The preliminary evaluation of differential characteristics and factor evaluation of the microbial structure of rural household toilet excrement in China

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Abstract

Recent studies on the microbial community composition of human excrement after rural household toilet treatment are unclear regarding the effects and risks of using recycled products as fertilizers in agriculture. In this study, we used Illumina high-throughput sequencing to investigate the microbial community structure of the excrement from 50 Chinese rural household toilets on a spatial scale, and we evaluated the impact of select geochemical factors on the bacterial and fungal communities in the human excrement. Multivariate analysis showed that there was a significant spatial differentiation of the human excrement in microbial communities after all toilet treatments. Twenty dry toilet samples and thirty septic tank samples had similar bacterial (Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes) and fungal phyla (Ascomycota and Basidiomycota), differing only in the proportions of the microorganisms. For both dry toilet samples and septic tank samples, the pH and ammonium nitrogen were found to be the major driving forces affecting the changes in bacterial community structures ($p < 0.05$), while there was no correlation found for the fungal community with environmental factors in China ($p > 0.05$), except in the northern regions, where the total phosphorus was found to be significantly correlated with the fungal community ($p < 0.05$). Network analysis confirmed that $\text{NH}_4^+\text{-N}$ had the most significant impact on the content of pathogens. Certain pathogens were still detected after toilet treatment, such as *Streptococcus*, *Bacteroides*, *Aspergillus*, and *Chrysosporium*, and the proportion of potential pathogenic bacteria in dry toilets was higher than that in septic tanks, suggesting that septic tanks were better than dry toilets in treating human excrement. These results provide an ecological perspective for understanding the large-scale geographic distribution of household excrement microbial communities in rural areas and for improving human excrement treatment technologies and avoiding the risks of agricultural applications.

Keywords Rural household toilets · Bacterial community · Fungal community · Pathogen · Spatial scale

Introduction

In recent years, choosing agricultural waste resources, such as livestock and poultry manure as well as straw, as a nutrient source for fertilizer application has been of great significance to ease resource consumption and provide environmental protection (Gupta and Jana 2017). Faced with a huge demand for

resources, the world is seeking other available resources to fulfil the sustainability agricultural mandate. The emergence of new sanitation systems, such as anaerobic treatments of human waste by septic tanks, provides an elegant method of obtaining useful products (nutrients) from undesirable components (pathogens and micro-pollutants) (Simha et al. 2017).

What people think of human excreta and how they manage it varies across different periods and regions (Liu et al. 2014). Joseph proposed that “Perhaps one reason we have taken such a head-in-the-sand approach to the recycling of human excrement is because we cannot even talk about it... This is the taboo topic, the unthinkable issue” (Joseph 2005). However, in China, especially in rural areas, the state vigorously promoted the disposal of human excrement from the source (households) redirected back to agricultural areas for use as crop fertilizer. As time progresses and paradigms shifts, people have gradually changed their perception of the treatment

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and use of human excreta. In many regions, source separated toilet wastes have been applied to the soil as crop fertilizers (Fan et al. 2017).

Some experts also believe that agricultural management and health planning can be transformed by placing a greater emphasis on recycling human waste (Simha et al. 2018). However, less is known regarding the essential composition of toilet waste after septic tank treatment and whether it meets the sanitary treatment standards. Currently, the Chinese government advocates the construction and renovation of hygienic toilets to improve the rural living environment. An important part of the modern development of sanitary rural toilets is the treatment of feces and fecal sewage, because the feces and fecal sewage disposal may not be treated in time and are incomplete, which easily results in surface water and soil contamination.

Previous studies found that the discharge of untreated human excrement into river ponds caused the surface water to be polluted seriously affecting the safety of drinking water for residents (Benjamin et al. 2018). Human excrement is a source of infection for many diseases, causing great harm to human health, such as bacterial diseases (bacterial dysentery, typhoid, and paratyphoid) as well as intestinal viral diseases (viral hepatitis, polio, etc.). Human excrement contains a large number of microorganisms, up to 1.5×10^{10} cfu/g (Rose et al. 2015). In spite of pathogenic bacteria accounting for less than 1%, they have a wide variety and high pathogenicity, and certain pathogenic bacteria can survive for several years. According to Cui et al. (2020), individual pathogens were detected in the feces of healthy people. Even if the patient has recovered, the feces still carry a large number of pathogens for a period of time. Common human pathogens of fecal origin include *Salmonella*, *Campylobacter*, and *Listeria monocytogenes*. *Salmonella* and *Campylobacter* are two of the most common bacterial causative agents for gastroenteritis illness in the USA (Scallan et al. 2011), while *L. monocytogenes* is less frequently reported but has a higher mortality rate (Hernandez-Milian and Payeras-Cifre 2014).

Fecal microbiota plays an essential role in diverse processes, including disease spread and agricultural activities that are critical to environmental safety. Sivaraman et al. (2016) reported that fecal coliforms and potential pathogenic bacteria were found in fish-processing industrial effluents. What is more noteworthy is that numerous diseases included the rampant coronavirus disease 2019 (COVID-19) recently, which may spread through the human excrement (Caruso and Freeman 2020). Due to the different treatment methods of toilet feces, the characteristics of the microbial community in rural household toilets excrement are still unknown, resulting in a certain risk of these feces being used in farmland.

Recent studies mainly focused on the improvement of toilet technology and epidemiology in feces (Hotta and Funamizu 2009; Liu et al. 2011). Khan et al. (2013) found that the risk of

children's intestinal pathogen infection caused by the random disposal of untreated human feces in the public areas of the community was much higher than that of regulated sanitary private toilets. Sintawardani et al. (2017) improved the bio-toilet degradation technology and found that the degradation efficiency was related to the moisture, temperature, pH, and microorganisms.

Unlike normal compost, such as animal manure, dry toilet and septic tank fermentation have unique production processes resulting from the long-term continuous presence of organic solids, such as feces. Judgments of the effluent after the toilet treatments on environmental pollution are typically measured by indicators of general fecal indicator bacteria, fecal source tracking markers, or the focused detection of specific pathogens of interest as indicators of the exposure risk (Ferguson et al. 2012). However, this single indicator bacteria lacks representativeness and cannot accurately measure the environmental impact of the microorganisms in feces. Therefore, it is urgent to profile the characteristic of microbial communities and their influencing factors in the household toilet excrement to ensure their safe use in agriculture.

The objectives of this study were to (i) determine the characteristics of microbial communities and the chemical component of household toilet excrement at the spatial scale of a Chinese rural area; (ii) reveal the major factors driving the differentiation of microbial communities; and (iii) assess the abundance and diversity of potential pathogens.

Methods and materials

Study location and sample collection

In 2019, the project team conducted a nationwide survey and collected samples of excrement from 50 rural household toilets from 10 provinces in China, of which 20 were traditional dry toilet samples (Fig. 1) and the other 30 were septic tank effluent samples (Fig. 2). The 10 provinces cover the four regions in northern, western, eastern, and southern China, namely A, B, C, and D (Table S1). A typical rural village was selected in each province, and each village selected five households, whose lifestyles and customs were consistent, as the research objects.

Physicochemical analysis

The physicochemical properties of the samples were determined using the following methods: The pH was measured using a pH meter (Hach, USA) on site. The total nitrogen (TN), nitrate nitrogen (NO_3^- -N), ammonium nitrogen (NH_4^+ -N), and total phosphorus (TP) were measured following the National Standard Methods (Yan et al. 2020).

Fig. 1 A picture of the types of the dry toilets we used in this research

Microbial community determination

The DNA was extracted using a Power Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) following the manual. The purity and quality of the genomic DNA were checked using 0.8% agarose gels. The V3–4 hypervariable region of bacterial 16S rRNA gene was amplified with the primers 338F (ACTCCTACGGGAGGCGAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) (Munyaka et al. 2015). For each sample, a 10-digit barcode sequence was added to the 5' end of the forward and reverse primers (provided by Allwegene Company, Beijing).

The PCR was carried out on a Mastercycler Gradient (Eppendorf, Germany). Three PCR products per sample were pooled to mitigate reaction-level PCR biases. The PCR products were purified using a QIAquick Gel Extraction Kit (QIAGEN, Germany), quantified using real-time PCR, and sequenced at the Allwegene Company, Beijing. Deep sequencing was performed on Miseq platform at Allwegene Company (Beijing) (Zhang et al. 2020a, b).

The fungal Internally Transcribed Spacer (ITS) region was amplified on an Eppendorf Master cycler Gradient Thermocycler (Germany), with the primers ITS1F (5-CTTGGTCATTTAGAGGAAGTAA-3) and ITS2 (5-TGCGTTCTTCATCGATGC -3) (Zhang et al. 2015).

Separate reactions were conducted to account for potentially heterogeneous amplification from the environmental template for each sample. The PCR products were purified using an Axygen Gel Extraction Kit (QIAGEN) and quantified using qPCR. An equimolar mix of all three amplicon libraries was used for sequencing at the Allwegene Company, China. The initial sequencing data were submitted to the NCBI Sequence Read Archive (SRA) with the Bioproject of PRJNA673785.

Data analyses

The spatial patterns of the geochemical factors and microbial communities were analyzed using partial least squares discrimination analysis (PLS-DA). Analysis of similarity statistics (ANOSIM) was used to test the significance of community differences among the four regions at the level of $p=0.05$. The overlap of the microbial communities was determined by the R values from the ANOSIM, with $R>0$ indicating significant differences between groups and $R<0$ indicating that the difference within the group was greater than the difference between the groups.

The reliability of the statistical analysis is represented by the p -value, and $p<0.05$ indicates statistical significance (Zhou et al. 2020). The interrelation between the physico-chemical properties and microbial communities among four regions was revealed by the redundancy analysis (RDA) using CANOCO software (version 4.5, Wageningen, the Netherlands). Alpha and beta diversity and partial least squares discrimination analysis (PLS-DA) were analyzed using “vegan” package in R. Heatmap was produced using “gplot” package. The Spearman correlation values were used to construct the network, of which significant ($p<0.05$) and robust ($|r|>0.5$) correlations were used to resolve the co-occurrence relationship between microbial communities and geochemical factors (Qu et al. 2019). Statistical analyses were performed using SPSS 20.0 (IBM SPSS statistics, USA).

Result and discussion

The diversity and abundance of microbial communities

Our study identified a total of $1,989,300 \pm 39,786$ high-quality bacterial sequences and $48,450 \pm 969$ high-quality fungal sequences from 50 samples. Among them, the numbers of high-quality bacterial and fungal sequences in the septic tank samples were 1,193,580 and 29,070, while the numbers of high-quality bacterial and fungal sequences in the dry toilet samples were 795,720 and 19,380, respectively. After random resampling and grouping, a mean of 6716 bacterial Operational Taxonomic Units (OTUs) at 97% identity cut off was

Fig. 2 Map of the samples of excrement from 50 rural household toilets

obtained, and the mean of 1879 fungal OTUs was also successfully assigned.

At the phylum level, the compositions of the bacterial communities in different regions were basically similar; however, the relative abundances were significantly different among samples ($p < 0.05$) (Fig. 3a). The dominant phyla detected in the four regions were Firmicutes (51.88–57.09%), Proteobacteria (14.57–20.10%), Actinobacteria (2.68–16.98%), and Bacteroidetes (3.61–18.74%). These results are similar to previous studies that also found Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes were the most dominant phyla in the five activated sludge samples from the wastewater treatment plants (Zhang et al. 2019).

The relative abundance of Actinobacteria in the eastern region of China was the lowest compared with the other three regions, whereas the relative abundance of Bacteroides was the highest, reaching up to 18.74%. Firmicutes could survive high temperatures ($>55\text{ }^{\circ}\text{C}$) and participate in various metabolic activities due to its formation of heat-resistant spores in high temperatures (Zhou et al. 2019), which can explain why Firmicutes was still the dominant bacteria phyla after toilet

fermentation. Members of Firmicutes comprise a wide variety of bacteria that decompose pollutants, which indicates that they play an important role in waste disposal. For instance, *Bacillus* (Firmicutes) species are oxic or facultatively anaerobic bacteria that produce a variety of enzymes decomposing organic matter in sewage (Zhang et al. 2019).

The diversity of the bacterial community had obvious spatial separations (Fig. 3b). The Shannon indexes in the C and D regions were higher than those in the A and B regions based on the mean value of all data in different groups, indicating that the diversity of the bacterial communities in the eastern and southern regions was higher than those in the western and northern regions. In particular, the northern region had the lowest bacterial diversity and abundance, which may be related to the local low temperature that inhibited the growth of certain microorganisms (Ernakovich and Wallenstein 2015).

Based on different types of toilet treatment, we found that the relative abundance of the bacterial phylum levels had significant differences ($p < 0.05$) (Fig. S1). The relative abundance of the dominant phylum in septic tanks was significantly higher than ($p = 0.027$) those in dry toilets. Despite these

Fig. 3 **a** Relative abundance of bacteria at phylum level in different regions; **b** alpha diversity of bacteria between groups measured with observed-species and Shannon indexes; **c** relative abundance of fungi at phylum level in different regions; **d** alpha diversity of fungi between

groups measured with observed-species and Shannon indexes. The A, B, C, and D represent north, west, east, and south sampling sites, respectively

differences, the samples of dry toilets and septic tanks shared a few common bacterial phyla with high dominance. The abundance of Synergistetes in the effluent samples of the septic tank was relatively high with an average of 4.3%, with the highest in Shandong Province.

According to the results of Zhang et al. (2020a, b), Synergistetes dominate under high salt levels. This was consistent with the high level of salt consumption in Shandong Province, which was related to the local salty eating habits. A survey carried out in 2011 showed that the per capita daily intake of salt was 12.5 g in the Shandong population, which was about two times greater than the recommended nutrient intake (Zhang et al. 2014).

At the genus level (Fig. S2), the five dominant genera of all samples were *Bifidobacterium* (0.03–32.05%), *Proteiniphilum* (0.02–17.78%), *Romboutsia* (0.02–28.49%), *Tissierella* (0–21.68%), and *Blautia* (0.01–21.68%), which was different from the dominant bacteria in the Zhu et al. study, which may be related to different raw materials (Zhu et al. 2019). However, the dominant genera and their relative abundance in septic tank samples were different from those in dry toilet samples. The dominant genera of septic tank samples were *Proteiniphilum* (2.71%), *Trichococcus* (2.34%), *Romboutsia* (1.88%), and *Tissierella* (1.86%), while the dominant genera of dry toilet samples were *Bifidobacterium*

(2.81%), *Blautia* (2.00%), *Eubacterium_hallii_group* (1.95%), and *Lactobacillus* (1.83%).

In addition, there were significant differences ($p < 0.05$) in the dominant genera among different regions. For instance, *Proteiniphilum* (8.03%) was the dominant genera in the eastern region. In the northern region, the dominant genera were *Eubacterium_hallii_group* (5.27%), *Bifidobacterium* (5.24%), and *Blautia* (5.02%). In the west area, the dominant genera were *Bifidobacterium* (6.59%) and *Trichococcus* (5.79%), while the southern region was dominated by *Romboutsia* (7.47%).

For fungi (Fig. 3c), the dominant phyla detected in the four regions were Ascomycota (29.09–60.21%) and Basidiomycota (7.01–15.04%), which was similar to the Awasthi et al. study, which showed that Ascomycota and Basidiomycota were the predominant phyla in the eighteen open windrows (Awasthi et al. 2017). This is likely due to the fact that spores of Basidiomycota and Ascomycota can survive in the composting process under high temperature and low moisture (Gu et al. 2017).

Based on different toilet treatment types, the relative abundance of Talaromyces from the dry toilets was higher than in septic tank effluent, which is similar to the report that Talaromyces dominated during the thermophilic stage of composting (Fig. S3) (Awasthi et al. 2017). Hence, great

importance must be attached to further improving the overall toilet treatment plans in China.

The spatial differentiation of microbial communities

The partial least squares discrimination analysis (PLS-DA) based on the Bray–Curtis distance was conducted in this study to analyze the distribution of the microbial communities. We found that the bacterial communities in the northern and eastern samples showed significant regional differences. The ANOSIM test also confirmed that there was a significant difference in the structure of the bacterial communities between the northern and eastern regions ($r=0.559$, $p<0.001$) (Table 1).

Unlike the clustered features of the bacterial communities (Fig. 4a), the fungal communities in the eastern and southern regions had significant differences, while the differences of the fungal communities in the northern and western regions were not significant (Fig. 4b). This finding suggested that factors in the environment may have caused the spatial divergence of the microbial communities. Recent studies on the major environmental predictors of microbial diversity indicated that no single factor could explain the biogeographic patterns of microorganisms (Guo et al. 2018; Meng et al. 2020; Zeb et al. 2019).

Likewise, no single environmental factor can consistently predict the shift of microbial communities at different spatial scales (Tian and Wang 2020; Upton et al. 2019; Wang et al. 2019), although sample properties and temperature were among the most important and consistent predictors of microbial diversity across large spatial scales (Cho et al. 2015; Gong et al. 2019; Song et al. 2020).

Role of geochemical variables in shaping microbial spatiotemporal distributions

Based on the ranking characteristics of the top 20 microorganisms in four regions and two different types of samples, redundant analysis (RDA) was performed to determine the relationship between geochemical variables (including pH, TP,

TN, OM, $\text{NH}_4^+\text{-N}$, and $\text{NO}_3^-\text{-N}$) and the microbiome. The physicochemical properties in the different samples are presented in Table S2 and Table S3.

For the northern region (Fig. 5a), RDA1 and RDA2 explained 23.9% and 8.8% of the total variation, respectively, and the results showed that $\text{NH}_4^+\text{-N}$ and pH had a major influence on the bacterial community ($p<0.05$). A previous study also found bacterial community clusters in a soil environment that appeared to be differentiated by pH (Liu et al. 2020). In the western region (Fig. 5b), RDA1 and RDA2 explained 41.9% and 12.4% of the total variation, respectively, and we observed that the TN, $\text{NH}_4^+\text{-N}$, and pH were significantly related to the bacterial community dissimilarity ($p<0.05$). In the eastern region (Fig. 5c), the OM, $\text{NO}_3^-\text{-N}$, $\text{NH}_4^+\text{-N}$, and TP showed a significant correlation with the bacterial communities ($p<0.05$). No correlation between the geochemical factors and bacterial community was found in the southern region ($p>0.05$) (Fig. 5d). In general, regardless of the spatial distribution, $\text{NH}_4^+\text{-N}$ demonstrated the greatest impact on the bacterial communities.

According to the RDA results of different toilet treatment types, we found that $\text{NH}_4^+\text{-N}$ and pH had a significant correlation with the bacterial community of dry toilet samples ($p<0.05$), similar to the influential relationship in the northern region (Fig. S4A). The RDA analysis of septic tank effluents showed that these physiochemical properties explained 22.9% of the total variation significantly ($p<0.01$) (Fig. S4B). *Fastidiosipila*, *Proteiniphilum*, *Tissierella*, and *Ruminococcus_2* were positively correlated with $\text{NH}_4^+\text{-N}$, TN, TP, and $\text{NO}_3^-\text{-N}$ ($p<0.05$), consistent with the influential factors of microorganisms in sewage treatment according to previous studies (Gao et al. 2016).

The three pathogenic bacteria, *Ignatzschineria*, *Pseudomonas*, and *Escherichia-Shigella*, all belong to Proteobacteria. The RDA demonstrated that the abundance of *Pseudomonas* was in direct proportion with the $\text{NH}_4^+\text{-N}$ in both the dry toilet samples and septic tank effluents, which was related to the fact that nitrogen was required for bacterial growth, with particularly high nutritional needs during the growth of Proteobacteria (Zoppini et al. 2010). Liu et al. (2016) also stated that increased $\text{NH}_4^+\text{-N}$ deposition enriched Proteobacteria, which was consistent with our finding. This was attributed to $\text{NH}_4^+\text{-N}$ being a direct utilization form for most bacteria, including Proteobacteria (Zhou et al. 2018a, b).

We performed network analysis to evaluate the relationship between the overall microbial community and the geochemical factors. The results also showed that $\text{NH}_4^+\text{-N}$ had the strongest collinearity with the bacterial composition and that *Streptococcus* had the most significant correlation with $\text{NH}_4^+\text{-N}$ ($p<0.05$, $r>|0.6|$) (Fig. 5e). The relationship between the $\text{NH}_4^+\text{-N}$ concentration and the bacterial community composition was previously observed (Zeng et al. 2016). The study of Liu et al. (2016) also found that the relative abundance of the

Table 1 ANOSIM test for differences between different regions

Method name	R statistic	p-value	Number of permutations	Group
ANOSIM	0.2176	0.001	999	A-B
	0.5593	0.001	999	A-C
	0.4281	0.001	999	A-D
	0.1328	0.042	999	B-C
	0.0553	0.186	999	B-D
	0.3632	0.001	999	C-D
	0.2784	0.001	999	all

Fig. 4 The partial least squares discrimination analysis (PLS-DA) of **a** bacterial community structure and **b** fungal community structure in four regions

Acidobacteria phyla was sensitive to the soil $\text{NH}_4^+\text{-N}$ concentration.

Shifts in the bacterial composition following N manipulation were previously explained by the copiotrophic hypothesis, in which copiotrophic groups (e.g., Actinobacteria and Firmicutes) that have fast growth rates are more likely to increase in nutrient-rich conditions (Zeng et al. 2016).

Therefore, while paying attention to the nutrient content of nitrogen in waste recycling, it is also necessary to emphasize its impact on the bacterial community structure and pathogens. Human excrement could be returned to the field after toilet treatment to synergize with soil microorganisms resulting in improvements of the soil fertility and a reduction in environmental pathogenesis.

Fig. 5 The correlation between environmental variables and the spatial pattern of top 20 genera of bacteria was shown by RDA double plot. The **a**, **b**, **c**, and **d** represents north, west, east, and south sampling sites,

respectively. **e** represents network analysis between environmental factors and top 20 genera of bacteria

The RDA analysis of the fungal abundance showed that, in addition to the significant effect of the TP on the fungal abundance in the northern region (Fig. S5A), there was no correlation ($p < 0.05$, $r > |0.6|$) between the geochemical variables and fungal abundance in other regions, suggesting that there may be factors other than the conventional physical and chemical indicators that affected the distribution of fungal abundance. The TP may impose physiological constraints on fungal survival and growth and, thereby, directly alter the fungal community composition.

Similarly, Zeng et al. (2020) found that the soil total phosphorus was the best factor for predicting the soil fungal diversity when studying the differences in the soil fungal community and diversity under different vegetation ecosystems on the Loess Plateau. In previous studies, the availability of nutrient resources (Waldrop et al. 2006), soil temperature (Andersen et al. 2013), and soil moisture (Peay et al. 2016) were the most important abiotic factors influencing the fungal diversity in terrestrial ecosystems. Other metal nutrients (e.g., Fe, Zn, and Ca) could also have significant impacts on the fungal diversity and composition.

Chen et al. (2020) also found that the changes in the fungal community composition were significantly correlated with the soil properties, particularly the available Zn. The contents of metal nutrients (Ca, K, and Fe) had positive correlations with the fungal community structure in the soils affected by coal mine exploitation (Wang et al. 2020). This may be due to the fact that the evolution of these fungal communities was easily driven by external input, and thus, it is necessary to explore the major factors affecting the fungal community in human excrement after household toilet treatment, which is beneficial to the regulation of the fungal community in farmland soil after application.

Pathogen

Although pathogens may be killed during thermophilic or anaerobic stages (Froeschle et al. 2015), little is known regarding the richness and diversity of pathogens after toilet manure treatment. In our study, pathogenic bacteria were detected in the top 20 genera, with a mean of 8.7% of the total genus, such as *Streptococcus*, *Bacteroides*, *Escherichia-Shigella*, and *Pseudomonas*, particularly in the northern region. As previously reported, *Streptococcus*, as an opportunistic pathogen, was commonly detected in several other environments, such as air, reclaimed water, and soil, which had border adaptation options (Jjemba et al. 2010). *Escherichia-Shigella* is a pathogenic bacteria genus often associated with diarrheal diseases and can cause deaths in children under 5 years of age (Zhou et al. 2018a, 2018b). *Escherichia coli*, a pathogen that causes diarrhea and other intestinal diseases, is widely used as an indicator of microbial quality in water and food. In Bangladesh, besides rotavirus, pathogenic *E. coli* is the second

leading cause of diarrhea (Mahmud et al. 2019). In addition, as the main intestinal symbiotic bacteria, multi-drug resistant *E. coli* may also cause common and severe bacterial infections, such as urinary tract infections and sepsis (Hutinel et al. 2019), which has attracted widespread attention in countries around the world.

Pseudomonas is one of the most common Gram-negative pathogens that can cause nosocomial pneumonia (Gaynes et al. 2005). *Erysipelothrix* is recognized as the etiological agent of swine erysipelas, which can seriously affect the health of livestock (pigs) (Jensen et al. 2010). In addition, scarlet fever is caused by the Gram-positive bacteria *Streptococcus pyogenes* (group A streptococcus, GAS), which also causes other diseases, including purulent pharyngitis, toxic shock, and necrotizing fasciitis, as a result of zoonotic diseases exposed to infected animal tissues or by-products (Clark 2015).

You et al. (2018) investigated the incidence data of all 31 provinces in mainland China from 2011 to 2016 and defined the western and northern regions as high-incidence areas, which was consistent with our findings, as evidenced indirectly by the abundance of toilet pathogenic bacteria in this study. However, there is a lack of long-term systematic investigations of high-risk environmental points, such as rural toilet pollution, and the incidence of pathogenic bacteria has been underestimated. The potential pathogens (5.53%) in the samples of dry toilets were significantly ($p < 0.05$) higher than those in the effluent samples of septic tanks (3.25%), suggesting that septic tanks were better than dry toilets in treating human excrement.

Fungal pathogens represent a substantial public health risk with more than one million attributable fatalities worldwide annually (Tischler and Hohl 2019). In our study, potential fungal pathogens accounted for 10.12% of the total number of fungi, such as *Mrakia*, *Stachybotrys*, *Scopulariopsis*, *Cladosporium*, *Chrysosporium*, and *Aspergillus*. Unlike the bacterial community, the proportion of fungi in dry toilets (4.73%) was significantly ($p < 0.05$) less than that in septic tank effluent (5.39%) (Fig. S6) indicating that dry toilet fermentation had a better effect on fungi.

In addition to the eastern region, the relative abundance of *Aspergillus* was three times higher than other regions, in the range from 4.72 to 11.16%. *Aspergillus* is related to pneumonia, specifically as a structural lung disease. *Chrysosporium* can infect human skin, manifested as a skin granuloma, soft tissue purulent perforation, etc., and, importantly, can also cause the infection of other human organs, causing pneumonia, osteomyelitis, pericarditis, pleurisy, etc. (Anstead et al. 2012).

Therefore, we must pay attention to the pathogenesis of human waste after toilet treatment, as the processed products will be used for agricultural utilization after toilet treatment, and human clinical infections may occur through agricultural processes that are exposed to the source of the disease or spread through the food chain (Clark 2015).

Conclusion

Our large-scale survey revealed that there were significant differences in the microbial community structure under the different toilet treatment types for human excrement as well as significant spatial differentiation. Redundancy analysis indicated that $\text{NH}_4^+\text{-N}$ and pH had strong links to bacterial community changes, while the differences in the fungal community structure may be due to the influence of other external factors rather than conventional physiochemical factors.

The proportion of pathogenic bacteria in dry toilet samples was higher than that of the effluent from septic tanks, reflecting that flushing toilets could be promoted when the rural economy and natural conditions permit in China to ensure environmental health and agricultural application safety. The future research on toilet improvements in rural areas may focus on the high-efficiency fermentation technology of dry toilets and the application scope of septic tanks.

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Data Availability The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval The work was based on environmental samples from rural areas of China, mainly from household toilets, all samples have been processed, such as septic tanks or dry toilet anaerobic fermentation treatment, belonging to environmental samples. There are no ethical issues, therefore, Ethics approval is not required for this paper.

Consent to Participate Not applicable

Consent to Publish Not applicable

Competing Interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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