Longitudinal disease-associated gut microbiome differences in infants with early food allergic manifestations

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1 **Title:**

2 **Longitudinal disease-associated gut microbiome differences in infants with early**
3 **food allergic manifestations**

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8 **Abstract (~150 words):**

9 Complex interactions between the gut microbiome and immune cells in infancy are thought to
10 be part of the pathogenesis for the marked rise in pediatric allergic diseases, particularly food
11 allergies¹⁻⁵. Food protein-induced allergic proctocolitis (FPIAP) is commonly the earliest
12 recognized non-IgE-mediated food allergy in infancy and is associated with atopic dermatitis
13 and subsequent IgE-mediated food allergy later in childhood^{6,7}. Yet, a large prospective
14 longitudinal study of the microbiome of infants with FPIAP (including samples prior to symptom
15 onset) has not been done. Here we analyzed 954 longitudinal samples from 160 infants in a
16 nested case-control study (81 who developed FPIAP, and 79 matched controls) from 1 week to
17 1 year of age by 16S rRNA ribosomal gene sequencing as part of the Gastrointestinal
18 Microbiome and Allergic Proctocolitis (GMAP) Study. We confirmed that vaginally delivered
19 infants had a greater abundance of *Bacteroides*, infants who received any breast milk had a
20 greater abundance of *Bifidobacterium*, and that overall bacterial richness rose over the first
21 year. We found key differences in the microbiome of infants with FPIAP, most strongly a higher
22 abundance of a genus of *Enterobacteriaceae* and a lower abundance of a family of *Clostridiales*
23 during the symptomatic period, as well as other key taxonomic differences across symptom
24 states including prior to symptom onset. This study contributes to the larger body of literature
25 examining structural development of the early life gut microbiome and provides a foundation for
26 more mechanistic investigation into the pathogenesis and microbial effects on FPIAP and
27 subsequent food allergic diseases in childhood.

28 **Introduction:**

29 Food protein-induced allergic proctocolitis (FPIAP) is a commonly recognized and
30 burdensome form of non-IgE-mediated food allergy of early infancy with rates as high as 17%
31 recently reported in the US from the GMAP study⁶. FPIAP is typically diagnosed clinically by the
32 presence of bloody and mucoid stools during the first few months of life in a generally healthy
33 infant that resolves with dietary restriction (most commonly of milk protein, and sometimes
34 soy)⁸. FPIAP can be seen in both children who are breastfed and formula fed, but exclusively
35 formula fed infants are at increased risk⁶. The pathophysiology of FPIAP is not well studied or
36 understood. It resolves for most affected patients within the first 12 months of life; however it
37 has been associated with an increased risk of developing both IgE-mediated food allergy
38 (IgE-FA)⁷ and Eosinophilic Esophagitis⁹. The rate of rise of food allergies strongly implicates
39 environmental factors (antibiotics, diet, and other exposures) resulting in dysbiosis (microbial
40 imbalance) which has been already associated with other forms of pediatric allergy^{1,2}. Complex
41 cross-talk between the intestinal microbiome, food antigens, intestinal inflammation, and the
42 innate immune system early in life likely contributes to the mechanisms responsible for either
43 healthy tolerance acquisition or food allergy development³. Given the early onset of FPIAP,
44 association with feeding practices and symptoms of the lower (and often also upper) GI tract,
45 we hypothesized that the developing infant microbiome plays a role in FPIAP development and
46 subsequent loss of oral tolerance.

47 There is growing evidence that the gut microbiome plays a key role in the development
48 of IgE and non-IgE mediated food allergy^{10–15}. Infants with IgE-mediated cow's milk allergy
49 (IgE-CMA) have key taxonomic differences when compared to healthy infant counterparts^{11,14,15}.
50 They have additionally been shown to have taxonomic differences once started on extensively
51 hydrolyzed formula and after achieving tolerance to milk protein, suggesting a role for the
52 microbiome in disease resolution^{14–16}. Literature on non-IgE-mediated cow's milk protein allergy
53 has similarly demonstrated taxonomic differences between healthy and allergic infants, infants
54 on an extensively hydrolyzed formula diet, and infants tolerant to milk protein^{10–13,17,18}. This
55 literature, however, has been limited by cross sectional design^{10,11}, lack of strict diagnostic
56 criteria for non-IgE-CMA^{12,13}, detection methods by culture^{12,13}, and pooling of non-IgE with

57 IgE-CMA^{11,15}. To our knowledge, there is no study that has analyzed samples longitudinally
58 including those collected prior to symptom or disease onset.

59 The Gastrointestinal Microbiome and Allergic Proctocolitis (GMAP) Study is the first
60 prospective observational healthy infant cohort study specifically designed to investigate the
61 epidemiology, clinical presentation, and potential role of the longitudinal microbiome in FPIAP⁶
62 and subsequent IgE-FA⁷. Over 1,000 healthy infants were enrolled at their first pediatrics visit in
63 the first week of life, stool samples were collected very frequently across the first year, and they
64 are being followed clinically with ongoing sample collection through age 18. 17% of these
65 healthy children went on to develop FPIAP⁶ and 6% went on to develop IgE-FA⁷. This large
66 cohort addresses the many limitations in the literature, as we enrolled infants in an unbiased
67 fashion, sampled infants frequently very early in life, and thus have sampling before, during, and
68 after disease onset. This provided us with a well-matched control population, dense early
69 sampling, and the ability to examine factors which may precede disease onset, herald symptom
70 progression, or be associated with disease resolution.

71 **Results:**

72 *Participant profiles*

73 From the GMAP cohort⁶, we selected 81 infants prospectively diagnosed with food
74 protein-induced allergic proctocolitis (FPIAP) and 79 matched controls with robust fecal
75 microbiome sampling across their first year of life (**Figure 1**). For infants who developed FPIAP,
76 samples were selected from before symptom onset, during their symptomatic period, and after
77 their symptoms resolved (**Figure 1**). The median number of samples in the first year per child
78 was 5 [3, 19]. A total of 954 samples met quality control criteria (**Methods**) when sequenced
79 using 16S ribosomal RNA gene sequencing. Data were analyzed using QIIME2 (see **Methods**),
80 generating a genus-level composition map for each sample. 45% were female, 65% were
81 delivered vaginally, 63% were initially exclusively breastfed, and 56% were perinatally exposed
82 to antibiotics (maternal 46%, perinatal infant 3%, or both 7%) (**Figure 1**). No significant
83 differences among any of these factors were noted between infants with FPIAP and controls,

84 with the exception of the initial infant diet which was more commonly formula in the children who
85 developed FPIAP as previously reported in the cohort at large⁶.

86 *Overall microbiome composition*

87 We first examined the microbiome collectively across all infants, looking for characteristic
88 features of the developing infant gut microbiome in the first year of life. We found a
89 predominance of *Bifidobacterium*, *Bacteroides*, *Enterobacteriales*, and *Clostridium* at the earliest
90 time points as has been previously shown^{19–22} (**Figure 2A**). We also saw the characteristic rise
91 in overall microbial richness from birth to age 1^{19,22,23} (**Figure 2B**). Consistent with existing
92 literature^{24,25}, we found that infants who were vaginally delivered had a greater abundance of
93 *Bacteroides* ($p=5.25 \times 10^{-7}$, **q-value = 5.06×10^{-6} , coefficient = 0.23**) compared to those delivered
94 by C-section, and infants who were exclusively breastfed had a greater abundance of
95 *Bifidobacterium* ($p=3.82 \times 10^{-4}$, **q= 2.25×10^{-3} , c=0.13**)(**Figure 2C**). Additionally, infants who
96 received probiotics in the first year (predominantly *Lactobacillus rhamnosus* GG in this cohort)
97 had a greater abundance of *Lactobacillus* (**Figure 2C**). Therefore, as we turned our attention to
98 exploring taxonomic features that differentiated infants with FPIAP compared to healthy
99 controls, we included age, mode of delivery, diet, and probiotic exposure in all of our multivariate
100 linear models (**Methods**) reported here. Only results that met an absolute coefficient threshold
101 of >0.05 and q-value <0.25 are reported. A complete table of all significant findings are available
102 in **Supplemental Table 1**.

103 *Taxonomic differences between FPIAP cases and controls*

104 In examining potential differences in the infant microbiome in children who developed FPIAP
105 compared to those who did not, we first looked for overall differences in community structure
106 across the first year. While the overall first year composition was similar between cases and
107 controls (**Supplemental Figure 1 (Composition plots by case status)**), we did find a greater
108 abundance of a single taxa in symptomatic FPIAP cases. We denote this taxa as
109 *Enterobacteriaceae_set1*, which is an unclassified genus of *Enterobacteriaceae* (most likely,

110 represented by *Escherichia*; **Methods**) and is found in greater abundance in symptomatic
111 FPIAP cases ($p=2.7\times 10^{-2}$, $q=1.13\times 10^{-1}$, $c=0.09$) when analyzing all samples (**Supplemental**
112 **Table 1**). We did not find any statistically significant differences between the overall microbial
113 richness or stability across the first year when comparing FPIAP cases and controls, using
114 shannon, chao1, and bray-curtis indices, though some pairwise differences were noted at 4 and
115 6 months (**Supplemental Figure 2**) between symptomatic cases and resolved cases and
116 controls respectively.

117 Given that FPIAP presents so early in infancy (median age of diagnosis = 1 month, >75%
118 diagnosed by 2.5 months), we took two approaches to narrow our focus on samples from
119 children at the times of most interest in this disease. First, we focused on all samples collected
120 between 0 and 2 months of age (when the majority of cases of FPIAP have presented). Second,
121 we made three key sample subsets: *Last pre-symptomatic* (where for each case of FPIAP we
122 chose their last pre-symptomatic sample and then the nearest age-matched control), *First*
123 *symptomatic* (where for each case of FPIAP we chose their first symptomatic sample and then
124 the nearest age-matched control), and *First resolved* (where for each case of FPIAP we chose
125 their first resolved sample and then the nearest age-matched control; **Supplemental Figure 3**).

126 In the 0-2 months sample subset, we again found a greater abundance of
127 *Enterobacteriaceae_set1* ($p=1.54\times 10^{-2}$, $q=0.11$, $c=0.140$), and a lower abundance of
128 *Enterobacteriaceae_set2* (unclassified at the genus level and most likely represented by
129 *Klebsiella*, *Salmonella*, or *Enterobacter*; **Methods**; $p=6.96\times 10^{-2}$, $q=0.23$, $c=-0.095$), and an
130 unclassified family of *Clostridiaceae* ($p=5.83\times 10^{-2}$, $q=0.22$, $c=-0.057$) in FPIAP cases compared
131 to controls (**Figure 3A**).

132 *Taxonomic differences between FPIAP disease stages and controls*

133 We next looked for taxonomic differences that might differentiate stages of FPIAP development
134 (pre-symptomatic, symptomatic, and resolved) from controls (**Methods**). Comparing infants with
135 FPIAP before they developed symptoms (“pre-symptomatic”) to matched controls over the first
136 two months of life, we found a greater abundance of the genus *Streptococcus* ($p=5.26 \times 10^{-2}$,
137 $q=0.23$, $c=0.056$) and *Enterobacteriaceae_set1* ($p=1.89 \times 10^{-2}$, $q=0.16$, $c=0.15$) and a lower
138 abundance of an unclassified family of *Clostridiales* ($p=3.41 \times 10^{-3}$, $q=5.21 \times 10^{-2}$, $c=-0.052$)
139 (**Figure 3B**). Within the *Last pre-symptomatic* subset, we saw the same enrichment of
140 *Enterobacteriaceae_set1* ($p=0.134$, $q=0.192$, $c=0.202$) in infants with FPIAP (**Figure 3A**). Next
141 we looked at the symptomatic FPIAP cases, and found a greater abundance of
142 *Enterobacteriaceae_set1* ($p=1.54 \times 10^{-2}$, $q=1.13 \times 10^{-1}$, $c=1.4 \times 10^{-1}$), and a lower abundance of an
143 unknown family of *Clostridiales* ($p=4.8 \times 10^{-6}$, $q=1.27 \times 10^{-3}$, $c=-6.33 \times 10^{-2}$) in FPIAP cases
144 compared to controls over the first two months. The same enrichment of
145 *Enterobacteriaceae_set1* and lower abundance of the same unclassified family of *Clostridiales*
146 was seen in the *First symptomatic* sample subset ($p=2.05 \times 10^{-3}$, $q=3.77 \times 10^{-2}$, $c=0.217$;
147 $p=5.99 \times 10^{-6}$, $q=5.5 \times 10^{-4}$, $c=-0.087$ respectively) (**Figure 3A**). Finally, looking at infants with
148 FPIAP once their symptoms had resolved using samples from 9-12 months of age compared to
149 controls, we did not find taxonomic differences which met our coefficient and q-value threshold
150 (though differences were seen at a less stringent coefficient threshold) (**Supplemental Figure**
151 **4**). When we examined the more restrictive *First resolved* sample subset, we found an
152 enrichment of the genus *Lactobacillus* ($p=5.86 \times 10^{-3}$, $q=7.17 \times 10^{-2}$, $c=0.07$) and a decreased
153 abundance of the genus *Blautia* ($p=1.22 \times 10^{-2}$, $q=0.105$, $c=-0.06$) in infants with FPIAP which
154 had resolved compared to controls (**Figure 3A**).

155 Next, we were interested in the trajectories over time of each of these most significantly
156 differential taxa as the infants’ disease progressed from pre-symptomatic, to symptomatic, to

157 resolved states. Looking at all samples across the first year, the unknown family of the
158 *Clostridiales* class was significantly lower in children with FPIAP, before they became
159 symptomatic, while they had symptoms, and after they had resolved when compared to controls
160 (**Figure 3C**) with a relatively static trajectory. The relative abundance of the genus
161 *Streptococcus* was statistically higher than controls when infants with FPIAP were
162 presymptomatic, but then came down to levels comparable to controls once they developed
163 symptoms and then resolved (**Figure 3C**). *Lactobacillus* on the other hand, is lowest when
164 infants are presymptomatic (comparable to controls), rises sharply as they become
165 symptomatic, and then reaches a significantly higher abundance than controls when infants'
166 symptoms have resolved (**Figure 3C**). Infants with FPIAP were more likely to receive
167 *Lactobacillus*-containing probiotics, so we next examined whether this could explain the
168 differential abundance of *Lactobacillus*. Stratifying by probiotic exposure in the first year, we
169 found that while indeed infants receiving probiotics had a higher abundance of *Lactobacillus*, the
170 differential trajectory was seen in both groups, regardless of probiotic use (**Supplemental**
171 **Figure 5**). The *Enterobacteriaceae_set1* also has an interesting trajectory: with a higher relative
172 abundance than controls even in the pre-symptomatic group, rising significantly higher than
173 controls as infants become symptomatic, and then decreasing but remaining higher than
174 controls even once symptoms have resolved (**Figure 3C**).

175 **Discussion:**

176 Food protein-induced allergic proctocolitis (FPIAP) is often the earliest manifestation of food
177 allergy in children, and yet little is known about its pathogenesis. Few studies have examined
178 the microbiome of infants with FPIAP, and to our knowledge none have dense longitudinal
179 sampling which includes samples prior to symptom onset. From the GMAP study we performed
180 16S rRNA gene sequencing on 954 longitudinal samples from 160 infants (81 with FPIAP and
181 79 matched controls) from 1 week to 1 year of age. While overall the composition and richness
182 of the infants' microbiome was similar in infants with FPIAP compared to controls, we identified

183 several taxonomic differences in infants with FPIAP across symptom states (from
184 pre-symptomatic to resolved).

185 We found an increased abundance of an unclassified genus of the *Enterobacteriaceae* family
186 which was most prominent when infants were symptomatic but was elevated even prior to
187 symptom onset. The *Enterobacteriaceae* family contains species known to bloom in other
188 inflammatory settings in the GI tract²⁶, and has previously been associated with cow's milk
189 allergy^{15,16} and other food allergies²⁷. We found a decreased abundance of an unclassified
190 family of *Clostridiales*. Some families within this order (*Peptostreptococcaceae*,
191 *Lachnospiraceae*, some *Clostridium* species) have been previously associated with decreased
192 relative abundance in IgE- and non IgE- food allergic children^{11,28,29} although many other
193 *Clostridiales* species are enriched. Previous reports suggest that specific species of *Clostridia*
194 drive the separation between allergic children and their non-allergic siblings and controls²⁸.
195 Among these families are species which are important for gut permeability/integrity as well as
196 butyrate production, both of which have important potential mechanistic roles in FPIAP^{10,30}. We
197 also found a greater abundance of members of the genus *Lactobacillus* as infants with FPIAP's
198 symptoms resolved compared to controls (irrespective of probiotic use). Other studies of infants
199 with a mix of IgE- and non-IgE-mediated cow's milk allergy have shown an increase in
200 *Lactobacillus* when infants were treated with hypoallergenic formula¹⁵, FMT³¹, or hypoallergenic
201 formula + LGG¹⁰. Finally, we found an unclassified genus of the *Clostridiaceae* family which was
202 significantly lower in the infants with FPIAP compared to controls overall, and similarly a lower
203 abundance of an unknown family of *Clostridiales* across symptom states in these allergic
204 infants. A few IgE-mediated food allergy studies have also shown decreased *Clostridia*
205 species^{16,32} in food allergic subjects.

206 One limitation inherent to studying the microbiome of FPIAP is how early in infancy the disease
207 presents. The microbiome composition in the first few months of life is highly dynamic, making it
208 more difficult to detect differences between clinical groups. This likely accounts for some of our
209 findings which appeared in some sample subsets but not others, but also underscores the
210 significance of those findings that were replicated across time. The limited number of samples in
211 our presymptomatic sample subset, due to very young age, also likely limited our power to
212 identify differences in that subset. Another challenge to understanding FPIAP pathogenesis with

213 respect to the microbiome is that diet often changes as infants are diagnosed (some with
214 maternal dietary elimination, some with changes to formula, some with changes across formula
215 types) which can have a significant impact on the microbiome and cannot be fully accounted for
216 in these models.

217 In summary, our unique longitudinal pediatric cohort enabled us to carefully investigate the role
218 of the microbiome in one of the earliest manifestations of allergy in children (FPIAP). Because of
219 the prospective healthy infant cohort design, we were able to identify key taxonomic differences
220 in children who developed FPIAP, even before they became symptomatic, as well as after their
221 disease resolved. We were also able to contribute to the growing body of literature describing
222 the early composition of the infant microbiome in non-allergic children and highlight several
223 potential areas of important further investigation. Our study provides a foundation for testable
224 hypotheses around the underlying role of potentially key taxa including *Enterobacteriaceae* and
225 *Clostridiales* in the pathophysiology of FPIAP. It remains important to further determine these
226 mechanisms as they may provide important opportunities for early targeted interventions to
227 prevent IgE and non-IgE-mediated food allergy more broadly, and perhaps to better support an
228 optimal pediatric microbiome promoting healthier non-allergic phenotypes.

229 **Methods:**

230 Patient characteristics

231 From the GMAP prospective observational healthy infant cohort, we selected infants diagnosed
232 with food protein-induced allergic proctocolitis (FPIAP) who had a minimum of 4 longitudinal
233 stool samples and selected matched controls for each who met the same sampling criteria. The
234 patient characteristics of this nested case-control subset were similar to those of the cohort at
235 large⁶.

236 Sample collection

237 Fresh stool samples were brought to each clinic visit, where stool was collected from diapers,
238 transferred to sterile tubes, stored immediately at -20 °C, then transferred to -80 °C.

239 DNA extraction and sequencing

240 We used the Qiagen DNeasy PowerSoil htp 96 (Cat No./ID: 12955-4) to extract stool samples
241 that were stored at -80 °C and “chipped” while frozen to obtain 100 - 150 mg of sample for
242 extraction. Bead beating was performed on a TissueLyzer at 20Hz for 10 min as per
243 manufacturer’s protocol.

244 16S rRNA gene libraries targeting the V4 region of the 16S rRNA gene were prepared by first
245 using qPCR to normalize template concentrations and determine optimal cycle number. Library
246 construction was performed in quadruplicate with the primers 515F
247 (5'-AATGATACGCGACCACCGAGATCTACACTATGGTAATTGTGTGCCAGCMGCCGCGGTA

248 A-3') and unique reverse barcode primers from the Golay primer set (Caporaso, 2011 and
249 2012). After amplification, sample replicates were pooled and cleaned via Agencourt AMPure
250 XP-PCR purification system. Prior to final pooling, purified libraries were normalized via qPCR in
251 two 25uL reactions, 2x iQ SYBR SUPERMix (Bio-Rad, REF: 1708880) with Read 1
252 (5'-TATGGTAATT GT GTGYCAGCMGCCGCGGTAA-3'), Read 2 (5'-AGTCAGTCAG CC
253 GGACTACNVGGGTWTCTAAT-3') primers. Pools were quantified by Qubit (Life Technologies,
254 Inc.) and sequenced on an Illumina MiSeq 300 using custom index
255 5'-ATTAGAWACCCBDGTAGTCC GG CTGACTGACT-3' and custom Read 1 and Read 2
256 primers mentioned above.

257 Raw sequencing data can be found on NCBI BioProject PRJNA730851.

258 16S rRNA gene sequencing analysis

259 Microbial communities were analyzed using QIIME2 version 2020.11.1³³. The DADA2
260 algorithm³⁴ was used for quality filtering and merging sequences with greater than 99%
261 similarity. Alignment was done by use of 'qiime feature-classifier fit-classifier-naive-bayes'. A
262 total of 1088 samples were collected. Samples that did not pass the sequencing process, had a
263 total frequency of less than 3000 reads, or those with an unclear diagnosis were dropped.
264 Additionally, features represented in only one sample or with a maximum relative abundance of
265 <0.03 were also dropped. A total of 954 samples remained for analysis. Relative abundance
266 data were normalised with QIIME2's tools. A reference Greengenes database version 13.5³⁵
267 was used to assign taxonomy features.

268 Some OTUs were not able to be classified on the genus, and were left as unclassified genera
269 within the Enterobacteriaceae family. For two of these sets that had significant results in our
270 data, we attempted to manually identify a better classification. We used blast-2.10.0+ to map the
271 OTU sequences against the NCBI nt database (downloaded on May 16 2017). We termed
272 these sets *Enterobacteriaceae_set1*, and *Enterobacteriaceae_set2*. Our results suggest that
273 *Enterobacteriaceae_set1* is most likely representing sequences from the *Escherichia* genus,
274 while *Enterobacteriaceae_set2* most likely represents sequences from the *Klebsiella*,
275 *Salmonella* and *Enterobacter* genera. In both sets there were many sequences that mapped to
276 "uncultured bacteria".

277 Statistical analysis

278 All analyses were performed in RStudio (Version 1.3.1093) run with R (version 4.0.2). Data
279 editing done with dplyr. Graphs were created using R packages ggplot2 and ggpubr. Alpha
280 diversity was calculated using the fossil package. Multivariate regression analysis was
281 performed using the MaAsLin2 R package. Mixed-effects linear models using a
282 variance-stabilizing arcsin square root transform on relative abundances are then used to
283 determine the significance of putative associations from among this reduced set. Nominal P
284 values across all associations were then adjusted using the Benjamini–Hochberg FDR method.
285 We reported microbial features with corrected $q < 0.25$ were reported. p values that did not
286 originate from the MaAsLin analysis were generated using the rstatix R package.

287 All boxplots were generated using ggplot2: The lower and upper hinges correspond to the first
288 and third quartiles (the 25th and 75th percentiles), and the upper and lower whiskers roughly
289 represent the 1.5*IQR from the hinge (default geom_boxplot function).

290 Composition and richness analysis

291 The composition and richness analysis were calculated using a subset of 808 samples, such
292 that each infant has a single sample at each time point. Richness was calculated using the
293 chao1 function in the fossil R package.

294 Multivariate models definition

295 To find a linear correlation between FPIAP and microbial features we used three different
296 models: (1) comparing samples from FPIAP cases to controls; (2) comparing samples based on
297 their allergic symptoms (pre-symptomatic, symptomatic, and resolved) to controls; and (3) as in
298 (2) but comparing the samples to the symptomatic group as a reference. Each model contains
299 the following fixed effects variables: (a) case/control or symptoms (b) mode of delivery (c) age at
300 visit (d) diet and (e) probiotics use in the first year of life. As we have multiple samples from
301 each child, all models also include the child ID as a random variable.

302 In general, we ran the models on six different subsets of samples, either subsetting by age or by
303 disease status:

304 Age subgroups -

- 305 a. All Samples - This group includes all samples (n=954).
- 306 b. 0-2 months - All samples with age visit < 3 months (n=473).
- 307 c. 9-12 months - All samples with age visit in the range [8.5-12.5] months (n=179).

308 Disease status subgroups (these subsets always contain a single sample per subject) -

- 309 d. *Last pre-symptomatic* - For each FPIAP case we chose the last sample collected prior to
310 symptom onset, and identified a matched control sample (n=114).
- 311 e. *First symptomatic* - For each FPIAP case we chose the first sample when the child was
312 symptomatic, and identified a matched control sample (n=144).
- 313 f. *First resolved* - For each FPIAP case we chose the first sample when the child was
314 asymptomatic, and identified a matched control sample (n=148).

315 Matched control samples were chosen by finding an optimal match between the FPIAP sample
316 times and all the control samples, making sure to include at most a single sample from each
317 control subject in each subset. We found the optimal match by defining it as a linear sum
318 assignment problem (LSAP) using the clue R package to solve it with the hungarian method.

319 Interactive visualization tool

320 All associations (significant and not significant) can be viewed on our interactive visualization
321 tool: https://www.cs.huji.ac.il/~morani/GMAP_data.html, enabling the comparison of multiple
322 models, and features, together with viewing the raw data and the statistical analysis results.

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410 collection; VMM and YVV curated and analyzed the metadata; HV and MY processed samples,
411 generated and processed raw sequencing data; ED, VMM, YVV HS, and MY performed data
412 analysis, interpretation, and figure generation; ED developed the online visualization tool; VMM,
413 HS, and MY wrote the paper, and all authors contributed to critical revisions and approved the
414 final manuscript.

415 **Figure Legends:**

416 **Figure 1. GMAP Study Cohort design, key clinical characteristics, and longitudinal**
417 **sampling.** (A) Key clinical and environmental features of the cohort analyzed including antibiotic
418 exposure, mode of delivery, and infant diet over time and the timeline for sample collection. (B)
419 Number of samples analyzed across the first year, binned by the age of the infant at the time of
420 sample collection and colored by whether that infant was a healthy control or a child with FPIAP.
421 Samples from infants with FPIAP are colored by their symptom state at the time of sample
422 collection. (C): Sample map showing the samples analyzed plotted by the age of the infant at
423 time of collection and colored by their disease and symptom state.

424 **Figure 2. Overall longitudinal microbiome composition.** (A) Composition plot of the full first
425 year of life showing the mean relative abundance of the top 15 taxa and their longitudinal
426 taxonomic assemblage over the first year (**Methods**). (B) Longitudinal microbial richness (using
427 the chao1 index) over the first year. The center line denotes the median, the boxes cover the
428 25th to 75th percentiles (see **Methods**). (C) Relative abundance (arcsine transformed, AST) of
429 key taxonomic differences mediated by important environmental factors: *Bacteroides* by delivery
430 mode, *Bifidobacterium* by infant diet, and *Lactobacillus* by probiotic use. Significant differences
431 and coefficients from MaAsLin 2 model across all samples (see **Methods**).

432 **Figure 3. Summary of key differential taxa between infants with FPIAP and healthy**
433 **controls.** (A) Significantly different taxa ($q < 0.25$), with absolute coefficient ≥ 0.05 , when
434 comparing infants with FPIAP to healthy controls when looking at the sample subsets: 0-2
435 months, *Last pre-symptomatic*, *First symptomatic*, and *First resolved*. Bars to the right are
436 enriched in infants with FPIAP, while bars to the left are enriched in the controls. (B)
437 Significantly different taxa ($q < 0.25$) when comparing infants with FPIAP to matched controls
438 before their symptom onset (top section), and then during the symptomatic period (lower
439 section) over the first 2 months of age. Association directionality is as in (A). (c) Relative
440 abundance trajectories of the key differential taxa between cases and controls identified in
441 panels (A) and (B) across symptom states (from pre-symptomatic to symptomatic to resolved).

442 **Supplemental Figure 1. Top 15 abundant taxa in infants with FPIAP compared to controls.**
443 Composition plots showing the mean relative abundance of the top 15 abundant taxa and their
444 longitudinal taxonomic assemblage over the first year in infants with FPIAP (left) compared to
445 controls (right).

446 **Supplemental Figure 2. Alpha diversity in infants with FPIAP compared to controls**
447 **examined longitudinally over the first year by symptom state.** Alpha diversity measured by

448 chao1 richness index in control infants compared to infants with FPIAP before symptom onset,
449 during the symptomatic period, and after symptom resolution over the first year.

450 **Supplemental Figure 3. Sample map.** Sample map showing longitudinal samples used for
451 each subset analyzed, axes and colors as in Figure 1.

452 **Supplemental Figure 4. Summary of additional key differential taxa between infants with**
453 **FPIAP and healthy controls.** Same analysis as Figure 2, with less stringent coefficient
454 threshold (>0.025). **(A)** Significantly different taxa when comparing infants with FPIAP to healthy
455 controls when looking at the sample subsets: 0-2 months, *Last pre-symptomatic*, *First*
456 *symptomatic*, and *First resolved*. Bars to the right are enriched in infants with FPIAP, while bars
457 to the left are enriched in the controls. **(B)** Significantly different taxa when comparing infants
458 with FPIAP to matched controls before their symptom onset (top section), and then during the
459 symptomatic period (lower section) over the first 2 months of age. Again, bars to the right are
460 enriched in infants with FPIAP, while bars to the left are enriched in the controls.

461 **Supplemental Figure 5. Differential trajectory of *Lactobacillus* across symptom states in**
462 **infants with FPIAP compared to controls, stratified by probiotic use.** Box plots of the
463 relative abundance (AST) trajectories of *Lactobacillus* across symptom states (from
464 pre-symptomatic to symptomatic to resolved) compared to controls, stratified by (largely
465 LGG-containing) probiotic use (panel A vs. panel B) across all samples.

466 **Supplemental Table 1. Summary table of all significant MaAsLin results.** All MaAsLin
467 results from 16S rRNA gene sequencing with q-values for significant taxa which met our
468 predetermined threshold for significance ($q < 0.25$). Mixed effects linear models using arcsine
469 transform on relative abundances were used to determine significance (see **Methods**). p-values
470 were adjusted for multiple comparisons using the Benjamini-Hochberg false discovery rate
471 (FDR) method with FDR set at < 0.25 .

Figures

Figure 1

GMAP Study Cohort design, key clinical characteristics, and longitudinal sampling. (A) Key clinical and environmental features of the cohort analyzed including antibiotic exposure, mode of delivery, and infant diet over time and the timeline for sample collection. (B) Number of samples analyzed across the first

year, binned by the age of the infant at the time of sample collection and colored by whether that infant was a healthy control or a child with FPIAP. Samples from infants with FPIAP are colored by their symptom state at the time of sample collection. (C): Sample map showing the samples analyzed plotted by the age of the infant at 423 time of collection and colored by their disease and symptom state.

Figure 2

Overall longitudinal microbiome composition. (A) Composition plot of the full first year of life showing the mean relative abundance of the top 15 taxa and their longitudinal taxonomic assemblage over the first year (Methods) . (B) Longitudinal microbial richness (using the chao1 index) over the first year. The center line denotes the median, the boxes cover the 25th to 75th percentiles (see Methods) . (C) Relative abundance (arcsine transformed, AST) of key taxonomic differences mediated by important environmental factors: Bacteroides by delivery mode, Bifidobacterium by infant diet, and Lactobacillus by probiotic use. Significant differences and coefficients from MaAsLin 2 model across all samples (see Methods).

Figure 3

Summary of key differential taxa between infants with FPIAP and healthy controls. (A) Significantly different taxa ($q < 0.05$), with absolute coefficient ≥ 0.05 , when comparing infants with FPIAP to healthy controls when looking at the sample subsets: 0-2 months, Last pre-symptomatic, First symptomatic, and First resolved. Bars to the right are enriched in infants with FPIAP, while bars to the left are enriched in the controls. (B) Significantly different taxa ($q < 0.05$) when comparing infants with FPIAP to matched controls before their symptom onset (top section), and then during the symptomatic period (lower section) over the first 2 months of age. Association directionality is as in (A). (c) Relative abundance trajectories of the key differential taxa between cases and controls identified in panels (A) and (B) across symptom states (from pre-symptomatic to symptomatic to resolved).

Supplementary Files

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