Changes in Potential Pathogens During Temperature-controlled Pig Manure Composting Process

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Abstract: With the development of intensive livestock and poultry breeding, problems associated with pathogenic microorganisms in animal manure are becoming more obvious. The pathogenic bacteria in pig manure may cause zoonotic transmission, which not only hinder the healthy development of livestock and poultry industry, but also harm people's health. In this study, the diversity and abundance of potential pathogens in the composting process of pig manure under three temperature-controlled treatments (high-temperature group, medium-temperature group and low-temperature group) were analyzed based on high-throughput sequencing technology. A total of 13 potential pathogenic bacteria were identified in the pig manure composting process, among which Enterococcus faecalis and Agrobacterium vitis accounted for the largest proportions (12%). Under different temperature-controlled treatments, the abundances of Pseudomonas stutzeri and Acinetobacter baumannii changed most obviously. Moreover, the diversity and abundance of potential pathogens under high-temperature treatment were significantly lower than those under medium-temperature and low-temperature treatment, indicating that high temperature condition of composting could effectively remove pathogenic bacteria and inhibit the growth and reproduction of pathogens.

Keywords: Pathogen, Swine manure, Compost, OTU sequence, Abundance.

1. Introduction

The rapid development of livestock and poultry industry has provided us with a variety of high-quality products in our daily life, and at the same time, the emission of manure from livestock and poultry farms has become increasingly large. In 2020, pork production accounted for 53.09% of the total production of livestock and poultry meat in that year, and the discharge of manure pollution was about 288 million tons. If the management of manure is not strengthened, it will lead to environmental pollution, soil degradation and other problems. These organic wastes cause serious environmental problems, including increased risks from the spread of pathogenic bacteria to humans and animals, increased emissions of toxic gases, and high nitrogen and phosphorus emissions leading to eutrophication.

Compost is often used for the harmless treatment of livestock manure. Composting is a complex biochemical process that relies on the interaction of highly diverse microbial communities to convert organic wastes into nutrient-rich, safe and stable organic fertilizers or soil amendments [1]. By controlling the moisture, oxygen content, pH, C/N ratio and temperature of compost, various complex organic nutrients can be converted into soluble nutrients and humus under the action of microorganisms. At the same time, the high temperature produced by accumulation (60-70℃) is used to kill bacteria, eggs and weed seeds in the raw materials, so as to achieve the purpose of harmless.

In this study, pig manure was selected as the research object, and the OTU sequence obtained by sequencing of bacterial flora structure in the composting process of pig manure was compared in NCBI database to further screen out potential pathogenic bacteria, and the influence of composting temperature on the diversity and abundance of potential pathogenic bacteria was analyzed.

2. Materials and Methods

2.1. Experimental procedure

Detailed information regarding the experimental design is described in our previous study [1]. In brief, the composting experiment was conducted on a 1 L cylinder composting reactor, containing the mixture of pig manure and corn stalk (C/N ratio about 25 and moisture about 60%). The reactor was placed in a water bath for temperature control and the composting process lasted for 42 days. Three groups of composting tests were carried out, with different maximum heating temperatures (Low: 40 °C; Medium: 54°C; High: 68°C). The composting procedures included heating (0-3 d), thermophilic (4-7 d), cooling (8-21 d) and maturation (22-42 d) periods. The forced intermittent ventilation and pile turning were used to supply oxygen for the composts. Samples were prepared by mixing subsamples from the top, middle and bottom layers of each pile. Temperature and pH were analyzed every day. Samples for microbial community and other physicochemical analysis were taken on days 0, 3, 7, 21 and 42.

2.2. High-throughput sequencing and potential pathogens analysis

The high-throughput sequencing of bacterial community was conducted on an Illumina MiSeq PE300 platform (Allwegene Co., Ltd, Beijing, China). The V3-V4 regions of bacterial 16S rRNA genes were amplified with the primers 338F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT), and the details of the

procedure are described in a previous study [1].

The qualified 16S rRNA sequences obtained from each sample were compared against the Pathogen Host Interactions database (http://www.phi-base.org/) and the previously constructed bacterial pathogens database by using BLASTN with an E-value $\leq 1 \times 10^{-20}$ [2]. The outputs were filtered according to an amino acid identity $\geq 98\%$ and alignment length ≥ 200 bp.

2.3. Data statistical analysis

Microsoft Excel 2010 was used for statistical analyses of the data. Principal component analysis (PCA), Venn diagram and correlation heatmap were conducted using R v.3.5.2.

3. Results and Discussion

3.1. Composition of potential pathogens

As shown in Fig. 1, a total of 13 potential pathogenic bacteria were detected in the pig manure pile. Enterococcus faecalis 12%, Agrobacterium vitis 12%, Corynebacterium jeikeium 10%, Bcillus cereus 10%, Pseudomonas stutzeri 10%, Escherichia coli 10%, Stenotrophomonas maltophilia 10%, Acinetobacter baumannii 9%, Salmonella enterica 8%, Serratica marcescens 3%, Bacteroides thetaiotaomicron 2%, Streptococcus parauberis 2%, Proteus mirabilis 2%. The percentages of Enterococcus faecalis and Agrobacterium vitis were higher than those of other pathogenic bacteria.

Enterococcus faecalis is a facultative anaerobic Gram-positive and hydrogen peroxide negative cocci, which is one of the main bacterial flora in the intestinal tract of humans and animals. It is often used as probiotics in breeding and production, and is also an important opportunistic pathogen, which can cause infection of multiple organs of humans and animals [3]. Bacillus cereus is a zoonotic foodborne opportunistic pathogen [4]. Under poor growth conditions, Bacillus cereus produces a "shell" called "spore" to protect itself from death. Escherichia coli [5,6] is ubiquitous in livestock and poultry feces, and is the most common opportunistic pathogen in zoonotic diseases, especially easy to cause disease in farmed animals. Acinetobacter baumannii is a lactose non-fermenting gram-negative cocci, which is widely found in natural environments and hospitals due to its low requirements for living conditions [7].
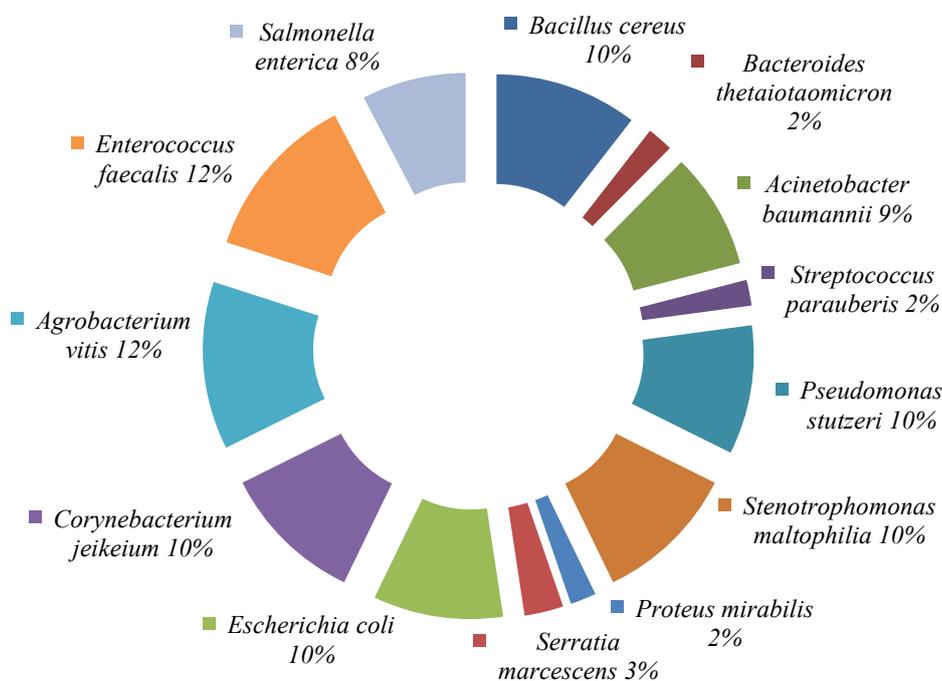

Figure 1. Percentages of potential pathogens during pig manure composting

In order to further analyze the differences of potential pathogenic bacteria in pig manure compost after treatment with different temperatures, the PCoA diagram was made from the sample data of D3, D7, D21 and D42 in the composting process. As can be seen from Fig. 2, the PCoA1 axis explained 50.65% of the sample variation, and the PCoA2 axis explained 27.26%. Different samples showed obvious clustering phenomenon, and the low temperature group (D3-L, D7-L, D21-L, D42-L) was more concentrated than the medium and high temperature groups (D3-H, D7 H, D21 H, D42 H, D7 M, D21 M, D42 M), and the colony structure of potential pathogenic bacteria was less different. The initial pig manure D0 was significantly different from the pile sample.
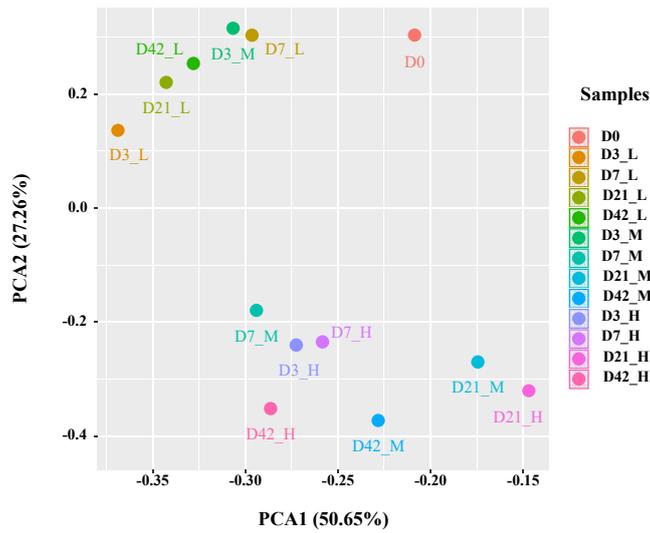
Figure 2. PCoA results of potential pathogens in pig manure composts

The Venn plot shows the difference between the potential pathogens in the initial pig manure and the final compost under different treatments. As can be seen from Fig. 3, the four samples share five kinds of the same potential pathogens. The D0 sample has a unique potential pathogen and shares four of the same potential pathogens with the D42-L sample. It can be seen that the potential pathogens in pig manure will be affected by temperature, and the potential pathogens will gradually decrease with the increase of temperature.
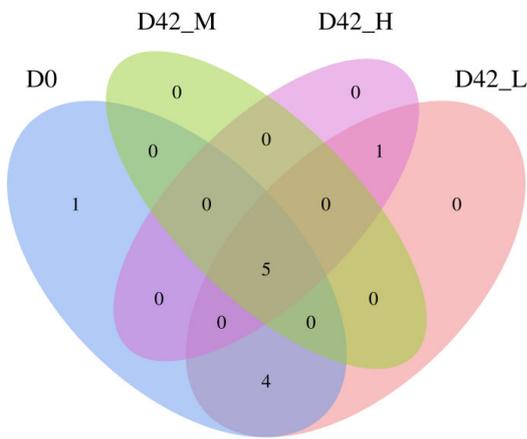

Figure 3. Venn diagram of potential pathogens in the initial pig manure and the final composts under different treatments

3.2. Relative abundance of potential pathogens

As the days of composting increased, the relative abundance of potential pathogenic bacteria generally showed a downward trend (Fig. 4). Under low temperature treatment, the species of potential pathogenic bacteria were more abundant than the other two groups. After H (high temperature) treatment, the relative abundance of potential pathogenic bacteria is minimal. Taking *Acinetobacter* baumannii as an example, which showed the most obvious variation in the abundance of 13 potential pathogenic bacteria,

its abundance range was 3.07×10^{-3}-1.14×10^{-2} under low temperature treatment, 0-1.07×10^{-2} under medium temperature treatment, and 0-1.08×10^{-4} under high temperature treatment. The abundance of the potential pathogenic bacteria in the high temperature treatment group was significantly decreased compared with that in the medium or low temperature treatment groups, indicating that the number of the potential pathogenic bacteria was significantly reduced under high temperature composting. The abundance of *Pseudomonas stutzeri* was 1.62×10^{-4} at D3H in the warming period, and then decreased to 0 at D7H in the high temperature period, but it rebounded to 1.09×10^{-2} at D21H in the cooling period. The abundance of potential pathogenic bacteria in the warming period was less than that in the cooling period, which again proved that high temperature could inhibit the inactivation of potential pathogenic bacteria

High temperature composting significantly reduced the number of potential pathogenic bacteria in fecal sources. In addition, the reduction effect of high temperature compost on potential pathogenic bacteria in fecal source mainly concentrated in the first 3 to 7 days, and the range of inhibition inactivation gradually reduced.
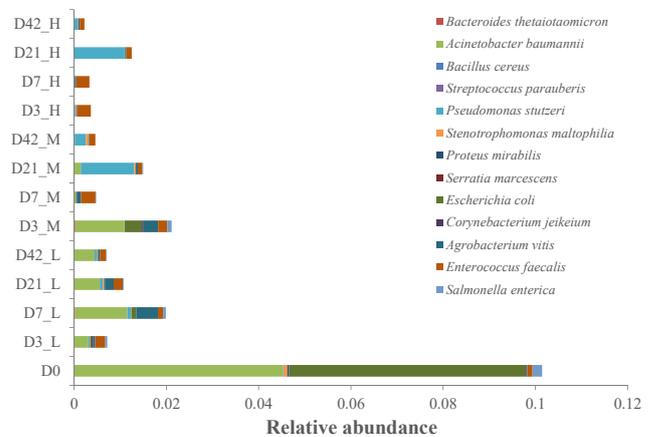

Figure 4. Relative abundances of potential pathogenic bacteria detected during pig manure composting

3.3. Impact of environmental factors on potential pathogens

Fig. 5 shows that the same environmental factor has different effects on different pathogenic bacteria. Temperature and *Stenotrophomonas maltophilia* had very significant negative correlation ($R = 0.86$, $P < 0.01$), but temperature had a very significant positive correlation with *Enterococcus faecalis* ($R = 0.07$, $P < 0.01$). *Corynebacterium jeikeium*, *Escherichia coli*, *Acinetobacter baumannii* and TN had a significant negative correlation ($0.57 \leq R \leq 0.67$, $P < 0.05$). *Bacillus cereus* and *Pseudomonas stutzeri* were positively correlated with TP ($0.56 \leq R \leq 0.57$, $P = 0.05$). There were also different correlations between the same pathogenic bacteria and different environmental factors. *Salmonella enterica* and TOC had extremely significant positive correlation ($R = 0.79$, $P = 0.01$), and *Salmonella enterica* and TP had a significantly negative correlation ($R = 0.61$, $P = 0.05$). *Corynebacterium jeikeium* and TP had a significantly negative correlation ($R = 0.79$, $P = 0.01$), and *Corynebacterium jeikeium* and TOC had a significant positive correlation ($R = 0.59$, $P = 0.05$).
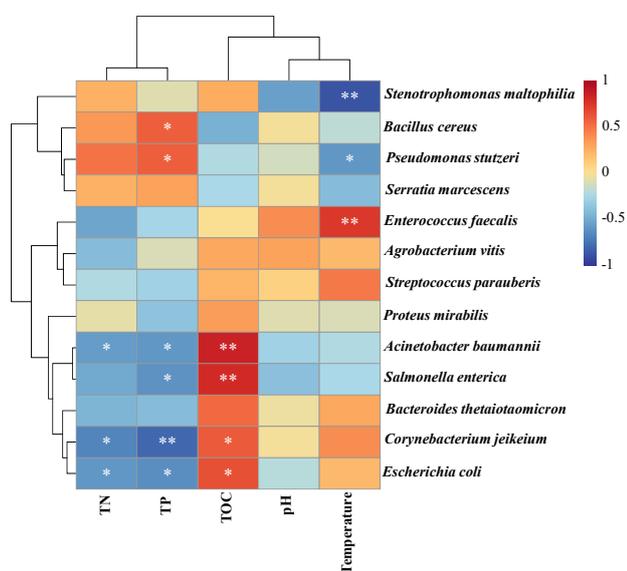

Figure 5. Spearman correlation analysis between environmental factors and potential pathogens. * $P < 0.05$, ** $P < 0.01$.

4. Summary

A total of 13 potential pathogenic bacteria were identified in the process of pig manure composting, among which *Enterococcus faecalis* and *Agrobacterium vitis* accounted for the largest proportion. Under different temperature treatments, the abundance values of *Pseudomonas Stutzeri* and *Acinetobacter Baumannii* changed the most obviously, and the diversity and abundance of potential pathogenic bacteria under high temperature treatment were significantly lower than those under medium-temperature and low-temperature treatment, indicating that the high temperature condition of compost can effectively remove pathogenic bacteria and inhibit the growth and reproduction of pathogenic bacteria.

Acknowledgment

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