groups are also summarized
729 by infant sex (male::female), mode of delivery (vaginal::caesarean), gestational ages and
730 maternal antibiotic exposure (yes::no). The number of individual stool samples used in the
731 analysis are also summarized by group.

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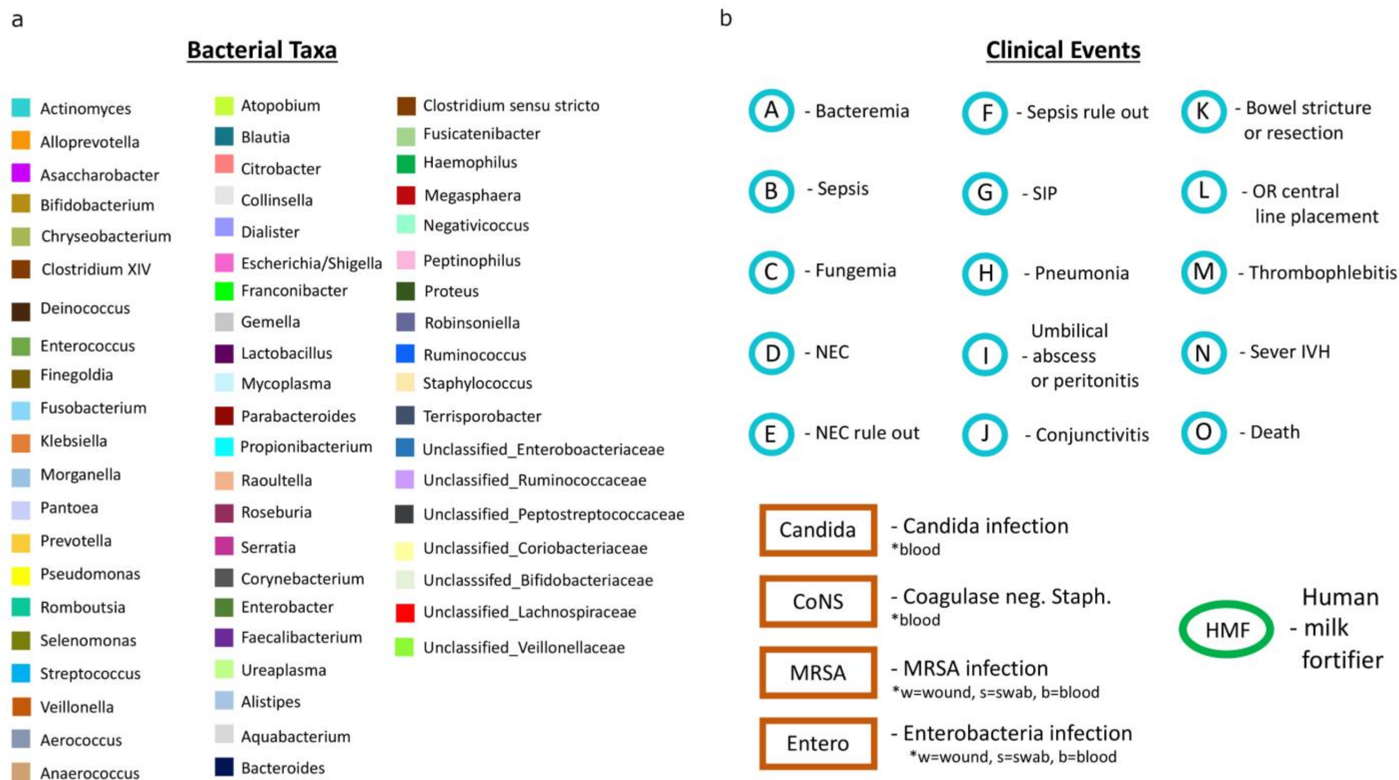

732

733 **Extended Data Fig. 2 – Bacterial taxa and adverse clinical event key for infant charts**

734 **(a)** Key for the color-coded bacterial taxa used in the stool 16S rRNA copy-number corrected
735 composition pie chart for each infant chart. **(b)** Key for the adverse clinical events (including
736 infections by body site) and the administration of human milk fortifier for each infant chart.

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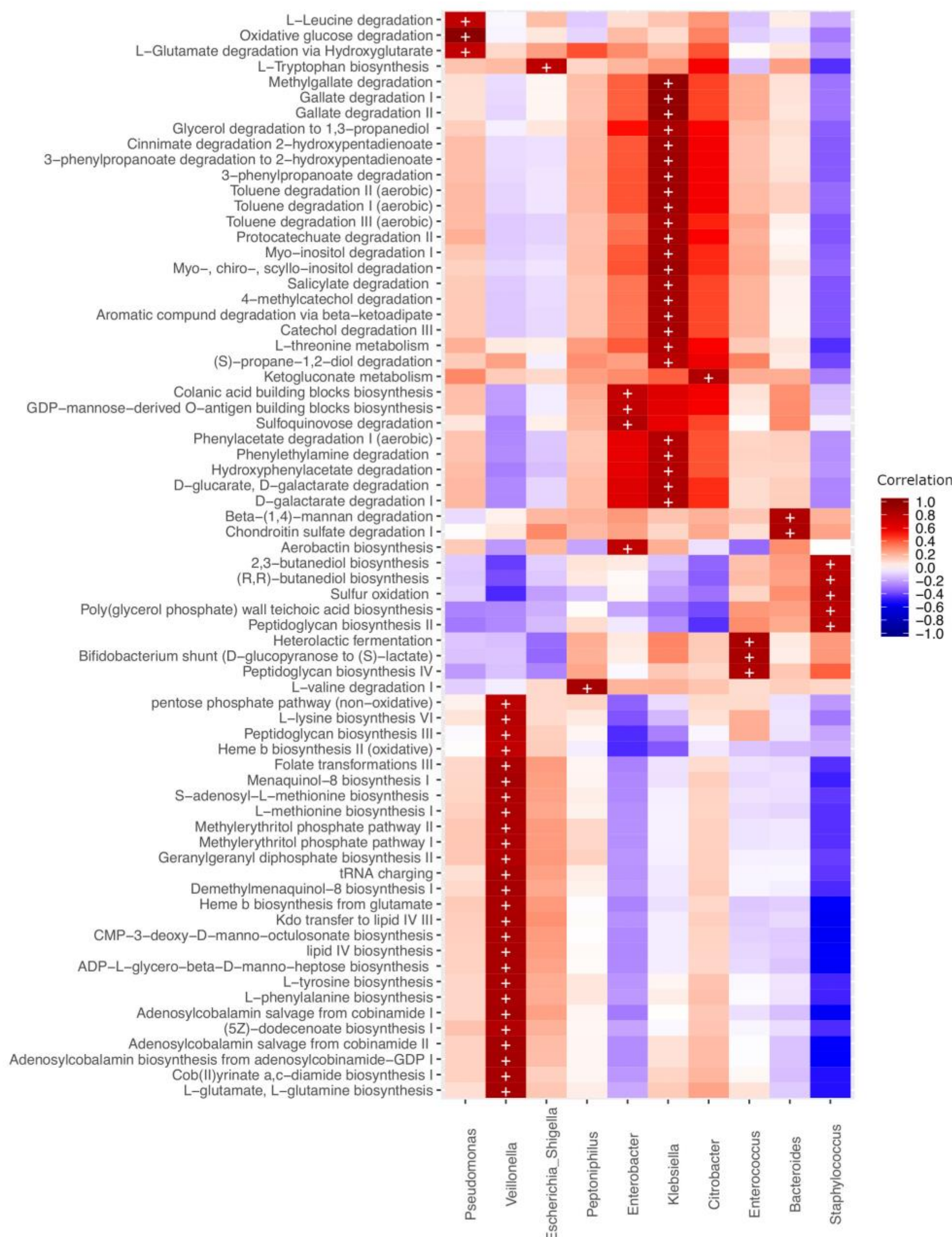

737

738 **Extended Data Fig. 3 – Abundance of top bacterial genera correlate with abundances of**

739 **inferred metabolic pathways**

740 Heatmap of Spearman correlation values between counts of the most highly abundant bacterial

741 genera and abundance of inferred metabolic pathways using PICRUSt2. Only bacterial genera

742 with at least one significant correlation are depicted (p-values < 0.05). n=90 stool samples.

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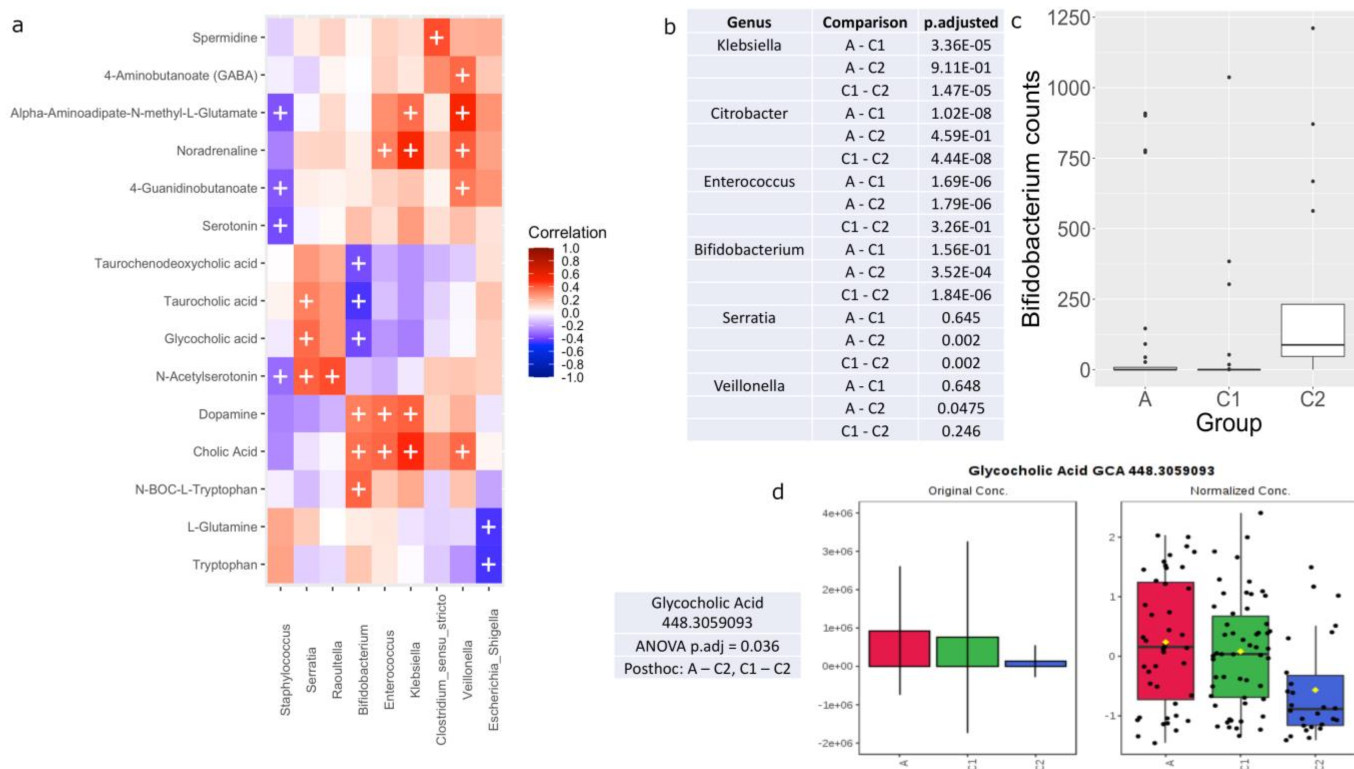

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	A - C2	0.002
	C1 - C2	0.002
Veillonella	A - C1	0.648
	A - C2	0.0475
	C1 - C2	0.246

743

744 **Extended Data Fig. 4 – Concentrations of glycocholic acid in stool by enrollment group**

745 **follow similar trends in *Bifidobacterium* abundance**

746 (**a**) Heatmap of Spearman correlation coefficients between peak response heights of identified

747 metabolites in stool and the top 10 bacterial genera from the same samples (n=90 stool samples),

748 of which 9 genera had at least 1 significant correlation with the metabolites shown (FDR-

749 corrected $p < 0.05$). Significant correlations are indicated by a '+'. (**b**) Bacterial genera which

750 had significantly different counts when comparing at least 2 enrollment groups (at least 1

751 pairwise comparison). P-values are FDR adjusted. (**c**) Boxplot comparing the number of

752 *Bifidobacterium* counts between the enrollment groups. (**d**) Original and normalized

753 concentrations of the conjugated bile acid glycocholic acid by enrollment group and significant

754 comparisons of normalized concentrations between groups by ANOVA.