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Comparison of a modified phenol/chloroform and commercial-kit methods for extracting DNA from horse fecal material

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ABSTRACT

There are many choices for methods of extracting bacterial DNA for Next Generation Sequencing (NGS) from fecal samples. Here, we compare our modifications of a phenol/chloroform extraction method plus an inhibitor removal solution (C3) (ph/Chl + C3) to the PowerFecal® DNA Isolation Kit (MoBio-K). DNA quality and quantity coupled to NGS results were used to assess differences in relative abundance, Shannon diversity index, unique species, and principle coordinate analysis (PCoA) between biological replicates. Six replicate samples, taken from a single ball of horse feces manually collected from the rectum, were subjected to each extraction method. The Ph/Chl + C3 method produced 100× higher DNA yields with less shearing than the MoBio-K method. To assess the methods, the two method samples were sent for sequencing of the bacterial V3-V4 region of 16S rRNA gene using the Illumina MiSeq platform. The relative abundance of *Bacteroidetes* was greater and there were more unique species assigned to this group in MoBio-K than in Ph/Chl + C3 ($P < 0.05$). In contrast, *Firmicutes* had greater relative abundance and more unique species in Ph/Chl + C3 extracts than in MoBio-K ($P < 0.05$). The other major bacterial phyla were equally abundant in samples using both extraction methods. Alpha diversity and Shannon Weaver indices showed greater evenness of bacterial distribution in Ph/Chl + C3 compared with MoBio-K ($P < 0.05$), but there was no difference in the OTU richness. Principle coordinate analysis (PCoA) indicated a distinct separation between the two methods ($P < 0.05$) and tighter clustering (less variability) in Ph/Chl + C3 than in MoBio-K. These results suggest that the Ph/Chl + C3 may be preferred for research to identify specific *Firmicutes* taxa such as *Clostridium*, and *Bacillus*. However; MoBio-K may be a better choice for projects focusing on *Bacteroidetes* abundance. The Ph/Chl + C3 method required less time, but has some safety concerns associated with exposure and disposal of phenol and chloroform. While the MoBio-K may be better choice for researchers with less access to safety equipment like a fume hood.

1. Introduction

The gut microbome (GM) of mammals is a diverse and complex community of microorganisms that are known to affect host health (Sekirov et al., 2010). Many of these microorganisms evade cultivation as demonstrated by plate count anomalies (Staley and Konopka, 1985) (Rappé and Giovannoni, 2003) but are detectable by Next Generations Sequencing (NGS) methods such as Illumina (Zhou et al., 2010). The ability of NGS to detect rare or recalcitrant bacteria depends on the extraction of a sufficient quantity (>20 ng/μl) of high quality, clean DNA from the entire microbial community. Inhibitors which co-purify during the extraction procedure, such as organic and phenolic compounds, divalent cations (e.g., Mg^{2+}, Ca^{2+}), and heavy metals can interfere with PCR based sequencing strategies (Wilson, 1997). This is important because animal feeds, especially for horses contain phenolic compounds (Dueñas et al., 2004; Naczk and Shahidi, 2006). Furthermore, fecal samples are generally rich with humic substances which can also affect the DNA quality and purity (Holben et al., 1988). In this study, two DNA extraction methods were compared: a phenol/chloroform method (Kerkhof and Ward, 1993; McGuinness et al., 2006; Männistö et al., 2009), which was modified for the present experiment, and a commercial DNA extraction kit, (MoBio PowerFecal® DNA Isolation Kit, catalog# 12830-50; MoBio Laboratories Inc.). Our PCoA results demonstrate less variability upon extracting horse fecal DNA using the modified ph/Chl + C3 method, where a primary modification was the addition of an inhibitor removal solution (C3) that precipitates non-DNA organic and inorganic substances such as humic acid, cell debris, and proteins (MoBio Laboratories Inc., Catalog# 12830-50-3). This

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suggests clean and stable DNA products were generated after inserting our modifications and using the inhibitor removal solution (C3) in the phenol chloroform extraction method.

2. Material and methods

2.1. Samples

Biological replicates were collected from one horse and the same fecal ball at the same time. The collection was made from the inner end of the rectum of the horse, ~45 cm of distance, using the rectal palpation method with aseptic procedures (Mueller and Moore, 2000). The fecal ball was placed on a sterile surface and opened with sterile forceps. After that, 0.25 g samples ($n = 6$ for each extraction method) were collected from the inner part of the ball and then placed in collection tube containing beads (MP Biomedical; (sku# 116914100) that had been preloaded with 300 μL of buffer solution containing cetyltrimethyl ammonium bromide (CTAB) (0.25 M phosphate buffer (pH 8), 5% CTAB in 1 M NaCl). Samples were then snap frozen in liquid nitrogen and then place in −80 °C for extraction.

2.2. DNA extraction methods

The (Ph/Chl + C3) method was modified as follows: Samples were subjected to 5 quick freeze/thaw cycles between liquid nitrogen and a 55 °C water bath. After these freeze/thaw cycles, 100 μl of Solution 1 (50 mM glucose, 10 mM EDTA, 25 mM Tris-Cl; pH 8.0), 50 μl of lysozyme solution (4 mg in 1 ml of Solution 1) and 50 μl of 500 mM EDTA were added to the frozen samples. The samples were then thawed and quickly combine with 50 μl 10% SDS and 800 μl phenol: chloroform: isoamyl alcohol; 25:24:1 (>pH 7.0). Samples were then disrupted using vortexing for 3 min and followed by centrifugation at (~16,000×g) for 3 min. After the aqueous phase was transferred to a new centrifuge tube, a second 800 μl phenol: chloroform: isoamyl alcohol; 25:24:1 (>pH 7.0) was performed. These tubes were vortexed for 1 min at maximum speed and centrifuged at about 16,000×g for 3 min. The aqueous layer was transferred to a new micro-centrifuge tube to which 200 μl of C3, inhibitor removal solution (MoBio Laboratories Inc., catlg# 12830-50-3) was added. The tubes containing the inhibitor removal solution and the DNA extract were vortexed briefly and then incubated in ice at 4 °C for 5 min. Following incubation, the tubes were centrifuged at 13,000×g for 1 min (as indicated by the manufacturer protocol), and the supernatant containing DNA was transferred to a sterile, DNAse, and RNAse free tube. The DNA was then assessed for purity and concentration using NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). DNA was visualized for shearing on agarose gel. DNA was checked for amplification of the 16S rRNA gene using the universal bacterial primers: 27F: AGAGTTTGATCCTGGCTCAG and 1522r: AAGGAGGTGATCCAICCGCA. We will refer to this method by (ph/Chl + C3) in the rest of this article.

For the MoBio-K extractions, we collected as described above (0.25 g of feces in 300 μL of buffer solution containing CTAB (0.25 M phosphate buffer (pH 8), 5% CTAB in 1 M NaCl)). We followed the exact and complete manufacturer's protocol for the Powerfecal kit.

2.3. Sequencing (Miseq)

To sequence the 16S rRNA genes within the fecal samples, the V3-V4 variable region of this gene was amplified with universal bacterial primers 341F, CCTACGGGAGGCAGCAG/785R, CTACCAGGGTATCTAATCC (Mühling et al., 2008), and barcodes on the forward primer in a 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA). Amplifications were performed under the following conditions: 94 °C for 3 min, followed by 28 cycles of 94 °C for 30 s, 53 °C for 40 s and 72 °C for 1 min, after which a final elongation step at 72 °C for 5 min was performed. PCR products were analyzed on a 2% agarose gel to identify the size and the relative intensity of bands. Samples were purified using calibrated Ampure XP beads. These purified PCR products were used to produce a DNA library using the Illumina TruSeq DNA library preparation protocol. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) using the Illumina MiSeq platform.

2.4. Bioinformatics

Sequence data were processed using MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA). In short, paired reads were joined and trimmed of barcodes. Sequences <150 bp and those with ambiguous base calls were removed. Sequences were denoised, Operational taxonomic units (OTUs) generated, and chimeras removed. OTUs were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn against a curated database derived from GreenGenes, RDPII and NCBI (www.ncbi.nlm.nih.gov, DeSantis et al. 2006, http://rdp.cme.msu.edu).

2.5. Statistical analysis

Alpha diversity was estimated by calculating relative abundance of bacteria at the phylum and species levels ($n = 6$ replicates/extraction method). *T* tests were used to compare the two extraction methods ($\alpha = 0.05$). Statistical analyses were performed using Prism 6 and Prism 7 (GraphPad, Inc.). Mean ± SEM was used to represent data. Shannon diversity index at the species level for each extraction method was calculated using the R software version 3.2.3 (Wooden Christmas Tree) with the packages: Vegan (Oksanen et al., 2016) and Mass (Venables and Ripley, 2002). The Shannon values were compared using *t*-test. Principle Coordinate Analysis (PCoA) was performed using the R software version 3.2.3 (Wooden Christmas Tree) with the

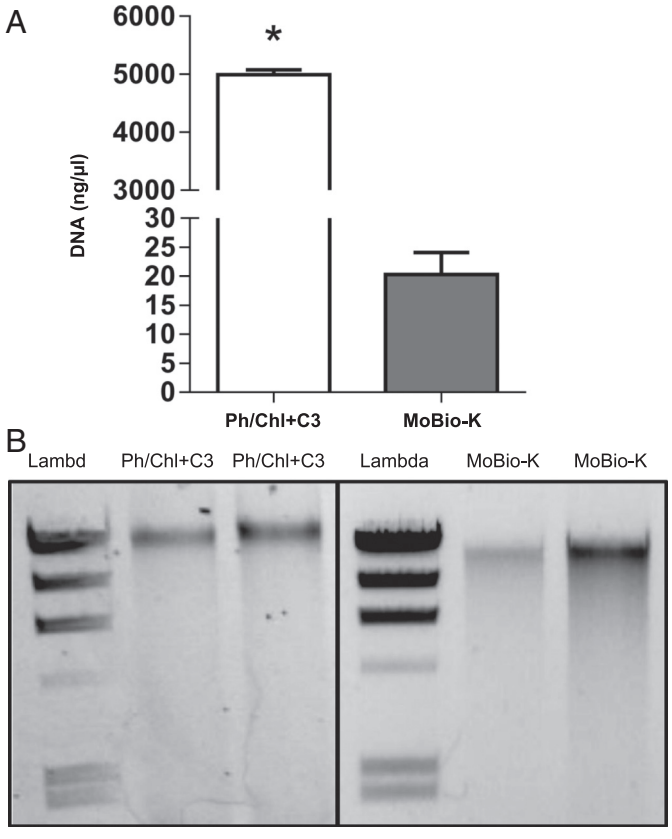

Fig. 1. Comparisons of DNA quantity and quality between the DNA extraction methods. A. Comparison between the two DNA extraction methods in the DNA concentrations, $n = 6$ replicates/method, (P value <0.0001). Error bars are SEMs. Asterisk represents significant difference. B. Comparison between the two DNA extraction methods in DNA shearing.

package Vegan (Oksanen et al., 2016), and scores were compared using *t*-test. Images generated in R software were converted to Tiff format using GIMP software version 2.8.16 (The GIMP team, 1997–2014). Unique species from each extraction method were identified by lists generated in Microsoft office excel.

3. Results

3.1. DNA quantity and quality

The DNA concentration, Fig. 1A, generated by Ph/Chl + C3 was significantly higher, ($P < 0.0001$), than the DNA concentration generated by MoBio-K. The 260/280 and 260/230 ratios for Ph/Chl + C3 were 1.84 and 1.81 respectively, while for MoBio-K, they were 2.11 and 1.94 respectively. Evident DNA shearing was greater for the MoBio-K method compared with the Ph/Chl + C3 method, Fig. 1B.

3.2. Bacterial taxa abundance

Phylum *Bacteroidetes* relative abundance, Fig. 2A, was significantly greater, ($p = 0.0328$), in MoBio-K than in Ph/Chl + C3 (39.83 ± 0.91 vs 37.06 ± 0.49). Genus *Barnsiella*, which belongs to phylum *Bacteroidetes*, relative abundance was significantly higher ($p = 0.0003$) in Ph/Chl + C3 than in MoBio-K (1.13 ± 0.08 vs 0.73 ± 0.08), Fig. 2B. In case of phylum *Firmicutes*, Fig. 2E, showed significantly higher relative abundance ($p = 0.0178$) in Ph/Chl + C3 than in MoBio-K (34.35 ± 0.31 vs 26.57 ± 2.15). Moreover; the relative abundance in 2 of the *Firmicutes* genera, *Clostridium* and *Bacillus*, Figs. 2F and G, were significantly higher

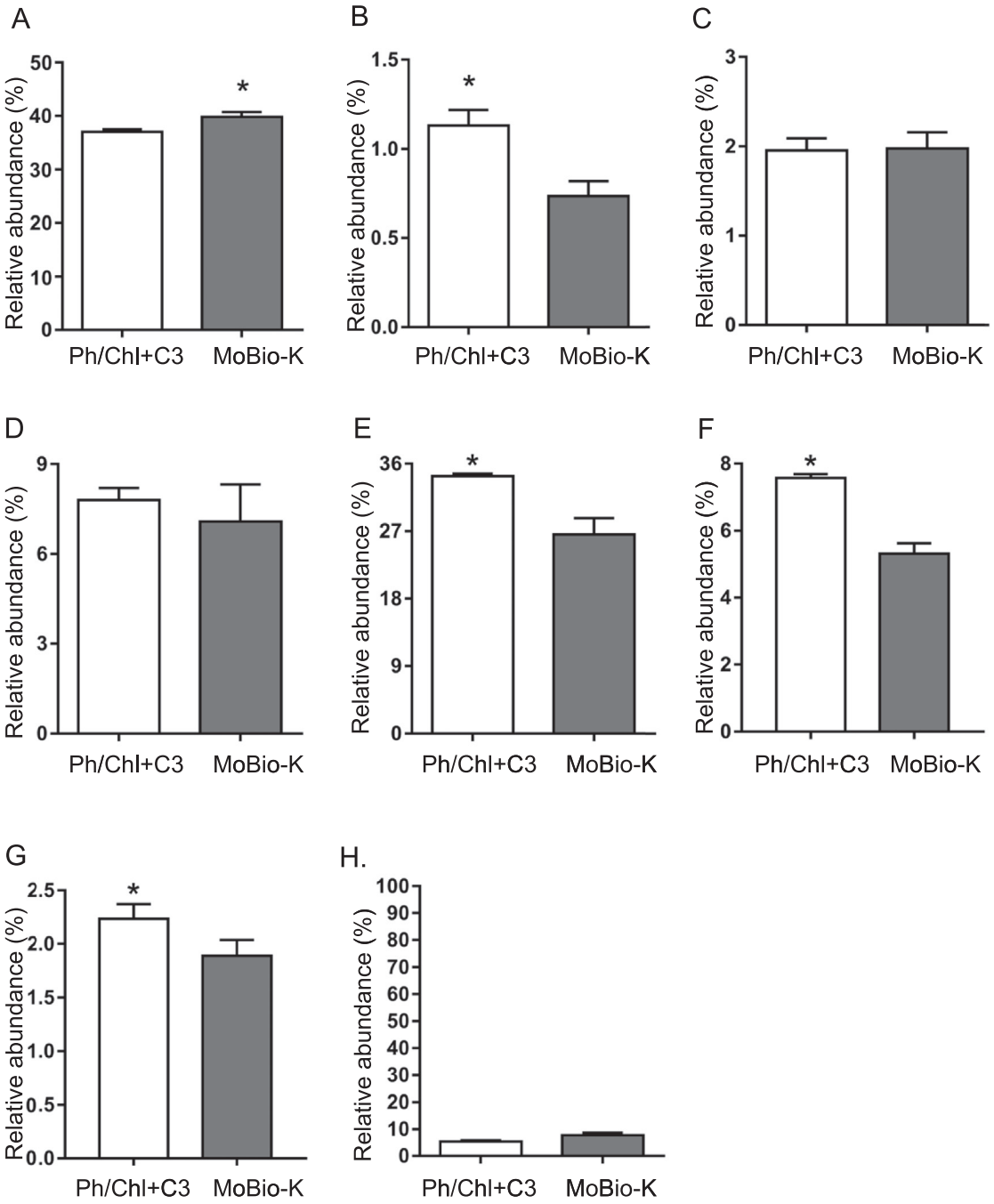

Fig. 2. Comparison between the two DNA extraction methods in the relative abundance (%) of some bacterial phyla and genera. A. *Bacteroidetes* B. Genus *Barnesiella*. C. *Proteobacteria* D. *Spirochaetes*. E. *Firmicutes*. F. Genus *Clostridium*. G. Genus: *Bacillus*. H. *Fibrobacteres*. $N = 6$ replicates/DNA extraction method. Error bars are SEMs. Asterisks represent significant differences.

($p = 0.0022$ and 0.0321 respectively) in Ph/Chl + C3 than in MoBio-K. Finally, there were no significant differences between the extraction methods in the relative abundances of Phylum *Proteobacteria*, *Spirochaetes*, or *Fibrobacteres*, Figs. 2C, 2D, and 2H ($p > 0.05$).

3.3. Shannon diversity index and principle coordinate analysis (PCoA)

At the species level, Fig. 3A, Ph/Chl + C3 showed significantly greater index ($p = 0.0161$) than MoBio-K (4.20 ± 0.02 vs 4.04 ± 0.04). The PCoA, Fig. 3B, showed significant separation ($p = 0.0464$) between Ph/Chl + C3 and MoBio-K (0.017 ± 0.003 vs 0.228 ± 0.079). The 6 replicates from Ph/Chl + C3 clustered together. There were thirteen unique species observed in each extraction method. Unique species from *Firmicutes* (7), *Actinobacteria* (4), and $_{\beta}$-and $_{\gamma}$-*Proteobacteria* (2) were identified in Ph/Chl + C3; while; species from *Bacteroidetes* (5), α-*Proteobacteria* (3), *Firmicutes* (3), δ-*Proteobacteria* (1), and *Planctomycetes* (1) appeared only in MoBio-K. All were low abundance species (<1%), Table 1.

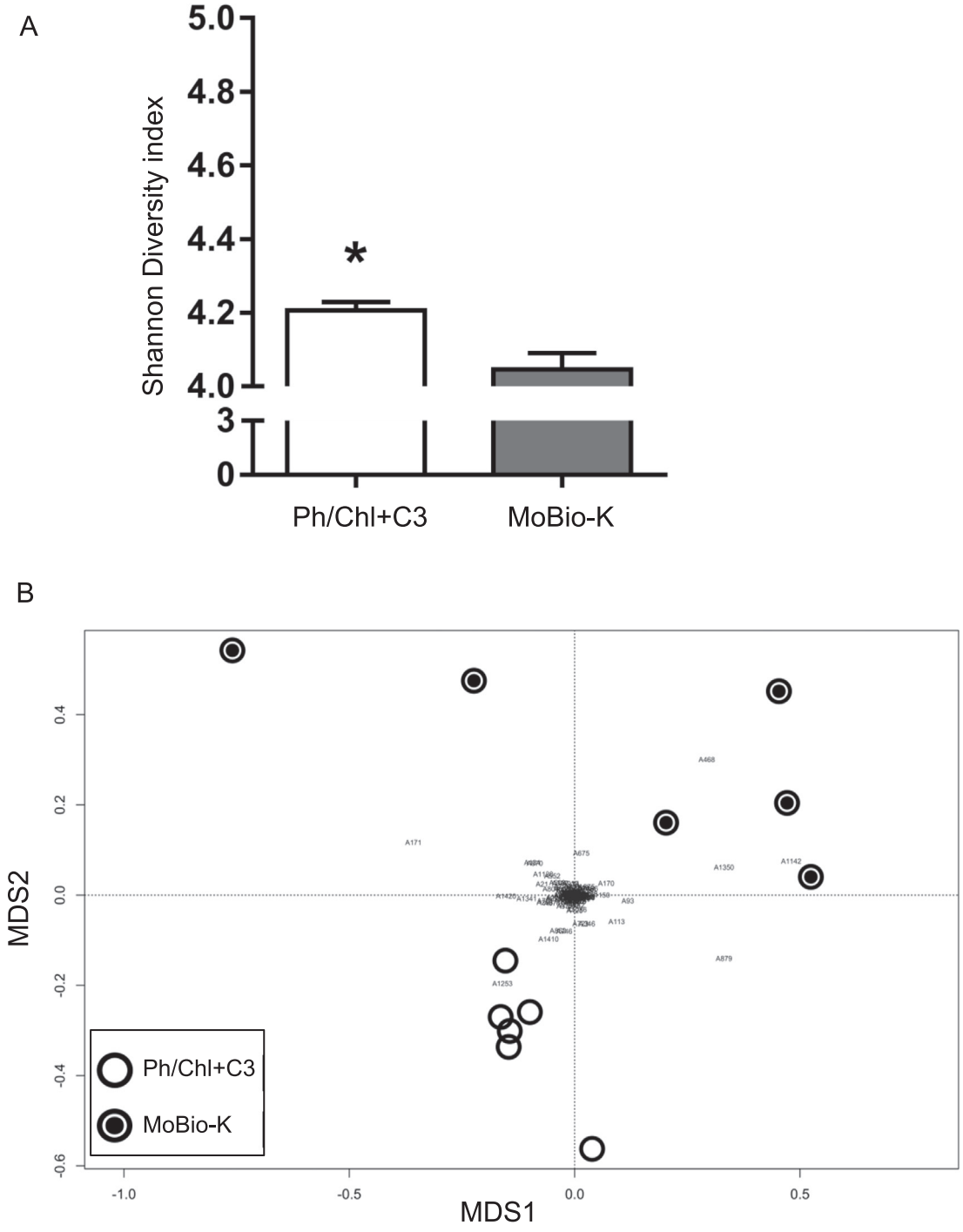

Fig. 3. Bacterial community ecological analyses. A. Shannon diversity index comparison between the two DNA extraction methods at the species level. $N = 6$ replicates/DNA extraction method. Error bars are SEMs. Asterisk represents significant difference. B. Principle coordinate analysis (PCoA) at the species level of the two DNA extraction methods. $N = 6$ replicates/DNA extraction method.

Table 1
Unique species in Ph/Chl + C3 or MoBio-K (relative abundance <1%).

Ph/Chl + C3 unique species	MoBio-K unique species	Phylum
	Bacteroides barnesiae	*Bacteroidetes*
Tepidimonas spp.		b-*Proteobacteria*
	Marivirga tractuosa	*Bacteroidetes*
Williamsia serinedens		*Actinobacteria*
Catenibacterium spp.		*Firmicutes*
	Dongia mobilis	a-*Proteobacteria*
	Devosia spp.	a-*Proteobacteria*
Williamsia sp.		*Actinobacteria*
Proteinivorax tanatarense		*Firmicutes*
	Eubacterium saburreum	*Firmicutes*
Robinsoniella peoriensis		*Firmicutes*
	Desulfovibrio vulgaris	d-*Proteobacteria*
Pseudomonas putida		g-*Proteobacteria*
Metabacterium polyspora		*Firmicutes*
Cellulosilyticum lentocellum		*Firmicutes*
	Alkaliflexus imshenetskii	*Bacteroidetes*
	Petrimonas spp.	*Bacteroidetes*
	Dysgonomonas capnocytophagoides	*Bacteroidetes*
Propionibacterium acnes		*Actinobacteria*
	Eubacterium contortum	*Firmicutes*
	Planctomyces maris	*Planctomycetes*
	Moorella spp.	*Firmicutes*
	Afipia sp.	a-*Proteobacteria*
Oceanirhabdus sediminicola		*Firmicutes*
Tindallia texcoconensis		*Firmicutes*
Eggerthella spp.		*Actinobacteria*

4. Discussion

The bacterial DNA extraction method will probably affect the observed bacterial community when analyzing fecal samples (Larsen et al., 2015). One criterion that should help decide whether a certain DNA extraction method is suitable is DNA yield. Some studies have shown that different modified phenol/chloroform methods yielded high amounts of DNA from different types of samples (Zhang et al., 2006; Kok et al., 2000). To our knowledge, no study has attempted to use a phenol/chloroform method to extract DNA from horse fecal samples for DNA extraction method comparison purposes. In the present study, our Ph/Chl + C3 method yielded a higher DNA concentration and higher molecular weight product (Fig. 1A) with less shearing in a shorter amount of time than the MoBio-K method (Fig. 1B). Hart et al. (2015) observed that the MoBio Power Fecal kit produced low amounts of DNA extracted from horse fecal samples. In our study, the MoBio-K also yielded a low concentration of DNA from horse fecal samples. These higher DNA concentrations and molecular weights of the Ph/Chl + C3 method might be a result of no prolonged and complicated processes such as multiple centrifugations involved in this method (Steffan et al., 1988). The second criterion is the downstream application that is needed to be used, such as NGS. Our NGS results showed that the relative abundance of the phyla Bacteroidetes and Firmicutes were impacted by the DNA extraction methods used in this study. In our study, more Bacteroidetes and less Firmicutes were recognized from MoBio-K by the NGS. In contrast, Peng et al. (2013) observed lower levels of Bacteroidetes and higher levels of Firmicutes in feces of rats using the UltraClean™ Fecal DNA kit (MoBio). This difference in sequence results could be from fecal material from rats and horses being very different in their nature. In our study, the Shannon diversity index was significantly lower in MoBio-K than Ph/Chl + C3 (Fig. 3A), indicating a major difference in overall DNA yield. However, both methods captured the same overall number of bacterial species. Peng et al. (2013) demonstrated using PCoA on fecal samples from rats that the UltraClean™ Fecal DNA kit (MoBio) behaved differently from other methods they used in their comparisons. They showed that MoBio kit showed different clustering and different microbial pattern on the PCoA. This confirms our results when our PCoA plot (Fig. 3B) showed significant separation between the two extraction methods and high scattering in the replicates of MoBio-K. For Ph/Chl + C3, replicates clustered closer together indicating higher reproducibility than MoBio-K. The Ph/Chl + C3 method requires a fume hood and hazardous material handling and disposal and fresh lysozyme solution needs to be prepared prior to each extraction. MoBio-K can be easily done on the benchtop with ordinary laboratory precautions, directly out of the box.

In conclusion, The Shannon and the PCoA analyses indicate that Ph/Chl + C3 is a reliable horse fecal DNA extraction method that could be used with high confidence of generating the same stable DNA extract products every round of extraction. This suggests that may be fewer factors could affect the DNA extracting during the use of this method. This research highlights the challenge in choosing a DNA extraction method for 16S rRNA sequencing that captures the rare and common bacterial taxa and is also economical and easy to implement.

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