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The need for stabilization: Short-term variation in microbiome profiles

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Introduction

Accurate analysis of the gut microbiome requires a complete snapshot of the microbial community contained in a fecal sample at the time of collection. A number of methods have been used in published studies for sample handling, including the use of ice packs or dry ice for temperature-controlled transportation or simply leaving samples at room temperature.[1,2] However, each of these methods has significant drawbacks. Room temperature transportation provides no stabilization of the sample, while ice packs maintain a cool temperature for only a short period of time; any delays in transport will result in temperatures incompatible with microbiome profile stabilization.[1] Transport on dry ice can maintain a suitable stabilization temperature for longer; however, the handling of dry ice poses a safety risk and logistical challenge to study participants. In addition, the sample requires transport by hazardous materials couriers, which substantially increases handling complexity and shipping costs.

DNA Genotek's OMNIgene®•GUT is an all-in-one system that enables study participants to volumetrically collect fecal samples in the comfort of their own homes.[3] The collection system is easy to use for untrained participants and has built-in sample homogenization and stabilization of microbial DNA from fecal samples, making it ideal for microbiome profiling by downstream metagenomic sequencing.

In this study we demonstrate the importance of sample stabilization by evaluating the change in microbiome profile after simulated short-term transport under a variety of common conditions. We show that changes in a fecal microbiome profile occur rapidly and progressively at room temperature (within 24 hours) and that an insulated box with ice packs only marginally mitigates the effects, with significant changes at 48 hours after collection. In contrast, OMNIgene•GUT shows a stable profile within a 7-day window from time of collection and has demonstrated stabilization of the microbiome profile in human stool for 60 days at ambient temperature.[4]

Materials and methods

Sample collection, storage and DNA extraction

Nineteen (19) healthy adult study participants were asked to collect a bulk fecal sample into a plastic specimen container and transport it to the lab in an insulated box (sample placed between 2 frozen ice packs) within 3 hours of collection.

Upon receipt in the lab, approximately 2 g of bulk fecal sample was immediately sub-sampled into four 5 mL tubes. From one of these tubes, approximately 500 mg of fecal sample was collected into an OMNIgene•GUT device, according to the manufacturer's instructions.

In order to evaluate the microbial profile as close to the time of collection as possible (T0 samples), DNA was immediately extracted from a 50 mg portion of fecal sample from each of the four tubes and from 250 µL of the OMNIgene•GUT fecal sample using the QIAamp® PowerFecal® Pro DNA Kit (QIAGEN®).

To simulate the various conditions that a sample may encounter during transport or prior to processing in the lab, three of the 5 mL tubes were placed into different storage conditions. One tube was left at room temperature (20°C to 23°C), a second was placed in a -80°C freezer, and the third was placed into an insulated box with 2 frozen ice packs and a calibrated Temp101A ambient temperature data logger (MadgeTech). The samples collected using OMNIgene•GUT were stored at room temperature.

At subsequent designated time points (T24h, T48h, T72h and T7d, described in Figure 1), DNA was extracted from 50 mg of the stored raw samples and 250 µL of the OMNIgene•GUT samples. The -80°C samples and the OMNIgene•GUT samples were extracted in triplicate only at the 7-day time point. After removing the portion of sample to be extracted at each time point, the tubes were returned to their storage locations.

All extracted DNA was quantified using a Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo Fisher Scientific).

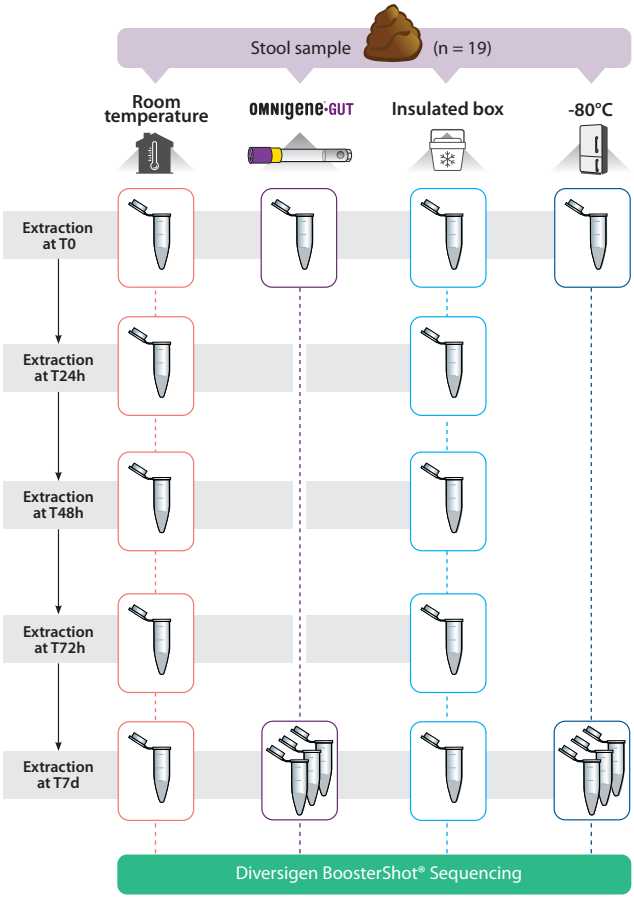

Figure 1. *Schematic illustrating storage conditions and sampling time points. Each microcentrifuge tube represents an aliquot of sample extracted.*

Shotgun metagenomic sequencing and data analysis

All extracted samples were sequenced using BoosterShot® methodology (Diversigen®). Briefly, libraries were prepared using a modified procedure adapted from the Nextera® XT DNA Library Preparation Kit (Illumina®). Libraries were sequenced on a NovaSeq™ 6000 (Illumina®). Sequenced read QC, read filtering and taxonomic assignment were obtained using Diversigen's Core Analysis™ pipeline. For analysis, read count data were transformed to centred log-ratios (CLR), and changes in microbiome composition and relative abundance were evaluated using the Aitchison distance metric for whole microbiome comparisons, and by differential abundance analysis using ALDEx2 at aggregated levels of taxonomy (order, family, genus). Taxa were considered significantly different with an FDR-corrected p-value < 0.05 (Welch's t-test) and an effect size > 1. Changes in whole microbiome composition by Aitchison distance were statistically evaluated using a Kruskal-Wallis non-parametric test between time points and collection conditions. In each comparison, sample replicates at T0 baseline served as the comparative group for statistical evaluation (e.g., baseline versus OMNIgene•GUT at 24 hours).

Over time after sample collection, increasing changes were observed for samples held in an insulated box with ice packs and samples held unstabilized at room temperature (Figure 2A and 2B). These changes were detectable as early as 24 hours after collection (unstabilized room temperature), and by 48 hours in an insulated box with ice packs, corresponding to an increase in temperature over the time period (Figure 2C). The amount of change (measured by Aitchison distance) increased over each 24-hour window of measurement with significant differences in microbiome composition at ≥ 24 hours at room temperature, and at ≥ 48 hours for samples in an insulated box with ice packs. By day 7, there was a significant change in overall microbiome composition for these samples, while samples at the same time point held at -80°C or at room temperature in OMNIgene•GUT had no significant change from T0 baseline (Figure 2D). In addition, samples stored for 7 days in OMNIgene•GUT had a lower variability compared to samples unstabilized at room temperature or in an insulated box with ice packs (Median distance +/- IQR: 65.52 +/- 5.62 in OMNIgene•GUT versus 87.78 +/- 21.11 at unstabilized room temperature, 90.43 +/- 26.35 in an insulated box with ice packs).

The most striking changes in overall microbiome composition were noted at day 7 (Figure 2D), and so taxonomic changes at this time point were evaluated by differential abundance analysis compared to T0 baseline (Table 1). The largest changes were apparent at the order level, with *Lactobacillaceae* (not shown) and *Enterococcaceae* (Figure 3) showing the largest differences in relative abundance (twofold to ninefold change at room temperature and in the insulated box, respectively). In contrast, no significant changes were detected for samples held at -80°C nor in OMNIgene•GUT after 7 days of storage. In addition to the changes noted in Figure 3 occurring across the majority of the participants, there were individual-specific changes in microbiome profile for several study participants, driven by their specific microbial composition. The relative abundance profile of a representative participant is shown in Figure 4, where there was an increase in relative abundance of *Enterobacteriaceae* after the sample had been held 7 days in an insulated box with ice packs, whereas after 7 days of room temperature storage in OMNIgene•GUT, the sample composition and taxonomic relative abundances were preserved when compared to the T0 starting profile for the same participant sample.

Results

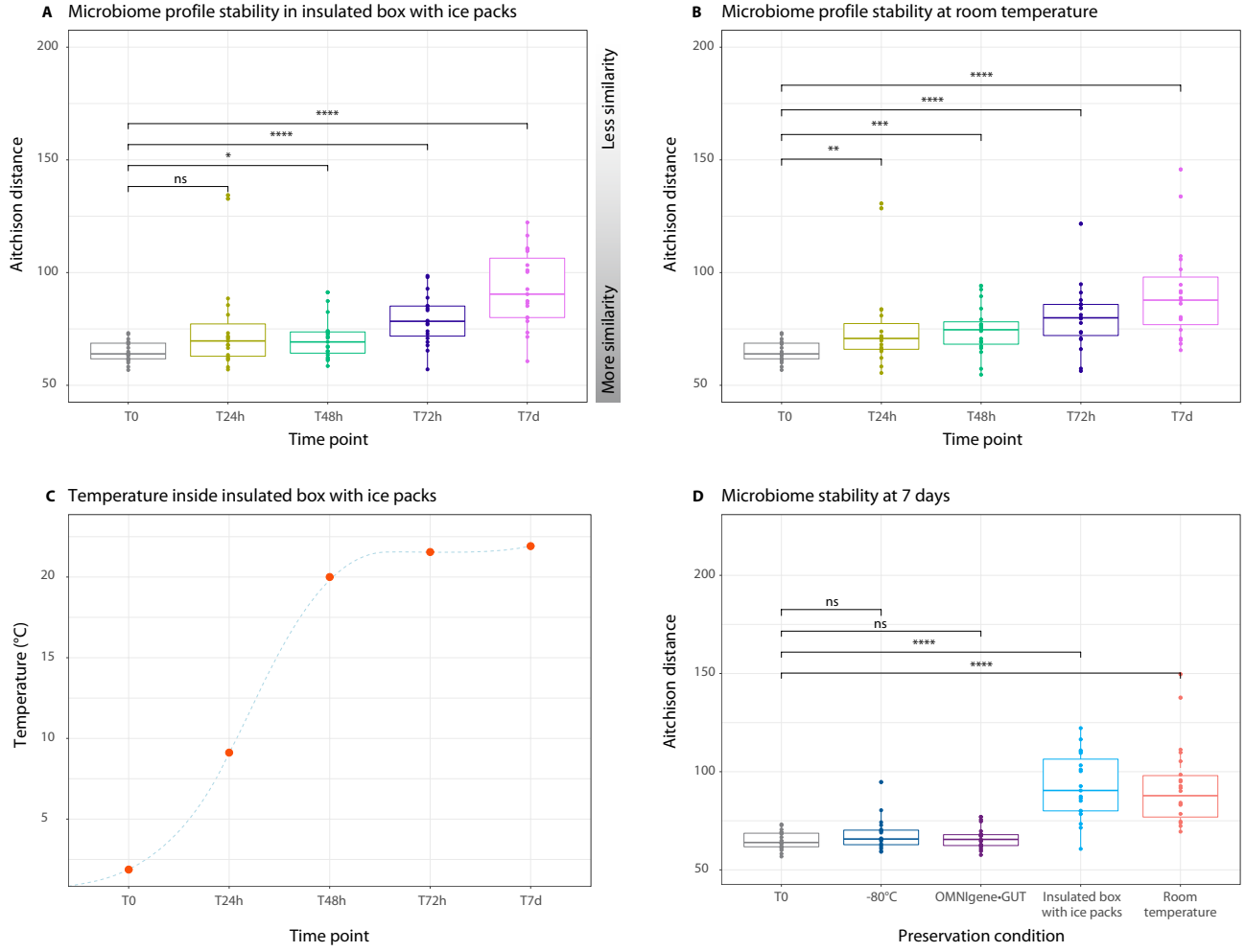

Figure 2. Change in total microbiome composition over time.
Differences between samples are plotted as Aitchison distance (panels A, B and D), with each point being a sample pair comparison (N = 19 participants) (A: changes over 7 days in an insulated box with ice packs, B: changes over 7 days at room temperature, C: measured temperature of the sample in an insulated box with ice packs corresponding to panel A, and D: changes at the 7-day endpoint for all conditions). The Aitchison distance measures a change in microbiome profile (composition and relative abundance) with a lower distance representing more similarity, and a higher distance being less similar. The distance between T0 baseline replicates (first box plot in panels A, B and D) serves as the baseline variation for every subsequent statistical comparison of condition and time point. In each box plot after the baseline, a point is a participant sample compared to its T0 baseline. Significant differences between groups were measured with a Wilcoxon rank-sum test ('ns': P > 0.05, '': P ≤ 0.05, '**': P ≤ 0.01, '***': P ≤ 0.001, '****': P ≤ 0.0001).*

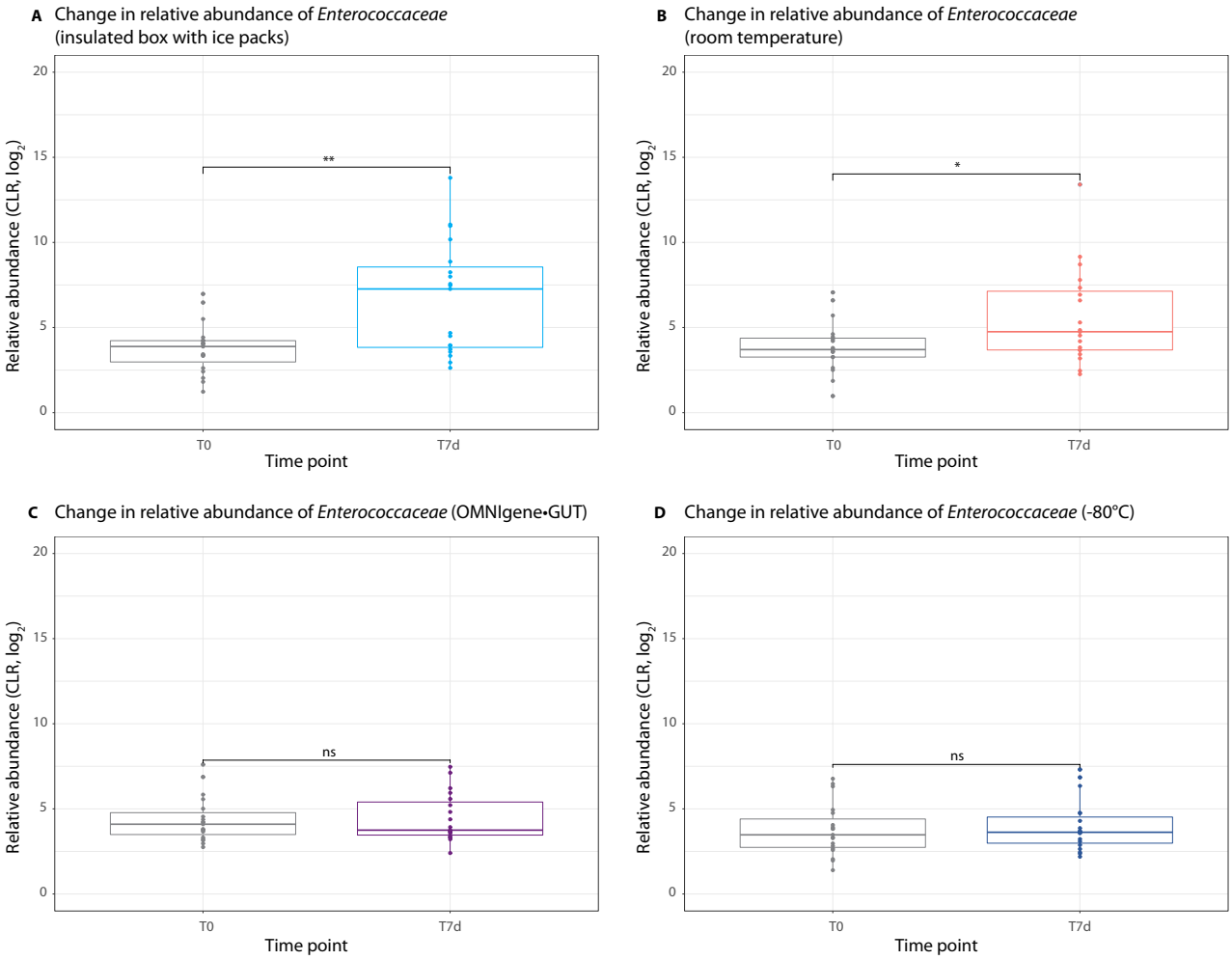

Figure 3. Changes in Enterococcaceae relative abundance affected by storage conditions.
Relative abundance (CLR transformed, log_2 scale) of Enterococcaceae plotted for samples at T0 baseline and the same samples at 7 days for the respective conditions (A: insulated box with ice packs, B: unstabilized room temperature, C: OMNIgene•GUT, D: -80°C). Differential abundance analysis showed a large change in relative abundance for unstabilized room temperature (twofold) and samples held in an insulated box for 7 days (ninefold) (A and B above). Significant differences by Wilcoxon rank-sum test are indicated ('ns': P > 0.05, '': P ≤ 0.05, '**': P ≤ 0.01).*

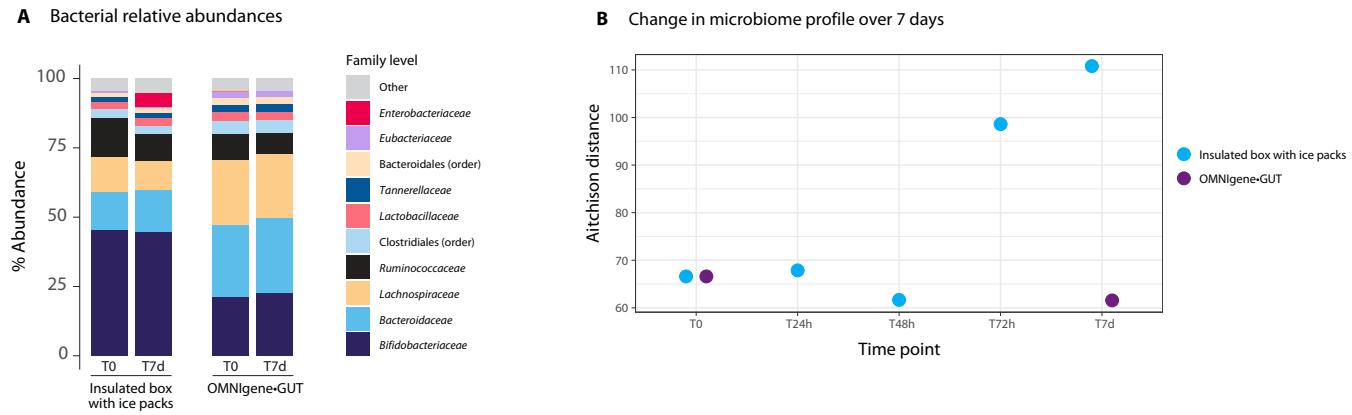

Figure 4. Representative participant: changes over 7 days in an insulated box with ice packs. *A: Relative abundance stacked bar plot showing the taxonomic profile for top 10 family-level taxa over 7 days in an insulated box with ice packs compared to OMNIgene•GUT, and B: the same samples showing change in microbial profile measured in Aitchison distance over time held in an insulated box with ice packs compared to OMNIgene•GUT samples held at room temperature.*

Table 1. Significantly changing taxa for samples stored unstabilized in an insulated box with ice packs or unstabilized at room temperature. *List of taxonomic features at the order, family and genus level that were significantly different at 7 days compared to T0 baseline (Welch's t-test, p-value < 0.05) between samples stored in an insulated box and at room temperature. There were no taxa found to be significantly different in either the -80°C or OMNIgene•GUT stabilized samples.*

	Insulated box with ice packs	Room temperature
Order	Lactobacillales	Lactobacillales
	Erysipelotrichales	Burkholderiales
	Burkholderiales	
	Enterobacterales	
Family	*Bacteroidaceae*	*Coriobacteriaceae*
	Barnesiellaceae	*Enterococcaceae*
	Odoribacteraceae	*Lactobacillaceae*
	Tannerellaceae	*Oscillospiraceae*
	Enterococcaceae	*Ruminococcaceae*
	Lactobacillaceae	*Sutterellaceae*
	Streptococcaceae	
	Oscillospiraceae	
	Ruminococcaceae	
	Erysipelotrichaceae	
	Sutterellaceae	
Genus	*Bacteroides*	*Bacteroides*
	Barnesiella	*Parabacteroides*

Conclusions

- An insulated foam shipping container with ice packs (cold-chain method) is a common form of sample transport for fecal samples and has been used in protocols such as the Human Microbiome Project. Results from this study using the cold-chain method show a similar amount of change as those samples held unstabilized at room temperature (the forced-failure condition).
- Changes to the fecal microbiome occur rapidly (within 24 hours) and progressively in the 1-6 days following, resulting in negative impacts on downstream discovery and the power to detect biological effects in the microbiome.
- Overall, these results suggest that an insulated shipping box with ice packs is not an effective method for stabilization of the taxonomic profile and that stabilization is essential if you are unable to process fecal microbiome samples within 24 hours.
- In contrast, samples stored at -80°C and those stored at room temperature in OMNIgene•GUT show no significant change in taxonomic profile over the 7-day period. However, when compared with dry ice, OMNIgene•GUT does not incur the expense and logistical challenges associated with shipping a hazardous material.
- In summary, at-home sample collection using OMNIgene•GUT removes many of the logistical challenges related to cold-chain transport, improves user experience and increases compliance. Additionally, the unique design of the OMNIgene•GUT collection kit ensures that the sample is collected volumetrically, properly homogenized and stabilized at the time of collection, resulting in a more consistent, reliable and reproducible microbiome profile.[5]

References

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