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Research Article

Variations in Gut Microbiome Profiles Based on Different DNA Extraction Methods: A Comparative Study

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Objectives. To compare four DNA extraction methods for recovering bacterial DNA from mammalian faecal samples. *Methods and Results*. Three commercial kits from QIAamp, TIANamp, and MAGEN, together with the classic cetyltrimethylammonium bromide (CTAB) method, were evaluated for their performance in extracting bacterial DNA from the gut microbiota of five mammals, including humans (*Homo sapiens*), macaques (*Macaca mulatta*), dogs (*Canis lupus familiaris*), golden hamsters (*Mesocricetus auratus*), and mice (*Mus musculus*). First, we assessed the efficiency of the four methods based on DNA yield, purity, and integrity. Then, we investigated the impact of these methods on microbial composition and diversity and examined the relative abundance of dominant phyla bacteria based on 16S rRNA gene sequencing and real-time quantitative PCR. Our results showed that the CTAB method yielded relatively larger amounts of DNA, while the MAGEN kit yielded more Firmicutes DNA and reflected the true status of the microbiota more accurately. *Conclusions*. Of the four methods tested, the traditional CTAB method and commercial MAGEN kit accomplished the best performance in terms of DNA concentration and Firmicutes abundance across most of the tested species. As the CTAB method and MAGEN kit exhibited different advantages, we further tested and compared their DNA extraction performance on a defined microbiota comprising six strains from four dominant phyla to see which one better reflected the true status of the microbiota. In conclusion, the MAGEN kit was found to be superior to the CTAB method, as the testing results were closer to that of the defined microbiota.

1. Introduction

The gut microbiota is recognized as a "forgotten organ", which is capable of secreting various enzymes and producing a variety of metabolites that functionally impact host metabolism [1, 2]. In recent years, alterations in the taxonomic composition and abundance of the gut microbiota have been investigated in the pathogenesis of various human diseases, including obesity [3, 4], type 2 diabetes [5, 6], cardiovascular diseases [7, 8], neurodegenerative diseases [9], and cancer [10, 11]. A growing number of studies have demonstrated that faecal microbiota transplantation [12], prebiotics [13], probiotics [14], and postbiotics [15] supplementation could restore the diversity of gut microbiota and potentially offer health benefits, underscoring the important impacts of studying the gut microbiota on disease diagnosis and treatment [16].

Typically, information on the gut microbiota is obtained by analyzing faecal samples due to their availabilities and noninvasive collections. Microbial culture [17] and high-throughput sequencing [18] are two commonly used methods to study gut microbiota. Microbial culture determines the type of organism and bacterial interactions, but only a small percentage of gut microbes are culturable from gastrointestinal biopsies or faeces [19]. Culture-independent strategies, such as extraction

of nucleic acids and high-throughput sequencing, enable high-resolution and in-depth analysis of microbial taxonomic composition and decode microbial genomes to investigate their functional repertoires [20]. However, the experimental design and sample pretreatments, including collection, storage, transportation of faeces, DNA extraction, and sequencing platforms, can affect the analysis of the composition and abundance of faecal microbiota.

The extraction process may introduce technical biases related to the efficacy of cell lysis on the method employed. For instance, lysis techniques that prefer complex remnants such as mammalian DNA, food residues, and diverse endogenous inhibitors may introduce eukaryotic DNA into the extract, thus compromising the quality of microbial DNA [21, 22]. Furthermore, gram-positive and gram-negative bacteria possess different properties owing to their distinct structural characteristics. Therefore, researchers must aim for representative lysis of microbial cells embedded within faecal samples, particularly for gram-positive bacteria with thicker cell walls, which confounds the extraction results. While commercial DNA extraction kits provide high-throughput and standardized protocols for streamlined sample processing, their different lysis protocols may induce technical variation and thus become a confounding factor that disrupts researchers to compare sequencing information between different extraction methods. Therefore, not all extraction protocols applied for faecal samples from diverse mammalian species produce acceptable quantities and quality, and such methodological biases have been reported in several studies [23, 24].

Hence, we focused on a traditional but commonly used DNA extraction reagent, cetyltrimethylammonium bromide (CTAB), and compared its extraction efficacy to that of three commercial stool DNA extraction kits (QIAamp Fast DNA Stool Mini Kit, TIANamp Stool DNA Kit, and HiPure Stool DNA Kit) for DNA extracts from humans and four experimental mammal species (macaques, dogs, golden hamsters, and mice) (Figure 1). These four approaches were selected in this study since they employ different combinations of chemical and mechanical disruption to lyse cells. The initial screening parameters included DNA yield, purity, and integrity, as these metrics are essential indicators of amplification amenability. Subsequently, the extracted DNA was subjected to 16S rRNA gene sequencing to investigate the potential microbial community bias resulting from different extraction approaches. Among the tested methods, the traditional CTAB method was suggested for its high DNA concentration, while the MAGEN kit was recommended for its high yield in Firmicutes, which are surrounded by thick cell walls and recognized as challenging to lyse. Finally, a simplified microbial community consisting of six common bacterial strains was introduced to mimic the complex composition of the gut microbiota *in vivo* and to validate the efficacy of the optimal DNA extraction method. As a result, the MAGEN kit was recommended for its ability to extract DNA with a similar phylum level to the microbiota.

2. Materials and Methods

2.1. Ethics Approval. Written informed consent was obtained from human participants. The study was approved by the Research Ethics Committee of the Shanghai Institute of Materia Medica. With regard to the animal experiments, all procedures were conducted in accordance with institutional ethical guidelines on animal care and approved by the Institute Animal Care and Use Committee at the Shanghai Institute of Materia Medica.

2.2. Sample Collection and Preparation. Male C57BL/6 J mice (8 weeks old, Jackson Laboratories, Bar Harbor, ME, USA) were housed upon arrival and provided free access to food and water. Fresh faecal samples were collected from three healthy volunteers and three mice in a 50 mL sterile conical tube or a 2 mL centrifuge tube, respectively, and transferred to -80°C as soon as possible. The faecal samples from macaques, dogs, and golden hamsters were kindly provided by Professor Xiaoyan Chen and Jingya Li from the Shanghai Institute of Materia Medica. The samples were stored at -80°C until DNA extraction. For each mammal species, faecal samples from three individuals were combined into a single sample and homogenized to the best of our ability.

2.3. DNA Extraction. DNA was extracted in triplicate from each faecal sample using four distinct methods, encompassing a widely used CTAB method and three common commercial kits (Table 1). For the CTAB method, the faecal sample, weighing between 50 and 200 mg, was homogenized with 3 mm glass beads and suspended in CTAB lysis solution (2% CTAB, 1.4 mol/L NaCl, 0.02 mol/L EDTA, and 0.1 mol/L Tris-HCl). This suspension was then incubated for 30 min at 70°C, supplemented with 5 μL lysozyme (50 mg/mL) and 10 μL proteinase K (20 mg/mL, Sinopharm, China). Following this, the extracts were purified using phenol-chloroform extraction, and DNA was subsequently recovered by isopropanol precipitation. The pelleted DNA was washed twice with cold 75% ethanol, allowed to air dry, and resuspended in 50 μL of TE buffer. Additionally, three commercial faecal DNA extraction kits were evaluated in this study: QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany, #51604), TIANamp Stool DNA Kit (TIANGEN biotech, China, #DP328), and HiPure Stool DNA Kit (MAGEN, China, #D3141-02). The DNA extraction procedures for the commercial kits were performed according to the manufacturer's instructions, which are briefly summarized in Table 1.

2.4. Quantification and Assessment of DNA Purity. The yield (concentration) and purity (the absorption ratio of A_{260}/$A_{280\,nm}$) of the DNA samples were determined by a Nanodrop 300 (Allsheng Instruments Co., Ltd., China). The integrity of the DNA samples was assessed by nucleic acid electrophoresis.

2.5. Agarose Gel Electrophoresis. Agarose gels were performed using a 1% (w/v) solution of agarose in buffer TAE (40 mmol/L Tris-acetate, 1 mmol/L EDTA). Electrophoresis was performed at 100 mV for 45 min to facilitate the separation of samples based on their respective molecular weights at the nucleic electrophoresis apparatus (Tanon EPS-100, China). Subsequently, the DNA bands were visualized and recorded using an automatic electrophoresis image managing system (Tanon-1600, China).

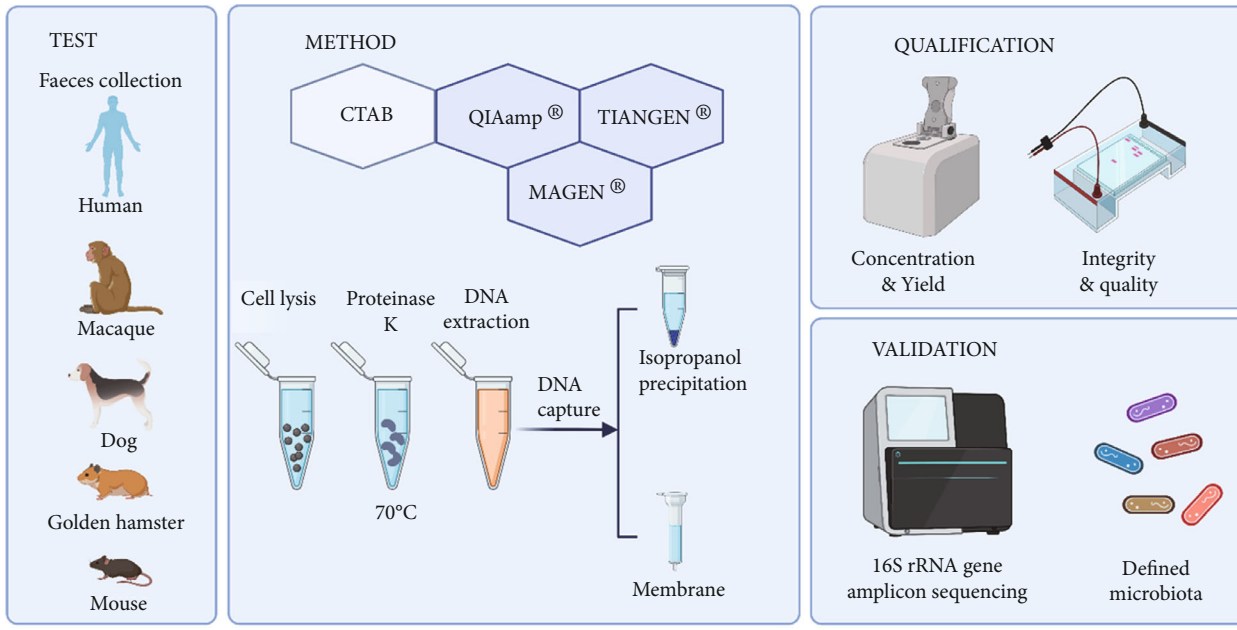

Figure 1: Workflow of experimental design. Four DNA extraction methods (CTAB, QIAamp, TIANGEN, and MAGEN) were compared for frozen faecal samples from five mammals (human, macaque, dog, golden hamster, and mouse). The bacterial DNA extracted using these methods was tested for concentration, purity, and integrity and then subjected to 16S rRNA gene sequencing for microbial community analysis. Finally, a certain community of defined microbiota was introduced to mimic the complex composition of the gut microbiota *in vivo* and to validate the efficacy of the optimal DNA extraction method.

Table 1: The brief protocol of each commercial kit used in this study.

Protocol	CTAB	QIA	TIAN	MAGEN
Full name of method/kit	Cetyltrimethylammonium bromide method	QIAamp® Fast DNA Stool Mini Kit	TIANamp® Stool DNA Kit	MAGEN® HiPure Stool DNA Mini Kit
Manufacturer details	—	Qiagen, Valencia, CA, USA	TIANGEN, Beijing, China	MAGEN, Guangzhou, China
Lysis method	Beads Lysis solution Heat	Lysis solution Heat	Beads Lysis solution Heat	Beads Lysis solution Heat
Bead size	3 mm	—	1 mm	0.1-0.6 mm
Proteinase K	0.5-1%	4-7.5%	2.5%	2%
Heating time	70°C, 30 min	70°C, 5 min/70°C, 10 min	70°C, 15 min	65°C, 15 min/70°C, 10 min
RNase	6%	—	2%	1%
DNA capture	Phenol-chloroform extraction, isopropanol precipitation	Silica membrane	Silica membrane	Glass fiber filter membrane
Elution buffer	Buffer TE	Buffer ATE	Buffer TB	Buffer AE

2.6. 16S rRNA Gene Sequencing. The microbial 16S rRNA profiles of the faecal DNA extracts were analyzed using HiSeq 16S rRNA gene sequencing methods (Illumina HiSeq-PE250, Novogene, China) targeting the V3-V4 hypervariable regions of the 16S rRNA gene [25]. In brief, the DNA samples extracted by the CTAB method and QIA, TIAN, and MAGEN kits were applied to amplify the V4 region of the 16S rRNA gene with specific primers 515F-806R. An amplicon library was constructed based on the characteristics of the amplified 16S region, and the libraries were quantified by Qubit and qPCR before being paired-end sequenced using NovaSeq6000.

2.7. Sequence Processing. To ensure high-quality data from Illumina NovaSeq sequencing, splicing and quality control were performed to obtain clean tags, followed by chimera filtering to obtain effective tags suitable for subsequent analysis The chimera sequences were detected using the UCHIME

algorithm [26]. To study the species composition of each sample, the tags were clustered into operational taxonomic units (OTUs) with a 97% threshold by using Uparse software (Uparse v7.0.1001) [27]. The first sequence clustered into each OTU was used as the reference sequence for that OTU field. The remaining sequences were classified using the SILVA SSU Ref database (v.119) [28], and sequences corresponding to eukaryotes, mitochondria, and "unknown" lineages were discarded.

2.8. Bacteria Strains and Cultivation. *Lachnospiraceae bacterium* (BNCC 354474), *Clostridium ramosum* (ATCC 25582), *Limosilactobacillus reuteri* subsp. *reuteri* (ATCC 23272), *Bacteroides acidifaciens* (BNCC 353574), *Escherichia coli* (ATCC 25922), and *Bifidobacterium animalis* subsp. *lactis* (JCM 10602) were used in this study. *Limosilactobacillus reuteri* subsp. *reuteri* and *Lactococcus lactis* subsp. *lactis* (ATCC 19435) were grown at 37°C under anaerobic conditions (anaerobic gas mixture, 80% N_2, 10% CO_2, and 10% H_2) in MRS broth. *Lachnospiraceae bacterium*, *Clostridium ramosum*, and *Bacteroides acidifaciens* were grown at 37°C under anaerobic conditions in trypticase soy broth with defibrinated sheep blood. *Escherichia coli* was aerobically grown at 37°C in an LB medium. *Bifidobacterium animalis* subsp. *lactis* was grown at 37°C under anaerobic conditions in a BBL medium. The six bacterial strains were mixed together in a ratio of 20% *Lachnospiraceae bacterium*, 40% *Clostridium ramosum*, 10% *Limosilactobacillus reuteri* subsp. *reuteri*, 25% *Bacteroides acidifaciens*, 3% *Escherichia coli*, and 2% *Bifidobacterium animalis* subsp. *lactis* based on OD_{600} and adjusted to a final concentration of 10^9 CFU/mL.

2.9. Real-Time PCR. Real-time PCR was performed using SYBR Premix Ex Taq (Vazyme, Jiangsu, China) on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad, CA, USA). The results were analyzed using the ΔCt method. The sequence of primers is shown in Table S1.

2.10. Statistical Analysis. Sample sizes were chosen based on pilot experiments that ensured adequate statistical power with similar variances. In our current study, data obtained from experiments were shown as means or means ± SEM, and differences between groups were normalized by the Shapiro-Wilk test and calculated by one-way analysis of variance (ANOVA) or Kruskal-Wallis test using GraphPad Prism version 8.0.1 (GraphPad Software, San Diego, USA). In section 3.4, differences were calculated by a two-tailed Student's *t*-test. *P* values < 0.05 were considered statistically significant. Bioinformatic analysis was performed using the OmicStudio tools.

3. Results

3.1. Assessment of DNA Yield, Purity, and Integrity. Microbiome sequencing requires sophisticated preprocessing to confirm the quality of the DNA product, verify that the DNA concentration is sufficient, and ensure the reliability of the sequencing data obtained from the faecal nucleic acid [29]. Thus, we conducted an evaluation of different methods for extracting microbiome DNA from faecal samples of five mammals. Our assessment included measuring the DNA concentration, purification ($A_{260}/A_{280\,nm}$), and integrity (Figure 1). We found that the CTAB method consistently yielded higher DNA concentrations than the other kits, making it a suitable choice for high-yield DNA extraction (Figure 2(a)). On the other hand, the MAGEN method showed comparable higher DNA concentration among the three commercial kits. We assessed the purity of the extracted DNA by measuring the absorption ratio of A_{260} to $A_{280\,nm}$. The extracted DNA had acceptable ratios ranging from 1.8 to 2.0 for all four methods, except for DNA extracts using the TIAN kit from macaques, which had a slightly lower ratio (Figure 2(b)). We also used agarose gel electrophoresis to evaluate DNA integrity. The CTAB and QIA methods extracted DNA with better integrity than the other two methods, with the CTAB method yielding DNA with excellent integrity (Figures 2(c)–2(g)). The QIA method extracted DNA with good integrity from human and golden hamster faeces, while the TIAN method did so from human and macaque faeces. The MAGEN method could extract integral DNA from all five species except the golden hamster. Overall, our results suggested that most extraction methods have minimal impact on DNA quality, and the CTAB method is the most effective in yielding abundant DNA with satisfactory quality.

3.2. DNA Extraction Methods Have Minimal Impact on Bacterial Diversity. The diversity of bacterial communities is a crucial determinant in studying gut microbiota, as it affects our health and can contribute to the development of diseases [30–32]. To evaluate bacterial diversity, we sequenced 60 DNA samples from five mammals using various extraction methods, with triplicates for each, and obtained a total of 4,976,882 sequences. It can be inferred from the rarefaction curve that the sequencing depth was sufficient to encompass a majority of the bacterial diversity in each sample (Figure 3(a), Figure S1). The CTAB method was found to yield the highest abundance of OTUs in four of the mammals, with the exception of mice (Figure 3(b)). Additionally, the alpha diversity of the faecal microbiota varied depending on the extraction method and mammal species. QIA yielded the lowest alpha diversity of microbiome DNA, while the other three methods showed similar results in humans. In other mammals, three commercial kits demonstrated similar alpha diversity, except for golden hamsters (Figures 3(c) and 3(d)).

We then conducted beta diversity analysis based on principal component analysis (PCA) and found that the gut microbiota provides a clearer reflection of the host's phylogenetic relationship, revealing the evolutionary characteristics and genetic traits between hosts [33]. In contrast, the faecal microbiota is more influenced by the host's diet, resulting in some coincidental dietary similarities in our study. We observed that DNA samples from the same mammalian species clustered together, differentiating them from other mammal species. However, dogs and macaques showed partial overlap (Figure 4(a)). The differences between methods across species were greater than the differences within the same species, implying that various extraction approaches used on the same species have minimal impact on faecal microbiota beta diversity (Figure 4(b)).

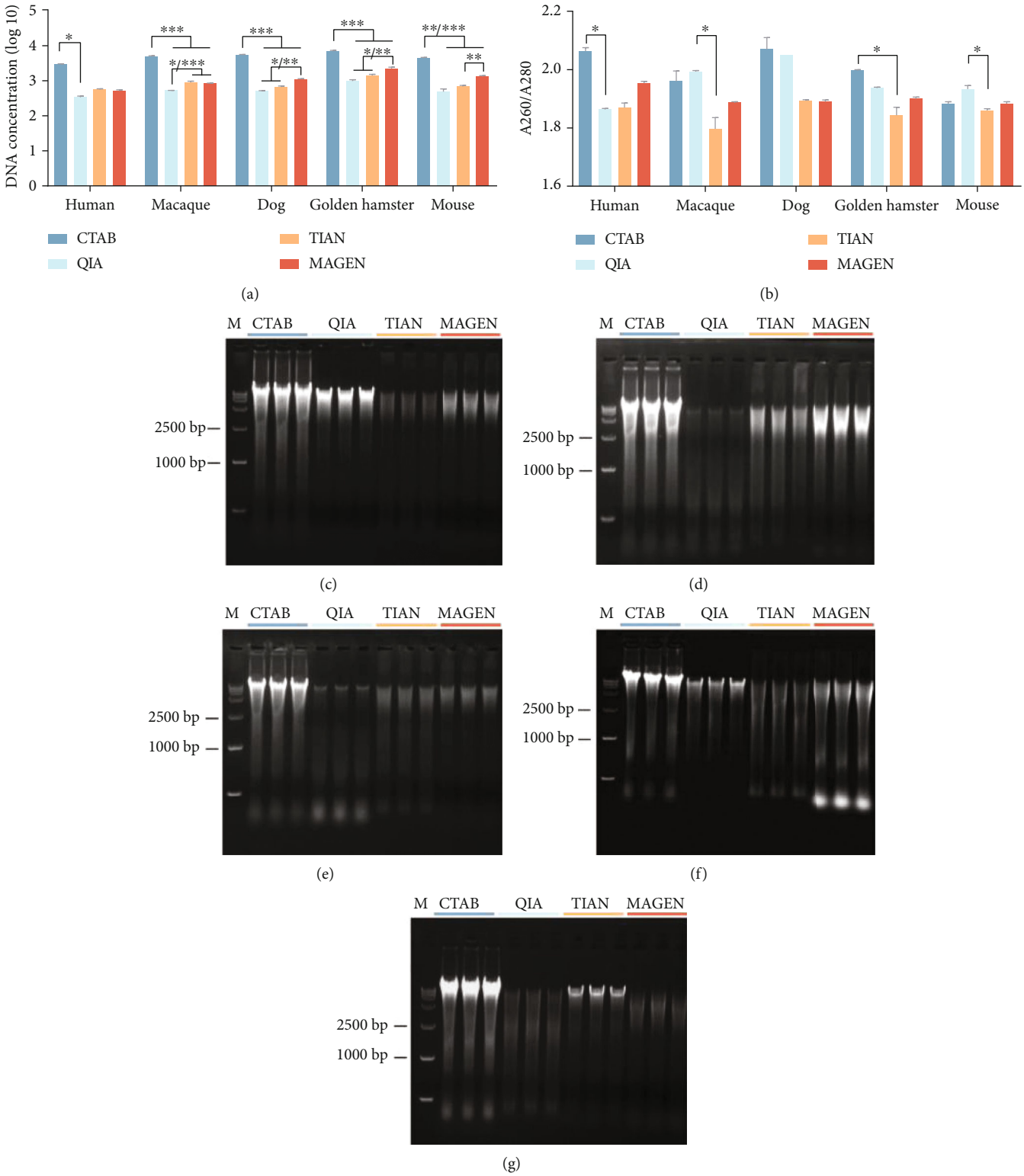

Figure 2: The impact of different extraction methods on DNA yield, quality, and integrity. (a) Concentration, (b) ratio of A_{260}/A_{280}, and (c–g) integrity of DNA extracted from five mammals (human, macaque, dog, golden hamster, and mouse). $n = 3$ per group. Data are expressed as the mean ± SEM. Graph bars marked with different quantities of star sign on top represent statistically significant results based on one-way ANOVA or Kruskal-Wallis test ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

Overall, our results indicate that host phylogeny has a more profound impact on gut microbiota than the DNA extraction method.

3.3. DNA Extraction Methods Influence Microbiota Composition. After investigating the variation of different extraction methods on microbial diversity, we proceeded to

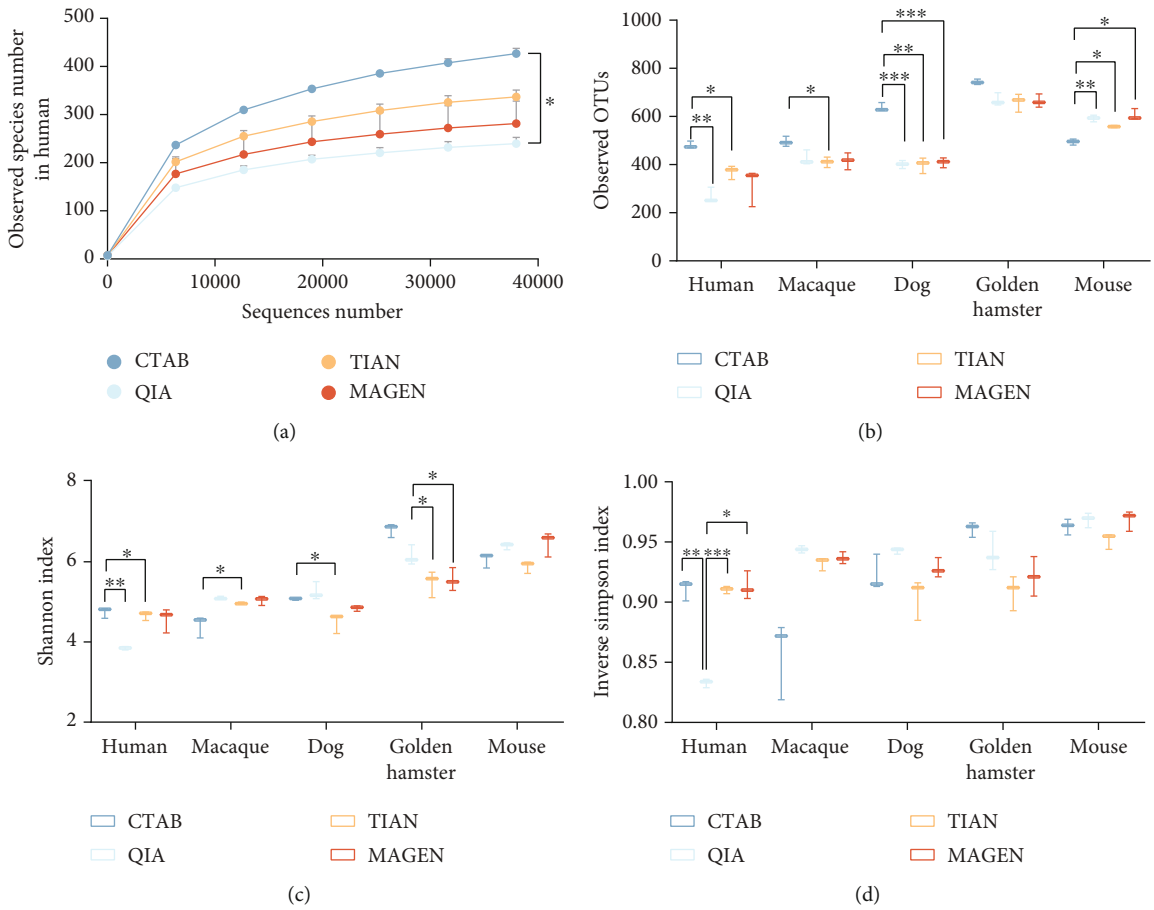

FIGURE 3: The impact of different extraction methods on bacterial diversity. (a) Observed species number in humans, (b) observed OTUs, (c) Shannon index, and (d) inverse Simpson index in five mammals. $n = 3$ per group. Data are expressed as the mean ± SEM. Graph bars marked with different quantities of star sign on top represent statistically significant results based on one-way ANOVA or Kruskal-Wallis test ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

evaluate the effect of different mammalian species and extraction methods on the detailed microbiota composition. At the phylum level, the results revealed that the inherent differences in microbiota among species were more significant than those between extraction methods (Figure 4(c)). For instance, Fusobacteriota was highly abundant in humans, macaques, and dogs, whereas it was relatively low in golden hamsters and mice. Meanwhile, Proteobacteria were the most prevalent in macaques, while Bacteroidota was more abundant than Firmicutes in all other mammals except golden hamsters, where Firmicutes accounted for the largest proportion. This trend has been observed in previous studies as well [34]. Furthermore, we compared the relative abundance of Bacteroidota, Firmicutes, and Proteobacteria in the top 35 genera of gut microbiota obtained through different extraction methods. The MAGEN kit performed well in extracting Firmicutes from various mammalian species, except for mice, whereas TIAN methods obtained a relatively high abundance of Proteobacteria in five mammal species (Figure 5, Figure S2-5).

At the phylum level, the impact of different extraction methods on the abundance of altered bacteria varied among mammals, with the phylum Firmicutes consistently being the most affected. A comparison between the CTAB method and the QIA kit showed that the extracted 16 genera with lower Firmicutes abundance in the human sample, while 7 genera showed higher abundance. The TIAN kit extracted 9 genera with lower Firmicutes abundance and 14 genera with higher abundance, whereas the MAGEN kit extracted 10 genera with lower Firmicutes abundance and 13 genera with higher abundance. Intriguingly, the MAGEN method extracted the highest abundance of Firmicutes from all mammals, except for the mouse, which might be attributed to the smaller bead size used in this method (Table 1). Overall, our assessment revealed that different extraction methods significantly impacted species-specific abundance variation, highlighting the need for a simulated microbial community to determine which method could extract DNA that closely resembles the actual microbial community.

3.4. The MAGEN Method Extracts DNA That Closely Reflects the Actual Composition of the Microbiome. We created a simplified microbial community consisting of six common bacterial strains, including *Lachnospiraceae bacterium*, *Clostridium ramosum*, *Limosilactobacillus reuteri* subsp. *reuteri*, *Bacteroides acidifaciens*, *Escherichia coli*, and *Bifidobacterium animalis* subsp. *lactis* (Figure 6(a)) [35]. We then

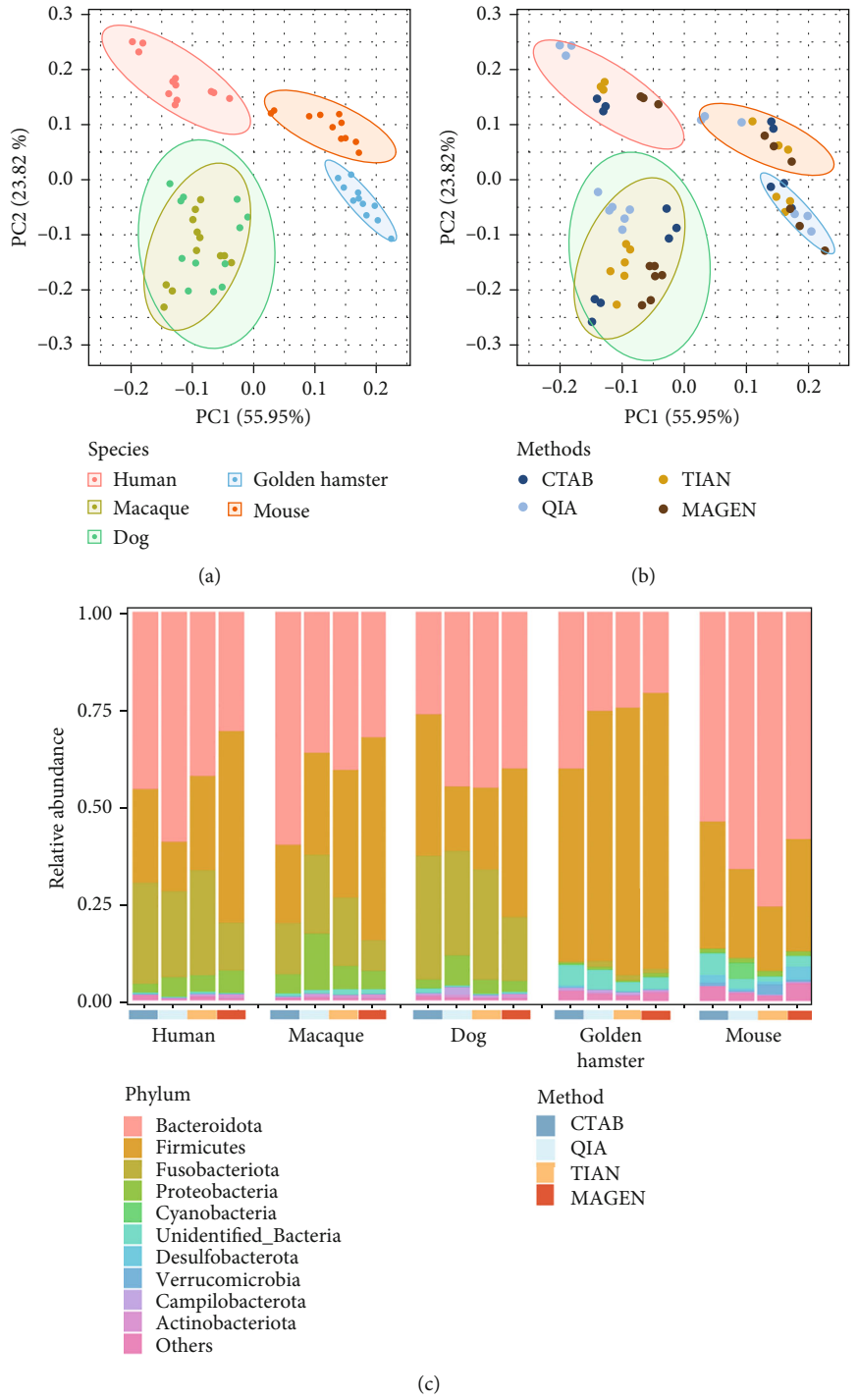

FIGURE 4: The similarity of DNA extracted from the same mammals using different extraction methods. (a) PCA based on different mammal species. (b) PCA based on different extraction methods. (c) Relative abundance of the top 10 phyla. $n = 3$ per group.

selected two methods—the CTAB and MAGEN methods—to extract DNA from the defined microbial community. The CTAB method previously yielded the highest DNA concentration, while the MAGEN method had extracted the highest abundance of Firmicutes. We found no significant differences in the $A_{260}/A_{280\,\mathrm{nm}}$ ratios, which ranged from 1.8 to 2.0 (Figures 6(b) and 6(c)). To compare the difference in the phylum abundance between the extracted DNA and the true phylum composition, we employed real-time quantitative PCR. Consistent with previous findings, DNA extracted using the MAGEN kit exhibited a significantly higher Firmicutes abundance than the CTAB method, whereas the Bacteroidota abundance showed no significant difference (Figure 6(d)). These results suggested that the MAGEN method is more effective in extracting DNA that more closely represents the actual microbiome composition.

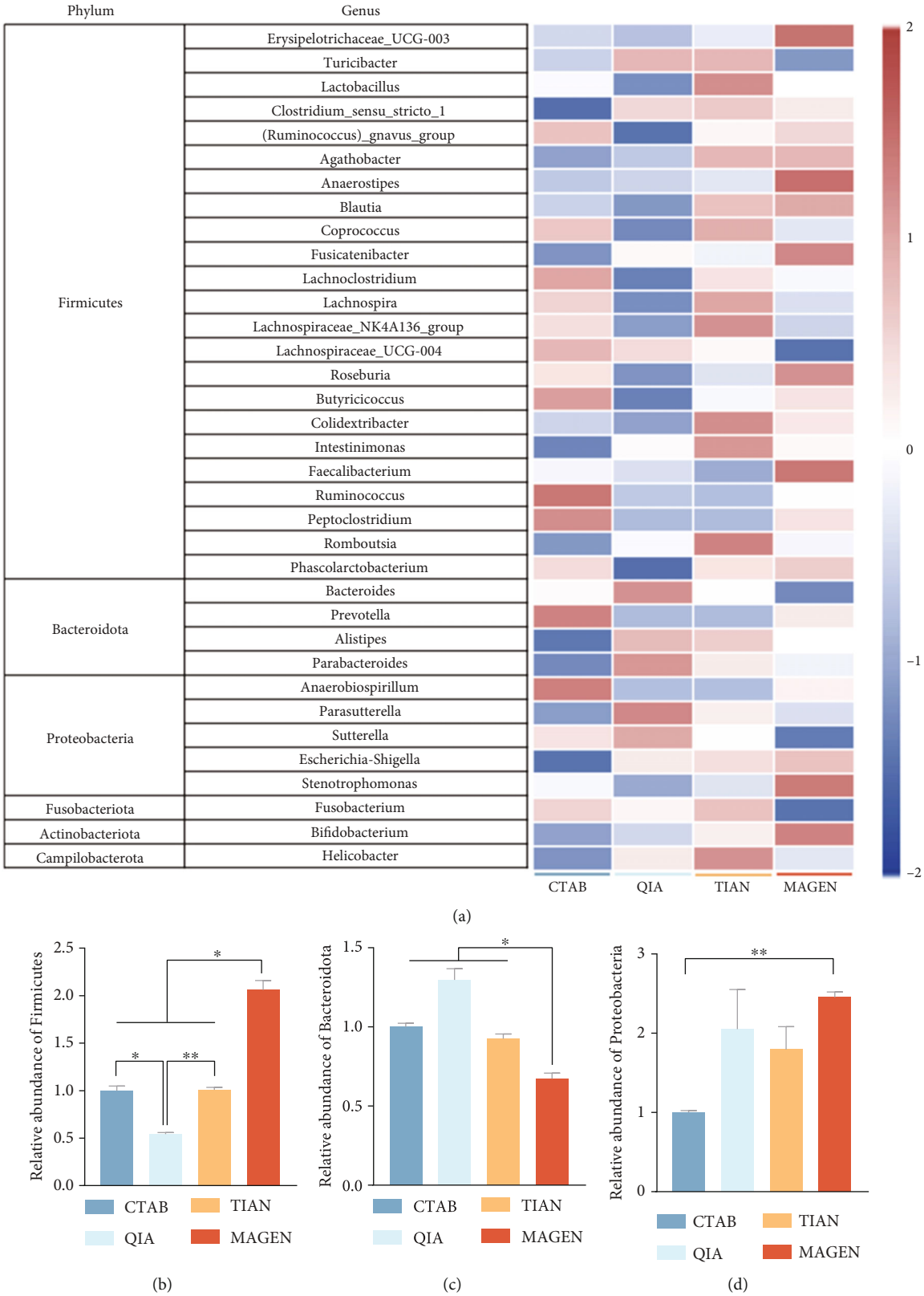

Figure 5: The differences of DNA extracted from human faeces using different extraction methods. (a) Heatmap for genus abundance in faecal DNA samples using different extraction methods. The relative abundance of (b) Firmicutes, (c) Bacteroidota, and (d) Proteobacteria in faecal DNA samples using different extraction methods. $n = 3$ per group. Data are expressed as the mean ± SEM. Graph bars marked with different quantities of star sign on top represent statistically significant results based on one-way ANOVA ($^{*}P < 0.05$ and $^{**}P < 0.01$).

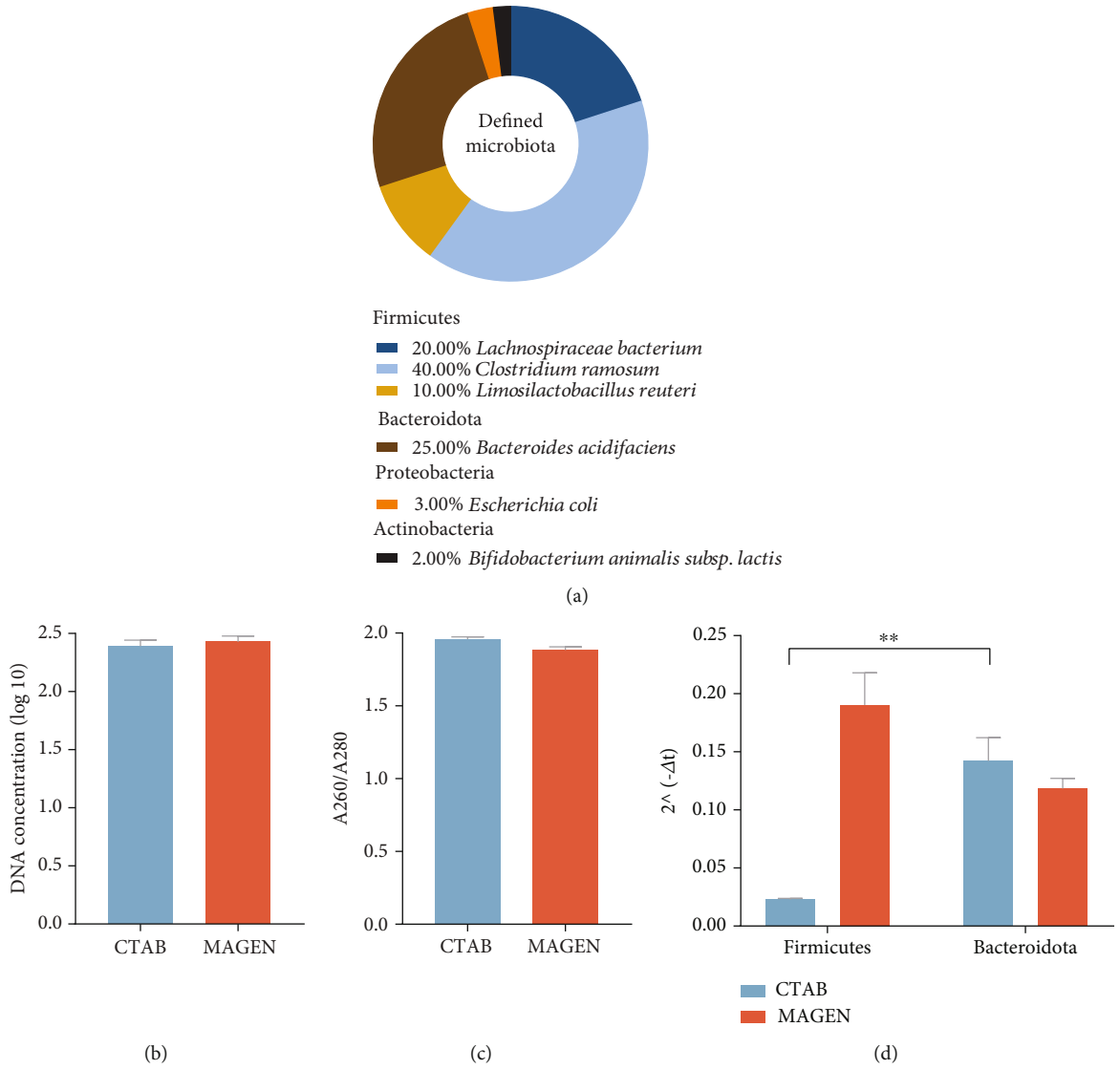

Figure 6: Comparing the CTAB method and MAGEN kit for bacterial DNA extraction from mammalian faecal samples using a simplified microbial community. (a) Composition and proportions of the simplified microbial community. (b) DNA concentration. (c) The ratio of A_{260}/A_{280}. (d) 2^(-Δt) of Firmicutes and Bacteroidota. $n = 3$ per group. Data are expressed as the mean ± SEM. Graph bars marked with different quantities of star sign on top represent statistically significant results based on two-tailed Student's t-tests ($^{**}P < 0.01$).

4. Discussion

The gut microbiota has received more and more research concern for its association with various human diseases [3–11]. A variety of experimental animals have been used to investigate the gut microbiota [36], especially rodents and nonhuman primates [37–40]. However, it is essential to compare the similarities and differences between these models and human gut microbiota. Some studies have systematically compared the composition of mouse, rat, and human gut microbiota, utilizing the same sequencing platform [41]. In the current study, we selected humans and four other widely used mammals as experimental subjects. The first thing that needs to be determined during the experimental design process is the sample size of the experiment. In several methodological studies, the sample size used was three [42, 43] while other similar studies chose a larger sample size of $n = 4$ or 5 to enhance the persuasiveness of the results [44, 45]. Through pilot experiments, the final sample was chosen as $n = 3$ while maintaining statistical power. In the case of a limited sample, individual differences among samples may confound the results of the experiment or even outweigh differences among different extraction methods. Therefore, to better represent the variation of different methods of DNA extraction, faecal samples from different individuals could be mixed to minimize individual differences among samples. After a thorough consideration, we chose to mix the samples to avoid differences among individuals as well as to ensure the persuasiveness of the experimental results. For each species, feacal samples from three individuals were mixed into a single sample and divided into three triplicates.

Then, we comprehensively evaluated the effects of established extraction methods on DNA yield, purity, integrity,

and variability in microbial composition. The four extraction protocols we compared differed in several aspects. Like all culture-independent techniques, data quality depends on the effectiveness of extracting microbial DNA. Different DNA extraction methods generate different microbial profiles in the same faecal samples due to different methods of cell wall fragmentation [46–48]. For instance, QIA does not include ceramic bead peening, while the size of the ceramic beads is in the order of CTAB (3 mm) > TIAN (1 mm) > MAGEN (0.1 – 0.6 mm). In addition, QIA does not use RNase alone, with the concentration of RNase A in the order of CTAB (6%) > TIAN (2%) > MAGEN (1%). All the kits use the adsorption method to enrich DNA by silica adsorption column, while the CTAB protocol uses the chloroform extraction and isopropanol precipitation method. Generally, all established methods yielded DNA concentrations within the acceptable range. While QIA, TIAN, and MAGEN kits produced similar DNA concentrations, the CTAB method yielded extracts with the highest DNA concentration, which is likely attributed to overnight isopropanol precipitation. The precipitation method allows for a more complete retention of DNA than the kit by adsorption columns. In addition, the time of lysis at high temperature may also be a contributing factor to the higher DNA yield with CTAB, as CTAB thermal cracking is conducted at 70°C for 30 min, QIA and TIAN at 70°C for 15 min, and MAGEN cleaved at 65°C for 15 min or 70°C for 10 min. Our results indicate that the CTAB method could extract the highest amount of DNA in all mammals, which is an advantage when the sample size is limited.

The ratio of $A_{260}/A_{280\,nm}$ is a widely used indicator for assessing DNA quality, with a range of 1.7 and 1.9 being considered optimal [49, 50]. RNA contamination can occur when the ratio is above 1.9 [45]. In this study, most of the extracted DNA had ratios within the optimal range, although the CTAB method had a slightly higher ratio of 2.1, which could be attributed to the use of a different dissolution buffer [51]. The species-level differences in gut microbiota composition were also observed, with Bacteroides being more abundant in mice and Firmicutes dominating in golden hamsters. These differences can be explained by inherent diversity among species [34], and related species exhibit more similar gut microbiota [52]. Moreover, differences in lysis efficiency among the four extraction methods could also explain the variation in sequencing results obtained. The MAGEN kit yielded more Firmicutes, likely due to its high efficacy such as the use of 0.1-0.6 mm glass beads and greater efficiency of fragmentation during its specific lysis procedure.

Microbial community analysis revealed that despite being extracted by different methods, DNA samples from each mammalian species were clustered separately. In addition, most spots overlapped through PCA clustering for each method, which highlighted their similarity in the microbial community. This similarity was further confirmed at the phylum and genus levels, suggesting that host phylogeny had a more significant impact on the gut microbiota than the DNA extraction method. Overall, it suggests that the selected extraction method is unlikely to introduce significant biases in microbial community composition in a study of distinguishing between different mammalian species.

To validate the efficacy of DNA extracted by different methods in terms of reflecting the true status of gut microbiota, a defined microbiota containing six species from different phyla was established. Based on previous research results, the CTAB method, which yielded the highest DNA concentration, and the MAGEN method, which extracted the highest abundance in Firmicutes, were chosen to conduct real-time quantitative PCR. The results demonstrated that the DNA extracted by the MAGEN method was more consistent with the actual ratio of phylum abundance, probably due to the utilization of smaller beads (0.1-0.6 mm) in this method, which enhances the extraction efficiency of Firmicutes bacteria with thick cell walls.

Our study provided a comprehensive evaluation on the impacts of four distinct methods on gut microbial profile using 16S rRNA gene sequencing data obtained from faecal samples of five mammalian species. CTAB and MAGEN methods demonstrate the advantages of each. Despite being time-consuming, the CTAB offers unique benefits such as low cost and high yield compared with other commercial kits. The MAGEN kit is recommended for obtaining a higher abundance of Firmicutes and reflecting the true status of the gut microbiota. The fact that diverse extraction methods can lead to statistically significant differences based on the research focus suggests that mixing different DNA extraction procedures within one experiment should be avoided. It also underlines the need to evaluate various DNA extraction methods before conducting formal experiments and advocates for a standard DNA extraction method.

5. Conclusions

In the current study, three commercial kits and a classic cetyltrimethylammonium bromide (CTAB) method were evaluated based on their performance in extracting bacterial DNA from the gut microbiota of five mammal species. The CTAB method is recommended when large amounts of DNA are required since it yields the maximum abundance of DNA. When the actual microbiome composition is investigated, a MAGEN kit is recommended for its ability to extract DNA reflecting the true status of the gut microbiota. Therefore, it is important to choose a proper DNA extraction method based on the specific research question and microbial group.

Data Availability

The datasets presented in this article are available on a reasonable request.

Ethical Approval

The experiments were conducted under the Guidelines for Animal Experiment of the Shanghai Institute of Materia Medica, and the protocol was approved by the Institutional Animal Ethics Committee.

Conflicts of Interest

No conflict of interest was declared.

Authors' Contributions

Yameng Liu, Xianchun Zhong, and Yibin Wang contributed equally to this work and share the first authorship.

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Supplementary Materials

The following are provided as supplementary supporting information: Tables S1: sequences of primers used for quantitative real-time PCR. Figure S1: the impact of different extraction methods on bacterial diversity. Figure S2: the differences of DNA extracted from macaque faeces using different extraction methods. Figure S3: the differences of DNA extracted from dog faeces using different extraction methods. Figure S4: the differences of DNA extracted from golden hamster faeces using different extraction methods. Figure S5: the differences of DNA extracted from mouse faeces using different extraction methods. *(Supplementary Materials)*

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