A validated study protocol to compare microbiome and mycobiome profiles of Inflammatory Bowel Disease patients in remission and active flare

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Abstract

Several large cohort studies of the gut bacterial composition of patients with Inflammatory Bowel Disease (IBD) have been published in recent years. While these studies have provided intriguing insights into the disease and promising clues for treatment options, they are often challenged by low enrollment and compliance rates. Low rates are largely influenced by donor perception of self-collection of the severe diarrhea samples common for IBD patients in flare. In collaboration with Crohn's and Colitis Canada we launched a research study with two aims: to adapt and improve current practices for stool collection, preservation, processing and analysis and to use these optimized methods to compare bacterial and fungal profile differences in IBD patients in remission to those in active flare.

To optimize methods for self-collection of IBD stool samples we adapted OMNIgene®•GUT (OMR-200, DNA Genotek), a validated gut microbiome self-collection kit that provides ambient temperature stabilization, to donors with severe diarrhea by pairing OMR-200 with a sampling spoon (OM-AC2, DNA Genotek). To optimize sample processing, we compared common extraction methodologies, both literature based "home-brew" methods and commercially available extraction kits. 16S and ITS amplicon sequencing was performed on Illumina's MiSeq platform to interrogate diversity and relative abundance differences for bacterial and fungal taxons. We evaluated donor compliance, ease of use, accuracy of the recovered microbial profile, and sample preservation over time, in addition to investigating microbial and fungal profile differences in a cohort of IBD patients who were either in remission or experiencing flare.

Donors with severe diarrhea reported that OMR-200 when combined with OM-AC2, provided an intuitive, easy to use method for sample self-collection, with a sample return rate of 92%, 96% of donors reporting the method as easy to very easy and a 100% sample utilization rate. Comparison of extraction methodologies found significant differences in discovery of diversity, particularly in the Blautia and Granulicatella genera, and total nucleic acid yields. Our preliminary profile analysis suggests trends in diversity and abundance of the bacterial and fungal microbiome, between IBD patients in remission or experiencing flare. Future work will expand on these associations between disease state and taxonomic communities in IBD patients.

We have established a study protocol for effective collection, storage and processing of stool from IBD patients. As researchers rapidly move towards gut microbiome collections from larger cohorts and unique sample types, an effective and validated means of collection and processing becomes an essential aspect to maximize value of the generated data and donor compliance.

Materials and methods

Sample collection, stabilization, DNA extraction and sample storage for fecal studies

OMNIgene•GUT kits were used by naïve donors recruited through Crohn's and Colitis Canada to self-collect fecal samples. Performance of naïve collections were determined through survey data and mass of sample collected. In addition, bulk samples from donors were collected and stored at 4°C overnight and then distributed among three OMNIgene•GUT kits, and two 5 mL tubes without stabilizer solution. Baseline extractions and post-storage extractions were performed using an adapted Repeat Bead Beating protocol (Yu and Morrison, 2004)[1]. For all extractions, a 0.35 mL aliquot of stabilized sample or 60 mg of unstabilized/fresh sample was processed. DNA concentration was determined using the Quant-iT™ PicoGreen™ dsDNA Reagent (Invitrogen).

Sequencing, bioinformatics and biostatistics

Library preparation, sequencing and bioinformatics were conducted using 16S V3-V4 hypervariable regions (bacterial) and ITS 1/2 (fungal) paired-end amplicon sequencing, with PE-300 V3 kit on an Illumina MiSeq platform. Paired-end sequencing reads were merged, screened for length, and filtered for quality using proprietary DNA Genotek scripts. Filtered sequences were aligned to the GreenGenes (16S) and UNITE (ITS 1/2) reference databases at 97% identity using NINJA-OPS. Samples were rarefied between 20,000 and 25,000 reads for 16S and between 5,000 and 50,000 for ITS 1/2 libraries. Operational Taxonomic Units (OTUs) that were not present with more than 10 counts/per sample in any sample were removed, and all remaining OTUs were collapsed at the species-level (L7) when possible, otherwise were assigned highest available taxonomic resolution. Shannon alpha diversity and Bray-Curtis dissimilarity, were calculated using QIIME on the collapsed OTU tables.

Cohort recruitment

Cohort source	Donors recruited through Crohn's and Colitis Canada Self reported chronic IBD symptoms
Cohort characteristics	23 subjects recruited Metadata, including Bristol Type, identified differences compared to healthy North American human cohorts **(Figure 1)**
Remission and flare sub-cohorts	Samples from recruited donors were split into two sub-cohorts based on their Bristol type (1-5, remission and 6-7, flare)

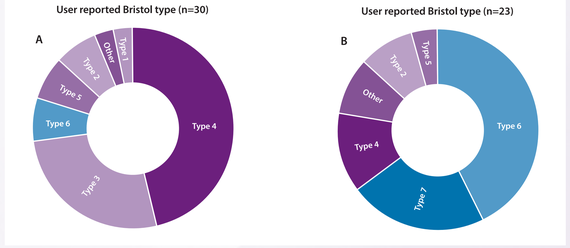

***Figure 1 (A)**: Distribution of Bristol stool type in previously established healthy NA human cohort. **(B)** Distribution of donor-reported Bristol stool type within this study. 65% of donors identified as Bristol type 6 or 7. 'Other' encompasses donors that identified more than a single type on the survey.*

Sample processing methodology

Methodology	Means of evaluation
Stool sampling by donor	Evaluation of physical designs for mass of stool collected and consistency across multiple donors, in addition to ease of use feedback by naïve donors **(Figure 2)**
Extraction methodology	Evaluation of commercial and academic extraction methods, measurement of yields, as well as, lysis and sequencing compatibility (data not shown) **(Figure 3)**
Bacterial sequencing	Utilization of established V3/V4 16S rRNA sequencing protocol
Fungal sequencing	Evaluation of 3 academic ITS1 and ITS2 gene region primers and their amplification protocols, measurement of sequencing performance as well as diversity classification **(Figure 4)**

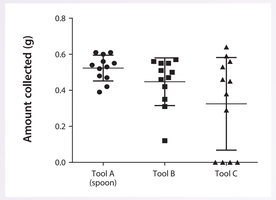

***Figure 2**: Different prototype collection devices were evaluated for their performance in collecting a volumetric amount of stool from the donor cohort. 500 mg collection mass was targeted for optimal sample to stabilization chemistry ratio, in addition, consistency of collection was measured through standard deviation (n = 12).*

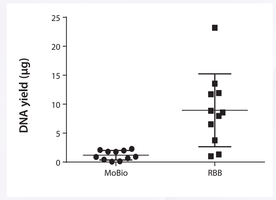

***Figure 3**: Comparison of yield performance between two common microbiome extraction technologies, MoBio PowerFecal kit and Repeat Bead Beating protocol. Samples from 11 donors were extracted in parallel and yield per purification aliquot (250 µL) was measured using PicoGreen fluorescence dye.*

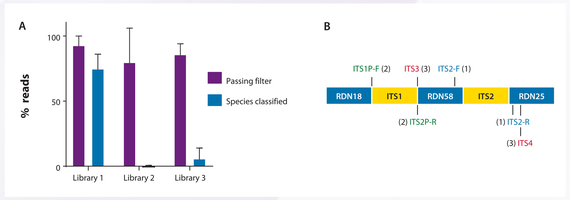

***Figure 4 (A)**: Library preparation/sequencing and diversity detection performance of 3 different library preparation methods was evaluated by % reads passing filter (Q score and amplicon size) and, subsequently, % reads that were successfully classified to species level. **(B)** Primer binding map, not to scale, for library 1 (ITS2-F/Ri)[2], library 2 (ITS1P-F/ITS2P-R)[3] and library 3 (ITS3/4)[4], amplicon sizes were 280 bp, 480 bp, and 420 bp, respectively. Recommended amplification strategies, master mix and cycling conditions, were followed for each primer set.*

Methodology testing results:

- The spoon was identified as an appropriate collection tool through prototype testing
- Significant differences were found between extraction methodologies
  - RBB method showed increase in yield, as well as, lysis efficiency (data not shown)
- Fungal sequencing methodology testing identified ITS2-F/R primers and their amplification conditions as optimal for gut fungal classification

Validated method for collection and stabilization

Physical device design and results

OMNIgene•GUT: Collection device for donors with dysbiosis

OMNIgene•GUT enables naïve donors to self-collect volumetric stool into a DNA stabilization/preservation chemistry.

- Spatula collected: 560 ± 160 mg (mean ± SD)
- Spoon collected: 580 ± 120 mg (mean ± SD)

The Instructions for Use (IFU) and tool choice allows donors from Bristol type 1-7 to easily and effectively collect a sample **(Figure 5)**. This cohort had a sample return rate of 92%, with all returned samples successfully collected **(Figure 6)**. Sample homogenization is achieved through physical mixing with the stainless steel ball.

Stabilization chemistry results

OMNIgene•GUT provides DNA yield sufficient for downstream sequencing for donors experiencing flare

A single OMNIgene•GUT sample provides more than sufficient DNA for various downstream assays **(Table 1)**.

- Total DNA yield from OMNIgene•GUT samples was 31.43 ± 25.67 µg (mean ± SD)
- Average DNA yield per OMNIgene•GUT extraction aliquot was 4.49 ± 3.67 µg (mean ± SD)

Table 1: Number of sequencing assays per OMNIgene•GUT sample. Based on mean DNA yield and an assumption of 7 extraction aliquots per sample.

	16S rRNA sequencing	WGS metagenomic	PCR-free WGS metagenomic
Assay input per sample	~5 ng	~100 ng	1-2 µg
Number assays per OMR-200 aliquot	>800	>40	4
Number assays per OMR-200 tube	>6000	>300	31

Microbiome profile is accurately captured by OMNIgene•GUT in donors experiencing flare

Microbial profiles of OMNIgene•GUT collected samples were compared to fresh, *in vivo*, samples. Bray-Curtis dissimilarity measured differences between collected and fresh were not significantly different from biological variability **(Figure 8A)**. Microbiome stabilization within OMNIgene•GUT does not introduce any significant bias in profile recovery.

Microbiome profiles of donors experiencing flare are preserved for up to 60 days in OMNIgene•GUT

OMNIgene•GUT samples were kept at room temperature for 60 days and 50°C for 3 days. Additionally, fresh sample aliquots were kept at -80°C and room temperature as positive and negative controls for profile change. OMNIgene•GUT samples collected from donors experiencing flare show significantly less profile change over time than fresh samples held at -80°C **(Figure 8B)**.

Remission vs. flare study

Bacterial and fungal profile differences were observed between donors experiencing remission and flare

Samples collected with OMNIgene•GUT were analyzed at T0 using 16S and ITS based amplicon sequencing.

- Samples from recruited donors were split into two sub-cohorts based on their Bristol type (1-5, remission and 6-7, flare).

Novel and previously published[5,6] differences were identified in species and genus level classifications between the remission and flare sub-cohorts **(Figure 9A)**. In particular, *Bacteroides fragilis* and *Bacteroides ovatus* were depleted in donors undergoing flare.

In addition, a preliminary assessment of mycobiome profiles of the two sub-cohorts suggests stark differences in relative abundance of select fungal species **(Figure 9B)**. Our evidence suggests that changes in mycobiome representation should be evaluated in future gut sample studies of IBD/Crohn's/Colitis cohorts.

Taken together, these results showcase the application of OMNIgene•GUT device to facilitate collection in gut dysbiosis studies.

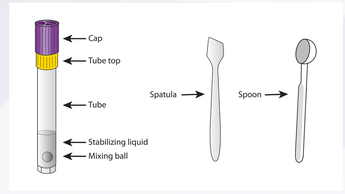

***Figure 5**: Illustration of OMNIgene•GUT device with spatula and spoon optimized for collection and stabilization of stool samples from healthy and dysbiosis donors. IFU not shown.*

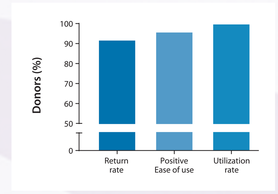

***Figure 6**: Our self-collection method resulted in a 92% return rate. While 96% of donors reported the method as easy to very easy, there was a 100% utilization rate.*

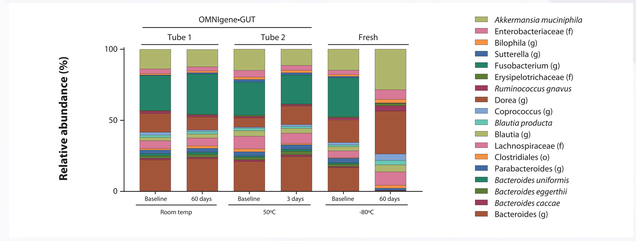

***Figure 7**: Stability of the bacterial profile from a representative Bristol Type 7 donor collected in OMNIgene•GUT and stored at room temperature for 60 days or 50°C for 3 days. Measureable profile change was observed for fresh samples stored at -80°C for 60 days.*

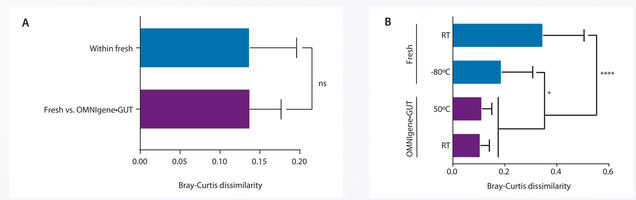

Figure 8 (A)**: No significant difference is seen in the dissimilarity between fresh and OMNIgene•GUT extractions and the dissimilarity between fresh biological replicates. **(B)** OMNIgene•GUT samples show significantly less profile change over time than fresh samples held at -80°C (*p≤0.05) or room temperature (*p≤0.0005).*

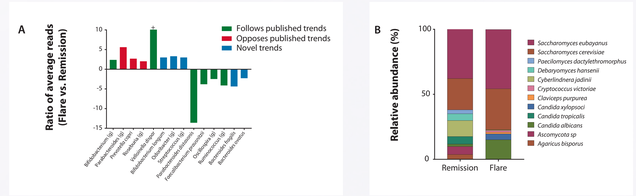

***Figure 9 (A)**: Measurement of bacterial taxonomic differences seen between donors experiencing remission versus flare. Averages in classified read count differences follow and oppose published trends for IBD and Crohn's and Colitis datasets, marked in green and red, respectively. In addition, several novel observed differences were identified that have not previously been reported in literature (marked in blue). V. dispar marked with (+) increases beyond the axis limits, due to near absence in remission cohort rather than abundance shift. **(B)** Preliminary results highlight average fungal profiles of donors experiencing remission versus flare.*

Conclusions

- OMNIgene•GUT enables easy self-collection and high return rates for donors with dysbiosis
- Volumetric collection and homogenization of fecal samples provides consistent DNA yield sufficient for downstream sequencing applications
- The *in vivo* microbiome profile is accurately maintained in OMNIgene•GUT samples
- Microbiome profiles are preserved during ambient transport when collected into OMNIgene•GUT
- OMNIgene•GUT can facilitate collection in gut dysbiosis studies

References

1 Yu, Z. and Morrison, M. (2004). Improved extraction of PCR-quality community DNA from digesta and fecal samples. *BioTechniques*, 36(5): 808–812.
2 Heisel T, Podgorski H, Staley CM, Knights D, Sadowsky MJ, Gale CA (2015) Complementary Amplicon-Based Genomic Approaches for the Study of Fungal Communities in Humans, *PloS ONE* 10(2): e0116705.
3 Chloe E. Huseyin, Raul Cabrera Rubio, Orla O'Sullivan, Paul D. Cotter and Pauline D. Scanian (2017) The fungal frontier: a comparative analysis of methods used in the study of the human gut mycobiome. *Front. Microbiol.*, 31 July 2017.
4 Andrea K. Nash et al., (2017) The gut mycobiome of the human microbiome project healthy cohort, *Microbiome* 5:153.
5 Walters, W.A., Xu, Z. and Knight, R. (2014). Meta-analyses of human gut microbes associated with obesity and IBD. *FEBS Letters*, 588.
6 Sartor, R.B. and Mazmanian, S.K. (2012). Intestinal microbes in inflammatory bowel diseases. *Am. J. Gastroenterol.* Suppl., 1: 15-21.

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