IRON DEFICIENCY ANEMIA-RELATED GUT MICROBIOTA DYSBIOSIS IN INFANTS AND YOUNG CHILDREN: A PILOT STUDY

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Nutritional iron deficiency (ID) causes not only anemia but also malfunction of the entire human organism. Recently, a role of the gut microbiota has been hypothesized, but limited data are available especially in infants. Here, we performed a pilot study to explore the gut microbiota in 10 patients with iron deficiency anemia (IDA) and 10 healthy controls aged 6–34 months. Fresh stool samples were collected from diapers, and the fecal microbiota was profiled by next-generation sequencing of the V3–V4 hypervariable region of the 16S rRNA gene. Except for diet diversity, the breastfeeding status at the enrollment, the exclusive breastfeeding duration, and the introduction of complementary foods did not differ between groups. Distinct microbial signatures were found in IDA patients, with increased relative abundance of *Enterobacteriaceae* (mean relative abundance, patients vs. controls, 4.4% vs. 3.0%) and *Veillonellaceae* (13.7% vs. 3.6%), and reduced abundance of *Coriobacteriaceae* (3.5% vs. 8.8%) compared to healthy controls. A decreased *Bifidobacteriaceae/Enterobacteriaceae* ratio was observed in IDA patients. Notwithstanding the low sample size, our data highlight microbiota dysbalance in IDA worth for further investigations, aimed at unraveling the ID impact on the microbiome trajectory in early life, and the possible long-term consequences.

Keywords: iron deficiency anemia, gut microbiota, dysbiosis, infants, children

Introduction

Anemia is a common disease found all over the world [1]. Half of the anemia cases are caused by iron deficiency (ID), and children are most

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vulnerable to this disease due to high iron needs [1, 2]. According to the World Health Organization, anemia is a moderate public health problem in Lithuania. The disease prevalence was 26% in children aged 6–59 months in 2011 [1]. Moreover, previous study suggests that almost 50% of children in the first 2 years of life may experience ID [3]. These figures are of particular concern, as ID significantly disturbs functions of the brain, heart, and skeletal muscles even in non-anemic stages [4]. The long-lasting health consequences are associated with delayed cognitive and social–emotional development [5–7].

Based on recent evidence [8–13], ID and ID anemia (IDA) may also be featured by unfavourable changes of the gut microbiota. Two studies addressing the effect of iron fortification on the intestinal microbiota in African children showed higher levels of potentially pathogenic enterobacteria than bifidobacteria and lactobacilli at the baseline [14, 15]. These findings are particularly relevant, as the gut microbiota is a key regulator of the host metabolic homeostasis [16], an integral component of the immune system [17–19], and essential for central nervous system development [20, 21]. Unbalanced microbial configurations have been found to result in lifelong consequences, including increased risk of metabolic and immunological diseases [16, 17, 20, 22].

We performed a pilot study in a homogenous cohort of 10 infants and young children with nutritional IDA compared to 10 healthy controls (HCs) to provide insights into the early-life alterations in the intestinal microbial ecosystem.

Methods

Subjects enrollment and sample collection

Ten infants and young children were recruited at a tertiary university hospital (Vilnius, Lithuania) with diagnosis of nutritional IDA (serum hemoglobin <110 g/L, ferritin <12 μg/L, and/or reticulocyte-hemoglobin equivalent <28 pg) meeting the following criteria: (1) anemia diagnosed for the first time in life, (2) otherwise healthy and not taking medicines for at least 4 weeks, (3) singleton pregnancy, and (4) vaginally delivered. Ten healthy children (HC) meeting the same (2–4) criteria served as controls. The study was approved by the Vilnius Regional Committee for Biomedical Research Ethics. Informed consent was obtained from the parents of all individual participants included in the study.

The parents of the subjects filled a questionnaire survey and provided information on their child's nutrition. Fresh stool samples were collected from diapers and immediately frozen at −20 °C. Samples were delivered to the laboratory (Bologna, Italy), where they were stored at −80 °C until processing.

Microbial DNA extraction

Microbial DNA was extracted from feces using the repeated bead-beating plus column method, as previously described [23]. Briefly, 250 mg of sample were suspended in 1 ml of lysis buffer (500 mM NaCl, 50 mM Tris-HCl, pH 8, 50 mM EDTA, and 4% sodium dodecyl sulfate) and bead-beaten thrice in a FastPrep instrument (MP Biomedicals, Irvine, CA) at 5.5 movements/s for 1 min, in the presence of four 3-mm glass beads and 0.5 g of 0.1-mm zirconia beads (BioSpec Products, Bartlesville, OK). Samples were heated at 95 °C for 15 min and then centrifuged at 13,000 rpm for 5 min. Two hundred and sixty microliters of 10 M ammonium acetate were added to the supernatant, followed by 5-min incubation in ice and 10-min centrifugation at 13,000 rpm. One volume of isopropanol was added to each sample and incubated in ice for 30 min. Precipitated nucleic acids were washed with 70% ethanol, resuspended in 100 μl of TE buffer, and treated with 2 μl of 10 mg/ml DNase-free RNase at 37 °C for 15 min. Proteinase K treatment and DNA purification were performed using the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany). DNA concentration and quality were evaluated using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

16S rRNA gene sequencing

The V3–V4 hypervariable region of the 16S rRNA gene was amplified using the 341F and 805R primers with added Illumina adapter overhang sequences, as previously described [24]. Polymerase chain reaction (PCR) products were cleaned up with Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA). Indexed libraries were prepared by limited-cycle PCR using Nextera technology, further cleaned up as described above, and pooled at equimolar concentrations. The sample pool was denatured with 0.2 N NaOH and diluted to 6 pM with 20% PhiX control. Sequencing was performed on Illumina MiSeq (Illumina, San Diego, CA) platform using a 2×250 bp paired-end protocol, according to the manufacturer's instructions.

Bioinformatics and statistics

Raw sequences were processed using a pipeline combining PANDAseq [25] and QIIME [26]. High-quality reads were clustered into operational taxonomic units (OTUs) at 97% similarity using UCLUST [27]. Taxonomy was assigned using the Ribosomal Database Project classifier against Greengenes database

(released on May 2013). Singleton OTUs were discarded to exclude chimera sequences. Alpha rarefaction was performed using the Faith's phylogenetic diversity, observed OTUs, and Shannon index metrics. Beta diversity was estimated by computing weighted and unweighted UniFrac distances.

All statistical analysis was performed using R software version 3.3.2. UniFrac distances were plotted using the vegan package, and data separation in the principal coordinates analysis (PCoA) was tested using a permutation test with pseudo-F ratios (function adonis). Significant differences in alpha or beta diversity as well as in taxon-relative abundances were assessed by Wilcoxon rank-sum test. Continuous clinical variables are provided as mean and standard deviation (SD). They were compared using Student's t-test or Wilcoxon rank-sum test based on normality. Categorical variables are provided as absolute numbers and compared using the Pearson's χ^2 test. A p value <0.05 was considered as statistically significant.

Results

Ten infants and young children suffering from nutritional IDA (7 females, aged 6–32 months) and 10 HCs (2 females, aged 7–34 months) were recruited. No significant differences in baseline characteristics were found between the two study groups, except for hemoglobin levels and birth weight, which are both higher in the control group (Student's t-test, $p < 0.05$). With regard to diet, less diversity is observed for IDA patients compared to HC, with a significantly lower proportion of subjects consuming fish (5 IDA infants vs. 10 HC; Pearson's χ^2 test, $p = 0.0098$) and a tendency toward a lower number of individuals fed with meat, cereals, and eggs (Table I).

For the gut microbiota analysis, fecal samples were analyzed by next-generation sequencing of the 16S rDNA V3–V4 hypervariable region. The sequencing generated 1,234,956 high-quality reads (mean = 61,748; SD = 9,362) that were clustered into 4,610 OTUs at 97% identity. According to common alpha diversity metrics (i.e., the phylogenetic diversity – PD_whole_tree, the Shannon index for biodiversity and observed OTUs), no difference was found between IDA and HC infants (Supplementary Figure 1). On the other hand, the PCoA of both weighted and unweighted UniFrac distances reveals a significant segregation between the two sample groups (permutational multivariate ANOVA, $p = 0.02$) (Figure 1).

The most abundant phyla are Firmicutes (mean relative abundance, 54.0%), Actinobacteria (23.4%), and Bacteroidetes (14.2%). Even if statistical significance is not achieved (Wilcoxon rank-sum test, $p > 0.05$), it is worth

Table I. Baseline characteristics of study participants

	IDA ($n = 10$)	HC ($n = 10$)	p value
Clinical data			
Hemoglobin (g/L)[a]	102 (5)	123 (8)	<0.0001
Age (months)[b]	13 (8)	16 (8)	0.1281
Gestational age (weeks)[b]	38 (2)	40 (1)	0.0631
Birth weight (g)[a]	3,252 (613)	3,808 (309)	0.0271
Exclusive breastfeeding duration (months)[b]	4 (2)	4 (2)	0.6696
Plant-based food introduction (months)[b]	5 (0)	5 (1)	1.0000
Animal-based food introduction (months)[b]	6 (1)	6 (1)	0.5267
Dietary data at the enrollment			
Breast milk[c]	8	7	0.6056
Vegetables and fruits[c]	10	10	1.0000
Cereals[c]	8	10	0.1360
Meat[c]	7	10	0.0603
Fish[c]	5	10	0.0098
Eggs[c]	4	7	0.3613
Dairy products[c]	7	9	0.2636

Note: Baseline characteristics of iron deficiency anemia (IDA) group and healthy controls (HCs). Data are expressed as mean (standard deviation) or numbers.
[a]Normal distribution, Student's t-test applied.
[b]Non-normal distribution, Wilcoxon rank-sum test applied.
[c]Pearson's χ^2 test applied.

noting that, compared to HC, IDA patients show an enrichment of Bacteroidetes (IDA vs. HC, 18.8% vs. 9.4%) and Proteobacteria (5.2% vs. 3.9%), with a corresponding reduction of Actinobacteria (19.3% vs. 27.5%) and Verrucomicrobia (0.7% vs. 3.0%) (Figure 2A). At family level, *Lachnospiraceae* (17.4%), *Bifidobacteriaceae* (17.2%), *Ruminococcaceae* (13.3%), and *Bacteroidaceae* (11.9%) are the most represented taxa in the gut microbiota of the entire cohort. The gut microbial community of IDA patients appears significantly depleted in *Coriobacteriaceae* (3.5% vs. 8.8%; $p = 0.004$) and enriched in *Veillonellaceae* (13.7% vs. 3.6%; $p = 0.009$) and *Enterobacteriaceae* (4.4% vs. 3.0%; $p = 0.04$) compared to HC (Figure 2B). Accordingly, we found a significantly decreased *Bifidobacteriaceae* to *Enterobacteriaceae* ratio, which is a recently proposed index of gut health [15] in IDA patients (19.5 vs. HC, 93.3; $p = 0.02$). At the genus level, the differences in the microbiota profiles are mainly attributable to an overrepresentation of *Veillonella* (mostly the *Veillonella dispar* and *Veillonella parvula* species) and diminished proportions of *Collinsella, Bifidobacterium* (*B. bifidum*), *Dialister*, and *Dorea* in IDA patients compared to HC (Figures 2C and 3). Although largely subdominant, the species *Clostridium neonatale* was identified only in IDA patients (Figure 3).
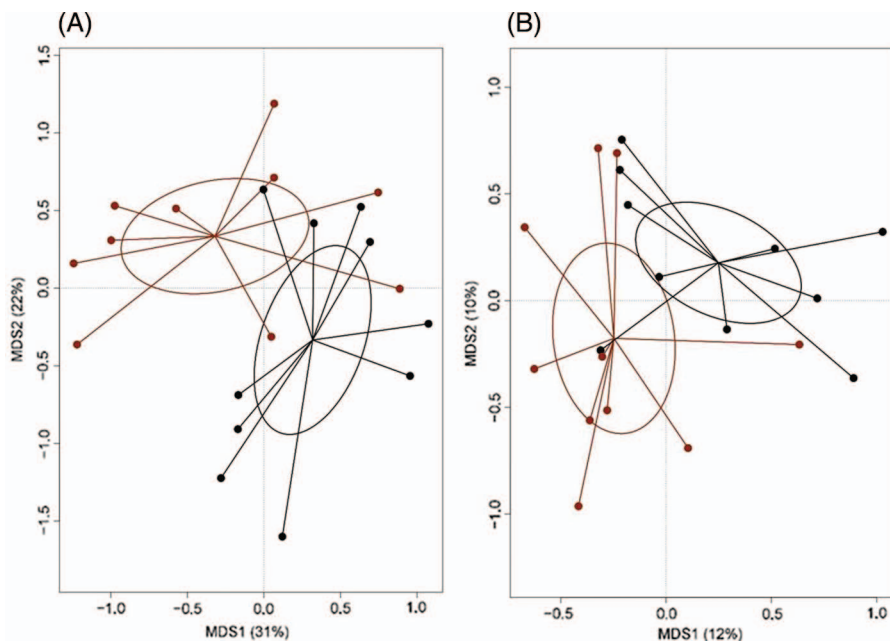
Figure 1. Gut microbiota community structure in infants suffering from iron deficiency anemia (IDA) and healthy controls (HCs). PCoA of Unweighted (A) and weighted (B) UniFrac distances shows significant segregation between infants with IDA (red) and HC (black). Permutational multivariate ANOVA based on distance matrices (adonis), $p = 0.02$

Discussion

To date, only a few studies have investigated the impact of ID and IDA on the intestinal microbiota, in animal experiments [9, 10], *in vitro* colonic fermentation models [11, 12], Indian women [13], and African infants, and school-aged children [14, 15]. Unlike these, this study included infants and young children living in a European town and aged 6–34 months, thus representing the most vulnerable age group, i.e., when a child exhausts endogenous iron stores and becomes dependent on iron-containing foods [28]. It is well established that this is a crucial time window for the child's long-term health, characterized by a peculiar developmental trajectory of the gut microbiota, which undergoes distinctive compositional shifts, as solid foods are progressively introduced in the diet [29]. Microbiota alterations during this critical developmental window may impair the programing of the infant's physiological systems, with long-term host metabolic and immunological effects [16, 17, 20, 22].
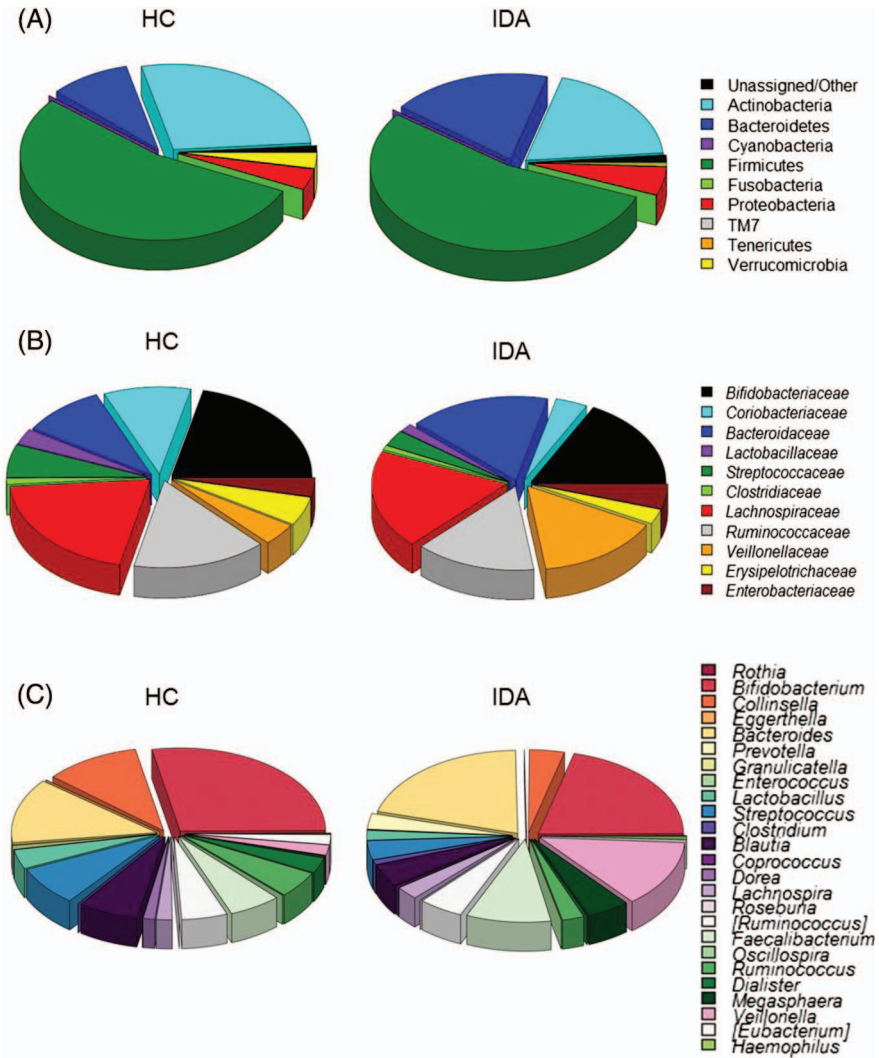
Figure 2. Taxonomic composition of intestinal bacterial communities in iron deficiency anemia (IDA) infants and healthy controls (HCs). Relative abundance of the most abundant phyla (A), families (B), and genera (C) in the gut microbiota of IDA infants and HCs. Only taxa with relative abundance >0.02% in at least 15 samples were included

In line with the literature, the gut microbiota of the infants in this study cohort (all receiving complementary foods but the majority of them still breastfed) mainly consists of Firmicutes (with Ruminococcaceae, Lachnospiraceae, and Streptococcaceae as the most abundant families), *Actinobacteria* (dominated by
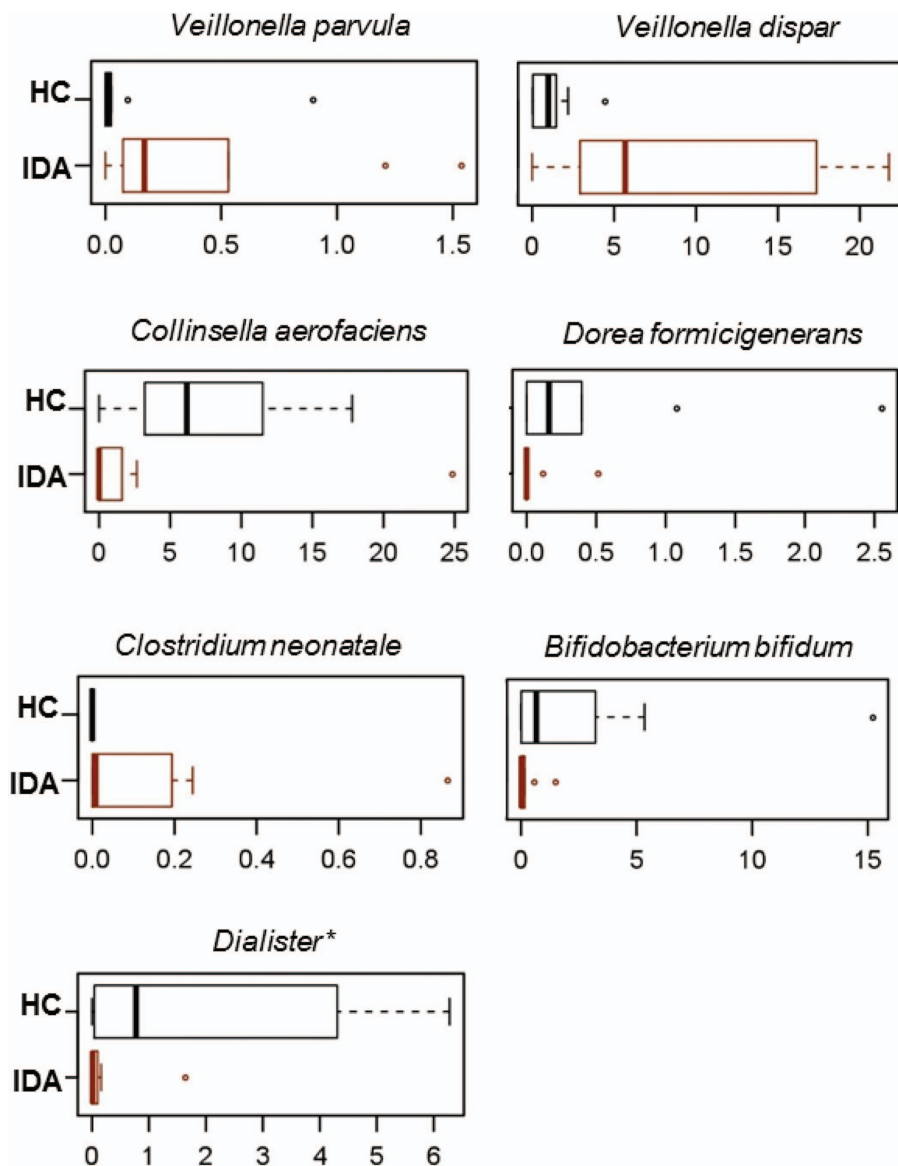
Figure 3. Discriminant species-level taxa between iron deficiency anemia (IDA) infants and healthy controls (HCs). Box plots showing the distribution of relative abundance values of discriminant species between IDA infants and HCs. Only taxa with relative abundance >0.02% in at least 15 samples were included. Wilcoxon rank-sum test, $p < 0.05$

Bifidobacteriaceae), and Bacteroidetes (represented by Bacteroidaceae). Interestingly, distinctive microbial signatures were identified in IDA patients compared to HC, especially an increase in *Enterobacteriaceae* and *Veillonella*, and a reduction in *Collinsella, Dialister, Bifidobacterium*, and *Dorea*. Such compositional traits could be partly accounted for by different iron needs for bacterial growth and metabolism.

Iron-low conditions are recognized to cause major shifts in microbial composition, leading to increased growth of bacteria, which are good iron scavengers (e.g., the health-promoting bifidobacteria and mainly *Enterobacteriaceae*, a family that includes known enteropathogens) or have no need for iron at all (e.g., *Lactobacillaceae*) [30]. Recently, these data have been confirmed in *in vitro* colonic fermentation models inoculated with immobilized fecal microbiota from children aged 2.5 [11] and 6–10 years [12]. Specifically, ID conditions were found to result in decreased relative abundance of short-chain fatty acid producers (*Lachnospiraceae, Ruminococcaceae*, and *Bacteroidaceae*), and increased proportions of *Bifidobacteriaceae, Enterobacteriaceae*, and *Lactobacillaceae* [11, 12].

On the contrary, experiments in animal models showed that the gut microbiota composition depends not only on the dietary iron but also on the host iron homeostasis. In particular, a work with iron-replete rats receiving an iron-deficient diet revealed only minimal changes in the microbiota (i.e., decreased relative abundance of *Bilophila* spp., *Eubacterium hallii*, and *Coprococcus* spp.) [10]. The authors speculated that the fecal iron content was also determined by iron degradation from sloughed enterocytes, thus explaining the stable microbiota composition observed in low-dietary iron conditions. These results are in agreement with findings that genetic modifications of iron metabolism in mice affect the gut microbial community [31]. Researchers concluded that higher iron availability in the intestinal lumen increased the relative abundance of *Lactobacillus murinus* and *Lactobacillus intestinalis.* Nevertheless, they could not exclude positive effects on *Lactobacillaceae* expansion of other metals and minerals (e.g., manganese) that were increased in feces along with iron. In light of this, it is doubtful whether the decreased relative abundance of *Lactobacillus acidophilus* in IDA women in South India is actually related to host ID [13]. Similar results were obtained in African populations with high prevalence of anemia (non-IDA in most cases) and systemic inflammation. In fact, school-aged Ivorian children had higher relative abundance of enterobacteria (including *Shigella* spp., enteroinvasive *E. coli*, and/or *Salmonella* spp.) than bifidobacteria and lactobacilli [14]. Six-month-old Kenyan infants also had high prevalence of enteropathogens, and the anemic group showed decreased abundance of

Actinomycetales and *Streptococcus* [15]. These findings could be associated with poor sanitation and hygiene. Conversely, our study included iron-deficient anemic, but otherwise healthy children from middle-class families; nevertheless, it showed a similar dysbiotic profile, with increased relative abundance of *Enterobacteriaceae* and altered *Bifidobacteriaceae/Enterobacteriaceae* ratio compared to HC. Such alterations (i.e., the expansion of enterobacteria) could reflect an altered intestinal environment created by host inflammatory responses [32]. Indeed, ID negatively affects cell-mediated and humoral immunity in children [33, 34]. An impaired functioning of the host immune system may result in dysbiosis of the gut microbiota, as that observed in our IDA infant cohort, which, in turn, may further impair immunological responses, nurturing the inflammatory process and contributing to disease development [35].

An expansion of *Enterobacteriaceae* is typically found in inflammatory bowel disease (IBD), likely representing a hallmark of inflammation in the global population [36, 37]. Interestingly, IBD patients also usually have increased levels of *Veillonellaceae* [37] and lower relative abundances of *Coriobacteriaceae* [38], as we observed in IDA infants. *Veillonella* spp. are lactate-utilizing bacteria normally present in human microbiomes, especially in those of breast-fed infants [39], which may aid the immune system development in the first few months of life [40]. However, the abundances found in our work (mainly of *V. dispar*, mean relative abundance in IDA is 9.0%) are far greater than those recently reported in a Swedish cohort of about 100 infants (1.5% in 1-year-old vaginally delivered infants) [39], suggesting an unusual succession of gut microbial communities in IDA. Further supporting these observations, other typical signatures of the infant microbiota, such as *Bifidobacterium* (mainly *B. bifidum*) and *Collinsella*, were found to be differentially represented between IDA infants and HC. IDA infants were also highly depleted in the short-chain fatty acid producer *Dorea*, as well as in *Dialister*. The latter was found to be negatively correlated with infant morbidity in the first weeks after birth [41], and completely absent in the infant gut microbiota prior to the onset of type-I diabetes [42]. *C. neonatale*, a recently described lactose-utilizing lactate-producing species, isolated from the microbiota of neonates [43], was only detected in IDA infants.

Although the analysis was conducted at a single time point and on a small sample size, our data highlight a significant dysbalance of the gut microbiota in IDA infants. These findings pave the way for further, possibly longitudinal investigations, aimed at unravelling how the ID status may affect the microbiome developmental trajectory in early life, and the possible long-term consequences on the child's health.

Conflict of Interest

The authors declare no conflict of interest.

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