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Evaluation of different methods for the effective isolation of high-quality DNA from rodent faecal samples

Nik Amirah Auni Nik Mohd Asri, Zarina Amin* and Yew Chee Wei

Biotechnology Research Institute, Universiti Malaysia Sabah, Jalan UMS, 88400, Kota Kinabalu Sabah, Malaysia.
Email: zamin@ums.edu.my

ABSTRACT

Aims: The inefficient lysis of recalcitrant bacterial cell wall and subsequent isolation of DNA from environmental samples can lead to a bias in the qualitative and quantitative assessment of bacteria present in the sample. Thus, the selection of an optimum DNA isolation method is the important first step for biosurveillance and metagenomic analyses. This study aims to determine the optimal DNA isolation method out of four commercial DNA isolation kits (A, B, C and D) and two conventional methods (E and F), for rodent faecal droppings. The key selection criterion is the general bacterial diversity contained in the isolated DNA, as evaluated by the Shannon-Weaver index based on the maximal number of PCR-amplicons of partial 16S rRNA gene, derived from each method. The amplicons were separated in accordance with their difference in nucleotide sequences via DGGE.
Methodology and results: Five faecal samples of wild rodents were collected from different sites and preserved in DNA/RNA shield reagent (Zymo Research). Each sample was extracted, and the DNA extracts were then subjected to amplification of the bacterial 16S rRNA and DGGE separation of the amplicons. Method E showed a higher yield of DNA (average 324.22 ng/µL) as compared to the other methods. However, the majority of the DNA extracts showed partial degradation. The DGGE profiles showed the highest number of amplicons were generated from DNA extracted from Method A and B with a total of 168 and 167 respectively. This is indicated by the Shannon-Weaver index which were 0.306 and 0.305, respectively.
Conclusion, significance and impact of study: Method A is the optimum DNA isolation method for rodent faecal samples as its isolated DNA contains the most diverse bacteria. The isolated DNA can then be used for PCR-biosurveillance or metagenomic sequencing and analyses.

Keywords: 16S rRNA, DNA extraction, PCR-denaturing gradient gel electrophoresis (DGGE), rodent faecal samples

INTRODUCTION

Molecular studies on the bacterial diversity in faecal samples are exponentially exploited to fill in the gaps in current knowledge of the gut microbiota and molecular diagnosis of zoonotic diseases. There are a plentiful of DNA isolation methods from environmental samples such as soil, water and faeces, that are naturally admixed or containing diverse bacterial species. Meanwhile, these environmental samples also contain PCR inhibitors such as humic acid and polysaccharides. Several factors associated with difficulties in extracting faecal samples are diverse sample composition of host cells and pathogens, and the richness of microbial load. Moreover, different bacteria have distinct cell wall structures thereby leading to variable efficiency in DNA extraction. Apart from that, the presence of inhibitors, such as humic acids may interrupt the downstream PCR analysis (Kikuchi *et al.*, 2010; Acharya *et al.*, 2017). Therefore, determining an optimal DNA isolation method for a type of sample is an important first step in bacterial diversity studies.

Selection of an optimal DNA isolation method should thus prioritise obtaining the highest number of bacteria within an environmental sample. Previous studies suggested that different DNA isolation methods unavoidably have a bias in lysis of recalcitrant cell walls in diverse species of bacteria. In order to minimise this circumstance, it is recommended that an optimal DNA isolation method should be determined out of a number of methods based on the criterion that the isolated DNA contains the highest number of different bacteria. As this can only be directly measured using deep sequencing and metagenomic analyses, an alternative proxy is to measure the number of amplicons separated by denaturing gradient gel electrophoresis (DGGE) and evaluated by Shannon-Weaver index. This can be achieved by amplifying the V2-V3 regions of the 16S rRNA gene as it is conserved in all bacteria and sequence variations within have been utilised for species identification (Coenye and Vandamme, 2003; Chakravorty *et al.*, 2007; Nakatsu, 2007). Using this feature, the number of bacteria species can be estimated once the

*Corresponding author

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16S rRNA amplicons are separated into individual amplicons by DGGE. The PCR-DGGE analysis is a molecular technique that separates the identical size of PCR amplicons by their differential mobility across the denaturant gradient on the polyacrylamide gel based on their sequence variations and melting temperature (Schäfer and Muyzer, 2001). This approach is valuable as it offers a monitoring tool in variations of microbial community in various environmental samples, as well as assessing the quality of the isolated DNA (Wilson *et al*., 1997).

Previous studies evaluated several DNA extraction methods on various sample types, such as human faecal samples (Ariefdjohan *et al.*, 2010; Mirsepasi *et al*., 2014), avian (Eriksson *et al.*, 2017; Hou *et al.*, 2021) and plants (Abdel-Latif and Osman, 2017). Regardless, there is limited study evaluating isolation methods specifically on rodent faecal samples. This study aims to fill the gaps by assessing the performance of six different DNA extraction methods based on DNA yield, applicability of DNA extracts for PCR analysis, and representation of bacterial diversity via DGGE analysis. Additionally, this study facilitates future research in the fields of microbiome research, zoonoses threat, as well as ecological study of rodent populations.

MATERIALS AND METHODS

Collection of rodents' faecal samples

Faecal droppings were collected from five different areas in Kota Kinabalu, Sabah, Malaysia in October-November 2021. The samples were collected around market and shop lots areas in Alamesra (S1; 6°1'56.78" N, 116°8'5.75" E), Indah Permai (S2; 6°3'41.52" N, 116°9'3.63" E), Menggatal S3; 6°1'27.63" N, 116°9'23.4" E), Inanam (S4; 5°59'29.55" N, 116°8'6.21" E) and Tanjung Lipat (S5; 6°0'32.69" N, 116°6'40.1" E). Each sample was collected in a 2.0 mL microcentrifuge tube containing 200 µL of DNA/RNA Shield Reagent (Zymo Research, USA). The samples were then pulverised into finer particles from which 10-20 mg was aspirated in new tubes before the samples were stored at -20 °C to prevent nucleic acid degradation, following the manufacturer's instructions.

DNA extraction and quantification of the rodent faecal samples

DNA from five random rodent faecal samples were extracted using six different methods. DNA extraction using commercial kits were performed according to the manufacturer's protocols with some minor modifications. Protocols recommended higher input of starting materials (>200 mg), however, the amount of faeces used in this study was 10-20 mg only. For three kits (A, B and D) that required bead-beating steps, the samples were homogenised using BeadBug™ 3 Position Bead Homogenizer (Thomas Scientific, USA). Both Method A and D were lysed at 878× *g* for 15 sec, then paused for 15 sec and continued for 15 sec, while Method B was homogenised at 878× *g* for 15 sec. For Method C, the samples were lysed using homogenizer pestle until fully dissolved. Then, the DNA was extracted according to the instructions and eluted in a 60 µL elution buffer provided.

For conventional methods, lysis buffers for Method E and F were prepared according to previous studies. Lysis buffer for Method E contains 4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% (wt/vol) *N*-laurosylsarcosine and 0.1 M 2-mercaptoethanol (Chomczynski and Sacchi, 2006), whereas Method F contained 2% CTAB, 100 mM Tris-HCl (pH8.0), 2 M NaCl and 20 mM EDTA (Tang *et al.*, 2008). The same number of faecal samples was also used in conventional methods. The faeces were lysed using homogenizer pestle in respective lysis buffers. Then, 500 µL of phenol, pH 7.8 was added to the samples and mixed well. The samples were centrifuged, and the upper aqueous solution was transferred to clean tubes. All centrifugation steps were done at 13000× *g* for 10 min in both methods. An equal volume of chloroform: isoamyl alcohol (49:1) was added and mixed well. The samples were centrifuged, and upper aqueous solutions were transferred to clean tubes. The DNA was precipitated using equal volume of isopropanol and incubated at -20 °C for at least 1 h. Then, the tubes were centrifuges and supernatants were removed. The pellets were washed using 75% cold-ethanol twice and finally, the DNA was eluted using a 60 µL TE buffer.

A single drop spectrophotometer (NanoDrop™, Thermo Scientific, USA) was used to quantify the concentration of the extracted DNA. The quality of the DNA extracts was determined using 1× Tris-Acetate-EDTA (TAE) at 100 V for 30 min. Then, the gel was stained with 0.5 µg/µL ethidium bromide (Sigma, USA) for 5 min and de-stained in water for 5 min. The gel was visualised under Molecular Imager Gel Doc™ XR System (BioRad, USA).

Determination of rodents' species of the faeces

The extracted DNA samples were subjected to DNA barcoding for rodent species identification by PCR of the *cytochrome oxidase I* (COI) gene using primers COI-BatL5310 (5′-CCT ACT CRG CCA TTT TAC CTA TG-3′) and COI-R6036R (5′-ACT TCT GGG TGT CCA AAG AAT CA-3′) (Robins *et al*., 2007). The expected amplicon size is 726 bp. The PCR reaction was performed using KOD-Plus-Neo (Toyobo, Japan) containing 1× buffer KOD-Plus-Neo, 0.2 mM dNTPs, 1.5 mM MgSO4, 0.2 µM both forward and reverse primers, 0.5 U Kod-Plus-Neo in a total 25 µL reaction volume. The DNA amplification was performed in a PCR thermocycler (MJ Research PTC-200) at conditions 95 °C for 3 min, 30 cycles of 98 °C for 25 sec, 56 °C for 10 sec and 68 °C for 30 sec and a final extension 68 °C for 1 min with a holding temperature at 10 °C. The PCR amplicons were separated on 1.5% (w/v) agarose gel in 1× TAE buffer (Vivantis, Malaysia), stained with 0.5 µg/µL ethidium bromide (Sigma, USA) for 5 min and de-stained in water for 5 min. The gel was visualised

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Table 1: Comparison of DNA extraction protocols for each respective extraction method.

Extraction method/steps	A	B	C	D	E	F
Type of bead used	Type unknown	Glass bead, 0.1 mm	None	Type unknown, 0.1 and 0.5 mm	None	None
Cell lysis and homogenization	BeadBug Homogenizer (2800 rpm for 15 sec, pause 15 sec, cont. 15 sec)	BeadBug Homogenizer (2800 rpm for 15 sec)	Homogenizer Pestle	BeadBug Homogenizer (2800 rpm for 15 sec, pause 15 sec, cont. 15 sec)	Homogenizer Pestle	Homogenizer Pestle
Adsorption/ removal of inhibitors	Solution CD2	Solution IRS	Proteinase K and Buffer APL2	Zymo-Spin III-HRC filter	Phenol, chloroform, isoamyl alcohol	Phenol, chloroform, isoamyl alcohol
Approximate time to completion (min)	45 to 60	60 to 80	60 to 80	45 to 60	95 to 160	95 to 160

under Molecular Imager Gel DocTM XR System (BioRad, USA). The samples were considered as derived from rodents if a visible amplicon within the expected size range was observed.

The COI amplicons were subjected to Sanger sequencing. Low quality sequences at the terminal ends were trimmed using BioEdit software. The trimmed DNA sequences were compared with the nucleotide collection (nr/nt) database at the NCBI GenBank using the Basic Local Alignment Search Tool (BLAST). The identity of the rodent species which was the source of the faecal sample was confirmed based on the similarity as evidenced from the BLAST result.

Amplification of 16s rRNA bacterial diversity

Each isolated DNA from different methods was subjected to PCR that amplified the partial V2-V3 region of 16S rRNA gene, using a primer set DG-GC338F (5'-ACT CCT ACG GGA GGC AGC AGT-3') and DG-61R (5′-GTA TTA CCG CGG CTG CTG GCA C-3'). A GC-clamp (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G-3') was added to the forward primer (Mirsepasi *et al*., 2014). The PCR components were similar to those mentioned earlier with cycling conditions at 95 °C for 3 min, 30 cycles of 98 °C for 25 sec, 60 °C for 10 sec and 68 °C for 30 sec and a final extension 68 °C for 1 min with a holding temperature at 10 °C.

Assessing bacterial diversity by denaturing gradient gel electrophoresis

The DGGE analysis was conducted according to Foong *et al*. (2010) protocols using the D-Code Universal Mutation Detection System (BioRad, USA). Two 16S rRNA PCR amplicons from each DNA sample were pooled and purified using Wizard SV Gel and PCR Clean-Up System (Promega, USA) according to the manufacturer's protocol. The purified products were quantified using Qubit 2.0 Fluorometer (Invitrogen, California). An 8% (wt/vol) polyacrylamide gel (acrylamide/bisacrylamide, 37:1) in 1× TAE buffer with a 35-75% denaturant gradient (100% denaturant was 7 M urea and 40% formamide) was prepared. The solution was delivered to gel cast using Model 475 Gradient Delivery System (BioRad, USA) and an appropriate comb was inserted. The gel was allowed to polymerize for at least 2 h. After that, 100 ng of the purified products were loaded onto the gel and the electrophoresis was carried out at 75 V for 18 h in 0.5× TAE buffer at 60 °C. After electrophoresis, the gel was stained with the SYBR Gold nucleic acid stain (Molecular Probes) for 30 min. The image was observed under Molecular Imager Gel Doc™ XR System (BioRad, USA).

DNA sequencing of DGGE amplicons

Six (6) distinct amplicons were excised using a sterile blade and incubated in sterile water at 4 °C overnight. The eluate was reamplified using the same primer set without GC clamp. Then, the PCR amplicons were subjected to Sanger's sequencing. The resultant sequences were then trimmed for quality and length using BioEdit software and used in a BLAST search of GenBank analysis to assess sequence similarity to identify the bacterial species.

Statistical analysis

DGGE profile analysis was conducted to compare the bacterial diversity using the Shannon-Weaver index using GelCompar II software (Applied Maths, Belgium). Shannon index *(H')* was calculated with the formula

$$H' = -\sum_{i=1}^{S} p_i \, ln \, ln \, p_i$$

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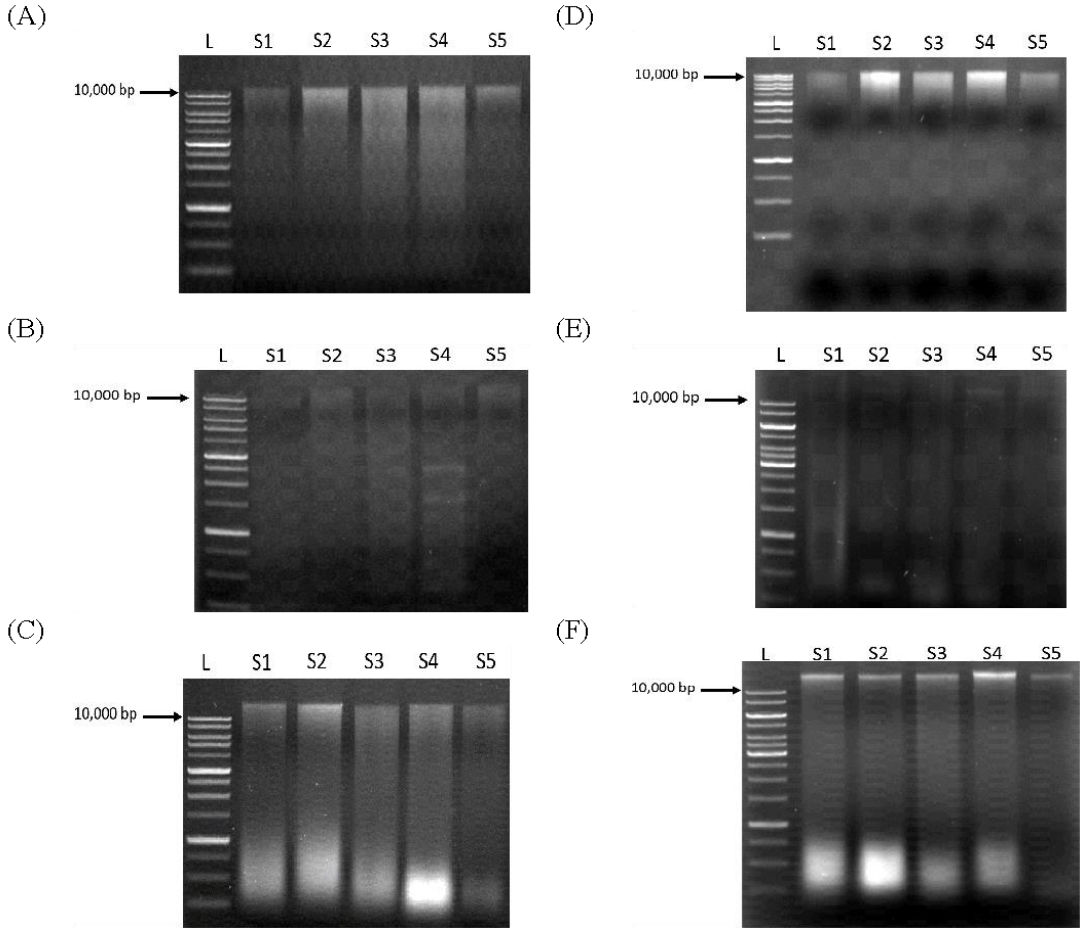

Figure 1: Genomic DNA of rodent faecal samples yielded using different extraction methods, in which (A) Method A; (B) Method B; (C) Method C; (D) Method D; (E) Method E; and (F) Method F. Lane L: 1 kb DNA ladder (Promega, USA).

Where s was the number of extraction methods and p_i was the proportion of the amplicons yielded to the total number of amplicons (Nolan and Callahan, 2006).

RESULTS

DNA extraction of the rodents' faecal samples

All tested methods were successful in extracting DNA from the rodents' faecal droppings. Majority of the DNA extracts from four methods, except Method B and E showed intact DNA. Method B and C methods yielded the highest concentration of DNA with 324.22 ng/µL and 220.74 ng/µL, respectively. However, the majority of the DNA was degraded when visualised using agarose gel electrophoresis (Figure 1). Judging on the possibilities of PCR inhibitors being carried over during DNA extraction, each DNA was diluted 2 to 8-fold prior to PCR amplification to avoid the inhibitory effect of the contaminants.

Identification of rodents' species

Out of five samples, four were detected as *Rattus norvegicus* and one sample was *Rattus tiomanicus* with identity of >99% and E-value of 0.0.

DGGE profiling and bacterial diversity

DGGE profiling results showed a number of DNA amplicons separated across the polyacrylamide gel for all samples. Extraction methods containing the highest number of amplicons were Method A (168) and Method B (167), while Method E (147) had the lowest number of amplicons. These are reflected in their Shannon-Weaver index as shown in Figure 2.

DGGE amplicons analysis

Six distinct amplicons of the DGGE were analysed and their sequences exhibited the highest similarity to *Prevotella* spp. with query coverage ranging from 96 to 100% and identity exceeding 94%. Among the extraction

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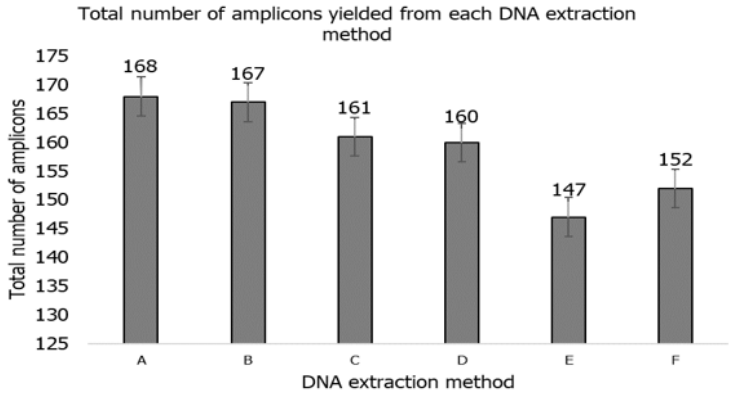

Extraction method	Shannon-Weaver index
A	0.306
B	0.305
C	0.300
D	0.299
E	0.288
F	0.293

Figure 2: The analysis of amplicons from six DNA extraction methods using GelCompar II software. The bar graph on the left shows the total number of amplicons separated from different DNA extraction methods. The table on the right shows the Shannon-Weaver diversity index for each extraction method. DNA extracted from Method A yielded the highest bacterial diversity based on this PCR-DGGE analysis, while Method E method yielded the lowest bacterial diversity.

Table 2: Average DNA yield obtained using six DNA extraction methods.

Sample	Concentration (ng/µL)					
	A	B	C	D	E	F
S1	24.6	48.5	188.7	26.4	381.7	210.6
S2	46.9	32.9	249.0	47.6	364.1	259.7
S3	91.3	61.8	195.7	44.7	253.1	148.6
S4	87.5	84.9	355.0	54.9	385.8	146.3
S5	34.6	36.2	115.3	37.4	236.4	63.5
Average	56.98	52.86	220.74	42.2	324.22	165.74
Standard deviation	30.66	21.22	88.87	10.83	73.24	74.10

Table 3: The presence of amplicons yielded from each extraction method.

Extraction method/Amplicon	A	B	C	D	E	F
Amplicon 1	/	/	/	/	/	/
Amplicon 2	/	/	/	/	/	O
Amplicon 3	/	/	O	O	O	/
Amplicon 4	/	/	/	/	/	O
Amplicon 5	/	/	/	/	O	/
Amplicon 6	/	O	O	/	O	O
Total amplicons yielded	6	5	4	5	3	3

Note: / indicates present; O indicates absence.

methods, Method A had successfully extracted all six amplicons, while both Method B and D extracted five amplicons. The Method C method yielded four amplicons, while both conventional methods extracted three amplicons.

DISCUSSION

In this study, six DNA extraction methods were assessed using rodent faecal samples subjected to PCR-DGGE analysis. All extraction methods successfully isolate the DNA from the faecal samples but differ in quality output. Based on the NanoDrop™ value (Table 2) and agarose gel images (Figure 1), Method A and D yielded the most intact DNA bands with least degradation as compared to the other methods. Although Method E showed highest concentration based on NanoDrop™ reading, the quality of DNA was poor. This could be due to the inaccuracy reading from NanoDrop™ that measures other compounds at the same absorbance wavelength such as RNA or contaminants. Mechanical lysis of the faecal samples using bead-beating (Method A, B and D) yielded less degradation across the gel images, except for Method E. This finding is supported by previous study on mice faecal samples (Ariefdjohan *et al*., 2010; Ferrand *et al*., 2014).

The sequencing analysis of COI gene revealed two species of rodents were detected in the faecal samples.

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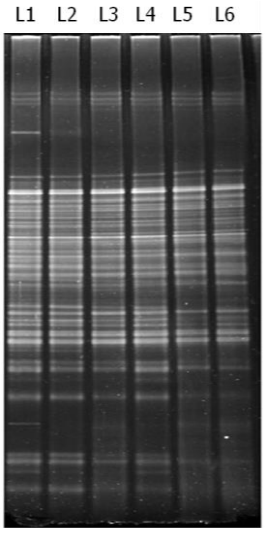

Figure 3: DGGE profiles of 16S rRNA amplified products from a DNA sample extracted using six methods tested. Lane L1: Method A; L2: Method B; L3: Method C; L4: Method D; L5: Method E; L6: Method F.

Four of the samples were from *Rattus norvegicus* and one from *Rattus tiomanicus*. This finding is supported by the fact that this commensal rodent species is very common in urbanised areas as they dwell around human settlements due to their ecological and behavioural traits (Gardner-Santana *et al*., 2009; Palmeirim *et al*., 2014; Morand *et al*., 2015).

The bacterial diversity in the extracted DNA was assessed using PCR-DGGE analysis based on their 16S rRNA partial gene sequences. DNA extracts from three methods (Method C, E and F) required dilutions prior to PCR amplification of 16S rRNA. This could be explained due to heavy smearing on the gel indicating the presence of inhibitory components being carried over during the extraction process. Diluting the DNA could help in reducing the concentration of PCR inhibitors present in the DNA samples, allowing amplification of the targeted gene (Mirsepasi *et al*., 2014).

The overall DGGE profiles analysis in Figure 2 showed that DNA samples extracted using commercial kits yielded a higher number of amplicons compared to conventional methods. The highest yield of the commercial kits was obtained from Method A, followed by Method B and Method C, and lastly Method D. This could be attributable to the strength of both mechanical and chemical disruption of the cell wall during lysis step to release the DNA from various bacterial types (Tang *et al*., 2008). Despite the use of common mechanical (homogenizer pestle), the yield of Method C method has proven to provide a relatively high number of amplicons compared to Method D, even though the yield difference is not significant. Briefly, the efficiency of lysing the cell wall of the bacteria, especially from Gram-positive, is highly dependent on both chemical lysing buffer and mechanical disruption. Nevertheless, since the lysis buffers contain manufacturer's proprietary components in each kit, the chemical disruption could not be assessed and compared.

It is worth reporting that DNA isolation can be time consuming and laborious which could result in delayed subsequent analysis. In summary, Method A shows the most efficient DNA isolation method for rodent faecal samples as the process can be completed within an hour, yet still provide high quality DNA and a more diverse bacterial community in its DGGE profiles.

CONCLUSION

The work described in this study has identified the optimal DNA isolation method for rodent faecal samples. Out of the six DNA isolation methods tested, Method A was found to be able to extract DNA from rodent faecal metagenomes, as assessed by PCR-DGGE. More diverse bacteria communities were yielded, apart from time effectiveness of the isolation process. However, this finding is limited to the performance of the tested DNA extraction methods on rodent faecal samples. Future studies are recommended to assess the DNA extraction methods tailored to sample types prior to downstream processes and analyses.

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CONFLICTS OF INTEREST

The authors declare that the research was carried out in the absence of any commercial or financial relationships, therefore, conflict of interests does not exist.

INFORMED CONSENT

There were no animals involved in this study.

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