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Using Faecal Immunochemical Test (FIT) undertaken in a national screening programme for large-scale gut microbiota analysis.

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We read with great interest the article by Gudra *et al*[1] reporting a Faecal Immunochemical Test (FIT; OC-Sensor, Eiken Chemical) commonly used in colonic neoplasia screening as a reliable sampling device for microbiome profiling when compared with immediately frozen samples from whole stool. The potential to use the FIT routinely completed by approximately 3.5 million participants annually as part of the English Bowel Cancer Screening Programme[2] to understand the role of gut microbiome in colorectal neoplasia holds great promise, not least because of the convenience to individuals, cost-savings associated with use of routinely-collected samples, and methodological advantages of samples collected before microbiome-altering procedures (e.g., bowel cleansing[3]).

We aimed to validate and expand upon the finding of Gudra *et al*[1] by investigating performance of the BCSP FIT, analysing more subjects, testing longer-term storage, investigating different methods of concentration, and comparing with OMNIgene.GUT (OG; DNAGenotek), a widely used research device for stool DNA preservation at ambient temperature[4]. We considered bacterial profile stability over time, mimicking real-world research scenarios with storage of FIT samples for up to 20 days prior to DNA extraction. We also explored whether concentration of samples by either speed vacuum (SV) or Lyophilisation (LY) is necessary to generate sufficient DNA yield from the FIT device.

We collected faecal samples from 10 healthy volunteers. Samples were homogenised immediately, aliquoted and stored/processed according to 11 different test conditions (**Table 1**), before extracting DNA on the corresponding day using the PowerSoil PowerLyzer DNA kit (QIAGEN). Faecal samples collected into a FIT tube adhered to the BCSPinstructions. 125 samples including negative and positive controls underwent V4 16S rRNA gene sequencing. All samples were rarefied to 10,000 reads.

Alpha-diversity (richness, FDR P=0.9; Shannon diversity, false discovery rate (FDR) P=0.44) was consistent within individuals regardless of test condition (**Figure 1A and 1B**). Beta-

diversity based on Bray-Curtis dissimilarity showed that samples grouped by patient (PERMANOVA P<0.001) and not test condition (PERMANOVA P=0.327) (**Figure 1C**), which was consistent with the presence/absence Jaccard index (patient PERMANOVA P<0.001; condition PERMANOVA P=0.99) (**Figure 1D**). EnvFit analysis further showed individual subject to be the only significant co-variate (P<0.001) (test condition (P=0.201); duration of storage (P=0.15)).

While overall microbiota profiles were consistent within individuals, the relative abundance of eight genera (3% of 245 total genera) were significantly different between fresh, OG day 10, and FIT day 10 conditions. *Blautia*, *Anaerostipes*, *Bifidobacterium*, and *Lachnospiracea* were higher in FIT samples stored for 10 days at room temperature, with *Parabacteroides*, *Bacteroides*, and *Sutterella* lower (Kruskal-Wallis FDR P>0.05) (**Figure 1E**). Storage of FIT samples over 20 days resulted in no significant difference in alpha or beta-diversity, but *Parabacteroides* relative abundance reduced significantly between day 0 (mean 0.9% relative abundance) and day 20 (mean 0.2% relative abundance; FDR P=0.006). Storage at -80ºC and concentrating samples by SV or LY had no effect on alpha-diversity, beta-diversity, or taxonomic profiles.

In concordance with other studies exploring the potential of FITs for microbiome sequencing[1,5–8], we found that faecal microbiome diversity and taxonomic profiles were consistent across test conditions. Notably, the changes in a small number (3%) of genera between fresh and FIT samples were consistent across all subjects. In addition, and contrary to the findings of Gudra *et al*[1], we showed that combining bead-beating based DNA extraction with amplicon sequencing negated the need to concentrate samples prior to analysis, increasing the sample throughout.

These important results demonstrate the potential of FIT, as obtained through a national screening programme, to provide a convenient, representative (i.e., sample obtained before bowel cleansing), and cost-effective means of studying faecal microbiota in a large population.

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Table 1. Summary of the different test conditions that each faecal sample underwent

Test condition label	Test	Days after defecation before extraction or storage	Storage temperature	Sample concentrator	Description
Fresh Day 0	Fresh faeces	0	RT	NA	Fresh sample
FIT Day 0	FIT*	0	RT	NA	Sample added to FIT
FIT Day 0 SV	FIT	0	RT	Speed vacuum	Sample added to FIT and speed vacuumed for 2 hours
FIT Day 0 LY	FIT	0	RT	Lyophilisation	Sample added to FIT and lyophilised overnight
OG Day 10	OMNIgene .GUT*	10	RT	NA	Sample added to OMNIgene.GUT and left at RT for 10 days
FIT Day 10	FIT	10	RT	NA	Sample added to FIT and left at RT for 10 days
FIT Day 10 -80	FIT	10	RT then -80°C	NA	Sample added to FIT, left at RT for 10 days, then stored in -80°C for 1 month
FIT Day 10 -80 SV	FIT	10	RT then -80°C	Speed vacuum	Sample added to FIT, left at RT for 10 days, stored in -80°C for 1 month, then speed vacuumed for 2 hours
FIT Day 10 -80 LY	FIT	10	RT then -80°C	Lyophilisation	Sample added to FIT, left at RT for 10 days, stored in -80°C for 1 month, then lyophilised overnight
-80 1M	Fresh faeces	10	RT then -80°C	NA	Fresh sample stored at RT for 10 days then frozen at -80°C for 1 month
FIT Day 20	FIT	20	RT	NA	Sample added to FIT and left at RT for 20 days

FIT, faecal immunochemical tests; NA, nonapplicable; RT, room temperature.
*use as per manufacturer's instructions

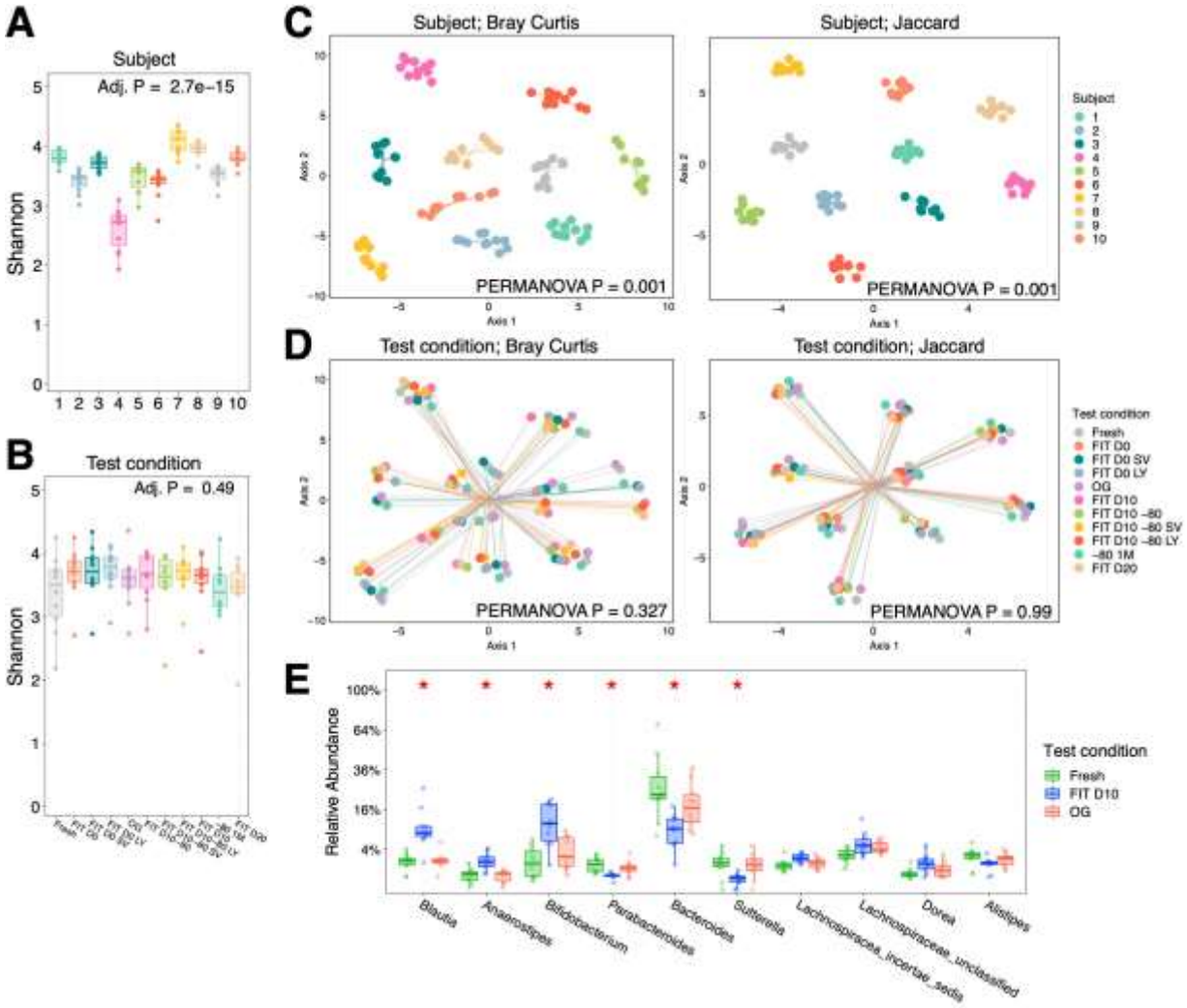

Figure 1. Microbiota by subject and test condition. Panels A (subject) and B (test condition) display box plots for the alpha diversity by both richness (observed OTUs) and Shannon diversity, showing diversity is subject specific and not affected by the test condition. Displayed P values based on Kruskal-Wallis and adjusted using FDR. Samples. Panels C (subject) and D (test condition) display the beta diversity by both weighted Bray curtis and unweighted Jaccard indices. Ordination based on t-SNE. The faecal microbiota clusters by subject regardless of test condition. Panel E displays box plots comparing the relative abundance of bacterial genera from fresh day 0, FIT day 10, and OMNIgene GUT day 10. Genera are ordered by significance based on Kruskal-Wallis with FDR adjustment. Only the 10 most significantly different genera are shown. Red asterisk denote genera with a P value between 0.01 and 0.05.