Development and Preliminary Validation of a Feasible Procedure for Isolating RNA from Fiber-Adherent Bacteria in Human Stool

Authors' Contribution:
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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Intestinal bacterial communities are not homogenous throughout the gastrointestinal tract. Human research on the gut microbiome often neglects intra-intestinal variability by relying on a single measurement from stool samples. One source of complexity is the adherence to undigested, residual fiber. Currently, no procedure exists to extract RNA from distinct bacterial subpopulations in stool samples.

Material/Methods: A serial centrifugation procedure was developed in which bacterial RNA could be extracted from distinct stool-fractions – fiber-adherent and non-fiber-adherent bacteria. To test whether the separation procedure yielded distinct bacterial subpopulations, a set of RT-qPCR assays were developed for a fiber-adherent bacterial species, *Bifidobacterium adolescentis*, then a within-subject repeated-measures study was conducted with 3 human subjects undergoing 4 dietary regimens. At each timepoint, between-fraction differences in gene expression were evaluated.

Results: The RNA isolation procedure was able to isolate intact RNA in 20 of 24 samples in the fiber-adherent fraction. *PurB* and *sdh* were identified as suitable reference genes for *B. adolescentis* RT-qPCR assays. When subjects were provided a high resistant starch diet, bacterial fractions exhibited different expression of the trp operon (p=0.031).

Conclusions: Our study provides human gut microbiome researchers a novel tool for evaluating functional characteristics of bacterial subpopulations in human stool. Moreover, these experiments provide modest support for the existence of a functionally unique fiber-adherent subpopulation of *B. adolescentis*. Until a more thorough evaluation of the adherent and non-adherent fraction can be performed, researchers should be cautious when generalizing functional data derived solely from unfractionated stool samples.

MeSH Keywords: Bifidobacterium • Microbiota • Tryptophan

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Background

Variability in the gut microbiome exists in several forms; for example, the small intestine and colon harbor unique species of bacteria [1], and the composition of bacteria embedded within intestinal mucus is different from that of luminal bacteria [2]. Functional variability is also observed across these bacterial subpopulations; bacteria from mucosal and luminal populations exhibit differential patterns of gene expression [2].

Another source of variability is dependent on the adherence to biological surfaces. Some bacteria, including adherent-invasive *Escherichia coli*, physically adhere to intestinal epithelial cells, while other bacteria attach to specific nutrients such as dietary fiber [3]. If distinct subpopulations of nutrient-adherent and non-adherent bacteria exist within the gut, each subpopulation may have a unique relationship with host physiology. From an experimental perspective, if these relationships are maintained in stool samples, then a procedure for analyzing these fractions separately would allow researchers to discover new relationships between the gut microbiome and host physiology.

From human stool samples, nutrient-adherent bacteria have been operationally defined as the bacteria that remain adherent to residual fiber after a series of steps involving agitation and washing [4]. Two lines of evidence support the theory that adherent and non-adherent bacterial populations are functionally distinct. Circumstantially, bacteria grown on different energy sources in cell cultures exhibit altered patterns of gene expression [5,6]. More directly, using human stool samples in which adherent and non-adherent fractions are separated, transferred to cell cultures, and provided the same nutritional input, different metabolic output was observed [4,7,8]. However, it could be that the observed metabolic alterations were an artifact of the differential procedure required for collecting and transferring each fraction to cell culture. Culture-independent methodologies, such as RT-qPCR, allow functional attributes to be more directly analyzed without additional processing. These methods could provide another perspective on functional attributes of bacterial communities that may be more indicative of microbial function within the intestine.

RNA extraction methods from stool samples have been reported by several groups, but each has limitations. Some methods proceed to RNA extraction based on unfractionated stool samples [9]. This method is well established, yielding high-concentration, chemically pure RNA. However, this method could be averaging across functionally distinct subpopulations of bacteria. Other protocols use centrifugation to separate and remove residual fiber prior to RNA extraction [10,11]. This procedure also yields high-abundance, chemically pure, non-degraded RNA. However, it neglects fiber-adherent bacterial

cells [12]. There is no existing protocol that extracts RNA solely from residual fiber.

In this study, a protocol was developed to extract RNA from residual fiber in human stool samples. Then, a series of RT-qPCR assays were developed for a pair of known fiber-adherent bacteria – *Bifidobacterium adolescentis* and *Eubacterium rectale* [13,14]. Finally, fecal samples were collected from 3 subjects undergoing 4 dietary regimens, and gene expression differences were evaluated between the adherent and non-adherent fractions. Taken as a whole, these experiments address an important technical consideration for processing fecal samples in preparation for analyzing gene expression, which may provide better insight into the role of bacterial subpopulations in mediating aspects of host physiology.

Material and Methods

Dietary Intervention

Informed consent was obtained for 3 healthy subjects to take part in a four-week dietary intervention consisting of 4 one-week dietary regimens (Figure 1). Week 1 – resistant starch (RS) depletion: Subjects were instructed to avoid foods containing RS (Supplementary Material 1), Week 2 – Potato/Corn Starch: In addition to their habitual diet, subjects were instructed to consume 24 grams of resistant starch per day (potato starch [48 g – 50% type II RS by weight, Bob's Red Mill] or corn starch [40 g, 60% RS by weight, Hi-Maize 260 product]), Week 3 – RS depletion, Week 4 – Potato/Corn Starch. Subjects provided stool samples on the final 2 days of each dietary period. Upon providing the sample, subjects were asked to indicate which Bristol Stool Scale type most closely resembled their sample [15].

Fecal sample collection and RNA extraction

Subjects were instructed to empty their bladder, flush the toilet, apply collection paper to the toilet seat, deposit a fecal sample on the collection paper, and transfer 1–3 scoops of stool to the collection tube (Sarstedt, Nümbrecht, Germany), which was pre-filled with 5 mL RNALater. Subjects were instructed to shake the tube vigorously to distribute the sample. Once fecal samples were received at the lab, they were vortexed, aliquoted into 950-uL portions (100–300 mg fecal solids), and stored at –80°C.

Based on the procedure used to separate residual-fiber-adherent and non-adherent fractions for DNA profiling [14], a protocol was adapted to isolate intact RNA. This procedure separates fractions by centrifugation, followed by a series of washes of the residual-fiber-fraction using PBS then PBS+Tween-20. Development was iterative, and involved modifying multiple
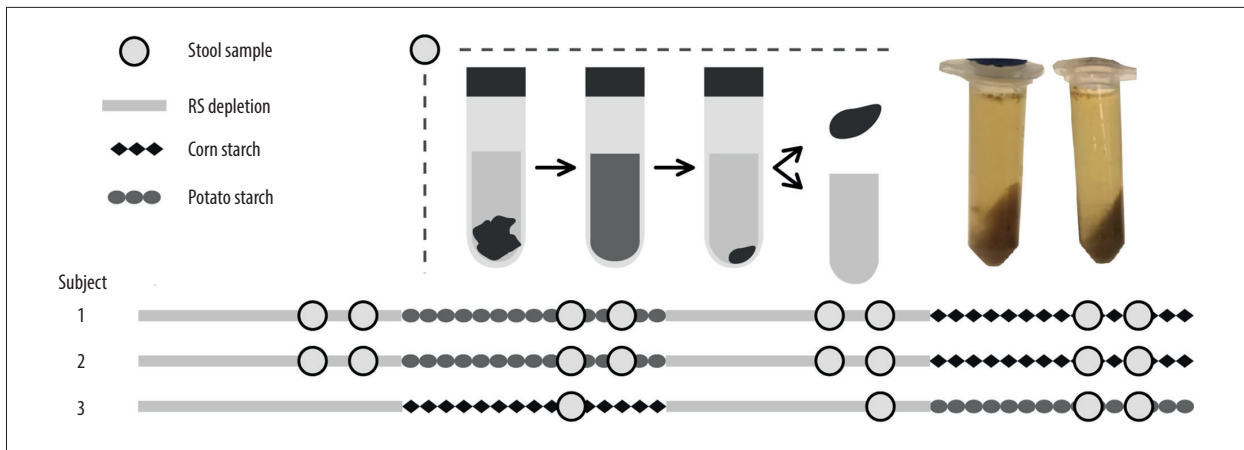
Figure 1. Study schematic: (**Top right**) Outlines the collection and wash procedure, in which stool samples were collected in a liquid storage media, distributed throughout the media, then separated via centrifugation. Not illustrated are several additional washing steps to ensure definitive removal of non-adherent bacteria from the pellet. (**Bottom**) Outlining each subject's dietary procedure. Samples that yielded degraded RNA, or those that did not pellet during centrifugation, were excluded from analysis.

parameters including centrifugation speed and time, wash buffer composition, and the order of washes.

For the final procedure, fecal aliquots were diluted in 500 µL RNALater, vortexed for 30 s, centrifuged at 1700g for 1 min, and separated into supernatant (non-adherent fraction) and pellet (precursor to fiber-adherent fraction). Supernatant was set aside at room temperature while pellets were further processed. Pellets were washed 3 times by resuspension in 1 mL RNALater, vortexing, and centrifugation at 1700g for 1 min. Samples were then washed twice in PBS+0.1% TWEEN-20. From this final wash, pellets were resuspended in RNALater, and along with the supernatants, were centrifuged at 9000 g for 5 min to pellet all bacteria.

Samples were homogenized using 0.1-mm glass beads, using 2 cycles of 5000 rpm, for 45 s, in a Precellys homogenizer (Bertin, Montigny-le-Bretonneux, France). RNA extractions using the MoBio power RNA extraction kit (Qiagen, Venlo, Netherlands), which includes a DNase digestion step. Informally, we tested DNA contamination with RT-qPCR by using a control well in which the reverse transcriptase was heat-inactivated (no-RT control). Consistently, we observed that DNase processing reduces DNA to concentrations >10 Ct's (>1000×) lower than RNA concentration. Because of this observation, and the reliance on standardized manufacturer protocols, we did not include any no-RT controls in these experiments. Finally, samples were eluted into 50 µL nuclease free water. To evaluate sample degradation, approximately 100 ng of extracted nucleic acid was run on an agarose gel.

Primer design

All primers were designed to target a set of germane genes from strains previously identified as fiber-adherent. *Bifidobacterium adolescentis*: strains 22L, BBMN23, and ATCC 15703, genes measured: *trpA* (tryptophan synthase alpha chain), BADO_1572 (RS degrading enzyme). *Eubacterium rectale*: strains DSM17629, M104/1, ATCC 22656**,** genes measured: *trpB* (tryptophan synthase beta chain), and *amy13B* (EUR_01860 – RS degrader). All primers and probes were designed according to directions in the TaqPath 1-step RT-qPCR manual (Applied Biosystems, Foster City, CA). To design primers across strains, a target sequence from one primary strain was input into primer BLAST. If BLAST identified all other target strains, all strains were selected and primers were designed to fit each strain. If BLAST did not identify other target strains, a representative sequence was obtained from each strain, and these sequences were aligned in Jalview to generate a consensus sequence [16]. The consensus sequence was input back into primer BLAST as the new target sequence. If each target strain was then identified, primer design would proceed, if not, a different gene was selected with greater sequence similarity across strains. Probes (IDT 6FAM/ZEN Iowa Black® FQ combination) and primers were ordered from IDT (Marlton, NJ) and stored in 0.1×TE buffer. Primers were stored at 4.5 µM concentration, while probes were stored at 1.6 µM.

Reaction optimization

Optimal primer and probe concentrations were determined based on evaluating the combination of 3 dilutions of each primer, then 3 dilutions of each probe, and selecting the combination with the lowest cycle threshold (Ct) and highest
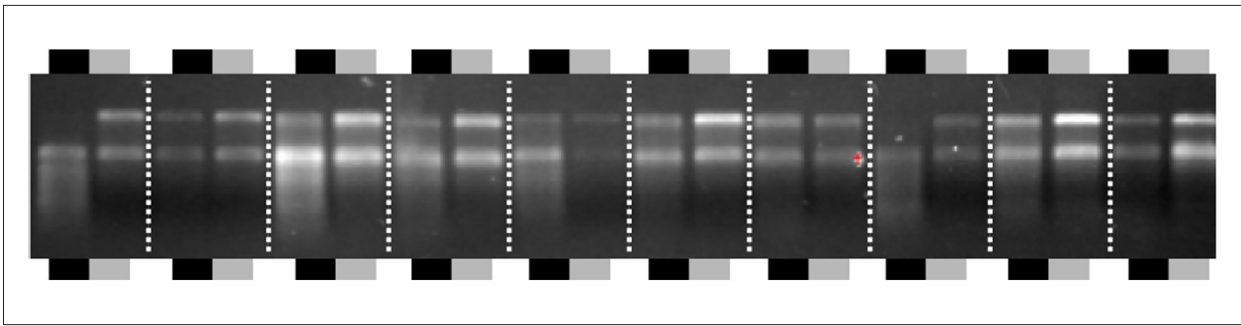
Figure 2. Sample gel trace of 10 independent stool samples. All solid and liquid fraction pairs were run next each other, and separated from other pairs with a dotted white line. Black bars underlie RNA derived from the residual-fiber-fraction, gray bars underlie RNA derived from the non-adherent fraction. Many samples show no evidence of RNA degradation; however, samples such as on the far left and third from the right exhibit some degradation. All gel traces are included in Supplementary Materials 1–5.

fluorescence. Eight-point efficiency curves were performed for each primer set; all reaction sets were ranged in efficiency between 0.92 and 1.15, with an R^2 for the efficiency curve >0.96 (Supplementary Material 2). Final concentrations of primer and probe sets are indicated in Supplementary Material 2.

RT-qPCR assays

One week prior to experimentation, primers and probes were combined into a single tube of working stock at 500 nM for probes and 600 nM for primers. This mixture comprised 50% of the final reaction volume of 8 μL, bringing the final reaction to 250 nM probe and 300 nM for each primer (for all genes). The final reaction also contained 2 μL RTqPCR master mix, 1.4 μL nuclease water, and 0.6 μL RNA with variable concentrations ranging from 0.3 to 43 ng/μL. For each 96-well plate, 4 samples with 8 genes each were measured in triplicate. All reactions were conducted using an Agilent Mx3005p instrument (Agilent, Santa Clara, CA) and the following cycling parameters: 25°C for 2 min, 50°C for 15 min, 95°C for 2 min, and 40 cycles of 95°C for 15 s and 60°C for 60 s. Efficiency was calculated as $-1+10^{\wedge}(-1/slope)$, with slope calculated as $\log_2$ concentration on the X-axis and cycle threshold (Ct) on the Y-axis. M values for reference gene variability were calculated as in Vandesompele [17].

Statistics

All statistics were performed using ANOVA on rank transformed data using JMP statistical software (SAS Institute, Cary, NC). Differences between dietary conditions were evaluated using delta-Ct's, with dietary condition, fraction, timepoint, and subject as factors. Within each dietary condition, differences in gene expression between fractions were evaluated on delta-Ct's, using fraction, timepoint, and subject as factors.

Results

Procedure development for isolating fiber-adherent-bacterial-RNA

Starting with a procedure developed to isolate residual-fiber-adherent-bacterial-DNA [14], a set of modifications were tested, and it was determined that exchanging the wash buffer for RNALater yielded the highest quality RNA. From a preliminary set of 24 samples collected from 3 subjects, intact RNA was successfully isolated from both the adherent and non-adherent fraction in 20 samples (Figure 2, see Supplementary Material 3). The average total RNA yield was 5.5 ug, and no differences were observed between the solid and liquid fraction (Figure 3).

The major limitation to this processing procedure is that RNALater is more viscous than PBS; therefore, for some samples, centrifugation failed to produce a substantial pellet of residual fiber. Differences between samples in the ability to form a pellet in RNALater were not explained by the self-reported Bristol Stool Scale number [15], and the Bristol number also failed to explain differences in concentration or chemical purity of extractions from either the adherent or non-adherent fraction.

RT-qPCR assay development and validation

Primers were designed for 2 fiber-adherent organisms – *Bifidobacterium adolescentis* and *Eubacterium rectale*. Three candidate reference genes were selected from Stenico [18] and Rocha [19], and tested in a set of 20 samples from subjects recruited for a separate dietary fiber intervention. Samples were processed as in Zoetendal [10], by removing residual fiber. For each species, 2 pairs of genes were selected, as they exhibited low variability (M values corresponding to 0.77 and 1.34 for *B. adolescentis* and *E. rectale*, respectively), and were not differentially expressed during fiber supplementation.
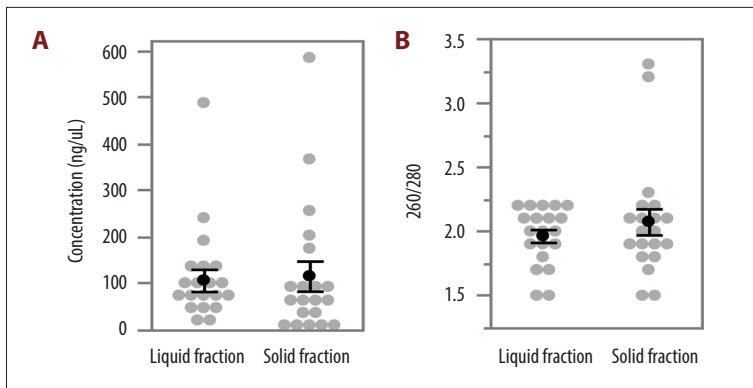
Figure 3. Concentration and purity data measured with nanodrop 2000. (**A**) RNA concentration derived from each sample in a final volume of 50 uL. The average total yield was 5.5 ug. (**B**) Protein contamination, as measured by the 260: 280 ratio. All error bars represent standard error.
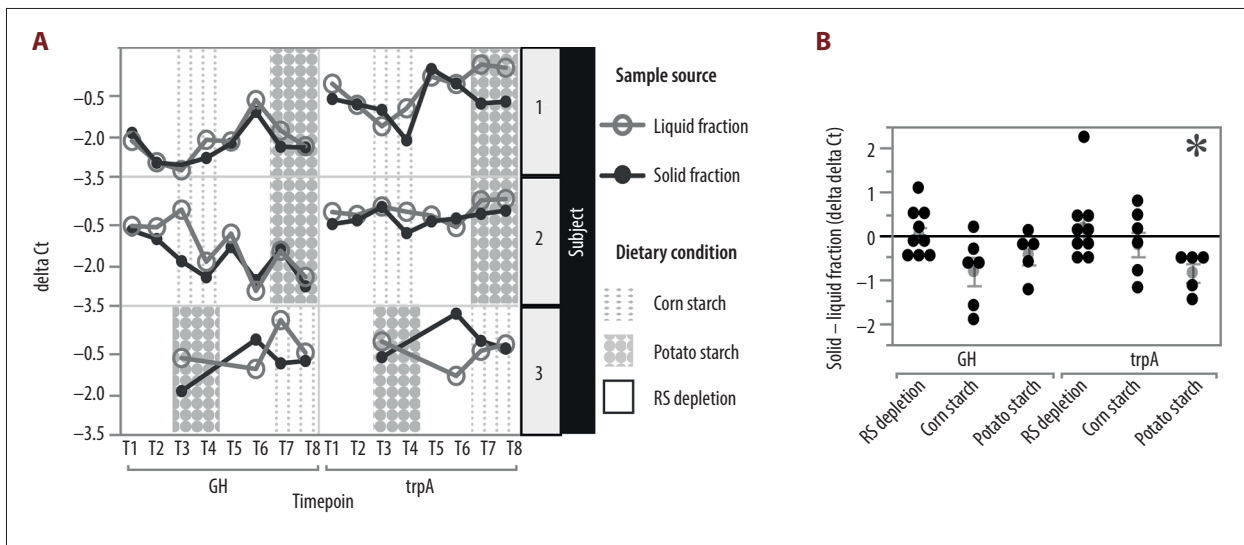

Figure 4. (**A**) Changes in gene expression, relative to reference genes, throughout the supplementation. GH=BADO_1572 (starch-degrading gene), trpA=tryptophan synthase alpha chain (trp operon). (**B**) Within each dietary condition, dots represent the difference between the fiber-adherent and non-adherent fraction for each gene. All measures are derived from *B. adolescentis*. * p<0.05 measured by repeated-measures ANOVA based on ranks.

From the subjects recruited for this study, reference gene variability was evaluated in the fiber-adherent and non-adherent fraction from 20 stool samples. There were no significant differences in pairwise variation between adherent and non-adherent fraction in any of the dietary conditions, and M values for the following genes were: *B. adolescentis* (*purB*, *sdh*): 0.58, *E. rectale* (*secA*, *dnaG*): 2.54. *E. rectale* was excluded from further analyses due to high reference gene variability.

Functional differences between adherent and non-adherent fractions

Three subjects alternated between one-week periods of resistant starch depletion (a type of dietary fiber), and one-week periods of no dietary restrictions plus a daily dose of either potato starch or corn starch (Figure 1 and Supplementary Material 1).

Considering gene expression across dietary conditions, a main effect of dietary condition was observed for the trp operon (*TrpA*) (p=0.034), driven by a change in expression in the non-adherent fraction (p=0.023), but not the adherent fraction (Figure 4). There was no effect of diet on expression of the fiber-degrading gene (*BADO_1572*)

Considering both starch conditions together, *BADO_1572* exhibited increased expression in the adherent fraction in 9 of 11 samples (p=0.055), corresponding to a 53% increase (Figure 4). *TrpA* exhibited increased expression in 8 of 11 samples (p=0.031), but its expression was variable between starch conditions. The corn starch condition yielded variable differences between the 2 fractions, but in the potato starch condition all 5 samples exhibited an upregulation in the solid fraction, corresponding to an 80% increase (p=0.015). In the starch depletion dietary regimen, the differences between fractions were inconsistent between samples and tended to cluster around zero.

Discussion

The protocol

Stool sampling is a central feature for many studies of the gut microbiome, as it does not rely on invasive collection procedures. However, there are drawbacks to this methodology. To the best of our knowledge, there are no stool sample processing procedures permitting RNA isolation from bacterial subpopulations. Overcoming this limitation could prove important, as bacteria derived from distinct intestinal environments exhibit differential patterns of gene expression [2], and therefore could have distinct relationships with host physiology. In this study, a protocol was developed that successfully isolated intact RNA from residua- fiber-adherent bacteria, allowing functional attributes of this population to be considered separately from non-adherent bacteria. This advance may offer researchers the opportunity to discover new relationships between gut bacteria and host physiology.

Improving the protocol

There is still room for improvements in the procedure. First, 17% of samples derived from the residual-fiber fraction were degraded. The degradation likely occurred during the washing steps using PBS. We chose not to exclude this washing step because samples formed a clearer pellet when using this washing buffer, thereby permitting a more thorough removal of non-adherent bacteria from the fiber fraction. However, it is possible that a washing procedure using entirely RNALater could yield more consistently non-degraded RNA. Secondly, some samples did not pellet in RNALater; therefore, adherent and non-adherent communities could not be separately evaluated in these samples. This limitation, however, highlights the possibility that new isolation procedures could separate fiber types based on viscosity, and therefore further fractionate bacterial subpopulations.

RT-qPCR assays

Performing RNA-seq on mixed bacterial community samples is a powerful method for evaluating gene expression, but it is limited primarily by cost, the availability of equipment, and having access to computational resources. As an alternative, RT-qPCR allows for inexpensive and targeted analysis, and is particularly useful when attempting to measure low-abundance genes. From this study, we provide data on cross-strain-compatible primers and probes for *Bifidobacterium adolescentis* and *Eubacterium rectale*, with optimized concentrations and acceptable reaction efficiency.

Instead of normalizing target genes to a conventionally used reference gene, these studies used the comparatively more rigorous approach of independently validating reference genes. While the set of reference genes from *E. rectale* proved too variable for further consideration, those from *B. adolescentis* (*purB* and *sdh)* exhibited negligible variability relative to each other. Further studies should evaluate the stability of these reference genes in a larger sample set and alongside a larger pool of prospective reference genes.

Are subpopulations distinct

Most samples exhibited higher expression of the fiber-degrading gene *BADO_1572* in the fiber-adherent fraction, consistent with previous observations in cell culture [6]. Moreover, during potato starch supplementation, all 5 samples exhibited higher expression of the trp operon. While this study only evaluated one gene and one species, and was conducted in only 3 subjects, these data nonetheless provide additional evidence in support of the theory that residual-fiber-adherent bacteria are functionally distinct from their non-adherent counterparts. Although this study was significantly hampered by a small sample size, this evidence bolsters the findings from metabolic studies performed in cell culture [4].

Conclusions

The protocol developed in this study provides a simple, feasible method for improving sampling resolution of human gut microbiome. To the best of our knowledge, the procedure represents the first attempt to extract RNA from bacterial subpopulations in stool samples. Evaluating RNA, rather than profiling functional characteristics from cell culture, provides researchers the opportunity to evaluate novel functional characteristics of bacterial subpopulations while reducing artificial conditions introduced in the culturing process. However, there is still opportunity to improve stool fractionation procedures; for example, by deriving more consistently intact RNA, or by achieving further subpopulation delineation through novel fractionation procedures.

The dietary intervention, although limited by sample size, provides modest validation that the RNA extraction procedure reflects *in vivo* gene expression, but replication is clearly needed. Until a more thorough evaluation of bacterial subpopulations can be performed, researchers should be cautious when generalizing data derived solely from unfractionated stool.

Conflict of interest

None.

HUMAN STUDY

Supplementay Data

Supplementay Material 1. Subject instruction packet.

Supplementay Material 2. Primer/probe sequences and reaction efficiency data.

Supplementay Material 3. Gel traces.

Supplementay Material 4. RNA extraction data.

Supplementay Material 5. RTqPCR data.

Supplementary/raw data available from the corresponding author on request.

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