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# Optimisation of a bead-beating procedure for simultaneous extraction of bacterial and fungal DNA from pig faeces and liquid feed for 16S and ITS2 rDNA amplicon sequencing

J.T. Cullen [a], P.G. Lawlor [b], P. Cormican [c], F. Crispie [d], G.E. Gardiner [a,*]

[a] Eco-Innovation Research Centre, Department of Science, Waterford Institute of Technology, Co. Waterford X91 K0EK, Ireland
[b] Pig Development Department, Teagasc Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork P61 C996, Ireland
[c] Animal Bioscience Research Centre, Teagasc Grange, Dunsany, Co. Meath C15 PW93, Ireland
[d] Teagasc, Food Research Centre, Moorepark, Fermoy, Co. Cork P61 C996, Ireland

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ABSTRACT

Efficient cell lysis is critical for the extraction of DNA from difficult-to-lyse microorganisms such as Gram-positive bacteria and filamentous fungi. A bead-beating **(BB)** step is usually included in DNA extraction protocols to improve cell lysis. However, there is no consensus on the duration of BB that is necessary for complete lysis of the microbial communities present in complex microbial ecosystems, but which will still maintain the integrity of DNA released from easy-to-lyse microbes. Another consideration is that most protocols are tailored to one particular target group of microbes, typically either bacteria or fungi, in a given sample matrix. In this study, we investigated the impact of five BB durations (0, 3, 10, 15 and 20 min) during DNA extraction with the QIAamp® Fast DNA Stool Mini Kit, on the bacterial and fungal communities of single pig faecal and liquid feed samples, extracted in triplicate, with the objective of determining a suitable 'catch-all' method. Both sample types were subjected to the BB durations in triplicate, followed by 16S (bacterial) and ITS2 (fungal) rDNA amplicon sequencing. The performance of the different BB durations was assessed based on the quantity of total DNA extracted, alpha- and beta-diversity analyses of the resultant microbial communities and differential abundance of bacterial and fungal taxa. Our results suggest that 20 min of BB is most appropriate for maximising the lysis of difficult-to-lyse bacteria and fungi in both pig faeces and liquid feed, while minimising the negative impact on easier-to-lyse microbes. Total DNA yield increased with BB duration for both sample types; however, the yield from faeces decreased after 20 min of BB. Despite this, DESeq2 analysis indicated that changes in the differential abundances of the dominant taxa at this point were limited, which was supported by the Shannon diversity results. Maximising the BB duration appeared to be necessary in order to obtain a representative profile of the Gram-positive bacteria, particularly in liquid feed, and of the filamentous fungi present in both sample types. However, considering the small sample size, along with the reliance on differential as opposed to absolute abundances to validate increases or decreases in taxa, a larger-scale study is necessary to verify the findings of the present study.



## Implications

Considering the variability of bead-beating duration and intensity used in sequence-based microbiome studies, this study highlights the impact of bead-beating on DNA recovery from difficult-to-lyse bacterial and fungal taxa present in pig faeces and liquid feed. Although additional studies are warranted, our results will inform the optimisation and standardisation of DNA extraction procedures so as to obtain a representative profile of liquid feed and porcine gut microbiota in future studies. By demonstrating the practicality of using a single DNA extract for simultaneous bacterial and fungal amplicon sequencing, the findings could also improve the cost- and time-effectiveness of microbiome studies.

* Corresponding author.
*E-mail address:* Gillian.Gardiner@setu.ie (G.E. Gardiner).

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**Specification table:**

| | |
|---|---|
| Subject | *Nutrition* |
| Type of data | Amplicon sequencing data |
| How data were acquired | Illumina MiSeq platform |
| Data format | Raw Fastq files for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under project accession number PRJEB49004 (https://www.ebi.ac.uk/ena/browser/view/PRJEB49004). |
| Parameters for data collection | Five different bead-beating (BB) durations were tested during DNA extraction from pig faeces and liquid feed to assess the impact of BB on the simultaneous recovery of DNA from bacterial and fungal communities. DNA yield, as well as alpha-diversity, beta-diversity and differential abundance of microbial taxa were compared for each BB duration. |
| Description of data collection | Data (sequence reads) were obtained following 2 × 300 bp paired-end sequencing on an Illumina MiSeq platform. Fastq files for each sample were generated by demultiplexing, where sequence reads were assigned to each sample based on unique barcodes associated with each sample, which were added during library preparation. |
| Data source location | Institution: Waterford Institute of Technology<br>City/Town/Region: Waterford<br>Country: Ireland |
| Data accessibility | Repository name: European Nucleotide Archive<br>Project accession number: *PRJEB49004*<br>The QIIME2 and R scripts used for analysis are available at: https://github.com/JamesTCullen/Cullen_et_al_2022_animal_open_space. |

## Introduction

Bias can be introduced via a number of sources during sequencing workflows, from DNA extraction to library preparation, sequencing and bioinformatic analysis. During DNA extraction, efficient lysis of the microbial cell wall is critical in order to obtain a representative yield of good quality DNA from both easy- and difficult-to-lyse microbes. Gram-positive bacteria in particular pose a challenge for complete lysis due to their thick cell walls, while fungal cell walls are more complex and can also be very difficult to lyse. Cell lysis methods include mechanical, chemical and enzymatic disruption of the microbial cell wall, and different methods are often combined (Islam et al., 2017; Frau et al., 2019). Although mechanical disruption methods such as bead-beating **(BB)** can enhance nucleic acid yield, excessive mechanical lysis may also shear microbial DNA into smaller fragments, which may impact success in subsequent downstream applications.

The most appropriate duration of BB necessary for complete lysis of the microbial communities present in complex microbial ecosystems, while also preserving the integrity of the DNA for downstream applications, remains open to debate. Most protocols are tailored to one particular target group of microbes, typically either bacteria or fungi, in a given sample matrix, although some studies have investigated procedures for simultaneous lysis of both bacteria and fungi (Fiedorová et al., 2019; Pérez-Brocal et al., 2020). This study aims to find the most appropriate BB duration, used in conjunction with the QIAamp® Fast DNA Stool Mini kit (Qiagen, Helden, Germany), to simultaneously extract both bacterial and fungal DNA from two different sample types; pig faeces and liquid feed. The performance of five different BB durations, often used across sequence-based microbiome studies, was assessed on the basis of the quantity of total DNA extracted, alpha- and beta-diversity estimates of the resultant microbial communities and the differential abundance of bacterial and fungal taxa across BB durations following 16S and ITS2 rDNA amplicon sequencing.

## Material and methods

### *Experimental design*

This study investigated the impact of five BB durations on the simultaneous extraction of bacterial and fungal DNA from both pig faeces and liquid feed. A faecal sample from an individual finisher pig and a single liquid feed sample were used throughout this study in order to avoid variability between individual pigs or different liquid feed batches to accommodate comparison of the BB

**Table 1**
Details of the bead-beating durations implemented during DNA extraction from pig faeces, liquid feed and a mock community standard using the QIAamp® Fast DNA Stool Mini Kit.

| Bead-beating (BB) duration | Bead-beating time, frequency and rest | Total time required (min) | Description/Reference |
|---|---|---|---|
| BB0 | 0 mins | 0 | Control (no bead-beating) |
| BB3 | 1 min (3 cycles); 1 min rest between | 5 | Scanlan and Marchesi (2008); Huseyin et al. (2017) |
| BB10 | 3 min (2 cycles) + 4 min (1 cycle); 1 min rest between | 12 | McCormack et al. (2019); Torres-Pitarch et al. (2020) |
| BB15 | 5 min (3 cycles); 5 min rest between | 25 | ZymoBIOMICS® Gut Microbiome Standard (Catalog No. D6331) Protocol (Zymo Research Corporation., 2021) |
| BB20 | 5 min (4 cycles); 5 min rest between | 35 | ZymoBIOMICS® Gut Microbiome Standard (Catalog No. D6331) Protocol (Zymo Research Corporation., 2021) |

durations. Differences between BB durations were evaluated based on the quantity of total DNA extracted, microbial diversity estimates and whether bacterial and fungal taxa were differentially abundant between BB durations following 16S and ITS2 rDNA amplicon sequencing. A gut microbiome mock community standard was also used to investigate the potential lysis bias of each BB duration.

### *Sample collection and storage*

Freshly voided faeces was sampled from an individual finisher pig (~120 kg live weight) on the day prior to slaughter. A wheat-barley-soybean meal liquid pig diet, prepared and fed using a liquid feeding system (HydroMix, BigDutchman, Vechta, Germany), was sampled from a trough in the finisher section. Both samples were immediately put on ice and stored at −80 °C until DNA extraction.

### *Bead-beating procedures and DNA extraction*

Five different BB durations selected from the literature are detailed in Table 1 (BB0, BB3, BB10, BB15 and BB20). DNA extractions were performed using the QIAamp® Fast DNA Stool Mini kit, following the 'Isolation of DNA from Stool for Pathogen Detection' protocol as per McCormack et al. (2019) with modifications to the following steps:

**Steps 5–7:** 540 μL of supernatant was added to 37.5 μL of proteinase K, followed by 500 μL of buffer AL for protein digestion.

**Step 9:** 500 μL of ethanol was added for DNA precipitation.

**Step 10:** 750 μL of lysate was applied to the spin column, followed by centrifugation for 2 min at 10 000*g*.

**Step 11:** Centrifugation performed at 10 000*g* for 2 min.

**Step 13:** Centrifugation performed at full speed for 5 min.

**Step 14:** 30 μL of Buffer ATE was applied to the membrane, incubated at room temperature for 5 min and centrifuged for 2 min at 10 000*g* to increase DNA yield.

The sample (0.25 g) was added to a 2-mL screw-cap tube containing 0.25 g of sterile zirconia beads (0.125 g of 0.1 mm and 0.125 g of 1.0 mm, a single bead of 2.5 mm; Stratech Scientific, Ely, UK). InhibitEX® buffer was then added (Step 2), and the relevant BB duration was used. All BB was performed using a MiniBeadbeater-24 (BioSpec Products, Bartlesville, OK, U.S.A.) at 3 000 RPM, and samples were stored on ice between BB cycles. Triplicate DNA extractions were performed on the faecal and liquid feed samples for each BB duration, along with negative controls (tubes containing only the beads and no sample). As a positive control, a mock community standard, ZymoBIOMICS® Gut Microbiome Standard (Zymo Research, Irvine, CA, U.S.A.), was also extracted in triplicate for each respective BB duration.

### *Library preparation and amplicon sequencing*

#### *16S rDNA*

Bacterial communities were profiled via amplicon sequencing of the V3-V4 hypervariable region of the 16S rRNA gene on the Illumina MiSeq platform, according to the Illumina 16S Metagenomic Sequencing Library Preparation Guide, with some modifications. The procedure was performed as described by Fouhy et al. (2015), except that 25 ng of input DNA was used (or 5 μL of neat extracts for the mock community standard and negative controls) for the initial amplicon PCR, and 30 cycles were used instead of 25. The cleaned indexed PCR products were quantified on a Qubit® 3.0 Fluorometer using the Qubit® dsDNA HS Assay Kit (Bio-Sciences, Dublin, Ireland) and were pooled in an equimolar fashion.

#### *ITS2 rDNA*

Fungal profiling was performed by amplifying the nuclear ribosomal ITS2 region with the following locus-specific primers (in bold) containing Illumina overhang adapters at the 5′ ends: forward primer ITS3 (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GCATCGATGAAGAACGCAGC-**3′) and reverse primer ITS4 (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**TCCTCCGCTTATTGATATGC-**3′) (White et al., 1990). Each reaction contained either 50 ng or 100 ng of DNA template for liquid feed and faeces, respectively (amounts of the mock community and control extracts were as described for the 16S rDNA protocol), 0.3 μM forward primer, 0.3 μM reverse primer, 5 μL 5x HiFi buffer, 0.03 mM dNTPs and 0.02 U of KAPA HiFi HotStart DNA polymerase (KAPA HiFi HotStart + dNTPs kit; Roche Diagnostics, West Sussex, U.K.) and nuclease-free PCR grade water in a final reaction volume of 25 μL. PCR parameters were as follows: 95 °C × 3 min, 35 cycles of 98 °C × 30 s, 65 °C × 30 s, 72 °C × 30 s, then 72 °C × 5 min and held at 4 °C.

PCR products were visualised and cleaned as described above for the 16S rDNA protocol, and 5 μL of the cleaned PCR product was dual indexed via a limited cycle PCR (using the same parameters as the ITS2 amplicon PCR except that the number of amplification cycles was reduced from 35 to 8). The index PCR reactions contained 5 μL cleaned PCR product, 5 μL index 1 primer (N7xx), 5 μL index 2 primer (S5xx), 10 μL 5x HiFi buffer, 0.03 mM dNTPs and 0.02 U of KAPA HiFi HotStart DNA polymerase (KAPA HiFi HotStart + dNTPs kit; Roche Diagnostics, West Sussex, U.K.) and nuclease-free PCR grade water in a final reaction volume of 50 μL. Indexed ITS2 PCR products were visualised, cleaned, quantified and pooled as described above.

The final library was quantified by qPCR, diluted, denatured and sequenced using a 2 × 300 cycle V3 kit in the Teagasc sequencing facility as described by Fouhy et al. (2015) in accordance with standard Illumina sequencing protocols.

### *Bioinformatics and statistical analysis*

The QIIME2 and R scripts used for this analysis are available at: https://github.com/JamesTCullen/Cullen_et_al_2022_animal_open_space. Demultiplexed paired-end 16S and ITS2 rDNA sequences (available at: https://www.ebi.ac.uk/ena/browser/view/PRJEB49004) were imported (in Casava 1.8 demultiplexed paired-end format) into QIIME2 v.2020.8.0 (Bolyen et al., 2019), which was installed on a virtual machine (VirtualBox 6.0). Forward and reverse reads were quality assessed using the 'qiime demux summarize' command, FastQC v.0.11.5 and MultiQC v.1.9. The 16S and ITS2 primers were removed from reads using the cutadapt plugin (Martin, 2011). The QIIME2 DADA2 (Callahan et al., 2016) plugin was used for filtering and dereplication, chimera removal, merging paired-end reads and to infer amplicon sequence variants **(ASVs)** in each sample after truncating reads to remove low-quality bases. Read 1 and read 2 of the 16S rDNA sequences were truncated at 267 and 183 bp, respectively, while ITS2 sequences were truncated at 266 and 187 bp, respectively. For bacterial sequences, taxonomy was assigned to each ASV using a Naive Bayes classifier trained on 16S V3-V4 sequences from the Silva database (Version 138) with the 'q2-feature-classifier' plugin, while taxonomy was assigned to fungal ASVs using a Naive Bayes classifier trained on full-length ITS sequences from the UNITE v.8.3 database (Kõljalg et al., 2013).

QIIME artifacts (taxonomy, ASV table, metadata and phylogenetic tree) were imported into R v.4.0.2 as a phyloseq (McMurdie and Holmes, 2013) object with the qza_to_phyloseq () function in the qiime2r package (Bisanz, 2018). Contaminant bacterial and fungal ASVs identified using the decontam package (Davis et al., 2018) were removed prior to further analysis. For

the 16S rDNA sequences, one liquid feed sample (BB3) replicate with a low number of reads compared to the other two replicates ($n$ = 11 006) was excluded from the analysis, while one liquid feed sample (BB10) was excluded from the ITS analysis for the same reason ($n$ = 9 507 reads). Alpha-diversity indices (Shannon diversity) and beta-diversity (Bray–Curtis), based on unrarefied sequences, were calculated using the phyloseq package (McMurdie and Holmes, 2013) in R. Shannon diversity was subsequently plotted using the alpha_boxplot function in the 'amplicon' package in R, which performs an ANOVA followed by Tukey's honestly significant difference **(HSD)** test (Liu et al., 2021), while Principal coordinate analysis **(PCoA)** plots were plotted using the ggplot2 package (Wickham, 2016). Differential abundance testing was performed using the DESeq2 package (Love et al., 2014) in R. Bacterial and fungal genera that were present at <1% mean relative abundance for each BB duration across either faecal or liquid feed samples were filtered from the unrarefied read counts prior to DESeq2 analysis. Log2 fold changes between BB durations with an adjusted $P$-value <0.05 were considered significant.

## Results

### *Impact of bead-beating on DNA yield*

Fig. 1 shows the concentrations of total DNA extracted from faeces, liquid feed and the mock community standard. DNA concentrations were highest for faeces. All samples, except for one BB0 and one BB10 replicate, had >100 ng/μL total DNA. After 3 min BB, the mean total faecal DNA concentration increased substantially from ~100 ng/μL without BB to ~350 ng/μL. The DNA yield for BB10 was lower, on average, than that of BB3; however, this was influenced by one replicate having a low yield, while the other two BB10 replicates had a yield similar to that of BB3. There was a subsequent increase in DNA yield from faeces after BB15. Finally, a notable drop in the total DNA yield occurred after BB20; however, the concentration was still higher compared to no BB. It should be noted that the total DNA concentration is not fully representative of microbial DNA as this figure will also include any host or plant DNA extracted.

The total DNA yields for liquid feed were lower compared to those obtained from faeces. Interestingly, BB did not appear to have an impact on the DNA yield until after 15 min. At BB15, the DNA concentration increased from ~35 ng/μL (for BB0, BB3 and BB10) to ~50 ng/μL, and further increased to ~60 ng/μL at BB20, albeit these increases were not as large as those observed in the pig faeces. The DNA yields from the mock community are much lower than the faecal and liquid feed samples because it contains only a mixture of 18 bacterial strains, 2 fungal strains, and 1 archaeal strain. Although yields were variable between BB durations, the most notable difference is that all samples processed with BB resulted in a higher total DNA yield compared to no BB, indicating improved lysis efficiency of the mock community with BB.

### *Alpha-diversity estimation*

The Shannon diversity of the pig faecal and liquid feed microbiota and mycobiota are displayed in Fig. 2. The microbial communities in liquid feed were much less diverse compared to those in the faeces.

In faeces, the bacterial Shannon diversity decreased after BB10 ($P$ < 0.05) but remained similar thereafter up to 20 min of BB ($P$ > 0.05). Conversely, the diversity of fungi in the faeces increased after 3 min of BB compared to the samples with no BB ($P$ < 0.05), with additional numerical increases up to BB20. Alpha-diversity of the bacterial and fungal communities in liquid feed were almost the inverse of those in faeces. Bacterial Shannon diversity was the same for BB0 and BB3 but was higher after BB10 ($P$ < 0.05), although there were no further increases after 15 and 20 min. Despite the samples that were bead beaten for 10 min appearing to be outliers in the data, the fungal diversity in liquid feed showed a general decrease with increased BB, with BB15 and BB20 having lower diversity than samples that were not bead beaten ($P$ < 0.05).

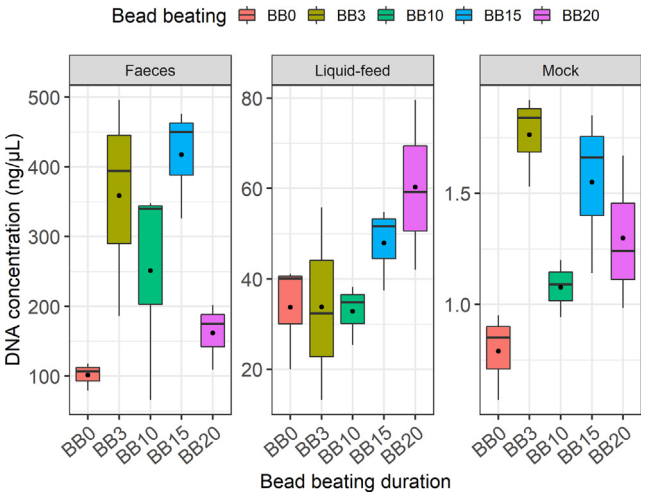


**Fig. 1.** Boxplots displaying total DNA concentration (ng/μL; y-axis) from pig faeces, liquid feed and a mock community after DNA extraction using five different bead-beating durations [no bead-beating (BB0); 3 min bead-beating (BB3); 10 min bead-beating (BB10); 15 min bead-beating (BB15); 20 min bead-beating (BB20); x-axis]. For each respective sample type, each bead-beating duration represents triplicate DNA extractions ($n$ = 3). The black dot in each box represents the mean DNA concentration for each bead-beating duration.

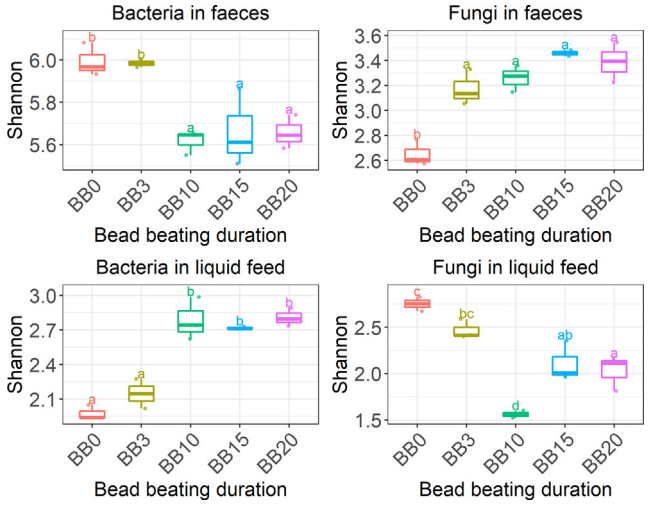


**Fig. 2.** Boxplots displaying mean bacterial and fungal Shannon diversity estimates (y-axis) for pig faeces and liquid feed after DNA extraction using five different bead-beating durations [no bead-beating (BB0); 3 min bead-beating (BB3); 10 min bead-beating (BB10); 15 min bead-beating (BB15); 20 min bead-beating (BB20); x-axis]. For each respective plot, each bead-beating duration represents the mean Shannon diversity of triplicate samples ($n$ = 3), except that one liquid feed sample (BB3) was excluded from the bacterial alpha-diversity analysis ($n$ = 2) and one liquid feed sample (BB10) was excluded from the fungal alpha-diversity analysis ($n$ = 2). $^{a–d}$For each respective plot, bead-beating durations that do not share a common letter are significantly different ($P$ < 0.05).

*Beta-diversity*

The bacterial and fungal beta-diversity was assessed using PCoA plots based on Bray-Curtis distances at the genus level to evaluate the between-sample dissimilarities for faeces and liquid feed (Fig. 3). For bacterial beta-diversity in faeces, the samples generally clustered by BB duration, except for BB15 where the triplicate extracts were more dissimilar. The faecal samples that were bead beaten for 10 min or more clustered away from BB0 and BB3, indicating differences in the faecal microbiota composition at BB10 and beyond, which was supported by the decreased bacterial alpha-diversity observed in faeces after BB10. A similar trend was observed for the bacterial diversity of liquid feed, where after 10 min of BB, samples generally clustered together, indicating similar bacterial compositions for BB10, BB15 and BB20. In contrast to faeces, the liquid feed bacteriome differed between BB0 and BB3, indicating that even 3 min of BB had a dramatic effect on bacterial composition of the liquid feed.

The impact of BB on the fungal communities in faeces is apparent from Fig. 3 where all BB durations clustered away from the samples with no BB. Although the other BB durations were distinctly different from BB0, they also displayed distinct clustering from each other, suggesting that the fungal communities in faeces differed at each of the BB durations. The mycobiota in liquid feed showed similar findings, although there was more inter-replicate variability between these samples. Nonetheless, the fungal composition of faeces and liquid feed appeared to be highly influenced by the different durations of BB.

*Differential abundance analysis*

In order to investigate whether specific bacterial or fungal taxa in pig faeces and liquid feed were differentially abundant between the different BB durations, differential abundance testing was performed using DESeq2. In order to focus on the microbes that predominated in the samples, only the taxa present at >1% mean relative abundance at each BB duration in either faecal or liquid feed samples were analysed. Log2 fold changes (adjusted *P*-value < 0.05) between BB durations in faeces and liquid feed for the most abundant bacterial and fungal genera are presented in Tables 2 and 3, respectively.

The results for the differentially abundant bacterial genera in faeces were variable, with a similar number of tested taxa being differentially increased and decreased between the different BB durations. It was noted, however, that fewer taxa were impacted by the increased BB time at BB20, compared to the other durations. This may suggest that at BB20, the faecal bacteriome is sufficiently lysed. Importantly, moving to BB20 did not show a large degree of decreased differential abundance of taxa, as might be expected due to the excessive heat generation and potential shearing of DNA. For liquid feed, which was dominated by only 5 genera, BB3 decreased the abundance of *Lactobacillus* and *Weissella* compared to BB0 (adjusted *P*-value <0.05), while *Pediococcus* was enriched at BB10 compared to BB3 (adjusted *P*-value <0.05).

A number of fungal genera were enriched at BB3 compared to BB0, which included the difficult-to-lyse moulds *Mucor* and *Monascus* (Scharf et al., 2020). However, differential abundance data must be interpreted with caution. For example, the results show that *Debaryomyces* had a log2 fold change of −26.30. This suggests that

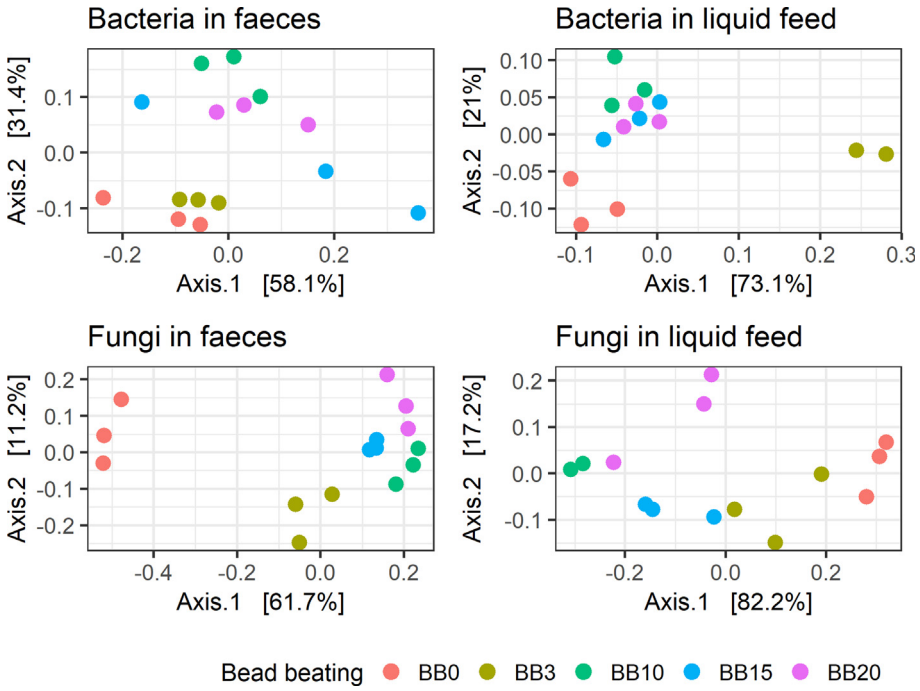


**Fig. 3.** Beta-diversity of bacterial and fungal communities in pig faeces and liquid feed after DNA extraction using five different bead-beating durations [no bead-beating (BB0); 3 min bead-beating (BB3); 10 min bead-beating (BB10); 15 min bead-beating (BB15); 20 min bead-beating (BB20)]. Principal coordinate analysis plots display beta-diversity based on Bray-Curtis distances at the genus level. For each respective plot, each bead-beating duration (coloured) represents triplicate samples ($n$ = 3), except that one liquid feed sample (BB3) was excluded from the bacterial beta-diversity analysis ($n$ = 2) and one liquid feed sample (BB10) was excluded from the fungal beta-diversity analysis ($n$ = 2).

**Table 2**
Differential abundance (log2 fold changes)[1] of bacterial genera, present at >1% mean relative abundance across each bead-beating duration[2] for pig faecal or liquid feed samples.[3]

| Genus | Log2 fold changes at the genus level between different bead-beating durations<br>Faeces | | | |
|---|---|---|---|---|
| | BB3 vs BB0 | BB10 vs BB3 | BB15 vs BB10 | BB20 vs BB15 |
| *Prevotella* | −0.18 | – | 0.12 | – |
| *Clostridium_sensu_stricto_1* | 0.63 | – | −0.70 | – |
| *Prevotellaceae_NK3B31_group* | 0.21 | 2.61 | – | – |
| *Muribaculaceae* | –[4] | 0.62 | −0.23 | – |
| *Lactobacillus* | – | 2.00 | −0.48 | – |
| *Treponema* | – | −1.03 | 0.66 | −0.24 |
| *Alloprevotella* | – | 0.76 | −0.41 | – |
| *Rikenellaceae_RC9_gut_group* | 0.45 | – | −0.19 | – |
| *Phascolarctobacterium* | −0.67 | 1.51 | −0.59 | – |
| *Oscillospiraceae UCG-005* | – | – | – | 0.29 |
| *Oscillospiraceae UCG-010* | −0.23 | −0.54 | – | −0.36 |
| *Anaerovibrio* | −0.45 | −0.45 | 0.35 | – |
| *Terrisporobacter* | 0.59 | 1.63 | −0.50 | – |
| *Christensenellaceae_R-7_group* | – | 0.68 | −0.50 | – |
| *Clostridia_vadinBB60_group* | −0.61 | −1.70 | 0.88 | −0.44 |
| *Streptococcus* | 0.43 | 2.02 | −0.54 | 0.40 |
| *Blautia* | −0.37 | −0.52 | 0.68 | – |
| *Succinivibrio* | – | −1.10 | 1.05 | – |
| *Clostridia_UCG-014* | – | −0.23 | – | – |
| *WCHB1-41* | −0.56 | −1.68 | 0.98 | – |
| *Oscillospiraceae UCG-002* | −0.22 | – | −0.24 | −0.30 |
| *Faecalibacterium* | −0.36 | −0.36 | 0.38 | – |
| *Oscillospiraceae NK4A214_group* | – | −0.46 | 0.24 | – |
| *Campylobacter* | – | −1.48 | 1.08 | −0.39 |
| *Ruminococcus* | −0.21 | −0.39 | 0.34 | – |
| *Subdoligranulum* | – | 0.28 | – | 0.39 |
| *Prevotellaceae_UCG-003* | 0.47 | 0.34 | −0.34 | – |
| *Gastranaerophilales* | −0.49 | −1.55 | 0.80 | – |
| *Megasphaera* | 1.13 | 2.42 | −0.75 | 0.34 |
| *Eubacterium_coprostanoligenes_group* | – | 0.52 | −0.36 | – |
| *Parabacteroides* | – | 0.25 | −0.40 | – |
| Genus | Log2 fold changes at the genus level between different bead-beating durations<br>Liquid feed | | | |
| | BB3 vs BB0 | BB10 vs BB3 | BB15 vs BB10 | BB20 vs BB15 |
| *Lactobacillus* | −0.81 | −1.18 | – | – |
| *Weissella* | −0.61 | −0.50 | – | – |
| *Pediococcus* | – | 0.40 | – | – |

[1] The adjusted *P*-value cut-off for Log2 fold changes was set at 0.05.
[2] Bead-beating durations during DNA extraction: no bead-beating (BB0); 3 min bead-beating (BB3); 10 min bead-beating (BB10); 15 min bead-beating (BB15); 20 min bead-beating (BB20).
[3] For DESeq2 analysis of each respective sample type, each bead-beating duration represented triplicate samples ($n$ = 3), except that one liquid feed sample (BB3) was excluded from the analysis ($n$ = 2).
[4] Dash (–) indicates that the log fold change of the genus between the two bead-beating durations was not statistically significant (adjusted *P*-value >0.05).

BB had a negative impact on the abundance of *Debaryomyces*; however, the large fold change was because this genus was present in the BB0 samples, but not in the BB3 faecal samples. However, *Debaryomyces* was detected again in the BB10, 15 and 20 samples, with increasing mean relative abundance, and therefore, it is difficult to say based on these data, that BB was responsible for the initial decrease in differential abundance.

Another surprising result was that *Malassezia*, a genus of difficult-to-lyse yeast (Diaz et al., 2017), appeared to be less abundant after BB20 compared to BB15. The relative abundance data support this as the relative abundance of *Malassezia* from BB0 to BB15 decreased from 2.8 to 0.1%, and the genus was not present after BB20 (data not shown). Despite this, inferring changes in abundance based on relative abundance data can be problematic as the decreased relative abundance of *Malassezia* may be a result of an increase in the absolute abundance of a different fungal taxon with increasing BB (Morton et al., 2019). For this reason, it may be more appropriate to quantify absolute abundances of taxa of interest using real-time PCR to determine the impact of BB on their lysis efficiency.

*Author's points of view*

Based on the aim of this study, which was to optimise a 'catch-all' workflow incorporating BB for optimal bacterial and fungal DNA recovery from both pig faeces and liquid feed, we recommend that 20 min BB (BB20) be used for simultaneous extraction of DNA from the bacterial and fungal communities present in pig faeces and liquid feed samples. Following an increase in total DNA yield from faeces with increasing BB time, the decrease in yield after BB20 indicated that a loss of DNA from easy-to-lyse microbes may have occurred. The fragile nature of the mammalian cell membrane compared to that of bacteria and fungi may also, at least in part, be responsible for the lower yield from BB20, as host DNA that was released early on may have been degraded, possibly in addition to DNA from easier-to-lyse microbes.

Despite this, DESeq2 analysis suggested that there were few changes in the differential abundances of the dominant taxa between BB15 and BB20. This was supported by the similar bacterial alpha-diversity with BB10 compared to BB20 for faeces. Despite a decrease in bacterial alpha-diversity after BB10, increas-

**Table 3**
Differential abundance (log2 fold changes)[1] of fungal genera, present at >1% mean relative abundance across each bead-beating duration[2] for pig faecal or liquid feed samples.[3]

| Genus | Log2 fold changes at the genus level between different bead-beating durations<br>Faeces | | | |
|---|---|---|---|---|
| | BB3 vs BB0 | BB10 vs BB3 | BB15 vs BB10 | BB20 vs BB15 |
| *Mucor* | 6.43 | – | – | – |
| *Peniophora* | −11.87 | – | – | – |
| *Trichosporon* | 22.29 | – | – | – |
| *Debaryomyces* | −26.30 | 22.16 | – | – |
| *Kazachstania* | −2.08 | −1.26 | – | – |
| *Gamsia* | 4.09 | – | – | – |
| *Scopulariopsis* | 4.22 | – | – | – |
| *Monascus* | 11.04 | – | – | – |
| *Pichia* | –[4] | −1.43 | – | – |
| *Cladosporium* | – | −2.90 | – | – |
| *Alternaria* | – | −1.99 | – | – |
| *Malassezia* | – | – | – | −22.20 |
| Genus | Log2 fold changes at the genus level between different bead-beating durations<br>Liquid feed | | | |
| | BB3 vs BB0 | BB10 vs BB3 | BB15 vs BB10 | BB20 vs BB15 |
| *Pichia* | – | 2.23 | −1.18 | – |
| *Kazachstania* | 1.97 | 2.50 | −1.25 | – |
| *Saccharomyces* | – | 2.33 | −1.63 | 0.58 |
| *Fusarium* | – | −0.56 | 0.59 | – |
| *Gibberella* | – | −0.81 | – | – |
| *Monographella* | – | −0.76 | 0.67 | – |

[1] The adjusted *P*-value cut-off for Log2 fold changes was set at 0.05.
[2] Bead-beating durations during DNA extraction: no bead-beating (BB0); 3 min bead-beating (BB3); 10 min bead-beating (BB10); 15 min bead-beating (BB15); 20 min bead-beating (BB20).
[3] For DESeq2 analysis of each respective sample type, each bead-beating duration represented triplicate samples ($n$ = 3), except that one liquid feed sample (BB10) was excluded from the analysis ($n$ = 2).
[4] Dash (–) indicates that the log fold change of the genus between the two bead-beating durations was not statistically significant (adjusted *P*-value >0.05).

ing the BB time is justified to increase the alpha-diversity of fungi in faeces, which increased with more BB. Additionally, beta-diversity analysis indicated a distinct microbiota and mycobiota associated with each BB duration and although differential abundance analysis suggested that one yeast genus was under-represented at BB20, we conclude that BB20 maximises the lysis of difficult-to-lyse microbes and does not appear to negatively impact easier-to-lyse microorganisms in faeces to a large extent.

Total DNA recovery from liquid feed was optimal at BB20; however, similar to the situation with host DNA in the faeces, it is also possible that plant DNA released from feed components by excessive BB contributed to the increased total DNA yields. Nonetheless, the liquid feed samples were dominated by Gram-positive lactic acid bacteria, which are more difficult to lyse. Therefore, considering that species richness was quite low for bacteria in liquid feed, the increased Shannon diversity may be explained by increased evenness in the samples i.e. with BB, a greater proportion of DNA was released from some previously under-represented difficult-to-lyse bacteria. Despite a lower fungal alpha-diversity in liquid feed with increased BB, several taxa appeared to benefit from increased BB. A decrease in the evenness of fungi may have been responsible for decreased Shannon diversity in liquid feed. While the yeast communities may have been sufficiently lysed, increased lysis of filamentous fungi, which are more difficult to lyse than yeast, may have influenced this.

For these reasons, we propose that maximising the BB duration at 20 min generates a more accurate representation of the true bacterial and fungal communities in pig faeces and liquid feed. This method development work will inform the optimisation and standardisation of DNA extraction procedures to help minimise lysis bias in future liquid feed and pig gut microbiota studies. By demonstrating the practicality of using a single DNA extract for simultaneous bacterial and fungal amplicon sequencing, the findings could also improve the cost- and time-effectiveness of microbiome studies. Nonetheless, the limitations of this study, which include a small sample size and a lack of absolute abundance data to validate increases or decreases in microbial abundance, justify a larger-scale study which would complement the dataset from this study and provide further insights into lysis bias within these communities.

In conclusion, to optimise simultaneous bacterial and fungal DNA extraction from pig faeces and liquid feed with the QIAamp® Fast DNA Stool Mini kit, our results suggest that BB for 20 min is the most appropriate duration to minimise lysis bias of Gram-positive bacteria and filamentous fungi. However, a larger-scale study is necessary to validate these findings considering the small number of samples used in this study, in addition to the fact that we performed differential abundance testing as opposed to quantification of absolute abundances of relevant taxa.

## Ethics approval

Not applicable.

## Author ORCIDs

**James T. Cullen:** https://orcid.org/0000-0003-2435-3567
**Peadar G. Lawlor:** https://orcid.org/0000-0003-1571-2919
**Paul Cormican:** https://orcid.org/0000-0001-7692-7011
**Fiona Crispie:** Not applicable
**Gillian E. Gardiner:** https://orcid.org/0000-0003-0262-395X

## Author contributions

**James T. Cullen:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original draft preparation.
**Peadar G. Lawlor:** Conceptualization, Methodology, Writing - Review and Editing, Supervision.

**Paul Cormican:** Conceptualization, Methodology, Writing - Review and Editing, Supervision.

**Fiona Crispie:** Formal analysis, Investigation, Writing - Review and Editing.

**Gillian E. Gardiner:** Conceptualization, Methodology, Writing - Review and Editing, Supervision.

## Declaration of interest.

None.

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## Reader comments

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