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Video Article

A Hybrid DNA Extraction Method for the Qualitative and Quantitative Assessment of Bacterial Communities from Poultry Production Samples

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Abstract

The efficacy of DNA extraction protocols can be highly dependent upon both the type of sample being investigated and the types of downstream analyses performed. Considering that the use of new bacterial community analysis techniques (e.g., microbiomics, metagenomics) is becoming more prevalent in the agricultural and environmental sciences and many environmental samples within these disciplines can be physiochemically and microbiologically unique (e.g., fecal and litter/bedding samples from the poultry production spectrum), appropriate and effective DNA extraction methods need to be carefully chosen. Therefore, a novel semi-automated hybrid DNA extraction method was developed specifically for use with environmental poultry production samples. This method is a combination of the two major types of DNA extraction: mechanical and enzymatic. A two-step intense mechanical homogenization step (using bead-beating specifically formulated for environmental samples) was added to the beginning of the “gold standard” enzymatic DNA extraction method for fecal samples to enhance the removal of bacteria and DNA from the sample matrix and improve the recovery of Gram-positive bacterial community members. Once the enzymatic extraction portion of the hybrid method was initiated, the remaining purification process was automated using a robotic workstation to increase sample throughput and decrease sample processing error. In comparison to the strict mechanical and enzymatic DNA extraction methods, this novel hybrid method provided the best overall combined performance when considering quantitative (using 16S rRNA qPCR) and qualitative (using microbiomics) estimates of the total bacterial communities when processing poultry feces and litter samples.

Video Link

The video component of this article can be found at <http://www.jove.com/video/52161/>

Introduction

When analyzing complex clinical or environmental samples (e.g., feces, soils), there are two main methodologies used for the extraction of DNA. The first is a mechanical disruption of the matrix using an intense bead-beating step, while the second is an enzymatic disruption of the matrix to chemically release bacterial cells and inhibit PCR inhibitors from the matrix simultaneously. Given the different means by which these two types of extraction methods work, it is not surprising that previous studies demonstrated that the appropriate DNA extraction method is both highly sample and analysis dependent. Comparative DNA extraction studies previously showed that some methods are more appropriate for improved DNA quality and quantity from environmental samples¹⁻³, while others demonstrated improvements for community-level analyses such as denaturing gradient gel electrophoresis (DGGE)⁴⁻⁶, terminal restriction fragment length polymorphism (T-RFLP)⁷, automated ribosomal intergenic spacer analysis (ARISA)⁸, and phylogenetic microarrays⁹. Therefore, appropriate DNA extraction methods need to be used, or developed, according to the types of environmental samples and the types of analyses being performed on those samples, especially given the recent advancements in bacterial community analyses.

Next generation sequencing, in conjunction with more quantitative community assessments (e.g., quantitative PCR (qPCR)), is becoming more prevalent in the environmental and clinical sciences, however, very little research has been performed to determine the effect of DNA extraction methods on these data sets. Most DNA extraction comparison studies dealt with microbiomic community estimates from human or human model samples^{10,11}, not agricultural animal samples. The few poultry-focused next generation sequencing studies dealt with specific metagenomic^{12,13} or microbiomic¹⁴ questions; they did not discuss the effect of DNA extraction method on the resulting microbiomic analyses. Considering the complex nature of environmental samples related to poultry production (e.g., feces, litter/bedding, pasture soil), DNA extraction methods need to be carefully selected. Poultry-related environmental samples are known to contain large numbers of PCR inhibitors and up to 500-fold DNA extract dilutions have been required for PCR and subsequent downstream analysis¹⁵⁻¹⁷. Therefore it is essential that DNA extraction methods be

optimized for these types of samples in order to not only physically disrupt the matrix, but also to be able to reduce/eliminate the large number of inhibitors that are present.

The QIAamp DNA Stool Mini Kit, an enzymatic extraction method, has been considered the “gold standard” when extracting DNA from difficult gut/fecal samples^{1,18,19} and has been applied successfully to poultry environmental samples^{8,14}. The enzymatic removal of PCR inhibitors through the use of a proprietary matrix is one of the greatest advantages of using this method for these types of environmental samples, as is the ability to significantly improve throughput (and reduce sample processing error) using automated workstations. One major disadvantage is the lack of a mechanical homogenization step to physically disassociate bacterial cells from the environmental matrix. When testing gut and fecal samples of non-poultry origin, the addition of a bead-beating or mechanical disruption step within a DNA extraction protocol significantly increased extraction efficiency⁹, DNA yield/quality^{1,4,5} and significantly improved downstream community analyses in terms of richness, diversity, and coverage^{5,6,11}. These studies compared not only mechanical bead-beating methods to the “gold standard” enzymatic method, but some also added the mechanical bead-beating step to the enzymatic protocol to improve results^{6,9,11}.

According to the results from the above studies, bacterial community analyses (both qualitative and quantitative) could be improved from poultry-related environmental samples through the addition of a mechanical homogenization step to the enzymatic method. Therefore, the goal of this study was twofold: (1) to develop a novel DNA extraction technique that utilizes the most desirable aspects of both the mechanical (powerful homogenization step) and enzymatic (PCR inhibitor removal and automation) extraction methods and (2) compare the quantitative (via qPCR) and qualitative (via microbiomics) bacterial community assessments of this novel method to representative mechanical and enzymatic methods.

Protocol

1. Mechanical Homogenization of Environmental Poultry Production Samples

1. Prior to extraction, set a water bath to 95 °C and allow the water bath time to reach that temperature.
2. Weigh out 0.33 g of soil or fecal material into a 2 ml Lysing Matrix E tube.
 1. Do not exceed 0.33 g of sample in the tube, since this will cause the following solutions to exceed the capacity of the tube.
 2. Thaw frozen samples to RT prior to weighing.
 3. In order to analyze a total of 1 g of soil/feces, weigh out 3 replicate 0.33 g samples for each individual environmental sample.
 4. Store samples at -20 °C within the conical matrix tubes prior to extraction, if needed.
3. Add 825 µl Sodium Phosphate Buffer and 275 µl of PLS solution to a sample tube. Mix using a vortex for ~15 sec and then centrifuge the samples at 14,000 x g for 5 min.
4. Decant the supernatant and add 700 µl of Buffer ASL. Mix using a vortex for 5 sec.
 1. Ensure that there is headspace (~10% total volume) available in the conical tube at this point. If there is no headspace, the tubes will have a tendency to leak during the next homogenization step which could lead to cross-contamination and/or sample loss.
5. Place the samples into a FastPrep 24 Instrument, and homogenize the samples at a speed of 6.0 m/sec for 40 sec.
6. Centrifuge the homogenized sample at 14,000 x g for 5 min. Transfer the supernatant to a sterile 2 ml microcentrifuge tube.
7. To maximize the DNA recovery from the sample, repeat steps 1.4 to 1.6, combining the supernatants into the same sterile, 2 ml microcentrifuge tube.

2. Enzymatic Inhibition of Inhibitors from Sample Homogenates

NOTE: This protocol uses the QIAamp DNA Stool Mini Kit.

1. Incubate the supernatant in a 95 °C water bath for 5 min to maximize DNA recovery from any remaining cells within the supernatant.
 1. Incubate at 70 °C for samples containing mostly Gram-negative organisms. However if Gram-positive organisms are present (which is the case with poultry fecal samples), incubate at 95 °C.
 2. Use plastic locking clips on the microcentrifuge tubes to ensure that the tubes will not “pop” open and potentially lose sample volume as pressure can build up in these sealed microcentrifuge tubes.
2. Open each microcentrifuge tube to release the pressure, re-cap the microcentrifuge tubes and mix using a vortex for 15 sec.
3. Centrifuge the sample at 14,000 x g for 1 min, remove 1.2 ml of the supernatant and place it into a new sterile 2 ml microcentrifuge tube.
4. Add 1 InhibitEx tab to each sample, and mix using a vortex until the sample becomes a uniformly white/off-white liquid.
 1. Avoid touching the InhibitEx tab while placing it into the microcentrifuge tube containing the sample. To accomplish this, place the blister pack containing the tab directly over the open microcentrifuge tube and gently push the tab out of the blister pack and into the microcentrifuge tube.
5. Incubate the sample for 1 min at RT (~ 25 °C) and centrifuge at 14,000 x g for 5 min.
6. Transfer all liquid to a sterile 1.5 ml microcentrifuge tube and centrifuge at 14,000 x g for 5 min.
 1. Avoid any remaining particulates that may have pelleted at the bottom of the microcentrifuge tube at the end of step 2.5 when transferring the liquid.

3. Automated DNA Purification Using the QIAcube Robotic Workstation

NOTE: The number of plastic consumables, the arrangement of the sample rotor adapters within the centrifuge, and the required volumes of the buffers/solutions are dependent on the number of samples that are being run.

1. Add elution tubes and filter tubes to the appropriate slots within the rotor adapters. For each sample, add 400 μ l to the middle slot of the rotor adapter. Place the rotor adapters in the workstation centrifuge in the correct arrangement according to the number of samples being purified.
 1. Make sure that all of the microcentrifuge tube lids are properly secured within the rotor adapter since a failure to do so could result in shearing during one of the centrifugation steps of the purification protocol.
2. Add the required number of 1,000 μ l and 200 μ l filter-tips to the workstation, and fill the supplied buffer bottles with the required volume of buffers.

NOTE: The buffers required for this purification protocol (AL, AW1, AW2, and AE) are all contained within the QIAamp DNA Stool Mini Kit. The user needs to supply the 100% ethanol that is needed for the AW buffers and as a solution used in the purification process.
3. Add the required volume of the supplied proteinase K solution into a sterile 1.5 ml microcentrifuge tube and place it into slot A on the workstation. Also, add the required number (equal to the number of samples being purified) of 2 ml safe-lock microcentrifuge sample tubes RB to the shaker section of the workstation.
 1. Ensure that the lids of the sample tubes are securely placed into the appropriate slots on the workstation, since a failure to do so will result in an error when the machine initially scans the workstation to make sure all needed plastics and liquids are available for the requested run.
4. Using the touchscreen on the workstation, select the DNA Stool – Human Stool – Pathogen Detection Protocol, and read through the subsequent screens to ensure that the workstation was loaded correctly. Once all check screens are passed, select Start to run this protocol.
 1. If extracting DNA from more than 12 samples, begin the homogenization process (Step 1) for the next set of samples, since a run of 12 samples takes ~72 min to complete on the workstation.
5. Remove the samples from the rotor adapters, cap them, and place at -20 °C until needed for subsequent downstream analyses.
 1. At this point, combine the 3 replicate purifications for an individual sample (total analyzed amount = 1 g) using a centrifugation/evaporation-based system. Combine the replicates and re-elute to a final volume of 100 μ l of Tris-ETDA buffer.

Representative Results

For this study, fresh fecal droppings and litter samples were recovered from a commercial broiler house (~25,000 birds) in the southeastern US. The broilers (*Gallus gallus*) were Cobb-500 crosses, and they were 59 days old at the time of sampling. Fresh fecal and litter samples were recovered from four distinct areas within the house (near cooling pad, near the waterer/feeder lines, in between the waterer/feeder lines, and near exhaust fans), and samples from each of these areas contained five pooled samples from within that area. Samples from the four areas of the house were extracted and quantitatively/qualitatively analyzed separately according to sample type (fecal or litter). The data presented below represent the mean values for the entire house for both environmental sample types.

Total 16S rDNA abundances, an estimate of the total bacterial community contained within a sample, were determined using a previously published qPCR protocol²⁰, and values were normalized using the amount of DNA recovered from each extraction method (based on fluorometric analysis described previously²¹). Of the three extraction methods, **Figure 1** shows that the mechanical method consistently provided the highest total bacterial normalized gene abundance estimates for both the fecal and litter samples (5.85 ± 0.16 and $5.56 \pm 0.08 \log_{10}$ 16S rDNA copies ng^{-1} DNA extracted, respectively); whereas, the enzymatic method consistently provided the lowest fecal and litter estimates (4.83 ± 0.54 and $4.61 \pm 0.13 \log_{10}$ 16S rDNA copies ng^{-1} DNA extracted, respectively). The novel hybrid extraction method yielded normalized 16S rDNA abundances that were found to be significantly ($p \leq 0.05$) higher than the enzymatic method, but statistically similar to the mechanical method for both the fecal and litter samples (5.50 ± 0.16 and $5.23 \pm 0.14 \log_{10}$ 16S rDNA copies ng^{-1} DNA extracted, respectively). Therefore, both the mechanical and hybrid extraction methods provided a greater qualitative estimate of the total bacterial communities within these samples than the enzymatic method.

In order to qualitatively assess the total bacterial communities from each sample type and extraction method, the microbiomic workflow as reviewed in Navas-Molina *et al.* (2013)²² was used. In short, the V4 region of the 16S rRNA gene was PCR amplified with primers containing MiSeq sequencing adapters and Golay barcodes and sequenced on the Illumina MiSeq platform^{23,24}. Raw sequencing data was then processed in QIIME 1.7.0-dev^{25,26,27} using the default parameters. Sequence data processing included: quality-filtering; OTU clustering (open-reference at 97% threshold) using UCLUST²⁷; OTU abundance filtering level of $<0.005\%$ ^{22,31}; taxonomy assignment using the RDP Classifier against the Greengenes 13_5 reference database²⁸; sequence alignment with PyNAST²⁹ against the Greengenes core set²⁸; phylogenetic tree construction using FastTree³⁰. The *core_diversity_analyses.py* script was used to run all alpha beta diversity metrics and to generate all plots, charts, and statistics at a sequencing depth of 7865 sequences per sample.

The chosen DNA extraction method exhibited a clear influence on the recovered fecal and litter microbiomes. Starting at the phylum-level (**Table 1**), the overall data (accounting for both fecal and litter samples) revealed that methods possessing the highly disruptive homogenization step (mechanical, hybrid) typically yielded communities with higher abundances of Gram-positive phyla (98.0 and 97.49%, respectively) relative to the enzymatic method (81.74%). Conversely, Gram-negative phyla represented a much greater part of the overall bacterial community recovered from the enzymatic method (17.49%) compared to either the mechanical (1.89%) or hybrid (2.38%) methods. This differential extraction effect on the overall microbiomic profiles and the prevalence of Gram-positive and Gram-negative organisms was effectively demonstrated at the genus level (**Figure 2**). The mechanical and hybrid methods produced relatively similar microbiomic profiles, while the enzymatic method microbiome showed a different distribution of the relative abundance of the recovered taxa. For example, Gram-negative *Alistipes* spp. in the feces samples (the red block with the A; 7.71% of the total community) extracted using the enzymatic method is severely reduced in the fingerprints for the

other two extraction methods (red arrows; 0.47-0.81%), but the Gram-positive *Lactobacillus* spp. (large purple block with the L) was much more prevalent is reduced when using the enzymatic method (42.71%) as compared to the mechanical or hybrid methods (57.22% and 61.85%, respectively). Nonetheless, while the relative abundances of the taxa exhibited some differences between the three extraction methods, the total number of taxa discovered were similar for all three methods for both the fecal (92 of 112 taxa) and litter (96 of 112 taxa).

The enzymatic method consistently yielded bacterial communities with the greatest richness, diversity, and evenness (using the chao1, Phylogenetic Diversity, and equitability metrics, respectively) for both the fecal and litter samples, with the mechanical method consistently showing the lowest estimates for these ecological parameters (Table 2). The hybrid method consistently demonstrated ecological metrics intermediary to the other two methods. Statistically, the ecological estimates from all samples, regardless of extraction method, were found to be similar, although enzymatic method did result in a significantly richer ($p = 0.045$) microbiome compared to the mechanical method when analyzing the litter samples. While no other significant differences were found between extraction methods, the greatest differences between the ecological parameters were typically found between the enzymatic and mechanical methods, with the hybrid method performing equally well to the other two methods; similar to the quantitative assessment of these extraction methods (Figure 1).

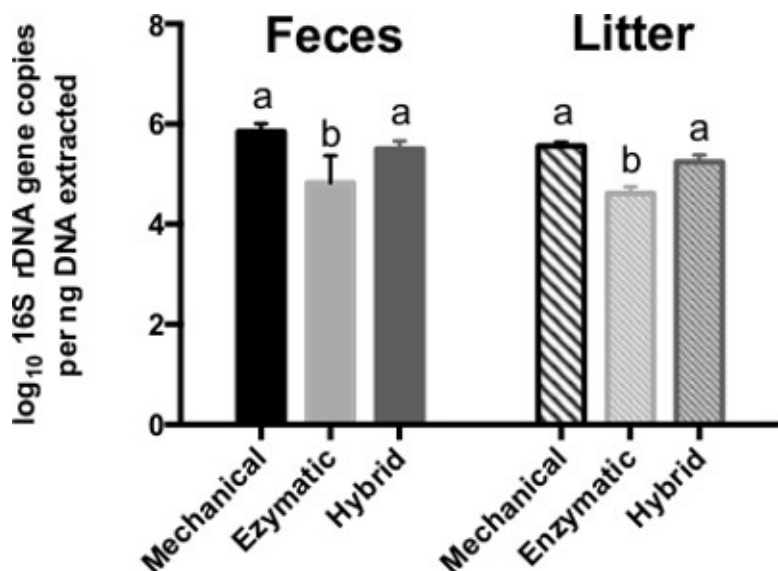


Figure 1. Qualitative bacterial community comparison based on DNA extraction methods. Columns represent the mean \log_{10} -transformed 16S rRNA gene copies as determined by qPCR normalized against the amount of DNA recovered from fecal (solid bars) or litter (dashed bars) sample (based on fluorometric determination). The DNA extraction method used to obtain these values is indicated on the x-axis. The error bars represent the standard deviation between 4 replicate samples. For each sample type (Feces or Litter), the letters above the columns represent significantly different means based on one-way ANOVA analysis using the Tukey's multiple comparison test ($p = 0.05$).

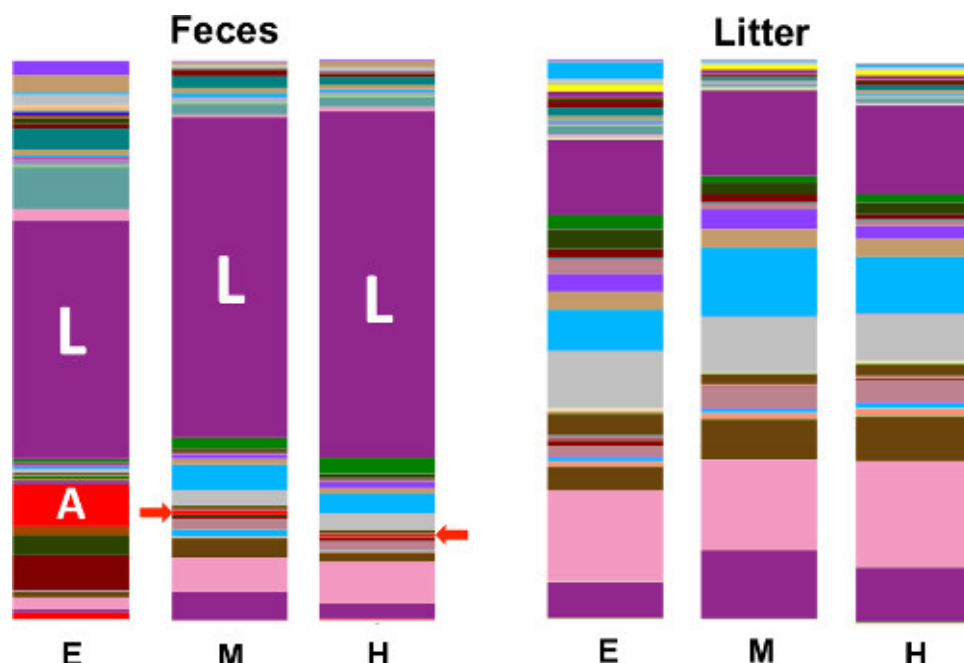


Figure 2. Genus-level microbiomic comparison of the DNA extraction methods. Each column represents the average total bacterial community (based on 16S rRNA gene Illumina MiSeq data) of recovered taxa for each extraction method for the feces (first three columns) and litter (last three columns) samples. Each column represents the average of four different samples from distinct areas within the broiler house. The letter at the base of the columns indicates the extraction method (M = Mechanical, E = Enzymatic, H = Hybrid). The different colors in each column represent the relative abundance of a genus within the overall community, and color for each genera is the same for all columns.

Phylum	Feces			Litter		
	Enzymatic	Mechanical	Hybrid	Enzymatic	Mechanical	Hybrid
Actinobacteria	4.00	18.11	13.97	30.91	41.54	43.26
Firmicutes	65.37	78.98	82.84	63.19	57.43	54.90
Bacteroidetes	20.30	1.68	1.22	1.49	0.27	0.60
Proteobacteria	6.32	0.85	1.51	3.99	0.63	0.97
Tenericutes	2.61	0.27	0.29	0.27	0.08	0.16

Table 1. Phylum-level microbiomic comparison of DNA extraction methods. Relative abundances (% total recovered sequences) for each DNA extraction method of the 2 Gram-positive (Actinomycetes and Firmicutes) and 3 Gram-negative (Bacteroidetes, Proteobacteria, Tenericutes) phyla recovered from the microbiomic analysis from both samples types.

Sample Type	Extraction Method 1	Extraction Method 2	Richness (chao1)			Diversity (Phylogenetic Diversity)			Evenness (Equitability)		
			Method 1	Method 2	p-value	Method 1	Method 2	p-value	Method 1	Method 2	p-value
Feces	Mechanical	Ezymatic	453.73 (80.75)	472.08 (21.50)	1.000	24.17 (3.66)	28.78 (1.35)	0.285	0.517 (0.117)	0.623 (0.106)	1.000
	Mechanical	Hybrid	453.73 (80.75)	462.90 (74.71)	1.000	24.17 (3.66)	23.86 (3.83)	1.000	0.517 (0.117)	0.536 (0.085)	1.000
	Ezymatic	Hybrid	472.08 (21.50)	462.90 (74.71)	1.000	28.78 (1.35)	23.86 (3.83)	0.525	0.623 (0.106)	0.536 (0.085)	1.000
Litter	Mechanical	Ezymatic	423.81 (45.25)	546.61 (38.74)	0.045	21.92 (2.27)	28.59 (2.68)	0.165	0.638 (0.028)	0.679 (0.015)	0.435
	Mechanical	Hybrid	423.81 (45.25)	463.04 (66.45)	1.000	21.92 (2.27)	23.91 (3.93)	1.000	0.638 (0.028)	0.643 (0.028)	1.000
	Ezymatic	Hybrid	546.61 (38.74)	463.04 (66.45)	0.99	28.59 (2.68)	23.91 (3.93)	1.000	0.679 (0.015)	0.643 (0.028)	0.525

Table 2. Pairwise comparisons of richness, diversity, and evenness estimates for the three DNA extraction methods based on genus-level microbiomic bacterial community analyses using QIIME. For both sample types, pairwise comparisons were performed for the three possible extraction method combinations. The total bacterial community parameters assessed include richness (α -diversity based on the chao1 statistic), diversity (β -diversity based on the Phylogenetic Diversity statistic), and evenness (based on the equitability statistic). Values represent the mean (standard deviation) of 4 distinct area samples within the broiler house, and an $\alpha = 0.05$ level was used to determine significance between pairwise comparisons. [Please click here to view a larger version of this table.](#)

Discussion

The DNA extraction method used effected the quantitative and qualitative total bacterial community estimates for both the fecal and litter samples, supporting the sample analyses dependent nature of DNA extraction methods seen previously^{1,3,6}. For both the fecal and litter samples, the order of performance of the DNA extraction methods was different for the quantitative (Mechanical > Hybrid > Enzymatic) and the qualitative (Enzymatic > Hybrid > Mechanical) total bacterial community estimates. While the hybrid method did not produce the highest quantitative or qualitative estimates, in both cases the hybrid method produced results that were statistically similar to the highest performing

extraction method (**Figure 1, Table 2**), thereby producing the best combination of qualitative and quantitative total bacterial community estimates of the three extraction methods tested. It should be noted that bacterial community profiles can be strongly affected by DNA extraction method, as seen in the results from this study (**Figure 2, Table 1**) and other previous studies using environmental samples^{4,5,8,9,11,12}, and these differential effects may strongly influence the analysis and interpretation of the study data. Knowing this, researchers must strongly consider the appropriate extraction method for their sample types and experimental goals on a study-by-study basis.

A key step to the effectiveness of the novel hybrid method is the powerful homogenization step to greatly aid in not only the release of bacterial cells from the complex fecal/soil matrices, but also to break down the tougher cell walls of Gram-positive community members. The higher quantitative estimates (**Figure 1**) and the higher proportion of Gram-positive Actinobacteria and Firmicutes within the microbiomes for both extraction methods that used this homogenization step (**Table 1**) are both excellent examples of the effectiveness of this homogenization step compared to the enzymatic method. These results are in agreement with previous studies using fecal samples that have shown the improvement of DNA quantity and improved community analyses when a mechanical homogenization step was included^{1,2,4-6,9}. Once the cells and/or DNA are disassociated/released from these matrices, the use of the "gold standard" enzymatic methodology in the hybrid method efficiently purifies the DNA from these samples. The inclusion of the inhibitor-binding step from the enzymatic method within the hybrid extraction protocol allows for a more efficient removal of the inhibitors present within the complex environmental samples used in this study. This step that is absent from the mechanical extraction protocol. Therefore, the hybrid extraction method, with the addition of a mechanical homogenization step in conjunction with the enzymatic purification of the released DNA, is an effective method for the assessment of total bacterial communities from environmental poultry production samples.

This method, as described, contains some specific requirements of which users will need to be aware. First is the use of a high-powered homogenizer as well as some components of a commercially available extraction kit. Previous studies demonstrated that other mechanical bead-beating steps or high-powered homogenizers improve DNA extraction efficiency and yield^{4,5,9}; therefore, a different type of mechanical homogenization step could be integrated into the hybrid extraction method (although the use of different specialized equipment may be required). The hybrid method described here also uses the bead-beating tubes that are specifically designed for the homogenizer and specific for environmental samples due to the success of this lysing matrix in previous community-based studies from poultry samples^{15,31-33}. Finally, the "gold standard" enzymatic DNA extraction method (QIAamp DNA Stool Mini Kit) is required for the enzymatic purification of the sample DNA in the hybrid protocol, since it has been shown to be the most effective enzymatic extraction method when using fecal samples^{3,18,19}. While the use of the robotic workstation is not required (instructions with the kit can be performed by hand), the use of the robotic workstation greatly increases throughput, minimizes processing error, and results in higher quality DNA for further downstream analysis⁷. Automation of this type, using the existing mechanical kits is not currently available, which represents another advantage of the hybrid method.

Currently, the robotic workstation described in this protocol is limited to processing a maximum of 12 samples in 72 min, but preliminary work has shown that this method works equally well in the higher throughput workstation that can process 96 samples within 40 min (data not shown). Further testing of this much higher throughput option will greatly enhance the efficacy of the hybrid method since more samples could be processed in a semi-automated manner within a typical day. Also, preliminary data using this novel DNA extraction method has shown that it is effective for other poultry-related samples (e.g., skin/feather rinses, carcass rinses, cecal content homogenates) as well as other types of environmental samples (e.g., agricultural soils, swine feces, horse feces, goat feces, bovine feces). Therefore, future studies will need to determine the efficacy of this hybrid method for the extraction of DNA from many agricultural and environmental samples. Further validation of this method by future studies would potentially allow for a wider adoption of this method for the assessment of total bacterial communities from a variety of agricultural and environmental settings.

Disclosures

The authors have nothing to disclose.

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References

1. Maukonen, J., Simoes, C., & Saarela, M. The currently used commercial DNA-extraction methods give different results of clostridial and actinobacterial populations derived from human fecal samples. *FEMS microbiology ecology*. **79**, 697-708, doi:10.1111/j.1574-6941.2011.01257.x (2012).
2. Tang, J. N. *et al.* An effective method for isolation of DNA from pig faeces and comparison of five different methods. *Journal of microbiological methods*. **75**, 432-436, doi:10.1016/j.mimet.2008.07.014 (2008).
3. McOrist, A. L., Jackson, M., & Bird, A. R. A comparison of five methods for extraction of bacterial DNA from human faecal samples. *Journal of microbiological methods*. **50**, 131-139, doi:10.1016/s0167-7012(02)00018-0 (2002).
4. Ariefdjohan, M. W., Savaiano, D. A., & Nakatsu, C. H. Comparison of DNA extraction kits for PCR-DGGE analysis of human intestinal microbial communities from fecal specimens. *Nutrition journal*. **9**, 23, doi:10.1186/1475-2891-9-23 (2010).
5. Carrigg, C., Rice, O., Kavanagh, S., Collins, G., & O'Flaherty, V. DNA extraction method affects microbial community profiles from soils and sediment. *Applied microbiology and biotechnology*. **77**, 955-964, doi:10.1007/s00253-007-1219-y (2007).

6. Smith, B., Li, N., Andersen, A. S., Slotved, H. C., & Krogfelt, K. A. Optimising Bacterial DNA Extraction from Faecal Samples: Comparison of Three Methods. *The Open microbiology journal*. **5**, 14-17, doi:10.2174/1874285801105010014 (2011).
7. Claassen, S. *et al.* A comparison of the efficiency of five different commercial DNA extraction kits for extraction of DNA from faecal samples. *Journal of microbiological methods*. **94**, 103-110, doi:10.1016/j.mimet.2013.05.008 (2013).
8. Scupham, A. J., Jones, J. A., & Wesley, I. V. Comparison of DNA extraction methods for analysis of turkey cecal microbiota. *Journal of applied microbiology*. **102**, 401-409, doi:10.1111/j.1365-2672.2006.03094.x (2007).
9. Salonen, A. *et al.* Comparative analysis of fecal DNA extraction methods with phylogenetic microarray: effective recovery of bacterial and archaeal DNA using mechanical cell lysis. *Journal of microbiological methods*. **81**, 127-134, doi:10.1016/j.mimet.2010.02.007 (2010).
10. Peng, X. *et al.* Comparison of direct boiling method with commercial kits for extracting fecal microbiome DNA by Illumina sequencing of 16S rRNA tags. *Journal of microbiological methods*. **95**, 455-462, doi:10.1016/j.mimet.2013.07.015 (2013).
11. Yuan, S., Cohen, D. B., Ravel, J., Abdo, Z., & Forney, L. J. Evaluation of methods for the extraction and purification of DNA from the human microbiome. *PLoS one*. **7**, e33865, doi:10.1371/journal.pone.0033865 (2012).
12. Qu, A. *et al.* Comparative Metagenomics Reveals Host Specific Metavirulomes and Horizontal Gene Transfer Elements in the Chicken Cecum Microbiome. *PLoS one*. **3**, e2945, doi:10.1371/journal.pone.0002945 (2008).
13. Sekelja, M. *et al.* Abrupt temporal fluctuations in the chicken fecal microbiota are explained by its gastrointestinal origin. *Applied and environmental microbiology*. **78**, 2941-2948, doi:10.1128/AEM.05391-11 (2012).
14. Oakley, B. B. *et al.* The Poultry-Associated Microbiome: Network Analysis and Farm-to-Fork Characterizations. *PLoS one*. **8**, e57190, doi:10.1371/journal.pone.0057190 (2013).
15. Cook, K. L., Rothrock, M. J., Jr., Eiteman, M. A., Lovanh, N., & Sistani, K. Evaluation of nitrogen retention and microbial populations in poultry litter treated with chemical, biological or adsorbent amendments. *Journal of environmental management*. **92**, 1760-1766, doi:10.1016/j.jenvman.2011.02.005 (2011).
16. Rothrock, M. J., Jr., Cook, K. L., Warren, J. G., Eiteman, M. A., & Sistani, K. Microbial mineralization of organic nitrogen forms in poultry litters. *Journal of environmental quality*. **39**, 1848-1857, doi:10.2134/jeq2010.0024 (2010).
17. Rothrock, M. J., Jr., Cook, K. L., Warren, J. G., & Sistani, K. The effect of alum addition on microbial communities in poultry litter. *Poultry science*. **87**, 1493-1503, doi:10.3382/ps.2007-00491 (2008).
18. Li, M. *et al.* Evaluation of QIAamp DNA Stool Mini Kit for ecological studies of gut microbiota. *Journal of microbiological methods*. **54**, 13-20, doi:10.1016/S0167-7012(02)00260-9 (2003).
19. Dridi, B., Henry, M., El Khechine, A., Raoult, D., & Drancourt, M. High prevalence of *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* detected in the human gut using an improved DNA detection protocol. *PLoS one*. **4**, e7063, doi:10.1371/journal.pone.0007063 (2009).
20. Harms, G. *et al.* Real-time PCR quantification of nitrifying bacteria in a municipal wastewater treatment plant. *Environmental science and technology*. **37**, 343-351, doi:10.1021/es0257164 (2003).
21. Rothrock, J., M.J. Comparison of microvolume DNA quantification methods for use with volume-sensitive environmental DNA extracts. *Journal of natural and environmental sciences*. **2**, 34-38 (2011).
22. Navas-Molina, J. A. *et al.* in *Methods in Enzymology*. Vol. Volume 531 (ed F. DeLong Edward) 371-444 Academic Press (2013).
23. Caporaso, J. G. *et al.* Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME journal*. **6**, 1621-1624, doi:10.1038/ismej.2012.8 (2012).
24. Caporaso, J. G. *et al.* Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences of the United States of America*. **108 Suppl 1**, 4516-4522, doi:10.1073/pnas.1000080107 (2011).
25. Caporaso, J. G. *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nature methods*. **7**, 335-336, doi: 10.1038/nmeth.f.303 (2010).
26. Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., & Knight, R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics*. **27**, 2194-2200, doi: 10.1093/bioinformatics/btr381 (2011).
27. Edgar, R. C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*. **26**, 2460-2461, doi: 10.1093/bioinformatics/btq461 (2010).
28. DeSantis, T. Z. *et al.* Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and environmental microbiology*. **72**, 5069-5072, doi:10.1128/AEM.03006-05 (2006).
29. Caporaso, J. G. *et al.* PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics*. **26**, 266-267, doi: 10.1093/bioinformatics/btp636 (2010).
30. Price, M. N., Dehal, P. S., & Arkin, A. P. FastTree 2—approximately maximum-likelihood trees for large alignments. *PLoS one*. **5**, e9490, doi:10.1371/journal.pone.0009490 (2010).
31. Cook, K. L., Rothrock, M. J., Jr., Lovanh, N., Sorrell, J. K., & Loughrin, J. H. Spatial and temporal changes in the microbial community in an anaerobic swine waste treatment lagoon. *Anaerobe*. **16**, 74-82, doi:10.1016/j.anaerobe.2009.06.003 (2010).
32. Cook, K. L., Rothrock, M. J., Jr., Warren, J. G., Sistani, K. R., & Moore, P. A., Jr. Effect of alum treatment on the concentration of total and ureolytic microorganisms in poultry litter. *Journal of environmental quality*. **37**, 2360-2367, doi:10.2134/jeq2008.0024 (2008).
33. Lovanh, N., Cook, K. L., Rothrock, M. J., Miles, D. M., & Sistani, K. Spatial shifts in microbial population structure within poultry litter associated with physicochemical properties. *Poultry science*. **86**, 1840-1849, doi:10.1093/ps/86.9.1840 (2007).