1. In Korteganas et al. (2020), water source was significantly associated with several gut microbiota outcomes in the study population. However, a follow-up randomized interventional study is necessary to further parse out the effect of water source on child gut microbiota. I hypothesize that access to piped drinking water (compared to water accessed from a borehole, well, river, or lake) would be associated with more diversity in the child gut microbiota.
2. My proposed study would be a randomized control trial which would test the effect of piped water interventions on child gut microbiota. There would be a temporal aspect as piped water interventions would be delivered first, then child stool samples would be collected later. Also, there would be longitudinal follow-up as I would collect these samples at multiple time points.
3. The study would take place in the same geographical area as the iLiNS-DYAD trial. I want to recruit participants from the same area because the effects of water source on gut microbiota may not persist in other areas. I am interested in measuring the stool samples of children only, because their microbiota composition changes across their developmental stages. Because this is an infrastructural intervention, I would randomize at the neighborhood level; half of the neighborhoods would receive clean water through pipes, and the other half would continue using water only from boreholes, wells, rivers, or lakes. Only neighborhoods using these natural water sources would be included in the study; those already using piped water would be excluded. To reduce selection bias, participants would be recruited widely throughout the study area and randomization would be done by a computer. Ideally, child retention rates during follow-up would be high, as would total sample size. Participants would be incentivized to continue with the study by receiving food and cash benefits at every stool sample collection visit.
4. The exposure would be the water source (piped water in the intervention group or preexisting natural water source in the control group). Exposure would be measured directly by delivering the intervention or control and monitoring children using their water sources. The outcome would be child microbiota diversity at ages 1, 6, 12, 18 and 30 months measured with fecal samples. Since fecal samples are surrogate measures of the mucosal large intestine, it will be important to perform a validity study to make sure that fecal samples are good indicators for studying the gut microbiota. This validity study would be done before the study in the same population, but with a smaller sample size. Ideally, we would compare fecal samples to the gold standard of mucosal large intestine biopsies and see if the microbiota outcomes are similar in both. Assuming they are, we would collect child fecal samples at multiple time points (at the ages listed above) to reduce within-person variability. Fecal samples would be collected at home for the convenience of participants, then stored at -80C until analysis. We would assess the samples with 16s rRNA sequencing and use the V4 region since it is well-suited for assessing the gut microbiota. However, only using 16s may pose systematic measurement error; since 16s sometimes does not capture all species, I would design a validity study comparing 16s to metagenomic sequencing to make sure that the results are consistent across both methods. This would be done during the study with participants’ samples. By comparing to metagenomics and validating the 16s method, we would reduce the risk of information bias via non-differential misclassification of outcome. If resources allowed and we could use metagenomics for the entire analysis, we could report microbiome function in secondary analyses as well. However, the aim of this paper is to first examine microbiota composition and diversity only. OTU and genus-level species would be reported from validated 16s methods. The statistical analysis methods would be the same as the original paper (i.e., using linear models) but we would add more α-diversity metrics; in addition to Shannon Index, we would measure the Simpson and Chao 1 indices to capture comprehensive quantitative and qualitative relative abundance information. It would be valuable to utilize multiple metrics to better understand the within-sample diversity. The β-diversity measures would remain unchanged; weighted and unweighted UniFrac distances in PERMANOVA models are rigorous methods. However, we would add negative controls in the form of sterile water and positive controls in the form of synthetically derived bacterial communities to validate our analytical methods. To reduce the source of measurement random error, we would have a reliability study in which technicians would perform sequencing on the same samples multiple times—this would reduce technician variability and reduce the risk of information bias via non-differential misclassification of outcome. Finally, household SES, crowding, animal ownership, sanitary facility type, parents’ education level, exact gestational age, child sex, and season of fecal sample collection are potential covariates for this analysis and would be controlled for in all analyses.
5. While we cannot be sure what the results of this hypothesized study would be, it is certain that the methods are more rigorous than the original paper. There are many strengths in this proposed study—there are two validity studies and one reliability study, rigorous methods, temporality and follow-up, and ideally high retention rates and sample size. The limitation is that it is very resource-intensive and would require significant human and financial investment. However, if our results support the hypothesis, and there is an association between piped water intervention and child gut microbiota diversity, the implications of this study are very strong given its rigorous methods. It is certainly biologically plausible that a cleaner water source would lead to improved child gut microbiota outcomes, as existing literature supports this hypothesis. Moreover, the study results would be generalizable to children living in rural Malawi. With rigorous randomization methods, it is unlikely that selection bias would be present in this study. If the results are strong and in the direction that we would expect, the main policy implication is that access to piped water must become widespread to improve child health in rural Malawi.

**References**

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