Postprint: Spatial Distribution and Diversity of Bacillus in Microbial Fermentation Beds for Swine Production

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Abstract

To understand the diversity and spatial distribution patterns of Bacillus in the bedding material of microbial fermentation beds for large-scale pig farming, laying a foundation for fermentation bed management, exploration of novel Bacillus resources, and microbial agent development. The fermentation bed was divided into 32 quadrats (4 rows × 8 columns), and samples from each quadrat were obtained using the five-point sampling method. Culture-based methods were employed to isolate Bacillus strains from the 32 samples, and the isolated Bacillus species were preliminarily identified using 16S rRNA gene sequencing. Aggregation indices and regression analysis were utilized to analyze the spatial distribution pattern of Bacillus in the quadrats. Spatial distribution diversity of Bacillus in the microbial fermentation bed was revealed through analysis of Shannon-Wiener diversity index, Simpson dominance index, Hill index, and richness index. A total of 452 Bacillus strains were obtained from the 32 samples, and 16S rRNA gene identification results indicated that they belonged to 2 families, 8 genera, and 48 species within the class Bacilli. Among them, the genus with the most species was Bacillus (30 species); Lysinibacillus (6 species); Paenibacillus (5 species); Brevibacillus (3 species); and Ornithinibacillus, Oceanibacillus, Paucisalibacillus, and Gracilibacillus each had 1 species. Bacillus species exhibited substantial spatial distribution differences in the fermentation bed. Based on their spatial occurrence frequency, they could be classified as widespread species, such as Bacillus licheniformis; oligotopic species, such as B. rhizosphaerae; and rare species, such as B. flexus. Based on their abundance, they could be categorized into high-abundance dominant populations, such as B. licheniformis; medium-abundance common populations, such as Lysinibacillus halotolerans; oligotopic populations with low abundance, such as B. rhizosphaerae; and rare populations with minimal abundance, such as B. humi. Analysis of spatial distribution patterns using aggregation indices and regression analysis indicated that

Bacillus exhibited an aggregated distribution pattern in the microbial fermentation bed. The total content of Bacillus species in the fermentation bed bedding material reached as high as 4.41×10^8 cells/g, with species content ranging from 0.01-94.1×10^6 cells/g (mean 8.96×10^6 cells/g). The richness index (D), dominance index ( ), Shannon-Wiener index (H'), and evenness index (J') were 0.4928, 0.2634, 1.3589, and 0.9803, respectively, with Bacillus licheniformis being the single Bacillus species with the highest Shannon index. Based on cluster analysis of Bacillus species diversity indices, when the Euclidean distance =17, the samples could be divided into high-richness-high-content and low-richness-low-content types. The microbial fermentation bed exhibited rich Bacillus species diversity and high abundance, representing a natural "fermenter" for microbial agents, with potential for direct application as microbial inoculants in fields such as soil improvement, crop disease control, and pollution remediation.

Full Text

Spatial Distribution Pattern and Community Diversity of Bacillus-like Species in a Fermentation Bed System for Pig Rearing

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Abstract

To provide a foundation for microbial fermentation bed management, new resource mining of Bacillus-like species, and microbial agent development, we investigated the spatial distribution pattern and community diversity of Bacillus-like species in the litters of a pig-on-litter system. The microbial fermentation bed was divided into 32 square areas of 4 rows × 8 columns. A sample from each area was obtained using a five-point sampling mode. Bacillus-like bacteria were isolated from the 32 samples using a culture method and preliminarily identified based on 16S rRNA gene sequences. The spatial distribution pattern of the Bacillus-like bacteria was analyzed using a concentration index and regression analysis. Moreover, the Shannon-Wiener diversity index, Simpson dominance index, Hill index, and richness were separately calculated to reveal the community structure and diversity of the Bacillus-like bacteria in the microbial fermentation bed. A total of 452 Bacillus-like isolates were obtained from the 32 litter samples, and identified as 48 species belonging to eight Bacillus genera and two families (Bacillaceae and Paenibacillaceae) within the Bacilli by 16S rRNA gene sequences. The species numbers for the genera were as follows: 30 for *Bacillus* and one each for *Ornithinibacillus*, *Oceanobacillus*, *Lysinibacil-*

lus, *Paenibacillus*, *Brevibacillus*, six for *Paucisalibacillus*, five for *Gracilibacillus*, and three for *Bacillus.* There was a large difference in the distribution of Bacillus-like species in the fermentation bed. On the basis of their occurrence frequency, the Bacillus-like species could be discriminated as widespread species, including *B. rhizosphaerae* and *B. flexus*; sparsely distributed species, including *Bacillus licheniformis*; and rare species, including *Bacillus humi.* According to the abundance of each species, the Bacillus-like species could be classified into high-content dominant populations, including *Lysinibacillus halotolerans*; medium-content common populations, including *B. rhizosphaerae*; low-content oligo populations, including *B. humi*; and occasional populations. The concentration class and regression analysis results indicated that the spatial distribution pattern of the Bacillus-like species in the microbial fermentation bed was an aggregated distribution. In the litters, the content range, total content, richness, Simpson index, Shannon-Wiener index, and Pielou' s evenness index of the Bacillus-like species were 0.01–94.1 × 10 cfu/g, 4.41 × 10 cfu/g, 0.4928, 0.2634, 1.3589, and 0.9803, respectively. Moreover, *B. licheniformis* appeared to have the highest Shannon-Wiener index value. On the basis of the diversity index cluster analysis, all the Bacillus-like species could be divided into a high richness-high content group and a low richness-low content group. The Bacillus-like bacteria can actively inhabit microbial fermentation beds and have rich species and abundant contents. Thus, the microbial fermentation bed can be considered as a natural bacterial "fermentation tank," which is expected to be directly used as a microbial inoculum and applied in the fields for purposes including soil improvement, crop disease prevention and control, and pollution management.

Keywords: Bacillus-like species; piggy bedding; 16S rRNA gene; spatial distribution; diversity

Introduction

Pig production causes severe environmental pollution. Taking Fujian Province as an example, with 12.978 million pigs in stock in 2011, and assuming each pig excretes approximately 25 kg of feces, urine, and pen-washing wastewater daily, the annual discharge volume reaches 118.42425 million tons, making it one of the major sources of agricultural non-point source pollution [1]. Microbial fermentation beds applied to pig farming use sawdust and other materials inoculated with microbial agents to achieve in-situ fermentation of pig manure, thereby realizing zero-emission pig production and solving pollution problems [2-4].

The concept of microbial fermentation bed pig farming can be traced back to China 400 years ago, with Zhang Lüxiang' s compilation documenting pig pen bedding with straw [5]. Modern microbial fermentation bed research first appeared in Japan, where the first wood-chip-based fermentation bed was established in 1970 [6]. Tiquia et al. studied the effects of turning fermentation bed litter [7], while Kaufmann et al. investigated nutrient balance in fattening pig fermentation beds [8]. Connor [9] introduced a straw-based fermentation bed system in 1995. Margarita et al. [10] discussed the advantages, disadvantages, and development prospects of pig fermentation beds in Croatia. Knecht et al. [11] found that parasite prevalence was lower in fermentation bed pig houses than in slatted-floor houses. Liu Bo et al. [12] designed a 2,100 m² large-pen fermentation bed intelligent pig farming system.

Research on fermentation bed microbial characteristics has been limited. Liu Bo et al. studied microbial community fatty acid biomarker diversity in fermentation beds [13]. Xue Fang et al. analyzed microbial sub-community differentiation in fermentation bed litter [14]. Zhang Xuefeng et al. revealed indigenous stable microbial flora at different depths of fermentation bed litter [15]. Wang Di isolated and identified Bacillus from fermentation beds [16]. Lin Lili et al. studied the distribution of microorganisms in fermentation bed pig houses and on pig body surfaces [17]. Zhang Qingning screened Bacillus capable of decomposing pig manure from microbial fermentation beds [18]. Liu Rang et al. screened Bacillus as fermentation strains [19].

Microbial fermentation beds have certain biocontrol effects on pig diseases. Zheng Xuefang et al. found that the stable microbial community structure formed in fermentation bed substrate layers could inhibit the growth of *E. coli*, particularly pathogenic strains [20]. Bi Xiaoyan studied microbial dynamics in pig fermentation bed litter and its effects on pig immunity [21]. Previous studies have shown that Bacillus, as the dominant flora in fermentation beds, has strong growth advantages, can produce various enzymes related to pig manure degradation [18], inhibit pathogens [22], and eliminate odors [23]. With extended fermentation bed feeding time, the antibiotic resistance of *E. coli* isolated from corresponding pigs gradually decreases [18].

Studying the characteristics of fermentation bed Bacillus and understanding their spatial distribution diversity is important for investigating fermentation bed microbial community functions and pig disease biocontrol mechanisms. However, no studies have been reported on the species structure of Bacillus-like microorganisms in fermentation beds. This study used a 2,100 m² microbial fermentation bed large-pen pig farming system at the Fuqing Modern Facility Agriculture Sample Engineering Demonstration Base of Fujian Academy of Agricultural Sciences as the research object. The fermentation bed litter had been used for 2 years, with a building area of 2,100 m² (35 m × 60 m), excluding two isolation pens, and a breeding area of 1,910 m². The fermentation bed depth was 80 cm, with a breeding density of 1,600 m². The litter consisted of coconut husk and rice husk. This study analyzed the species distribution and spatial distribution diversity of Bacillus in the fermentation bed, evaluated the spatial distribution characteristics of Bacillus in pig fermentation beds using diversity indices, and provided a research foundation for fermentation bed management, pig manure resource utilization, and pig disease biocontrol through spatial pattern sampling, spatial distribution pattern analysis, and diversity assessment.

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1. Sample Collection

The large-pen fermentation bed was divided into 4 rows in the width direction and 8 columns in the length direction, creating 32 spatial samples. Each pen used a five-point sampling method to collect samples from the 0–20 cm layer, which were mixed to obtain the litter sample. Samples were placed in sterile polyvinyl chloride plastic bags, brought back to the laboratory, and immediately used for Bacillus strain isolation and preservation.

[Figure 1: see original paper] The piggy bedding

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2. Instruments and Reagents

Instruments included a constant temperature incubator, PCR instrument, and gel imaging analyzer. Reagents included 2×PCR Master Mix. Primers were synthesized by Shanghai Baishang Biotechnology Co., Ltd.

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3. Isolation and Identification of Bacillus from Microbial Fermentation Bed

Bacillus were isolated from fermentation bed samples using gradient dilution plating. Based on colony morphology characteristics, Bacillus species were counted and purified, then preserved using glycerol freezing at -80°C. Genomic DNA was extracted using the saturated phenol method described by Liu et al. [24]. The 16S rRNA gene was amplified using universal bacterial primers: 27F (5 -AGAGTTTGATCCTGGCTCAG-3) and 1492R (5 -GGTTACCTTGTTACGACTT-3). Strains with detectable bands were sent to Shanghai Baishang Biotechnology Co., Ltd. for sequencing. Sequences were analyzed on the Korean website EzTaxon-e (http://ezbiocloud.net) [25] for preliminary taxonomic identification. When similarity was >97.0%, strains were defined as the same taxonomic unit [26].

[Figure 2: see original paper] Samples information collected from fermentation bed in the piggrey

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4. Spatial Distribution of Bacillus Species and Quantity in Fermentation Bed Samples

The microbial fermentation bed was divided into 32 spatial samples. Bacillus species and quantities in each spatial sample were isolated, identified, and counted. The changes in Bacillus species and quantities among fermentation bed spatial samples were compared, and histograms were drawn to analyze the occurrence frequency and quantity of Bacillus species in the fermentation bed spatial samples.

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5. Spatial Distribution Pattern of Bacillus in Fermentation Bed

Based on the 4×8 spatial sampling scheme, a data matrix was constructed. Aggregation indices and regression analysis were used to analyze the spatial distribution pattern of Bacillus samples. The average and variance of Bacillus quantities in each row of samples were calculated.

Distribution index of Bacillus-like species in the piggy bedding

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2. Results and Analysis

2.1 Isolation and Identification of Bacillus Species from Fermentation Bed A total of 452 Bacillus strains were isolated from 32 microbial fermentation bed spatial samples. Based on 16S rRNA gene sequence alignment analysis, all Bacillus strains showed >97.0% similarity and belonged to eight genera within Bacilli: *Bacillus* (30 species), *Brevibacillus*, *Gracilibacillus*, *Lysinibacillus*, *Ornithinibacillus*, *Oceanobacillus*, *Paenibacillus*, and *Paucisalibacillus*, and two families (Bacillaceae and Paenibacillaceae).

Isolation and identified of Bacillus-like species from the piggy bedding

2.2 Spatial Distribution of Bacillus Species and Quantity in Fermentation Bed The microbial fermentation bed was divided into 32 spatial samples. Bacillus species and quantities varied significantly among spatial samples. Some Bacillus species were distributed across multiple spatial samples, such as *Bacillus aryabhattai* (distributed in MF-1-3, MF-6-3, MF-5-3), while some species were only found in specific spatial samples, such as *Brevibacillus limnophilus* (only in MF-4-1). The maximum quantity of a single Bacillus strain was *Bacillus circulans* at 50.0×10 cfu/g (MF-1-4), while the minimum was *Bacillus isronensis* at 0.01×10 cfu/g (MF-4-3).

The content of Bacillus-like species from the piggy bedding

2.3 Spatial Frequency Distribution of Bacillus in Fermentation Bed Analysis of occurrence frequency showed significant differences among the 48

Bacillus species across 32 spatial samples. Based on distribution breadth, they could be classified into: (1) Widespread species (distributed in >20 samples), including *B. licheniformis* (29 samples), *B. kochii* (23 samples), *B. methylotrophicus* (21 samples), *B. altitudinis* (18 samples), *B. aryabhattai* (16 samples), *B. circulans* (14 samples), *B. clausii* (10 samples), and *Lysinibacillus halotolerans*; (2) Sparsely distributed species (distributed in 8–13 samples), including *B. rhizosphaerae*, *B. halosaccharovorans*, *B. subtilis*, *B. amyloliquefaciens*, *B. siamensis*, and *B. cereus*; (3) Rare species (distributed in <8 samples). Notably, *Paenibacillus* and *Brevibacillus* belonged to rare species, while common *B. cereus* and *B. subtilis* were sparsely distributed.

[Figure 3: see original paper] Occurrence frequency of Bacillus-like in the piggy bedding

2.4 Quantity Distribution of Bacillus in Fermentation Bed Quantity distribution results showed that among the 48 Bacillus species, the maximum quantity was 94.1×10 cfu/g for *B. circulans*, and the minimum was 0.1×10 cfu/g for *Paucisalibacillus globulus*. Based on quantity distribution, they were classified into: (1) High-content group (17–94×10 cfu/g), including *B. circulans*, *B. aryabhattai*, *B. methylotrophicus*, *Paenibacillus ginsengiterrae*, *B. licheniformis*, and *B. altitudinis* (16.7% of species); (2) Medium-content group (8–13×10 cfu/g), including *B. tequilensis*, *B. clausii*, *B. nealsonii*, *Paenibacillus illinoisensis*, *B. subtilis*, and *P. globulus* (14.6% of species); (3) Low-content group (3–5×10 cfu/g), including *B. rhizosphaerae*, *B. amyloliquefaciens*, *Gracilibacillus marinus*, *B. siamensis*, *B. cereus*, and *Lysinibacillus composti* (18.76% of species); (4) Rare-content group (2–0.1×10 cfu/g), including *B. isronensis*, *B. halosaccharovorans*, *B. oleronius*, *Ornithinibacillus scapharcae*, and others.

[Figure 4: see original paper] Distribution diversity of Bacillus-like species in the piggy bedding

2.5 Spatial Distribution Pattern of Bacillus in Fermentation Bed Statistical analysis of Bacillus quantities in 32 spatial sample units showed significant differences. The highest quantity was 97.6×10 cfu/g in sample M-7-4, while the lowest was 0.800×10 cfu/g in sample M-4-1. Spatial distribution pattern indices are shown in Table 5. Aggregation measurements indicated: I index = 1.4212 > 0 (aggregated distribution); CA index = 2.4212 > 1 (aggregated distribution); diffusion coefficient C = 22.6183 > 1 (aggregated distribution); negative binomial distribution K index = 23.6183 > 1 (aggregated distribution). The m-*m regression equation was m* = -47.1208 + 5.9076m (r = 0.7795), with = -47.1208 < 0, indicating aggregated distribution. Taylor' s power law analysis gave lg(m*) = -1.2876 + 3.1966lg(m) (r = 0.8233), with b = 3.1966 > 1, confirming aggregated distribution.

The distribution index of Bacillus-like species in the piggy bedding

2.6 Diversity Indices of Bacillus in Fermentation Bed Overall diversity indices are shown in Table 6. Bacillus content ranged from 0.01–94.1×10 cfu/g, with total content of 48 species reaching 4.41×10 cfu/g. Richness index was 0.4928, Simpson index 0.2634, Shannon-Wiener index 1.3589, and evenness index 0.9803.

Diversity index of Bacillus-like species in the piggy bedding

2.7 Species Distribution Diversity of Bacillus in Fermentation Bed Species diversity indices based on Table 4 showed: Shannon-Wiener index range 0–2.88 (highest in *B. licheniformis*, lowest in *B. haikouensis*); Simpson index range 0.08–1 (highest in *B. marisflavi* and *B. oceanisediminis*, lowest in *B. licheniformis*); Hill index range 1–17.75 (highest in *B. marisflavi* and *B. oceanisediminis*, lowest in *B. haikouensis*); richness index range 0–7.71 (highest in *B. licheniformis*, lowest in *B. haikouensis* and *B. oceanisediminis*); evenness index range 0.08–1 (highest in *L. macroldes*, lowest in *B. marisflavi*).

2.8 Cluster Analysis of Bacillus Diversity Indices Cluster analysis using Euclidean distance and average linkage divided Bacillus species into: (1) High richness-high content type, including *B. methylotrophicus*, *L. halotolerans*, *B. clausii*, *B. aryabhattai*, *B. kochii*, *B. licheniformis*, *B. altitudinis*, *B. circulans*, *B. tequilensis*, *B. rhizosphaerae*, *B. subtilis*, *O. scapharcae*, and *B. halosaccharovorans*; (2) Lower richness-lower content type, subdivided into medium richness-medium content (*B. flexus*, *B. cereus*, *O. scapharcae*, *B. siamensis*, *B. rhizosphaerae*, *B. circulans*, *B. tequilensis*, *B. subtilis*, *B. halosaccharovorans*) and low richness-low content groups (*B. amyloliquefaciens* and others).

[Figure 5: see original paper] Distribution analysis of Bacillus-like species

3. Discussion

Microbial fermentation bed pig farming is a modern ecological pig production model developed in recent years. Microbial fermentation beds can efficiently decompose pig manure and convert it into artificial humus, which can be further developed into high-value products such as microbial fertilizers and biological seedling substrates, achieving both in-situ microbial treatment of pig pollution and resource utilization of pig manure. Microorganisms play a key role in this process. Our research group used high-throughput metagenomic analysis to study microbial diversity in fermentation bed litter (data unpublished) and found extremely rich microbial diversity, including diverse Bacillus-like species.

Bacillus are important microbial resources that can produce various antibacterial substances such as lantibiotics [27]. Bacillus have antibacterial and disease-prevention functions for pig herds and potential as probiotics. Manhar et al. found that *B. subtilis* has probiotic characteristics including strong acid resistance (pH 2.0), high antibacterial activity, anti-pathogen adhesion, and strong cellulose degradation ability [28]. As a feed additive, it can enhance animal cellulose digestion and intestinal health [28]. Lopetuso et al. reviewed the clinical application effects of probiotic *B. clausii* and its possible mechanisms of interaction with intestinal barriers and reconstruction of intestinal balance [29]. Numerous studies have shown that Bacillus can also efficiently degrade organic matter and pharmaceuticals in pig manure and reduce odor and ammonia emissions [30-34].

To further analyze the culturable Bacillus population and its spatial distribution patterns in fermentation bed litter, this study used dilution plating combined with 16S rRNA phylogenetic analysis. A total of 452 Bacillus strains were isolated from 32 fermentation bed samples, belonging to 8 genera within Bacillaceae and Paenibacillaceae. The fermentation bed litter showed extremely high Bacillus abundance and diversity. The total content of 48 Bacillus species reached 4.41×10 cfu/g, with individual species content ranging from 0.01-94.1×10 cfu/g. Dominant populations included *B. licheniformis*, *B. aryabhattai*, *B. circulans*, *B. methylotrophicus*, *B. altitudinis*, and *L. halotolerans*. The spatial distribution pattern was aggregated. These results indicate that Bacillus are important microbial populations in fermentation bed litter, and pig manure enrichment plays a role in their concentration.

Although few studies have reported on fermentation bed Bacillus populations and spatial distribution, similar results have been found in other pig manure-containing environments. Guo et al. used culturable methods, PCR-RFLP, and DGGE to analyze pig manure composting and found that culturable Bacillus numbers and temporal-spatial distribution characteristics changed significantly with temperature [35]. Yi et al. found that Bacillus diversity was lower during thermophilic composting, with highest Bacillus content in each layer during high-temperature phases and lowest in middle layers at all stages [35]. Guo et al. found that *Bacillus* was the dominant population in middle layers during cooling stages of pig manure composting, while *Clostridium* dominated bottom layers, with *B. subtilis* and *B. cereus* as dominant species [36]. Li et al. found that during thermophilic composting of cow manure, *Bacillus*, *Geobacillus*, and *Ureibacillus* were dominant genera, with *G. toebii*, *G. thermodenitrificans*, and *U. terrenus* as dominant species [37]. Guo et al. also found *Bacillus* was the most common population in all layers during pig manure thermophilic composting [38]. He et al. showed that Bacillus maintained high content at all stages of chicken manure composting [39].

Our results are not completely consistent with these studies. While the dominant genus in fermentation bed litter was also *Bacillus*, the dominant populations differed. We identified *B. licheniformis*, *B. aryabhattai*, *B. circulans*, *B. methylotrophicus*, *B. altitudinis*, and *L. halotolerans* as dominant, whereas *B. subtilis* and *B. cereus*, reported as dominant in pig manure composting, were not dominant in fermentation beds. The population structure differed significantly from that in pig manure. This study found *Lysinibacillus* as the second most abundant genus after *Bacillus*, while this genus was not dominant in pig or cow manure composting.

The uneven diversity indices of Bacillus species distribution in fermentation beds may be related to fermentation grade and species adaptability to litter components. Some Bacillus species became enriched as dominant species, such as *B. licheniformis*, which existed throughout the fermentation bed, while others like *B. haikouensis* existed only in certain locations, indicating that litter fermentation degree greatly influences microbial distribution. Xue Chao et al. found that continuous cropping creates selective pressure on soil microorganisms, causing population enrichment and reduction phenomena [40], leading to significant differences in microbial carbon source utilization capacity in different cropping sequences [41]. Fermentation bed litter consists mainly of high-fiber materials like coconut husk and rice husk. Ye Shaowen et al. analyzed enzyme activities at different depths of fermentation bed litter and found high cellulase and hemicellulase activities [42], revealing a survival space for fiber-degrading microorganisms. McCarthy et al. found that *B. licheniformis* was the dominant culturable microorganism during pig manure solid composting [45], confirming our finding that *B. licheniformis* is a dominant population in pig fermentation bed litter. Islas-Espinoza et al. found that *B. licheniformis* can degrade sulfonamide antibiotics in pig manure [32], indicating its important role in pig manure degradation.

B. altitudinis, another dominant species, is a multifunctional Bacillus that can produce alkaline protease [46,47]. The strong presence of dominant species *B. aryabhattai* and *B. altitudinis* may be closely related to fermentation bed health. *B. aryabhattai* has robust growth characteristics, wide distribution, and can be used as an inoculant for solid-state fermentation [47]. *B. circulans* is an important animal probiotic that can produce substances inhibiting pig pathogens and biosurfactants [48], and can serve as a composting fermentation degree indicator [49]. The dominant species *B. halosaccharovorans*, isolated from saline-alkali soil, has the characteristic of surviving in saline-alkaline environments [50]. Lan Jianglin et al. found that fermentation bed litter had high salt content and pH 8.0–9.0, with salt concentration and pH increasing over fermentation time [51], likely causing the presence of salt-alkali tolerant Bacillus species. The dominant species *B. kochii*, initially isolated from dairy products, has wide growth conditions (temperature 10–40°C, pH 6.0–10.5, NaCl 0%–10%) [52], allowing it to adapt to extreme condition changes during fermentation bed fermentation.

Microbial fermentation beds contain rich microbial resources, providing important sources for mining new functions and species. Further research is needed to explore the relationship between Bacillus population changes and fermentation bed ecological functions.

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