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"Fecal microbiome in epidemiologic studies" – Response

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We thank Drew *et al.* for their interest in our recent publication (1) and share in their enthusiasm for identifying "a straight forward self-collection procedure" for "multiple molecular analyses." Fecal collection and sampling have continued to be a topic of study, including our own published works (1–3).

In our reply to the comment by Drew *et al.*, we offer short clarification of some of their many salient points and address a list of important questions that are in need of additional study. The first point of clarification regards two minor errata with their interpretation of our "earlier reports." Two of our studies (2, 3) were taken as evidence for the ability of RNA*later* fixation to recapture the "gold standard" immediately frozen fecal samples. However, one of

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these previous studies did not compare RNA*later* to a "gold standard" (3) and in the other, we explicitly stated that "compared to specimens collected in RNA*later*-based media, specimens collected without media had significantly different microbial composition (2)." Moreover, Drew *et al.* refer to a manuscript by Aagaard *et al.* (4) as evidence that samples in RNA*later* did not change microbial diversity. In fact, this manuscript was describing the samples collected in the Human Microbiome Project and did not evaluate RNA*later*.

Secondly, we would like to point out that there are a number of advantages to using a fecal occult blood test (FOBT) card for gut microbiome studies, including the low cost, relative ease of use and shipping at ambient temperature, as well as minimal storage requirements for large prospective cohorts. The use of 5–10 mL of RNA*later* solution to preserve a fecal sample may be cost prohibitive for cohorts with hundreds of thousands of subjects, due to the higher cost of vials, RNA*later*, shipping, and storage. Most biosamples collected in cohorts are not used since only nested subsets are selected for processing years after banking, which means that minimizing collection and storage costs are crucial priorities.

We also would like to point out that clinical use of FOBT which requires dietary and medication modifications differs from the question of FOBT cards as a collection method for microbiome research purposes only.

Finally, we would like to thank Drew *et al.* for raising a number of important research questions. We are currently addressing many of these issues in our ongoing studies. This initial study (1) allowed us to narrow our sampling to only the better collection methods and to dedicate resources to where it matters most. Specifically, we have completed two additional studies with over 50 volunteers per study in both the US and a low-resource country in Asia to evaluate the robustness of our findings. We included a fecal immunochemical test (FIT) test kit in recognition of the changing trend from FOBT to FIT-based colorectal cancer screening.

With regards to the need for testing for other molecular analyses, we have conducted a study of metabolomics using fresh fecal samples, and fecal samples preserved using 95% ethanol, FOBT cards, and FIT tubes. We would like to note that a major US metabolomics company was unable to perform the metabolomics assay for fecal samples collected in RNA*later*. In addition, we are currently testing whole-genome shotgun sequencing in fecal sample collected by different methods. While it might be "premature to recommend FOBT cards" for all molecular analyses, it is unlikely that one individual method will be useable for all molecular analyses. Therefore, it may be necessary that each study or large cohort identify assay priorities and pick one or two methods for collecting and storing fecal samples. Alternatively, our findings could lead companies or research groups to develop and evaluate the efficacy of alternative fixative solutions to preserve the integrity of a variety of biomolecules.

In conclusion, we believe that there are multiple fecal sample collection methods appropriate for different assays, but from this specific analysis, we identified the FOBT card as the most accurate, stable, and cost-effective for 16S analyses. We are continuing to conduct methodologic work to address some of the important points raised by Drew *et al.* for

collecting fecal samples that will be valid and applicable for multiple assays in large cohorts within different populations.

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