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Bacterial diversity and microbiological risk analysis for the ready-to-eat meat products in China

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ABSTRACT

The global popularity of ready-to-eat (RTE) meat products continues to rise due to their convenience; however, this trend also brings potential risks, since RTE meat products can be susceptible to microbial contaminations by various foodborne pathogens or spoilage bacteria. This study is focused on comprehensively investigating the bacterial contamination of RTE meat products in Yangzhou, China with both cultivable and high-throughput sequencing (HTS) approaches. In addition, risk-related phenotypes of the strains in RTE meat products, including biofilm formation, bacterial motility, and spoilage enzyme production, were also explored. Results showed that 90 strains collected from 25 RTE samples belonged to 20 and 50 different genera and species. The genus of *Bacillus* was the most dominant, accounting for 40% of total isolates, followed by *Salmonella* (10%), *Pseudomonas* (10%), and *Staphylococcus* (7.8%). HTS analysis revealed a markedly distinct bacterial population, with *Streptococcus* and *Acinetobacter* being most dominant in RTE meat products. Most of the meat-derived strains were identified as strong biofilm formers with high swarming (0.87–5.35 cm) and swimming (0.88–7.89 cm) activity, and had good capacity to produce proteases (0.88–4.87 A/h·mL at 28 °C, and 0.82–1.21 A/h·mL at 7 °C) and lipases (4.25–83.12 nmol/min·mL at 28 °C, and 6.28–45.93 nmol/min·mL at 7 °C). This work offers novel insights into the potential microbiological risks of strains in RTE meat products, and further promotes the development of advanced methods for RTE meat product preservation.

1. Introduction

Ready-to-eat (RTE) meat products are pre-cooked meats that can be directly eaten or with minimal preparation. Their special flavor, low cost, and convenience have made them one of the most widely consumed foods globally. However, RTE meat products are vulnerable to spoilage and pathogenic bacteria contamination during processing, storage, and transportation[1-2]. As a result, concerns regarding their safety and quality have garnered widespread attention. The common foodborne pathogens in RTE meat products were identified as *Salmonella*, *Staphylococcus aureus*, and *Listeria monocytogenes*, which may pose serious health risks to human health[2-3]. On the other hand, spoilage microorganisms in meat products like *Bacillus*, *Pseudomonas*, *Brochothrix*, and *Acinetobacter* exhibited high resistance to extreme temperatures, pH levels, and salt concentrations, and a high prevalence of the above bacteria has been observed in RTE meat products[3-5]. The spoilage bacteria can lead to undesirable reactions that negatively impact flavor, odor, color, sensory, and textural properties by the production of proteases and lipases[6]. It is estimated that meat deterioration can be up to 40% of the total amount, which may cause huge economic losses to the food industry[6]. However, there is a relative lack of analysis on the bacterial diversity of RTE meat products in China.

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Issues caused by undesirable bacteria in the meat industry extend beyond spoilage potential and health risks. In microbial communities, bacteria typically shift from a planktonic mode to an aggregated state, produce extracellular polymeric substance (EPS), and then form biofilms[7]. Meat-derived spoilage bacteria or pathogens have been shown great capacity to rapidly form dense biofilms on various surfaces in meat-relevant environments[8-12]. The formed biofilms usually show their ability to survive traditional chemical and physical treatments, such as antibiotics, lactic acid, sodium hypochlorite, chlorine dioxide, and low-energy X-ray irradiation[13-15]. Therefore, such contaminated products frequently contact with different equipment surfaces to cause cross-contamination during production, transportation, and storage, and then act as continuous bacterial contamination to pose serious health risks to the consumers[16-17].

Exploring the microbial population in foods has heavily relied on culture-based methods, and the results are often biased towards certain microorganisms; however, the main concern for this method is that many microorganisms in meat products cannot be cultivated through cultivation methods, and it is usually time-consuming[18]. Recently, the wide use of high-throughput sequencing (HTS) provides a much more comprehensive and accurate taxonomic classification of the microbial consortium in foods with increased accuracy, including those that are uncultivable with the traditional culture-relied method[19-20]. To the best of our knowledge, there have been limited results reporting the analysis of bacterial contamination in RTE meat products in China using high-throughput HTS.

China has one of the highest annual consumption rates of RTE meat products, making it essential to monitor bacterial contamination to ensure both product quality and microbial safety. This study conducted a comprehensive analysis of the bacterial community in RTE meat products sourced from markets in Yangzhou, China by integrating culture-based and HTS methods. Additionally, we elucidated safety-related phenotypes of strains from these products, including biofilm formation, bacterial motility, and spoilage enzyme production. The findings of this work may enhance our understanding of bacterial contamination and improve quality management systems for RTE meat products globally.

2. Materials and methods

2.1 Collection of RTE meat product sampless

As shown in Table 1, a total of 25 RTE meat products (labeled from A to Z, except for B) were collected from local markets in Yangzhou from October 2023 to April 2024. According to the storage temperature of different products provided by the manufacturers, 5 samples (H, L, T, U, and X) were kept at room temperature and the other 20 samples were kept at low temperature until analysis, and all were within their use-by dates.

2.2 Enumeration, and identification of bacterial strains by culture-based method

Each RTE meat product was subjected to a traditional culture-dependent method. In brief, a total of 10 g of each meat product was blended in 90 mL buffered peptone water (0.1%, *m/V*) followed by serial dilutions. A 10-fold dilution was plated onto tryptic soya agar (TSA, Difco, Detroit, USA), followed by incubating at 37 °C for 24 h, at 28 °C for 48 h, and 7 °C for 7 days, respectively[21]. After incubation, the total viable bacterial counts of each product were recorded as lg (CFU/g).

A total of 90 bacterial colonies were chosen due to their differing morphological traits observed on TSA plates. DNA extraction was performed from each colony from tryptic soya broth (TSB, Difco, Detroit, USA) by manufacturer's instructions for DNA extraction kit (Axygen, Hangzhou, China), and was kept at –20 °C until use. Universal primers, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) were utilized for PCR amplification of a genomic fragment with 16S rRNA gene sequencing. PCR amplification was performed with a programmable thermal cycler (BioRad Inc., Berkeley, USA) using the following conditions: an initial denaturation at 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 59 °C for 50 s and 72 °C for 90 s, and followed by a final extension at 72 °C for 10 min[22]. Each PCR product was sequenced by a company (Shanghai Sangon Biotechnology Co., Shanghai, China), and identified by comparing them against BLASTN in NCBI to obtain the closest known bacterial species.

2.3 Motility assay of the strains

Motility assays were characterized by using 20 mL TSB in dishes (90 mm) solidified with 0.3% agar for swimming and 0.5% agar for swarming[23]. Briefly, 5 μL of overnight culture of each strain was spotted onto surfaces of swimming or swarming agar plates. After incubation at 28 °C for 48 h, each motility zone (cm) on the surface of plates was measured.

2.4 Measurement of biofilm formation ability of the strains

Biofilm formation of 90 meat-derived strains were determined by a microtiter plate method as provided by Liu et al.[24] with few modifies. In brief, each strain's overnight culture was diluted to an initial concentration at 10^4 CFU/mL in fresh TSB. A total of 200 μL each bacterial suspension were added to 5 wells of 96-well plates for incubation for 48 h at 28 °C and 7 days at 7 °C, respectively. TSB absent of bacterial suspension was treated as a negative control. After incubation, planktonic cells of each strain were discarded, and each well was carefully washed 3 times with sterile phosphate buffer saline (PBS, pH 7.4). The plates were air-dried at room temperature, and fixed by 200 μL methanol for 15 min. Subsequently, the fixed cells were stained with 200 μL of 0.05% (*m/V*) crystal violet (Aladdin Industrial Co., Shanghai, China) for 10 min. Excess dye was washed 3 times with PBS (pH 7.4). After drying, wells were solubilized using 200 μL of acetic acid (Aladdin Industrial Co., Shanghai, China) for 10 min. At last, the absorbance was measured at 594 nm ($OD_{594\ nm}$) using a microplate reader (Thermo Fisher, MA, USA).

2.5 Spoilage potential of the strains

The spoilage potential of each strain was assessed based on the production of proteases and lipases. Protease activity was determined from a method described by Shao et al.[25] with slight changes. Briefly, each strain's overnight culture was inoculated in TSB to achieve a cell concentration at 10^4 CFU/mL, and cultured at 28 °C for 48 h and 7 °C for 7 days.

Table 1
Total viable bacterial count of 25 RTE meat product samples collected from local markets in Yangzhou.

Sample number	Production region	Sample type	Total bacterial count (lg (CFU/g))			Shelf-life	Storage condition
			37 °C	28 °C	7 °C		
A	Anhui	Ham	3.47^{a}	3.42^{a}	3.44^{b}	60 days	0–4 °C
C	Guangdong	Ham	2.48^{b}	2.88^{d}	2.56^{e}	60 days	0–4 °C
D	Shandong	Ham	3.47^{a}	3.00^{c}	3.72^{a}	60 days	0–4 °C
E	Beijing	Steak	3.44^{a}	3.42^{a}	3.15^{c}	60 days	0–10 °C
F	Beijing	Ham	1.00^{g}	1.00^{g}	–	90 days	0–4 °C
G	Shandong	Sausage	1.00^{g}	–	–	60 days	0–4 °C
H	Hebei	Pigskin jelly	–	1.00^{g}	–	60 days	Room temperature
I	Heilongjiang	Drumstick	–	1.30^{f}	–	60 days	0–4 °C
J	Heilongjiang	Sausage	1.80^{c}	1.30^{f}	–	45 days	0–4 °C
K	Shandong	Ham	2.42^{c}	2.78^{e}	2.64^{d}	60 days	0–4 °C
L	Heilongjiang	Sausage	1.36^{d}	–	–	90 days	Room temperature
M	Heilongjiang	Meatloaf	1.20^{ef}	–	–	30 days	0–4 °C
N	Jiangsu	Ham	1.30^{ef}	1.30^{f}	–	90 days	0–4 °C
O	Jiangsu	Ham	1.00^{fg}	1.30^{f}	2.42^{f}	75 days	0–4 °C
P	Heilongjiang	Sausage	1.00^{g}	1.30^{f}	–	45 days	0–4 °C
Q	Liaoning	Roast pork	1.30^{ef}	–	–	90 days	0–4 °C
R	Heilongjiang	Sausage	1.00^{fg}	–	–	120 days	0–4 °C
S	Shandong	Sausage	1.20^{efg}	–	–	45 days	0–4 °C
T	Henan	Breasts	–	–	–	60 days	Room temperature
U	Hebei	Chicken strips	1.00^{g}	1.00^{g}	–	90 days	Room temperature
V	Liaoning	Pot stewed meat	–	–	–	90 days	0–4 °C
W	Heilongjiang	Pork tripe	1.30^{de}	1.00^{g}	–	30 days	0–4 °C
X	Jiangsu	Spiced pig's ear	–	–	–	30 days	Room temperature
Y	Jiangsu	Sausage	–	1.00^{g}	–	90 days	0–4 °C
Z	Heilongjiang	Drumstick	–	–	–	60 days	0–4 °C
Mean			1.22	1.08	0.71		

Note: "–" indicates that total bacterial count was not detected. Different lowercase letters indicate a significant difference ($P < 0.05$).

The supernatant was obtained following centrifugation at 12 000 × *g* for 10 min at 4 °C. Then, 100 μL of each supernatant was combined with 500 μL PBS (pH 7.4) and 100 μL of 1.5% (*m/V*) azocasein (Sigma, St. Louis, USA). The above mixture was incubated at 37 °C for 1 h, after which the reaction was subsequently quenched by the addition of 500 μL of 20% (*m/V*) trichloroacetic acid (TCA, Aladdin Industrial Co., Shanghai, China), and then centrifuged at 12 000 × *g* and 4 °C for 5 min. The absorbance at 366 nm ($OD_{366\ nm}$) was measured using a microplate reader (Thermo Fisher, USA). Lipolytic activity of each strain was determined by a lipase activity kit (Suzhou Grace Biotechnology Co., Ltd., Suzhou, China) based on the manufacturer's instructions.

2.6 HTS analysis of bacterial population in 25 RTE meat products

Analysis of bacterial community in 25 RTE meat products was performed by high-throughput sequencing described by Li et al[22]. Each RTE meat sample was homogenized and total bacterial DNA from 20 g of each RTE meat sample was extracted using the Omega Soil DNA Kit (Omega, Norcross, USA). DNA quality was examined using a 1.2% (*m/V*) agarose gel, while its concentration was determined with a NanoDrop 2000 (Thermo Fisher Scientific, USA). To target overall bacterial communities, primers 338F (5′-CCTACGGGGNGGCWGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) were used to amplify V3–V4 regions for the construction of PCR-based HTS libraries[26]. PCR assay conditions encompassed pre-denaturation at 95 °C for 3 min, succeeded by 27 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 45 s, and final extension at 72 °C for 10 min. Each amplification product was purified using the Illumina MiSeq system. Raw FLASH files were demultiplexed and filtered by Trimmomatic software (version 0.33) and merged with FLASH software (version 1.2.11). Quantitative insights into microbial ecology were performed using QQIME (version 1.9.1), USEARCH (version 7.1), and UPARSE software for identifying, pruning, and processing initiating sequences. α-Diversity (Chao1 richness, Simpson and Shannon indices) were evaluated by QIIME. Each operational taxonomic unit (OTU) with 97% similarity was clustered and analyzed using UPARSE. Identification and removal of chimeric sequences was performed with UCHIME. To analyze the classification of 16S rRNA gene sequence, the Ribosomal Database Project (RDP) classifier algorithm was applied against the Silva (SSU132) 16S rRNA database with a confidence threshold of 70%.

2.7 Statistical analysis

At least 3 replications were set up for each set of tests in this study, and the results of the data obtained were given as mean ± standard

deviation, and analyzed for the significance of differences using One-way analysis of variance (ANOVA) in SPSS 22.0 software (SPSS Inc., Chicago, USA). Only when $P < 0.05$ was considered as a significant difference.

3. Results and discussion

3.1 Identification of meat-derived strains by culture-dependent method

In this study, the total bacterial count and microbial population of each RTE meat product obtained from markets in Yangzhou are shown in Tables 1 and 2. Overall, the bacterial counts varied in different samples collected in Yangzhou, with a range from 1 to 4 lg (CFU/g), and a mean value of 1.22, 1.08, and 0.71 lg (CFU/g) at 37, 28, and 7 °C, respectively. The total bacterial count is commonly used as an indicator of hygiene, and it is usually affected by many factors like original bacterial loads in raw materials, storage conditions, product composition, and packaging style. In another study, about 60% of packaged RTE meat samples showed a total viable bacterial count exceeding 7 lg (CFU/g)[27]. Moreover, only 5 out of 25 samples had bacterial loads over 2 lg (CFU/g), which suggested relatively low bacterial contamination in RTE meat products in China. Psychrotrophic bacteria were only observed in 6 samples (A, C, D, E, K, and O) that were stored at low temperatures. Similarly, around 2 lg (CFU/g) of total viable count was observed in vacuum-packaged smoked bacon when cultured at 7 °C[28]. Psychrophilic bacteria can produce extracellular proteases and lipases. During the storage of refrigerated food, the inactivated enzymes will continue to decompose the protein and fat components in the food, which will seriously affect the quality of the food.

The culture-dependent method has been widely used to identify the prevalent microorganisms in RTE meat products[29]. In the present work, 90 bacterial strains were isolated from the 25 RTE meat samples, and these strains were grouped into 20 and 50 different genera and species (Table 2).

Table 2
Microbial population of 25 RTE meat product samples collected from markets in Yangzhou.

Sample number	Production region	Sample type	Incubation temperature	Isolate number	Strains
A	Anhui	Ham	37 °C	A-1	*B. aerius*
A	Anhui	Ham	37 °C	A-2	*Exiguobacterium profundum*
A	Anhui	Ham	37 °C	A-3	*B. licheniformis*
A	Anhui	Ham	37 °C	A-4	*Bacillus amyloliquefaciens*
A	Anhui	Ham	37 °C	A-5	*Acinetobacter lwoffii*
A	Anhui	Ham	37 °C	A-6	*A. lwoffii*
A	Anhui	Ham	37 °C	A-7	*Bacillus cereus*
A	Anhui	Ham	28 °C	A-8	*A. lwoffii*
A	Anhui	Ham	28 °C	A-9	*B. aerius*
A	Anhui	Ham	28 °C	A-10	*L. fusiformis*
A	Anhui	Ham	28 °C	A-11	*B. cereus*
A	Anhui	Ham	28 °C	A-12	*Acinetobacter baumannii*
A	Anhui	Ham	28 °C	A-13	*Bacillus velezensis*
A	Anhui	Ham	7 °C	A-14	*C. maltaromaticum*
A	Anhui	Ham	7 °C	A-15	*B. thermosphacta*
C	Guangdong	Ham	37 °C	C-1	*B. amyloliquefaciens*
C	Guangdong	Ham	37 °C	C-2	*B. subtilis*
C	Guangdong	Ham	37 °C	C-3	*Bacillus altitudinis*
C	Guangdong	Ham	37 °C	C-4	*B. safensis*
C	Guangdong	Ham	37 °C	C-5	*B. subtilis*
C	Guangdong	Ham	37 °C	C-6	*Bacillus mojavensis*
C	Guangdong	Ham	28 °C	C-7	*B. velezensis*
C	Guangdong	Ham	28 °C	C-8	*B. nakamurai*
C	Guangdong	Ham	28 °C	C-9	*B. subtilis*
C	Guangdong	Ham	28 °C	C-10	*B. safensis*
C	Guangdong	Ham	28 °C	C-11	*B. aerius*
C	Guangdong	Ham	28 °C	C-12	*B. mojavensis*
C	Guangdong	Ham	28 °C	C-13	*T. goriensis*
C	Guangdong	Ham	28 °C	C-14	*Acinetobacter pittii*
C	Guangdong	Ham	7 °C	C-15	*C. gossypii*
C	Guangdong	Ham	7 °C	C-16	*P. azotoformans*
C	Guangdong	Ham	7 °C	C-17	*P. knackmussii*
C	Guangdong	Ham	7 °C	C-18	*Psychrobacter alimentarius*
D	Shandong	Ham	37 °C	D-1	*S. enterica*
D	Shandong	Ham	37 °C	D-2	*P. lactis*
D	Shandong	Ham	28 °C	D-3	*S. enterica*
D	Shandong	Ham	28 °C	D-4	*P. azotoformans*
D	Shandong	Ham	7 °C	D-5	*P. lactis*
D	Shandong	Ham	7 °C	D-6	*S. enterica*
E	Beijing	Steak	37 °C	E-1	*Bacillus anthracis*
E	Beijing	Steak	37 °C	E-2	*P. lactis*

Table 2 (*Continued*)

Sample number	Production region	Sample type	Incubation temperature	Isolate number	Strains
E	Beijing	Steak	28 °C	E-3	*B. safensis*
E	Beijing	Steak	28 °C	E-4	*P. lactis*
E	Beijing	Steak	7 °C	E-5	*B. anthracis*
F	Beijing	Ham	37 °C	F-1	*Staphylococcus epidermidis*
F	Beijing	Ham	28 °C	F-2	*Microbacterium foliorum*
G	Shandong	Sausage	37 °C	G-1	*Peribacillus frigoritolerans*
H	Hebei	Pigskin jelly	37 °C	H-1	*P. frigoritolerans*
I	Heilongjiang	Drumstick	28 °C	I-1	*Bacillus australimaris*
I	Heilongjiang	Drumstick	28 °C	I-2	*Staphylococcus warneri*
J	Heilongjiang	Sausage	37 °C	J-1	*B. safensis*
J	Heilongjiang	Sausage	37 °C	J-2	*Staphylococcus epidermidis*
J	Heilongjiang	Sausage	37 °C	J-3	*B. australimaris*
J	Heilongjiang	Sausage	37 °C	J-4	*B. safensis*
J	Heilongjiang	Sausage	37 °C	J-5	*S. epidermidis*
J	Heilongjiang	Sausage	28 °C	J-6	*S. warneri*
J	Heilongjiang	Sausage	28 °C	J-7	*S. epidermidis*
K	Shandong	Ham	37 °C	K-1	*B. tropicus*
K	Shandong	Ham	37 °C	K-2	*S. enterica*
K	Shandong	Ham	28 °C	K-3	*S. enterica*
K	Shandong	Ham	28 °C	K-4	*Priestia aryabhattai*
K	Shandong	Ham	28 °C	K-5	*S. enterica*
K	Shandong	Ham	28 °C	K-6	*B. anthracis*
K	Shandong	Ham	28 °C	K-7	*P. lactis*
K	Shandong	Ham	28 °C	K-8	*P. yamanorum*
L	Heilongjiang	Sausage	37 °C	L-1	*B. haynesii*
M	Heilongjiang	Meatloaf	37 °C	M-1	*B. anthracis*
M	Heilongjiang	Meatloaf	37 °C	M-2	*Staphylococcus capitis*
N	Jiangsu	Ham	37 °C	N-1	*B. haynesii*
N	Jiangsu	Ham	28 °C	N-2	*S. enterica*
N	Jiangsu	Ham	28 °C	N-3	*Paenibacillus aquistagni*
N	Jiangsu	Ham	28 °C	N-4	*B. haynesii*
O	Jiangsu	Ham	37 °C	O-1	*E. hormaechei*
O	Jiangsu	Ham	7 °C	O-2	*E. hormaechei*
O	Jiangsu	Ham	7 °C	O-3	*Enterobacter cloacae*
P	Heilongjiang	Sausage	37 °C	P-1	*E. hormaechei*
P	Heilongjiang	Sausage	28 °C	P-2	*B. atrophaeus*
Q	Liaoning	Roast pork	37 °C	Q-1	*P. aryabhattai*
Q	Liaoning	Roast pork	37 °C	Q-2	*T. saccharophilus*
R	Heilongjiang	Sausage	37 °C	R-1	*B. haynesii*
S	Shandong	Sausage	37 °C	S-1	*Anaerobacillus alkalidiazotrophicus*
S	Shandong	Sausage	37 °C	S-2	*Shouchella rhizosphaerae*
S	Shandong	Sausage	37 °C	S-3	*Oceanobacillus sojae*
S	Shandong	Sausage	37 °C	S-4	*P. pulmonis*
U	Hebei	Chicken strips	37 °C	U-1	*Prolinoborus fasciculus*
U	Hebei	Chicken strips	28 °C	U-2	*P. fasciculus*
W	Heilongjiang	Pork tripe	37 °C	W-1	*B. velezensis*
W	Heilongjiang	Pork tripe	37 °C	W-2	*S. enterica*
W	Heilongjiang	Pork tripe	37 °C	W-3	*S. enterica*
Y	Jiangsu	Sausage	28 °C	Y-1	*B. haynesii*

Bacterial population in the 25 RTE meat samples was quite different, likely due to variations in slaughtering procedures, post-slaughter handling practices, and general hygiene standards maintained at different points of a processing chain[30]. At the genus level (Fig. 1A), *Bacillus* had the highest relative abundance (40%), followed by *Salmonella* (10%), *Pseudomonas* (10%) and *Staphylococcus* (7.8%). In previous works, *Bacillus*, *Pseudomonas*, *Salmonella*, and *Staphylococcus* have also been reported to be the abundant genera in RTE meat products[31-33]. Strains of both *Bacillus* and *Pseudomonas* are widespread in nature (soil, water, and dust) from where it easily spreads to the meat products. In general, *Bacillus* and *Pseudomonas* strains show great spoilage potential in raw meat and meat products, since they can produce proteases and lipases that lead to discolorations, off-odors, and slime formation of meat products[34-35]. In addition, *Bacillus* species may also carry toxin genes (*nheA*, *nheB*, *nheC*, *entFM*), and the food involved in outbreaks associated with bacillus is usually heat-treated, as it causes spores to germinate, and in the absence of competing flora, *Bacillus* grows well after cooking[36-37]. *Salmonella* is an important and frequently identified foodborne pathogen in raw meat and meat products, and can cause headache, gastroenteritis, nausea, and other salmonellosis symptoms to humans[10]. High prevalence of *Salmonella* was also reported to be 41% among 79 RTE meats in Taiwan region[38], and 28.4% of 257 RTE meat products in Ethiopia[30]. Some *Staphylococcus* strains, like *S. aureus*, are also the key foodborne pathogens identified in meat products, which can cause human disease by producing toxins[35]. Despite that the source and bacterial diversity of the raw meat were not addressed in this work, the high

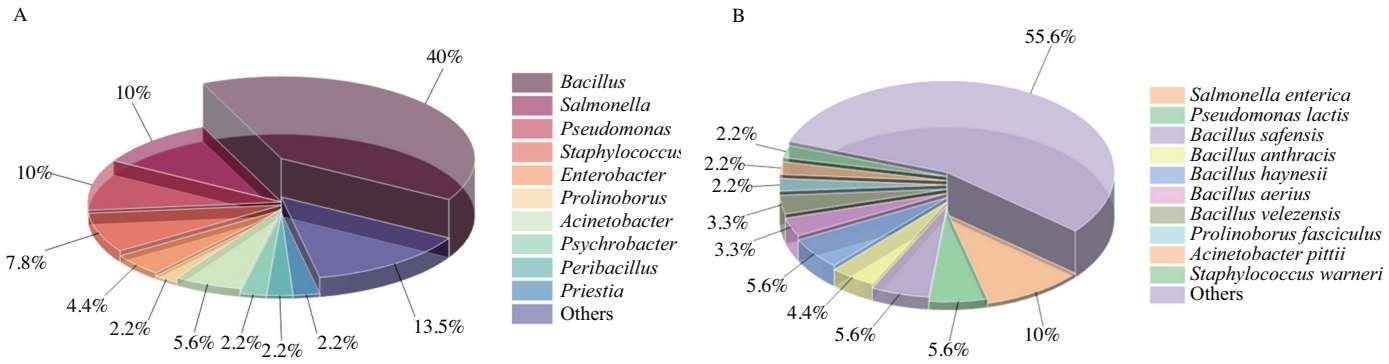


Fig. 1 Distribution of culturable bacteria in 25 RTE meat products obtained from Yangzhou at both (A) genus level and (B) species level.

levels of spoilage bacteria and foodborne pathogens in RTE meat could be caused by the high bacterial counts in the original raw meat. In addition, inadequate sterilization, cross-contamination from workers and unhygienic processing environments, and poor handling practices can also lead to low microbial quality of meat products.

At species level, *Salmonella enterica* (10%) was presented in 4 (sample D, K, N, and W) out of the 25 samples, followed by *Pseudomonas lactis* (5.6%), *Bacillus safensis* (5.6%), and *Bacillus haynesii* (5.6%) (Fig. 1B). High percentage of *S. enterica* has also been confirmed in raw meat samples and RTE meats in China and Namibia[31,39]. To sum up, results on bacterial diversity in this study indicate potential quality and safety risks of RTE meat during its shelf-life. The presence of the above spoilage bacteria and foodborne pathogens must be tightly monitored and controlled during production, transportation, and storage.

3.2 Biofilm formation by the meat-derived strains

In the meat industry, microorganisms commonly colonize surfaces, form biofilms, and exhibit resistance to conventional chemical and physical treatments, thereby serving as persistent reservoirs for spoilage and pathogenic bacteria[7-8]. Therefore, determination of biofilm formation by meat-derived strains is crucial for ensuring both microbial safety of meat products.

According to different storage conditions of 25 RTE meat products, biofilm formation by 90 strains were measured at both 7 and 28 °C using crystal violet staining. $OD_{594\ nm}$ values of 90 strains were in a range of 0.07–3.44 at 28 °C. The highest biofilm-forming ability was exhibited by *Bacillus atrophaeus* P-2 ($OD_{594\ nm}$ = 3.44), followed by *Bacillus subtilis* C-5 ($OD_{594\ nm}$ = 2.82) and *Bacillus licheniformis* A-3 ($OD_{594\ nm}$ = 2.72). In addition, the ability to form biofilms was confirmed to be in a strain-specific way, as 52, 10, 21, and 7 strains were identified as good, moderate, weak, and none biofilm formers, respectively (Fig. 2). Strong biofilm formation potential of *Bacillus* and other meat-derived strains have also been reported in previous studies, which suggests the need to optimize efficient biofilm control procedure in the food industry[40-41].

Meanwhile, the biofilm formation by 11 psychrophilic bacteria was measured at 7 °C. Higher biofilm formation of most strains was assessed by crystal violet staining when the biofilms were formed at the lower temperature. The $OD_{594\ nm}$ values of the 11 psychrophilic bacteria ranged from 0.82 to 2.31, with the highest value recorded

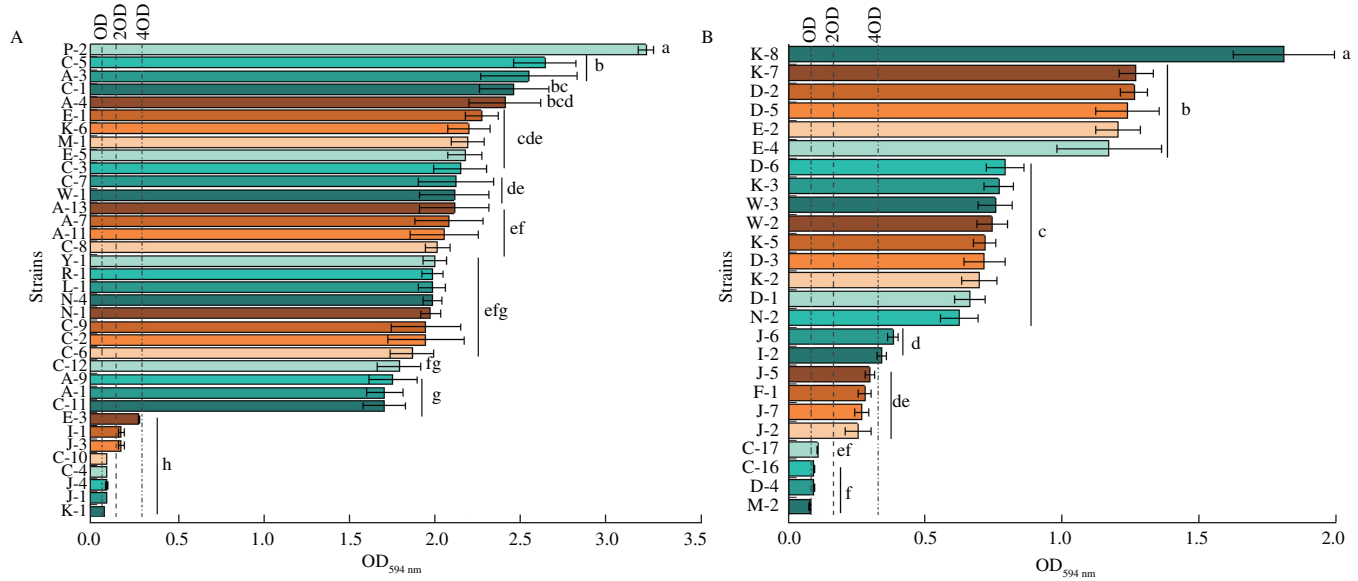


Fig. 2 Biofilm-forming capacity of the meat-derived strains in 96-well plates. (A) Biofilm formation by *Bacillus* strains at 28 °C for 2 days. (B) Biofilm formation by *Salmonella*, *Pseudomonas*, and *Staphylococcus* strains at 28 °C for 2 days. (C) Biofilm formation by other strains at 28 °C for 2 days. (D) Biofilm formation by 11 psychrophilic bacteria at 7 °C for 7 days. Different lowercase letters indicate a significant difference ($P < 0.05$).

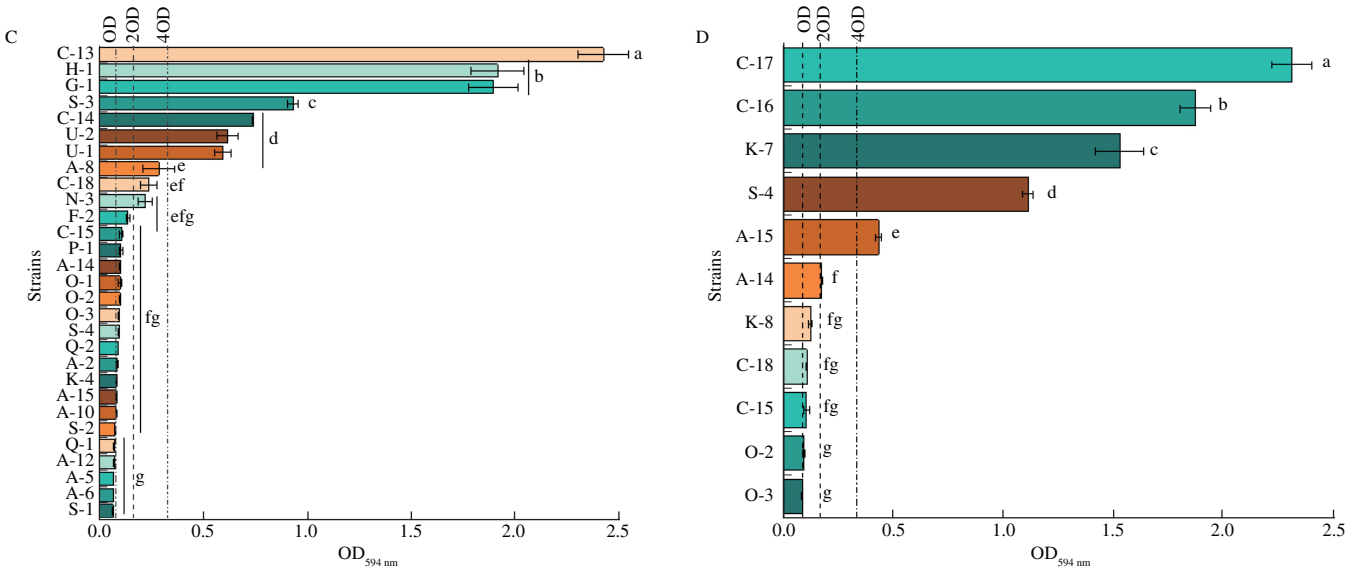


Fig. 2 (*Continued*)

for *Pseudomonas knackmussii* C-17 ($OD_{594\ nm}$ = 2.31) followed by *Pseudomonas azotoformans* C-16 ($OD_{594\ nm}$ = 1.87), and *P. lactis* K-7 ($OD_{594\ nm}$ = 1.53). In addition, 5, 1, and 5 strains were identified as strong, moderate, and weak biofilm formers, respectively (Fig. 2D). Low temperatures can be considered microbial stress to enhance EPS production, and the high biofilm-forming capacity of *Pseudomonas* were found at low temperatures[22,42]. Biofilm formation by *Pseudomonas* strains led to increased production of spoilage enzymes by sessile cells, and further protected pathogens from chemical disinfectants, creating serious challenges for the meat industry[43-44]. The result demonstrated the varying biofilm-forming abilities within a species or genus, which can enhance the understanding of the biofilm formation by bacteria that are prevalent in RTE meat products, and is important for developing strategies to control their biofilm formation in meat processing environment.

3.3 Motility assay of the meat-derived strains

Bacterial motility is a notable feature of microbial physiology that contributes to biofilm spread and formation[45]. In the present work, 90 isolates were evaluated for their swarming and swimming capacity at 28 °C (Figs. 3 and 4). The swarming motility of the strains was in a range from 0.87 (*P. lactis* E-4) to 5.35 cm (*Bacillus aerius* C-11). Unlike swarming ability, *Enterobacter hormaechei* O-2 exhibited the highest swimming capacity (7.89 cm), followed by *E. hormaechei* O-1 (7.88 cm), and *Lysinibacillus fusiformis* A-10 (7.22 cm), while the weakest swimming ability was recorded in *Terribacillus saccharophilus* Q-2 (0.88 cm). The findings indicated a poor correlation between biofilm formation and bacterial motility, since the relationship can be affected by factors like specific strains, temperature, cell concentration, and other environmental conditions[20].

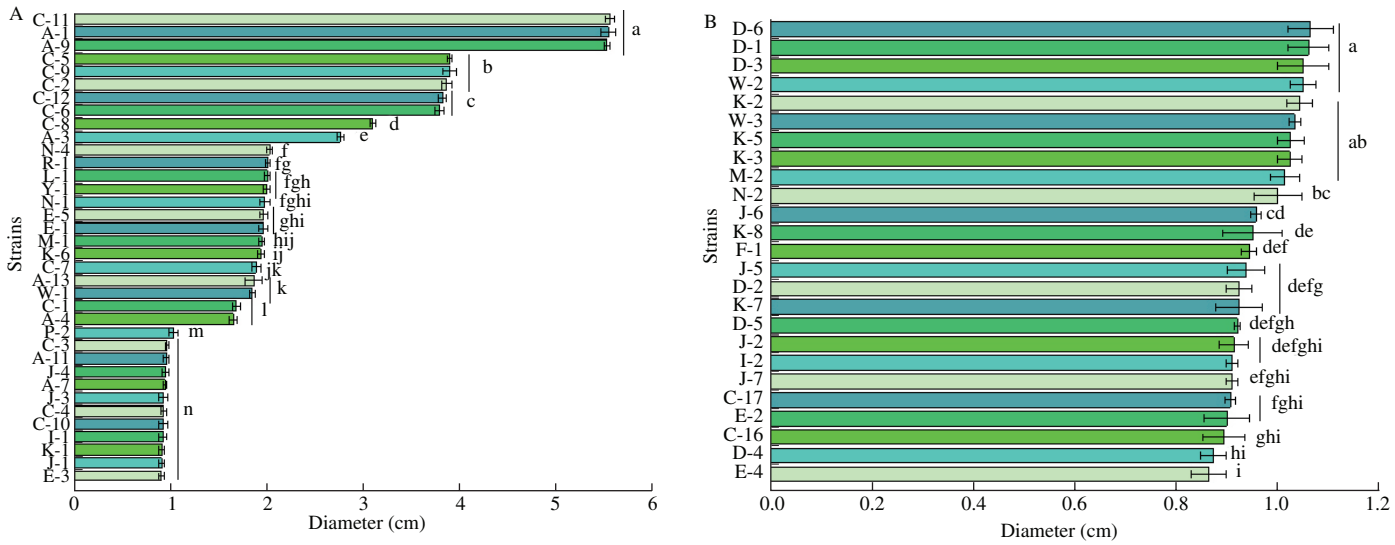


Fig. 3 Measurement of swarming motility of the meat-derived strains. (A) Swarming motility of *Bacillus* strains at 28 °C for 2 days. (B) Swarming motility of *Salmonella*, *Pseudomonas*, and *Staphylococcus* strains at 28 °C for 2 days. (C) Swarming motility of other strains at 28 °C for 2 days. Different lowercase letters indicate a significant difference ($P < 0.05$).

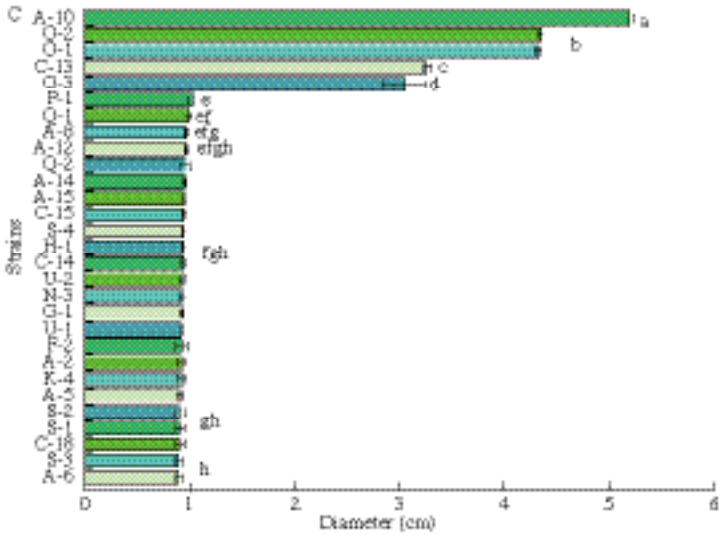
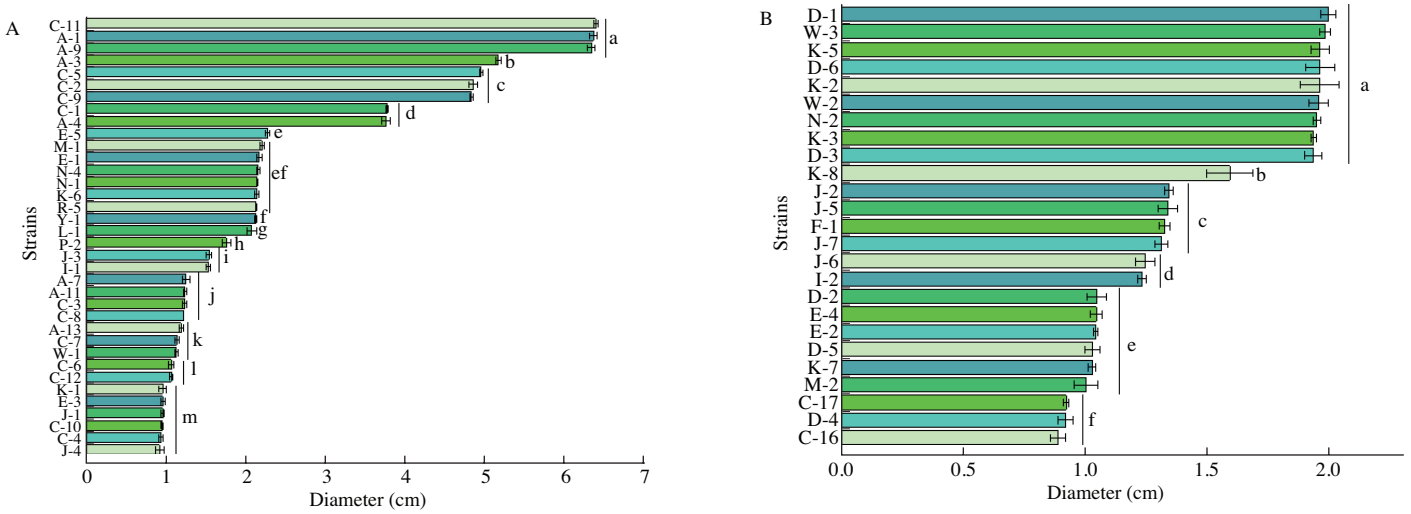


Fig. 3 (*Continued*)



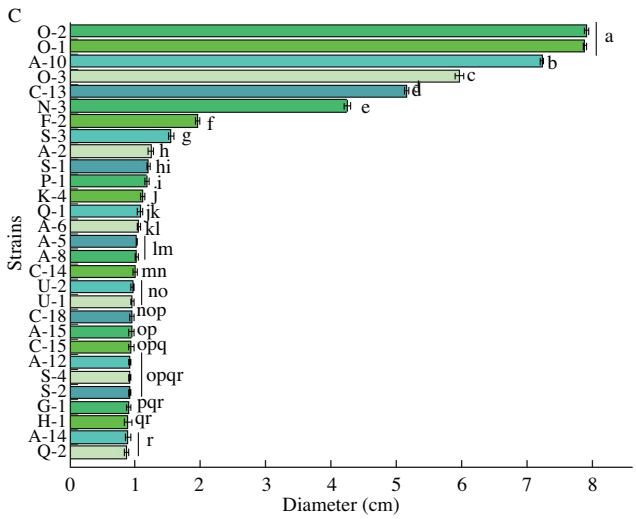


Fig. 4 Measurement of swimming motility of the meat-derived strains. (A) Swimming motility of *Bacillus* strains at 28 °C for 2 days. (B) Swimming motility of *Salmonella*, *Pseudomonas*, and *Staphylococcus* strains at 28 °C for 2 days. (C) Swimming motility of other strains at 28 °C for 2 days. Different lowercase letters indicate a significant difference ($P < 0.05$).

3.4 Protease and lipase production by the meat-derived strains

Meat products are typically rich in high-quality proteins, lipids, and other nutrients that are very beneficial to human nutrition. Extracellular proteases or lipases produced by spoilage bacteria play a significant role during food spoilage[6,25]. The spoilage enzymes can be secreted when the microbial population reaches 10^7 to 10^8 CFU/g, and breakdown of fats and proteins in meat can lead to the observation of off-odors, off-tastes, discolorations, and slime formation in meat products[46]. Proteases can degrade proteins and produce free amino acids, which can lead directly to nutrient loss and loss of freshness of meat, ultimately leading to meat spoilage. In this study, 81 strains were proved to have the ability to produce proteases at 28 °C, and the highest protease activity was observed in *Terribacillus goriensis* C-13 (4.87 ΔA/h·mL), followed by *Bacillus nakamurai* C-8 (4.63 ΔA/h·mL) and *B. amyloliquefaciens* C-1 (4.58 ΔA/h·mL) (Fig. 5). In addition, the protease production by the strains was significantly lower at 7 °C than at the temperature of 28 °C, with the highest protease activity by *Pseudomonas yamanorum* K-8 (1.21 ΔA/h·mL), *Psychrobacter pulmonis* S-4 (0.98 ΔA/h·mL) and *Brochothrix thermosphacta* A-15 (0.95 ΔA/h·mL) at 7 °C (Fig. 5D).

Unlike the protease activity, 81 isolates were identified to be lipolytic, and the maximum lipase activity was observed in *Bacillus tropicus* K-1 (83.12 nmol/min·mL), followed by *P. lactis* D-2 (56.22 nmol/min·mL) and *P. lactis* K-7 (56.89 nmol/min·mL) (Fig. 6). Also, relatively weak lipase activity of the strains at 7 °C was observed. The highest lipase activity was found in *P. azotoformans* C-16 (45.93 nmol/min·mL), followed by *Curtobacterium gossypii* C-15 (44.09 nmol/min·mL), and *Carnobacterium maltaromaticum* A-14 (42.98 nmol/min·mL) (Fig. 6D). The unpleasant odor detected in spoiled meat products is linked to lipases and therefore with lipid oxidation[47].

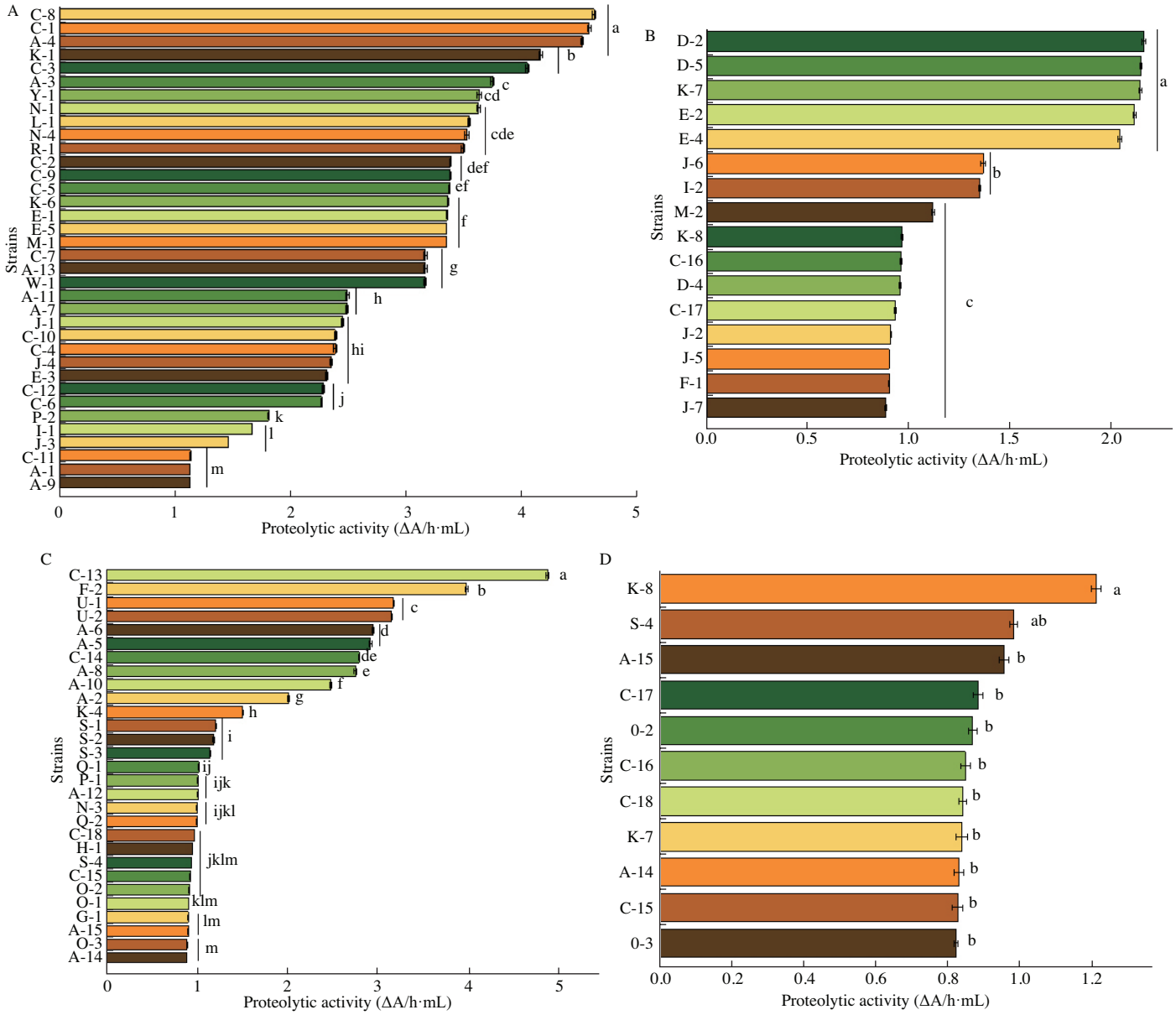


Fig. 5 Production of protease by the meat-derived strains. (A) Production of protease by *Bacillus* strains at 28 °C for 2 days. (B) Production of protease by *Pseudomonas*, and *Staphylococcus* strains at 28 °C for 2 days. (C) Production of protease by other strains at 28 °C for 2 days. (D) Production of protease by 11 psychrophilic bacteria at 7 °C for 7 days. Different lowercase letters indicate a significant difference ($P < 0.05$).

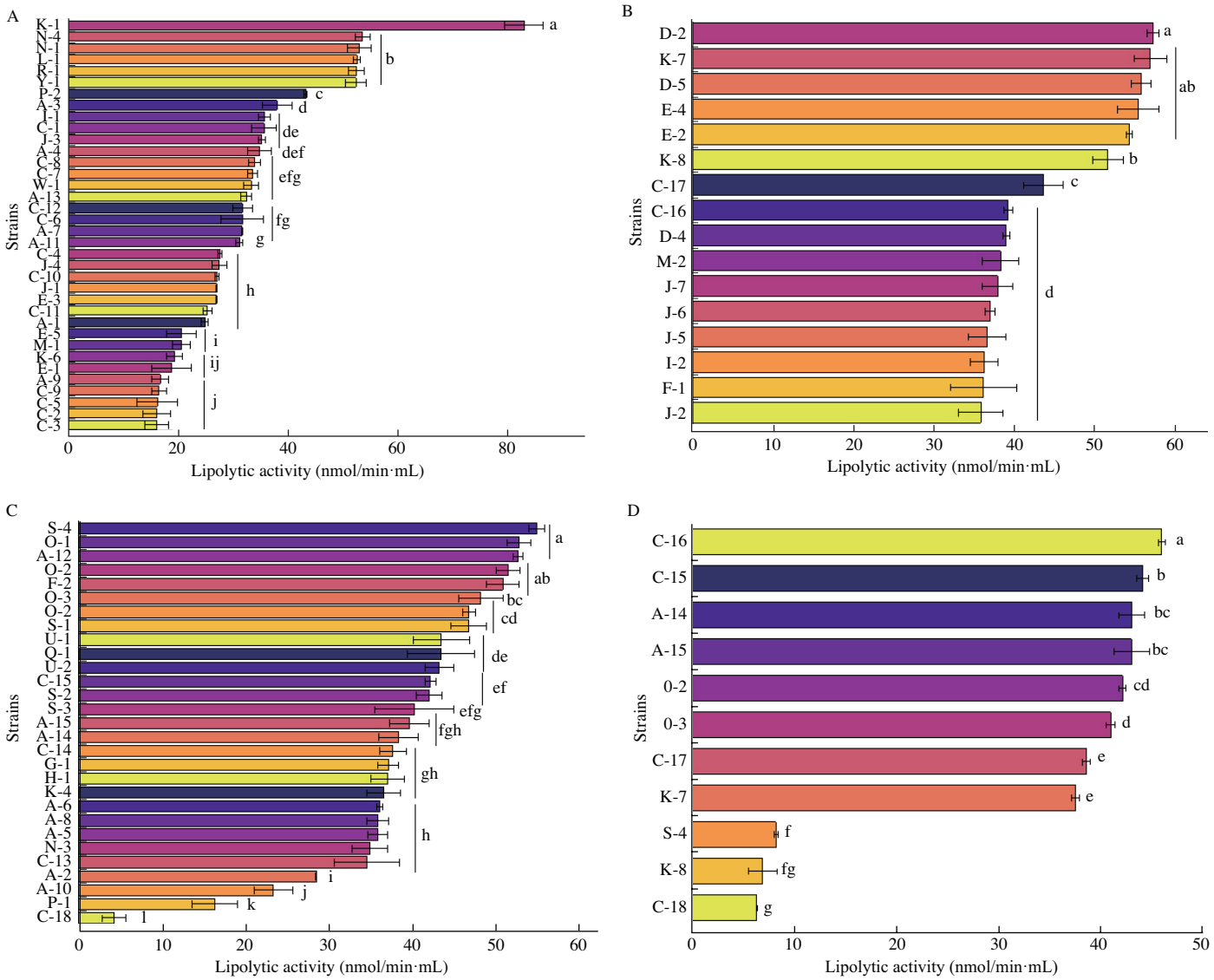


Fig. 6 Production of lipase by the meat-derived strains. (A) Production of lipase by *Bacillus* strains at 28 °C for 2 days. (B) Production of lipase by *Pseudomonas*, and *Staphylococcus* strains at 28 °C for 2 days. (C) Production of lipase by other strains at 28 °C for 2 days. (D) Production of lipase by 11 psychrophilic bacteria at 7 °C for 7 days. Different lowercase letters indicate a significant difference ($P < 0.05$).

As shown in Fig. 7, the differences in the microbiological risk properties between the strains were further compared. The horizontal axis represents the number of strains, while the vertical axis indicates the bacterial properties examined in this study. It is observed that *Bacillus* isolates pose a potential risk to the meat industry, as the strains exhibit strong biofilm-forming capacity, swimming and swarming motility, and protease production. Thus, this bacterial group should be of particular concern in the meat industry.

3.5 HTS analysis of bacterial diversity in RTE meat products

In the present work, HTS analysis was conducted to enable a comprehensive characterization of the bacterial community of 25 RTE meat samples. The α-diversity index (Chao1, Shannon, Simpson, and Coverage indices) for each sample in this work is presented in Table 3. Shannon index and Simpson index represent the species diversity, Chao1 index reflects species richness. The highest and lowest Shannon index was found in RTE meat products of S and Y, respectively, while the highest and the lowest Simpson indices were found in samples K and U, indicating that species diversity of sample K was higher than others. Coverage, a characterization index of sampling completeness, was all in a range from 0.99 to 1, indicating that the HTS is highly sensitive for detecting microbial communities in the collected 25 RTE meat product samples.

Venn diagram was applied to analyze bacterial distribution of OTUs in the 25 prepared meat products (Fig. 8A). However, no OTUs were shared among the meat products, indicating the very low biodiversity similarity among different prepared meat samples. To further explore the bacterial community composition in meat products, the β-diversity of 25 samples was analyzed by principal component analysis (PCA) analysis. The first 2 principal coordinates (PC1 and PC2) accounted for 56.70% of

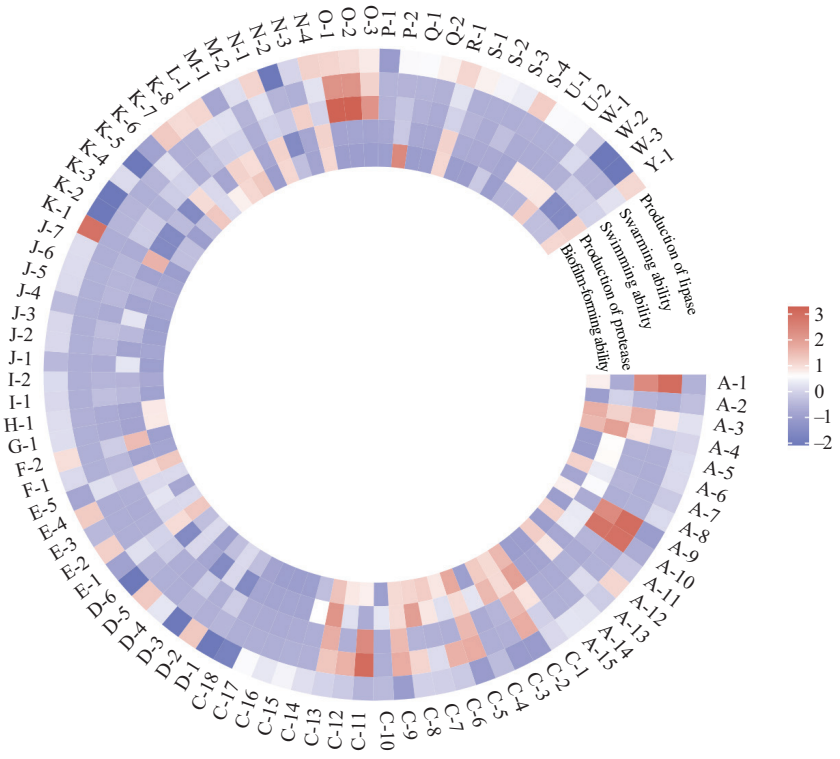


Fig. 7 Heatmap analysis of biofilm-forming ability, swimming and swarming ability, and protease and lipase production in meat-derived strains (n = 90).

Table 3
Sample sequencing information and α-diversity indices of RTE meat products.

Sample	Chao1 index	Simpson index	Shannon index	Coverage
A	310.261	2.757 03	0.616 671	0.997 684
C	103.13	4.810 25	0.944 802	0.999 441
D	658.869	5.239 22	0.887 779	0.996 325
E	339.688	3.247 2	0.733 701	0.997 789
F	630.908	5.447 83	0.920 58	0.995 832
G	615.881	5.325 63	0.891 18	0.997 887
H	722.068	5.615 89	0.905 283	0.995 161
I	827.427	6.345 46	0.950 269	0.996 985
J	931.213	5.764 23	0.900 176	0.997 496
K	523.889	7.195 8	0.986 273	0.999 414
L	903.593	6.272 34	0.957 042	0.994 165
M	984.752	5.722 93	0.898 275	0.994 125
N	686.696	6.203 41	0.952 356	0.997 973
O	892.701	7.036 25	0.976 993	0.994 098
P	750.258	5.011 05	0.824 544	0.995 805
Q	142.163	5.041 52	0.904 987	0.999 473
R	298.824	3.406 14	0.707 37	0.998 949
S	777.34	7.185 06	0.974 491	0.995 364
T	31.9783	3.190 74	0.863 776	0.999 789
U	32.9542	2.636 32	0.803 789	0.999 695
V	48.9357	3.359 93	0.878 642	0.999 652
W	642.355	6.027 12	0.951 081	0.996 731
X	46.686 7	3.130 51	0.862 585	0.999 605
Y	186.162	2.683 28	0.548 092	0.999 383
Z	309.105	2.834 39	0.644 236	0.997 473

variance in microbial assemblages, with PC1 explaining 33.4% by itself (Fig. 8B). Despite the great difference in the bacterial composition of the 25 samples, the products were clustered based on the storage condition, since the bacterial composition of meat products (H, L, T, U, and X) storage at room temperature was distinct from the other samples storage at low temperature (Fig. 8B). On the other hand, the meat processing method is another factor to affect the bacterial community of meat products, since the 2 fermented products (A and Z) were distant from the other 23 samples (Fig. 8C). It has also been found in the study that variations in ingredients and processing technologies can have a profound impact on microbial diversity[19]. Correlation loading plots of measured values for PC1 and PC2 are shown in Fig. 8D, which characterized the top 10 genera (*Latilactobacillus*, *Streptococcus*, *Pseudomonas*, *Acinetobacter*, *Enterococcus*, *Citrobacter*, *Diaphorobacter*, *Ralstonia*, and *Photobacterium*) that are different from each meat product sample. In addition, the unweighted pairwise group method and arithmetic mean (UPGMA) analysis were used to explore differences in bacterial composition in different prepared meat samples (Fig. 8E), and the results were consistent with those from PCA analysis, indicating that the processing method was the key to influence its bacterial diversity.

Further, the species classification of OTUs in the samples was carried out by comparing them with the database and characterizing the bacterial population in prepared meat product samples at phylum and genus levels, respectively (Fig. 9). Total sequences corresponded to 28 phyla, 619 genera, and 1 200 species in 25 meat products. At the phylum level (Fig. 9A), Firmicutes (44.92%) emerged as the most dominant phylum in all meat products, with a high percentage observed in the majority of samples. Proteobacteria (34.23%) was identified as the second dominant phylum, and its high abundance was found in samples C (65.43%), G (53.34%), K (58.17%), L (69.47%), N (52.73%), T (78.69%), U (94.62%), W (71.70%), and X (81.92%). The core microbiota at the phylum level is comparable to the primary microbial communities found in braised chickens and fermented sausage[48-50]. Under limited atmospheric conditions, the phylum Proteobacteria was categorized as the most active microorganisms associated with the deterioration of fresh and refrigerated pork, and the high percentage of Proteobacteria in the samples may be attributed to the cross-contamination of carcasses during slaughter and processing[4,51].

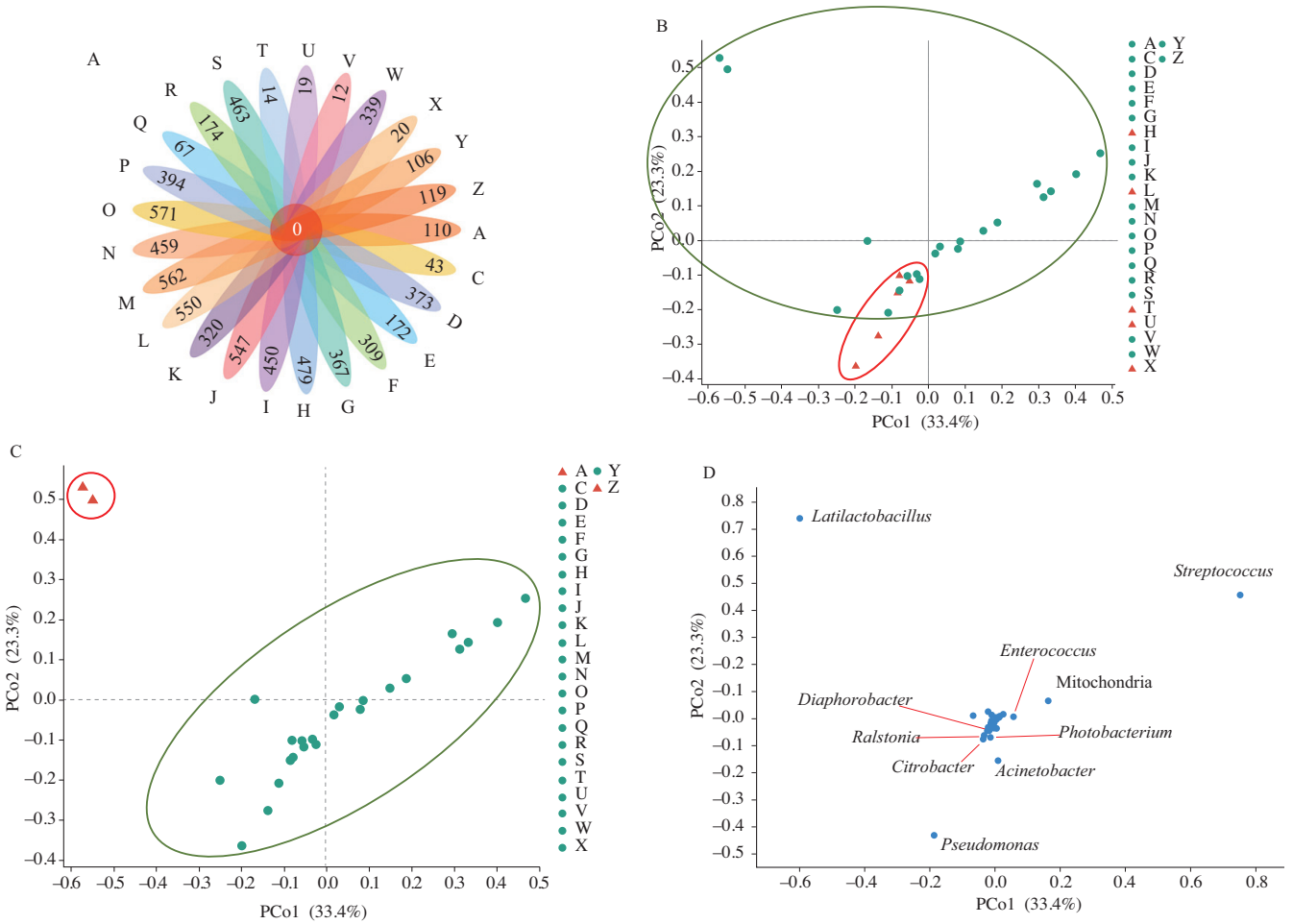
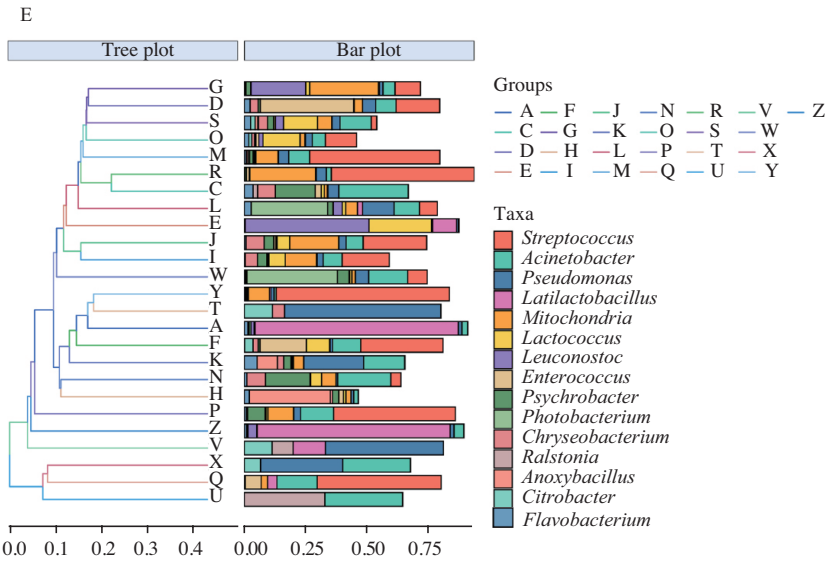


Fig. 8 Bacterial community analysis of the 25 RTE meat products by high-throughput sequencing method. (A) Venn diagram of 25 RTE meat products according to the bacterial biodiversity. (B) PCA of bacterial community in 5 RTE meat products stored at low temperature and 20 RTE meat products stored at room temperature. (C) PCA of the bacterial community in 2 fermented RTE meat products and 23 other RTE meat products. (D) Loadings plot. (E) The taxonomic composition of bacterial communities in RTE meat product samples was determined by UPGMA analysis.



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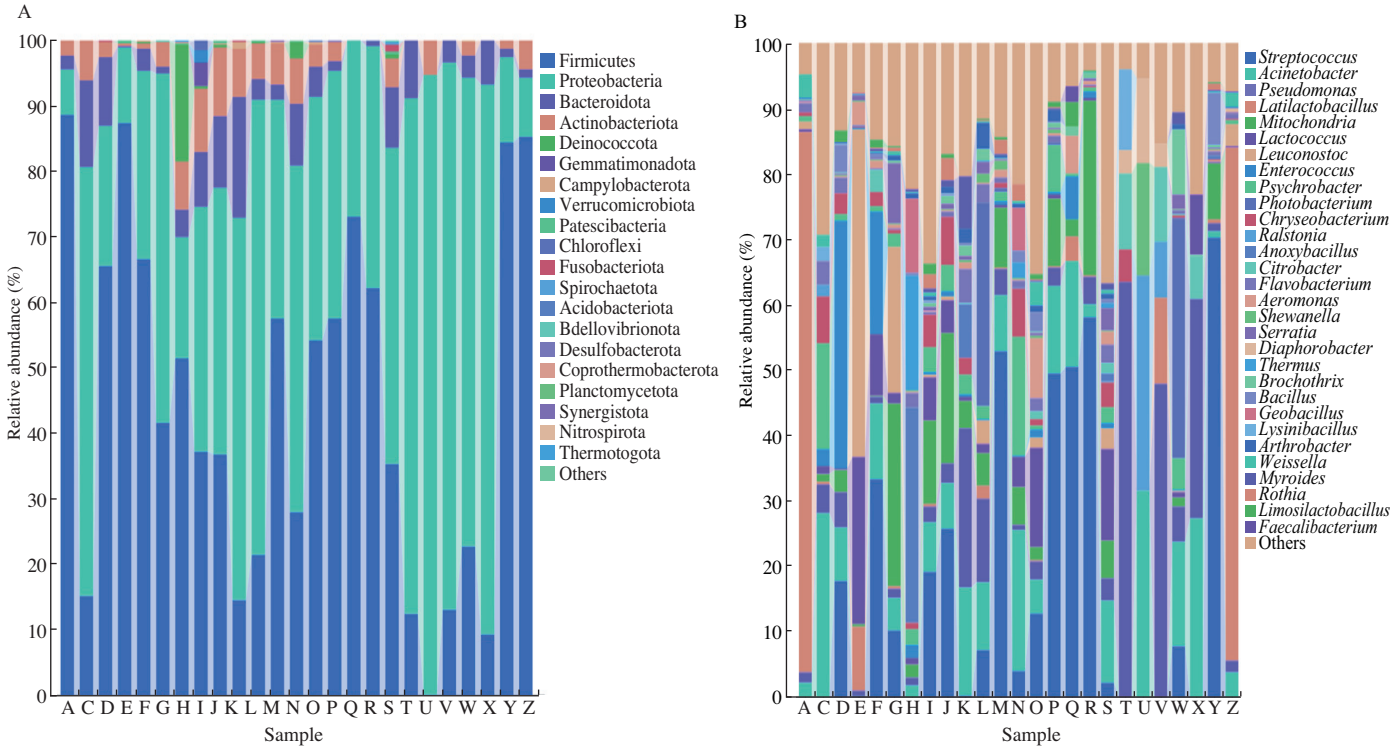

Fig. 9 Bacterial community analysis of 25 RTE meat product samples by high-throughput sequence analysis at both (A) phylum and (B) genus levels.

The great difference in bacterial composition of 25 RTE meat products was also observed at the species level (Fig. 9B), and dominant genera with high relative abundance were *Streptococcus* (16.92%), *Acinetobacter* (10.32%), *Pseudomonas* (9.16%), *Latilactobacillus* (7.70%), *Lactococcus* (3.56%), and *Leuconostoc* (3.48%), and these genera are commonly identified in processed meat products worldwide[52-53]. The highest level of *Streptococcus*, *Acinetobacter*, and *Pseudomonas*, were observed in samples Y (70.37%), U (31.60%), and T (63.53%), respectively. *Streptococcus*, *Acinetobacter*, and *Pseudomonas* were suggested as key indicators for accelerated meat spoilage, and usually form biofilms on contact surfaces in meat processing environment[52,54]. *Latilactobacillus* was higher in samples A (82.56%) and samples Z (78.40%). The identified lactic acid bacteria, like *Latilactobacillus*, *Lactococcus* (3.56%), and *Leuconostoc*, were considered technologically important for meat products, since they can lead to special texture and taste of meat products, and inhibit bacterial growth through acidification or producing antimicrobial compounds[52]. Overall, these genera above in the samples are different from the culture-dependent method, because the HTS method can directly extract and analyze the total DNA from a microbial assemblage, allowing for a more accurate reflection of the composition in complex microbial systems.

In addition, the heatmap presented the similarities and differences of bacterial communities in 25 samples of prepared meat products (Fig. 10). The color gradation from red to blue signifies an escalating relative abundance. Cluster analysis revealed that the

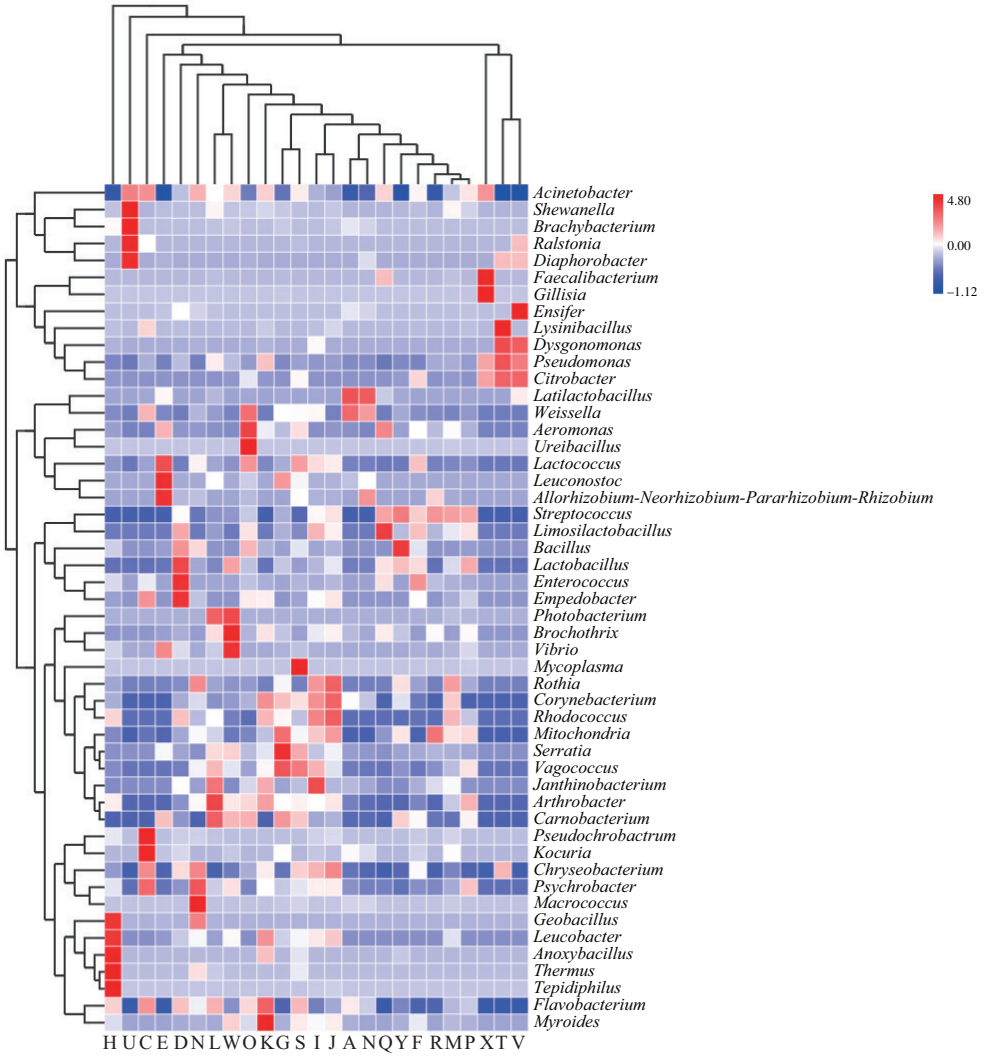

Fig. 10 Heatmap analysis of microbial diversity of 25 RTE meat product samples.

microbial community composition differed among different samples. Microorganisms present in meat products are influenced by factors including hygiene and handling of raw meat during processing, moisture, water activity, pH, oxygen availability, and storage temperature.

4. Conclusion

In the present work, we analyzed the bacterial diversity of the 25 RTE meat products by culture-dependent method and HTS analysis. As a result of the culture-dependent, 15 and 48 different genera and species were detected in all meat products, with the most strains belonging to *Bacillus*. HTS is superior for tracking the dynamic changes of bacterial populations in RTE meat products. *Streptococcus*, *Acinetobacter*, and *Pseudomonas* were the prevalent genera, providing a theoretical foundation for assessing the microbial risks of the meat products. Besides, the results regarding biofilm-forming capacity, bacterial motility, and enzyme production of isolates revealed potential health risks for human consumption and serious public safety incidents. To sum up, obtained results can offer novel insights into bacterial contamination, and further elevate the safety and quality of RTE meat products in the food industry. At last, further studies are still needed to confirm potential entry points for the meat-derived strains, and accordingly, stringent management, production guidelines, and efficient cleaning and disinfection techniques are required to limit bacterial contamination in meat processing industry.

Declaration of competing interest

All authors declared that they have no competing interests.

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