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Preparation of a standardised faecal slurry for *ex-vivo* microbiota studies which reduces inter-individual donor bias

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ABSTRACT

Background: *In-vitro* gut fermentation systems provide suitable models for studying gut microbiota composition and functionality. However, such methods depend on the availability of donors and the assumption of reproducibility between microbial communities before experimental treatments commence. The aim of this study was to develop a frozen standardised inoculum (FSI) which minimizes inter-individual variation and to determine its stability over time using culture-dependent and culture-independent techniques.
Results: A method for the preparation difference of a FSI is described which involves pooling the faecal samples, centrifugation and pelleting of the cell biomass and finally homogenising the cell pellets with phosphate buffer and glycerol. Using this approach, no significant difference in total anaerobe cell viability was observed between the fresh standardised inoculum (before freezing) and the 12 days post freezing FSI. Moreover, Quantitative PCR revealed no significant alterations in the estimated bacterial numbers in the FSI preparations for any of the phyla. MiSeq sequencing revealed minute differences in the relative abundance at phylum, family and genus levels between the FSI preparations. Differences in the microbiota denoted as significant were limited between preparations in the majority of cases to changes in percentage relative abundance of ±0.5%. The independently prepared FSIs revealed a high degree of reproducibility in terms of microbial composition between the three preparations.
Conclusions: This study provides a method to produce a standardised human faecal inoculum suitable for freezing. Based on culture-dependent and independent analysis, the method ensures a degree of reproducibility between preparations by lessening the effect of inter-individual variation among the donors, thereby making the system more suitable for the accurate interpretation of the effects of experimental treatments.

1. Background

The human gut microbiota plays a pivotal role in health endowing the host with an array of essential functions from fermentation of complex carbohydrates to immune modulation, energy harvesting, intestinal barrier function and metabolite generation (Jones et al., 2014). More recently, its potential role in brain development has come to the fore (Dinan et al., 2015). Hence, microbiota disruptions can have detrimental consequences for the host and have been linked to a variety of pathological disorders ranging from intestinal to neurological (Guinane and Cotter, 2013; Kelly et al., 2015). Factors known to disrupt or alter the composition of the gut microbiota include antibiotics and diet, among others (Jones et al., 2014). Therapeutic strategies to manipulate the composition of the gut microbiota in order to treat such conditions include the use of probiotics, prebiotics, faecal transplantations or antimicrobials as reviewed by Guinane and Cotter (2013). However, more research is warranted in order to further understand the factors which disrupt gut microbiota composition and to develop worthwhile intervention strategies. Unsurprisingly, *in-vivo* experiments identifying the effects of such interventions are limited due to the invasive nature of procedures required to access the large intestine alongside ethical and social concerns. In this respect, *in-vitro* gut fermentation models and animal models have proven effective substitutes for investigating microbiota composition and functionality (Fooks and Gibson, 2003; Sarbini et al., 2013; Turnbaugh et al., 2009).

Gut fermentation models are basically composed of vessels or compartments (single or multiple) inoculated with faecal microbiota and operated in an anaerobic atmosphere at temperature and pH values mimicking physiological conditions (for review see Payne et al., 2012). The complexity of the faecal fermentation is determined by the control

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system employed, the number of vessels used and the feeding system to each unit (Fooks and Gibson, 2003; Barroso et al., 2015; Feria-Gervasio et al., 2014; Minekus et al., 1999; Molly et al., 1994; Rea et al., 2011). Batch fermentation systems are often used to examine the effects of non-digestible oligosaccharides and other prebiotics on faecal microbiota (Sarbini et al., 2013; Rodriguez-Colinas et al., 2013), but are constrained by the faecal inoculum and nutrient limitations over time (Payne et al., 2012). Importantly, *ex-vivo* fermentation systems are not to be mistaken for an exact replica of the human/animal intestinal environment since applied fermentation conditions can never completely simulate those of the mammalian intestine (Payne et al., 2012). Frozen non-standardised faecal inoculum has also been used in gnotobiotic humanised mouse models to reproduce the human gut microbiota *in-vivo* (Turnbaugh et al., 2009). Fresh faecal samples are often used from a single donor and oral gavaged into a number of gnotobiotic mice (Turnbaugh et al., 2009; Respondek et al., 2013). Some studies, however do use a form of standardisation to prepare their samples prior to humanization of the mouse gut (Collins et al., 2015).

The standardisation of faecal material preparation is not a new concept, having been shown to significantly simplify practical aspects of faecal microbiota transplantation without compromising the clearing of *Clostridium difficile* infection in patients (Hamilton et al., 2012). In this case, the faecal material underwent homogenisation, filtration, centrifugation, immediate use or freezing in 10% glycerol at −80 °C. No significant difference was observed between fresh and frozen donor material in clearing the infection. Hamilton et al. 2013) later highlighted the effectiveness of the method using high-throughput sequencing analysis on faecal samples from treated patients (Hamilton et al., 2013).

In this study, we have examined the compositional stability and reproducibility of using pre-prepared, frozen faecal inoculum to be potentially used as the standardised microbiota for experimentation or as control samples for wider microbiota studies. The purpose of our study was therefore to establish a straight-forward repeatable method to produce a frozen and stable starting faecal inoculum. The efficiency of this frozen standardised inoculum (FSI) was examined using culture-dependent and culture-independent methods. The use of the FSI should allow researchers more experimental flexibility, reliability, repeatability and reduce the reliance on the availability of fresh faeces.

2. Results

We attempted to design a standardised faecal microbiota suitable for freezing referred to as the frozen standardised inoculum (FSI) for *ex-vivo* experimentation. In order to achieve this we assessed the effect of preparation on the microbiota along with the stability of the FSI following 12 days of freezing using culture-dependent techniques (as outlined in Fig. 1). We then compared the three independent preparations of FSI using culture-independent techniques, namely qPCR and Illumina Miseq compositional sequencing.

2.1. Total anaerobe enumeration

The effect of the different stages of FSI preparation on total anaerobic bacteria was examined using plate count data. The total anaerobic counts (Log10 CFU ml^{-1}) at the centrifugation step and in the fresh standardised inoculum (day 0, prior to freezing) were significantly different from the pooled sample ($n = 6$) ($p < 0.05$) as was the difference (<0.5 log) between the slurry and the 12 days post-freezing FSI samples ($p < 0.01$) (Fig. 2(a)). No significant difference was observed between the fresh standardised inoculum and the 12 days post-freezing FSI.

2.2. Quantitative PCR

Examination of the qPCR data revealed no significant differences in population numbers of the major phyla between the three FSI preparations. Total bacterial numbers between FSI preparations were estimated

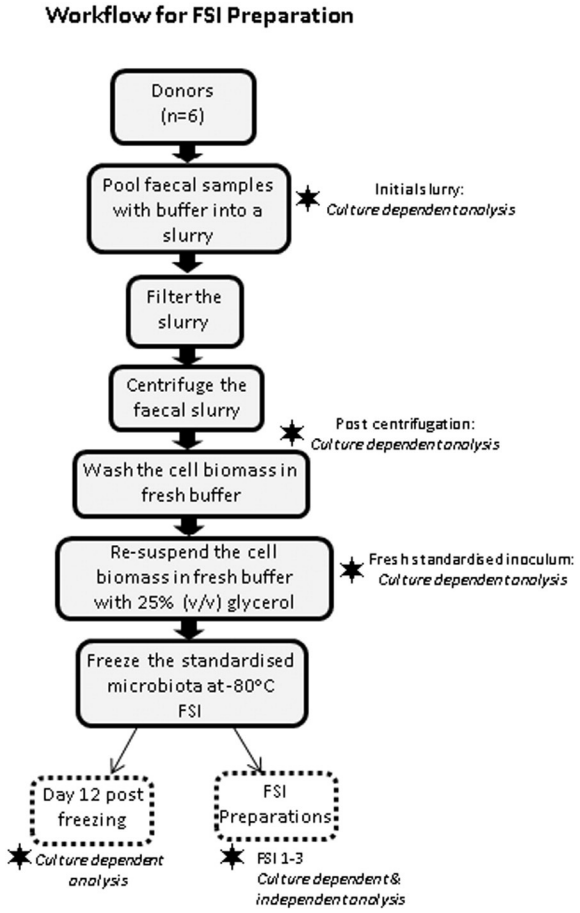

Fig. 1. Workflow for the frozen standardised inoculum (FSI). Sampling points are signified by ★ and types of analysis are indicated.

to be 8.2–8.5 log using the standard curve, however the small variance between FSI preparations was not statistically significant (data not shown). The relatively small variance (<0.3 log) between the FSI preparations was not significant.

Fig. 2(b) shows the stability of the major phyla across FSI preparations. Importantly, there were no significant differences between FSI preparations for tested phyla: *Firmicutes*, *Bacteroidetes* or *Proteobacteria*, highlighting the reproducibility of the FSI method in terms of microbial composition and diversity.

2.3. MiSeq compositional sequencing - dominant taxa FSI's

We generated a 16S rRNA dataset consisting of 5,313,070 raw sequencing reads and 4,063,716 high quality filtered reads. There were a mean number of 225,762 sequences per sample (Range: 37,140–519,957). Sequences identified from the three preparations of FSI clustered into 1260 OTUs. Rarefaction curves plateaued for all samples indicating that a sufficient sub-sampling was used (Supplementary Fig. 1).

The FSI's were dominated by the *Firmicutes* and *Bacteroidetes* phyla and to a lesser extent *Actinobacteria* and *Proteobacteria*. At the family level in the standardised preparations *Ruminococcaceae*, *Lachnospiraceae* and *Prevotellaceae* were dominant. Similarly, at the genus level *Lachnospiraceae Incertae Sedis*, *Prevotella*, *Faecalibacterium* and *Bacteroides* were dominant.

2.4. Taxonomy of the FSIs

Firmicutes and *Bacteroidetes*, the dominant phyla in the three preparations of FSI based on compositional sequencing accounted for ≥92% of