Succession of bacteria communities during production and application of dairy bedding by membrane-covered aerobic fermentation

Hongjie Yin[1†], Jianfei Zeng[2†], Chen Fang[1,3], Xueqin He[1], Ya Su[1], Jinpeng Xiong[1], Lujia Han[1], Guangqun Huang[1*]

(1. *College of Engineering China Agricultural University, Beijing 100083, China*;
2. *Institute of Environment and Sustainable Development in Agriculture, Chinese Academy of Agricultural Sciences, Beijing 100081, China*;
3. *College of Agriculture, Guizhou University, Guiyang 550025, China*)

Abstract: The cost of dairy manure treatment and bedding material purchase increases the operating cost of the dairy farm. Membrane-covered aerobic fermentation system has been widely used for dealing with dairy manure and recycling the final product as bedding material. However, the microbial safety in each processing step is still uncertain. To better understand the bacterial community dynamics during the whole bedding conversion process, a full-chain and large-scale experiment including 16-day membrane-covered aerobic fermentation and 11-day bedding material application was conducted. The results showed that the pile temperatures in the fermentation stage rapidly increased to 80°C and maintained >50°C for more than 11 d and the use of fermentation product as bedding material provided cows with a stable and comfortable bedding environment. The Chao1 and Shannon index decreased at the end of the fermentation stage and remained stable in the application stage, indicating that membrane-covered aerobic fermentation effectively killed some pathogenic bacteria and guaranteed both the maturity and stability of the final product. The dominant bacteria in the fermentation stage were *Acinetobacter*, *Thermus*, and *Rhodothermus* at genus level. Seven common potential pathogens of mastitis (*Staphylococcus*, *Enterococcus*, *Serratia*, *Pseudomonas*, *Corynebacterium*, *Mycobacterium*, and *Bacillus*) were found at the end of fermentation stage but the relative abundance was low (0.0025%-0.2727%). The dominant bacteria in the application stage mainly included *Acinetobacter*, *Pseudomonas*, and *Flavobacterium* at the genus level. The relative abundance of *Pseudomonas* increased in the application stage, which was a reminder to the dairy farm to pay attention to the disinfection and timely replacement of bedding material to prevent the occurrence of dairy mastitis. The results of this study contributed deep understanding of the microorganism-driven bedding conversion process and provide practical guidance and cautions for the bedding materials application.

Keywords: semi-permeable membrane, dairy manure, aerobic fermentation, bedding material, bacterial diversity
DOI: 10.25165/j.ijabe.20241701.8530

Citation: Yin H J, Zeng J F, Fang C, He X Q, Su Y, Xiong J P, et al. Succession of bacteria communities during production and application of dairy bedding by membrane-covered aerobic fermentation. Int J Agric & Biol Eng, 2024; 17(1): 232–240.

1 Introduction

The rapid development of dairy farming has resulted in the increasing production of dairy manure with high moisture content, which has become a huge expense in waste management for the farm. Improper treatment of dairy manure caused by the cost limit could contribute to serious pollution of the atmosphere, soil, and water[1,2]. Economical and environment-friendly treatment technology was and will always be the focus of researchers and farm managers. At the same time, dairy cattle spend an average of 10-13 h/d lying down on the bedding and the bedding is proven to be crucial for the well-being of cows in dairy farming, which helps to keep the cows clean, dry and comfortable, reducing the risk of intramammary infection, minimizing lameness and hock lesions, and ensuring the milk production[3,4]. Affected by the rising market price of traditional bedding materials such as rice husks, wood chips and sand, seeking new bedding material sourcing in high quality, comfort and low price has become a matter of urgency.

Bedding conversion, an effective combination of dairy manure deposal and bedding material production, has been widely used in dairy farms in China because it provides a way to dispose dairy manure directly inside the farm and transform and recycle them as bedding materials for cows, which not only solves the problem of dairy manure contamination, but also the problem of sourcing and cost of bedding material purchase[5]. After solid-liquid separation pretreatment, aerobic fermentation was thought to be one of the most commonly used method in bedding conversion process because of the highest safety and moderate costs when compared with direct drying[6,7]. It has been proved in the field of composting that aerobic fermentation by membrane-covered can effectively reduce greenhouse gas emissions[8], kill pathogenic bacteria[9] and shorten the fermentation cycle. Therefore, adopting membrane-

Received date: 2023-09-13 **Accepted date:** 2023-12-26
Biographies: Hongjie Yin, PhD candidate, research interest: agricultural biomass resource utilization, Email: yinhongjie@cau.edu.cn; **Jianfei Zeng**, PhD, Researcher, research interest: waste management/agricultural equipment, Email: zengjianfei@caas.cn; **Chen Fang**, PhD, Distinguished Professor, research interest: recycling and utilization of agricultural waste, Email: gzufangchen@163.com; **Xueqin He**, PhD, Associate Professor, research interest: agricultural equipment/biomass engineering, Email: hxq12@cau.edu.cn; **Ya Su**, PhD candidate, research interest: agricultural biomass resource utilization, Email: causuya@cau.edu.cn; **Jinpeng Xiong**, PhD candidate, research interest: agricultural biomass resource utilization, Email: xiongjinpeng@cau.edu.cn; **Lujia Han**, PhD, Professor, research interest: agricultural biomass engineering, Email: hanlj@cau.edu.cn.
†The authors contributed equally to this work.
***Corresponding author: Guangqun Huang**, PhD, Professor, research interest: agricultural equipment/biomass engineering. College of Engineering, China Agricultural University, Beijing 100083, China. Tel: +86-10-62737995, Email: huangguangqun@126.com.

covered aerobic fermentation in bedding conversion process will be of great significance for the green and sustainable development of dairy production.

Research into membrane-covered aerobic fermentation technology mainly focused on greenhouse gas emissions and microbial community dynamics. Ma et al.[10] studied the impacts of membrane-covered composting approach on bacterial communities and gas emissions in the aerobic composting of dairy manure. The results showed that the membrane-covered composting process could effectively decrease greenhouse gases and improve the relative abundance of actinomycetes, which are more conducive to the degradation of volatile solids. Microorganism is the key factor affecting the degradation of organic matter during the fermentation process, among which bacteria account for the largest proportion of the microbial community[11]. Thus, many scholars used 16SrRNA high-throughput sequencing technology to study bacterial diversity[12]. This method addresses the defects in environmental microbial information that research methods involving the isolation and culture of microorganisms cannot fully obtain[13]. Studies have shown that common bacterial phyla involved in the process of aerobic fermentation include *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria*[14]. These bacteria are key to polysaccharide hydrolysis and lignocellulosic degradation[15]. Recently, researches on the bacteria community dynamics related to bedding conversion mainly focus on the bedding production stage or the initial and final stage of bedding material application. Few teams pay overall attention to the whole process from bedding production to application.

Therefore, this study conducted a full-chain and large-scale bedding conversion experiment, including 16-days membrane-covered aerobic fermentation and 11-days bedding material application, to better understand the bacterial community dynamics during the whole process. Physicochemical characteristics of the pile in membrane-covered aerobic fermentation stage and those of the bedding materials in application stage were investigated and compared. High-throughput sequencing was used to analyze dynamics of the bacterial community composition, diversity and difference. The results of this study will contribute a deep understanding of the microorganism-driven membrane-covered aerobic fermentation process and providea theoretical basis and practical guidance for the bedding materials application process.

2 Materials and methods

2.1 Experimental design

The experiment was conducted at Jinyindao Dairy Farm (Beijing Sunlon Livestock Development Co., Ltd., China). The experiment was categorized into a bedding production stage (that is, the fermentation stage) and a bedding application stage. Solid substances in manure from the dairy farm after the solid-liquid separation were applied as the fermentation raw materials. To reduce the bedding material's moisture content, a drying stage was added between the fermentation stage and application stage. In the fermentation stage, a membrane-covered trough aerobic fermentation process was carried out with trough dimension of 8.5 m in length, 4 m in width, and 1.5 m in height. At the bottom of the trough, a ventilation pipe was provided for oxygen supply. Group A and Group B were set up in this stage and covered with a ZT1 membrane and ZT2 membrane, respectively (Qingdao Zhiteng Technology Co., Ltd., China). The two membranes were semi-permeable with certain differences in pore structure, and the core interlayers were made of expanded polytetrafluoroethylene (e-PTFE). The main performance parameters and scanning electron microscope images of the membranes have been presented in previous studies[6]. Continuous ventilation was applied from Day 0 to Day 10 with the ventilation rate of 200 m^3/h and intermittent ventilation was carried out for 5 min on and 5 min off from Day 11 to Day 16 with the ventilation rate of 400 m^3/h. In the bedding application stage, the final products of Group A and Group B were respectively applied in cowsheds A and B with 88 beds in each barn. These two treatments were labelled as Group PA and Group PB. The whole bedding application period lasted for 11 days, during which the bedding material was added and laid on Day 4 and Day 7, and Days 0-4, Days 4-7 and Days 7-11 in the application stage were marked as Period Ⅰ, Period Ⅱ and Period Ⅲ, respectively.

2.2 Methods of sample collection and analysis

In the fermentation stage, solid samples were gathered on Day 0, 1, 3, 5, 7, 10, 13, and 16, respectively. Each sample was a mixture of subsamples from the upper, middle, and lower layers of the pile. During the application stage, five beds were chosen for the collection of solid samples and the collection frequency was once a day. A sampling diagram is provided and presented in previous study[6]. Solid samples were kept at −20°C and −80°C to determine the basic physicochemical indices and to extract DNA. Measurement of the volatile solids (VS) and moisture content (MC) was conducted by referring to existing standard methods[16]. The pH value was determined by extracting and filtering the supernatant from a mix of the fresh sample and deionized water according to a solid-liquid ratio of 1:10 (w/v). A PT100 temperature sensor (32 208 551, Heraeus, Germany) was used to monitor the daily core temperature of the pile, and a mercury thermometer was used to monitor the daily ambient temperature. Oxygen concentrations in the center of the pile were monitored using a gas concentration detector (Biogas 5000; Geotechnical Instruments Ltd., U.K.).

2.3 DNA extraction and high-throughput sequencing

E.Z.N.A Mag-Bind Soil DNA Kit (Omega Bio-Tek, U.S.) was adopted for the extraction of DNA from samples. The DNA extracted was subsequently quantified with Qubit3.0 DNA test kit (Life Technologies, U.S.) to identify the amount of DNA to be added to the PCR reaction. The amplification target region was the V3-V4 region of 16S rRNA gene, and the primer sequences were 805R, GACTACHVGGGTATCTAATCC and 341F, CCTACGGGNGGCWGCAG. After amplification, library size was determined by 2% agarose gel electrophoresis, and its concentration was assayed with a Qubit3.0 fluorometer (Invitrogen, U.S.). Lastly, sequencing of the libraries was performed on an Illumina MiSeq platform.

2.4 Statistical analysis

This study used Usearch (version 11.0.667, http://drive5.com/usearch/) to cluster operating taxonomic units (OTUs) with 97% sequence similarity. A comparison of representative sequences was made with the RDP 16S database, and classification and analysis were performed in RDP classifier (http://rdp.cme.msu.edu/). Data processing and charting used Excel 2016 (Microsoft, USA), Origin 9.1 (OriginLab, USA), R 3.6.0 (https://www.r-project.org/), STAMP 2.1.3 (https://beikolab.cs.dal.ca/software/STAMP), and Adobe Illustrator CC 2019 (Adobe, USA).

3 Results and discussion

3.1 Physicochemical properties

Figure 1a shows that the pile temperatures of Group A and Group B in the fermentation stage both rose rapidly to more than 80°C on the first day and maintained high temperatures (>50°C) for

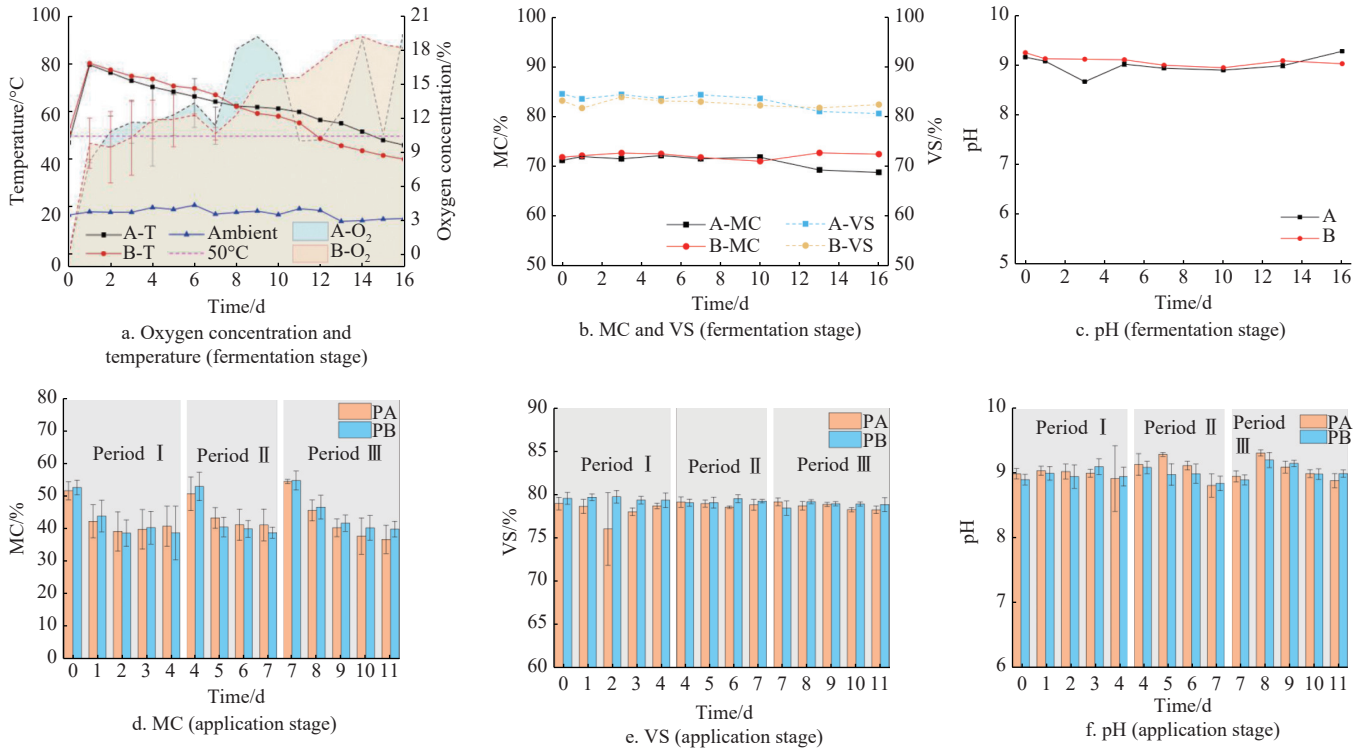
14 and 11 d, respectively. The oxygen concentrations of Group A and Group B were maintained at more than 10% (Figure 1a), suggesting that the micro-positive pressure environment in the pile created by the membrane covering system was conducive to the oxygen utilization by microorganism and kept the pile in a good aerobic fermentation condition[17]. Aerobic microorganisms were active under this circumstance and generated a lot of heat. Additionally, the semi-permeable membrane had the effect of heat preservation, resulting in the pile could maintain high temperatures for a long time. High temperatures can effectively kill pathogenic bacteria[18], so the fermentation products have a certain level of safety. As the water backs up under the membrane, the MC of the two groups of piles changed little throughout the fermentation process (Figure 1b). The decrease in VS content in the two groups was relatively low (Figure 1b), which may be due to the loss of volatile solid along with the liquid part by solid-liquid separation and the high content and limited degradation rate of lignocellulosic in the remained solids[19]. Both groups were kept in a slightly alkaline environment with a pH between 8.66 and 9.27 (Figure 1c).

Figure 1d shows that the MC of the bedding material gradually decreased in each period during the whole application stage, which may be attributed to the evaporation from the exposed bedding material in the open air. The VS content of the bedding material ranged from 76.02% to 79.70% (Figure 1e), and the pH value ranged from 8.83 to 9.30 (Figure 1f). Beales et al.[20] proposed that the pH of bacterial growth and survival should be between 4 and 8. Coupled with the low MC of the bedding material, conditions may not be favorable for bacterial development and colonization, which further ensured the safety of the bedding material. In addition to MC, the VS content and pH remained in relatively stable ranges during the application stage, indicating that the degradation degree of the bedding material was low in the application stage. The physicochemical indices did not change significantly in the application stage, which also suggested that the fermentation product had basically reached a mature and stable state. The use of the fermentation product as bedding material could provide cows with a stable and comfortable bedding environment. Using an aerobic fermentation process by membrane-covered for producing the bedding material was therefore concluded to be feasible.

3.2 Analysis of bacterial diversity

3.2.1 Analysis of alpha diversity indexes

As can be seen from Figure 2a, the Chao1 index and Shannon index of samples in Group A and Group B both decreased first and then increased over time during the aerobic fermentation stage. In the mesophilic phase of aerobic fermentation, the decreasing trend in the Chao1 index indicated that the community richness in the pile decreased. The reason might be that some bacteria that were not thermoduric were killed quickly by the rapid increase of pile temperature in the mesophilic phase of aerobic fermentation. At this time, thermophilic bacteria became the dominant bacterial community, and the bacterial richness of the community decreased[14]. The decline in the Shannon index at the beginning of aerobic fermentation suggested a decrease in microbial diversity in the pile. This is because the richness of the bacterial community decreased and the uniformity of the bacterial community decreased as thermophilic bacteria became the dominant bacterial community, thus reducing the diversity of the bacterial community in the pile[14]. The increase in the Chao1 index and Shannon index in the thermophilic and cooling phases of aerobic fermentation may have been due to the pile temperature gradually decreasing at this stage, thus some thermophilic bacteria became active, and the bacterial richness and the bacterial evenness increased. The bacterial diversity in the pile thus increased. The Chao1 index and Shannon index of the final materials of Group A and Group B were lower than those of the initial materials, indicating that moderate aerobic fermentation decreased the diversity and richness of bacterial community distribution in the materials. The decrease in the distribution richness of the bacterial community indicated that the



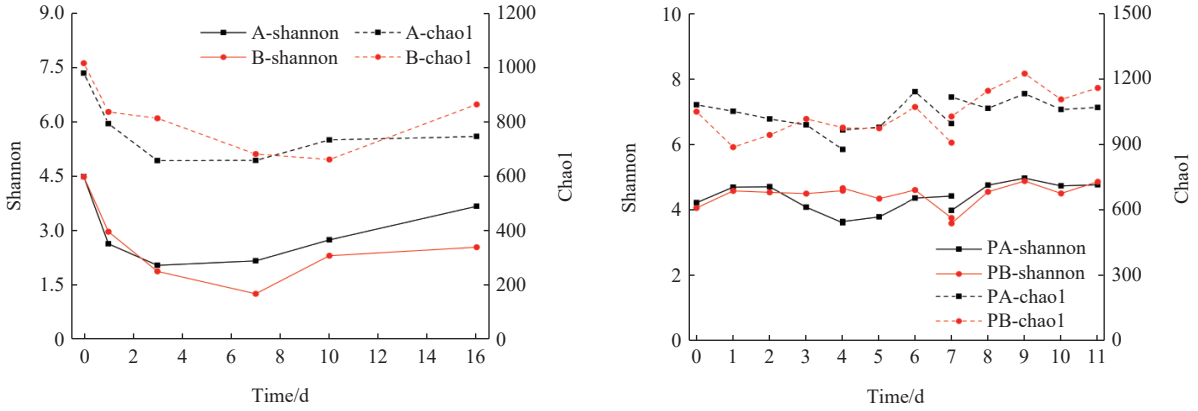
a. Oxygen concentration and temperature (fermentation stage)
b. MC and VS (fermentation stage)
c. pH (fermentation stage)
d. MC (application stage)
e. VS (application stage)
f. pH (application stage)

Note: ⊥: standard deviation. MC, moisture content; VS, volatile solids.

Figure 1 Dynamics of basic physicochemical properties during fermentation and application stage

aerobic fermentation process effectively killed bacteria such as *Salmonella*, *Klebsiella* and other pathogenic bacteria[21], and had a different dominant bacterial community at each stage of fermentation. Alpha diversity indexes were not markedly different between Groups A and B. In the final material, Group B had a greater Chao1 index and a smaller Shannon index than Group A, suggesting that Group B had a higher richness but lower diversity of bacterial communities compared to Group A. That is, at the end of the fermentation, the number of species in Group B was greater than that in Group A, but species distribution was more uniform in Group A.

From Figure 2b, it can be seen that the Chao1 index and Shannon index of samples from groups PA and PB fluctuated within certain ranges with time, and there was no obvious similar change during each period, which indicates that there were no significant bacterial community succession features in the bedding material in the application stage. The variation in bacterial community diversity in the bedding material may be related to the environmental conditions of the practical application. After new bedding material was laid, the Chao1 index of the bedding material generally increased, indicating that adding bedding material increased the bacterial community richness of the bedding material in the bed, i.e., the unused bedding material had higher bacterial community richness than the used bedding material. This may be related to the drying process after bedding production. During the drying process, a large area of the bedding material was in contact with the air, which probably led to an increase in bacterial community richness in the bedding material. In addition, there was no apparent difference in the bacterial richness and diversity between Group PA and Group PB during the application process.



a. Fermentation stage b. Application stage

Note: Community diversity was represented by the Shannon index, and the larger the Shannon index, the higher the community diversity. Chao1 was used to represent community richness, and the greater the value of Chao1, the greater the total number of species.

Figure 2 Dynamic changes of the alpha diversity indexes

3.2.2 Analysis of bacterial community composition at phylum level

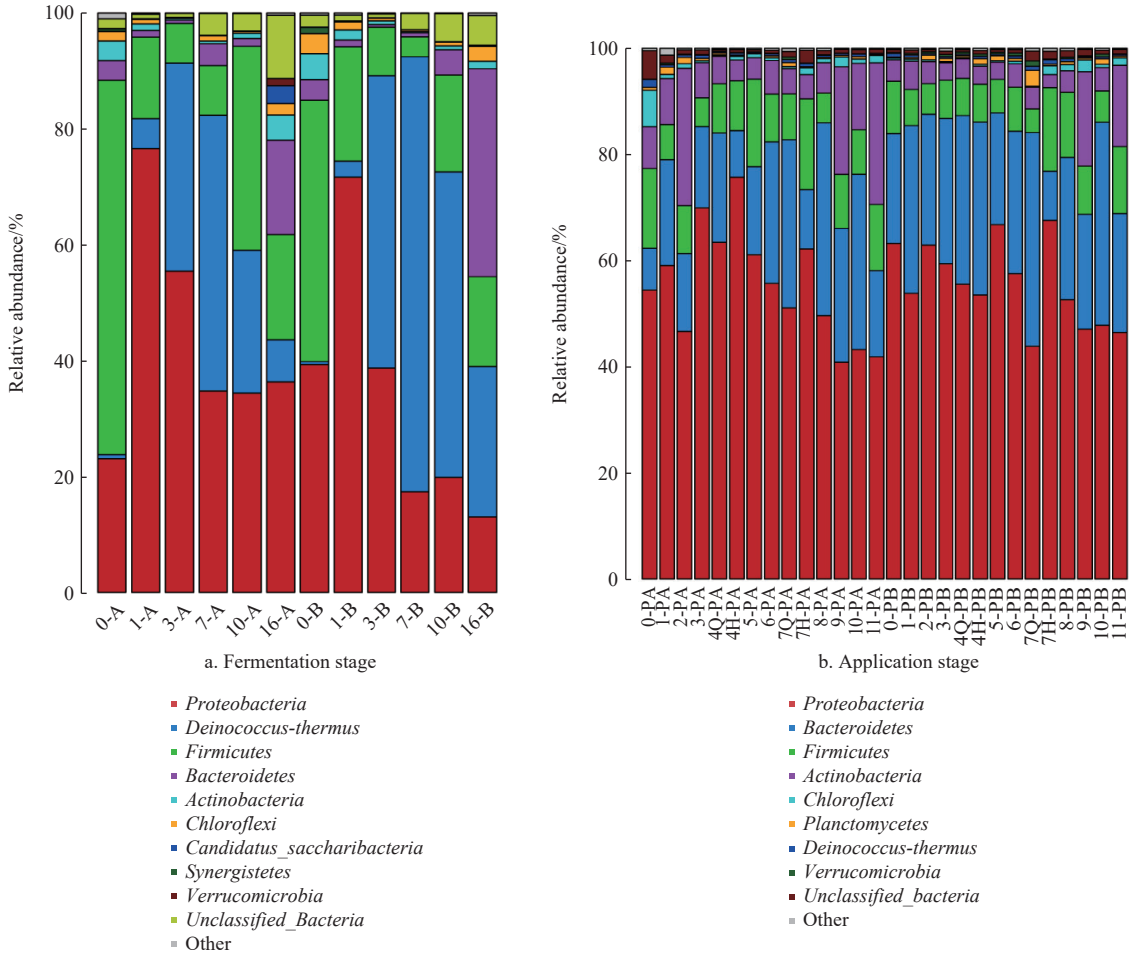
Figure 3a presents that the dominant bacteria in the fermentation stage in Group A and Group B were *Proteobacteria*, *Deinococcus-Thermus*, *Firmicute*, *Bacteroidetes*, *Chloroflexi*, and *Actinobacteria*, which is similar to the results of most studies[22,23]. The *Proteobacteria* relative abundance rose rapidly on the first day and then gradually decreased, which is consistent with the trend in temperature changes, indicating that *Proteobacteria* was related to the change in pile temperature[24]. In addition, *Proteobacteria* plays a key role in the degradation of small molecular organic matter[25]. The content of small molecular organic matter in the solid material was enough to support the rapid propagation of *Proteobacteria*, so *Proteobacteria* only increased rapidly on the first day, after that point the bacteria gradually decreased. The relative abundance of *Deinococcus-Thermus* was firstly raised to the peak on Day 7 and later reduced. On the first day of fermentation, the relative abundance of *Firmicutes* decreased significantly, and revealed a reducing and later raising trend throughout the fermentation stage. This result was contrary to most existing studies, which showed *Firmicutes* to be more common at the thermophilic phases[26]. *Firmicutes* are thought to play a significant role in the degradation of wood fiber components[27], and at the same time, *Firmicutes* can rapidly utilize carbohydrates as nutrients[28]. This trend may be attributed to the low lignocellulosic content in the mesophilic phase of fermentation and the increase in available carbohydrates in the cooling phase. The relative abundance of *Bacteroidetes* increased more obviously at the cooling phase of fermentation and reached a peak at the end of the fermentation. The variation in the *Bacteroidetes* population was related to temperature, and its optimal activity temperature was 25°C-45°C[29]. Therefore, when the temperature was higher than 50°C, the relative abundance of *Bacteroidetes* decreased. In the cooling phase of aerobic fermentation, its relative abundance increased with decreasing temperature. At the same time, *Bacteroidetes* are considered specialists in degrading high molecular weight compounds[30], and another research report also pointed out that in the cooling phase, *Bacteroidetes* became the dominant bacteria to degrade cellulose and hydrolyze polysaccharides[15]. Therefore, high molecular weight organic matter that was not completely degraded in the early phase and the hydrolyzed polysaccharide produced by the decomposition of organic matter in the fermentation process may have been degraded by *Bacteroidetes* in the cooling phase. Therefore, the reproduction of *Bacteroidetes* increased in the cooling phase, resulting in a marked rise in relative abundance. The relative abundance of *Actinobacteria* first decreased and then increased with fermentation time, a trend in agreement with previous studies[14]. It has been suggested that the increase in *Actinobacteria* accelerates the degradation of volatile solids[11]. In this study, the variations in the amounts of volatile solids and relative abundance of *Actinobacteria* with fermentation time were consistent with results in the literature. *Chloroflexi* was relatively abundant at the beginning and end of fermentation, which is consistent with previous studies. *Chloroflexi* was more abundant in the cooling

phase[26], which was related to the degradation of soluble microbial products and the removal of biological nutrients[31].

Figure 3b shows that the dominant bacteria in Group PA and Group PB at the application stage mainly included *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Chloroflexi*, and the sum of the relative abundance of the five dominant phyla reached more than 90% (92.11%-98.95%). Among them, *Proteobacteria* was the most dominant phyla, and its relative abundance ranged from 40.86% to 75.72%. Compared with the fermentation stage, the relative abundance of this dominant phyla was higher, which may have been because the environments for drying and bedding storage were conducive to the growth and propagation of *Proteobacteria*. Compared with the dominant bacteria in the fermentation stage, the abundance of *Deinococcus-Thermus* in the application stage was relatively low, which may be because the materials were spread out evenly for drying, which reduced the amount of accumulative heat in the materials. Therefore, conditions were not suitable for the survival of *Deinococcus-Thermus*. In addition, the relative abundance of *Planctomycetes* in the application stage was greater than 1% in some samples; all samples in the fermentation stage had less than 1% *Planctomycetes*. Studies have shown that the genus *Planctomycetes* has unique characteristics of anaerobic ammoxidation[32]. Anaerobic ammoxidation is carried out under anaerobic conditions, which explains the relative abundance variation law of the phylum *Planctomycetes* in the fermentation and application stages. Conditions in the fermentation stage were mainly aerobic. As conditions in the application stage were mainly anaerobic, *Planctomycetes* in this stage had more advantages for growth than those in the fermentation stage.

3.2.3 Analysis of bacterial community composition at genus level

Figure 4a shows that the top ten dominant bacteria genera (for specific known species of bacteria) in Group A and Group B were *Acinetobacter*, *Thermus*, *Rhodothermus*, *Clostridium_III*, *Ureibacillus*, *Caldicoprobacter*, *Psychrobacter*, *Povalibacter*, *Thermobacillus*, and *Thermaerobacter*. The bacterial species in the initial material were numerous and relatively uniform. After the beginning of the fermentation, the homogeneity of the bacterial community decreased, and dominant bacteria gradually emerged, which is consistent with the aforementioned analysis of the alpha diversity indexes. *Acinetobacter* and *Thermus* accounted for the largest proportions in Groups A and B, both of which exhibited an increasing and later declining trend. This is in line with the trend in phylum-level *Proteobacteria* and *Deinococcus-Thermus* in the fermentation stage (*Acinetobacter* belongs to *Proteobacteria*, and *Thermus* belongs to *Deinococcus-Thermus*). *Acinetobacter* and *Thermus* are both adapted to growth under aerobic conditions[19,33], and the trends in their relative abundances combined with the trend in oxygen concentration also indicated that the piles were in good aerobic conditions. Among the other dominant bacteria genera, most species were *Firmicutes*. The relative abundances of *Rhodothermus* in Group A and Group B were much higher and peaked at the end of fermentation compared with those in the mesophilic and thermophilic phases of fermentation. This trend was consistent with that of *Bacteroidetes* (*Rhodothermus* belongs to



Note: The “other” in the figure represents bacterial community phyla with relative abundances of less than 1%.

Figure 3 Analysis of bacterial community composition at phylum level

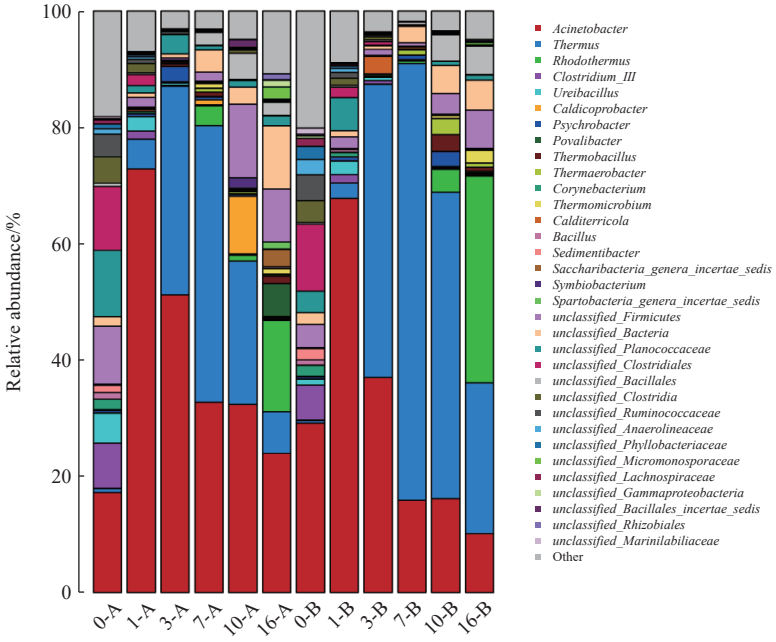
Bacteroidetes).

Figure 4b shows that the top ten dominant bacteria genera (for specific known types of bacteria) in Group PA and Group PB in the application stage mainly included *Acinetobacter*, *Pseudomonas*, *Flavobacterium*, *Luteimonas*, *Moheibacter*, *Proteiniclasticum*, *Sphingobacterium*, *Psychrobacter*, *Arthrobacter*, and *Cellvibrio*. The number of bacteria genera with relative abundance >1% in the application stage was much higher than that in the fermentation stage. This was probably because there was a drying stage between the fermentation stage and the application stage during which the materials were evenly spread and had more contact with microorganisms in the atmosphere. This exposure increased the number of bacterial genera and increased the richness and diversity of bacteria in the materials. The common potential pathogens of mastitis detected in samples at the application stage were basically the same as those detected in samples at the end of the fermentation, and the relative abundances of most of the pathogens were also small.

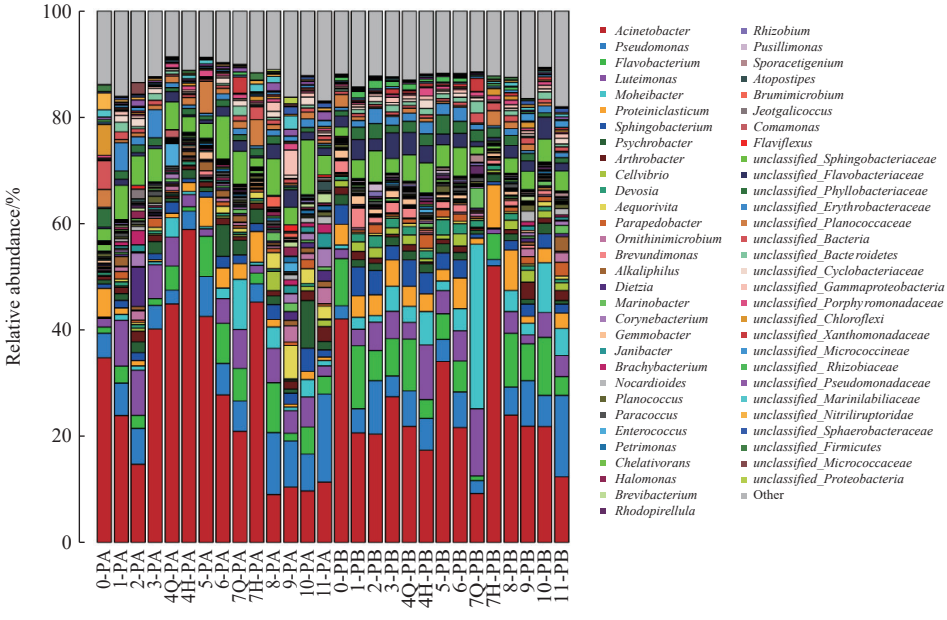
3.2.4 Principal component analysis

As Figure 5a displayed, Groups A and B were similar during the whole fermentation stage. The bacterial community succession for Group A and Group B basically varies with the fermentation time, which could be roughly divided into the mesophilic, thermophilic, and cooling phases. The fermentation periods are divided by green lines in Figure 5a.

Figure 5b presents that samples belonging to the same group were relatively concentrated, among which Group PA samples were primarily concentrated in the third and fourth quadrants, and Group PB samples were primarily concentrated in the first and second quadrants, illustrating that there were some differences in the bacterial community compositions of the samples from Group PA and Group PB. The samples from Group PA and Group PB had no



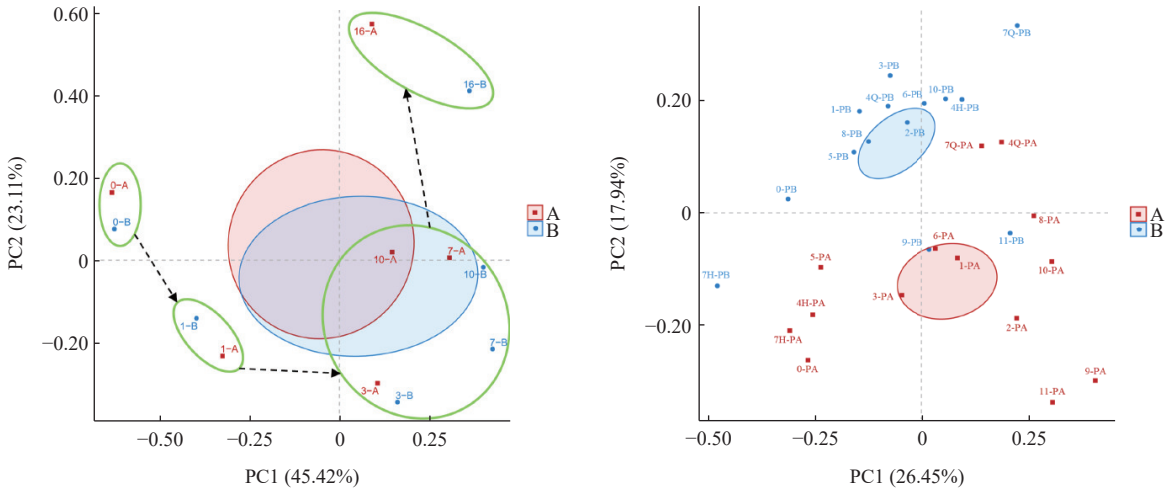
a. Fermentation stage



b. Application stage

Note: The "other" in the figure represents bacterial community genera with relative abundances of less than 1%.

Figure 4 Analysis of bacterial community composition at genus level



a. Fermentation stage b. Application stage

Figure 5 Principal component analysis at OTU level

obvious variation regularity over time, and there were large distances between the samples on adjacent days, indicating that there were big differences between timepoints. The changes in samples over time may have been related to the activities of the cows. The cows occupying different beds displayed different activities every day, so changes in the bacteria genera varied.

3.2.5 Welch's *t*-test difference analysis

Comparison of bacterial abundances between Group A and Group B at the fermentation stage was analyzed with Welch's *t*-test. The findings indicated that no bacteria differed remarkably at the level of phylum, but *Lysobacter* differed significantly at the level of genus (Figure 6a). Studies have pointed out that *Lysobacter* has strong bacteriostatic and antifungal effects and strong antagonistic activity against a variety of plant pathogenic fungi, bacteria and nematodes. Therefore, it is a new type of biocontrol bacteria with potential uses in biocontrol[34]. The relative abundance of *Lysobacter* in Group A was slightly higher than that in Group B, indicating that materials produced by Group A could be used as fertilizers in addition to bedding materials. Group A materials can more effectively inhibit the development of plant pathogens and prevent diseases compared with Group B[35].

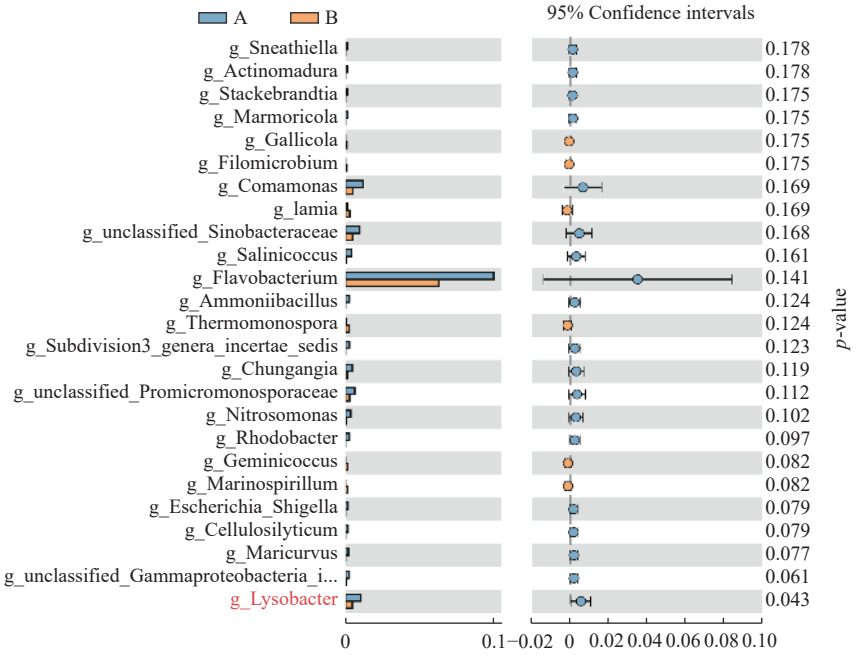
In the application stage, at the phylum level, *Cyanobacteria-Chloroplast* and *Verrucomicrobia* abundances were significantly different between Group PA and Group PB (Figure. 6b). The relative abundance of *Cyanobacteria-Chloroplast* in Group PA was slightly higher than that in Group PB, and the relative abundance of *Verrucomicrobia* in Group PA was slightly lower than that in Group PB, but in fact the relative abundance of these two bacteria were low, especially the *Cyanobacteria-Chloroplast*. *Verrucomicrobia* has been used to synthesize useful chemicals, such as antibiotics that can be used to fight off pathogens. *Verrucomicrobia* plays an important role in ecosystems in helping to improve soil fertility and water quality, as well as helping plants and animals absorb nutrients[36]. Therefore, bedding material with this genus can also be used as fertilizer to improve soil fertility, and benefit plant nutrition absorption. These results also provide ideas and a theoretical basis for the subsequent treatment of bedding material.

3.3 Prospects and challenges

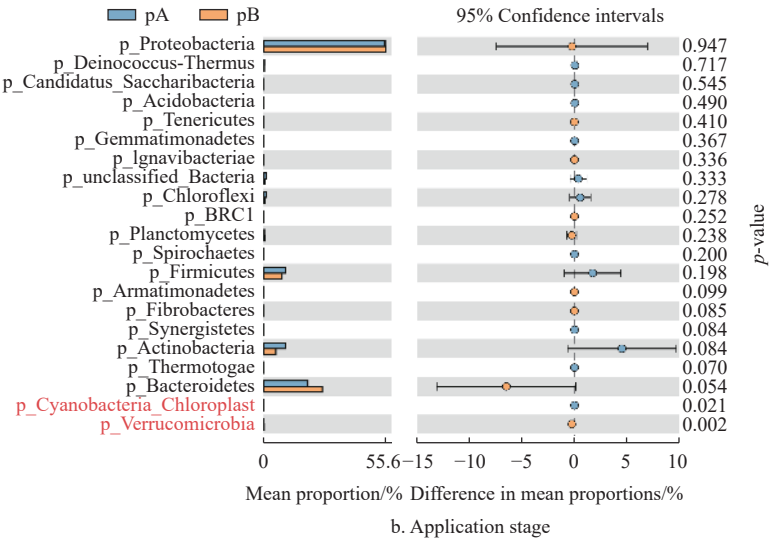
In our previous study, membrane-covered aerobic fermentation was proved to be safe, efficient, environment friendly and sustainable for waste management[8,11,19]. It has been reported that membrane-covered aerobic fermentation can effectively increase fermentation temperature and lengthen the thermophilic period[8,11,19], which contributes to killing the potential pathogenic bacteria related to cow diseases during the bedding application period. Moreover, the fermentation cycle was obviously shortened due to the high fermentation temperature and oxygen utilization efficiency, which considerably improved the waste treatment efficiency and lowered the treatment cost. Otherwise, the reduction of greenhouse gas emissions and odor emissions were also reported in the membrane-covered aerobic fermentation[8,11,19,37]. These advantages were strengthened in dairy manure fermentation because solid-liquid separation pretreatment optimized the MC, porosity and air permeability of the solid part of dairy waste and making the dairy manure as raw material more suitable for aerobic fermentation. The membrane-covered aerobic fermentation will be of good prospect in the bedding conversion process.

However, seven pathogens, i.e., *Staphylococcus*, *Enterococcus*, *Serratia*, *Pseudomonas*, *Corynebacterium*, *Mycobacterium*, and *Bacillus*, which were among the 20 common potential pathogens of mastitis described in previous studies[6], were found at the end of fermentation in this study despite the relative abundances of these seven pathogenic bacteria were low and tended towards 0 (0.0025%-0.2727% in Figure 4a). The main reason might be the inhomogeneous distribution of temperature even though the temperature of the core part was as high as 80°C or more[38]. Usually, the temperatures of the two ends and the surface of the pile were much lower than the temperature of the core part, which might create a certain risk that potential pathogens of mastitis would survive after fermentation. Furthermore, the relative abundance of *Pseudomonas* was presented to increase in the application stage and *Streptococcus*, *Actinomyces* and *Nocardia* were occasionally found in one or two samples during the application stage with their relative abundances approaching 0 (Figure 4b).

The existence of common potential pathogens of mastitis in fermentation stage, the rise of relative abundance of *Pseudomonas* and the occasional appearance of *Streptococcus*, *Actinomyces*, and *Nocardia* in the application stage was an important reminder to the dairy farm managers. Significant attention should be paid to the new methods or technologies to thoroughly kill the potential pathogenic bacteria that are related to cow diseases. For example, increasing fermentation temperature and lengthening the thermophilic period, or regularly mixing the pile were suggested. Moreover, the disinfection and timely replacement of bedding material were also considerable in preventing the occurrence of dairy mastitis.



a. Fermentation stage



b. Application stage

Figure 6 Welch's *t*-test difference analysis

4 Conclusions

In this study, membrane-covered aerobic fermentation could effectively kill some pathogenic bacteria and guarantee both the maturity and stability of final product. The use of fermentation product as bedding material provided cows with a stable and comfortable bedding environment. The dominant bacteria in fermentation stage were *Acinetobacter*, *Thermus*, and *Rhodothermus* at genus level. Seven common potential pathogens of mastitis (*Staphylococcus*, *Enterococcus*, *Serratia*, *Pseudomonas*, *Corynebacterium*, *Mycobacterium*, and *Bacillus*) were found at the end of fermentation stage but the relative abundance was low. The dominant bacteria in application stage mainly included *Acinetobacter*, *Pseudomonas*, and *Flavobacterium* at genus level. The relative abundance of *Pseudomonas* increased in application stage, which was a reminder to the dairy farm to pay attention to the disinfection and timely replacement of bedding material to prevent the occurrence of dairy mastitis. The results of this study contributed deep understanding of the microorganism-driven bedding conversion process and provided practical guidance and cautions for the bedding materials application. A deficiency of this study was that some pathogenic bacteria were not completely removed from the materials. Therefore, further research should be conducted on new methods or technologies that can more effectively remove pathogenic bacteria.

Acknowledgments

This research was supported by the China Agriculture Research System of MOF and MARA (CARS 36), Fundamental Research Funds for the Central Universities (Grant No. 2021TC039) and the National Natural Science Foundation of China (Grant No. 31771684).

[References]

[1] Ahmad T, Aadil R M., Ahmed H., Rahman U U, Soares B C V, Souza S L Q, et al. Treatment and utilization of dairy industrial waste: A review. Trends Food Sci Technol, 2019; 88: 361–372.

[2] Khoshnevisan B, Duan N, Tsapekos P, Kumar Awasthi M, Liu Z D,

Mohammadi A, et al. A critical review on livestock manure biorefinery technologies: Sustainability, challenges, and future perspectives. Renew Sust Energ Rev, 2021; 135: 110033.

[3] Robles I, Kelton D F, Barkema H W, Keefe G P, Roy J P, von Keyserlingk M A G, et al. Bacterial concentrations in bedding and their association with dairy cow hygiene and milk quality. Animal, 2020; 14(5): 1052–1066.

[4] Husfeldt A W, Endres M I, Salfer J A, Janni K A. Management and characteristics of recycled manure solids used for bedding in Midwest freestall dairy herds. J Dairy Sci, 2012; 95(4): 2195–2203.

[5] Varma V S, Parajuli R, Scott E, Canter T, Lim T T, Popp J, et al. Dairy and swine manure management-Challenges and perspectives for sustainable treatment technology. Sci Total Environ, 2021; 778: 146319.

[6] Yin H J, Fang C, He X Q, Yu H, Liang Y Y, Han L J, et al. Safety production and application of dairy bedding by membrane-covered aerobic fermentation: Insight into the evolution of mastitis pathogens and harmful gas emissions. J Environ Chem Eng, 2023; 11(3): 110002.

[7] Peng Y X, Li J W, Wang J F, Gao J W. Analysis of dried solid manure used as bedding for dairy farm. China Dairy Cattle, 2015; 2: 47–51. (in Chinese)

[8] Fang C, Yin H J, Han L J, Ma S S, He X Q, Huang G G. Effects of semi-permeable membrane covering coupled with intermittent aeration on gas emissions during aerobic composting from the solid fraction of dairy manure at industrial scale. Waste Manage, 2021; 131: 1–9.

[9] Robledo-Mahon, Gomez-Silvan C, Andersen G L, Calvo C, Aranda E. Assessment of bacterial and fungal communities in a full-scale thermophilic sewage sludge composting pile under a semipermeable cover. Bioresour Technol, 2020; 298: 122550.

[10] Ma S S, Xiong J P, Wu X Y, Liu H T, Han L J, Huang G Q. Effects of the functional membrane covering on the gas emissions and bacterial community during aerobic composting. Bioresour Technol, 2021; 340: 125660.

[11] Wang X F, Wan J X, Wei Z, Xu Y C, Shen Q R. Succession of microbial communities during livestock manure composting. Biotechnology Bulletin, 2022; 38(5): 13–21. (in Chinese)

[12] Zhao X, Li J, Che Z X, Xue L G. Succession of the bacterial communities and functional characteristics in sheep manure composting. Biology, 2022; 11(8): 1181.

[13] He X Q. Effect and mechanism of biochar addition on greenhouse gases emissions during pig manure aerobic composting. PhD dissertation. Beijing: China Agricultural University, 2019. (in Chinese)

[14] Jiang Z W, Lu Y Y, Xu J Q, Li M Q, Shan G C, Li Q L. Exploring the characteristics of dissolved organic matter and succession of bacterial community during composting. Bioresour Technol, 2019; 292: 121942.

[15] Xu S, Lu W J, Liu Y T, Ming Z Y, Liu Y J, Meng R H, et al. Structure and diversity of bacterial communities in two large sanitary landfills in China as revealed by high-throughput sequencing (MiSeq). Waste Manage, 2017; 63: 41–48.

[16] US Composting Council. Test Methods for the Examination of Composting and Compost. Bethesda: US Composting Council. 2002.

[17] Cáceres R, Coromina N, Malińska K, Marfà O. Evolution of process control parameters during extended co-composting of green waste and solid fraction of cattle slurry to obtain growing media. Bioresour Technol, 2015; 179: 398–406.

[18] Wang L L, Oda Y, Grewal S, Morrison M. Persistence of resistance to erythromycin and tetracycline in swine manure during simulated composting and lagoon treatments. Microb Ecol, 2012; 63(1): 32–40.

[19] Fang C, Su Y, Liang Y Y, Han L J, He X Q, Huang G Q. Exploring the microbial mechanism of reducing methanogenesis during dairy manure membrane-covered aerobic composting at industrial scale. Bioresour Technol, 2022; 354: 127214.

[20] Beales N. Adaptation of microorganisms to cold temperatures, weak acid preservatives, low pH, and osmotic stress: A review. Compr Rev Food Sci Food Saf, 2004; 3(1): 1–20.

[21] Manyi-Loh C E, Mamphweli S N, Meyer E L, Makaka G, Simon M, Okoh A I. An overview of the control of bacterial pathogens in cattle manure. Int J Env Res Public Health, 2016; 13(9): 843.

[22] de Gannes V, Eudoxie G, Hickey W J. Prokaryotic successions and diversity in composts as revealed by 454-pyrosequencing. Bioresour Technol, 2013; 133: 573–580.

[23] Su J-Q, Wei B, Ou-Yang W-Y, Huang F-Y, Zhao Y, Xu H-J, et al. Antibiotic resistome and its association with bacterial communities during sewage sludge composting. Environ Sci Technol, 2015; 49(12): 7356–7363.

[24] Olakanye A O, Ralebitso-Senior T K. Assessing subsurface decomposition and potential impacts on forensic investigations. In: Ralebitso-Senior T K (ed.), Forensic ecogenomics (First edition). Academic Press. 2018; pp.145–176.

[25] Awasthi M K, Zhang Z Q, Wang Q, Shen F, Li R H, Li D-S, et al. New insight with the effects of biochar amendment on bacterial diversity as indicators of biomarkers support the thermophilic phase during sewage sludge composting. Bioresour Technol, 2017; 238: 589–601.

[26] Zhong X-Z, Ma S-C, Wang S-P, Wang T T, Sun Z-Y, Tang Y-Q, et al. A comparative study of composting the solid fraction of dairy manure with or without bulking material: Performance and microbial community dynamics. Bioresour Technol, 2018; 247: 443–452.

[27] Pankratov T A, Ivanova A O, Dedysh S N, Liesack W. Bacterial populations and environmental factors controlling cellulose degradation in an acidic *Sphagnum* peat. Environ Microbiol, 2011; 13(7): 1800–1814.

[28] Liu L, Wang S Q, Guo X P, Zhao T N, Zhang B L. Succession and diversity of microorganisms and their association with physicochemical properties during green waste thermophilic composting. Waste Manag, 2018; 73: 101–112.

[29] Albuquerque L, Polónia A R M, Barroso C, Froufe H J C, Lage O, Lobo-da-Cunha A, et al. *Raineya orbicola* gen. nov., sp. nov. a slightly thermophilic bacterium of the phylum Bacteroidetes and the description of Raineyaceae fam. nov. Int J Syst Evol Microbiol, 2018; 68(4): 982–989.

[30] Zhu Y J, Wang Y Y, Jiang X X, Zhou S, Wu M, Pan M L et al. Microbial community compositional analysis for membrane bioreactor treating antibiotics containing wastewater. Chem Eng J, 2017; 325: 300–309.

[31] Fang C, Zhou L, Liu Y, Xiong J P, Su Y, Lan Z F, et al. Effect of micro-aerobic conditions based on semipermeable membrane-covered on greenhouse gas emissions and bacterial community during dairy manure storage at industrial scale. Environ Pollut, 2022; 299: 118879.

[32] Lage O M, Bondoso J, Viana F. Isolation and characterization of Planctomycetes from the sediments of a fish farm wastewater treatment tank. Arch Microbiol, 2012; 194(10): 879–885.

[33] Buchanan R E, Gibbons N E. Bergey's manual of determinative bacteriology (8th ed). Baltimore, Maryland: Williams and Wilkins, 1975.

[34] Wang T. Studies on the secondary metabolites and activities of two strains from the genus *Lysobacter*. Master dissertation. Yunnan: Yunnan University, 2019; 95p. (in Chinese)

[35] Sojka M, Saeid A. Chapter 10: Bio-based products for agriculture. in: Chojnacka K, Saeid A (Eds.), Smart agrochemicals for sustainable agriculture. Academic press. 2022; pp.279–310.

[36] Fuerst J A. Phylum Verrucomicrobia, in: Schmidt T M (Ed.), Encyclopedia of microbiology (Fourth edition). Academic press. 2019; pp.551–563.

[37] Li Y C, Liu Y D, Yong X Y, Wu X Y, Jia H H, Wong J W C, et al. Odor emission and microbial community succession during biogas residue composting covered with a molecular membrane. Bioresour Technol, 2020; 297: 122518.

[38] Zeng J F, Shen X L, Sun X X, Liu N Han L J, Huang G Q, et al. Spatial and temporal distribution of pore gas concentrations during mainstream large-scale trough composting in China. Waste Manage, 2018; 75: 297–304.