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Insight into the heterogeneity of spoilage potential in meat-borne *Serratia* spp.

Jing Yang [a], Qing Zhang [b], Yun Sun [a], Qiang Xiong [a], Xinfu Li [a, *]

[a] *College of Food Science and Light Industry, Nanjing Tech University, Nanjing, 211800, China*

[b] *Food Microbiology Key Laboratory of Sichuan Province, Xihua University, Chengdu, 611039, China*

ABSTRACT: *Serratia* spp. are common spoilage bacteria in meat. This study screened five *Serratia* strains with significant proteolytic and lipolytic activities (*Serratia bozhouensis* YB10, *Serratia myotis* YB16, *Serratia quinivorans* NC6, *Serratia liquefaciens* NC12, and *Serratia grimesii* NC17) from 34 meat-borne *Enterobacteriaceae* isolates. Their spoilage potential was evaluated by analyzing spoilage-related phenotypes and spoilage capacity in a vacuum-packaged pork model. Differences in spoilage phenotypes among strains, including proteolytic and lipolytic activities, growth characteristics, motility, extracellular polysaccharide (EPS) production, adhesion capacity, and biofilm formation, were observed and found to be regulated by temperature. The results indicated that 30 ℃ was more conducive than 37 ℃ for the growth and phenotypic expression of most *Serratia* strains. The motility of the strains was determined, with NC12 exhibiting the strongest motility. Comprehensive evaluation indicated that YB16 possessed relatively strong spoilage potential. It demonstrated the highest proteolytic and lipolytic activities at 37 ℃ and showed prominent adhesion capacity, EPS production, and biofilm formation at 4 ℃. In addition, it effectively degraded both sarcoplasmic and myofibrillar proteins. In the pork model, all strains colonized rapidly, significantly increasing total viable count (TVC), total volatile basic nitrogen (TVB-N), pH, and thiobarbituric acid reactive substances (TBARS) values, thereby accelerating protein and lipid deterioration. YB16 showed particularly vigorous growth and spoilage metabolism during the later storage period. These findings reveal interspecies heterogeneity in spoilage potential among *Serratia* strains, providing a novel theoretical basis for understanding species-specific spoilage mechanisms and offering insights for developing targeted preservation strategies to enhance meat quality and safety.

Keywords: *Serratia*, *Enterobacteriaceae*, Spoilage potential, Heterogeneity, Proteolytic activity

1. Introduction

Meat is highly susceptible to spoilage due to its high moisture and rich nutrient content[1]. Throughout the entire process from production to retail, meat is vulnerable to microbial spoilage, particularly due to the proliferation of spoilage microorganisms and enzymatic reactions driven by proteases or lipases[2]. Spoilage microorganisms can also secrete EPS, which gradually forms a protective barrier on the meat surface, enabling microorganisms to continuously grow and reproduce within the barrier, and further result in mucus production on the meat surface as well as meat softening[3]. In addition, the spoilage potential of microorganisms is associated with various factors, among which microbial motility and adhesion capacity

***Corresponding author**
lixinfu316@126.com

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are the key factors[4]. Strong motility and adhesion capacity can further promote biofilm formation, ultimately leading to microbial cross-contamination in meat. Therefore, a comprehensive study on the spoilage potential of microorganisms is crucial for ensuring the quality and safety of meat.

Enterobacteriaceae are widely regarded as indicator bacteria of microbiological quality and hygiene conditions in food production[5]. When growth conditions are favorable for their proliferation, some members of *Enterobacteriaceae* can become important spoilage flora in meat[6]. Their metabolic activities and the action of endogenous enzymes can lead to the accumulation of undesirable metabolites and even the production of harmful substances, thereby contributing to meat quality deterioration[7]. *Serratia* is a group of gram-negative bacteria within the family *Enterobacteriaceae* and is a typical spoilage organism in vacuum-packaged meat[8,9]. Currently, this genus comprises 22 species[10].

In previous studies, it has been demonstrated that *Serratia* can promote the degradation of proteins and lipids by secreting proteases and lipases, thereby directly contributing to the deterioration of meat texture and flavor[11,12]. For instance, Yang et al.[2] screened *Serratia liquefaciens* as the strain with the strongest protease-production capacity from 30 meat-borne spoilage bacteria. This strain was shown to effectively degrade myofibrillar proteins in chicken meat. Similarly, Wen et al.[13] reported that *Serratia proteamaculans* possesses strong lipid-degrading ability, which could significantly increase the total free fatty acid content in vacuum-packed lamb meat. In addition, some strains of the genus *Serratia* have been confirmed to possess biofilm-forming ability, and bacterial motility is also involved in the process of biofilm formation[14-16]. Ning et al.[17] found that meat-related *S. liquefaciens* exhibited moderate swimming motility and strong swarming motility, with a more prominent biofilm-forming ability at 30 ℃. This characteristic facilitates bacterial adhesion and colonization, endowing the strain with a stronger colonization potential and thus further exacerbating the risk of meat spoilage. This demonstrates the importance of conducting in-depth research into the spoilage potential of *Serratia*. However, studies on the spoilage properties of the genus *Serratia* have mainly focused on its spoilage phenotypes in milk[18,19], while research on its spoilage potential in meat has been limited to isolated aspects, such as protein/lipid degradation activities or biofilm formation. Therefore, systematic comparison of its spoilage phenotypes in meat remains significantly insufficient.

Therefore, this study aimed to systematically evaluate the spoilage potential of *Serratia* spp. Initially, a pre-screening was conducted to assess the spoilage potential of 34 different meat-borne *Enterobacteriaceae* strains using skim milk agar (SMA) and glycerol tributyrate medium (GTM). This screening identified five *Serratia* strains exhibiting both proteolytic and lipolytic capacities. Subsequently, we characterized these strains by determining their growth characteristics, motility, EPS production, adhesion capacity, biofilm formation, and muscle protein degradation capacity. Finally, to assess their practical impact on meat quality, the strains were inoculated into vacuum-packaged pork, and their effects on key meat-quality indices (TVC, TVB-N, pH, and TBARS) were investigated. This study provides theoretical support for systematically elucidating interspecific differences in the spoilage potential of *Serratia* spp.

2. Materials and methods

2.1. Bacteria strains

Thirty-four strains of *Enterobacteriaceae* (see Supplementary Information) were isolated from vacuum-packed chilled meat (pork, mutton, and beef). These strains were then stored at -80 ℃ in the laboratory of Nanjing Tech University (Nanjing, Jiangsu Province, China). Strains were grown on SMA and GTM media. The SMA medium contained: peptone 10 g/L, NaCl 10 g/L, yeast extract 5 g/L, skimmed milk powder 10 g/L, and agar 15 g/L[20]. The GTM medium contained: peptone 10 g/L, NaCl 5 g/L, yeast extract 5 g/L, tributyl glyceride 2 mL/L, and agar 20 g/L[21]. All cultures were incubated at 30 ℃ and 37 ℃ for 48 h. These temperatures were selected to represent the optimal growth temperature for some *Enterobacteriaceae* (30 ℃) and a common screening temperature for this family (37 ℃), respectively. The proteolytic and lipolytic activities of the isolates were assessed by calculating the ratio of the degradation zone diameter to the bacterial colony diameter (DZD/BCD), which served as an indicator of their spoilage potential (Table 1). In this study, five strains exhibiting both lipolytic and high proteolytic activities (defined by a DZD/BCD ratio greater than 3 at 30 ℃) were selected for subsequent experiments. All selected strains belonged to the genus *Serratia*: *S. bozhouensis* (YB10, accession number NR_169374.1), *S. myotis* (YB16, accession number NR_178702.1), *S. quinivorans* (NC6, accession number NR_037112.1), *S. liquefaciens* (NC12, accession number NR_122057.1) and *S. grimesii* (NC17, accession number NR_114576.1). Each *Serratia* strain was activated on tryptone soy agar (TSA, Qingdao Haibo Biotechnology Co., Ltd., China) plates incubated overnight at 30 ℃. Then, a single colony of each strain was transferred to 6 mL of tryptic soy broth (TSB, Qingdao Haibo Biotechnology Co., Ltd., China) and incubated at 30 ℃ for 24 h.

Table 1. Protease and lipase degradation tests of isolates.

***Enterobacteriaceae* Species**	**Isolates**	**Origin**	**Proteolytic activity**		**Lipolytic activity**	
			30℃	**37℃**	**30℃**	**37℃**
Klebsiella grimontii	ZA2	Pork	-	-	-	-
Enterobacter cloacae	ZA6	Pork	-	-	-	-
Providencia sneebia	ZA10, YB18	Pork, Mutton	-	-	-	-
Citrobacter freundii	ZA12, YB2	Pork, Mutton	+	+	-	-
Silvania hatchlandensis	YB3	Mutton	-	-	-	-
Raoultella ornithinolytica	YB5	Mutton	-	-	-	-
Obesumbacterium proteus	YB8	Mutton	-	-	-	-
Serratia bozhouensis	YB10	Mutton	+++++	+++++	+	+++
Kluyvera intermedia	YB11, NC19	Mutton, Beef	+	+	-	-
Hafnia paralvei	YB13, ZA7, NC16	Mutton, Pork, Beef	-	-	-	-
Shigella flexneri	YB14	Mutton	++	++	-	-
Serratia myotis	YB16, ZA9, NC9	Mutton, Pork, Beef	+++++	+++++	++	++++
Citrobacter arsenatis	YB19, NC7	Mutton, Beef	+	+	-	-
Lelliottia amnigena	YB20	Mutton	-	-	-	-
Buttiauxella massiliensis	NC5, YB1	Beef, Mutton	-	-	-	-
Serratia quinivorans	NC6, ZA8, YB12	Beef, Pork, Mutton	+++++	-	++	+
Rahnella ecdela	NC11	Beef	+	-	-	-
Serratia liquefaciens	NC12	Beef	+++++	++++	+++	++
Pantoea agglomerans	NC15	Beef	-	-	-	-
Serratia grimesii	NC17	Beef	+++++	-	+	-
Klebsiella pneumoniae	NC20, ZA13, YB17	Beef, Pork, Mutton	+	++	-	-

Proteolytic and lipolytic activities were assessed based on the DZD/BCD ratio and categorized as: "-" (No activity), "+" (1.0–1.5), "++" (1.5–2.0), "+++" (2.0–2.5), "++++" (2.5–3.0), and "+++++" (>3.0).

2.2. Growth curves

The experiment was conducted following the methodology outlined in reference[22], with certain modifications. Briefly, the concentration of the freshly prepared bacterial suspension in TSB was adjusted to 1×10^8–10^9 CFU/mL. Subsequently, the 1% (v/v) suspension was inoculated into a conical flask containing fresh TSB medium. The cultures were incubated under shaking conditions at 150 r/min at two different temperatures: 30 ℃ and 37 ℃. Samples were collected at 2 h intervals, and the optical density at 600 nm and viable colony count were determined immediately for each sample. Growth curves were then plotted based on the obtained data.

2.3. Motility assays

The swimming and swarming motility of the strains were assessed with reference to the study by Wang et al.[23], employing the plate diffusion method to measure the motility capacity of each strain, with certain modifications made. Specifically, 3 μL of bacterial suspension in the stationary phase was spotted at the center of both swimming agar plates (tryptone 10 g/L, NaCl 5 g/L, glucose 2.5 g/L and agar powder 3 g/L) and swarming agar plates (LB broth 25 g/L, glucose 0.5 g/L and agar powder 5 g/L). The plates were then left to stand at room temperature for 20 min. Subsequently, they were incubated at 30 ℃ and 37 ℃ for 24, 48, 72, and 96 h, respectively. The time point scheme (24–96 h) was adopted from Wang et al.[23] to enable dynamic monitoring and capture early strain differences. The two temperatures (30 ℃ and 37 ℃), chosen based on Hidalgo et al.[24], were used to assess temperature-dependent motility. The diameter of the diffused bacterial colonies formed by each strain was measured using a vernier caliper.

2.4. Adhesion capacity assays

The assessment of bacterial adhesion capacity was conducted in accordance with a previously established reference method[25], with minor modifications. A standard bacterial suspension was diluted in TSB to achieve a concentration of 10^4–10^5 CFU/mL, yielding the working cell suspension. Subsequently, 2 mL of the working suspension was transferred into wells of a 24-well polystyrene microplate. Sterilized stainless-steel plates (304, 10 mm× 10 mm × 1 mm), which had been pre-cleaned and sterilized, were carefully placed into each well. The plates were then incubated at 30 ℃ and 37 ℃ for 48 h, and separately at 4 ℃ for 7 d. Following incubation, the stainless-steel plates were gently rinsed with sterile PBS using sterile forceps to remove non-adherent cells. Thereafter, each plate was transferred into a test tube containing 10 mL of 0.85% (w/v) NaCl solution along with an appropriate quantity of sterile glass beads. The tubes were vortexed vigorously for 3 min to dislodge adherent bacteria. The resulting suspensions were serially diluted in 0.85% NaCl solution and plated onto TSA plates using the spread plate method. After 24 h incubation at 30 ℃, colonies were enumerated and expressed as log CFU/cm².

2.5. EPS production assays

The determination of EPS was conducted with partial modifications based on the method described by Li et al.[26]. Bacterial cultures were inoculated into TSB, and the cell density was adjusted to 10^6–10^7

CFU/mL. A 1% (v/v) inoculum was transferred into fresh TSB medium to achieve an initial concentration of 10^4–10^5 CFU/mL. Subsequently, 9 mL of the bacterial suspension was dispensed into 6-well polystyrene microplates and incubated at 30 ℃ and 37 ℃ for 48 h, and at 4 ℃ for 7 d. Following incubation, the cultures were centrifuged at 3,000 × g for 10 min, and the supernatant was collected. To this, four volumes of anhydrous ethanol were added, followed by precipitation at 4 ℃ for 12 h. The mixture was then centrifuged at 7,600 × g for 10 min, and the resulting precipitate was collected and dissolved in distilled water to obtain an EPS aqueous solution. The concentration of EPS was quantified using the phenol-sulfuric acid method. Specifically, 1 mL of the sample solution was mixed with 1 mL of 6% (v/v) phenol solution, followed by the addition of 5 mL of concentrated sulfuric acid. After thorough mixing, the reaction mixture was allowed to stand for 30 min, and absorbance was measured at 490 nm.

2.6. Biofilm formation assays

The biofilm formation assay was carried out using a modified method described by Salinas et al.[27]. Briefly, the turbidity of fresh bacterial cultures in TSB was adjusted to an OD600 of approximately 1.000, and 200 μL of the suspension was transferred into a 96-well polystyrene microplate. The plates were incubated at 30 ℃ and 37 ℃ for 48 h, and at 4 ℃ for 7 d, respectively. Sterile TSB served as the negative control. Upon completion of incubation, the culture medium was carefully removed, and the wells were washed three times with sterile PBS (pH 7.2). Subsequently, 200 μL of methanol was added to each well for fixation at room temperature for 15 min. After removal of methanol and air-drying, 200 μL of 0.1% (w/v) crystal violet solution was added to each well for staining over 15 min. Unbound stain was aspirated, and the wells were washed three times with PBS. To quantify biofilm formation, the bound crystal violet was solubilized in 200 μL of anhydrous ethanol for 30 min, and the absorbance was measured at 570 nm using a microplate reader (Infinite 200PRO, Tecan (Shanghai) Trading Co., Ltd., Shanghai).

2.7. Bacterial extracellular proteolytic activity in vitro assays

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to evaluate the total protein profile of bacterial extracellular protease degradation[23]. The bacterial suspension of each strain cultured to the logarithmic phase in TSB was centrifuged at 10,000 ×g for 5 min. The supernatant was then filtered through a 0.22 μm filter membrane for sterilization and used as the microbial extract for further detection. According to the description of Yu et al.[28], sarcoplasmic and myofibrillar proteins were extracted from pork. After determining the protein concentration using the binin chinonic acid (BCA) protein quantification kit (Beijing Leagene Biotechnology Co., Ltd., China), the concentration was adjusted to 2 mg/mL with 0.02 mol/L phosphate buffered saline (PBS, pH 6.5). The microbial extract (200 uL) was incubated with sarcoplasmic or myofibrillar proteins (1 mL) under static conditions at 30 ℃ for 24 h respectively, and then SDS-PAGE was performed according to the method of Paramithiotis et al.[29]. The assessment of extracellular proteolytic activity in bacteria is conducted by comparing the protein profiles of control samples (proteins incubated only with an equal amount of sterile TSB) with those of samples incubated with the selected strain.

2.8. Impact on pork freshness in situ

2.8.1. Sample treatment and storage

The pork loins were sourced from a local supermarket and transported to the laboratory within 1 h under refrigerated conditions. The samples were cut into pieces of approximately 25 g (3 × 3 × 3 cm^3) each, which were then placed in sterile stomacher bags and sterilized by irradiation at a dose of 6 kGy using a ^{60}Co source at the Irradiation Center of Nanjing University of Aeronautics and Astronautics (Nanjing, China). This dose is widely used for meat sterilization[30,31], effectively ensuring consistency in the initial microbial background, and has been shown to have little effect on the physicochemical properties of pork[32]. Following sterilization, the samples were immersed in bacterial suspension for 10–30 s to achieve a final inoculum concentration of 4.0 log CFU/g. Samples treated with sterile 0.85% NaCl solution under identical conditions served as the control group. After processing, all samples were vacuum-packaged and stored at 4 ℃ for a period of 12 d, during which microbiological and physicochemical analyses were performed at 2 days intervals (0, 2, 4, 6, 8, 10, and 12 d).

2.8.2. The measurement of TVC

On each sampling date, 10 g of meat samples were aseptically transferred into sterile stomacher bags containing 90 mL of 0.85% sterile sodium chloride solution for homogenization. One milliliter of the homogenate was serially diluted using appropriate diluents. A volume of 100 μL of the suitably diluted sample was spread onto Plate Count Agar (PCA, Qingdao Haibo Biotechnology Co., Ltd., Qingdao, China) plates, which were then incubated inverted at 37 ℃ for 24 h to determine the TVC. Colony-forming units were enumerated and expressed as log (CFU/g).

2.8.3. The measurement of TVB-N and pH

TVB-N levels were determined with an automatic Kjeldahl nitrogen analyzer (K9840, Haineng Future Technology Group Co., Ltd., Shandong, China) in accordance with the National Food Safety Standard of China (GB 5009.228-2016). The TVB-N content was expressed as mg per 100 g of sample. The determination of pH was performed following the method proposed by Chen et al.[33].

2.8.4. The measurement of TBARS

TBARS were evaluated using a published method with slight modifications[34]. A 1 g meat sample was accurately weighed and homogenized with 10 mL of 7.5% trichloroacetic acid solution (containing 0.1% disodium ethylenediaminetetraacetate). The mixture was thoroughly vortexed and then incubated in a constant temperature shaker at 50 ℃ for 30 min. After incubation, the mixture was cooled to room temperature and filtered through double-layer qualitative filter paper to collect the filtrate. Subsequently, 5 mL of the filtrate was mixed with an equal volume (5 mL) of 0.02 mol/L thiobarbituric acid solution, and the reaction mixture was heated in a 90 ℃ water bath for 30 min. The absorbance of the resulting solution was measured at a wavelength of 532 nm. The TBARS value is expressed by the standard curve as malondialdehyde (MDA) mg/kg sample.

2.9. Statistical analysis

All experiments were conducted in triplicate. Data analysis and graph plotting were performed using SPSS 16.0 and Origin 2021 software. Data are displayed as mean ± standard deviation (SD).

3. Results

3.1 Screening for proteolytic and lipolytic activities

The spoilage potential of bacteria was evaluated by assessing the degradation resulting from the secretion of proteases and lipases as spoilage indicators. As shown in Table 1, among 34 meat-borne *Enterobacteriaceae* strains incubated at 30 ℃ and 37 ℃, 20 exhibited varying proteolytic activities, whereas only 9 displayed lipolytic activity. Based on the results in Table 1, five strains (YB10, YB16, NC6, NC12 and NC17) with both lipolytic activity and strong proteolytic activity were selected, as illustrated in Fig. 1. Notably, Fig. 1 indicates that NC12 displayed the highest proteolytic and lipolytic activities at 30 ℃, with the corresponding DZD/BCD ratios reaching 3.63 and 2.00, respectively. In contrast, at 37 ℃, the DZD/BCD ratios of YB16 for proteolytic and lipolytic activities were 3.62 and 2.82, respectively, which were significantly higher than those of other strains ($P < 0.05$). In addition, NC17 exhibited remarkable temperature specificity: at 30 ℃, it possessed both enzymatic hydrolytic activities, with DZD/BCD ratios of 3.00 for proteolytic activity and 1.29 for lipolytic activity; however, at 37 ℃, both activities were completely disappeared, as reflected by the corresponding ratios dropping to 0, with no degradation zones observed. In contrast, NC6 lost its proteolytic activity at 37 ℃ but retained it at 30 ℃. The two enzymatic activities of YB10 showed opposite trends at 30 ℃ versus 37 ℃ (lipolytic activity increased with rising temperature, while proteolytic activity decreased accordingly).

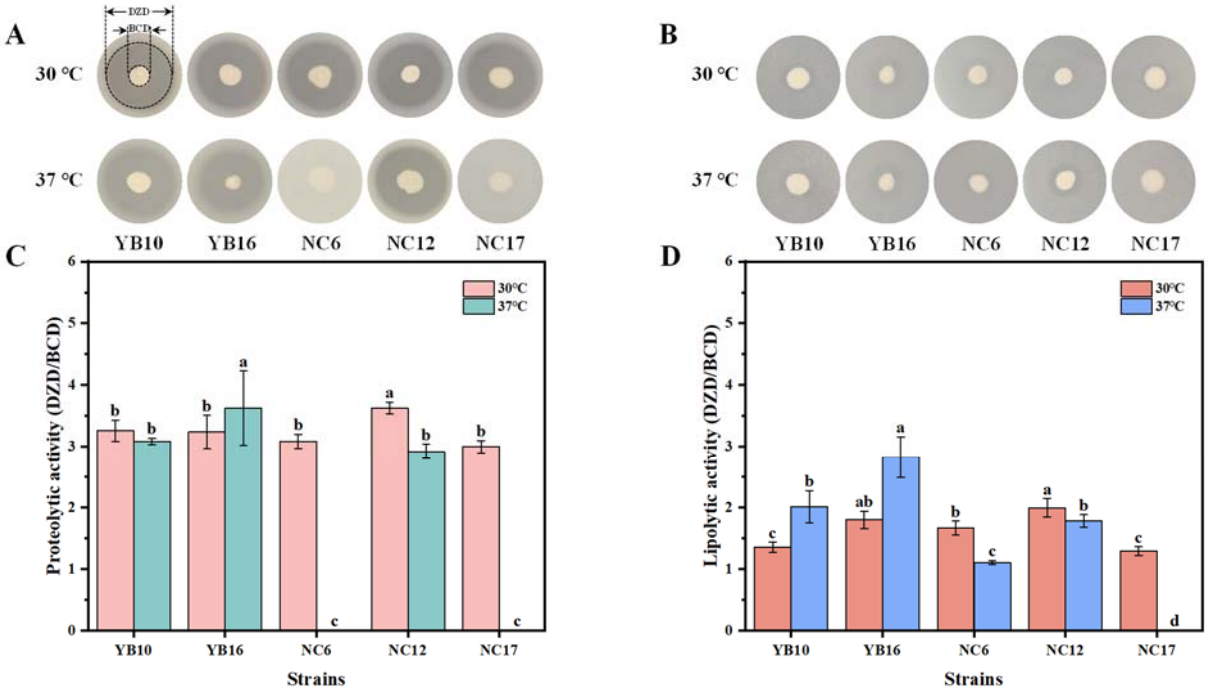


Fig. 1 Proteolytic zones on SMA (A), lipolytic zones on GTM (B), DZD/BCD ratios on SMA (C), and DZD/BCD ratios on GTM (D) of *S. bozhouensis* YB10, *S. myotis* YB16, *S. quinivorans* NC6, *S. liquefaciens* NC12, and *S. grimesii* NC17. All results were expressed as mean ± standard deviation; error bars represent the standard error of the mean (n=3). Different

lowercase letters (a-d) indicate significant differences ($P < 0.05$) in the DZD/BCD ratio among strains at the same incubation temperature.

3.2. Growth curves

The growth curves of strains YB10, YB16, NC6, NC12 and NC17 at 30 ℃ and 37 ℃ are shown in Fig. 2. Based on the changes in OD_{600} and viable colony count, it was observed that under both temperature conditions, the lag phase of the five *Serratia* strains (YB10, YB16, NC6, NC12, and NC17) lasted between 0 and 2 h. Subsequently, NC12 exhibited faster growth, while YB16 grew slower under both temperatures. At 30 ℃, YB10, YB16, NC6, and NC17 entered the stationary phase after 24 h of growth, whereas NC12 entered the stationary phase at 18 h (Fig. 2A and 2C). At this temperature, the maximum viable colony counts of YB10, YB16, NC6, and NC12 exceeded 10^{14} CFU/mL, while those of NC17 were approximately 10^{13} CFU/mL. In contrast, at 37 ℃, both the OD_{600} values and viable colony counts of the five strains during the stationary phase were lower than those at 30 ℃ (Fig. 2B and 2D), indicating that 30 ℃ was a more favorable temperature for the growth of these five strains.

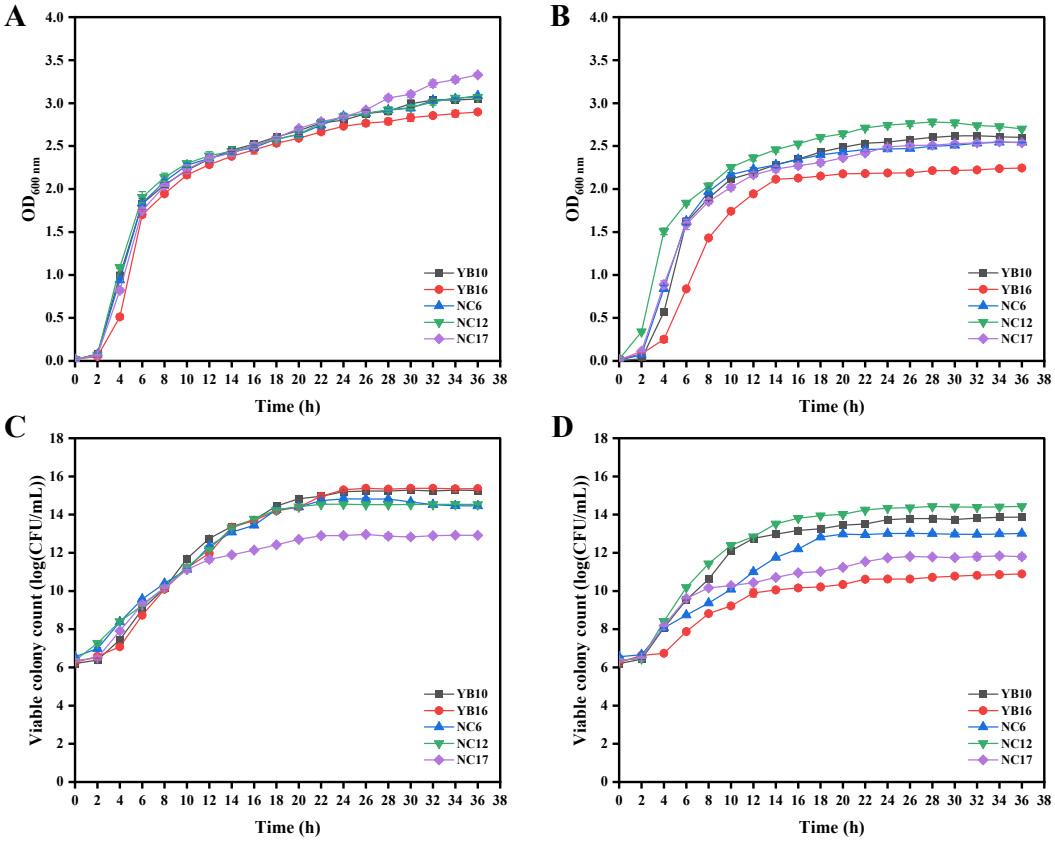


Fig. 2 Growth curves of *S. bozhouensis* YB10, *S. myotis* YB16, *S. quinivorans* NC6, *S. liquefaciens* NC12, and *S. grimesii* NC17 at 30 ℃ (A, C) and 37 ℃ (B, D). All results were expressed as mean ± standard deviation; error bars represent the standard error of the mean (n=3).

3.3. Evaluation of strain motility

The motility of *Serratia* strains was evaluated by measuring the diameter of the expansion zones. Fig. 3 shows that both swimming and swarming motility zones of all strains increased with prolonged incubation time, though differences were observed among strains. Among them, NC12 exhibited the strongest motility: after 96 h at 30 ℃ and 37 ℃, its swimming diameters reached 31.75 and 30.32 mm, and its swarming

diameters were 20.09 and 10.76 mm, respectively. YB10, YB16, NC6, and NC17 showed similar swimming motility performance at both temperatures (Fig. 3A and 3B). For example, the diameter increased by 3–6 mm from 24 h to 48 h of incubation, reaching a motility range from 11 to 16 mm at 48 h. Compared with their swimming motility, the swarming motility of the five strains was relatively weak and exhibited a time-dependent pattern (Fig. 3C and 3D). NC12 was the most significantly affected by temperature, and its swarming motility was attenuated at 37 ℃ compared with that at 30 ℃. Additionally, the swarming motility diameter of YB16 at 37 ℃ increased from 3.58 mm at 24 h to 5.78 mm at 96 h, which was significantly lower than that of other strains ($P < 0.05$). In contrast, the swarming motility diameters of the other four strains were substantially higher at the same time points; for instance, at 96 h, the diameters of YB10, NC6, NC12, and NC17 were 10.71, 9.17, 10.76, and 7.77 mm, respectively.

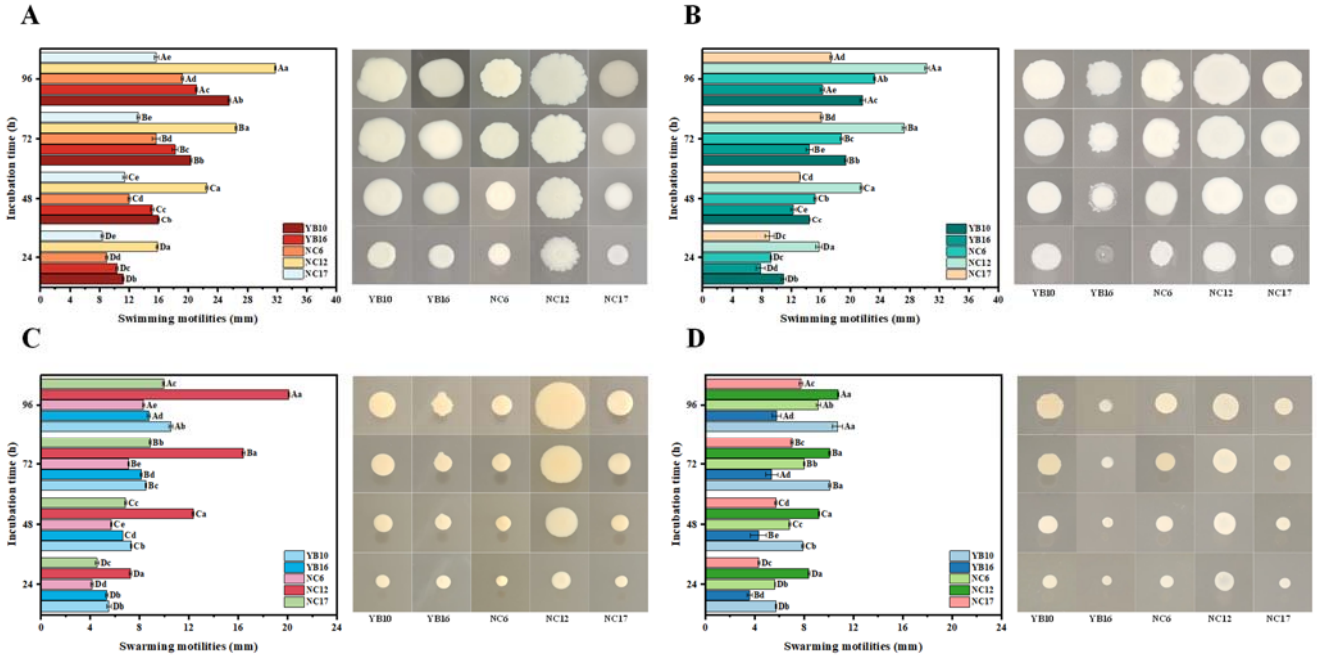


Fig. 3 Swimming and swarming motilities of *S. bozhouensis* YB10, *S. myotis* YB16, *S. quinivorans* NC6, *S. liquefaciens* NC12, and *S. grimesii* NC17. Swimming motility at 30 ℃ (A) and 37 ℃ (C); Swarming motility at 30 ℃ (B) and 37 ℃ (D). All results were expressed as mean ± standard deviation; error bars represent the standard error of the mean (n=3). Different lowercase letters (a-e) indicate significant differences ($P < 0.05$) among strains at the same time point; different uppercase letters (A-D) indicate significant differences ($P < 0.05$) in the same strain at different time points.

3.4. Evaluation of adhesion capacity

Fig. 4A shows the adhesion capacity of all strains to abiotic carriers after 48 h of incubation at 30 ℃ and 37 ℃, and 7 d at 4 ℃. After 7 d of cultivation on stainless steel surfaces at 4 ℃, the number of bacteria adhered by NC6 reached 7.26 lg CFU/cm², which was significantly higher than that of the other four strains ($P < 0.05$). Following 48 h of incubation at 30 ℃ and 37 ℃, the adhesion levels of all strains except YB16 (2.04 lg CFU/cm² at 37 ℃) ranged from 5.8 to 7.0 lg CFU/cm². Further analysis on the impact of temperature on the strains revealed that the bacterial adhesion amounts of YB16, NC6, and NC12 at 30 ℃ were all higher than those at 37 ℃, while the adhesion levels of YB10 and NC17 showed little difference between these two temperatures.

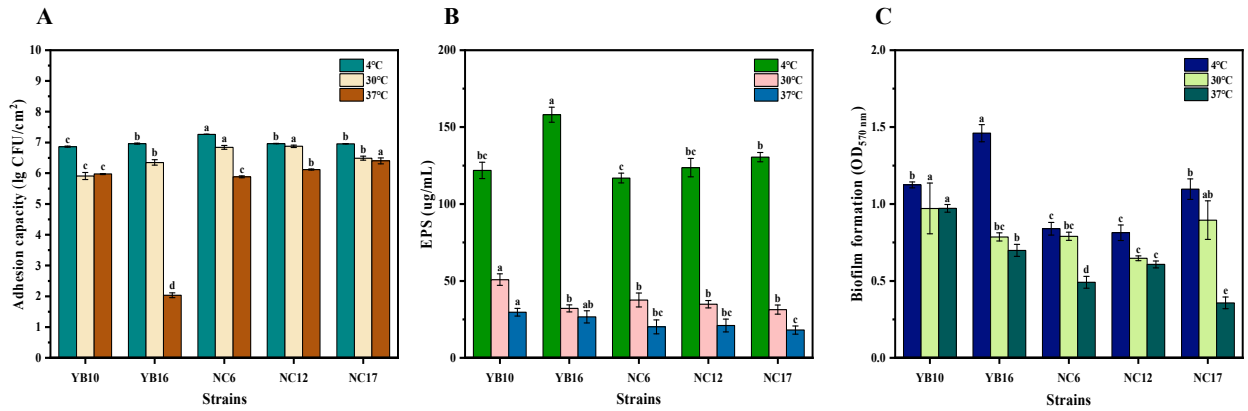


Fig. 4 Adhesion capacity (A), EPS production (B), and biofilm formation (C) of *S. bozhouensis* YB10, *S. myotis* YB16, *S. quinivorans* NC6, *S. liquefaciens* NC12, and *S. grimesii* NC17 under different incubation conditions (4 ℃ for 7 d, 30 ℃ or 37 ℃ for 48 h). All results were expressed as mean ± standard deviation; error bars represent the standard error of the mean (n=3). Different lowercase letters (a-e) indicate significant differences ($P < 0.05$) among strains at the same incubation temperature.

3.5. Evaluation of EPS production

Fig. 4B shows the EPS production of the five *Serratia* strains. All five strains were capable of producing EPS, and the trends in EPS production across different temperatures were both similar to and distinct from the trends observed in their adhesion capabilities. Specifically, after incubation at 4 ℃ for 7 d, YB16 exhibited the highest EPS yield, reaching 158.02 μg/mL, which was significantly higher than that of the other four strains ($P < 0.05$). Meanwhile, YB10, NC6, NC12, and NC17 also maintained relatively high EPS production levels, with yields of 121.81, 116.87, 123.66, and 130.47 μg/mL, respectively. When incubated at 30 ℃ for 48 h, YB10 showed the highest EPS production at 50.87 μg/mL, significantly exceeding the other four strains. In contrast, under 37 ℃ for 48 h, the differences in EPS yield among the strains were smaller. Furthermore, the EPS production capability of all five strains was higher at 30 ℃ than at 37 ℃.

3.6. Evaluation of biofilm formation

The biofilm formation capacity of five *Serratia* strains was assessed under two short-term culture conditions (48 h at 30 ℃ and 37 ℃) and one long-term culture condition (7 d at 4 ℃). Fig. 4C shows that all strains were able to adhere to the well-plate surface, but there were interspecific differences in the amount of biofilm formed, and this difference trend was consistent with the adhesion capacity and EPS production of each strain (see Fig. 4A and 4B). Specifically, when cultured at 4 ℃ for 7 d, the amount of biofilm formed by YB16 was significantly higher than that of the other strains ($P < 0.05$). Under the 48 h culture condition, YB10 and NC17 produced the most biofilm at 30 ℃; at 37 ℃, biofilm levels of the remaining four strains, except YB10, were lower than their respective levels at 30 ℃. Overall, the differences in biofilm formation among the strains were most significant after 48 h of culture at 37 ℃.

3.7. Evaluation of muscle protein degradation

The SDS-PAGE results of incubating extracellular enzymes from five *Serratia* strains with sarcoplasmic proteins and myofibrillar proteins are shown in Fig. 5. No significant differences were

observed between NC17 and the control group, whereas the other four strains (YB10, YB16, NC6, and NC12) exhibited varying degrees of degradation of the aforementioned proteins. Based on the molecular weight standards (10–250 kDa) run in lane M, specific changes in protein bands were analyzed. As shown in Fig. 5A, for sarcoplasmic proteins, the intensity of protein bands at approximately 20–25 and 75–100 kDa was lower in the four strains than in the control, indicating partial degradation of these proteins. In addition, YB16, NC6, and NC12 also exhibited slight degradation of the protein band at approximately 35 kDa, whereas YB10 showed no evident degradation at this molecular weight range. Among myofibrillar proteins, the band at approximately 55 kDa corresponds to actin, whereas those at approximately 80 and 94 kDa represent myosin and α-actinin, respectively. The band at approximately 245 kDa is derived from myosin heavy chain[30,35,36]. All four strains were able to degrade the above-mentioned myofibrillar proteins to a similar extent (Fig. 5B). Specifically, the protein bands at approximately 55 kDa, 130 kDa (unidentified), and 245 kDa disappeared completely, while the two bands at approximately 80 and 94 kDa were partially degraded.

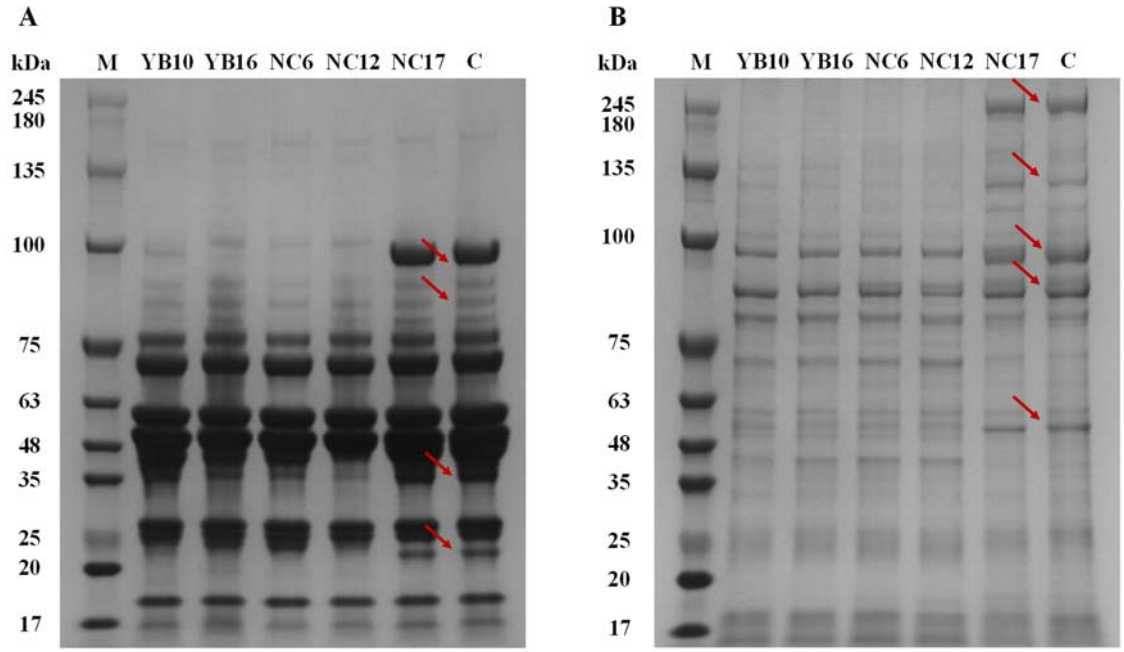


Fig. 5 SDS-PAGE of sarcoplasmic (A) and myofibrillar (B) proteins. Lane M: molecular weight marker; Lane YB10: *S. bozhouensis*; Lane YB16: *S. myotis*; Lane NC6: *S. quinivorans*; Lane NC12: *S. liquefaciens*; Lane NC17: *S. grimesii*; Lane C: control sample. The arrows indicate protein bands that are altered in the samples.

3.8. Evaluation of spoilage potential of Serratia strains

3.8.1. Microbiological analysis

Five *Serratia* strains were inoculated into vacuum-packaged pork and stored at 4 ℃ for 12 d (Fig. 6A). The initial TVC in all inoculated groups was approximately 4.00 log CFU/g, while the TVC of the control group remained unchanged throughout the storage period, indicating that irradiation effectively inactivated all microorganisms in the meat. The TVC of the inoculated groups increased continuously with storage time. Within the first 6 days, the YB10 group showed the fastest growth rate, reaching a TVC of 6.13 log CFU/g on day 6, which exceeded the commonly specified limit of 6.00 log CFU/g[8], suggesting the onset of meat

spoilage by that time. In comparison, the TVC of the YB16, NC6, and NC12 groups reached and exceeded the limit on days 8, 10, and 12 of storage, respectively, whereas the NC17 group exhibited relatively slow growth from day 2 of storage onward, with its TVC remaining below the limit for the entire 12-day period.

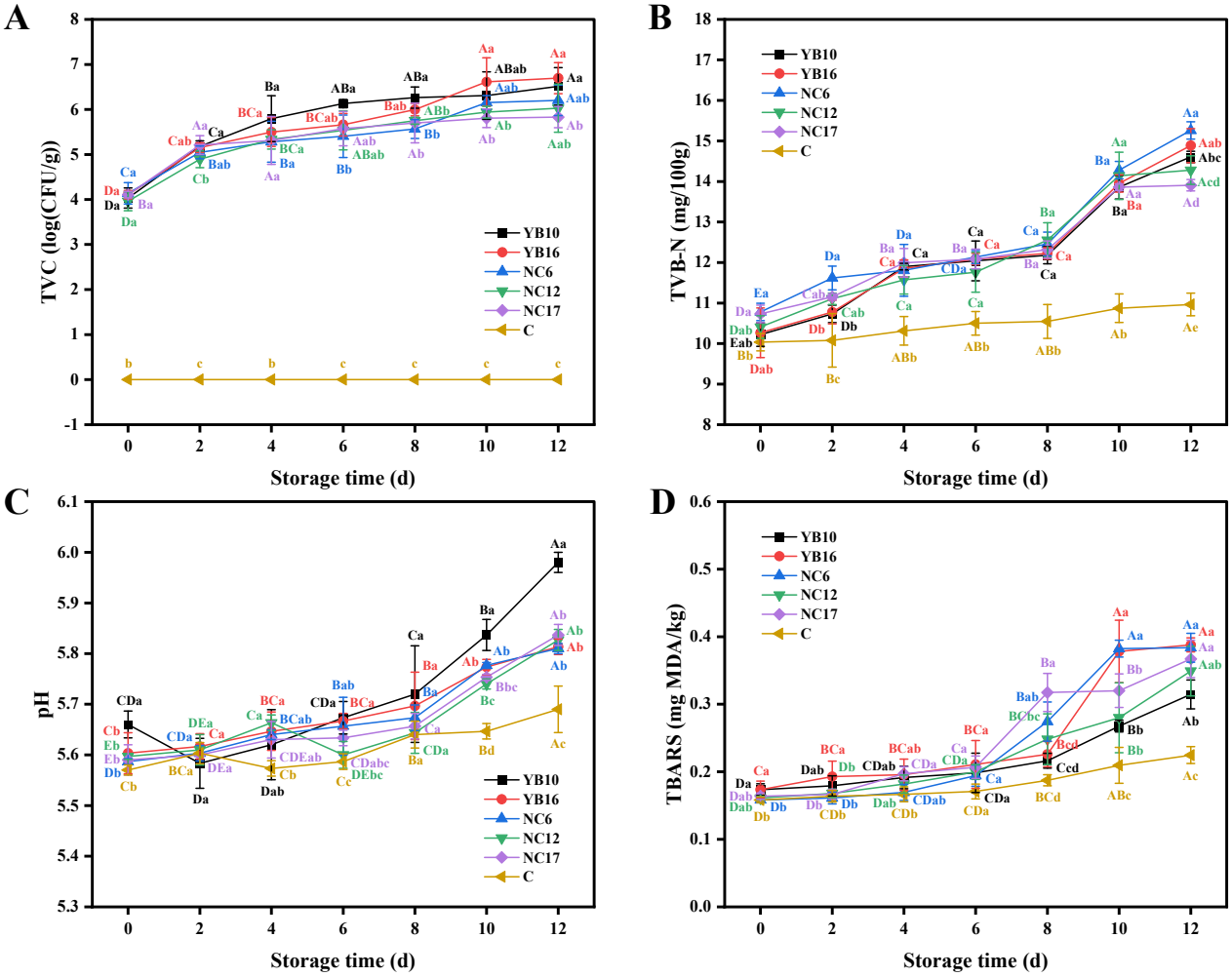


Fig. 6 Changes in TVC (A), TVB-N (B), pH value (C), and TBARS (D) of the sample during storage at 4 °C. All results were expressed as mean ± standard deviation; error bars represent the standard error of the mean (n=3). Different lowercase letters (a-e) indicate significant differences ($P < 0.05$) for the same storage time among different strains; different uppercase letters (A-E) indicate significant differences ($P < 0.05$) for the same strain at different storage times.

3.8.2. TVB-N and pH changes

TVB-N and pH are important indicators reflecting meat freshness. As shown in Fig. 6B, the initial TVB-N content of all groups ranged from 10 to 11 mg/100g. During storage, although the TVB-N content of each inoculated group grew at different rates, all of them began to exhibit a substantial increase starting from the 8th day. By day 12 of storage, the TVB-N content in all inoculated groups was significantly higher than that in the control group ($P < 0.05$). At this time, there was no significant difference in TVB-N content between the NC6 and YB16 groups ($P > 0.05$). However, the TVB-N content of the NC6 group was significantly higher than that of the YB10, NC12, and NC17 groups ($P < 0.05$). Among them, the NC6 group reached 15.26 mg/100g, exceeding the spoilage threshold of 15 mg/100g first. Both YB10 and YB16 groups showed similar growth trends, reaching 14.61 and 14.89 mg/100g by day 12, approaching but not exceeding the spoilage threshold. In contrast, the TVB-N content of the NC12 and NC17 groups changed

less significantly during the later storage period (days 10 to 12), with growth becoming slower. Fig. 6C shows the pH changes of all groups over the 12-day period. The pH values of the inoculated groups remained around 5.6 in the first two days, with minimal differences. However, as storage time extended, the pH values of all groups increased. Among them, the pH value of YB10 began to rise more rapidly from day 8, and the pH difference between YB10 and the other four inoculated strains gradually widened. From day 10 to day 12, the pH values of all inoculated groups were significantly higher than those of the control group ($P < 0.05$), and among the inoculated groups, the pH value of the YB10 group was significantly higher than that of the other four groups ($P < 0.05$). Meanwhile, the pH values of the YB16, NC6, NC12, and NC17 groups after 12 days of storage were 5.81, 5.81, 5.83, and 5.84, respectively, with minimal differences among these four groups. The pH value of the control group remained between 5.57 and 5.69 throughout the storage period.

3.8.3. TBARS changes

The results of TBARS content determination showed that the TBARS values of all groups increased over time throughout the storage period (Fig. 6D). Within the first 6 days of storage, the TBARS values of all inoculated groups increased slowly; after day 6, the growth rates of the NC6, NC12, and NC17 groups accelerated, while those of the YB10 and YB16 groups showed a faster increase starting from day 8. After 12 days of storage, the TBARS values of the YB10, YB16, NC6, NC12, and NC17 groups reached 0.31, 0.39, 0.38, 0.35, and 0.37 mg MDA/kg, respectively, which were significantly higher than that of the control group (0.22 mg MDA/kg) ($P < 0.05$). Among them, there was no significant difference in TBARS values among the YB16, NC6, and NC17 groups, but all three groups were significantly higher than the YB10 group ($P < 0.05$), while there was no significant difference between the NC12 group and the YB10 group ($P > 0.05$).

4. Discussion

In this study, five *Serratia* strains with both protease and lipase activities were screened, suggesting a stronger spoilage potential. This is consistent with previous findings that link such enzyme activities to spoilage[37,38]. Studies have shown that proteases degrade meat proteins to produce free amino acids, reducing meat freshness, whereas lipase-mediated degradation of meat fat induces lipid oxidation and offensive odors[39,40]. In addition, the expression of enzymatic activity is affected by temperature and exhibits clear temperature dependence, indicating that enzyme activity is maintained within a specific temperature range but significantly decreases or is lost when the temperature exceeds a certain threshold[41]. For example, Liu et al.[42] found that *Pseudomonas fragi* NMC206 exhibited proteolytic activity at relatively low temperatures but lost this activity when the temperature exceeded 25 ℃. Similarly, Huarcaya et al.[43] reported that the lipolytic activity of *Staphylococcus ureilyticus* LMB-06 significantly decreased after culture at temperatures above 30 ℃. Similar to these findings, the present study found that at 37 ℃, NC6 showed no proteolytic activity, and NC17 lost both proteolytic and lipolytic activities. Therefore, this study further characterized the spoilage-related phenotypes of the five screened *Serratia* strains under different

temperature conditions, and explored their effects on the quality of vacuum-packaged pork (one of their original isolation matrices), so as to comprehensively evaluate the spoilage potential of the strains.

Studying bacterial motility in a semi-solid environment can reveal their adaptive characteristics and transmission potential in food matrices[44]. In this study, NC12 exhibited strong motility at both 30 ℃ and 37 ℃. Combined with its rapid growth at the two temperatures, these characteristics indicated that NC12 possessed a strong environmental adaptability. This also implied that NC12 could quickly migrate to nutrient-rich regions in the matrix, which might greatly accelerate protein and lipid degradation[45]. Notably, its swarming motility was temperature-sensitive, consistent with a report on *Serratia marcescens*[46]. Bacterial motility is important for enabling microorganisms to approach surfaces during the early adhesion stage[47]; in particular, swarming motility is key for active colonization on solid surfaces such as meat[48]. In addition, since the agar concentration in motility plates is much lower than that in regular agar plates, strains with functional flagella can migrate through this medium to form migration zones[49]. Flagellum-mediated motility is implicated in *Serratia* biofilm formation[50], though the mechanistic link is unreported.

Bacterial biofilm formation is one of the root causes of microbial cross-contamination in the food industry, and strong adhesion often serves as a prerequisite for biofilm development[17,51]. This enables the strains to potentially form biofilms on meat surfaces by secreting extracellular polymeric substances, thereby worsening cross-contamination risks during meat processing. In this study, all five *Serratia* strains exhibited strong adhesion ability. Among these strains, YB16 exhibited a significant difference in adhesion capacity between 30 ℃ and 37 ℃, which might be attributed to the greater influence of culture temperature on this strain[52]. Bacterial adhesion to surfaces is followed by the secretion of EPS, which act as adhesives regulating attachment to abiotic surfaces such as stainless steel and thereby influence biofilm formation[53]. EPS yields were significantly higher at 30 ℃ than at 37 ℃ for all strains, a trend also observed by Vera Pingitore et al.[54]. This might be explained by a metabolic trade-off: at 30 ℃, bacterial metabolism favors protective EPS synthesis, while at 37 ℃, energy is prioritized for rapid proliferation. Biofilm formation protects bacteria from environmental stress and improves tolerance to external factors, allowing them to accelerate meat spoilage and become harder to eliminate from processing environments[55]. Furthermore, the variation trends of biofilm-forming capacity of the five *Serratia* strains with temperature were consistent with those of their adhesion capacity and EPS production. However, differences existed among the tested strains, which may be partially attributed to the interspecific heterogeneity in adhesion and EPS production among different species within the genus *Serratia*. Similar interspecific heterogeneity in biofilm-forming capacity has also been observed in meat-borne bacteria such as *Acinetobacter*, *Staphylococcus*, and *Salmonella*[56-58]. Notably, it was found that YB16 exhibited high adhesion capacity along with significant EPS production and biofilm formation at 4 ℃, compared to the other four strains. We speculate that this may be attributed to a cold-adaptive mechanism similar to that reported in *Shewanella* and *Pseudomonas aeruginosa*, where low temperature upregulates diguanylate cyclase genes, increasing intracellular c-di-GMP levels and thereby promoting adhesion, EPS production, and biofilm formation[59,60]. Although the low temperature-c-di-GMP

regulatory mechanism has not been reported in *Serratia*, future studies measuring c-di-GMP levels and related gene expression are needed to verify this hypothesis.

Spoilage microorganisms secrete proteases to hydrolyze muscle proteins, accelerating spoilage[61]. In this study, no protein degradation was observed in the control group (C), eliminating interference from endogenous muscle enzymes[62]. Except for NC17, the other four *Serratia* strains showed strong degradation capacities toward sarcoplasmic and myofibrillar proteins, indicating high spoilage potential. These results not only confirmed that the genus *Serratia* could participate in meat spoilage by secreting extracellular proteases to hydrolyze key muscle proteins, but also revealed strain-specific differences in protein degradation capabilities, which differ from previous reports showing weaker or limited activities in other *Serratia* strains[13,63]. Notably, some common meat-borne spoilage bacteria differ markedly from *Serratia* in protein degradation capacity. *Brochothrix thermosphacta* and *Acinetobacter* have been reported as key spoilage bacteria in meat[64-66]. However, Casaburi et al.[67] demonstrated that 33 strains of *B. thermosphacta* from raw meat could not degrade sarcoplasmic proteins, and Wen et al.[68] showed that lamb-derived *Acinetobacter* MN21 had little ability to degrade sarcoplasmic or myofibrillar proteins. In contrast, the four *Serratia* strains in this study strongly degraded both sarcoplasmic and myofibrillar proteins. Thus, *Serratia* plays a non-negligible role in meat protein degradation and deserves increased attention.

Microbial growth and proliferation can accelerate meat spoilage. The results showed that all tested strains were able to successfully colonize and proliferate in vacuum-packaged pork, which provided a prerequisite for exerting their spoilage effects. Previous studies have demonstrated that bacterial counts are generally strongly correlated with TVB-N, pH, and other spoilage-related indicators[69]. TVB-N is an alkaline, nitrogen-containing compound generated during protein degradation, serving as an indicator of meat spoilage[70]. The results showed that in the later stage of storage, the increases in TVB-N content and pH value in the groups inoculated with *Serratia* were significantly higher than those in the control groups, which was consistent with the findings of Wang et al.[63]. This may be attributed to the strong proteolytic activity of *Serratia* to promote protein degradation, producing alkaline by-products[39]. Changes in TBARS content reflect lipid oxidation and can serve as an indicator for evaluating the degree of oxidative spoilage in meat[71]. In this study, the TBARS values of all inoculated groups increased continuously over time, which might be attributed to the lipolytic activity of *Serratia* strains that promoted lipid oxidation in pork, leading to the production of malondialdehyde and thus an elevation in TBARS levels[72].

5. Conclusion

The five *Serratia* strains in this study (YB10, YB16, NC6, NC12 and NC17) all exhibited significant proteolytic and lipolytic activities, and showed varying degrees of motility, adhesion capacity, EPS production, biofilm formation and muscle protein degradation capacity. These phenotypes are strain-dependent and affected by temperature. Further inoculation tests on vacuum-packaged pork demonstrated that all strains could grow in meat, resulting in a significant increase in TVC, TVB-N, pH and TBARS after storage, thus accelerating the spoilage process. Among these strains, YB16 demonstrated

strong comprehensive spoilage potential, suggesting that special attention should be paid to such strains in actual production. Although this study has deepened the understanding of the spoilage potential of *Serratia* as meat spoilage bacteria, the research was only conducted on vacuum-packaged pork (one of the strain sources), without considering the effects of temperature fluctuations and the synergistic effects of other spoilage bacteria in actual meat processing. Future studies can further verify the spoilage potential of these strains in other meat and complex microbial communities.

Declaration of competing interests

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

Data availability

Data will be made available on request.

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