

OMNIGene™•GUT provides ambient temperature stabilization of pediatric fecal samples for microbiome profiling

Jean Macklaim, Alaya Mikalauskas, Laura Cunningham, Caroline Egloff and Evgueni Doukhanine
DNA Genotek, Ottawa, Ontario, Canada
2022-09-20

OMNIGene™•GUT (OM-200 and OMR-200) is an all-in-one system for easy self-collection and stabilization of microbial DNA from feces for gut microbiome profiling. In this paper, we demonstrate that OMNIGene•GUT is compatible with commonly utilized extraction technologies, collects sufficient input of fecal samples and stabilizes the microbiome profile of pediatric gut microbiomes for up to 60 days at ambient temperature.

Introduction

Pediatric fecal samples are often collected to investigate early microbial colonization of the gut with respect to physiological and external factors that may influence human maturation and disease outcomes throughout life.^{1,2} Accurate representation of pediatric gut microbiome relies on obtaining the in vivo representation of the richness and relative abundance of microbes during sample collection. Effective stabilization of fecal gut microbial profiles by OMNIGene•GUT has been demonstrated in adults³; however, the development of the gut during infancy presents unique challenges, in part due to the difference between adult and pediatric fecal microbiome composition. Children younger than 3 years of age have a significantly lower diversity index compared to adults, with roughly half of the microbial taxa present in the first year of life when compared to later life.^{1,2,4} In addition to differences in microbial diversity (e.g., relative dominance of Actinobacteria and Proteobacteria phyla) and matrix composition (dependent on factors such as diet and microbial activity),^{1,2,4} these characteristics have not previously been addressed with regard to the suitability of OMNIGene•GUT in preserving pediatric gut microbiome.

The ability to generate high-quality and reliable microbiome sequencing data from pediatric gut samples opens up avenues for novel research focusing on the transient changes of gut health during early development. In 2014, Arrieta et al. highlighted the

transient and dynamic nature of pediatric gut microbiome samples, while detecting consistent interplay between taxonomic diversity and interindividual variability (Figure 1).⁵ Accurate measurement of such changes and identification of key drivers require a high degree of confidence in the sample preservation method and compatibility with downstream processing and analysis of the sample. It is therefore crucial to use a method that will capture accurately, on the first attempt, those important exposure time points that will form, over time, an adult-like diverse and complex microbiome.

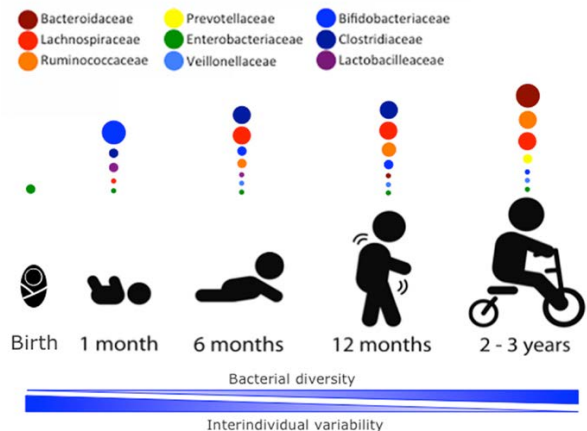


Figure 1: Stages of microbial colonization of the infant and child intestine. Adapted from "The intestinal microbiome in early life: health and disease," by Arrieta M-C, Stiemsma LT, Amenyogbe N, Brown EM and Finlay B, 2014, *Front. Immunol.* 5:427. (doi: 10.3389/fimmu.2014.00427). Copyright © 2014 Arrieta, Stiemsma, Amenyogbe, Brown and Finlay.

Arrieta et al. also observed that children undergo a substantial increase in microbiota diversity during the first few months of life. The results captured in this paper support these conclusions and further expand on the benefit of using OMNIGene•GUT for collecting pediatric fecal microbiome samples. In addition, through related and microbiome relevant measurements, our findings are in agreement with results published by Williams et al., whereby "For samples transported in ambient conditions, the limits of agreement showed

that the OMNIgene•GUT kit had the narrowest 95% limits of agreement with the frozen standard as measured by the number of operational taxonomic units and the Shannon diversity index.⁶ Such standardization of procedures is critical for allowing a wider number of researchers to perform impactful studies and compare results across experiments.

In order to evaluate OMNIgene•GUT performance on infant samples, we collaborated with Dr. Molly Fox at the University of California, Los Angeles (UCLA). We obtained access to a range of pediatric gut microbiome samples from children who ranged in age from newborn to 12 months. This cohort formed the core of the evaluation to determine impact of donor age, taxonomic diversity and sample matrix on OMNIgene•GUT performance. In addition, with the field of microbiome research progressing into metagenomic sequencing for analysis, we opted to include fecal samples collected in OMNIgene•GUT from an internally conducted pediatric study (involving children from 4 months to 45 months old) utilizing metagenomic sequencing (MGS) and taxonomic assignment following collection and storage.

Materials and methods

Sample collection

Fecal samples were collected from diapers into OMNIgene•GUT, according to the manufacturer's instructions. Through our collaboration with Dr. Fox,⁷ we used 48 OMNIgene•GUT samples to evaluate device compatibility for collection and extraction of pediatric fecal samples across a range of age groups (cohort 1). OMNIgene•GUT samples collected for the internal pediatric study were used to evaluate post-collection stabilization performance on a WGS (shotgun sequencing) platform (cohort 2). An aliquot of bulk feces from each donor in cohort 2 was collected in parallel and transported in an insulated box containing frozen ice packs.⁸

DNA extraction and sample storage

Samples in cohort 1 were extracted using a repeated bead beating (RBB) method adapted from Yu and Morrison.⁹ Briefly, the method involved processing fecal samples through one cycle of bead beating in lysis buffer, followed by ammonium acetate protein and inhibitor precipitation, DNA precipitation in isopropanol using Eppendorf® DNA LoBind (MilliporeSigma) microcentrifuge tubes and subsequent DNA purification by QIAamp® spin columns (QIAGEN®).

For baseline analysis of cohort 2 samples, an aliquot of OMNIgene•GUT and an equivalent mass of fecal sample were extracted from fresh samples using the

commonly utilized and efficient lysis method QIAamp® PowerFecal® Pro DNA Kit (QIAGEN) according to manufacturer protocol.¹⁰ The remaining OMNIgene•GUT samples were kept in the collection tubes for storage at ambient temperature ($23^{\circ} \pm 3^{\circ}\text{C}$) for 30 and 60 days.

DNA analysis

DNA concentration and yield were determined using the Quant-iT™ PicoGreen® reagent (Thermo Fisher Scientific). DNA integrity and stability over time was evaluated on a subset of samples by running approximately 75 ng of purified DNA on a 0.8% agarose gel and staining with ethidium bromide. UltraRanger 1 kb DNA ladder (Norgen Biotek Corp.) was used to determine the size of purified DNA.

Library preparation and metagenomic sequencing (MGS)

Libraries were prepared from extracted genomic DNA from cohort 2 samples using the Nextera® XT Library Preparation Kit (Illumina®) paired with Nextera XT Index Kit v2 (Illumina), according to the manufacturer's protocol for tagmentation, amplification and clean-up steps. Libraries were normalized using DNA concentration determined by Quant-iT™ PicoGreen™ fluorescence (Thermo Fisher Scientific) and average fragment size determined using the D5000 ScreenTape Assay (Agilent Technologies). The library pool was loaded onto the Illumina NextSeq™ 550 sequencing platform using the 300 cycles NextSeq 500/550 High Output Kit v2.5 (Illumina).

Bioinformatics data analysis

Demultiplexed FASTQ files were trimmed and quality filtered, and reads mapping to the human genome or 16S rRNA gene were removed. The resultant trimmed and filtered reads were submitted to the One Codex platform for taxonomic classification (database v. 2018). The mapped reads counts table was filtered to keep reads assigned to taxonomic bins occurring in at least 2 samples and having at least 10 mapped reads.

An appropriate percent abundance filter was applied for each analysis. For compositional data analysis, counts data were transformed using the centred log-ratio to generate relative abundance values. Differences in the microbiome between test conditions were evaluated using the Aitchison distance,^{11,12} and differential abundance analysis was performed on the species-level taxonomy using ALDEx2.^{13,14} All data analysis, visual representations and statistical analyses were performed using R¹⁵ and various package add-ons.

Results

OMNIgene•GUT is compatible with extraction techniques commonly used in the pediatric fecal microbiome field

When it comes to microbial community profiling from complex environments like fecal samples, there are several sources of technical variation. The choice of extraction methodology for obtaining sufficient and high-quality microbial nucleic acids is essential for meaningful and reliable insights into these complex communities. The potential for low microbial biomass within pediatric samples can further affect DNA recovery, ultimately limiting the use of downstream analytical applications for characterizing microbial profiles. Some sequencing applications require a substantial amount of DNA (> 500 ng), necessitating an extraction procedure that can maximize DNA recovery. To this end, we utilized the QIAamp PowerFecal Pro DNA Kit and RBB extraction method to verify that sufficient DNA yields can be obtained when processing fecal samples across a wide range of pediatric age groups. An expected increase in yield across the age range of the cohort was observed for OMNIgene•GUT collected samples extracted with RBB (Figure 2).

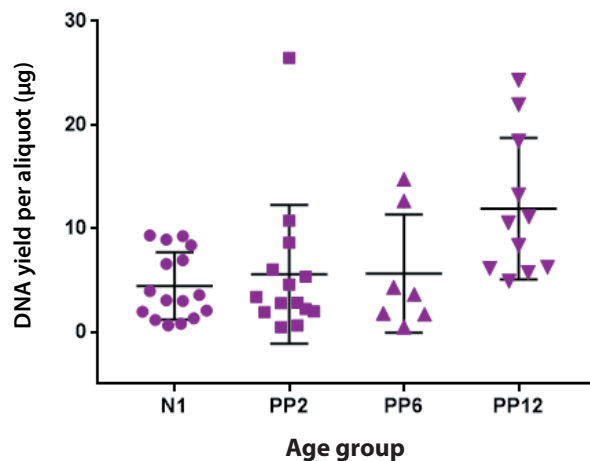


Figure 2: DNA yield for RBB extracted OMNIgene•GUT collected pediatric samples (N = 48) from 4 age groups: newborn to 3 weeks (N1), 2 months (PP2), 6 months (PP6) and 12 months (PP12). For each donor, samples were extracted at baseline (T0) after collection in an OMNIgene•GUT device. Error bars represent the mean value \pm standard deviation.

In addition to the importance of obtaining sufficient DNA yields across post-collection sample storage conditions, it is crucial to preserve the in vivo DNA

integrity in the collected samples. Preservation of high molecular weight DNA within the collection device supports optimal performance of long-range sequencing platforms, such as single-molecule real-time sequencing (PacBio® SMRT® Sequencing) and nanopore sequencing (Oxford Nanopore Technologies). DNA degradation caused by nuclease activity selectively decreases signals from lysed microbial cells, leading to a bias in the recovered microbiome profile. In DNA extracted from OMNIgene•GUT samples, high molecular weight DNA bands (> 10 kb on average) were identified across 3 donor age groups (Figure 3).

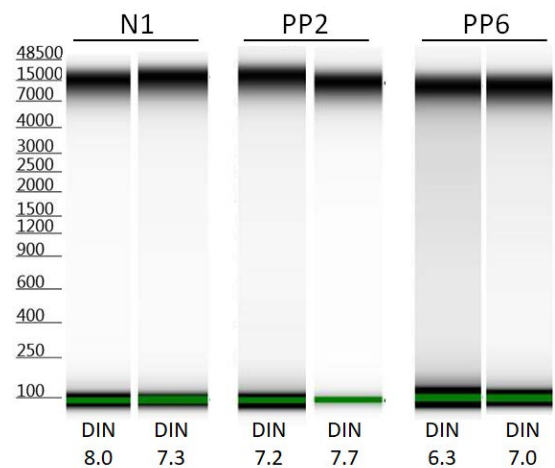


Figure 3: DNA integrity of OMNIgene•GUT collected pediatric samples. OMNIgene•GUT samples extracted by RBB at baseline from representative pediatric donors belonging to 3 age groups (newborn to 3 weeks (N1), 2 months (PP2), 6 months (PP6)) were run on TapeStation (Agilent).

OMNIgene•GUT maintains profile neutrality in pediatric microbiota at the point of collection

The pediatric gut is an environment of dynamic change, characterized by a general increase in microbial diversity and varying fecal composition throughout the child's development, resulting in a unique and challenging sample type. Obtaining the best in vivo representation of the gut microbiome at the exact moment of sample collection is the goal of current stabilization technologies. Preserving fecal microbiota at -80°C remains the gold standard; however, transportation or immediate access to freezers is required if collected in a clinical setting. Chemical stabilization buffers can offer independence from these expensive cold-chain preservation techniques. Ideally, these buffers should keep the collected material neutral by not promoting selective microbial growth or accelerating DNA degradation.

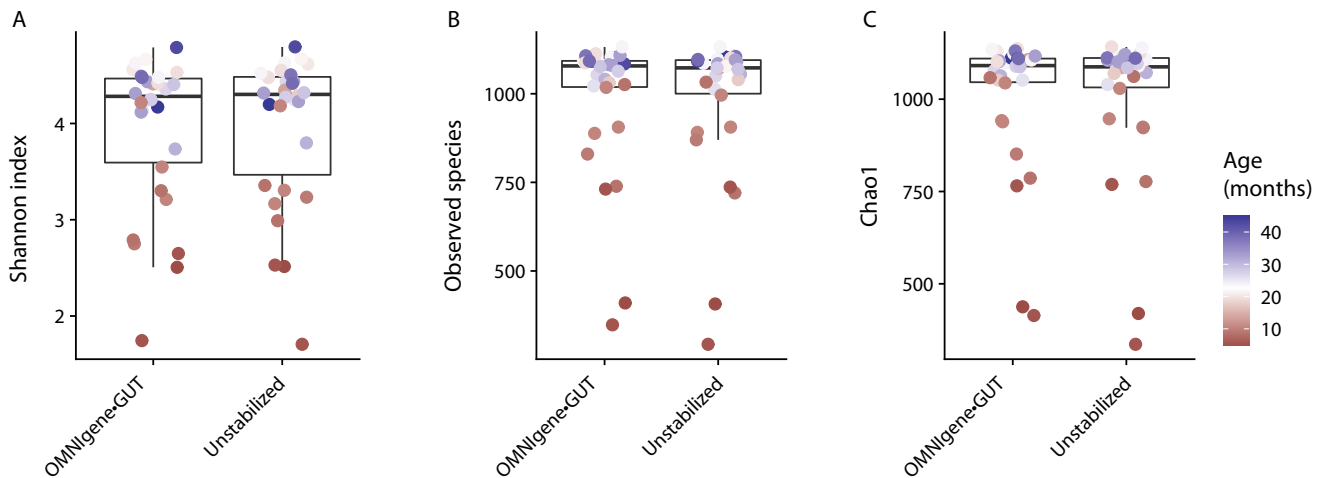


Figure 4: Neutrality assessment of OMNIgene•GUT stabilization chemistry by alpha diversity metrics. (A) Shannon index (B) Observed species and (C) Chao1 metrics were used to compare donor-paired OMNIgene•GUT and fresh unstabilized sample (N = 30, cohort 2). Each dot represents a donor-paired fresh unstabilized and OMNIgene•GUT sample where the color corresponds to age in months. No significant differences in alpha diversity were detected between the fresh unstabilized samples and OMNIgene•GUT samples (Wilcoxon signed-rank test).

To assess the neutrality of OMNIgene•GUT with this distinctive sample type, we collected fecal samples from donors ranging in age from 4 months to 45 months.

To evaluate the preservation of pediatric microbial profiles in OMNIgene•GUT samples, alpha diversity metrics (Shannon index, number of observed species and Chao1) were calculated at the species taxonomic level to assess species richness and evenness. OMNIgene•GUT samples and corresponding fresh samples were extracted from 30 donors in cohort 2. A Wilcoxon signed-rank test of values from each alpha diversity metric showed no significant difference between donor-paired OMNIgene•GUT samples and fresh samples, indicating that the richness and the microbial composition of the samples were not affected by the chemical stabilization of the OMNIgene•GUT (Figure 4).

Additional analysis at the beta diversity level (through the calculation of Aitchison distance) was performed on donor-paired samples collected in OMNIgene•GUT and fresh unstabilized samples (Figure 5). The distance between paired samples was far lower than the donor-to-donor distances, demonstrating there is no change to the sample profile at collection (sample neutrality). Taken together with alpha diversity results, the stabilization solution and the built-in features of the OMNIgene•GUT offer confidence in recovering an accurate representation of the microbial community.

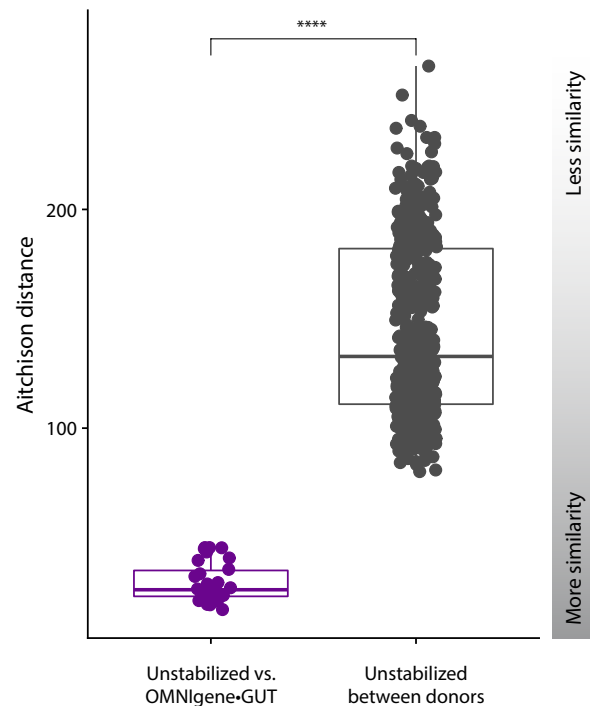


Figure 5: Beta diversity assessment of pediatric samples. Aitchison distance (beta diversity) for donor-paired samples collected in OMNIgene•GUT compared to fresh unstabilized samples at baseline (N = 30, cohort 2) shows significantly lower difference (Wilcoxon rank-sum test) compared to donor-to-donor differences between fresh unstabilized samples at baseline (****; $P \leq 0.0001$). These significantly low differences demonstrate that no change is occurring to the sample profile after collection in OMNIgene•GUT.

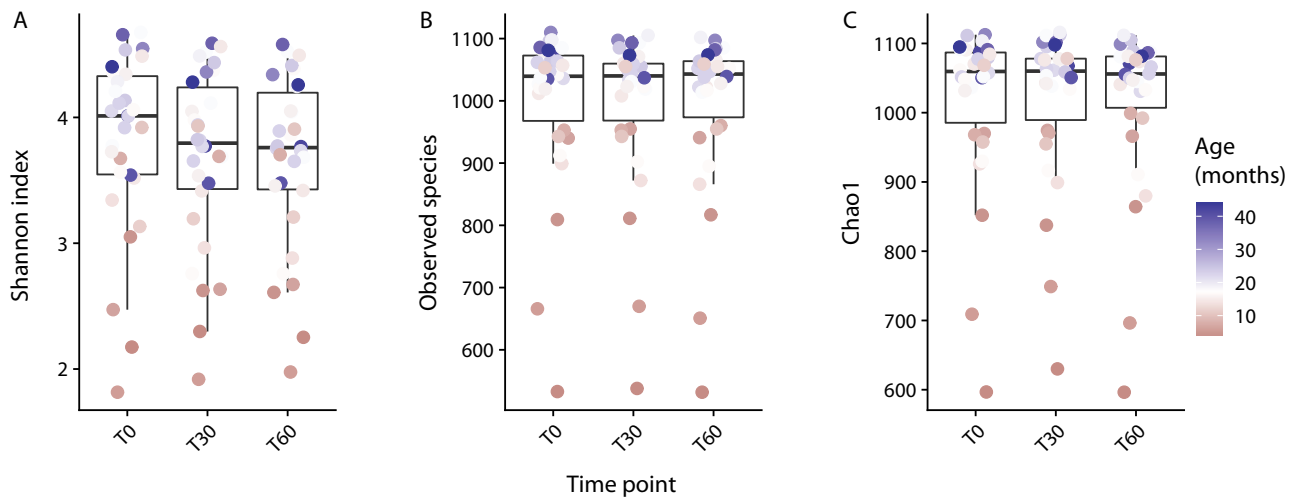


Figure 6: Alpha diversity metrics over 30 and 60 days compared to baseline. (A) Shannon index (B) Observed species and (C) Chao1 metrics were used to compare pediatric samples ($N = 30$, cohort 2) collected in OMNIgene•GUT at baseline (T0), 30 days (T30) and 60 days (T60) after collection. Each dot represents a donor sample collected in OMNIgene•GUT with color corresponding to age in months. No significant differences in alpha diversity were detected between time points (Kruskal-Wallis).

OMNIgene•GUT stabilizes pediatric microbiome profiles beyond the point of collection

To access the stability of samples collected in OMNIgene•GUT, we evaluated the changes in the microbiome at 30 and 60 days using alpha (Figure 6) and beta (Figure 7) analyses. Statistical measurement through Kruskal-Wallis did not show any significant difference in 30- and 60-day stored samples compared to baseline, suggesting a lack of time-dependent shifts during storage. In contrast, distances between pediatric donors showed a large magnitude of microbiome compositional differences between donors within the same cohort. This result is indicative of how effectively the OMNIgene•GUT device's stabilization chemistry prevents microbial profile changes during storage conditions, including a 60-day hold at ambient temperature.

Conversely, a highly significant difference (Figure 7) was observed when comparing beta diversity (Aitchison distance) between donors, highlighting the ability of OMNIgene•GUT devices to conserve the unique donor microbial profile even within samples held 60 days post-collection at ambient temperature.

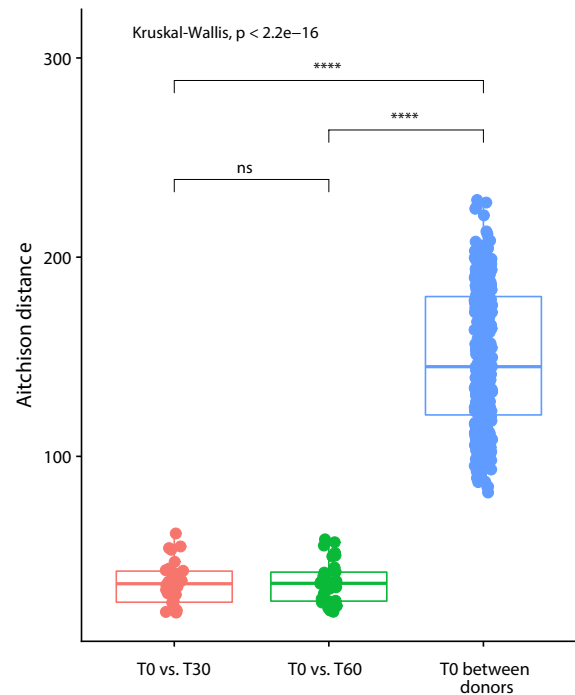


Figure 7: Beta diversity assessment of pediatric samples over time at ambient temperature. Each point is a donor sample at baseline (T0) compared to the same sample after storage at ambient temperature for 30 days (T30) or 60 days (T60) in OMNIgene•GUT. For statistical comparison, the final box shows the between-donor distance of baseline (T0) samples across all donor-to-donor permutations. The magnitude of change (distance) and the variability for each time-point group is far lower than the between-donor change, suggesting the microbiome profiles are stable over time in the OMNIgene•GUT device. Kruskal-Wallis non-parametric test was applied between groups ('****': $P \leq 0.0001$) and a Wilcoxon rank-sum test was applied for the two-group comparison ('ns': $P > 0.05$).

In addition to alpha diversity and microbiome composition distance measurements, differential abundance analysis was applied to compare OMNIgene•GUT collected samples at baseline to 30- and 60-day extractions to determine if there were any taxonomic species that consistently and significantly changed across the cohort. Our previous work highlighted rapid and progressive onset of microbiome profile change when gut samples are collected without a -80°C cold-chain transport or chemical stabilizer, even at time holds of 24 hours with ice packs.¹⁶ Owing to the effectiveness of the stabilization chemistry, we did not find any significantly different species relative abundances for any of the time points (Figure 8). These results confirm that the OMNIgene•GUT device can effectively stabilize pediatric gut microbiome samples post-collection without introducing any storage-induced bias in taxonomic recovery.

As a final assessment of performance, a taxonomic stacked bar plot at species-level resolution was constructed for a subset of cohort 2 OMNIgene•GUT stabilized samples following metagenomic sequencing results (Figure 9). The subset of donor-paired samples for representation was selected based on donor age range and represented from youngest to oldest within the study cohort. With our current scientific understanding of pediatric gut microbiome development, as depicted in Figure 1 from Arrieta et al., there is an expectation of increase in diversity and complexity of taxonomic presence as infants develop. The taxonomic profiles from pediatric donors from 3 age groups (Figure 9) demonstrate some of these expected changes in microbiome composition as environmental factors and age influence the microbiome. One of the first colonizers of the pediatric gut, *Bifidobacteriaceae*, becomes less abundant over time, and while microbiome diversity increases (more rare taxa represented in “Other”), interindividual variability decreases.

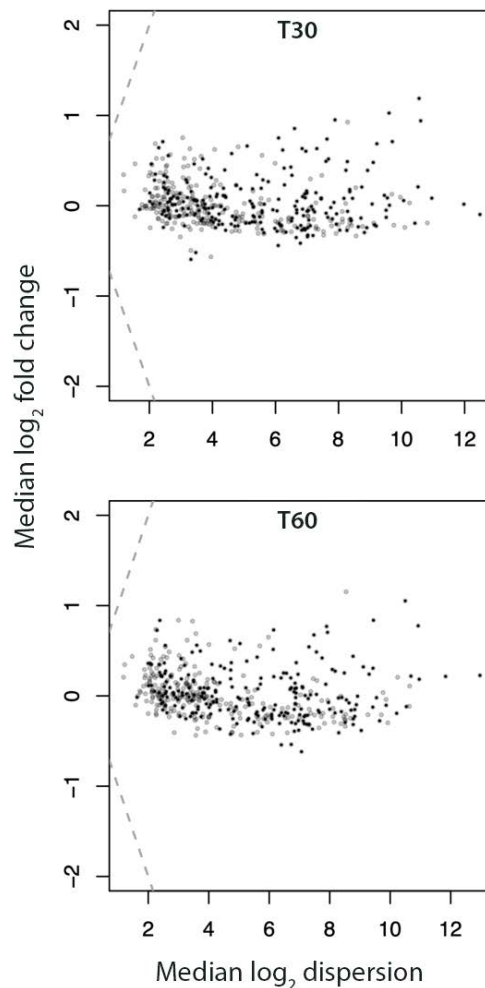


Figure 8: Effect plots for differential abundance analysis of pediatric baseline samples collected in OMNIgene•GUT versus the same samples stored at ambient temperature in OMNIgene•GUT for 30 or 60 days. Each dot is a taxonomic species, with the y-axis showing fold change (\log_2) between time points scaled by the within group variance (x-axis). An effect size > 1 , represented by the dashed lines, would indicate a significant change in relative abundance due to the fold change between samples exceeding the within group variance. No significant changes in species relative abundance were detected.

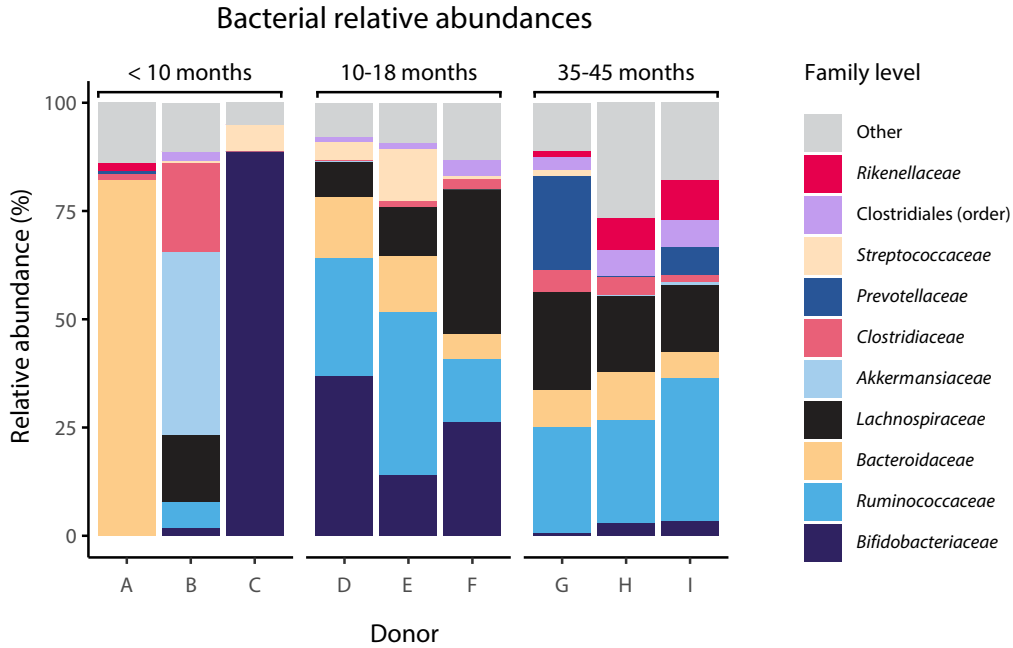


Figure 9: Relative abundance stacked bar plot showing the taxonomic profile for representative donors from cohort 2, grouped into 3 age ranges. The top 10 family-level taxonomies (by average relative abundance) are shown, with the remaining reads grouped as "Other."

Conclusions

Consideration of sample collection and stabilization chemistry methods is critical for obtaining accurate representation of the pediatric gut microbiome during various stages of life. As shown in Figure 9, distinct differences between pediatric gut samples from different age groups can be recovered when collected using OMNIgene•GUT. In-depth assessment of the collection device is required to confirm effectiveness and compatibility with:

- Extraction methods of processing a wide range of microbial bioload to recover sufficient nucleic acid for sequencing applications.
- A wide range of Bristol stool scale sample types.
- Matrix uniqueness across donors, particularly in early development stages.
- A wide range of microbial cell types.

We have demonstrated that OMNIgene•GUT stabilizes the gut microbiome of pediatric samples, collected from infants ranging in age from newborn to 45 months, without compromising sample integrity or microbial richness. Showcasing the utility of OMNIgene•GUT for assessing and quantifying complex microbial structures within pediatric studies, and powering scalable studies through an accessible at-home collection device.

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