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Storage and handling of human faecal samples affect the gut microbiome composition: A feasibility study

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ABSTRACT

Human gut microbiome analysis through faecal sampling typically involves five stages: sample collection, storage, DNA extraction, next generation sequencing and bioinformatics analysis. Of these, the first three are considered irreversible. This feasibility study describes an assessment of methodologies used for faecal DNA extraction and sample handling, using the parameters DNA yield, purity and resultant microbial profile. Six DNA extraction techniques, including commercially available kits and manual protocols were compared on human faecal samples ($n = 3$). Different extraction techniques produced significant variance in DNA yield (range 2.7–164 ng/mg faeces) and microbial diversity profiles, with considerable variation in phyla dominance (Firmicutes ($P < 0.001$), Bacteroidetes ($P = 0.003$), Actinobacteria ($P = 0.003$), One-way ANOVA). The most effective method, with the highest DNA yield, was a simple and inexpensive extraction technique named MetaHIT. Using this method, DNA was extracted from separate faecal samples ($n = 3$) and had been aliquoted to seven storage conditions including three stabilizing buffers and three temperature conditions, for a period of 120-h, with storage at −80 °C as a control treatment. DNA yield and purity was not statistically different between the control and remaining treatments. 16S rDNA-based diversity profile was largely comparable across the treatments with only minor differences in genera between samples stored at room temperature in air and − 80 °C control. Overall these results suggest that the choice of DNA extraction method has a greater influence on the resultant microbial diversity profile than the short-term storage method.

1. Introduction

The human gastrointestinal (GI) tract plays host to a large array of microorganisms collectively termed the gut microbiota. This community, primarily bacteria, assist in host digestion and absorption largely through the fermentation of partially digested dietary components (Macfarlane and Gibson, 1995). In recent years it has become apparent that this ecosystem may also contribute significantly to human health and risk of disease throughout life. Detrimental perturbations in the gut microbiota, termed dysbiosis, have been associated with a number of disorders including obesity (Ley et al., 2006; Turnbaugh et al., 2009), GI inflammatory diseases (Hold et al., 2014), type I and II diabetes (Kostic et al., 2015; Qin et al., 2012), colorectal cancer (Ahn et al., 2013) and depression (Winter et al., 2018).

Faecal sampling offers a simple, non-invasive method to acquire GI microbes for downstream analysis, principally microbial characterisation through next generation sequencing. This process typically involves faecal sample collection, sample storage (if required), DNA extraction, sequencing and bioinformatics analysis to generate a highly individualised snapshot of distal GI microbes and their relative abundance. It should be noted that the faecal microbiota have a limited capacity to provide information about the whole GI tract, given that microbial density and composition vary along it's length (Eckburg et al., 2005). However, it is still the most widely used sampling method for gut microbiome analysis in human based research today. A limitation of faecal sampling analysis lies in the significant impact that sample handling and next generation sequencing parameters have on the resultant microbial profile (Rintala et al., 2017). Considering the contrasting environmental conditions between the colonic lumen and the atmosphere, storage of samples prior to DNA extraction has the potential to significantly alter microbial composition (Choo et al., 2015; Gorzelak et al., 2015; Roesch et al., 2009). Furthermore, DNA

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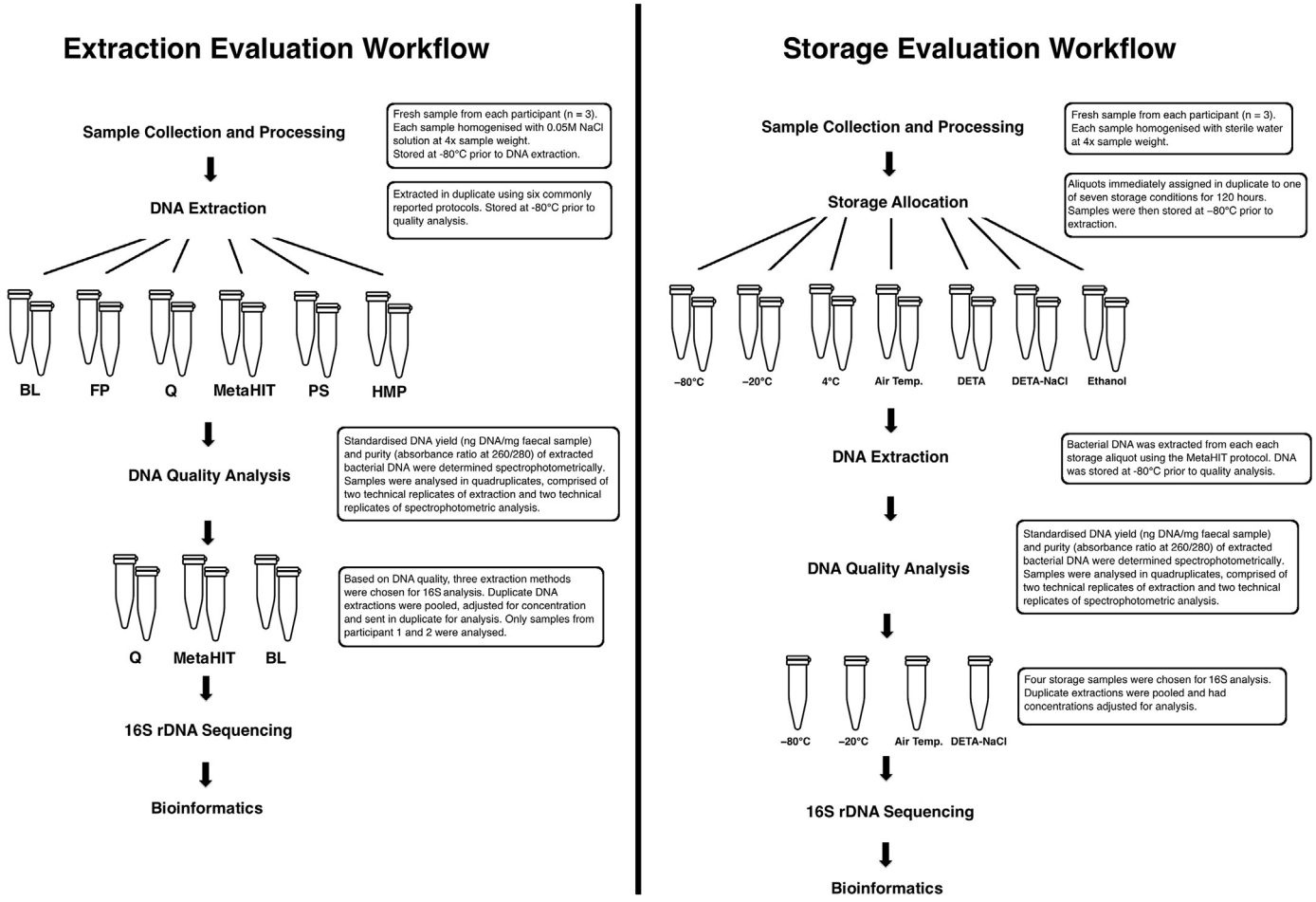

Fig. 1. Experimental design.

extraction methodologies have also demonstrated an ability to skew data (Claassen et al., 2013; Salonen et al., 2010; Wesolowska-Andersen et al., 2014). As such, it is critical to understand how post collection storage, sample handling, and DNA extraction influence microbial composition.

The current gold standard for faecal storage is freezing at −80 °C, with storage at this temperature demonstrating ability to maintain bacterial composition for up to 6 months (Carroll et al., 2012; Fouhy et al., 2015). However, this method is impractical for research participants collecting samples in the home environment. Previous investigations of higher storage temperatures provides conflicting evidence. Some studies show that DNA integrity and microbial composition is maintained with storage at room temperature up to 24 h (Carroll et al., 2012; Tedjo et al., 2015), 4 °C up to 24–72 h (Choo et al., 2015; Tedjo et al., 2015; Wu et al., 2010) and freezing at −20 °C for up to 7 days (Cardona et al., 2012; Tedjo et al., 2015). By contrast, other studies demonstrate that room temperature storage over 12–24 h (Choo et al., 2015; Roesch et al., 2009) and − 20 °C storage over 3–7 days results in significant changes to bacterial composition (Gorzelak et al., 2015). Some have even reported changes in the relative bacterial phyla after just 30 min of exposure to room temperature (Gorzelak et al., 2015). Freeze-thaw cycles, a consideration pertaining to domestic frost-free freezers or during the transfer of samples from home to a laboratory, have been associated with compositional changes by some (Cardona et al., 2012), but not others (Gorzelak et al., 2015). The use of nucleic acid stabilisation agents (in combination with room temperature storage) has also resulted in conflicting data with respect to compositional changes. Some report good stability overtime with use of RNAlater (although different to immediate storage at −80 °C) (Flores et al., 2015), while other report significant changes in DNA yield and quality, as well as bacterial diversity and relative abundance (Dominianni et al., 2014; Gorzelak et al., 2015; Nechvatal et al., 2008).

The confusion around sample storage is compounded by the range of sample handing and extraction procedures reported. Prior to DNA extraction, homogenisation of samples appears to be warranted, given that a single stool sample can vary in bacterial composition when subsampled from different regions (i.e. internal vs. external) (Gorzelak et al., 2015; Hsieh et al., 2016). The method of DNA extraction has also been shown to produce changes in relative bacterial abundance and diversity (Claassen et al., 2013; Salonen et al., 2010; Wesolowska-Andersen et al., 2014). One of the major factors contributing to this may be the ability of an extraction method to provide adequate cell lysis. The use of mechanical (bead beating) disruption of the bacterial cells appears to be important for adequate DNA recovery, bacterial diversity and minimising biased extraction of taxa based on differences in cell wall structure and integrity (Guo and Zhang, 2013; Salonen et al., 2010). While there is no gold standard for DNA extraction, the QIAamp Mini Stool Kit by Qiagen has been commonly used, particularly with the addition of mechanical lysis (Flores et al., 2015; Gorzelak et al., 2015; Hsieh et al., 2016; Zoetendal et al., 2012). However, the use of commercial kits is potentially financially restrictive. As such, large scale human studies such as the Human Microbiome Project (HMP) and European MetaHIT have tended to use other stand alone or modified kit extraction methods (Wesolowska-Andersen et al., 2014).

The conflicting data around sample storage, handling and extraction procedures presents a challenge for researchers aiming to design human-based microbiome trials. Additionally, there is a lack of evidence regarding lab- prepared protocols for faecal DNA extraction.

These methods present a low cost alternative that is often attractive for researchers due to the high cost involved in further downstream analysis of faecal microbial diversity. Therefore the aim of this experiment was two-fold. The first was to compare DNA yield, purity and bacterial composition across a range of DNA extraction methods. These included commercially available kits as well as stand-alone, lab made techniques previously used in large-scale human trials. The second was to compare the impact of a number of storage techniques on bacterial DNA over a 5-day period. The results of these feasibility experiments will support researchers in making informed decisions regarding cost effective experimental design for human microbiome trials that necessitate home storage collection.

2. Material and methods

2.1. Experimental approach

To determine the best approach to faecal sampling and handling, two independent experiments were conducted. The first aimed to examine the impact of different extraction methods on DNA quality, yield and bacterial composition (extraction method assessment). A following experiment aimed to examine the effect of sample storage conditions on DNA quality/yield, as well as faecal microbial community profiles (sample storage assessment). The results of the extraction method assessment directly influenced the DNA extraction method utilized in the sample storage assessment. Both procedures are outlined in Fig. 1.

2.2. Faecal sample collection and processing

2.2.1. Extraction method assessment

Faecal samples were collected from three healthy male volunteers (Participants (P) 1–3, mean age 33.7 ± 7.6 years) for the comparison of DNA extraction methods. Each sample was weighed and homogenised in a commercial grinder (Breville Coffee and Spice, BCG200, Sydney, NSW) with the addition of a sterile 0.05 M NaCl solution (4 °C) at a proportion of 4 times the sample weight. The resulting faecal slurry was separated into 800 mg aliquots and stored at −80 °C prior to extractions. All samples were processed within 30 min of collection.

2.2.2. Sample storage assessment

A further three samples were collected from separate male volunteers (P 4–6, mean age 26.7 ± 2.5 years) to investigate the impact of sample storage conditions of the faecal microbiota. Samples were homogenised as described above, with the exception of sterile milliQ water used in place of a saline solution. Two aliquots from each sample were assigned to one of seven storage conditions (a total of 14 samples per condition): freezing at −80 °C (control), freezing at −20 °C, refrigeration at 4 °C, re-suspension in a 7× volume dimethylsulfoxide-ethylenediaminetetraacetic acid solution (20% DMSO-0.25 M EDTA, pH 8.0)(DETA), re-suspension in a 7× saline saturated DETA solution (20% DMSO-0.25 M EDTA, pH 8.0,)(DETA-NaCl), re-suspension in a 7× volume of 95% ethanol, or storage at room temperature in a dry collection tube (air). Samples were stored in their respective conditions for 120 h and then frozen at −80 °C prior to DNA extraction to control for any impact that freezing may have on bacterial community composition. All samples were processed within 30 min of collection. No participants reported antibiotic exposure within the six months prior to collection.

2.3. DNA extraction methods

2.3.1. Extraction method assessment

Bacterial DNA was extracted in duplicate from each sample (P 1–3) using 6 extraction methods (Table 1). These included four commercially available kits (Bioline ISOLATE Faecal DNA Extraction Kit (BL), FavorPrep® Stool DNA Isolation Mini Kit (FP), PowerSoil® DNA Isolation Kit (PS) & Qiagen QIAamp® DNA Stool Mini Kit (Q) with extractions performed as per manufacturer instructions, and two laboratory developed protocols, Human Microbiome Project (HMP) and the European MetaHIT (MetaHIT) described below.

The HMP protocol utilises the Mobio PowerLyzer PowerSoil DNA isolation Kit (MO BIO Laboratories), with slight modifications as described by Wesolowska-Andersen et al. (Wesolowska-Andersen et al., 2014). In brief, samples were thawed and centrifuged at 14,000 ×*g* for 10 mins before removing the supernatant. Approximately 200 mg of the pellet was transferred to a bead beating tube containing 250 µL of kit lysis buffer and 200 mg of 0.1 mm silica beads. Samples were then heat treated in a dry block bath (Thermoline Scientific) to 65 °C for 10 mins, and then 95 °C for a further 10 mins. Bead beating was performed with two bouts of 5 min at 30 cycles/s, separated by a 10 min rest. The extraction was then performed as per the manufacturer's instructions.

The MetaHIT protocol followed methods outlined by Wesolowska-Andersen et al. (Wesolowska-Andersen et al., 2014) and Godon et al. (Godon et al., 1997) with slight modification. Samples were thawed and centrifuged at 14,000 x*g* for 10 min before removing the supernatant. Approximately 200 mg of the pellet was transferred to a new tube. To each sample 250 µL 4 M guanidine thiocyanate and 40 µL N-lauryl sarcosine (10%) was added before incubation at room temperature for 10 min. 500 µL of N-lauryl sarcosine (5%) was added before each sample was vortexed, briefly centrifuged and heat treated in a dry block bath (Thermoline Scientific) at 70 °C for 2 h. Following this, 300 µL of 0.1 mm silica beads were added and bead beating performed at 30 cycles/s for 5 min, followed by a 10 min rest and a further 5 min of bead beating. The remaining extraction was performed as described by Godon et al. (Godon et al., 1997). The extracted DNA was stored at −80 °C prior to yield, quality and compositional analysis.

2.3.2. Sample storage assessment

Bacterial DNA was extracted in duplicate from each sample (P 4–6) using the MetaHIT protocol outlined above. The extracted DNA was stored at −80 °C prior to yield, quality and compositional analysis.

2.4. DNA quality assessment

Both experiments underwent the same DNA quality control. Standardised DNA yield (ng DNA/mg faecal sample) and purity (absorbance ratio at 260/280) of extracted bacterial DNA were determined using the Nano Drop® 8000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Assessment of DNA quality/quantity via spectrophotometric analysis is a commonly reported method in literature comparing DNA extraction techniques (Claassen et al., 2013; Lee et al., 2010; Wesolowska-Andersen et al., 2014). Acceptable DNA purity was defined as having a 260/280 absorbance ratio ranging between 1.7 and 2.0 with pure DNA at an absorbance of 1.8 (Chen et al., 2010). To account for carryover contamination, each method in the extraction evaluation was also analysed using fluorescence quantification (Qubit 3.0, Invitrogen, Life technologies). The Qubit fluorometer calculates concentration based on the fluorescence of a dye bound to double stranded DNA (dsDNA). Samples were analysed in quadruplicates, comprised of two technical replicates of extraction and two technical replicates of spectrophotometric/fluorescence analysis.

2.5. 16S rDNA sequencing and analysis

2.5.1. Extraction method assessment

Duplicate DNA samples were evenly pooled and adjusted to a concentration of 10 ng/µL. Duplicate samples of the DNA extraction experiment were sent to the Australian Genome Research Facility (AGRF, Brisbane, Australia) for diversity profiling. The V3–V4 region of the 16S rRNA gene was amplified using primers 341F (5-CCTAYGGGRBGCASCAG-3) and 806R (5- GGACTACNNGGGTATCTAAT-3). Thermocycling

Table 1
DNA extraction methods utilized in this study.

Extraction method	Manufacturer	Kit Abbreviation	Recommended faecal starting amount (mg)	Elution volume (μl)
ISOLATE Faecal DNA Extraction Kit	Bioline	BL	150	100
FavorPrep® Stool DNA Isolation Mini Kit	Favorgen	FP	200	100
DNEasy Powerlyzer PowerSoil® DNA Isolation Kit	MoBio	PS	250	100
QIAamp® DNA Stool Mini Kit	Qiagen	Q	180–220	200
Human Microbiome Project	Modified PowerSoil® Kit	HMP	200	100
European MetaHIT	NA	MetaHIT	200	400

was performed on an Applied Biosystem 384 Veriti and using AmpliTaq Gold 360 mastermix (Life Technologies, Australia) under the following conditions: Initial denaturing at 95 °C for 7 min followed by 29 cycles of 94 °C × 45 s, 50 °C × 60 s, and 72 °C × 1 min. The reaction was terminated after a 7 min extension at 72 °C. Amplicons were then prepared and sequenced on the Illumina MiSeq platform, using the Illumina Nextera XT Index with paired end sequencing. Raw paired-end Illumina reads were trimmed using Cutadapt (https://cutadapt.readthedocs.io/en/stable/). Sequence analysis was performed using Quantitative Insights Into Microbial Ecology 2 (QIIME2-2018.4) (Caporaso et al., 2010). DNA sequences underwent quality filtering resulting in an average of 56,816 ± 24,970 reads per sample (56,834 ± 24,979 prior to filtering). Sequences were then clustered into operation taxonomic units (OTUs) following the default QIIME2 pipeline with referencing to 97% sequence similarity against the Greengenes database version 13.8 (DeSantis et al., 2006). For alpha and beta diversity, samples were rarefied to 6800 reads. Alpha diversity metrics included observed OTUs, Chao1, Shannon index, Simpson index and Faith phylogenetic diversity. Beta diversity was assessed with Bray-Curtis dissimilarity, unweighted UniFrac and weighted UniFrac metrics. Visualisation was performed using principal coordinated analysis (PCoA). Analysis and visualisation of relative abundance at the phylum/genus level was performed using R packages: "tidyverse"(Wickham, 2017), "qiime2R", "phyloseq" (McMurdie and Holmes, 2013), "DESeq2" (Love et al., 2014) and "ggplot2" (Wickham, 2016) (R 3.5.0). To ensure adequate coverage of sample taxa, sequences were also aligned to 99% similarity against the SILVA bacterial database (SILVA 132 release) (Quast et al., 2012). However, only minor variations were observed in taxonomic assignment to genus variance when compared to taxonomic assignment using the Greengenes database (Supplementary Fig. 1).

2.5.2. Sample storage assessment

Duplicate DNA samples were evenly pooled and adjusted to a concentration of 10 ng/μL. Samples were sent to the Ramaciotti Centre for Genomics (Sydney, Australia) for diversity profiling. The V4 region of the 16S rRNA gene was amplified using primers 515F 5-GTGCCAGCMGCCGCGGTAA-3) and 806R (5-GGACTACHVHHHTWTCTAAT-3). Thermocycling was performed on a Platinum Hot Start PCR Master Mix (ThermoFisher Scientific) under the following conditions: Initial denaturing at 94 °C for 3 min followed by 35 cycles of 94 °C × 45 s, 50 °C × 60 s, and 72 °C × 1 min 30 s. The reaction was terminated after a 10 min extension at 72 °C. Amplicons were then prepared and sequenced on the Illumina MiSeq platform, using the Illumina Nextera XT Index and paired end sequencing. Sequences were processed as described above. As above, sequences were also aligned to 99% similarity against the SILVA bacterial database (SILVA 132 release) to ensure adequate taxa coverage. An average of 176,607 ± 18,885 reads per sample were obtained (176,609 ± 18,886 prior to filtering). For alpha and beta diversity, samples were rarefied to 55,000 reads. To account for background reagent contamination, a negative control sample (blank extraction performed of DNA free water) was included in sequencing and analysis pipelines. A contribution from bacteria belonging to the Proteobacteria phylum was detected. However, the bacterial load detected using qPCR amplifying the 16S rRNA was 5 orders of magnitude lower in the reagent negative control compared to samples (5×10^1 vs 5×10^6, results not shown) and therefore the reagent contribution to the overall diversity profile was deemed as negligible.

2.6. Statistical analysis

Standard descriptive statistics and comparison of yield and purity was completed with SPSS Statistics for Windows (Version 22.0. Armonk, NY: IBM Corp). The Kolmogorov-Smirnov test was used to check the normality of data distribution. Comparisons of normally distributed data were made by paired *t*-tests for intra-group comparisons and un-paired tests for inter-group comparisons. One-way ANOVA was used to compare multiple means. Non-parametric data was compared using Mann–Whitney *U* tests (unpaired) and Kruskal-Wallis test for multiple means.

2.7. Ethics

This study was approved by the Human Research Ethics Committee of the University of New England, Australia (Approval no. HE15-294).

3. Results

3.1. Extraction method assessment

3.1.1. DNA yield and quality

Normalised yield rates (ng of DNA/mg starting faecal mass) for the six DNA extraction methods are presented in Fig. 2a. The MetaHIT extraction technique demonstrated a significantly higher DNA yield (MetaHIT mean yield ± SE = 131 ± 8.3 ng/mg) than the remaining methods (combined mean yield ± SE = 29.6 ± 4.7 ng/mg). Both the Q and HMP protocols resulted in significantly lower DNA yields when compared to BL (Q, $p = 0.006$; HMP, $p < 0.001$), PS (Q, $p < 0.001$; HMP, $p < 0.001$) and FP (Q, $p = 0.004$; HMP, $p < 0.001$) kits. Results presented here are based on fluorescence data.

With regard to DNA purity (260/280 absorbance ratio), the MetaHIT extraction protocol also resulted in superior quality (mean ratio ± SE = 1.80 ± 0.02) when compared to all other methods (Fig. 2b). The HMP protocol resulted in the largest degree of contamination (mean ratio 2.50 ± 0.15) and was significantly higher than the remaining kits (FP; $p = 0.02$, PS; $p < 0.001$, BL; $p < 0.001$, and Q; $p = 0.002$). The BL protocol demonstrated a significantly lower absorbance ratio (mean ratio 1.51 ± 0.05) when compared to FP ($p = 0.001$), PS ($p < 0.001$) and Q ($p < 0.001$).

3.1.2. Microbial diversity and composition

To determine the effect of extraction protocol on the microbial (bacterial) community composition, total DNA samples extracted by three different methods for participants one and two were analysed by 16S rRNA gene sequencing. Extractions performed with BL, MetaHIT and Q protocols were chosen based on the parameters of yield, purity and popularity of use. Bacterial alpha diversity indicated a trend towards increased diversity of BL and MetaHIT protocols when compared to Q over a number of metrics, however this trend was not statistically significant (Table 2).

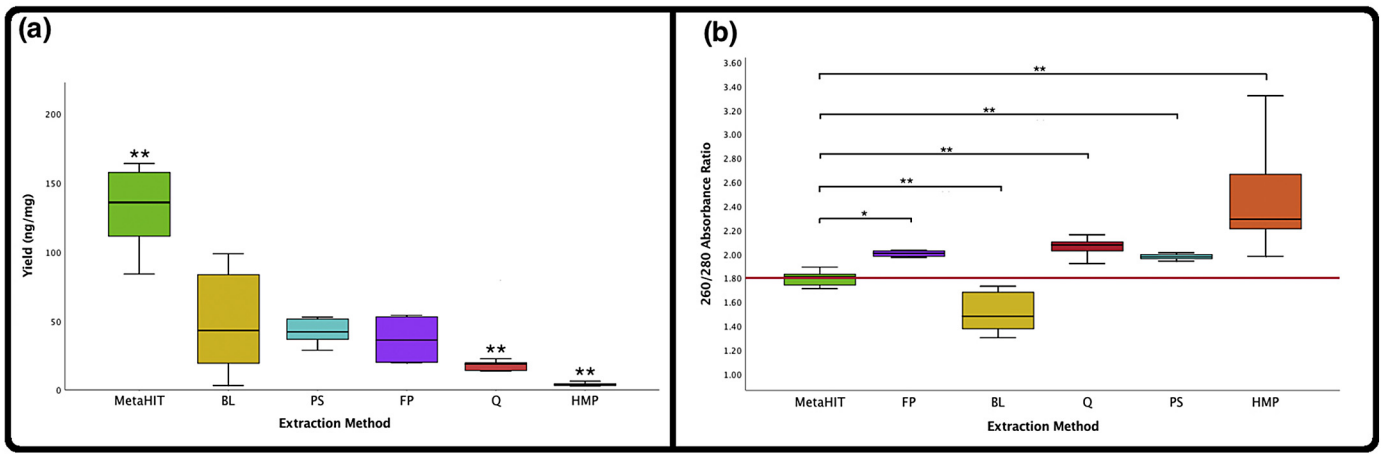

Fig. 2. DNA yield and quality for extraction method assessment. (a) DNA yield (normalised for 1 mg of starting faecal mass) between extraction methods. ** $p \leq 0.01$ to all other extraction methods. (b) DNA purity (260/280 absorbance ratio) of DNA isolated using various extraction methods. DNA was extracted in duplicate from each sample provided by participants 1–3. Extractions were analysed twice producing two technical replicates. Median values are indicated by the line within the box plot. The box extends form the 25th to 75th percentile with whiskers indicating maximum and minimum values. Data points considered outliers are represented by the circles. * $p < 0.05$; ** $p \leq 0.01$.

Principal Coordinates Analysis (PCoA) of Bray-Curtis distances revealed distinct separation according to participant (Axis 1, 60.23%) and extraction method (Axis 2, 23.39%) (Fig. 3a). Analysis of weighted UniFrac distances (taking into account OUT abundance as oppose to OUT presence/absence used in unweighted UniFrac calculations), found greater separation by extraction (PERMANOVA, p = 0.001) then by participant ($p = 0.051$) (Fig. 3b). With further analysis by pairwise PERMANOVA, this difference was evident when comparing BL to Q ($p = 0.027$) and BL to MetaHIT ($p = 0.037$).

Considerable variation was observed in the relative phyla contribution, with Firmicutes ($P < 0.001$), Bacteroidetes ($P = 0.003$) and Actinobacteria ($P = 0.003$) demonstrating significant differences across the three extraction methods tested (One-way ANOVA) (Fig. 3c). Consistent with weighted UniFrac analysis presented above, pairwise analysis revealed this difference across extractions to be driven by the BL method, with all phyla demonstrating significant differences to both MetaHIT and Q methods (data not shown). Refined taxonomic analysis revealed Actinobacteria (genera *Collinsella* & *Adlercreutzia*) and Firmicutes (genera *Oscillospira*, *Blautia*, *Streptococcus*, *Coprococcus* and *SMB53*) phylum were significantly more dominant in BL samples when compared to Q samples (Fig. 3d). MetaHIT demonstrated a smaller contribution from phyla Bacteroidetes (40.7 ± 5.2% vs 54.4 ± 7.5%; $P = 0.038$) and Proteobacteria (1.7 ± 0.5% vs 3.2 ± 1.3%; $P = 0.022$) when compared to Q, however further analysis did not define these phyla differences to specific genus.

3.2. Storage method assessment

3.2.1. DNA yield and quality

Following storage treatment, all samples were frozen at −80 °C before extraction with the MetaHIT protocol. DNA yield and purity were assessed to determine the impact of storage methods on these variables. Standardised yield (ng/mg faeces) for each storage treatment is presented in Fig. 4a. A comparison to the control treatment revealed no significant differences in yield, however higher temperature storage conditions did result in significantly higher DNA yield when compared to immediate buffer solutions (Fig. 4a).

DNA purity across storage treatments demonstrated no statistical differences (Fig. 4b). Acceptable purity was observed for most treatments with mean absorbance between 1.8 and 2.0 observed, although ethanol demonstrated a slightly higher absorbance value (mean absorbance = 2.02 ± 0.13) and variability.

3.2.2. Microbial diversity and composition

To determine the effect of sample storage conditions on microbial composition, four treatment methods for each participant were sent for 16S rRNA analysis. Storage treatments air, −20 °C and DETA-NaCl were chosen (along with −80 °C control) due to their ease of application to human research and because no apparent advantage was evident based on yield and purity data (described above). Bacterial alpha diversity of samples was not dependent on sample storage conditions (Table 3).

Relative phyla composition also demonstrated stability across storage methods (One-way ANOVA between treatments; Firmicutes, $P = 0.899$; Bacteroidetes, $P = 0.874$; Actinobacteria, $P = 0.592$; Proteobacteria, $P = 0.211$) and Other, $P = 0.998$ (Fig. 4c)). When analysed at a lower taxonomic level, two genera were significantly elevated with air storage, *Pseudomonas* (Proteobacteria) and *Solibacillus* (Firmicutes), when compared to −80 °C control (data not shown).

Stability in the bacterial community across different storage methods was re-enforced with PCoA of Bray-Curtis distances revealing distinct clustering according to participant (Fig. 4d). This finding was reflected in further PERMANOVA analysis, with significant differences observed between participants (unweighted UniFrac $p = 0.002$; weighted UniFrac $p = 0.001$), but not between storage methods (unweighted UniFrac $p = 0.974$; weighted UniFrac $p = 0.873$).

Table 2
Microbial diversity across selected extraction methods.

Extraction method	Qiagen	MetaHIT	Bioline	*p*-value (Kruskal-Wallis)
Observed OTUs (mean ± SD)	136 ± 13.4	147 ± 13.7	157 ± 22.3	0.246
Chao1 (mean ± SD)	149 ± 21.1	154 ± 13.9	167 ± 18.0	0.276
Shannon Index (mean ± SD)	4.92 ± 0.69	5.09 ± 0.34	5.22 ± 0.51	0.668
Simpson Index (mean ± SD)	0.913 ± 0.056	0.937 ± 0.023	0.943 ± 0.022	0.584
Faith PD (mean ± SD)	8.84 ± 1.05	9.40 ± 1.10	10.45 ± 1.48	0.147

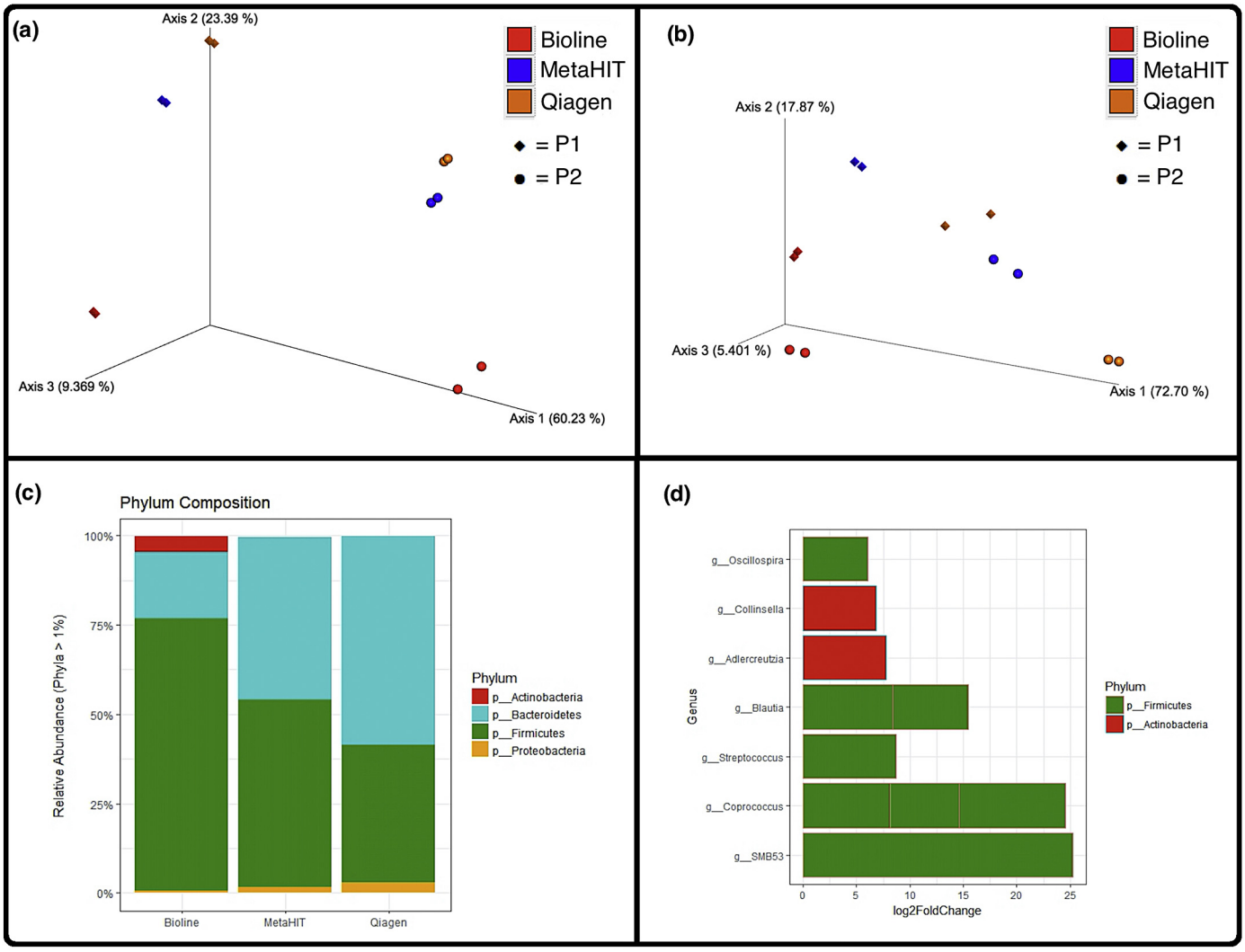

Fig. 3. Microbial beta diversity and composition compared across extraction methods. (a) sample clustering due to extraction method by PCoA (PC1 vs. PC2 vs. PC3), based on Bray-Curtis similarity distance. Combined coordinate axes represent 92.99% of the total variation between groups. (b) sample clustering due to extraction method by PCoA (PC1 vs. PC2 vs. PC3), based on weighted UniFrac distance. Combined coordinate axes represent 95.97% of the total variation between groups. (c) mean relative phylum distribution across selected extraction methods. (d) shows log 2 fold change of significantly increased genus (Wald test, adjusted (Bonferonni) $p < 0.001$) in BL samples compared to Q sample.

4. Discussion

Faecal sample storage and DNA extraction represent critical, yet irreversible factors that must be considered early in human microbiome trial design. The aim of this study was to explore the effect of commonly used faecal storage and DNA extraction techniques on the resultant microbial profile, to provide a guide for future human microbiome experiments. Overall, the results presented here indicate that the choice of DNA extraction method has a greater influence on the bacterial community structure revealed by 16S rDNA sequencing than the choice of short-term sample storage.

Quality of DNA extraction technique was assessed based on yield, purity and 16S rRNA gene sequencing. Decreased yield in genomic DNA has the potential to insufficiently characterise the microbial communities and importantly, impair ability to detect low quantity microbes of interest to human health and disease status (Claassen et al., 2013). Lower DNA yield with Q and HMP (which utilises PS kit) protocols has been previously reported (Ariefdjohan et al., 2010; Claassen et al., 2013; Scupham et al., 2007; Wesolowska-Andersen et al., 2014). This is of importance, given that the HMP protocol is a standardised institutional procedure (part of the U.S based National Institutes of Health) used in the characterisation of human microbes across a range of body habitats. Of the protocols tested here, MetaHIT demonstrated superior yield over the other tested methods (Fig. 2a), as previously reported when compared to the HMP protocol (Wesolowska-Andersen et al., 2014). Additionally, spectrophotometric analysis also revealed the MetaHIT protocol resulted in pure DNA extraction, although most other kits fell within acceptable purity limits (Chen et al., 2010). Based simply on DNA yield, purity and quality, these findings suggest that the relatively inexpensive MetaHIT protocol performs at least as good as expensive, commercially available kits. This finding was also reflected in rat-based data produced by the same lab (data not shown). To the authors' knowledge, this is the first instance where MetaHIT has been compared to commercial kits.

Sequenced based community analysis (BL, MetaHIT and Q only), revealed a slight increase in community diversity with BL and MetaHIT extractions over Q, although this result was not statistically significantly (Table 2). Furthermore, BL samples tended towards higher diversity despite a significantly lower yield than MetaHIT. Reassuringly, this suggests that sufficient DNA material was captured from all methods. Weighted UniFrac distances (taking into account OTU abundance) (Fig. 3b) did reveal the bacterial community extracted with the

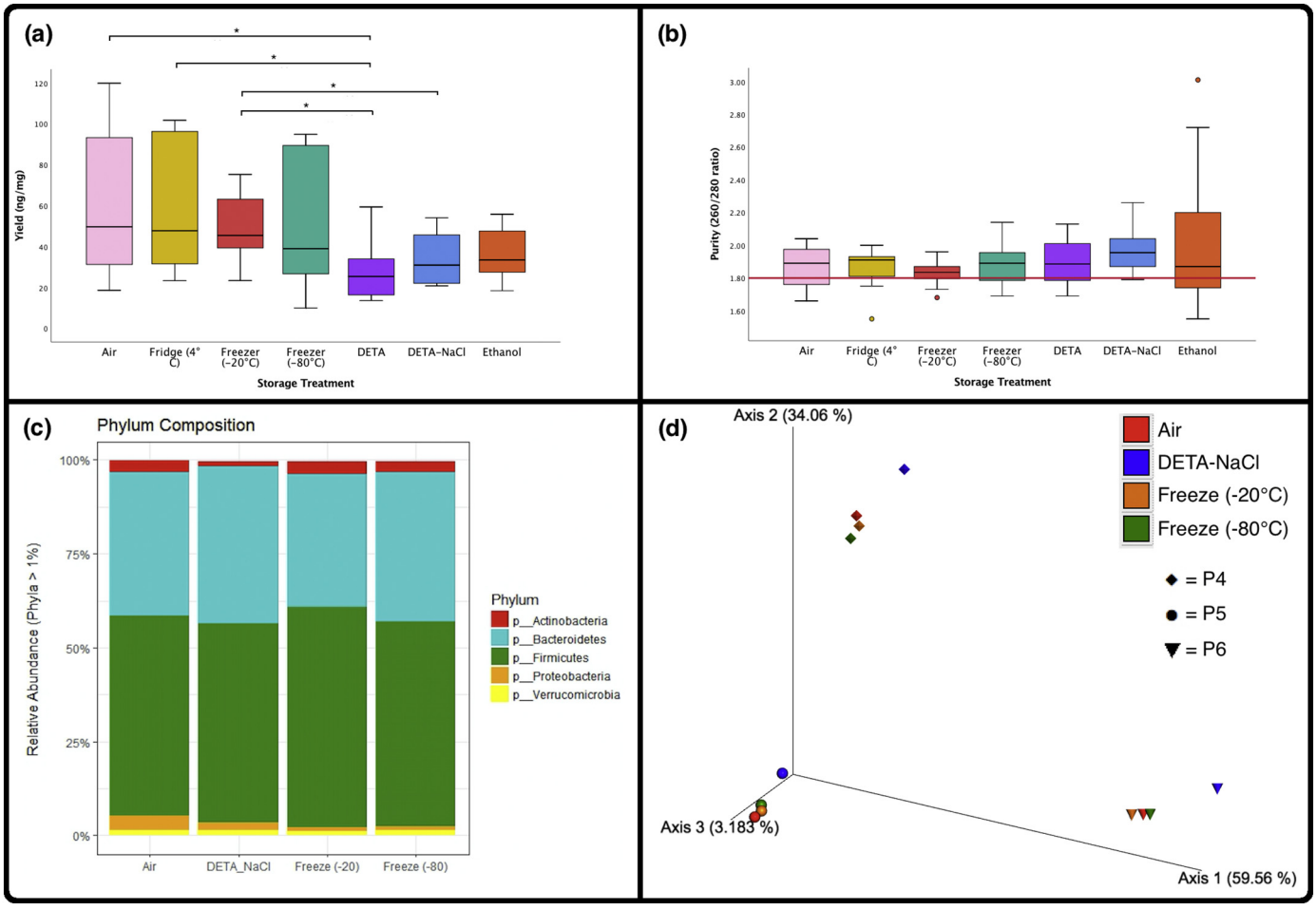

Fig. 4. DNA quality, microbial composition and beta diversity across storage methods. (a) DNA yield (normalised for starting faecal mass) of the tested storage methods. (b) DNA purity (260/280 absorbance ratio) of the tested storage methods. DNA was extracted in duplicate from each sample provided by participants 4–6. Extractions were analysed twice producing two technical replicates. Median values are indicated by the line within the box plot. The box extends form the 25th to 75th percentile with whiskers indicating maximum and minimum values. Data points considered outliers are represented by the circles. * $p < 0.05$; ** $p \leq 0.01$. (c) mean relative phylum distribution across selected storage methods. (d) sample clustering due to storage condition by PCoA (PC1 vs. PC2 vs. PC3), based on Bray-Curtis similarity distance. Combined coordinate axes represent 96.80% of the total variation between groups.

BL method to diverge from Q and MetaHIT samples. This finding was confirmed with genus level analysis indicating significantly increased taxa in BL samples when compared to Q samples (Fig. 3d). This difference was born out with increases in microbes belonging to Actinobacteria and Firmicutes phyla (data supported when aligned to the SILVA database – Sup. Fig. 1). To note, increased genus include genera such as *Oscillospira* and *Blautia*, which have been associated with health/gut status in humans (Hsiao et al., 2014; Konikoff and Gophna, 2016). The variability between extraction methods is of high importance when attempting to compare studies using alternate extraction methods. It is also prudent to remember that this is only one of the many factors affecting microbial diversity analysis and other factors such as variable region amplification, OTU assignment and 16S database selection has considerable weight on the resultant profile (Morgan and Huttenhower, 2012).

The second aim of this experiment was to compare a range of logistically viable storage solutions for human microbiome research. The findings presented here are in agreement with previous work (Carroll et al., 2012; Flores et al., 2015; Tedjo et al., 2015), with −20 °C and nucleic acid stabilisation agents (DETA-NaCl) demonstrating equivalent bacterial composition when compared to −80 °C storage for up to 7 days storage. Overall, sample storage conditions had little effect on DNA purity with minor differences observed in yield in comparison to the control treatment (Fig. 4a).

With regard to bacterial diversity and compositional analysis at the phyla level, no significant variance between Air, −20 °C, DETA-NaCl and − 80 °C storage solutions were observed. At the genus level, two bacterial groups showed increased abundance in Air samples

Table 3
Microbial diversity across storage treatment methods.

Data set	−80 °C	−20 °C	Air	DETA-NaCl	p-value (Kruskal-Wallis)
Observed OTUs (mean ± SD)	227 ± 40.5	238 ± 47.3	239 ± 40.5	238 ± 32.9	0.776
Chao1 (mean ± SD)	230 ± 40.2	241 ± 47.3	243 ± 37.3	240 ± 33.1	0.668
Shannon Index (mean ± SD)	5.35 ± 0.23	5.44 ± 0.21	5.42 ± 0.12	5.33 ± 0.23	0.764
Simpson Index (mean ± SD)	0.946 ± 0.015	0.954 ± 0.012	0.950 ± 0.005	0.944 ± 0.016	0.863
Faith PD (mean ± SD)	12.7 ± 1.76	13.3 ± 1.80	13.3 ± 1.75	13.5 ± 1.38	0.668

(compared to −80 °C); *Pseudomonas* (Proteobacteria) and *Solibacillus* (Firmicutes). This is not surprising given that both genus constitute aerobic microbes and hence have the potential to proliferate in this storage environment (Krishnamurthi et al., 2009; Palleroni, 2015). As such, these microbes should be taken into account by any studies using room temperature storage prior to sample collection and/or extraction. Overall, inter-individual variation far exceeded any composition changes present with sample storage (Fig. 4d).

The results of this experiment should be viewed in light of a number of limitations. While every effort was made to ensure reliability through technical replication, the sample size was nevertheless relatively small, with three biological samples used for each experiment. However, the authors are confident that the data presented here has real world application in terms of trial design. It should also be noted that these results relate to very specific sample analysis techniques such as variable region amplification, sequencing provider, OTU assignment, 16S database selection and bioinformatics processing. Of particular relevance to this data, sequencing providers differed between experiments. While no comparisons were drawn between these data sets, it is important to recognise the significant impact amplification and sequencing could have on results. As such, researchers dealing with large cohort data sets need to carefully consider the impact of these steps and be sure to include negative controls to account for any reagent contamination during extraction preparations, as well as positive controls to account for sequencing variance if batch analysis is required.

5. Conclusion

DNA extraction techniques and sample storage conditions are pivotal choices researchers must make prior to data collection as they are irreversible (unlike downstream analyses such as 16S rDNA sequencing and bioinformatic analyses) and have the potential to significantly alter microbial detection. As such, future study design should focus on standardising sample storage and handling techniques. Results presented here suggest that the relatively inexpensive extraction method provided by the MetaHIT protocol can produce bacterial DNA of sufficient yield, purity and comprised of a bacterial community composition comparable to more expensive, commercially available kits. Furthermore, low temperature storage solutions such as those seen in the home freezer environment, as well as lab made DNA buffer solutions both appear to be sufficient to maintain bacterial community composition for up to 5 days prior to long term storage and extraction. It is suggested that these findings could be used as a framework for cost effective human based microbiome research that requires home sample storage.

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Declaration of Conflict of Interests

The authors declare no conflict of interests.

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