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**Original Research Article**

# Isolation and Identification of Dominant Spoilage Bacteria in Fresh Pork During Refrigerated Storage

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**Abstract:** To clarify the main spoilage bacteria of refrigerated pork and their spoilage characteristics, and to provide theoretical support for the optimization of precision preservation technology for pork, this study focused on fresh pork refrigerated at 4 °C until the end of its spoilage stage. A total of 8 dominant spoilage bacterial strains were isolated and purified from the spoiled pork samples, including 6 Gram-positive bacilli and 2 Gram-negative bacilli. Strain identification was performed through morphological observation, Gram staining, and 16S rDNA gene sequencing. The identification results showed that 6 of the core strains were *Bacillus cereus*, and the other 2 belonged to the genus *Proteus*. Among the isolates, *Bacillus cereus* exhibited the highest abundance and the strongest spoilage potential, making it the dominant spoilage bacterium in the refrigerated pork. Subsequently, the screened core dominant spoilage bacterial strains were inoculated back into fresh pork tenderloin. The results indicated that the rising rates of pH value, water loss rate, and TVB-N content of the pork samples inoculated with this bacterium during the refrigerated period were significantly higher than those of the control group, and the degree of color deterioration was more severe. This study confirmed that *Bacillus cereus* is the key spoilage bacterium during the refrigerated storage of fresh pork.

**Keyword:** Fresh pork; Dominant spoilage bacteria; Isolation and identification; *Bacillus cereus*.



## INTRODUCTION

Pork is rich in proteins, fats, and other nutrients, and has a favorable water activity, making it highly susceptible to microbial contamination(Pereira & Vicente, 2013). The spoilage of pork is a complex microbial metabolic process(Yi *et al.,* 2024); spoilage microorganisms competitively consume nutrients while secreting large amounts of extracellular hydrolytic enzymes such as proteases and lipases, producing various secondary metabolites(Tang *et al.,* 2021). The undesirable metabolites produced by spoilage bacteria are the key factors leading to meat deterioration, and the degree of spoilage is closely related to the types, quantities, and metabolic pathways of the core microbiota(Hao *et al.,* 2025; X. Y. Wang *et al.,* 2025). Studies have shown that under modified atmosphere packaging conditions, the metabolic activities of the microbiota centered on *Pseudomonas* and *Serratia* significantly alter the original amino acid degradation and lipid oxidation metabolic networks of pork, resulting in a decline in sensory and quality attributes(X. Wang *et al.,* 2025). Meanwhile, spoilage bacteria in chilled pork also exhibit significant synergistic spoilage effects, jointly promoting the accumulation of spoilage products(Dou, Bassey, Li, Zeng, & Ye, 2025). When the concentration reaches the sensory threshold, it causes the chilled meat to produce an objectionable off-odor.

Common pork spoilage microorganisms mainly include *Bacillus*, *Pseudomonas*, *Lactobacillus*, *Acinetobacter* spp, etc. (Dorn-In, Mang, Cosentino, & Schwaiger, 2024; B. Zhang, Dou, Teng, & Ye, 2025). The dominant spoilage bacterial community structures vary significantly among pork from different sources and under different storage conditions(Zhao, Wei, Zhou, Kristiansen, & Wang, 2022). The cold chain is one of the main means to inhibit microbial growth and is crucial for maintaining the quality of pork products; however, traditional pork distribution methods in China often involve ambient temperature exposure, and environmental heat stress can sharply increase microbial metabolic activity, significantly accelerating the proliferation of microorganisms and the deterioration of

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pork quality(Han *et al.,* 2025). During refrigeration, transient temperature fluctuations can significantly shorten the shelf life of fresh pork. Furthermore, under refrigerated conditions, microorganisms such as Pseudomonas, Carnobacterium, and Psychrobacter still exhibit cold adaptation and high spoilage potential in pork(Y. Zhang, Yang, Peng, Liao, & Wang, 2024). It is worth noting that Bacillus cereus can form biofilms, possessing good tolerance to low-temperature environments and repeated freeze-thaw stress in cold chain logistics, enabling it to survive for a long time in cold-stored meat products(Guo *et al.,* 2021). At the same time, Bacillus cereus can form highly resistant spores, exhibiting a certain tolerance to conventional physical sterilization technologies such as heat treatment and ultrasound, which significantly increases the difficulty of microbial control during pork processing(Owusu-Ansah *et al.,* 2020). Studies have pointed out that the positive detection rate of Bacillus cereus in commercially available meat and meat products is as high as 26.37%; the isolated strains not only generally carry key virulence genes such as nheABC and hblACD, but also exhibit multidrug resistance to five or more classes of antibiotics, and the food safety risks caused by Bacillus cereus have become a global public health issue(Ghosh, Dhar, Mukhopadhyay, & Bhattacharya, 2024; Kong *et al.,* 2021; Rahnama *et al.,* 2023).

Refrigeration is the primary method for pork preservation, but low-temperature storage conditions can enrich cold-adapted microorganisms. Concurrently, pork can also be contaminated by personnel, equipment, machinery, or the slaughter environment during the slaughter and processing stages(Zwirzitz *et al.,* 2020). Currently, considering the microbial-induced pork spoilage under refrigerated conditions and related public health issues, there have been studies on the isolation and identification of pork spoilage bacteria, but most focus on microbial community identification without clarifying the actual spoilage capacity of key strains through re-inoculation. Based on this, the present study focused on pork refrigerated at 4 °C until spoilage, and Plate Count Agar (PCA) and Pseudomonas CFC selective agar were used to isolate and purify the main spoilage bacteria in pork; strain identification was completed through morphological observation, Gram staining, and 16S rDNA molecular biology methods. Subsequently, the identified key spoilage bacterial strains were inoculated back into fresh pork tenderloin, and the pH value, water loss rate, color difference, TVB-N content, of the pork during storage at 4 °C were measured to clarify the spoilage capacity of the isolated strains. This study aims to determine the main spoilage bacteria in refrigerated pork and their spoilage characteristics, providing theoretical support for the optimization of precision preservation technologies against pork spoilage.

# MATERIALS AND METHODS

## Materials

Fresh pork tenderloin was purchased from Rongrong Supermarket chain in Yibin City and stored at 4 °C for future use. The bacterial genomic DNA extraction kit was purchased from Tiangen Biotech (Beijing) Co., Ltd. The 2×Taq PCR MasterMix was purchased from Takara Biotechnology (Dalian) Co., Ltd. The Gram staining kit was purchased from Beijing Solarbio Science & Technology Co., Ltd.

## Isolation and Purification of Main Spoilage Bacteria in Pork

Fresh pork was cut into pieces, packaged in bags, and stored in a refrigerator at 4 °C until spoilage. An appropriate amount of spoiled pork was placed on an ultra-clean workbench and chopped aseptically. Then, 25 g of the sample was weighed and added to 225 mL of sterile physiological saline. The mixture was homogenized and subjected to gradient dilution. Three suitable dilution gradients were selected, and 100 μL of the bacterial suspension from each gradient was evenly spread on PCA and CFC media for cultivation. Typical colonies with good growth and different colonial characteristics were selected from each plate. Subculture purification was performed on nutrient agar plates, and single colonies were selected and inoculated into nutrient broth, incubating at 37 °C and 220 r/min for 18 h. The bacterial culture broth was mixed evenly with 50% mass fraction glycerol at a 1:1 ratio and stored at -80 °C for future use.

## Morphological Identification of Spoilage Bacteria

The purified and preserved strains were activated, and the bacterial suspension was streaked onto nutrient agar plates and incubated for 24 h to isolate single colonies. The colony morphology of the well-grown isolated and purified strains was observed and recorded, and the colonies were stained using a Gram staining kit, examined microscopically, and recorded.

## Genomic DNA Extraction and Sequence Amplification of Spoilage Bacteria

Bacterial genomic DNA of the isolated strains was extracted using a bacterial genomic DNA extraction kit. Subsequently, universal primers 27F (5'-GAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') for bacterial 16S rDNA amplification were selected, and PCR amplification was performed using 2×Taq PCR Master Mix, after which the PCR products were stored in a 4 °C refrigerator. A 5,000 bp DNA Marker was used, and 5 μL of the PCR product was sequentially added into the sample wells of a 1% agarose gel. The gel was then placed in an electrophoresis tank containing 1× TAE buffer, and electrophoresis was started with the voltage set to 120 V; after electrophoresis, the target bands were detected using a gel imaging system, and the PCR products meeting the required size of 1,500 bp were sent to Sangon Biotech (Shanghai) Co., Ltd. for sequencing.

**Re-inoculation Verification of Spoilage Bacteria**

Fresh pork tenderloin was soaked in 75% alcohol for 1 min on an ultra-clean workbench, then rinsed clean with sterile water. It was then soaked in a 2.5% sodium hypochlorite solution for 5 min, rinsed again with sterile water, and cut into uniform square meat slices of approximately 10 g. They were subsequently placed in sterile plastic petri dishes and stored at 4 °C. Fifty microliters of the activated bacterial suspension (adjusted to an OD600 of 0.5, corresponding to approximately $1\times10^8$ CFU/mL) was inoculated, while inoculation with 50 μL of physiological saline served as the control. The sensory and physicochemical indicators of the meat slices were measured on days 0, 1, 3, 5, 7, and 9.

**Determination of pH Value**

Referring to GB5009.237—2016. 90 mL of 0.9 g/100mL NaCl solution was added to a 10 g minced pork sample, homogenized for 2 min, filtered, and the filtrate was collected. The pH value of the filtrate was measured and recorded using a calibrated digital pH meter.

**Determination of Weight Loss Rate**

After wiping off the surface moisture, the meat sample was weighed and recorded as $m_0$. During cold storage at 4 °C, samples were taken periodically, wiped free of surface juices, and weighed, recorded as $m_t$.

Water loss rate (%) = $[(m_0 - m_t) / m_0] \times 100\%$

**Determination of Color Difference**

Three points were randomly selected on a 5 g sample, and the lens of the calibrated colorimeter was placed vertically on the meat surface, tightly pressing against it (without light leakage), avoiding intramuscular fat and connective tissue as much as possible. A D65 light source was selected to measure and record the lightness (L*), redness (a*), and yellowness (b*) values of the meat samples respectively.

**Determination of Total Volatile Basic Nitrogen (TVB-N)**

Referring to the method in GB5009.228—2016 "National Food Safety Standard - Determination of Volatile Basic Nitrogen in Foods", a 10 g minced pork sample was mixed with 100 mL of distilled water, ultrasonicated (100 W) for 15 min, and then filtered. 10 mL of the filtrate was placed in a distillation tube, and 10 mL of 10 g/L MgO solution was added. Kjeldahl distillation parameters were set as follows: boric acid 25 mL, dilution water 10 mL, distillation time 5 min, and washing water 30 mL.

**STATISTICAL ANALYSIS OF DATA**

The sequencing results were compared using BLAST on the NCBI website, and combined with homologous sequences in the database, a phylogenetic tree was constructed using the Neighbor-Joining method in MEGA software. Excel 2024 and SPSS software were used for significance analysis of experimental data, with $P < 0.05$ considered significantly different. Graphs were plotted using Origin Pro 2024 software, and the results are expressed as the mean of 3 replicates ± standard deviation.

## RESULTS AND DISCUSSION

**Morphological Identification of Spoilage Bacteria**

Through observing the appearance and odor of the pork stored at 4 °C, it was found that as the storage period extended, the fresh pork emitted an undesirable odor. This is because characteristic spoilage markers such as pentanal, dimethyl trisulfide, and dimethyl disulfide are gradually produced during storage, presenting an obvious spoilage odor(Lv *et al.,* 2025). On the 12th day of storage, the chilled pork had a distinct sour and putrid smell, and its color turned green, marking the end of the spoilage deterioration stage, at which point spoilage bacteria were screened. Eight spoilage bacterial strains were isolated and cultured on Pseudomonas CFC selective media and PCA media, labeled A, B, C, D, E, F, G, and H, respectively. The Gram staining results (Figure 1) showed that among the 8 strains, A, B, C, D, E, and F were Gram-positive bacteria ($G^+$), all rod-shaped, arranged singly or in chains; G and H were Gram-negative bacteria ($G^-$), rod-shaped, arranged in short chains and singly, respectively.

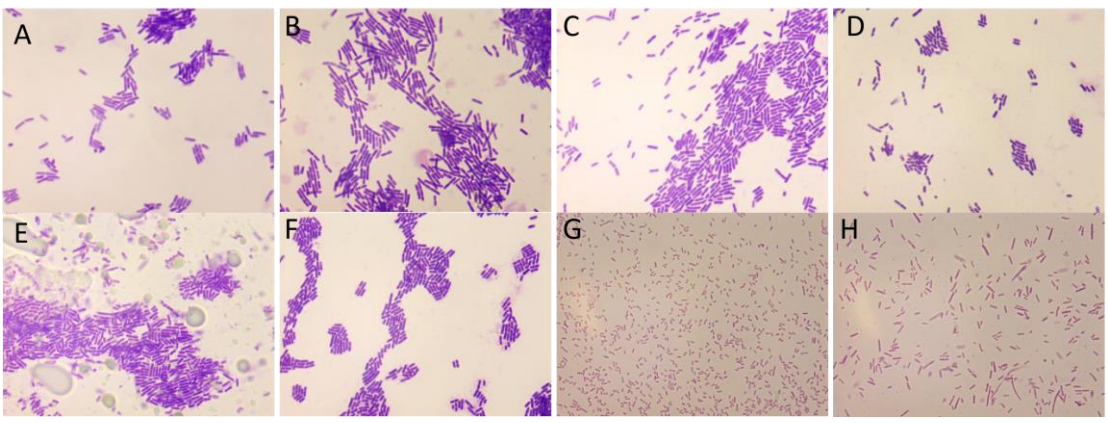



**Figure 1: Gram staining results of eight spoilage bacterial strains**

**Molecular Biological Identification of Spoilage Bacteria**

DNA was extracted from the eight screened spoilage bacterial strains for further PCR amplification. The PCR products were detected by 1% agarose gel electrophoresis, and as shown in Figure 2, all 8 strains exhibited clear specific bands at approximately 1500 bp, which is consistent with the expected size of the 16S rDNA target fragment. The bands had no obvious non-specific amplification and showed good brightness, indicating that the extracted DNA was of high quality, the PCR amplification reaction had strong specificity, and the amplification products could meet the needs of subsequent sequencing.

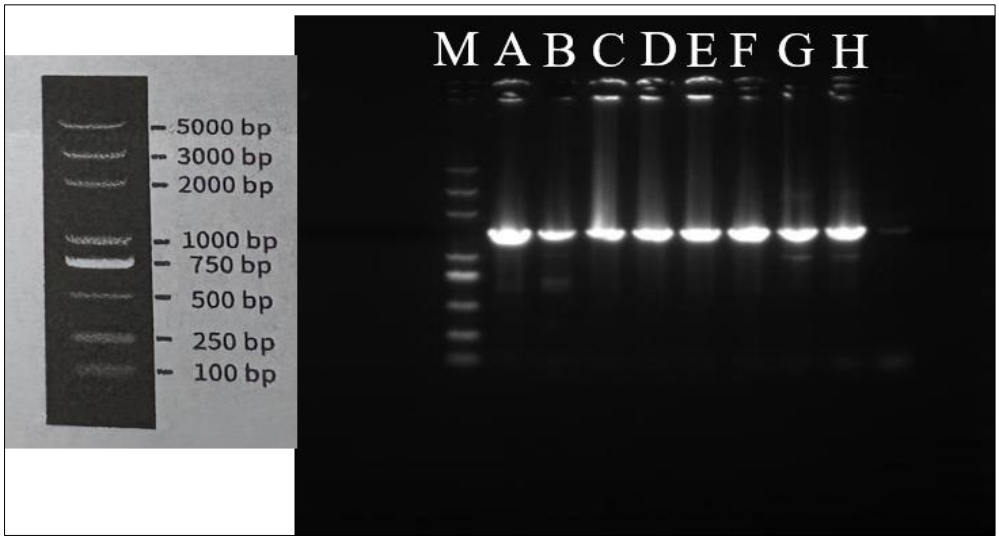



**Figure 2: Agarose gel electrophoresis results after PCR**

The 16S rRNA gene sequences of each isolated strain were submitted to the National Center for Biotechnology Information (NCBI) database for BLAST homology comparison to clarify their taxonomic status. As shown in Figure 3, strains A to F all belonged to the genus *Bacillus* and formed an independent monophyletic evolutionary branch with the *Bacillus cereus* reference strain, with Bootstrap support values > 99%, indicating reliable clustering results; strains G and H belonged to the genus *Proteus*, where strain G had the closest phylogenetic relationship with the *Proteus penneri* reference strain CIFRI P4, with a Bootstrap support value of 100%; strain H had the closest phylogenetic relationship with the *Proteus vulgaris* reference strain W2, with a Bootstrap support value of 100%. Combined with the isolation frequency statistics, the results indicate that the culturable dominant spoilage bacteria in this batch of refrigerated pork were mainly *Bacillus*; given that *Bacillus cereus* accounted for the highest proportion among the isolated strains, a representative *Bacillus cereus* strain was selected as the target spoilage bacterium for subsequent pork spoilage re-inoculation verification experiments.

**Re-inoculation Verification of Spoilage Bacteria**

The identified dominant spoilage bacterium, *Bacillus cereus*, was inoculated back into fresh pork, and the appearance results are shown in Figure 4. During storage at 4 °C for 0 to 9 days, the sensory quality of the pork samples inoculated with *Bacillus cereus* showed significant differences compared to that of the blank control group. In terms of color, both groups of pork maintained a fresh light pink color at the initial stage of storage, with a glossy surface and no obvious difference. As the storage time extended, the color of the inoculated group gradually turned yellow, and the gloss faded significantly; on the 7th day of storage, the surface color turned dull yellow, and slight fat exudation appeared. The blank group only showed a slight darkening of color throughout the storage period, with no obvious yellowing phenomenon. In terms of texture, both groups were firm and elastic with smooth cut surfaces at the beginning of storage. With prolonged storage time, the surface of the inoculated group gradually became sticky, and the elasticity continued to decline; on the 5th day of storage, the texture became soft and mushy, stickiness was significant, and the cut surface was blurred. This is similar to the research results of Li *et al.,* (Li *et al.,* 2019), where untreated pork stored at 4 °C exhibited significant color changes, off-odors, and slime by the 5th day. The blank group only softened slightly, maintaining basic firmness with no obvious stickiness. It can be seen that *Bacillus cereus* caused spoilage effects on fresh pork during refrigerated storage at4 °C.

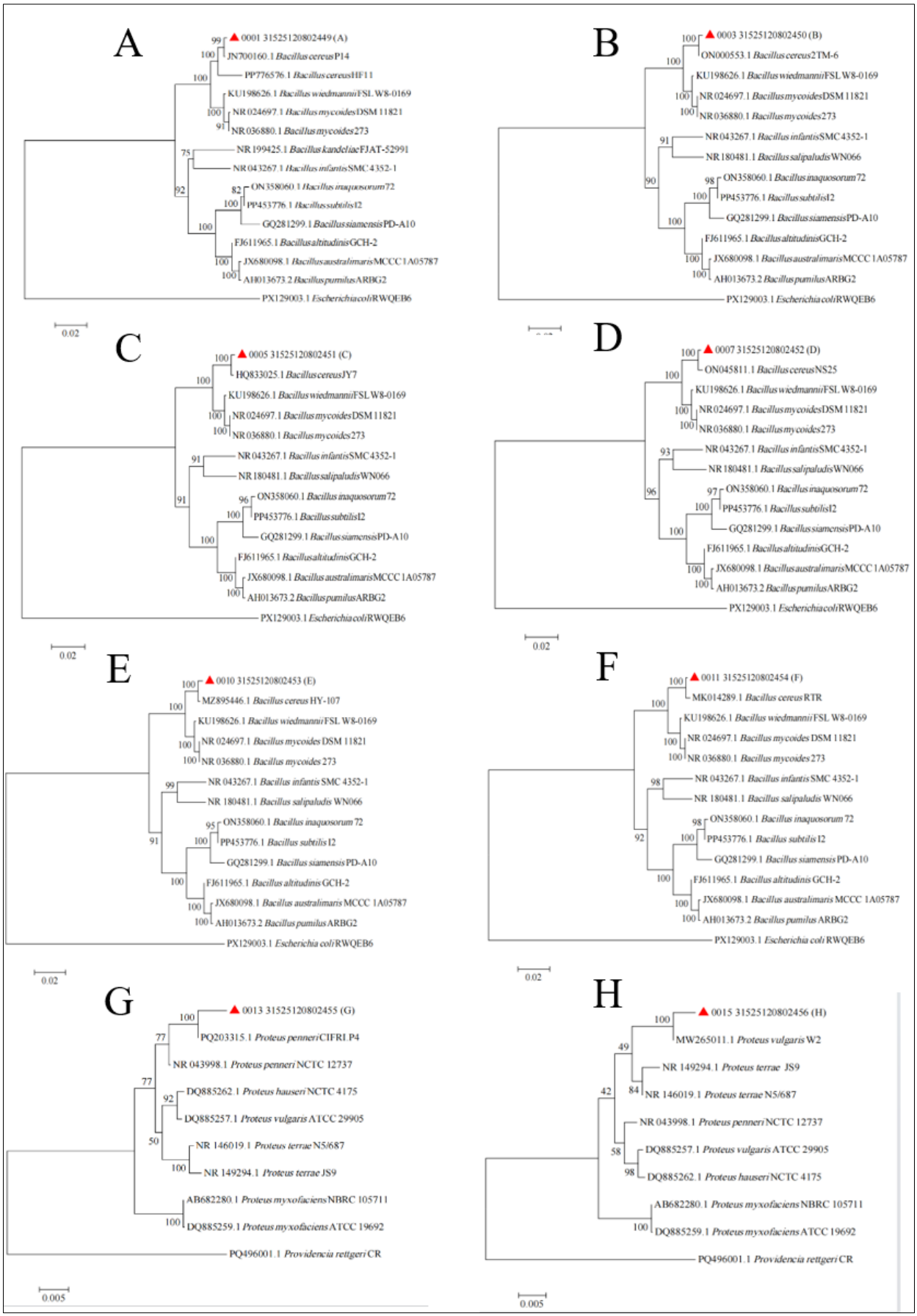



**Figure 3: Phylogenetic tree of dominant spoilage bacteria**

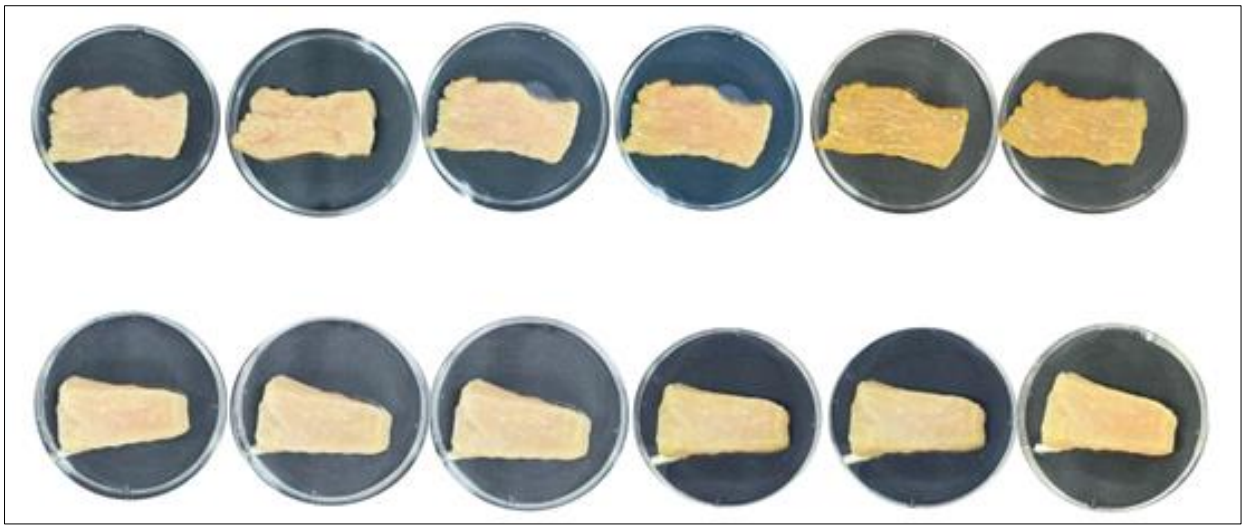


**Figure 4: Appearance comparison of pork samples inoculated with *Bacillus cereus* and blank control group during storage at 4 ℃**

**Changes in pH Value**

As shown in Figure 5, by monitoring the pH changes of pork over a 9-day storage period, it was found that the pH values of both groups of samples showed an upward trend, reflecting the pattern of alkaline substance generation from protein decomposition during storage. The pH value of the inoculated group rapidly and continuously increased from an initial ~5.5 to ~7.25 on the 9th day; while the pH of the blank group (added with sterile physiological saline only) slowly increased from 5.5 to ~5.95, which is consistent with the findings of Bruckner et al. (Bruckner, Albrecht, Petersen, & Kreyenschmidt, 2012). The significant difference in pH values between the two groups indicates that the inoculated target spoilage bacterium can significantly accelerate pork protein degradation and the generation of alkaline amines(Pogacic *et al.,* 2015).

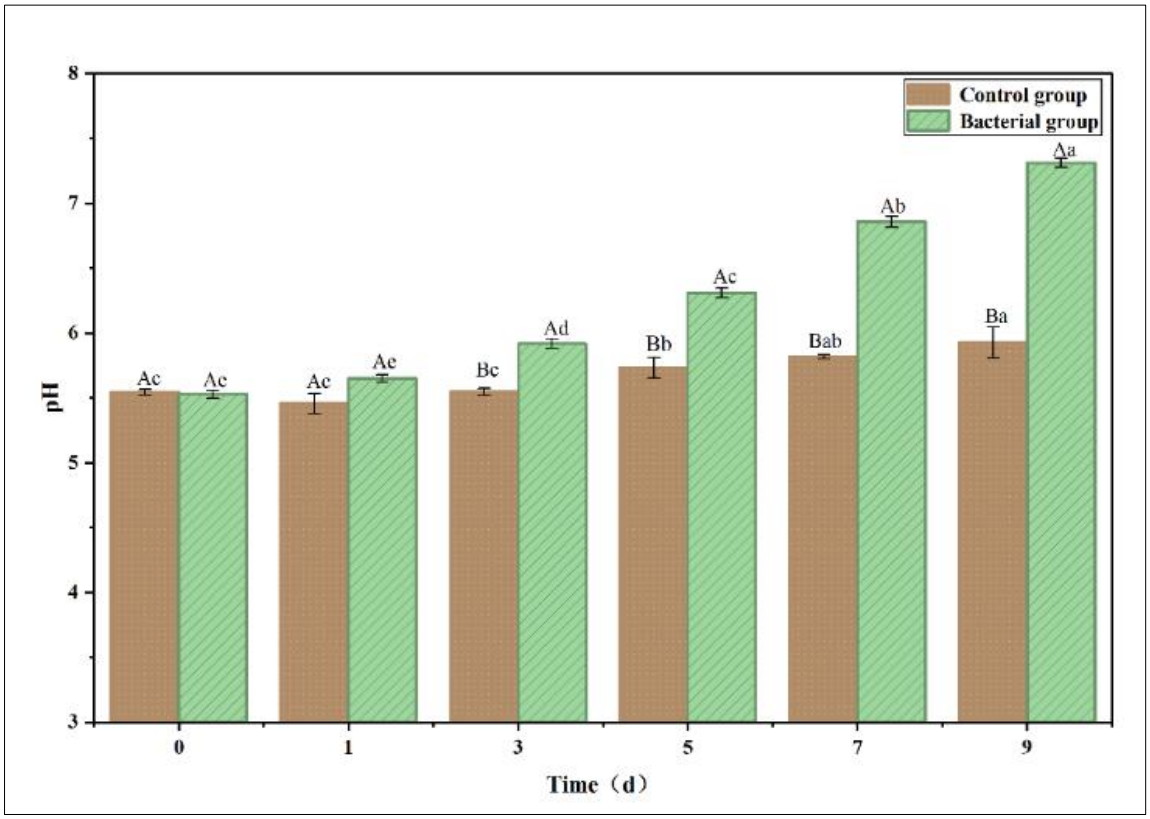



**Figure 5: Changes in pH value during storage**

**Notes:** Significant differences (P<0.05) for the same treatment at different time points are indicated by superscript lowercase letters, while differences between different treatments at the same time point are indicated by superscript capital letters (P<0.05).

**Changes in Weight Loss Rate**

From Figure 6, it can be seen that with prolonged storage time, the weight loss rate of both the blank group and the inoculated group showed a continuous upward trend. The weight loss rate of the inoculated group was consistently and significantly higher than that of the blank group ($P < 0.05$). On the 9th day of storage, the weight loss rate of the inoculated group reached 11.50%, which is approximately 1.77 times that of the blank group (6.52%). This indicates that the introduction of exogenous spoilage bacteria significantly accelerated pork moisture loss and exacerbated the decline in meat water-holding capacity.

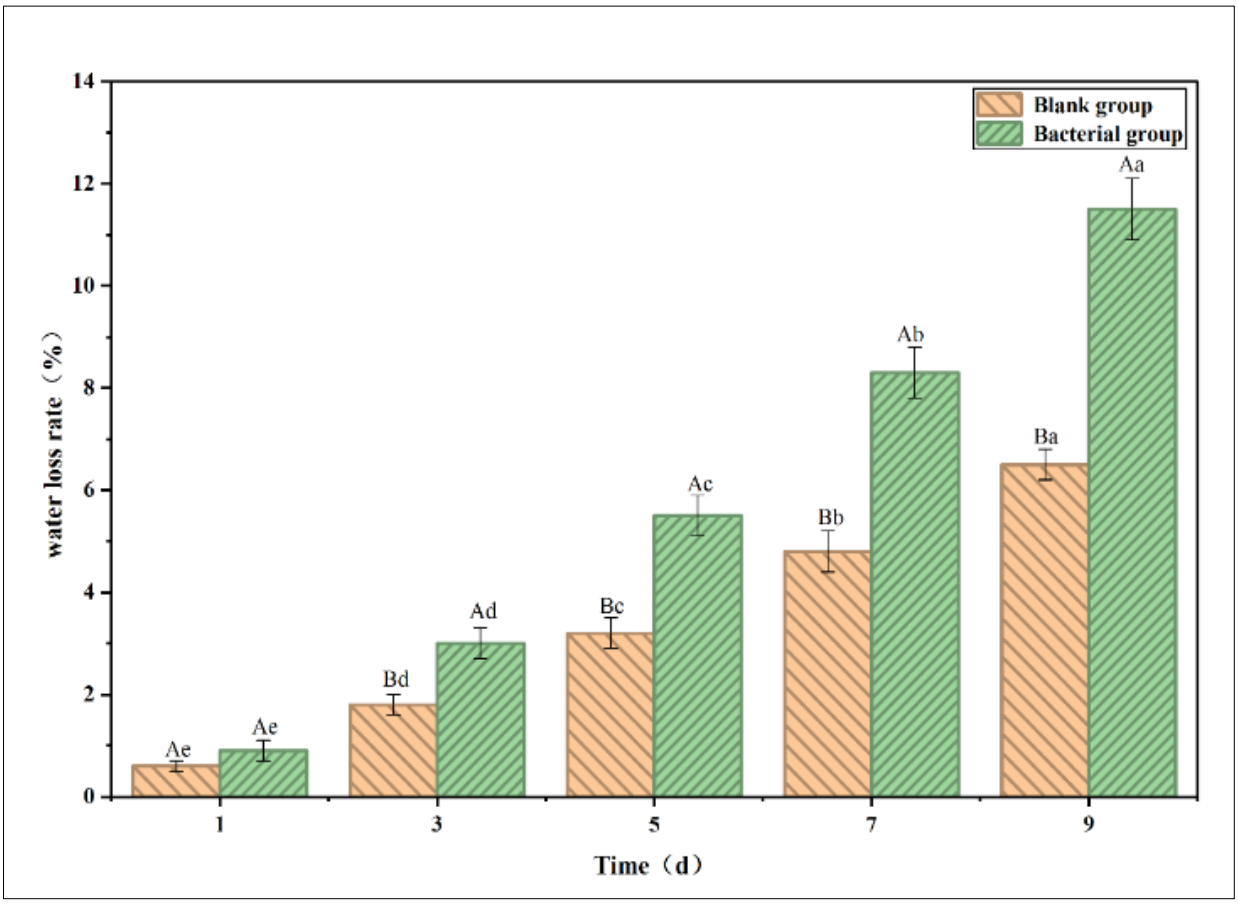



**Figure 6: Effect of storage time on weight loss rate of pork**

**Notes:** Significant differences ($P<0.05$) for the same treatment at different time points are indicated by superscript lowercase letters, while differences between different treatments at the same time point are indicated by superscript capital letters ($P<0.05$).

**Changes in Total Volatile Basic Nitrogen (TVB-N)**

As shown in Figure 7, with the extension of storage time, the TVB-N contents of both groups of pork showed a continuous upward trend, and the growth rate of the inoculated group was significantly faster than that of the blank group ($P < 0.05$). On the 3rd day of storage, the TVB-N content of the inoculated group samples approached the acceptability limit of 15 mg/100g for fresh meat. By the 9th day, the TVB-N content of the inoculated group reached 40.2 mg/100g, significantly higher than that of the blank group (22.3 mg/100g), indicating that the inoculation of exogenous spoilage bacteria significantly accelerated pork protein decomposition and TVB-N accumulation, aggravating the meat spoilage process. In the early stage of storage, microorganisms need to adapt and proliferate, and bacteria prefer carbohydrates as an energy source, thus the increase in TVB-N levels is relatively slow(Pellissery, Vinayamohan, Amalaradjou, & Venkitanarayanan, 2020). In the middle and late stages of storage, as the proliferation and metabolic activities of *Bacillus cereus* accelerate, the TVB-N level enters a stage of rapid increase(W. Wang *et al.,* 2025).

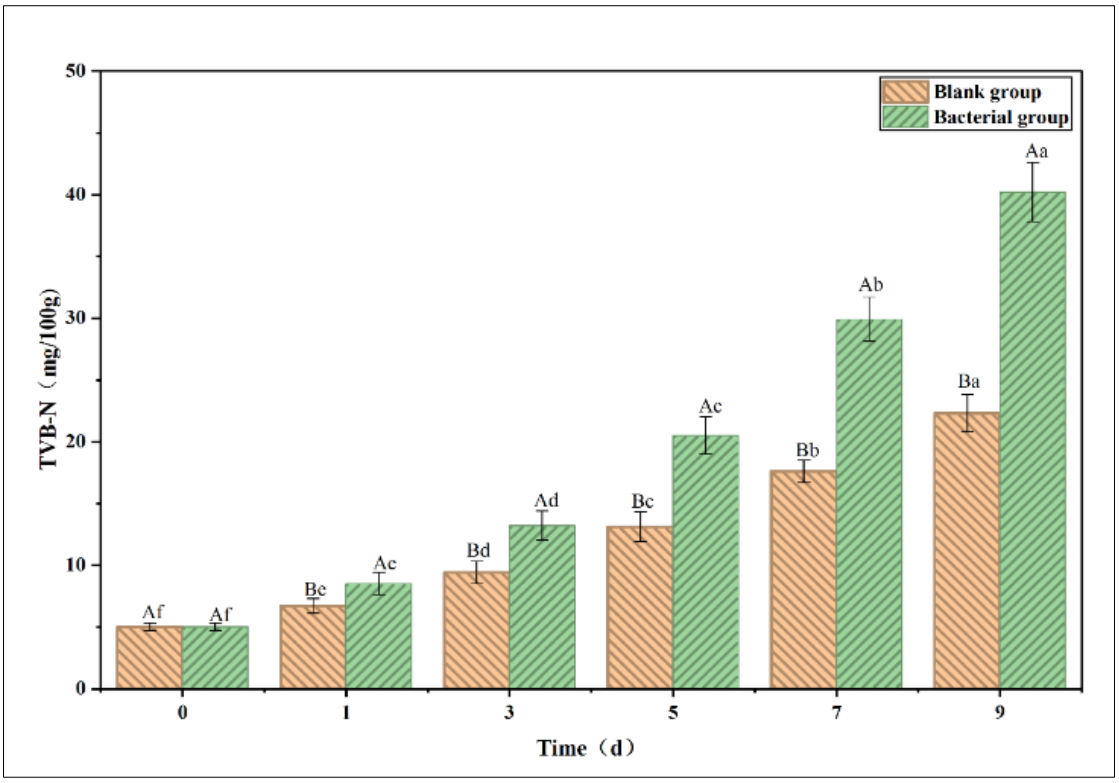
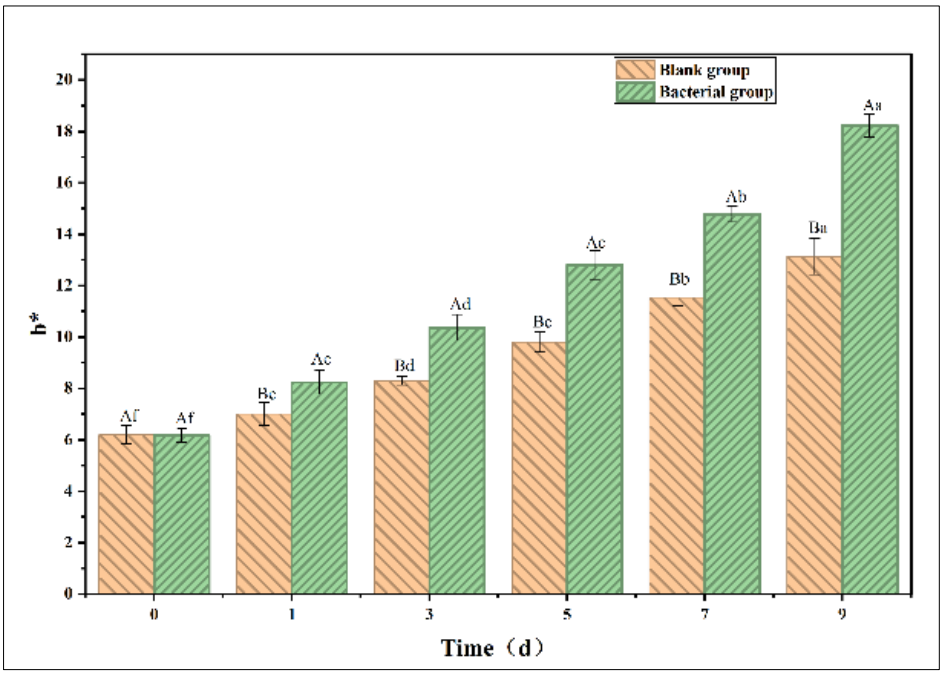



**Figure 7: Effect of storage time on TVB-N content of pork**

**Notes:** Significant differences (P<0.05) for the same treatment at different time points are indicated by superscript lowercase letters, while differences between different treatments at the same time point are indicated by superscript capital letters (P<0.05).

**Changes in Color Parameters**

As shown in Figure 8, with the extension of storage time, the L* (lightness) and a* (redness) values of both groups of pork continuously decreased, while the b* (yellowness) value continuously increased, and the magnitude of change in the inoculated group was significantly greater than that in the blank group (P < 0.05). By the 9th day of storage, the inoculated group exhibited lower lightness and redness, and higher yellowness, demonstrating that the introduction of spoilage bacteria accelerated meat color deterioration, causing the pork to darken, fade, and turn yellow more quickly.



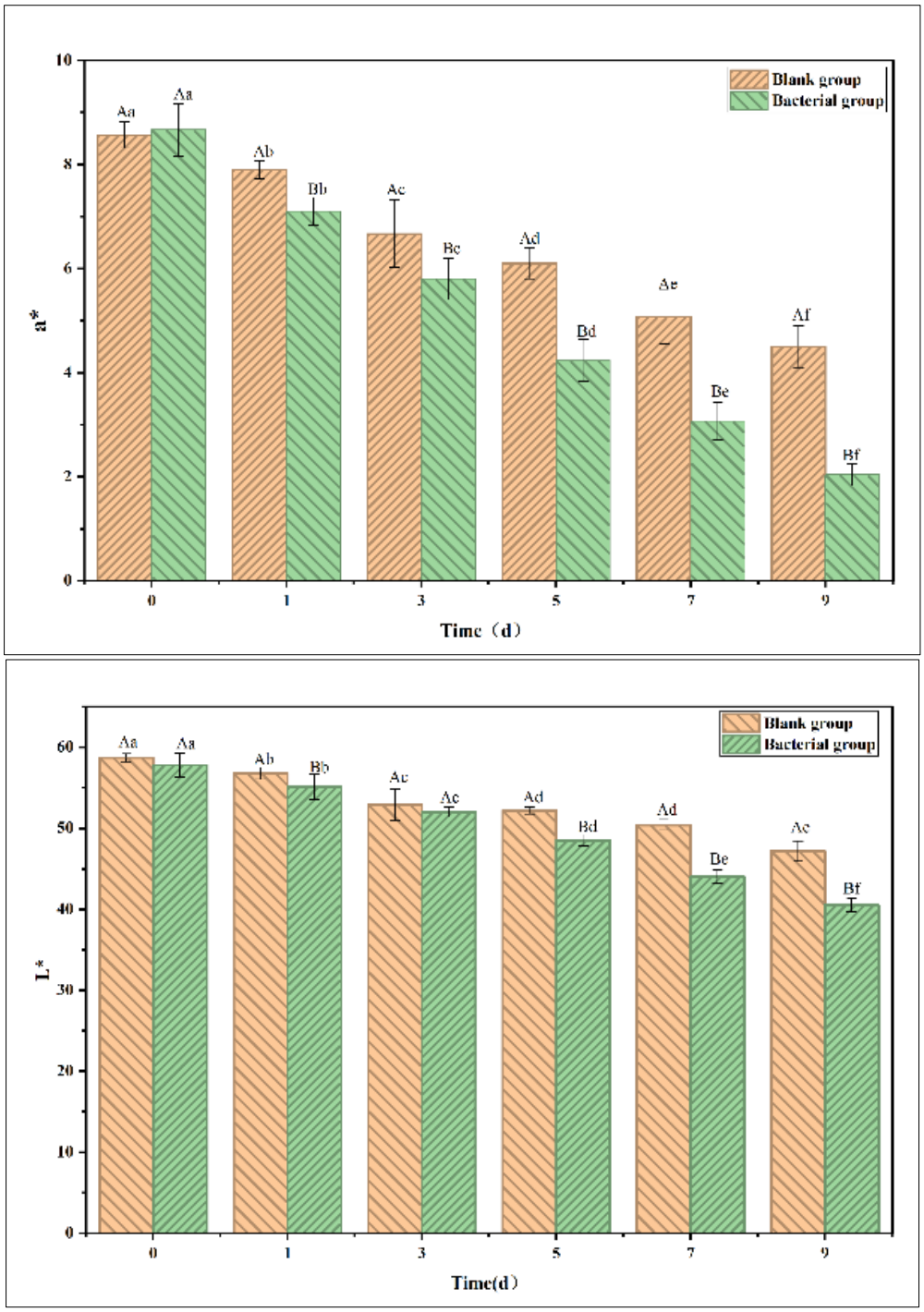



**Figure 8: Effect of storage time on color parameters (L*, a*, b*) of pork**

**Notes:** Significant differences (P<0.05) for the same treatment at different time points are indicated by superscript lowercase letters, while differences between different treatments at the same time point are indicated by superscript capital letters (P<0.05).

## CONCLUSIONS

This study focused on fresh pork refrigerated at 4 °C until the end of spoilage, completing the isolation and purification, species identification, and spoilage capacity verification of dominant spoilage bacteria. A total of 8 dominant spoilage bacterial strains were isolated and purified from spoiled pork samples; Gram staining microscopic examination revealed that 6 strains were Gram-positive bacilli and 2 were Gram-negative bacilli. Through 16S rDNA gene sequencing and phylogenetic tree analysis, 6 core strains were identified as *Bacillus cereus*, and the other 2 belonged to the genus *Proteus*, clarifying that the culturable dominant spoilage bacteria in this batch of refrigerated pork were mainly *Bacillus*. The re-inoculation verification experiment of *Bacillus cereus* showed that during a 9-day refrigerated storage cycle at 4 °C, the pork samples inoculated with

this strain exhibited significantly higher rising rates in pH value, water loss rate, and TVB-N content, as well as greater color deterioration, compared to the sterile physiological saline control group, confirming that the strain is the key spoilage bacterium during pork refrigeration.

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