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Optimisation of a propidium monoazide based method to determine the viability of microbes in faecal slurries for transplantation

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Abstract

The efficacy of faecal microbiota transplantation (FMT) as a therapeutic intervention may depend on the viability of the microorganisms in faecal slurries (FS) prepared from donor stool. However, determining the viability of these organisms is challenging. Most microorganisms in stool are refractory to culture using standard techniques, and culture-independent PCR-based methods derive signal from both viable and non-viable cells. Propidium monoazide (PMA) treatment has been shown to be effective in preventing PCR amplification of DNA from non-viable bacteria in a range of contexts. However, this methodology can be sensitive to factors such as bacterial load and sample turbidity. We describe the optimisation of a PMA treatment methodology for FS that restricts quantitative PCR-based bacterial enumeration to viable cells. When applied to concentrated FS (10-25% stool content), PMA treatment at 100 µM concentration was ineffective in preventing DNA amplification from heat-killed cells. Efficacy was not significantly improved by doubling the PMA concentration. However, PMA treatment efficacy was improved markedly following 10-fold sample dilution, and was found to be optimal at 100-fold dilution. Substantial reductions in viable bacterial load could be observed following both freeze-thaw and heat-treatment of FS. This method successfully prevented DNA amplification of heat-killed *Pseudomonas* and *Staphylococcus* spiked into stool and could reliably determine the proportion of live bacteria and viable *E. coli* counts present in fresh and heat-treated stool. With appropriate sample dilution, PMA treatment excluded >97% of non-viable cells from amplification in all assays, without significantly affecting the amplification of DNA from viable cells. This method can be applied to optimise sample processing of FMT donor material, and to characterise bacterial viability within faecal samples more widely.

Key words: bacterial viability, fecal microbiota transplantation, qPCR

1. Introduction

Faecal microbiota transplantation (FMT) is a therapeutic intervention in which stool from one or more healthy donors is processed into a faecal slurry (FS) and delivered to the lower intestinal tract of the recipient. FMT is an established therapy for *Clostridium difficile* colitis (van Nood et al., 2013) and shows promise as a therapeutic intervention in inflammatory conditions such as ulcerative colitis (Costello et al., 2017). It is postulated that the efficacy of FMT is dependent on the ability of beneficial commensal bacteria from the donor to proliferate within the recipient (Khoruts et al., 2010; Seekatz et al., 2014; van Nood et al., 2013). This requires those microbes to be viable at the time of transplantation. The ability to accurately determine the viability of bacteria in donor faecal samples is critical to developing appropriate protocols for the preparation and standardisation of FMT material.

Previous efforts to assess the viability of microorganisms in faecal material used for FMT have been limited either by the use of culture methods, that can readily isolate only a small subset of the total gut microbiota (Costello et al., 2015), or molecular methods that lack the capacity to distinguish between DNA from viable cells, non-viable cells, and the extracellular environment (Fouhy et al., 2015). A potentially effective strategy to overcome these challenges is to combine quantitative (q)PCR-based bacterial enumeration with propidium monoazide sample treatment (PMA-qPCR). PMA is a red fluorescent dye that is excluded from viable cells by the energised membrane of an intact cell wall. When the cell wall is compromised, PMA enters the cell and intercalates into DNA (Nocker et al., 2007). The monoazide group allows PMA to covalently bind DNA upon exposure to light, thus limiting PCR amplification to DNA present within viable cells (Nocker et al., 2006).

The combination of PMA treatment with PCR-based analysis has been shown to be effective in a range of contexts, including the assessment of bacterial viability in samples with mixed populations, such as in waste water or sputa (Bae and Wuertz, 2009; Cuthbertson et al., 2015; Rogers et al., 2008). However, its efficiency may be reduced when applied to samples in which levels of non-viable bacterial DNA and extracellular DNA are high, or where sample turbidity impedes light penetration (Bae and Wuertz, 2009; Varma et al., 2009).

The use of PMA in combination with 16S rRNA gene amplicon sequencing has been reported previously in the assessment of viable bacterial composition of FS for FMT (Chu et al., 2017). Chu

and colleagues subjected undiluted simulated faecal transplant material from a single participant to various processing conditions including exposure to oxygen and freeze thaw cycles prior to treatment with PMA (Chu et al., 2017). However, this study did not include a validation of the methodology.

We report here the optimisation of PMA-qPCR to determine the viable bacterial content of faecal slurries for FMT. In doing so we provide a method that can reliably be applied to optimise sample processing methodologies for FMT donor material, as well as the characterisation of bacterial viability within faecal samples more widely.

2. Materials and Methods

2.1 FMT faecal slurry (FS) processing

Stool was collected with informed consent from participants being screened as FMT donors for a clinical trial (Australia New Zealand Clinical Trials Registry, 2018). Fresh stool from 3 faecal donors were collected on separate occasions and processed immediately. Stool was collected on site and processed with 15 minutes. Stool was blended with normal saline (NS) and glycerol to produce a FS consisting of 25% (wt/vol) stool, 65% NS, and 10% glycerol, as previously described (Costello et al., 2016). Stool blending and PMA treatment were performed within an anaerobic chamber. Remaining stool was frozen at -80^{o}C in either 50 mL centrifuge tubes or 250 mL sterile pots. To assess the effects of freeze-thaw, a 50mL aliquot of FS was stored at -80 ^{o}C for 48 hrs and then allowed to thaw at room temperature within the anaerobic cabinet. Heat killing was performed by subjecting a 1 mL aliquot of thawed FS to 99 ^{o}C for 30 minutes in a heating block.

2.2 Dilution and PMA treatment of fresh, frozen and thawed, and heat-killed FS.

Fresh, freeze-thawed and heat-treated FS was tested at four different dilutions. Neat FS (25% stool content) was serially diluted in phosphate buffered saline (PBS) 10, 100, and 1000-fold. Neat FS and each dilution were treated with PMA or control in triplicate, as described in Section 2.3.

2.3 PMA treatment

Stock solution was prepared by dissolving 1 mg of PMA (Biotium Inc., Fremont, CA, USA) in 1 mL of 20% dimethyl sulfoxide. For PMA treatment, 5 µL of PMA was added to 95 µL of sample to achieve 100 µM final concentration of PMA in 100 µL (Bae and Wuertz, 2009). All samples were prepared in clear RNase-free 1.5mL tubes (Ambion®, Thermo Fisher Scientific, Waltham MA, USA). Following a 30 min incubation at room temperature in the dark (Nkuipou-Kenfack et al., 2013), samples were exposed to an LED light (1.5 W, Model AL329, Aqua Zonic, Singapore) at a distance of 15 cm for 20 min.

2.4 Spiking of stool with heat-killed Pseudomonas aeruginosa and dilution of stool prior to PMA treatment.

P. aeruginosa ATCC 27863 was cultured onto horse blood agar (HBA, bioMerieux, Australia) for 24 hours. Colonies were dispersed in 1 mL PBS and diluted 40-fold to give a suspension of 3.35 McFarland units (~$1x10^9$ CFU/mL). Heat-killing of the neat suspension was performed by heating 1 mL aliquots to 99 °C for 30 min.

To assess the effect of stool concentration on the exclusion of non-viable bacteria through PMA treatment, donor stool was spiked with heat-killed *P. aeruginosa* to produce FS consisting of 25% (vol/vol) heat-killed *P. aeruginosa*, 10% (wt/vol) stool suspended in PBS. This suspension was further serially diluted 10-fold in PBS to produce suspensions of 1%, 0.1% and 0.01% stool. Each dilution of spiked stool was separated into six 95 µL aliquots. Three aliquots were treated with PMA (as described in section 2.3) and three used as untreated controls.

As the presence of stool in specimens could result in PCR inhibition or affect the performance of PMA, the performance of the *P. aeruginosa* qPCR assay in pure culture alone was compared to its performance in stool specimens. Therefore, the heat-killed *P. aeruginosa* culture was diluted to 25%, 2.5%, and 0.25% in PBS to mirror the concentration of spiked bacteria in the stool samples and treated with PMA in an identical manner.

2.5 Spiking of viable and non-viable Staphylococcus aureus into 1/100 diluted FS

Fifty µL of *S. aureus* ATCC 29213 overnight culture in cerebrospinal fluid (CSF) enrichment broth (bioMerieux, Australia) was inoculated into 3 mL CSF broth, which was grown at 37 °C with shaking for 6 hours prior to use to ensure log-phase of growth. Two 1.5 mL culture aliquots were centrifuged for 2 min at 10,000 *g*. The pellet was washed twice in NS and resuspended to provide a suspension of 4 McFarland units (~1.2 x10^9 CFU/mL).

To confirm that PMA treatment was effective in preventing PCR amplification of DNA from non-viable cells, while not affecting amplification of DNA from viable cells, dilute FS was spiked with live and heat-killed *S. aureus* cells as well as with a combination of live and heat-killed cells in a 1:1 ratio. For each, 100 µL was spiked into 900 µL of diluted (1/100) FS. The three spiked samples were treated in triplicate with PMA or PBS control. Pure bacterial cells (live, heat-killed, and combined) were similarly PMA-treated.

To determine whether a higher PMA concentration would provide greater efficacy in concentrated stool, 100 µL heat-killed *S. aureus* was spiked into 900 µL neat FS and divided into nine aliquots (three used as controls, three treated with 100 µM PMA as described above, and three treated with 200 µM PMA).

2.7 Assessing performance of PMA qPCR over a defined range of viable concentrations

To determine the performance of this method over a range of viable/dead concentrations, FS was prepared fresh as described in section 2.1, diluted 100-fold in PBS, and mixed with heat-killed FS in defined proportions. The following FS mixtures were prepared in 1mL aliquots: 100% fresh, 80% fresh/20% heat-killed, 60% fresh/40% heat-killed, 40% fresh/60% heat-killed, 20% fresh/80% heat-killed and 100% heat-killed. Each mixture was treated with and without PMA in triplicate in 100 µL aliquots as described in section 2.3. The proportion of total bacteria viable in each specimen was determined using PMA qPCR targeting the 16S rRNA gene, as described in section 2.8. Estimated *Escherichia coli* colony forming units (CFU/µL) were quantified using a probe based qPCR targeting the *tuf* gene as described previously (Maheux et al., 2009). For each FS mixture, 100 µL of sample was plated onto three MacConkey with salt agar plates (Thermo Fisher Scientific, Therbarton SA,

Australia) at three 10-fold dilutions to select for single colonies of coliforms. Plates were incubated aerobically at 37°C for 24 hr. The heat-killed aliquot was also cultured under the same conditions and demonstrated no growth. Single coliform colonies were counted and confirmed to be *E. coli* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Daltonik MALDI Biotyper, Bruker Biosciences Pty Ltd, Preston VIC, Australia).

2.8 DNA extraction and qPCR

DNA was extracted from samples using the PowerLyzer® PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carslbad, CA, USA) in accordance with the manufacturer's instructions and stored at 20^{o}C.

Levels of total bacteria, *P. aeruginosa*, and *S. aureus*, were determined using previously described qPCR assays (Denman and McSweeney, 2006; Feizabadi et al., 2010; Thomas et al., 2007) on a QuantStudio 6 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Total bacterial and *P. aeruginosa* qPCR assays were performed using sybr green fluorophore reagents (PowerUp™ SYBR™ Green Master Mix, Applied Biosystems, Foster City, CA, USA). *S. aureus* and *E. coli* specific qPCR was performed using probe-based assays (KAPA PROBE FAST ROX Low MasterMix, Kapa Biosystems, Japan). All qPCR assays were performed in triplicate and mean CT values were converted to log10 cell/µL.

Proportions of live cells were determined by dividing the quantity of cells amplified in the presence of PMA by the quantity of cells amplified in matching untreated controls. Statistical significance (p-value <0.05) was determined using paired t-tests for parametric data and the Wilcoxon matched-pairs single rank test for non-parametric data.

3. Results

3.1 *Effect of stool concentration on PMA-qPCR efficacy in fresh, freeze-thawed, and heat-treated samples*

The efficacy of PMA treatment was reduced in both neat and 10-fold diluted FS (2.5% stool content) compared to 100-fold and 1000-fold diluted FS. In neat FS, no difference in amplification between PMA-treated and control samples was observed for fresh (p=0.496), frozen (p=0.203) or heat-treated samples (p=0.203) (Figure 1A). In 10-fold diluted FS (Figure 1B), amplification of bacterial DNA was significantly reduced in fresh (p=0.004) and heat-killed FS (p=0.004) following PMA-treatment compared to controls, but not in frozen FS (p=0.074). In 100-fold diluted FS (Figure 1C), amplification of bacterial DNA was significantly reduced following PMA-treatment compared to controls (p=0.004 for all three treatment conditions). While reductions in bacterial DNA amplification were also observed in 1000-fold diluted FS (p=0.004 for all three treatment conditions, Figure 1D), at this dilution the FS is so dilute that amplification levels are near the threshold of the assay even without PMA treatment. Overall, the best separation of heat-killed control and PMA treated samples is observed at the 100-fold dilution of FS (Figure 2, arrow)

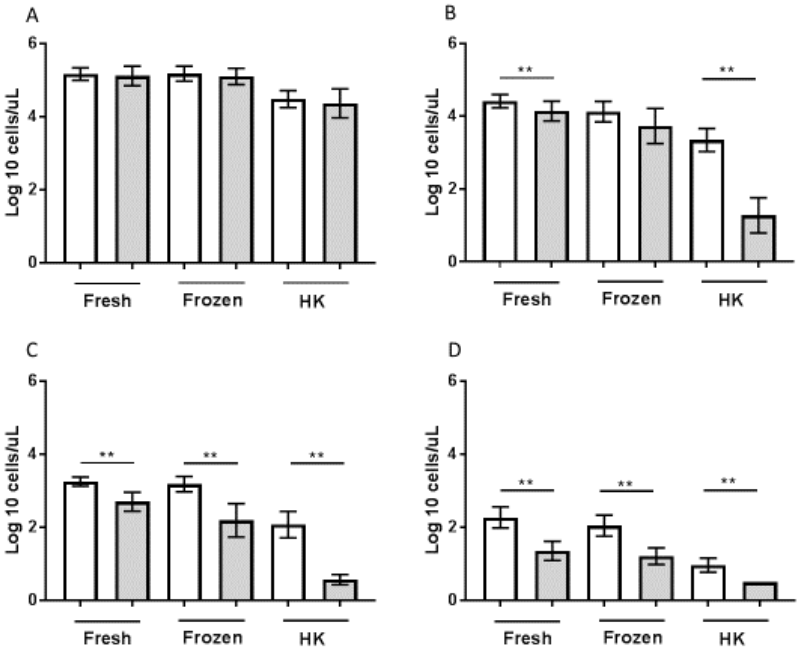

Figure 1. Effect of faecal slurry (FS) dilution on the ability of PMA to exclude non-viable cells from amplification in FS material processed as fresh, frozen or heat-killed (HK). **A.** Neat FS (25% stool) **B.** 1/10 diluted FS (2.5% stool) **C.** 1/100 diluted FS (0.25% stool) **D.** 1/1000 diluted FS (0.025% stool)

Bars depict the mean (+/- standard deviation) log cells/µL amplified by 16S qPCR from three donors, each with three replicate samples. Statistical comparisons are made between amplification from PMA treated samples (shaded bars) compared to untreated control samples (clear bars) (**= p<0.01).

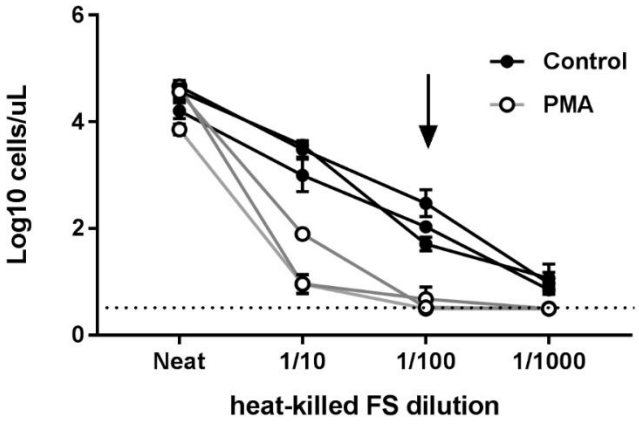

Figure 2. Effect of faecal slurry (FS) dilution on the ability of PMA to exclude non-viable cells from amplification in heat-killed FS material. Circle symbols depict the mean (±standard deviation) log cells/µL amplified by 16S qPCR from three donors, each with three replicate samples. The 1/100 dilution shows the best separation between control and PMA treated specimens (arrow). Dotted line represents the assay's threshold of detection.

3.2 Effect of stool concentration on the ability of PMA to exclude spiked non-viable P. aeruginosa cells from qPCR amplification.

PMA treatment did not prevent the amplification of DNA from non-viable *P. aeruginosa* cells in the presence of 10% stool, with no significant difference observed between PMA-treated and control samples (Table 1). Furthermore, the level of amplification of *P. aeruginosa* DNA in spiked FS was significantly reduced compared to an equivalent level of *P. aeruginosa* DNA in pure culture (3.45 ± 0.04 vs 5.99 ± 0.05 log cell/µL p<0.0001, Figure 3), suggesting inhibition of PCR by components of stool DNA extract. Inhibition was also observed to a smaller degree in the 1% stool samples (4.34 ±

0.02 vs 4.81 ± 0.12; p=0.02), but not in the 0.1% stool sample (Figure 3). No *P. aeruginosa* DNA was detectable in unspiked FS.

In all samples with 1% or less stool concentration, amplification of *P. aeruginosa* DNA in the PMA-treated samples was significantly reduced compared to controls ($p < 0.005$), consistent with the successful limitation of DNA amplification to viable cells (Table 1).

When viability was assessed as a proportion of total bacterial cells (Table 1), no significant difference was observed between spiked samples with ≤1% stool content. More than 97% of DNA from heat-killed cells was excluded from amplification following PMA treatment. The performance of PMA-qPCR in samples with a stool concentration of ≤1% did not differ significantly from the performance of PMA on cells from pure culture alone.

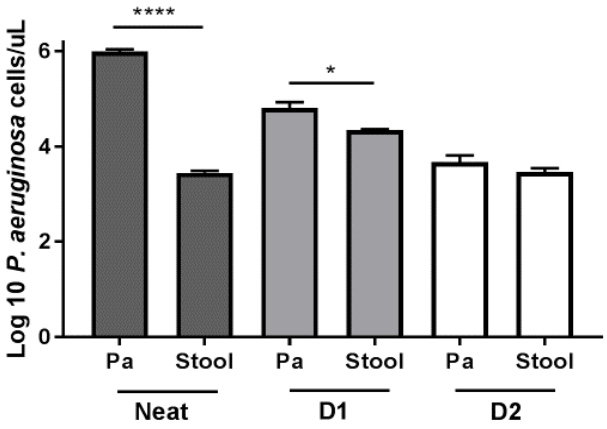

Figure 3. Effect of stool dilution on amplification of heat-killed *P. aeruginosa* cells without PMA treatment. The amplification in *P. aeruginosa* cells alone (Pa) is compared to amplification of the same cells at the same concentration in spiked in FS (*= $p<0.05$; ***= $p<0.001$). The neat spiked sample consists of 10% stool, dilution 1 (D1) of 1% stool and dilution 2 (D2) of 0.1% stool

Table 1. Effect of stool dilution on PMA's ability to exclude heat-killed *P. aeruginosa* DNA from amplification using *P. aeruginosa* specific qPCR.

	Stool content (%)	*P. aeruginosa* cells detected[e]		Difference (p-value)	Proportion viable[f] (PMA /control)
		Control	PMA		
Neat[a]	10%	3.45 ± 0.04	3.80 ± 0.17	0.1	>100%
D1[b]	1%	4.34 ± 0.02	2.80 ± 0.17	0.005	3.0%
D2[c]	0.1%	3.46 ± 0.08	1.74 ± 0.08	0.002	2.0%
D3[d]	0.01%	2.19 ± 0.12	0.53± 0.13	0.0006	2.2%

[a]Heat-killed *P. aeruginosa* cells (5.8 log10 cells/µL) were spiked into a sample consisting of 10% stool (Neat). [b,c,d]This sample was then serially 10-fold diluted: 1/10 dilution (D1), 1/100 dilution (D2), 1/1000 dilution (D3). [e]All *P. aeruginosa* cells detected (log10 cells/µL, mean± SD of 3 replicate samples) in spiked stool samples without PMA treatment (Control) were compared to viable cells detected in corresponding PMA-treated samples (PMA). [f]The proportion of heat-killed *P. aeruginosa* cells detected as viable is determined by dividing viable cells detected in PMA treated samples by total cells detected in the control samples.

3.3 *Efficacy of PMA-qPCR in discriminating live and dead S. aureus cells spiked into 100-fold diluted FS*

Our initial experiment suggested that 100-fold diluted FS (0.25% stool) was optimal for PMA-qPCR determination of viable bacterial load. We sought to confirm this finding by assessing whether PMA treatment could reliably eliminate amplification from non-viable cells, while not significantly affecting the amplification of viable cells. 100-fold diluted FS was spiked with live, heat-killed, or a 1:1 ratio of live and heat-killed *S. aureus* cells, to a concentration of ~ 1.2 x10^9 CFU/mL. The use of

S. aureus also allowed an assessment of the efficacy of PMA treatment when applied to a Gram positive organism and a different, probe-based qPCR assay.

PMA treatment did not significantly affect amplification from viable cells from culture (p= 0.33, data not shown), while still providing optimal (>99%) exclusion of DNA from heat-killed cells from amplification (p= 0.004). No amplification of *S. aureus* DNA was observed with unspiked FS.

PMA treatment reduced amplification of DNA from non-viable cells in FS samples spiked with 1:1 heat-killed: viable cells (p=0.003) and 100% heat-killed cells (p<0.001) when compared to FS spiked with live cells (Figure 4).

We also examined whether the inefficiency of PMA treatment in concentrated stool could be overcome by doubling the concentration of PMA used. However, in neat FS piked with heat-killed *S. aureus* there was no difference in amplification between PMA treated and control samples regardless of PMA concentration (control vs 100 µM PMA, p=0.47; control vs 200 µM PMA p= 0.51).

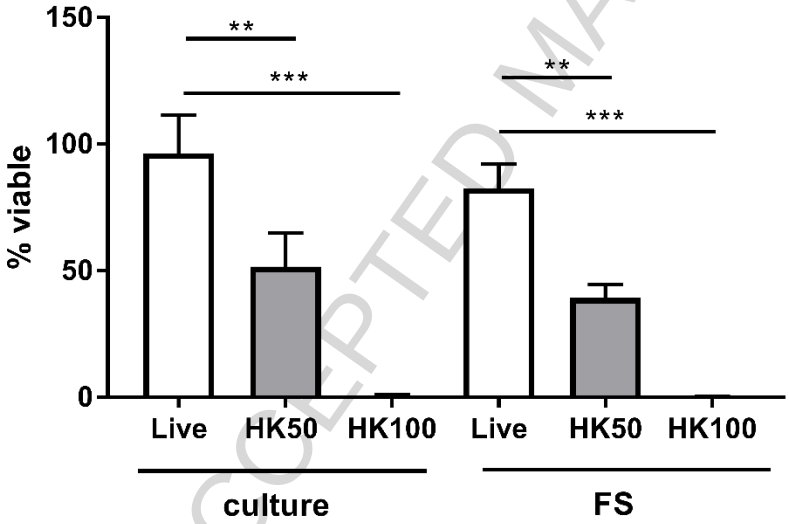

Figure 4. Proportion of cells determined to be viable *S. aureus* cells (mean ± SD of 3 replicate samples). Proportion of viable cells was determined by dividing viable cells amplified in PMA-treated samples over total number of cells amplified in non-PMA treated control samples. *S. aureus* cells consisted of live culture (Live), a mixture of 50% live culture and 50% heat-killed culture (HK50), or 100% heat-killed culture (HK100). The ability of PMA to exclude dead cells from amplification was

assessed in *S. aureus* culture alone, or in *S. aureus* spiked into diluted faecal slurry consisting of 0.25% stool (FS) (**= p<0.01; ***= p<0.001).

3.4 Performance of PMA-qPCR in varying ratios of fresh and heat-killed FS.

The PMA-qPCR method performed well in predicting the proportion of live bacteria in the sample with a strong linear correlation (Figure 5, R^2= 0.966). As the proportion of fresh FS increased there was an increase in the variability between replicate samples, with the most variability in the 100% fresh sample (mean proportion viable 0.55 +/- SD 0.18). PMA-qPCR was used to determine *E. coli* CFU/µL and these results were compared to corresponding colony counts on selective agar. Estimates of viable bacterial load, as determined by PMA-qPCR, were closely correlated with bacterial colony counts (Figure 6). PMA-qPCR performed comparably to culture in identifying the proportion of live *E. coli* in the sample, with a strong linear correlation (Figure 6 panel A: *E. coli* culture R^2= 0.900, panel B: *E. coli* PMA qPCR R^2= 0.978).

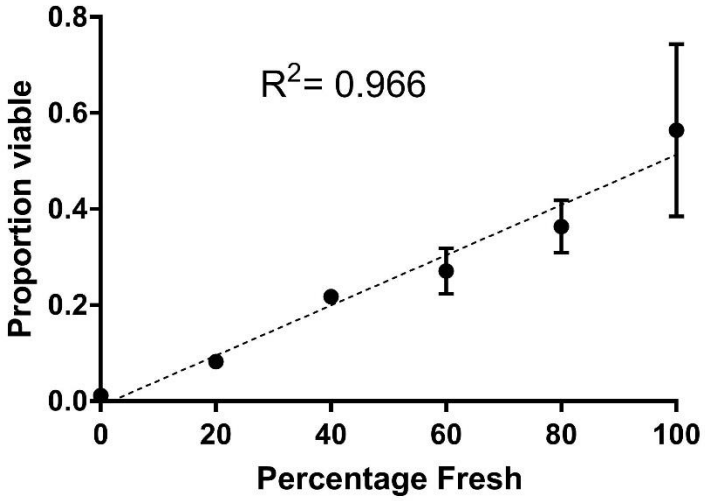

Figure 5. Performance of PMA-qPCR when applied to defined ratios of fresh and heat-killed FS. Data points and error bars represent the proportion of viable bacterial cells detected in FS after 16S qPCR (mean ± SD of 3 replicate samples). Proportion viable was determined by dividing cells/ µL amplified in PMA treated samples by the amplification of the non-PMA treated in the 100% fresh sample.

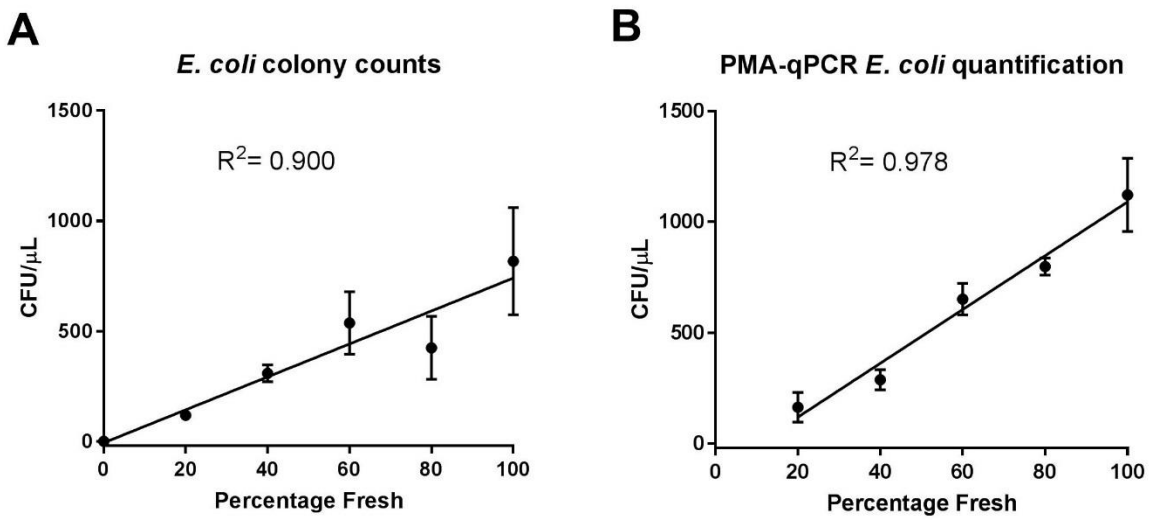

Figure 6. Performance of PMA qPCR when applied to defined ratios of fresh and heat-killed FS. Data points and error bars represent the CFU/ µL after selective culture (panel A) and CFU/ µL of bacterial cells determined to be viable after *E. coli*-specific qPCR (panel B) (mean ± SD of 3 replicate samples).

4. Discussion

A methodology that can reliably determine the viability of stool bacteria, a substantial portion of which are refractory to standard culture techniques, is essential for the accurate assessment and optimisation of stool processing protocols for FMT. While the use of PMA treatment in conjunction with PCR-based bacterial enumeration has shown promise in other contexts, a failure to consider the reduced efficacy of this approach when applied to undiluted faecal slurries may have confounded previous applications in this context. We describe the optimisation of this approach and demonstrate the importance of sample dilution in achieving efficient exclusion of DNA from non-viable cells.

Stool is an inherently heterogeneous and variable material, with substantial variation in water content between samples. The degree of stool dilution incorporated into standard protocols must therefore allow effective PMA treatment on even the densest stool samples. Our results suggest that 1/100 dilution of stool or faecal slurry to ~0.25% stool content is necessary to achieve optimal results following PMA treatment. As a guide, the optical density of the FS specimens used at 0.25% stool

content was 3.76 -4 McFarland units. At this dilution, PMA reliably inhibited amplification from non-viable cells in all experiments.

Although PMA treatment also performed well when stool content was further diluted, over-dilution of specimens is inadvisable, particularly when attempting to amplify targets that are already present at low concentrations (as illustrated in Figure 2). PMA treatment also performed well in excluding DNA from spiked non-viable cells at 1% stool content. However, at this concentration, PCR assay inhibition was observed, a phenomenon that is well-described in relation to DNA extracts from stool (Monteiro et al., 1997).

The inefficiency of PMA treatment at stool contents of ≥10%, could not be overcome by doubling the concentration of PMA used to 200 µM. This observation suggests that factors such as light penetration, rather than PMA concentration, limit the effectiveness of this approach in concentrated stool samples.

PMA-based methods might, under certain circumstances, be prone to underestimating numbers of non-viable bacteria (Nebe-von-Caron et al., 2000). Therefore, our method employs a relatively high PMA concentration (100 µM), long incubation time (30 min) and long period of light exposure (20 min). These parameters are conservative, based on available evidence to optimise the elimination of non-viable cells from amplification (Bae and Wuertz, 2009; Nkuipou-Kenfack et al., 2013). By applying our method to live as well as heat-killed spiked *S. aureus* cells we confirmed that our method did not significantly affect live cells while still excluding more than 99% of DNA from non-viable cells from amplification (Figure 4).

The application of our methodology demonstrates that a single freeze-thaw cycle renders the majority of bacteria in FS non-viable (Figure 1). These results differ from those reported by Chu et al., who did not detect a significant difference in cell viability in FS for FMT after 20 freeze-thaw cycles (Chu et al., 2017). However, in contrast to our optimised approach, they applied PMA treatment to undiluted FS, which we have shown significantly impairs its efficacy. Similarly, Young and colleagues also reported applying PMA treatment to concentrated stool samples (Young et al., 2017). In addition, they analysed samples that had already been frozen, a process which is problematic given the impact that this has on the pool of viable bacteria within the sample.

Commensal intestinal bacteria are increasingly being recognised as important mediators of both human health and disease (Honda and Littman, 2016). Many of these organisms are only readily detectable using molecular methods. PMA-based methodologies have an important role to play in determining the viability of a wide-range of organisms in stool. Application of the optimised methodology described here will allow standardisation of appropriate preparation protocols for FMT-based therapeutics.

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Optimisation of a propidium monoazide based method to determine the viability of microbes in faecal slurries for transplantation

Highlights

- PMA treatment can prevent PCR amplification of DNA from non-viable cells
- Standard PMA protocols are ineffective when applied to faecal slurries
- Pre-treatment dilution of slurries effectively excludes non-viable bacteria
- Freeze-thaw substantially reduces bacterial viability in faecal transplant slurries