Assessment of stool collection and storage conditions for in vitro human gut model studies

Assessing collection and storage conditions for stool samples to be used for *in vitro* human gut model studies

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Abstract

Background: The role of the gut microbiota in health and disease is becoming increasingly apparent. Faeces is the most accessible sample to collect from human volunteers for studying the gut microbiota. However, the impact of stool collection and storage conditions on microbial and metabolic profiles have not been fully evaluated. By understanding the effect of different stool collection and storage conditions on microbial and metabolic composition, we can consider these parameters in the design of *in vitro* fermentation studies.

Methods: Stool samples from 3 volunteers were stored under 5 different conditions to mimic methods that researchers may use to collect and store stool samples for study of the gut microbiota, including: fresh sample used within 10 minutes; stored on wet ice (4°C) for 60 minutes; stored in an anaerobic chamber in a temperature-controlled bag (4°C) for 60 minutes; freezing at -20°C for 60 minutes and freezing at -20°C for 60 minutes and then at -80°C for 2 weeks. The stored samples were added to basal medium in batch culture fermenters alone (negative control) or with 5 g 2'-Fucosyllactose (2'FL) Human Milk Oligosaccharide (HMO) (as a positive fermentation control). Samples were collected at 3 timepoints (0, 12 and 24hours) for analysis by Flow Cytometry-Fluorescent *In Situ* Hybridisation (FC-FISH) and ^{1}H-Nuclear Magnetic Resonance (NMR) spectroscopy to assess the impact on microbial and metabolic profiles, respectively.

Results: Freezing stool significantly impacted microbial numbers and activity during *in vitro* fermentations, whereas storing the stool on wet ice (4°C) or in an anaerobic chamber at 4°C for 60 minutes had minimal effects on microbial and metabolic profiles throughout the 24 hour fermentation batch cultures.

Discussion: For *in vitro* batch culture fermentation studies where it may not be practical or possible to use fresh stool, either storing the stool on wet ice (4°C) or in an anaerobic chamber at 4°C for 60 minutes could be plausible alternatives to maintain microbial and metabolic profiles for analysis.

Keywords: Gut microbiota, gut metabolome, stool storage, *in vitro* batch cultures, flow cytometry-fluorescent *in situ* hybridisation (FC-FISH), ^{1}H-NMR spectroscopy

1. Introduction

Interest in microbes that make up the gut microbiome, as well as the metabolites they produce, has increased as their importance and impact on human health and disease has become more evident. Associated diseases vary from gut disorders such as gastroenteritis, Irritable Bowel Syndrome (IBS) and Inflammatory Bowel Disease (IBD); to neurological disorders such as depression and autism; and other systemic conditions such as diabetes and arthritis as well as many others (Ghaisas et al., 2016).

Stool samples are generally considered the reference standard (Fair et al., 2019) to study the gut microbiota as they are non-invasive (Fu et al., 2016) and can be analysed for microbes and metabolites (Um et al., 2019). There have been studies to compare the effect that collection and storage methods have on gut microbial composition (Cardona et al., 2012; Choo et al., 2015; Fouhy et al., 2015; Gorzelak et al., 2015; Roesch et al., 2009; Tedjo et al., 2015; Um et al., 2019; Williams, et al., 2019; Wu et al., 2010) and metabolic profiles (De Spiegeleer et al., 2020; Gratton et al., 2016; Liang et al., 2020; Mandal et al., 2020), however, our searches did not find studies that had analysed the effect of collection and storage conditions on the microbial and metabolic profiles of stool collected for studying *in vitro* human gut model fermentation experiments. Standardisation of methods to study the human microbiome is important to achieve results that can be compared between different studies (Fu et al., 2016).

Our study aims to determine the effect of different storage conditions on microbial and metabolic profiles of stool that could allow flexibility for volunteers and researchers involved in *in vitro* human gut microbiome studies. Objectives were to determine which storage condition impacted on microbial and metabolic profiles least and resembled results from the fresh stool in subsequent *in vitro* human gut model fermentations.

2. Method

2.1. Materials and substrates

The 2'-Fucosyllactose (2'FL) Human Milk Oligosaccharide (a fermentable substrate) was used as a positive control in this study and obtained from Glycom (Hørsholm, Denmark). Custom DNA oligonucleotides, which were designed to target typical bacteria species found in the gut, had fluorescent probes attached (Table 1) and were obtained from Eurofins Genomics (Ebersberg, Germany).

All other materials and substrates were obtained from Sigma-Aldrich (Dorset, UK) and Fisher Scientific (Leicestershire, UK). Phosphate-buffered saline (PBS) solution was prepared to a final concentration 1 x PBS (pH 7.4).

Short name	Probe name	Target species	Fluorescence	Sequence (5' to 3')	Reference
NON-EUB	Non Eub	Negative control	Alexa488 & Alexa 647	ACTCCTACGG GAGGCAGC	(Wallner et al., 1993)
EUB	Eub338 Eub338 II Eub338 III	Total bacteria	Alexa488 & Alexa 647	GCTGCCTCCC GTAGGAGT GCAGCCACCC GTAGGTGT GCTGCCACCC GTAGGTGT	(Daims et al., 1999)
BIF	Bif164	Most *Bifidobacterium*	Alexa647	CATCCGGCATT ACCACCC	(Langendijk et al., 1995)
LAB	Lab158	*Lactobacillus and Enterococcus*	Alexa647	GGTATTAGCAY CTGTTTCCA	(Harmsen et al., 1999)
BAC	Bac303	*Bacteroides and Prevotella*	Alexa647	CCAATGTGGGG GACCTT	(Manz et al., 1996)
EREC	Erec482	*Clostridium* clusters XIVa and XIVb	Alexa647	GCTTCTTAGTC ARGTACCG	(Franks et al., 1998)
RREC	Rrec584	*Roseburia* and *Eubacterium* group (subgroup of cluster XIVa)	Alexa647	TCAGACTTGCC GYACCGC	(Walker et al., 2005)
ATO	Ato291	*Atopobium* cluster	Alexa647	GGTCGGTCTCT CAACCC	(Harmsen et al., 2000)
PROP	Prop853	*Clostridium* cluster IX	Alexa647	ATTGCGTTAAC TCCGGCAC	(Walker et al., 2005)
FPRAU	Fprau655	*Faecalibacterium prausnitzii*	Alexa647	CGCCTACCTCT GCACTAC	(Devereux et al., 1992)
DSV	DSV687	Most *Desulfovibrionale*	Alexa647	TACGGATTTCA CTCCT	(Hold et al., 2003)

		s and many *Desulfuromonales*			
CHIS	Chis150	*Clostridium* clusters I and II	Alexa647	TTATGCGGTAT TAATCTYCCTTT	(Franks et al., 1998)

Table 1. Probes used for flow cytometry-fluorescence in situ hybridisation (FC-FISH) analysis of bacterial populations. Sequences were selected in a literature review for high specificity and low cross-reactivity. Fluorescent dyes, Alexa488 and Alexa647, were tagged to the 5' end to identify the controls and specific groups of microorganisms, respectively.

2.2. Stool sample preparation

2.2.1. *Collection*

This study was conducted under ethical approval granted by the University of Reading's Research Ethics Committee (UREC 15/20). Following consent, fresh stool samples for evaluation in this study were provided by three healthy donors (D1-3). Participants recruited were all female, aged between 26 and 29 years old and had not recently taken antibiotics.

2.2.2. Storage

To evaluate the impact of storage parameters on stool used in *in vitro* human gut model studies, an aliquot of each donor's stool sample was stored under the following conditions:

C1. Fresh (sample used within 10 minutes).

C2. Wet ice (4°C) for 60 minutes which could be equivalent to storing the stool in a temperature-controlled freezer bag with an ice pack (4˚C) or storing in the fridge (4˚C) for an hour after collection. This was considered a practical approach for volunteers who could not transport a sample immediately to the laboratory.

C3. Anaerobic chamber in a temperature-controlled bag (4°C) for 60 minutes. This was considered another practical approach for volunteers who could not transport a sample immediately to the laboratory. The preservation of anaerobic bacteria is important, and the anaerobic chamber provides one such mechanism to do this.

C4. Frozen at -20°C for 60 minutes which could be stored in a domestic or laboratory setting and thawed on wet ice (4°C) prior to use which mimics the option for the volunteer to transfer the sample in a temperature-controlled bag with an ice pack. Freezing is another approach that has been demonstrated to preserve bacterial communities.

C5. Frozen at -20°C for 60 minutes and then at -80°C for 2 weeks and thawed on wet ice (4°C) prior to use. This experimental condition was designed to mimic scenarios where it may not be possible to analyse samples immediately or shortly after transport to the laboratory and may require a short period of storage. Deep freezing at -80˚C is considered an optimal approach for maximum bacterial viability (De Paoli, 2005).

2.3. Batch culture vessels preparation

Sterile batch culture vessels containing 135 ml autoclaved basal medium [2 g/l peptone water; 2g/l yeast extract; 0.1 g/l NaCl; 0.04 g/l K_2HPO_4; 0.04 g/l KH_2PO_4; 0.01 g/l $MgSO_4.7H_2O$; 0.01 g/l $CaCl_2.6H_2O$; 2 g/l NaHCO3; 0.5 g/l L-Cysteine HCl; 0.5 g/l bile salts; 0.05 g/l hemin (dissolved in a few drops of 1 mol/l NaOH); 2 ml/l Tween 80; 0.01 ml/l vitamin K and 4 ml/l resazurin solution (0.025

155g/100 ml)] were inoculated with 15 ml faecal slurry. Faecal slurry was prepared using stool from each of the donors after being stored by each condition (C1-5) which was then diluted in 1 x PBS (pH 7.4) with 10% (w/v) dilution and mixed in a stomacher for 2 minutes.

Thirty batch culture vessels in total for 3 donors, 2 controls and 5 conditions were set up. Odd-numbered vessels (V1/3/5/7/9) were negative controls with no substrate added, and even-numbered vessels (V2/4/6/8/10) were positive controls with 5 g of 2'FL added. Faecal slurries from stool samples stored in various conditions were then added to the vessels: V1 and V2 had fresh stool used within 10 minutes (C1) added; V3 and V4 had stool that had been stored on wet ice (4°C) for 60 minutes (C2) added; V5 and V6 had stool stored in an anaerobic chamber in a temperature-controlled bag (4°C) for 60 minutes (C3) added; V7 and V8 had stool that had been frozen at -20°C for 60 minutes (C4) added and V9 and V10 had stool that had been frozen at -20°C for 60 minutes and then at -80°C for 2 weeks (C5) added. The study design illustrating which stool storage conditions were used for each vessel is shown in Table 2.

Negative control	Positive control	Condition
V1	V2	C1. Fresh sample used within 10 minutes
V3	V4	C2. Stored on wet ice (4°C) for 60 minutes
V5	V6	C3. Stored in an anaerobic chamber in a temperature-controlled bag (4°C) for 60 minutes
V7	V8	C4. Frozen at -20°C for 60 minutes
V9	V10	C5. Frozen at -20°C for 60 minutes and then at -80°C for 2 weeks

Table 2. Conditions (C1-5) that human stool samples were stored in prior to being added to anaerobic batch culture vessels (V1-10). The odd-numbered vessels (V1/V3/V5/V7/V9) represent the negative controls where no additional substrates were added, whereas the positive-numbered vessels (V2/V4/V6/V8/V10) represent the positive controls which had 5g 2'FL HMO carbohydrate was added. The above vessels were set up for each donor (D1-3).

These vessels were kept at 37°C using a water jacket and pH was controlled consistently at 6.8. A flow of nitrogen gas (15 ml/h) was used to maintain an anaerobic environment and contents of the vessels stirred throughout the experiment with magnetic stirrers. A volume of 5 ml was taken at 0, 12 and 24 hours for analysis by FC-FISH and ^{1}H-NMR spectroscopy.

2.4. Flow cytometry-fluorescent in-situ hybridisation (FC-FISH)

2.4.1. *Probe design, selection and validation*

Specific details of all probes used are shown in Table 1 which were selected from a review of the literature for their high specificity and lack of cross-reaction with other species. All probes were labelled with Alexa488 or Alexa647 fluorescent dyes to their 5' end, prepared to yield 50 ng/µl and kept at -20°C.

Probes were selected for major bacterial groups which are commonly found in the human gut. BIF, LAB, BAC, EREC, RREC, ATO, PROP, FPRAU, DSV and CHIS were chosen to quantify *Bifidobacterium*, *Lactobacillus/Enterococcus, Bacteroides/Prevotella, Clostridium* clusters XIVa and XIVb, *Roseburia/Eubacterium, Atopobium* cluster, *Clostridium* cluster IX, *Faecalibacterium prausnitzii, Desulfovibrionales/Desulfuromonales* and *Clostridium* clusters I and II, respectively (Table 1).

2.4.2. *Sample preparation*

Fixation of microbial cells. 750 µl of batch culture samples from 0, 12 and 24 hours post-inoculation was centrifuged at 11,337 *g* for 5 minutes. The supernatant was discarded, and pellet re-suspended in 375 µl of 1 x PBS (pH 7.4). 1125 µl of cold 4% (w/v) paraformaldehyde (PFA) was added to this, vortexed and kept at 4°C for between 4 and 8 hours. Samples were centrifuged at 11,337 *g* for 5 minutes at room temperature and washed twice in 1 ml cold 1 x PBS (pH 7.4). The pellet was re-suspended in 150 µl 1 x PBS (pH 7.4), mixed with 150 µl of ethanol and stored at -20°C.

Permeabilisation of the cell walls. 75 µl of defrosted and vortexed fixed samples was added to 500 µl 1 x PBS (pH 7.4) and centrifuged at 11,337 *g* for 3 minutes. The supernatant was removed, and pellet re-suspended in 100 µl TE-FISH [1 Tris/HCl 1M (pH8): 1 EDTA 0.5M (pH8): 8 H_2O] containing lysozyme (1 mg/ml), then incubated in the dark for 10 minutes. Samples were vortexed, centrifuged at 11,337 *g* for 3 minutes and washed with 500 µl 1 x PBS (pH 7.4).

In situ hybridisation. The pellet was re-suspended and washed using 150 µl hybridisation buffer [180 µl/ml NaCl 5M; 20 µl/ml Tris/HCl 1M (pH 8); 300 µl/ml formamide; 499 µl/ml ddH_2O and 1 µl/ml 10% sodium dodecyl sulphate (SDS)]. The pellet was re-suspended and homogenised in 1 ml hybridisation buffer and kept at -4°C until required.

Samples and probes were defrosted for use whilst the probes were restricted to light exposure as much as possible. 4 µl of each probe described in Table 1, was added, vortexed and incubated with 50 µl of each sample at 35°C overnight. Samples were washed and centrifuged at 11,337 *g* for 3 minutes in 130 µl hybridisation buffer. The pellet was homogenised and incubated at 37°C with 200 µl washing buffer [12.8 µl/ml NaCl 5M; 20 µl/ml Tris/HCl 1M (pH8); 10 µl/ml EDTA 0.5M (pH8); 956.2 µl/ml ddH_2O and 1 µl/ml 10% SDS] for 20 minutes. Samples were centrifuged at 11,337 *g* for 3 minutes and 300 µl 1 x PBS (pH 7.4) added to the pellet and kept covered at -4°C.

2.4.3. *Microbial profiling analysis*

Samples were well vortexed and analysed using a BD Accuri™C6 Flow Cytometer and bacterial populations were quantified using the Accuri™ CFlow Sampler software.

2.5. ^{1}H-Nuclear Magnetic Resonance (^{1}H-NMR) Spectroscopy

2.5.1. *Sample preparation*

From the batch culture samples, 400 µl of supernatant was taken after centrifugation at 11,337 *g* for 5 minutes and added to 200 µl of 0.2M sodium phosphate buffer solution (pH 7.4) made in 100% deuterium oxide. The buffer solution also contained 0.01% of sodium 3-(trimethylsilyl) [2,2,3,3-2H4] propionate (TSP), as an internal reference standard for calibration of acquired spectral profiles, and 3mM NaN_3 as a preservative. Samples were organised into a randomised order and 500 µl of sample/buffer mixture transferred into 5 mm diameter NMR tubes. Quality control (QC) samples were produced using equal volumes of all samples in the study, pooled together to create a composite sample, and prepared in the same way as described above.

2.5.2. *Metabolic profiling analysis*

A 500 MHz ^{1}H-NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) was used to acquire global metabolic profiles of all batch culture vessel samples collected at different time-points (0, 12 and 24 hours). Spectra were acquired using a standard 1D pulse sequence with water pre-saturation. Parameters were optimised using the QC sample and ran at 300 K (27°C) using 128 scans.

Processing of the acquired ^{1}H-NMR spectra was carried out using the TOPSPIN 3.2 software package (Bruker Biospin, Rheinstetten, Germany). FIDs were transformed into a spectrum by Fourier

transformation. Spectra were automatically phased, baseline corrected and calibrated to the TSP signal at δ0.0. Spectral regions that were excluded from multivariate statistical analyses were the TSP peak (-1-0.5 ppm); water peak (4.5-5.15) and spectral regions above 8.51 ppm, since these did not contain biological information, then the data was normalised using the probabilistic quotient method (Dieterle et al. 2006).

2.6. Statistics

Multivariate statistical models were calculated from the data using the SIMCA-P software programme (Umetrics, Umea, Sweden). Principal component analysis (PCA) is an unsupervised modelling approach conducted on the data first for identification of patterns and trends. Microbial numbers were then averaged, and each condition statistically analysed using paired t-tests compared to fresh stool (C1) to assess the impact of different storage conditions on microbial numbers per timepoint and control. The PCAs were used to indicate vessels with metabolic differences with which were then interrogated further by overlaying their ^{1}H-NMR spectra in Topspin to identify key metabolites contributing to differences. Integrals for these key metabolites were calculated and averaged to analyse each condition statistically with paired t-tests compared to fresh stool (C1) to assess the impact of different storage conditions on metabolites per timepoint and control.

3. Results

Preliminary multivariate statistical modelling showed that time and control were the main influencing factors driving differences between samples for both microbial and metabolic profiles (Figures S1-6). Therefore, these profiles were stratified by timepoint (T0, T12 and T24) and control (negative and positive), as seen in Figures 1A and 1B.

It was evident that differences in microbial profiles were more influenced by donor than vessel in both negative (Figure 1A) and positive controls (Figure 1B) at T0, but over time the vessel had a stronger influence on the microbial differences (Figures 1A and 1B). Donor 1 was most microbially different at T0, whereas donor 3 had the most different microbial profile to the other donors by T24 in positively controlled vessels. By T24, negative and positive controls for freezing samples at -20˚C for 60 minutes followed by -80˚C for 2 weeks (C5) (V9 and V10) were most microbially different to their comparative fresh stool samples (V1 and V2), respectively (Figures 1A and 1B). Microbial profiles of stool stored in an anaerobic chamber in a temperature-controlled bag (4°C) for 60 minutes (C3) and frozen at -20°C for 60 minutes (C4) remained the most similar to the fresh stool (C1) in negative controls throughout the fermentation (Figure 1A). In positive controls, the same conditions (C3 and C4) as well as storing the stool on wet ice (4°C) for 60 minutes (C2) were the most similar microbially to the fresh stool (C1) (Figure 1B).

Bar plots illustrating average numbers of microbes enumerated by FC-FISH in each vessel are shown in Figure 2. Effects of the conditions on average numbers of microbes were compared to those detected in fresh stool (C1: V1 and V2) for each timepoint per control. No condition was found to significantly affect the outcome on the number of BIF, LAB, BAC, EREC, RREC, FPRAU and CHIS at any timepoint. Freezing the stool at -20˚C for 60 minutes followed by -80˚C for 2 weeks (C5) significantly reduced total numbers of bacteria (EUB) (p=0.049, 95% C.I.) from 9.462 log10 cells/ml in fresh stool sample (C1) to 8.598 log10 cells/ml and *Clostridium* cluster IX (PROP) (p=0.004, 99% C.I.) from 7.421 log10 cells/ml in fresh stool sample (C1) to 6.142 log10 cells/ml detected at 12 hours in the positively controlled batch culture vessels (V2 versus V10). This storage condition also significantly increased *Atopobium* cluster (ATO) (p=0.007, 99% C.I.) from 6.664 log10 cells/ml in fresh stool sample (C1) to 7.170 log10 cells/ml detected at T24 in the negatively controlled batch culture vessel (V1 vs. V9).

Storing the stool in an anaerobic chamber at 4˚C for 60 minutes (C3) significantly increased *Desulfovibrionales/Desulfuromonales* (DSV) (p=0.037, 95% C.I.) from 5.632 log10 cells/ml in fresh stool sample (C1) to 6.218 log10 cells/ml detected at T24 in the positively controlled batch culture vessel (V2 vs. V6).

PCA models generated using metabolic profile data, as seen in Figures 3A and 3B, showed neither the donor nor vessel to be a distinctive influencing factor for metabolic profiles at T0. Over time, it could be argued that the vessels were slightly more responsible for metabolic differences between the samples, but all samples are generally clustered with a few exceptions (Figures 3A and 3B).

The sample for donor 1 that was stored in an anaerobic chamber in a temperature-controlled bag (4°C) for 60 minutes (C3) and the sample belonging to donor 3 that was frozen at -20°C for 60 minutes and then at -80°C for 2 weeks (C5), V6 and V10 respectively, demonstrated largest metabolic differences compared to other samples at t12 in the positive control (Figure 3B). The sample frozen at -20˚C for 60 minutes followed by -80˚C for 2 weeks (C5) for the negative control (V9) was most metabolically distinct from the fresh stool (C1) control vessel (V1) at T24 (Figure 3A). However, there were less metabolic differences between the positive control samples at T24, apart from outliers both belonging to donor 3 for the samples stored in an anaerobic chamber at 4˚C for 60 minutes (C3) and frozen at -20˚C for 60 minutes (C4), V6 and V8 respectively (Figure 3B). An overlay of spectroscopic data generated from these samples identified key metabolites contributing to differences between the samples as butyrate (t, 0.9; d, 1.56; t, 2.16), propionate (t, 1.06; q, 2.19), ethanol (t, 1.18; q, 3.66), lactate (d, 1.33; q, 4.11), acetate (s, 1.92), succinate (s, 2.41), trimethylamine (s, 2.88), trimethylamine-N-oxide (s, 3.27) and formate (s, 8.46) (Figure S7).

Bar plots illustrating the average integral for key metabolites that were different between samples can be seen in Figure 4. Effects of conditions on the average integral of each metabolite were compared to those detected in fresh stools (C1: V1 and V2) for each timepoint per control. No condition was found to significantly affect the outcome of propionate, ethanol, lactate, acetate, succinate and formate at any timepoint. Freezing the stool at -20°C for 60 minutes (C4) and at -20˚C for 60 minutes followed by -80˚C for 2 weeks (C5) significantly reduced butyrate detected in positive control samples at 12 hours. Butyrate was reduced from an integral of 6.846 detected in the fresh stool sample (C1) in V2 to 2.367 (p=0.015, 95% C.I.) and 2.274 (p=0.014, 95% C.I.) in V8 and V10, respectively. Conversely, freezing at -20˚C for 60 minutes followed by -80˚C for 2 weeks (C5) significantly increased trimethylamine-N-oxide from 0.067 in the fresh stool sample (C1) in V2 to 0.370 (p=0.014, 95% C.I.) in V10 by T24 in positive controls. Trimethylamine was significantly reduced at T0 from 0.036 in the fresh stool (C1) in V2 to 0.009 (p=0.047, 95% C.I.) in the stool sample kept on wet ice (4°C) for 60 minutes (C2) in V4 (Figure 4).

Overall, as the fermentations progress to T12 and T24, metabolic profiles of the samples that had been stored on wet ice (4°C) for 60 minutes (C2), in an anaerobic chamber at 4˚C for 60 minutes (C3) and frozen at -20˚C for 60 minutes (C4) were all clustered with the fresh stool sample (C1) in the negatively controlled vessels and, therefore, had similar metabolic profiles(V3, V5 and V7, respectively, versus V1) (Figure 3A). However, in the positive control, the sample that had been stored on wet ice (4°C) for 60 minutes (C2) was most metabolically similar to the fresh stool sample (C1) as the fermentation progressed for both timepoints T12 and T24 (V4 and V2, respectively) (Figure 3B).

4. Discussion

This study showed that differences in stool collection and storage conditions prior to study in *in vitro* human gut fermentation systems does impact upon microbial and metabolic profiles. We found that

inoculating anaerobic batch cultures fermenters using stool samples stored under different conditions did have an impact on the growth of microbes over time, both in the negative and positive controls, but not when analysed at 0 hours pre-fermentation where there was no significant impact on the microbes seen for any of the storage conditions compared to fresh stool. This is in line with results seen by Al et al. (2018) who found no significant alteration of microbial diversity or composition by storing stool at -80, 7, 22 and 37˚C for 3 or 7 days. Whilst this is reassuring for studies focussing only on stool microbial profiling (e.g. microbiomics, metataxonomics), in fermentation experiments storage conditions did have impacts on microbial numbers and activity. Freezing the stool at -20°C for 60 minutes and then at -80°C for 2 weeks (C5) had the most profound effect on microbial growth, distinguishing them the most from the other samples over time. This difference was driven by significantly fewer total bacteria (EUB) and *Clostridium* cluster IX (PROP) at 12 hours in the positive controls. It is possible that because cells were not stored in a cryoprotectant, such as glycerol, which protects microorganisms against damage due to freezing, therefore, significantly affecting cell viability and re-growth capabilities. Cell damage experienced in the cryofreezing process could explain their inability to grow like the bacteria in the fresh stool sample in the positive controls. Furthermore, the thawing process should be rapid following storage, potentially in a water bath at 37˚C, whereas all samples thawed in this study were defrosted on wet ice (4 ˚C). This condition (C5) also significantly increased *Atopobium* cluster (ATO) at 24 hours in the negative control compared to the fresh stool alone for an unknown reason (V9 versus V1) (Figure 2). Further investigation revealed that donor 1 may be responsible for skewing the significant increase of ATO seen in vessel 9 at 24 hours. As seen in Figure S8, the number of *Atopobium* cluster cells (ATO) in vessel 9 for donors 2 and 3 follow a similar pattern to that seen in vessel 1 over time. However, there was a notable increase in number of *Atopobium* cluster cells (ATO) seen at 24 hours in vessel 9 for donor 1 which suggests this could be an anomaly. Furthermore, when the result for donor 1 was removed from this analysis, no significant difference was calculated for ATO when comparing vessel 9 and vessel 1 at 24 hours (Figure S8). We found that different storage conditions did have an impact on the changes seen in metabolites in batch culture vessel fermentations, but significant differences were only detected in positive controls over time. Storage conditions did not significantly affect integrals for key metabolites over time compared to fresh stool samples which is not surprising as changes in metabolic profiles would rely on the growth of active bacteria driven by 2'FL HMO in the positive control.

Stool storage conditions that had a significant impact on metabolic profiles in positive controls were freezing the stool at -20°C for 60 minutes (C4) and at -20˚C for 60 minutes followed by -80˚C for 2 weeks (C5) (V8 and V10 respectively) which reduced the amount of butyrate detected after 12 hours compared to the fresh stool sample. This suggests that freezing of stools may have a detrimental effect on butyrate-producing bacteria. The majority of butyrate-producing bacteria found in human stool belong to *Clostridium* clusters IV and XIVa (Louis and Flint, 2009; Van Immerseel, et al., 2010) which also produce the highest concentrations of butyrate (Eeckhaut, et al., 2011). *Clostridium* cluster XIVa found in human stool convert lactate to butyrate (Duncan, et al., 2004). Therefore, it is plausible that the reduced numbers of *Clostridium* cluster XIVa, represented by EREC and RREC, seen in V8 and V10 at 12 hours were responsible for the higher levels of lactate and significantly lower levels of butyrate detected, although results for EREC, RREC and lactate were not significant. Furthermore, bacteria from *Clostridium* cluster IX (detected by PROP probe) can also convert lactate to butyrate (Eeckhaut, et al., 2011). PROP was significantly reduced at 12 hours in V10 which could explain higher levels of lactate and significant reduction in butyrate seen in the same vessel.

Trimethylamine-N-oxide (TMAO) was significantly higher after 24 hours in the stool sample subjected to storage at -20˚C for 60 minutes followed by -80˚C for 2 weeks (C5) than fresh stool

sample (C1) in the positive control, V10 versus V2. These results suggest that freezing negatively impacted bacteria that utilise TMAO. Hoyles, et al. (2018) found that Enterobacteriaceae predominantly convert TMAO to trimethylamine (TMA) in the gut microbiota of mice (Hoyles, et al., 2018). Although Enterobacteriaceae were not detected by probes in this study, increases in TMA mirror decreases in TMAO over time in different vessels which complements this theory.

A significant reduction in trimethylamine was seen in the positively controlled sample that had been stored on wet ice (4°C) for 60 minutes (C2) compared to fresh stool at 0 hours, V4 versus V2. However, the same was not seen for the negative control (V3 versus V1) which would be expected if this was a genuine result as it is at 0 hours when the results from the positive and negative controls should be the same, therefore suggesting the significant difference could be an anomaly (Figure 4).

Storing the stool in an anaerobic chamber in a temperature-controlled bag (4°C) for 60 minutes (C3) caused a significant increase in the positive control with *Desulfovibrionales/Desulfuromonales* (DSV) (V6) at 24 hours compared to the fresh stool (V2). Although not significant, more DSV was detected in all the vessels with stool that was stored (C2-C5) by 24 hours compared to the fresh stool sample vessel (C1). *Desulfovibrio* reportedly competes with butyrate-producing bacteria for lactate to convert it to propionate (Finegold, 2011; MacFabe, 2012). This is in line with the lower levels of RREC, which detects the butyrate-producing *Clostridium* cluster XIVa as previously discussed (Duncan, et al., 2004), seen in vessels that underwent storage conditions (C2-C5) compared to the fresh stool (C1) at 24 hours in the positive controls. Furthermore, although not significant, propionate was elevated in V6 compared to V2 at 24 hours which is in line with more propionate-producing *Desulfovibrionales* in the same vessel. Therefore, it is possible that *Desulfovibrionales/Desulfuromonales* (DSV) may grow due to reduced competition from less butyrate-producing bacteria (RREC) which contributes towards the conversion of lactate to propionate.

The storage condition that had most distinct microbial and metabolic profiles from the fresh stool were samples frozen at -20°C for 60 minutes and then at -80°C for 2 weeks (C5). Therefore, this stool storage method is not advisable for *in vitro* human gut model studies.

Freezing samples at -20°C for 60 minutes (C4) resembled microbial profiles of fresh stools in both controls and in all timepoints which is consistent with other outcomes from research in this area (Cardona et al., 2012; De Spiegeleer et al., 2020; Gorzelak et al., 2015; Gratton et al., 2016). However, significant differences observed in positively controlled vessels for butyrate make it unsuitable as a storage method for *in vitro* human gut model studies when analysing metabolites. However, it could be an option if the study was only interested in the changes in microbial profiles. Furthermore, it is possible that storage time at -20˚C could be extended if longer than 60 minutes were required, provided that freeze-thaw cycles were kept to a minimum. Gorzelak et al. (2015) suggested a possible storage time at -20˚C of up to 3 days. They stored a homogenised stool sample at -20˚C and tested bacterial composition using qPCR at 0, 3, 7, 14 and 30 days. The authors found a decrease of Firmicutes by day 3, significant decrease of Bacteroidetes by day 14 as well a significant decrease of *Bifidobacterium* spp. and *Lactobacillus* spp. by day 30. However, Enterobacteriaceae significantly increased by day 14 (Gorzelak et al., 2015). This is in line with conclusions from Bahl et al. (2012) who saw a significantly increased Firmicutes:Bacteroidetes ratio in stool samples frozen at -20˚C for 53 days compared to the fresh stool samples (Bahl et al., 2012).

Gorzelak et al. (2015) also suggested that bacterial abundances of the gut microbiota from stool samples were not affected for up to four freeze-thaw cycles. However, it is important to note that they analysed abundances rather than actual numbers and used an instant snap freeze-thaw

method in liquid nitrogen which would most likely not be practical in studies where volunteers store their own specimens. By the fifth freeze-thaw cycle, significant decreases in Enterobacteriaceae and increases in Bacteroidetes were observed. Furthermore, they also noted that unreported data showed increased variation when freeze-thaw cycles exceeded 10 minutes (Gorzelak et al., 2015). Research by Gratton et al. (2016) and De Spiegeleer et al. (2020) both advised avoidance of freeze-thaw cycles for metabolic analyses of stool samples (De Spiegeleer et al., 2020; Gratton et al., 2016). This suggests a storage time of below 3 days at -20˚C ideally with only one freeze-thaw cycle (possibly more if samples were snap frozen) could be a possible storage solution, however, both variables would need further investigation for optimal recommendations.

Freezing stool samples at -80˚C without pre-freezing at -20˚C also warrants further study as it has been suggested that freezing at a faster rate (i.e. -80˚C) forms smaller ice crystals and limits mechanical destruction, although there is conflicting evidence for this theory (Haines, 1938). Al et al., (2018) found that storing stool samples at -80˚C for 3 or even 7 days had no significant difference on the microbial diversity of composition. However, it is important to note that these samples were not tested in fermentation studies following storage. Also, samples were analysed using 16S rRNA sequencing and therefore did not require viable or intact cells for analysis, unlike FC-FISH. Furthermore, De Spiegeleer et al. (2020) reported stool metabolites to be more stable when stored at -80˚C than -20˚C (De Spiegeleer et al., 2020).

Another consideration is time limit of freezing the stool sample post-defecation. Gorzelak et al. (2015) detected bacterial abundance in stool samples at room temperature 15 and 30 minutes post-defecation and found a significant decrease of Bacteroidetes and increase of Firmicutes after 30 minutes compared to 15 minutes. Therefore, they advised to freeze stool samples within 15 minutes of the sample being taken (Gorzelak et al. (2015).

Overall, microbial and metabolic profiles of samples stored anaerobically in a temperature-controlled bag (4°C) for 60 minutes (C3) mimicked fresh stool sample the most closely, other than a significant increase seen in *Desulfovibrionales/Desulfuromonales* (DSV) in the positive control (V6) at 24 hours and a few anomalies for donor 3 in metabolic data. However, observing the vessels individually, there were higher levels of *Desulfovibrionales/Desulfuromonales* than for the stool that was kept anaerobically in a temperature-controlled bag (4°C) for 60 minutes (C3).

Similarly, other than the odd anomaly seen in the PCAs, the sample kept on wet ice (4°C) for 60 minutes (C2) performed well and there were no significant differences observed for microbes or metabolites compared to fresh stool samples (C1).

Overall, our study suggests that storing stool on wet ice (4°C) (C2) or in an anaerobic jar in a temperature-controlled bag (4°C) (C3) for up to 60 minutes were the most suitable storage conditions for stool to be used in *in vitro* human gut model fermenters to be analysed for microbes and metabolites over a 24 hour time period. Freezing samples at -20°C for 60 minutes (C4) should also be considered for *in vitro* human gut models when only interested in microbial profiles as it is possible that storage time could be extended providing the sample is still only thawed once, which would provide more flexibility to sample collection and storage for studies. Furthermore, although freezing at -20°C for 60 minutes and then at -80°C for 2 weeks (C5) should not be considered for stool storage before use in *in vitro* studies, further investigation into freezing at -80°C only may provide a faster freezing rate to reduce microbial cell damage and have a more similar outcome to the fresh stool, as well as providing a longer-term storage solution for researchers.

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References

Al, K.F., Bisanz, J.E., Gloor, G.B., Reid, G., Burton, J.P., 2018. Evaluation of sampling and storage procedures on preserving the community structure of stool microbiota: a simple at-home toilet-paper collection method. Journal of Microbiological Methods. 144, 117-121. https://doi.org/10.1016/j.mimet.2017.11.014

Albenberg, L., Esipova, T.V., Judge, C.P., Bittinger, K., Chen, J., Laughlin, A., Grunberg, S., Baldassano, R.N., Lewis, J.D., Li, H., Thom, S.R., Bushman, F.D., Vinogradov, S.A., Wu G.D., 2014. Correlation between intraluminal oxygen gradient and radial partitioning of intestinal microbiota. Gastroenterology. 147 (5), 1055-1063. https://doi.org/10.1053/j.gastro.2014.07.020.

Bahl, M.I., Bergström, A., Licht, T.R., 2012. Freezing fecal samples prior to DNA extraction affects the Firmicutes to Bacteroidetes ratio determined by downstream quantitative PCR analysis. FEMS Microbiology Letters. 329 (2), 193-197. https://doi.org/10.1111/j.1574-6968.2012.02523.x.

Cardona, S., Eck, A., Cassellas, M., Gallart, M., Alastrue, C., Dore, J., Azpiroz, F., Roca, J., Guarner, F., Manichanh, C., 2012. Storage conditions of intestinal microbiota matter in metagenomic analysis. BMC Microbiology. 12, 158. https://doi.org/10.1186/1471-2180-12-158.

Choo, J., Leong, L., Rogers, G., 2015. Sample storage conditions significantly influence faecal microbiome profiles. Scientific Reports. 5, 16350. https://doi.org/10.1038/srep16350.

Daims, H., Brühl, A., Amann, R., Schleifer, K.H., Wagner, M., 1999. The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. Systematic and Applied Microbiology. 22(3), 434-444. https://doi.org/10.1016/S0723-2020(99)80053-8.

De Paoli, P., 2005. Biobanking in microbiology: From sample collection to epidemiology, diagnosis and research. FEMS Microbiology Reviews. 29(5), 897-910. https://doi.org/10.1016/j.femsre.2005.01.005.

De Spiegeleer, M., De Graeve, M., Huysman, S., Vanderbeke, A., Van Meulebroek, L., Vanhaecke, L., 2020. Impact of storage conditions on the human stool metabolome and lipidome: preserving the most accurate fingerprint. Analytica Chimica Acta. 1108, 79-88. https://doi.org/10.1016/j.aca.2020.02.046.

Devereux, R., Kane, M.D., Winfrey, J., Stahl, D.A., 1992. Genus- and group-specific hybridization probes for determinative and environmental studies of sulfate-reducing bacteria. Systematic and Applied Microbiology. 15(4), 601-609. https://doi.org/10.1016/S0723-2020(11)80122-0.

Duncan, S.H., Louis, P., Flint, H.J., 2004. Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. Applied and Environmental Microbiology. 5810-5817. https://doi.org/10.1128/AEM.70.10.5810-5817.2004.

Eeckhaut, V., Van Immerseel, F., Croubels, S., De Baere, S., Haesebrouck, F., Ducatelle, R., Louis, P., Vandamme, P., 2011. Butyrate production in phylogenetically diverse Firmicutes isolated from the chicken caecum. Microbial Biotechnology. 4(4), 503-512. https://doi.org/10.1111/j.1751-7915.2010.00244.x.

Fair, K., Dunlap, D.G., Fitch, A., Bogdanovich, T., Methé, B., Morris, A., McVerry, B.J., Kitsios, G.D., 2019. Rectal swabs from critically ill patients provide discordant representations of the gut microbiome compared to stool samples. mSphere. 4, e00358-19. https://doi.org/10.1128/mSphere.00358-19.

Finegold, S.M., 2011. *Desulfovibrio* species are potentially important in regressive autism. Medical Hypotheses. 77(2), 270-274. https://doi.org/10.1016/j.mehy.2011.04.032.

Fouhy, F., Deane, J., Rea, M.C., O'Sullivan, Ó., Ross, R.P., O'Callaghan, G., Plant, B.J., Stanton, C., 2015. The effects of freezing on faecal microbiota as determined using MiSeq sequencing and culture-based investigations. PLoS One. 10(3), e0119355. https://doi.org/10.1371/journal.pone.0119355.

Franks, A.H., Harmsen, H.J.M., Raangs, G.C., Jansen, G.J., Schut, F., Welling, G.W., 1998. Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. Applied and Environmental Microbiology. 64, 3336-3345. https://doi.org/10.1128/AEM.64.9.3336-3345.1998.

Friedrich, U., Van Langenhove, H., Altendorf, K., Lipski, A., 2003. Microbial community and physicochemical analysis of an industrial waste gas biofilter and design of 16S rRNA-targeting oligonucleotide probes. Environmental Microbiology. 5(3), 183-201. https://doi.org/10.1046/j.1462-2920.2003.00397.x.

Fu, B.C., Randolph, T.W., Lim, U., Monroe, K.R., Cheng, I., Wilkens, L.R., Le Marchand, L., Hullar, M.A.J., Lampe, J.W., 2016. Characterization of the gut microbiome in epidemiologic studies: the multiethnic cohort experience. Annals of Epidemiology. 26(5), 373-379. https://doi.org/10.1016/j.annepidem.2016.02.009.

Ghaisas, S., Maher, J., Kanthasamy, A., 2016. Gut microbiome in health and disease: linking the microbiome–gut–brain axis and environmental factors in the pathogenesis of systemic and neurodegenerative diseases. Pharmacology & Therapeutics. 158, 52-62. https://doi.org/10.1016/j.pharmthera.2015.11.012.

Gorzelak, M. A., Gill, S. K., Tasnim, N., Ahmadi-Vand, Z., Jay, M., Gibson, D. L., 2015. Methods for improving human gut microbiome data by reducing variability through sample processing and storage of stool. PLoS One. 10(9), e0139529. https://doi.org/10.1371/journal.pone.0139529.

Gratton, J., Phetcharaburanin, J., Mullish, B.H., Williams, H.R.T., Thursz, M., Nicholson, J.K., Holmes, E., Marchesi, J.R., Li, J.V., 2016. Optimized sample handling strategy for metabolic profiling of human feces. Analytical chemistry. 88, 4661-4668. https://doi.org/10.1021/acs.analchem.5b04159.

Haines, R.B., 1938. The effect of freezing on bacteria. Proceedings of the Royal Society of London. 124(837), 451-463. https://doi.org/10.1098/rspb.1938.0005.

Harmsen, H.J.M., Elfferich, P., Schut, F., Welling, G.W., 1999. A 16S rRNA-targeted probe for detection of *Lactobacilli* and *Enterococci* in faecal samples by fluorescent *in situ* hybridization. Microbial Ecology in Health and Disease. 11(1), 3-12. https://doi.org/10.1080/089106099435862.

Harmsen, H.J.M., Wildeboer-Veloo, A.C.M., Grijpstra, J., Knol, J., Degener, J.E., Welling, G.W., 2000. Development of 16S rRNA-based probes for the *Coriobacterium* group and the *Atopobium* cluster and their application for enumeration of *Coriobacteriaceaein* human feces from volunteers of different age groups. Applied and Environmental Microbiology. 66, 4523-4527. https://doi.org/10.1128/AEM.66.10.4523-4527.2000.

Hold, G.L., Schwiertz, A., Aminov, R.I., Blaut, M., Flint, H.J., 2003. Oligonucleotide probes that detect quantitatively significant groups of butyrate-producing bacteria in human feces. Applied and Environmental Microbiology. 69, 4320-4324. https://doi.org/10.1128/AEM.69.7.4320-4324.2003.

Hoyles, L., Jiménez-Pranteda, M.L., Chilloux, J., Brial, F., Myridakis, A., Aranias, T., Magnan, C., Gibson, G.R., Sanderson, J.D., Nicholson, J.K., Gauguier, D., McCartney, A.L., Dumas, M.E., 2018. Metabolic retroconversion of trimethylamine N-oxide and the gut microbiota. Microbiome. 6(73). https://doi.org/10.1186/s40168-018-0461-0.

Langendijk, P.S., Schut, F., Jansen, G.J., Raangs, G.C., Kamphuis, G.R., Wilkinson, M.H., Welling, G.W., 1995. Quantitative fluorescence in situ hybridization of *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. Applied and Environmental Microbiology. 61, 3069-3075. https://doi.org/10.1128/AEM.61.8.3069-3075.1995.

Liang, Y., Dong, T., Chen, M., He, L., Wang, T., Liu, X., Chang, H., Mao, J.H., Hang, B., Snijders, A.M., Zia, Y., 2020. Systematic analysis of impact of sampling regions and storage methods on fecal gut microbiome and metabolome profiles. mSphere 5. e00763-19. https://doi.org/10.1128/mSphere.00763-19.

Louis, P., Flint, H.J., 2009. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. FEMS Microbiology Letters. 294(1), 1-8. https://doi.org/10.1111/j.1574-6968.2009.01514.x.

Lozupone, C.A., Stombaugh, J.I., Gordon, J.I., Jansson, J.K., Knight, R., 2012. Diversity, stability and resilience of the human gut microbiota. Nature. 489, 220–230. https://doi.org/10.1038/nature11550.

Mandal, R., Cano, R., Davis, C.D., Hayashi, D., Jackson, S.A., Jones, C.M., Lampe, J.W., Latulippe, M.E., Lin, N.J., Lippa, K.A., Piotrowski, P., Da Silva, S.M., Swanson, K.S., Wishart, D.S., 2020. Workshop report: toward the development of a human whole stool reference material for metabolomic and metagenomic gut microbiome measurements. Metabolomics. 16(119). https://doi.org/10.1007/s11306-020-01744-5.

Manz, W., Amann, R., Ludwig, W., Vancanneyt, M., Schleifer, K.H., 1996. Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. Microbiology. 142, 1097-1106. https://doi.org/10.1099/13500872-142-5-1097.

Roesch, L.F., Casella, G., Simell, O., Krischer, J., Wasserfall, C.H., Schatz, D., Atkinson, M.A., Neu, J., Triplett, E.W., 2009. Influence of fecal sample storage on bacterial community diversity. The Open Microbiology Journal. 3, 40-46. https://doi.org/10.2174/1874285800903010040.

Tedjo, D.I., Jonkers, D.M.A.E., Savelkoul, P.H., Masclee, A.A., van Best, N., Pierik, M.J., Penders, J., 2015. The effect of sampling and storage on the fecal microbiota composition in healthy and diseased subjects. PLoS One. 10(5), e0126685. https://doi.org/10.1371/journal.pone.0126685.

Um, C., Lichtman, C., Stevens, V., Gapstur, S., Patel, A., McCullough, M.A., 2019. Pilot study to assess stool sample collection methods for gut microbiome research in large prospective cohort studies. Current Developments in Nutrition. 3, nzz036. https://doi.org/10.1093/cdn/nzz036.FS07-01-19.

Van Immerseel, F., Ducatelle, R., De Vos, M., Boon, N., Van De Wiele, T., Verbeke, K., Rutgeerts, P., Sas, B., Louis, P., Flint, H.J., 2010. Butyric acid-producing anaerobic bacteria as a novel probiotic

treatment approach for inflammatory bowel disease. Journal of Medical Microbiology. 59(2). https://doi.org/10.1099/jmm.0.017541-0.

Wallner, G., Amann, R., Beisker, W., 1993. Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry. 14(2), 136-143. https://doi.org/10.1002/cyto.990140205.

Walker, A.W., Duncan, S.H., McWilliam Leitch, E. C., Child, M. W., Flint, H. J., 2005. pH and peptide supply can radically alter bacterial populations and short-chain fatty acid ratios within microbial communities from the human colon. Microbial Ecology. 3692-3700. https://doi.org/10.1128/AEM.71.7.3692–3700.2005.

Wesolowska-Andersen, A., Bahl, M.I., Carvalho, V., Kristiansen, K., Sicheritz-Pontén, T., Gupta, R. Licht, T. R., 2014. Choice of bacterial DNA extraction method from fecal material influences community structure as evaluated by metagenomic analysis. Microbiome. 2(19). https://doi.org/10.1186/2049-2618-2-19.

Williams, G.M., Leary, S.D., Ajami, N.J., Chipper Keating, S., Petrosin, J.F, Hamilton-Shield, J.P., 2019. Gut microbiome analysis by post: evaluation of the optimal method to collect stool samples from infants within a national cohort study. PLoS One. 14(6), e0216557. https://doi.org/10.1371/journal.pone.0216557.

Wu, G.D., Lewis, J.D., Hoffmann, C., Chen, Y., Knight, R., Bittinger, K., Hwang, J., Chen, J., Berkowsky, R., Nessel, L., Li, H., Bushman, F.D., 2010. Sampling and pyrosequencing methods for characterizing bacterial communities in the human gut using 16S sequence tags. BMC Microbiology. 10, 206. https://doi.org/10.1186/1471-2180-10-206.