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Diversity and characterization of spoilage-associated psychrotrophs in food in cold chain

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ABSTRACT

In this work, psychrotrophs known to cause food spoilage were isolated from commercial food products. Further, temperature sensitivities and volatile organic compounds of the representative strains were characterized to evaluate the population heterogeneity. A total of 490 isolates belonging to 38 genera of 20 families were identified from 30 psychrotroph-positive samples, among which Gram-negative bacteria occurred frequently. The genus *Pseudomonas* exhibited a clear predominance, especially *Pseudomonas fragi*, followed by *Psychrobacter*, *Brochothrix*, *Serratia*, and *Stenotrophomonas*, with the dominant bacteria varying with origin. Aquatic products related to *Hafnia* and quick-frozen food corresponding to *Stenotrophomonas*, as well as livestock products were shown to be good ecological niches for growth of psychrotrophs. The genus *Pantoea* was shown to have an intimate relationship with fruits. While in bean, cereal grain and dairy products, only *Pseudomonas* was present. The fits of the growth curves demonstrated good adaptability and tolerance of the tested strains under 4 °C, and multifarious growth also reflected intra-species differences and phenotypic diversity. Various kinds of esters, aromatic compounds, alcohols, and ketones were frequently detected by GC–MS. High alcohols were seen in *Psychrobacter*, but hydrocarbons and ethers were more often found in *Pseudomonas*. In particular, since high amounts of isophorone were only discovered in bacteria samples, it is speculated to be the characteristic substance of psychrotrophs.

1. Introduction

In the cold chain, diversified food can be divided into primary cereals, vegetables, aquatic products, cold milks, eggs, poultries and meats, frozen foods and fast-food materials, and other processed foods (Mercier et al., 2017). An efficient cold chain stops or reduces the rate at which microbiological changes occur in food, provides a guarantee for food safety and freshness, promotes the prosperity of the food industry while maximizing commercial potential, and improves life quality (James and James, 2010). However, the cold chain has limits because microbes active at low temperatures can still cause microbial spoilage of food and pose a potential threat to customers, while also inflicting economic damage. In order to deal with this problem, more attention should be given to the control and improvement of the quality and safety of all stages of the cold chain (Stahl et al., 2015).

In 1887, for the first time, Forster (1887) isolated a microorganism from frozen fish that could reproduce at 0 °C, which opened the way for research on low-temperature microbes. Depending on the growth temperature, low-temperature microbes were divided into psychrophiles and psychrotrophs (Bowman et al., 1997; Russell et al., 1990). Since the growth of psychrophiles is tightly limited by environmental factors, even in the harsh cold niches, most of the identified isolates were psychrotrophs. Therefore, it can be considered that the bacteria isolated from the food in cold chain usually belong to the genera *Pseudomonas*, *Psychrobacter*, and *Arthrobacter* (Bowman et al., 1997; Rodrigues et al., 2009), which are commonly psychrotrophs, also known as cold-resistant bacteria.

Microorganisms are key contributors to spoilage processes. Spoilage is a serious challenge, especially for fresh, chilled, and frozen food, which are not be subjected to high temperature treatments or other forms of sanitization or preservation (Stellato et al., 2017). The residential psychrotrophs attach on food surfaces and spread through growth, ultimately resulting in full spoilage of the food or cross-contamination when they multiply to a certain amount (Møretrø and Langsrud, 2017). Many studies have explored cold-resistant bacteria in food, and it is recognized that *Pseudomonas*, *Enterobacteriaceae*, and

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Brochothrix thermosphacta are the most common organisms associated with spoilage, which typically cause slime, damaged food texture, malodor, and off-flavors (Gram et al., 2002; Wang et al., 2017), which can be responsible for decreased food quality and lower consumer acceptability.

In microbiological studies, cell growth modeling and curve parameters estimation is commonly performed and ranges from basic research to predictive model (Huang, 2011; Veríssimo et al., 2013). Thus, several mathematical models based on differential and algebraic equations have already been developed and are widely used in the field of food science. Here, the free software environment "R" has been explored for both visualizing and fitting curves. The growth curves resulting from temperature tests can be fit by model-based and spline-based approaches using the "grofit" package in R (Kahm et al., 2010).

Bacteria are known to produce a range of volatile organic compounds (VOCs) which are thought to evolve as products or by-products of metabolic pathways. Headspace solid-phase micro-extraction coupled with gas chromatography mass spectrometry (HS-SPME-GC–MS) is a widely used analytical method for the study of VOCs. In some cases, VOCs from the same species of bacteria have differed hugely between studies due to the differences in culture media (Tait et al., 2014). Therefore, this study used a preliminary culture medium that was developed to eliminate this interference.

A comprehensive assessment of psychrotrophs was investigated in the most extensive list of food products. Microbially-active temperature ranges for the dominant individuals were evaluated, and the VOCs of typical potential spoilage bacteria were monitored to predict their spoilage performance. The present study aims to provide a more integrated view of cold-resistant bacteria in potentially contaminated food in the cold chain that can be applied widely under a broad range of conditions, expanding the scope of such models substantially, and providing key understanding for the improved handling and storage of frozen foods.

2. Materials and methods

2.1. Sample collection and preparation

Forty-one samples including fruits, livestock products, aquatic products, bean products, cereal grain products, dairy products as well as quick-frozen food were purchased from three markets in Shaanxi, China (Table S1). All samples were collected at the beginning of their shelf lives. For the 37 solid samples, a total of 1 kg from five locations on each food item was collected separately, then homogenized to obtain a single sample. For the four liquid samples, i.e. 250 mL packaged dairy products, four units were obtained from each location and homogenized. Samples were collected aseptically in a time-efficient manner, using standardized methods, as follows:

Solid samples: samples were cut into uniform pieces on a clean surface with a sterile scalpel, mixed thoroughly, and quickly put into an aseptic sample box. This procedure was performed in duplicate. Liquid samples: under aseptic conditions, the four bags for each sample were opened carefully. After combining and mixing uniformly, the liquid was divided into two portions.

After pretreatment, the original temperatures of samples (4 °C or − 20 °C) were guaranteed before bacterial isolation, to minimize the effects of temperature fluctuations on microbial diversity. The bacterial isolations of samples were completed within one month. Finally, all samples were stored at −20 °C.

2.2. Isolation and identification of psychrotrophs

2.2.1. Isolation, enumeration, and purification of bacteria

Three kinds of medium were selected for the separation of the residential bacteria comprised of plate count agar (PCA) for total aerobic and facultatively anaerobic colony counts, *Pseudomonas* CFC selective agar (CFC), with 5 mL of dehydrated culture medium supplement per liter, and violet red bile dextrose agar (VRBDA) for the *Enterobacteriaceae*. PCA was chosen as a general reference medium for the enumeration of microbiota on all food samples. Despite the rare incidence of *Enterobacteriaceae*, once it occurs, spoilage grows rampantly, so *Enterobacteriaceae* is considered a considered key indicator of food safety (Nychas et al., 2008).

Ten grams of samples were soaked in 90 mL sterile saline solution and kept for 1 h to elute the microbes at 4 °C. A series of ten-fold dilutions was performed, and aliquots of 100 μL of the appropriate dilution were spread in duplicate on the three media. The plates were incubated for 10 d at 4 °C until the colonies grew. To measure the degree of contamination, total viable counts (TVCs) of cultivable microorganisms in each matrix were counted by the plate count method, if the number of colony forming units (CFU) was between 30 and 300. The concentration of bacteria was expressed as log10 (CFU/g) (Chaillou et al., 2015). After incubation, colonies were randomly selected from plates and re-streaked on nutrient agar to obtain pure colonies (Zhang et al., 2013). Next, a single colony was selected and placed into nutrient broth (NB), and actively growing strains were preserved in NB supplemented with 30% glycerol at −80 °C.

2.2.2. Molecular identification

DNA extraction was carried out using a Biospin Bacteria Genomic DNA Extraction kit (Hangzhou Bioer Technology Co., Ltd., Hangzhou, China) according to the manufacturer's instructions. Subsequently, a portion of their 16S rRNA gene was amplified using the primers 27F(5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R(5′-GGYTACCTTGTTACGACTT-3′), and the PCR reaction program was conducted by a previous study (Zhang et al., 2013). The 50 μL reaction system was performed by combining the following: 25 μL of Premix Taq (Takara, supplied by Takara Biotechnology Co., Ltd., Dalian, China), 2 μL of each primer (10 μmol/L, Invitrogen), 2 μL of template DNA, and 19 μL of distilled water. Then, 3 μL of PCR products was analyzed by 1.5% agarose electrophoresis to make sure there was a 1.5 kb fragment. The purification and sequencing of the fragment were conducted by Sangon Biotech Co., Ltd. (Shanghai, China).

The Basic Local Alignment Search Tool (BLAST) was used to complete sequencing alignment between the sequences of the isolates and those deposited in the GenBank DNA database (http://www.ncbi.nlm.nih.gov/blast). The similarity of > 98% was considered to be the same species, and between 95% and 98% was determined to one genus (Carrión et al., 2011).

2.3. Temperature sensitivity test

To evaluate the cold tolerance of the strains, ninety-eight isolates covering 67 *Pseudomonas*, 24 *Psychrobacter*, and seven type strains (purchased from CICC and CGMCC, China) were selected.

Six temperature gradients were set: 4 °C, 15 °C, 20 °C, 28 °C, 37 °C, and 45 °C. The tested strains were incubated at different temperatures in 96-well plates with each well containing 250 μL of inoculum (10^6 cells/well). The growth was monitored by measuring the optical density at 600 nm (iMark Microplate Reader, Bio-Rad, Hercules, CA, USA) at fixed time intervals. All tests were repeated three times. The grofit package in R software was used for growth curve fitting with four parametric models: Logistic, Gompertz, modified Gompertz, and Richards, and the optimal model was chosen according to the Akaike criterion (Kahm et al., 2010). After fitting, the optimal value of the three characteristic parameters, namely, the length of lag phase λ, the growth rate μ, and the maximum cell growth A, were obtained to evaluate the growth at different temperatures.

2.4. HS-SPME-GC–MS analysis

Thirty-five representative cold-resistant bacteria (22 of *Pseudomonas*, 11 of *Psychrobacter*, and two of type strains) were selected for determination of VOC generation by a GC–MS QP2010 Ultra system (Shimadzu USA Manufacturing Inc., Kyoto, Japan). In parallel, the abiotic release of odors from nutrient broth media was investigated simultaneously as a control. All strains were incubated at 4 °C for 72 h, and then 5 mL of the bacterial liquid was removed for centrifugation. The supernatant was collected and placed in a 20-mL glass vial with 1.5 g NaCl to facilitate the evolution of volatiles. For semi-quantification, 3-octanol was subsequently added as an internal standard.

The specific processes were as follows: volatiles were extracted by HS-SPME, samples were equilibrated at 40 °C for 15 min, and an SPME injector equipped with a 50/30 μm DVB/CAR/PDMS fiber (Supelco, Bellefonte PA, USA) was exposed for 10 min at 40 °C to extract the analytes. A DB-17MS fused-silica capillary column (60 m × 0.25 mm × 0.25 μm) was used to separate the volatiles. One microliter of sample was injected at 250 °C with a Shimadzu AOC-6000 automatic sampler under the splitless injection mode. High purity helium was used as the carrier gas at a column flow of 0.96 mL/min. The column oven temperature program was set from 40 °C (held for 3 min) to 120 °C at 4 °C/min and finally reached 240 °C (maintained for 9 min) at a rate of 6 °C/min. Mass spectrometry was operated in electron impact ionization mode at 70 eV. The temperature of ion source and interface was 230 °C. The data was collected at a scanning mode and the total ion current was in the range 35–500 *m/z*. The VOCs were identified by comparing their mass spectra with available information in the NIST14 library. The relative content of each compound was calculated based on the internal standard (i.e. the content of 3-octanol was taken as 1) and the relative content was calculated by the ratio of the peak area of the volatiles to the peak area of the 3-octanol.

2.5. Data analysis

In order to eliminate the effect of the sampling number on result analysis, the average bacterial occupancy (ABO) of samples was used to measure the mean number of isolates. In this method, each category of food was treated as an analytical unit, and the populations of isolates in one category were divided by the number of samples in this classification, thus obtaining the ABO.

Frequency percentage (FP) analysis was performed to study the distribution of dominant bacteria in the various food samples. This method required calculation of the number of times that the target bacteria were detected instead of the number of isolates. Then, the number of times was divided by the number of positive samples, and the results were expressed as a percentage.

In the GC–MS analyses, the data of the relative content were first standardized, and then the R software was used for analysis, with heatmap generated subsequently. One-way analysis of variance and Duncan's multiple-range test ($p < 0.05$) were carried out with SPSS 22.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Scanning of isolation

A total of 490 bacterial isolates spanning 38 genera of 20 families were cultured and identified via 16S rRNA sequencing, among which 423 (86.3%) were Gram-negative bacteria. The populations of isolates varied with the type of food (Table S1). The Gram-negative bacteria were found extensively on the surfaces of various types of chilled foods, which was congruent with previous results (Møretrø and Langsrud, 2017).

On the whole, 30 out of 41 samples (73.2%) were positive for psychrotrophs. Specifically, livestock products and aquatic products showed a higher incidence of growth; while cereal grain products, fruits, and dairy were observed to show evidence of cold-tolerant microbial growth at a lesser extent (Table 1). From a nutritional point of view, meats are rich in high-quality protein, all essential amino acids, minerals, and vitamins with high bioavailability (Chaillou et al., 2015; Wang et al., 2017), which provide a good substrate for microbial growth and attachment. To our surprise, the average bacterial occupancy (ABO) value of quick-frozen food and bean products was somewhat pronounced, especially with bean products, with a leading value of 20.5, substantially exceeding the average level (11.95).

Table 1
Analysis of food by categories.

Category	Number of samples	Positive samples	Gram-negative	ABO	Frequency		
					Pse	*Pse. fragi*	*Psy*
Livestock	12	11	173	16.75	7	7	5
Aquatic	10	10	134	15.5	7	6	10
Quick-frozen	5	3	58	14.8	3	2	2
Bean	2	2	41	20.5	2	2	0
Cereal	2	1	11	6	1	1	0
Fruits	6	2	5	1	1	1	0
Dairy	4	1	1	0.25	1	0	0

Note: Pse = *Pseudomonas*; Psy = *Psychrobacter*.

3.2. Total viable count and bacterial distribution in three media

Total viable count (TVC) values of all batches had a broad range, from 0 to 7 (Fig. 1a). The mean and median values of TVC on PCA were higher than those on the two selective media, except for dairy products, which showed no colony on PCA and CFC plates. Also, most of the box charts showed a phenomenon of 0 value as the lower quartile, indicating the presence of negative samples of these groups, which was consistent with the previous analysis (Section 3.1). Clearly, the counts of bean products on PCA and CFC matrix were the highest observed values in all groups, which demonstrated a possible high level of microbial contamination in this category. However, few studies have been aimed at bean products, and only one study was found to report intermittent detection of *Pseudomonas aeruginosa* in bean sprouts (Curran et al., 2005). In addition, for dairy products and fruits, these two groups deserve special attention, as only a fluctuation was observed on VRBDA and PCA, respectively. However, in our research, the commercial milk products were selected in sampling, and the fruits were also as fresh food, so these foods should be safe and pollution-free. A small amount of detected microorganisms may be due to the slight temperature fluctuation from sampling to strain separation.

When discarding the negative samples (Fig. 1b), the TVC value of bean products, cereals, and aquatic products still had the largest counts on PCA. Quick-frozen food also joined this group. Compared to the counts on PCA, the fruits class showed a higher value on CFC and VRBDA, which were previously identified as outliers because of small positive sample size (i.e. only Yali pear). As for livestock products, when considering only positive samples, the highest counts on VRBDA were observed, illustrating the number of samples been positive for VRBDA was small, but the counts values were high. In fact, some of the bacteria, especially *Enterobacteriaceae*, originate from the animal's intestinal tract as well as the environment that the animal came into contact with before or during slaughter. These microorganisms can spread to the work surfaces and may survive and exist in the end products, thus causing contamination (Chaillou et al., 2015; Nychas et al., 2008).

Next, the differences in number and species of isolates on the three media were analyzed (Fig. 3b). Overall, the number of bacteria isolated on PCA, CFC, and VRBDA was 287, 127, and 76, respectively. A wide variety of species occurred on PCA plates. When focusing on the CFC

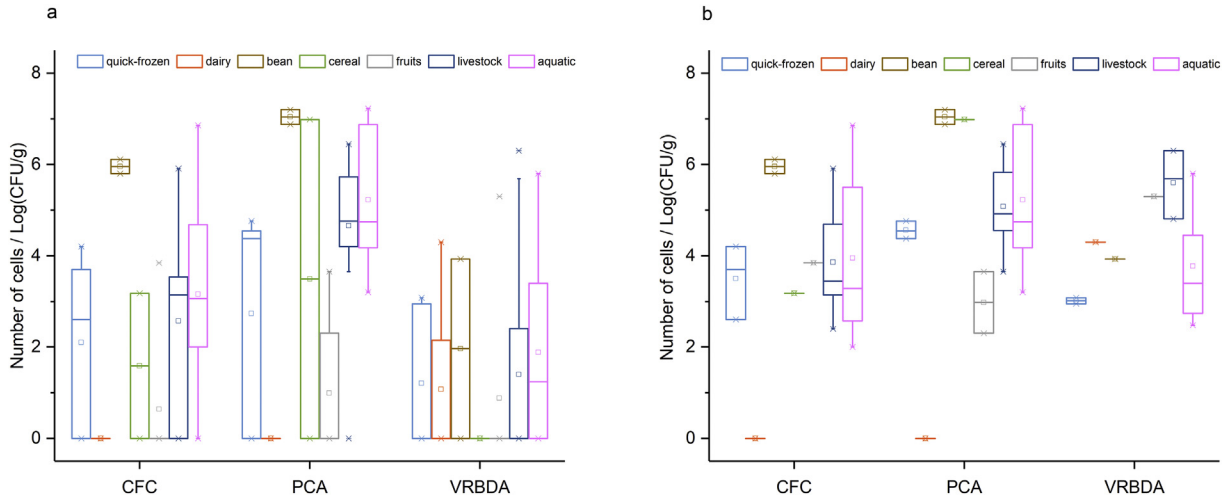

Fig. 1. Total viable count of bacteria from different food products on three types of media: (a) all samples, (b) positive samples.
Quick-frozen = quick-frozen food; dairy = dairy products; bean = bean products; cereal = cereal grain products; livestock = livestock products; aquatic = aquatic products.

plates, almost all strains were identified as *Pseudomonas*, reaching 93%. This is due to the selectivity of the culture medium and a widespread distribution of *Pseudomonas* in cold-chain foods. However, on VRBDA plates, the *Pseudomonas* count was still the highest, accounting for 48.68%, and *Enterobacteriaceae* ranked second with a 35% proportion. All species identified on VRBDA plates presented as Gram-negative.

3.3. The phylogenetic distribution of psychrotrophs

In this section, all 490 strains were classified by family and genus. Fig. 2a shows the distribution at the family level (< 1% for others). The proportion of *Pseudomonadaceae* was more than half, reaching 50.41%. Environment may exert a selective pressure on bacterial communities, so that a well-adapted flora will have an advantage, become dominant, and thrive (Wang et al., 2017). Although a portion of contaminants were animal-derived microbiota (i.e. livestock), they were less abundant than the psychrotrophs mainly originating directly from the natural environment (Chaillou et al., 2015).

Further, the isolates were distributed based on genus as shown in Fig. 2b (< 1% for others). Despite the large number of microorganisms that compose the initial microbiota, only a few species were dominant enough to cause spoilage and reduce shelf-life (Casaburi et al., 2015; Huang et al., 2017; Parlapani et al., 2017). Clearly, *Pseudomonas* (50.41%) was dominant, in agreement with aforementioned findings. The species was usually isolated from soil, water, plant surfaces, and raw materials and may be continually introduced into end food

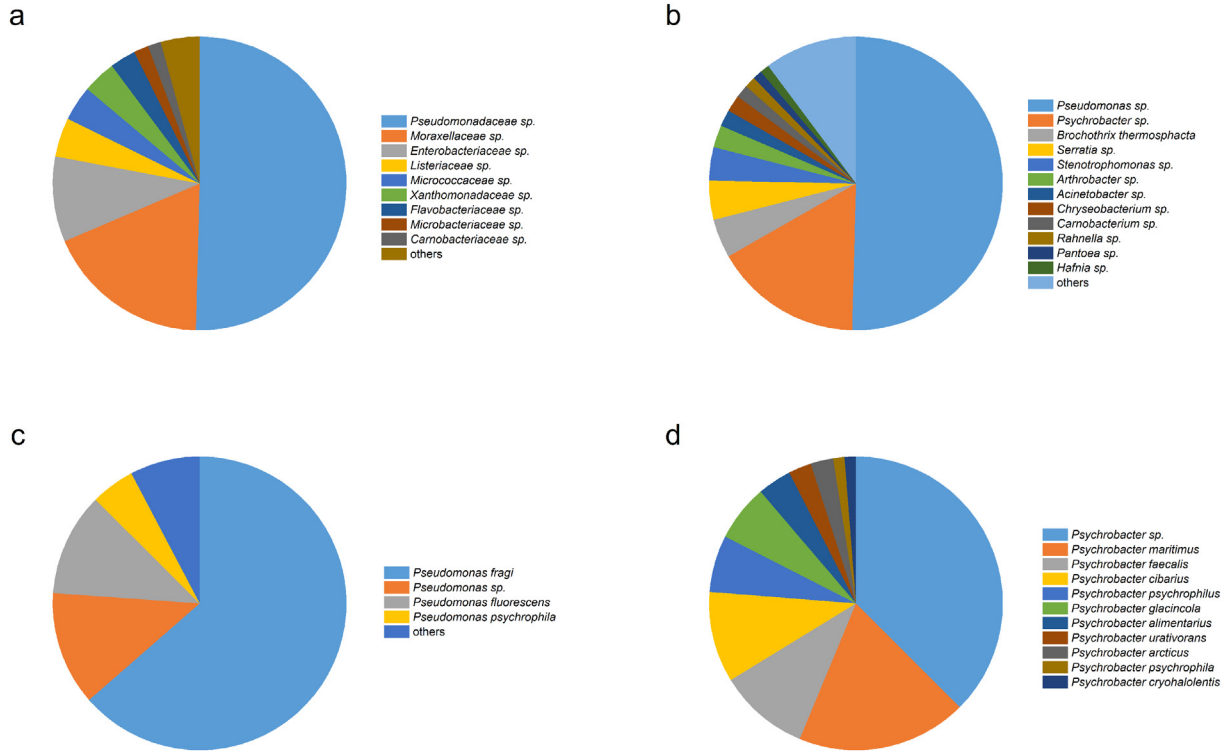

Fig. 2. Distribution of psychrotrophs: (a) and (b) represent the family and genus level, respectively, (c) and (d) represent the distribution of dominant species of *Pseudomonas* and *Psychrobacter*, respectively.

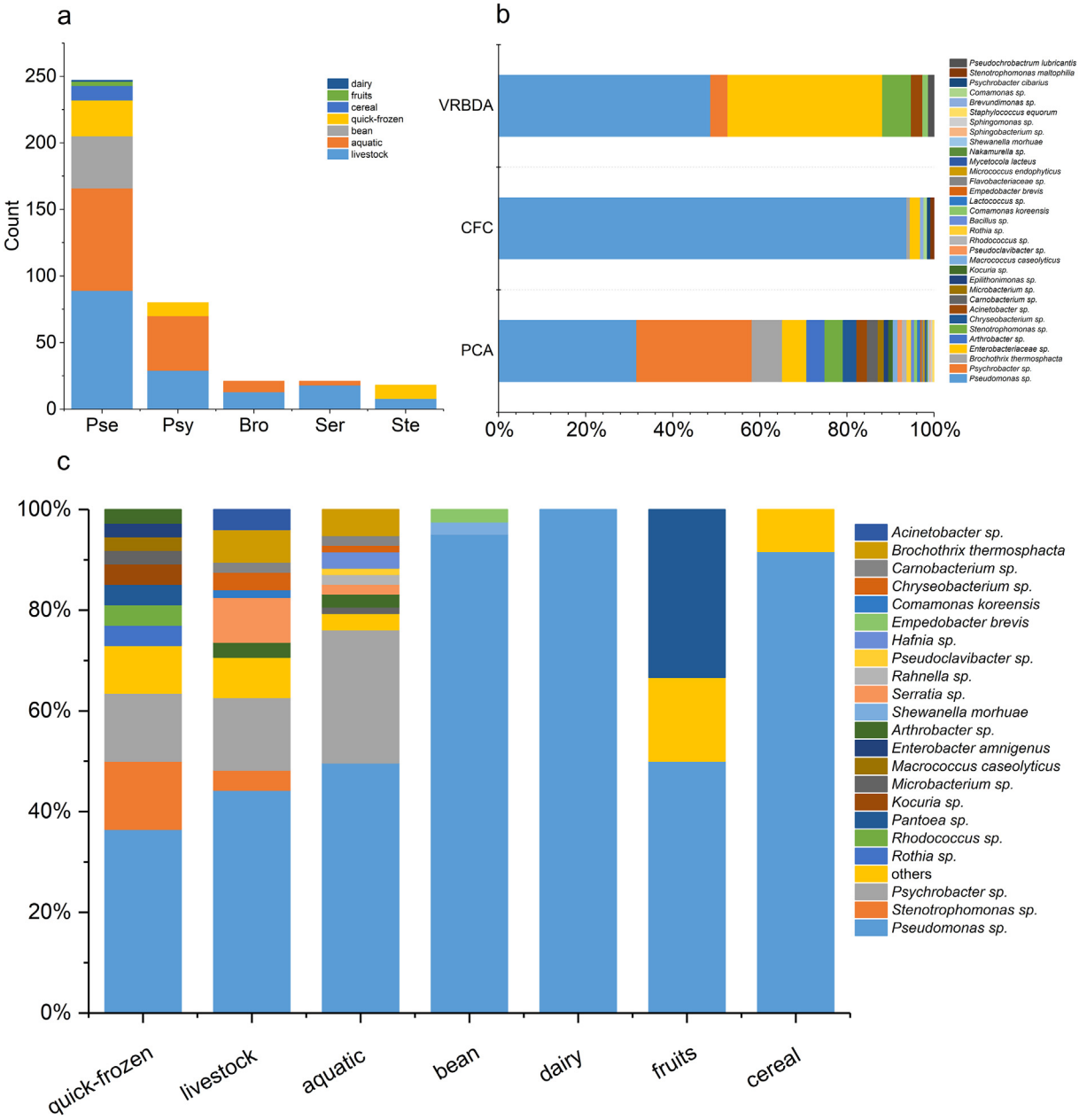

Fig. 3. Diversity of psychrotrophs: (a) distribution of dominant species in various food. (b) strain abundance in three media. (c) biodiversity of different food categories.
Pse = *Pseudomonas*; Psy = *Psychrobacter*.; Bro = *Brochothrix thermosphacta*; Ser = *Serratia*; Ste = *Stenotrophomonas*.

products through various routes (Møretrø and Langsrud, 2017). They are known as the fastest growing bacteria under cold or normal storage conditions and can easily and rapidly develop into the dominant species (Parlapani et al., 2017), especially in proteinaceous foods, and lead to spoilage flooding (Gram et al., 2002). *Psychrobacter*, accounting for 16.33% of all strains, was also a member of the dominant cold-resistant bacteria detected in this study, followed by *B. thermosphacta* (4.29%), and *Serratia* (4.29%). *Serratia* possesses strongly spoilage potential and is well-known as a spoilage bacterium in cooled poultry and meat (Casaburi et al., 2015; Wang et al., 2017).

The top five most dominant bacteria were selected to discuss their distribution in various types of food (Fig. 3a). The genus *Pseudomonas* was pervasively ubiquitous in all food types. *Psychrobacter* appeared on livestock products, aquatic products, and quick-frozen food. This was consistent with the report that fresh or salted meats, poultry, and marine products may be good niches for *Psychrobacter* ascribed to the psychrotolerant and halotolerant character of the bacteria (Møretrø and Langsrud, 2017). In addition, *B. thermosphacta* and *Enterobacteriaceae* families, especially *Serratia liquefaciens*, were usually found in vacuum-packed meat and fish due to their facultative anaerobic feature and associated with food spoilage (Gram et al., 2002; Gribble et al., 2014; Wang et al., 2017). While for *Stenotrophomonas*, a bacterium mainly observed in livestock products and quick-frozen food, few studies have been previously reported and they were only found in Sichuan paocai brine and some refrigerated milks (Boubendir et al., 2016; Cao et al., 2017; Rasolofo et al., 2010).

Next, for *Pseudomonas* (Fig. 2c) and *Psychrobacter* (Fig. 2d), the two most dominant flora in the cold-chain, further analysis was performed (< 1% for others). It was clear that *Pse. fragi* possessed of 63.56%, firmly occupying a dominant role. In addition, *Pse. fragi* achieved a proportion of 30.61% of all isolates. Furthermore, *Pseudomonas fluorescens* and *Pseudomonas psychrophila* were also contaminants that often

appeared in food. In earlier studies, it was established that *Pse. fragi*, *Pse. fluorescens*, and *Pseudomonas lundensis* were the most important potential species under aerobic storage (Casaburi et al., 2015; Nychas et al., 2008). Also, *Pse. fragi* was detected in nearly all poultry samples under different low temperature conditions and was dominant over time (Wang et al., 2017). With respect to *Psychrobacter*, 37.5% of the strains could not be identified more specifically, while the rest of the species were homogeneously distributed.

The frequency percentage (FP) of the predominating population (calculated from Table 1) was studied. It was found that the FP of *Pseudomonas* was as high as 73.33%, and the FP of *Psychrobacter* was 56.67%. Moreover, we specifically investigated the FP of *Pse. fragi*, and it was surprising to find out that this species, with an occurrence of 63.33%, was present in nearly all kinds of food except dairy products.

3.4. Diversity analysis on different food substrates

The microbial flora assemblage associated with spoilage was shaped to a certain extent by the nutritional value of different food matrices (Chaillou et al., 2015). Livestock products, aquatic products, and quick-frozen food had a rich variety of biodiversity and species diversity (Fig. 3c). Specifically, the isolates in quick-frozen food included 18 species, of which the more numerous species were *Pseudomonas*, *Psychrobacter*, and *Stenotrophomonas*. Regarding the livestock products, 23 species identified in them. The first four were *Pseudomonas*, *Psychrobacter*, *Serratia*, and *B. thermosphacta*. Due to the high correlation between the number of *Pseudomonas* and the sensory index, the acceptability of this species was considered a freshness indicator of livestock products. (Bruckner et al., 2012; Nychas et al., 2008). The aquatic products had a clear advantage of *Pseudomonas* and *Psychrobacter*, followed by *B. thermosphacta* and *Hafnia*. This result was slightly different from that reported in the literature. It has been well documented that *Psychrobacter* is dominant in the initial stage of fish and shrimp storage (Broekaert et al., 2013; Lan and Xie, 2012), while *Pseudomonas* and *Shewanella* become the dominant microorganisms toward the end of storage (Kostaki et al., 2009; Lan and Xie, 2012). Contradictory to previous studies, we only sporadically found *Shewanella* in bean products rather than aquatic products. As for the other four categories, spoilage-associated microbiota was simplistic, as the dominant contaminants were more pronounced in these groups. In fact, the fresh milk circulating at low temperatures could not completely eliminate the breeding of psychrotrophic bacteria, such as *S. liquefaciens*, *Pseudomonas*, and *Stenotrophomonas*, which were responsible for the spoilage of milk (Boubendir et al., 2016; Machado et al., 2015; Rasolofo et al., 2010).

At the same time, the first twelve genus groups, which accounted for 89.8% of the total, were selected for correspondence analysis with seven food categories (Fig. 4). Gram-positive bacteria appeared only in the third quadrant. Fruit and *Pantoea* were characteristic points observed farthest from the center, and they were highly correlated. The points of bean products, cereal grain products, and dairy products coincided, since they only contained *Pseudomonas*. Regarding the other three food categories, the representative bacteria of these were not singular, expressed as a high density aggregation of points. The aquatic products were highly correlated with *Hafnia*, a psychrotolerant *Enterobacteriaceae* that is non-pathogenic but has a high potential for spoilage (Newton et al., 1978), and the aquatic products also associated with *Pseudomonas*, *Psychrobacter*, and *Rahnella*. Quick-frozen foods were closely linked to the occurrence of *Stenotrophomonas*, and somewhat linked to *Pantoea*. However, the presence of microbes in livestock products was complex; genus groups were correlated with this category except for the *Pantoea* and *Hafnia*.

3.5. Temperature sensitivity

Covering all types of food sampled, 98 isolates belonging to the top

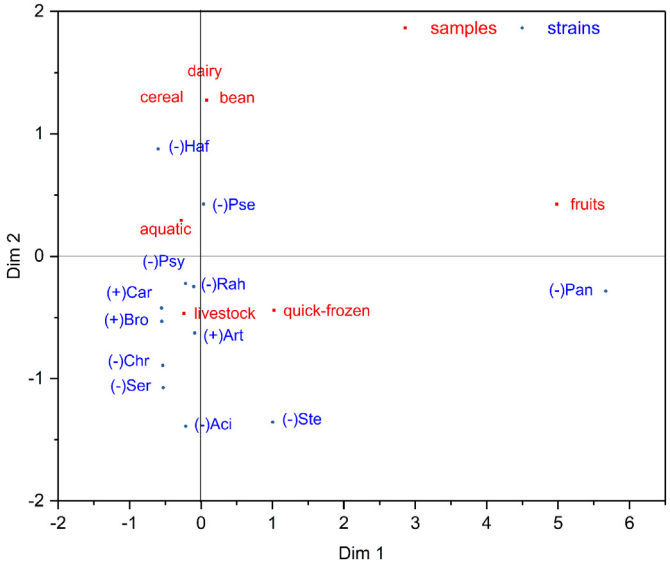

Fig. 4. Correspondence analysis. (+) Gram-positive; (−) Gram-negative.
Quick-frozen = quick-frozen food; dairy = dairy products; bean = bean products; cereal = cereal grain products; livestock = livestock products; aquatic = aquatic products.
Haf = *Hafnia*; Rah = *Rahnella*; Car = *Carnobacterium*; Chr = *Chryseobacterium*; Art = *Arthrobacter*; Aci = *Acinetobacter*; Pan = *Pantoea*.

two genera were randomly selected for a temperature tolerance trial. The growth curves of the tested strains under different temperatures were generally well-fit with the four models in R, except for 45 °C (Fig. 5). The Gompertz and Logistic models were broadly applied in the growth curve fitting of bacteria including *Pseudomonas* (Giannuzzi et al., 1998; Singh et al., 2015). Importantly, the modified Gompertz model was one of the earliest models adopted by food scientists for predictive microbial growth (Huang, 2011).

For the well-fit strains, we performed a statistical analysis of their three parameters under optimal fitting. In the box plots (Fig. 6), *Pseudomonas* showed a longer lag phase at 4 °C and a slower growth rate, but the final A value occupied the highest level. However, when cultured at 37 °C, the strains responded with a smaller A value, showing the limited growth at this temperature, although a shorter lag phase and a larger growth rate were given. The temperatures of 15, 20, and 28 °C were more suitable for *Pseudomonas* growth. As for *Psychrobacter* species, the average λ at 4 °C was significantly higher than those at other temperatures, yet there was no significant difference in the growth rate under any situation. Combined with A, a similar consequence with *Pseudomonas* was reached, in terms of the response and tolerance to temperature. Moreover, the μ values of *Pseudomonas* showed a high level overall when compared to those of *Psychrobacter*. This discovery shows that as soon as *Pseudomonas* adapts to the circumstances, they will breed at a relatively fast pace and then dominate in a cold environment. Especially at low temperatures, the strong competitiveness of *Pseudomonas* was prominent compared to most other food-associated bacteria, which may be due to the unaffected biofilm formation (Liu et al., 2015). Regarding 45 °C, it can be seen from the figure that despite the growth curves of two selected species being in line with the model, the extremely low level of A indicates very little viability of these cold-tolerant bacteria.

Naturally, there were always special cases in the test (data not shown), suggesting that different strains of the same species could have different adaptation efficiency, resulting in resilient bacterial associations (Stellato et al., 2017). The 41-3 (*Psychrobacter*) was lacking in vitality and could slowly grow in colder conditions; the growth even remained in a stagnant state at 28 °C, 37 °C, and 45 °C. In addition, the high temperatures of 37 °C and 45 °C were not conducive to the

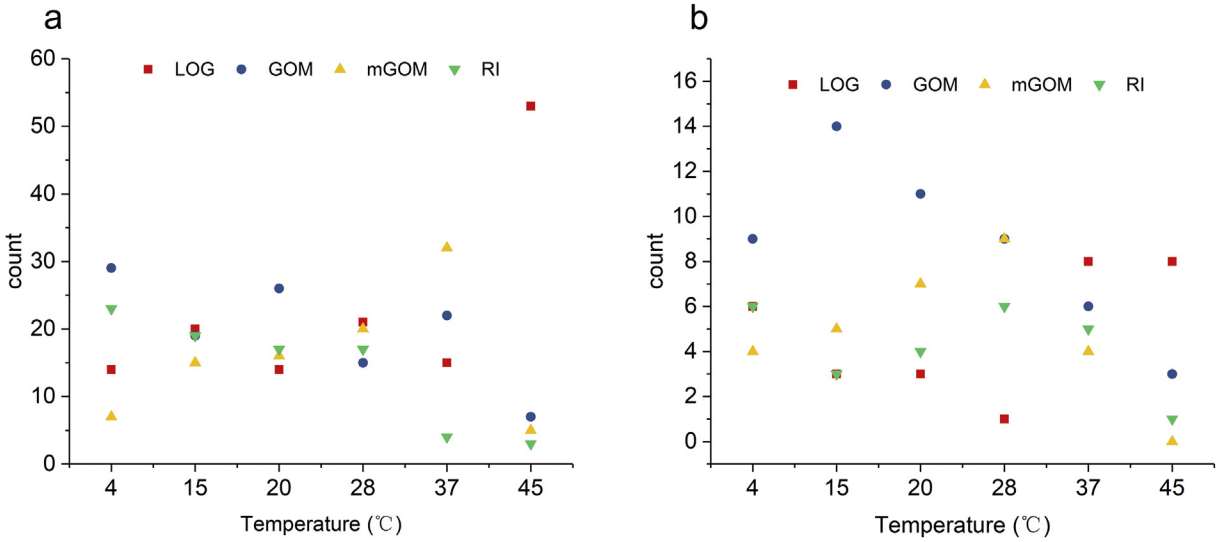

Fig. 5. Bacterial count that can be fitted with different models at different temperatures: (a) *Pseudomonas*, (b) *Psychrobacter*.
LOG = Logistic; GOM = Gompertz; mGOM = modified Gompertz; RI = Richards.

proliferation of 32-3 (*Psychrobacter urativorans*). At 45 °C, most *Pseudomonas* expressed a certain tolerance but low biological activity; only 7-1 (*Pse. fluorescens*), 10-28 (*Pse. fluorescens*), 23-24 (*Pse. fluorescens*), 36-16 (*Pse. fragi*), and CICC 21620 stopped growing. Regarding the *Psychrobacter* species, more than half of the strains did not grow any more, confirming that the strains had poor tolerance to heat. It is particularly worth mentioning the super adaptability of 19-7 (*Psychrobacter faecalis*) to temperature: it could remain active in the wide range of 4 °C

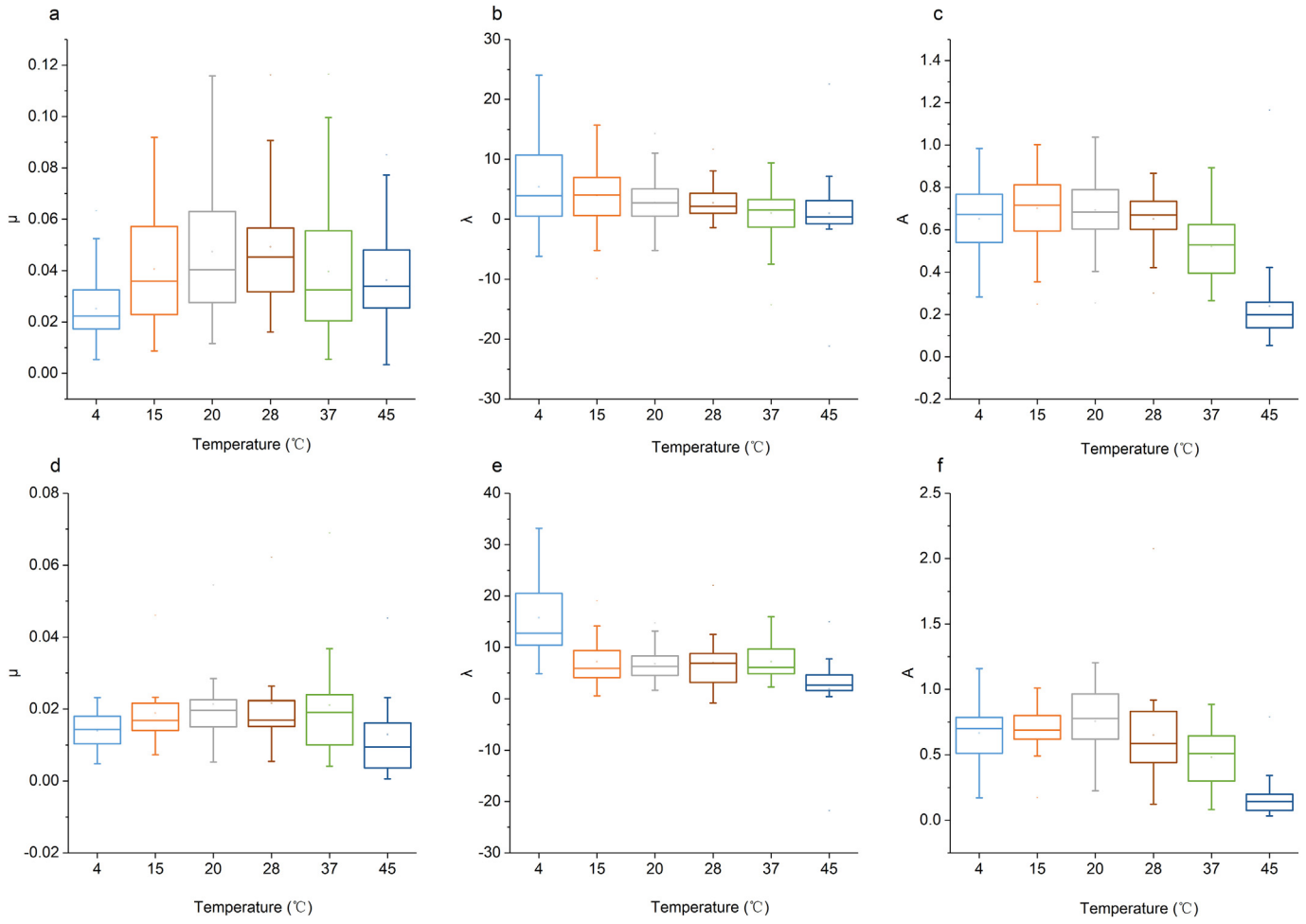

Fig. 6. The optimal estimated parameters of the growth rate μ, the lag phase λ, and the maximum cell growth A: (a, b, c) represent *Pseudomonas* and (d, e, f) represent *Psychrobacter*.

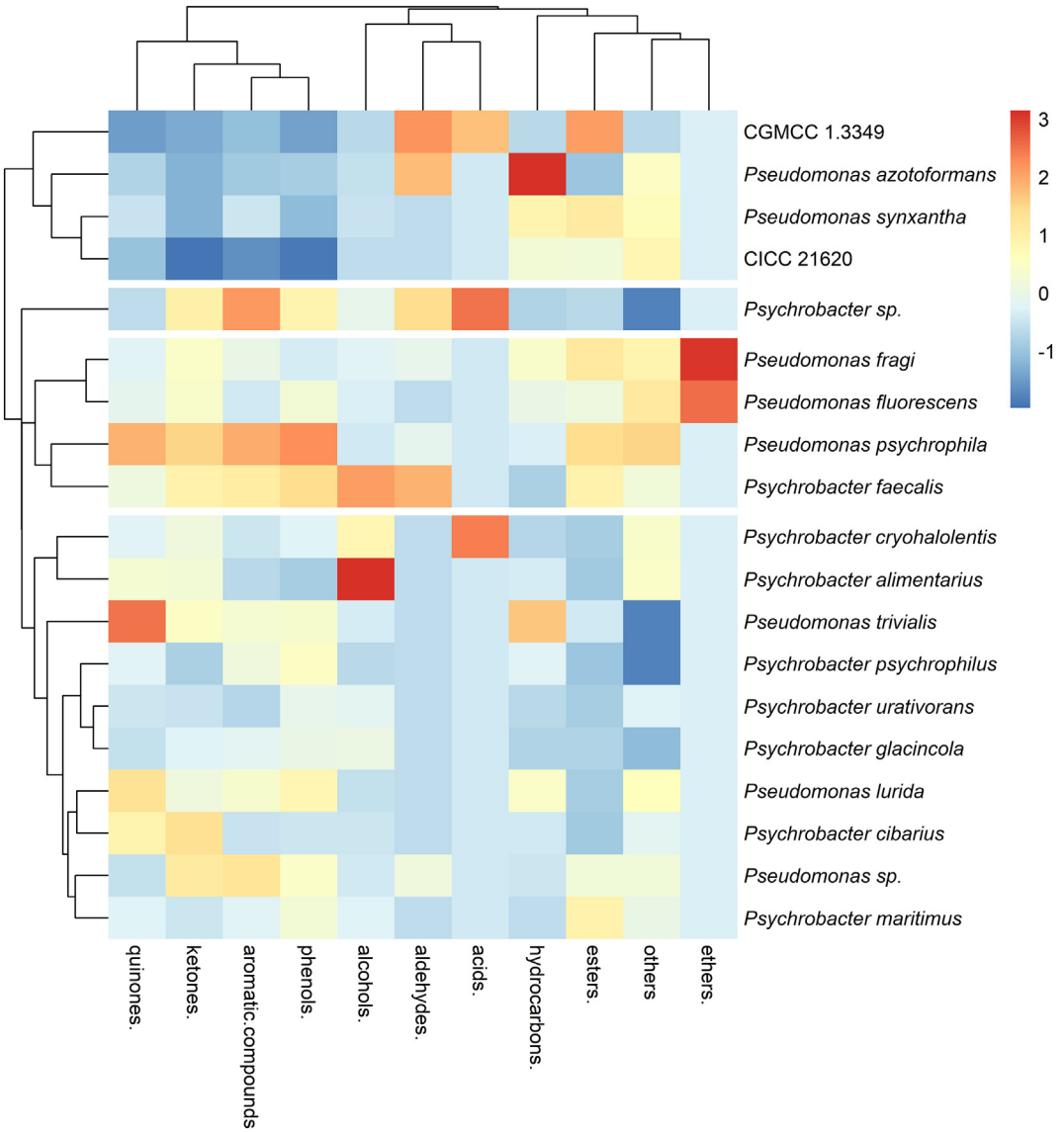

Fig. 7. Heatmap showing the relative content of various aroma categories in isolates.

to 45 °C. The characterization of growth response to various temperature levels revealed the high phenotypic diversity of strains and species.

3.6. HS-SPME-GC–MS analysis

Numerous VOCs derived from bacterial metabolism have been reported in many previous studies and related to undesirable odors characteristics during spoilage (Wang et al., 2017). A total of 194 volatile compounds were detected in the 35 samples as well as the negative media, and they were divided into 11 categories (Table S2). In the tested strains, the number of aroma matter was identified in the range of 30 to 40 for almost all bacteria, and the compounds were mainly concentrated on the top five categories, namely, esters, aromatic compounds, ketones, alcohols, and hydrocarbons. However, there were only three samples including acids, and ethers were only present in four strains. In addition, more than half of the samples did not show the presence of aldehydes. The hydrocarbons were presumed to be closely linked to the metabolic activity of the strain itself, while the aldehydes were mostly caused by the interaction of microorganisms and food matrices with high protein content (Casaburi et al., 2015; Ercolini et al., 2010). By the way, although the aromas of the media were relatively rich in diversity, production of these substances was detected at micro to trace levels.

Analysis was conducted by comparing the mean value of the tested strains classified at the species level. In the heatmap (Fig. 7), the relative content of various aroma categories in 19 groups were shown intuitively. According to the color distribution, a marked difference is apparent. Overall, the groups were separated into four classes which were clustered by the distinction of the volatiles. Belonging to one branch, the group of CGMCC1.3349, *Pseudomonas azotoformans*, *Pseudomonas synxantha*, and CICC21620 showed ultra-low degrees of aromatic compounds, phenols, ketones, and quinones. While *Pseudomonas psychrophila* and *Psy. faecalis* presented relatively high levels in these categories, they also contained higher levels of esters. Additionally, *Psy. faecalis* occupied a large proportion of alcohols and aldehydes. When observing the distribution of alcohols, high content was present in the genus of *Psychrobacter*, compared to the genus of *Pseudomonas*, especially *Psychrobacter alimentarius*, *Psychrobacter cryohalolentis*, and *Psy. faecalis*. Meanwhile, *Psy. cryohalolentis* was also rich in acids, a category that only appeared in CGMCC 1.3349, *Psychrobacter*, and *Psy. cryohalolentis*. As for hydrocarbons, they were centrally distributed in the genus of *Pseudomonas*, particularly focused on *Pse. azotoformans*. The content of aldehydes in this group was observed to be high as well. CGMCC 1.3349 exhibits high levels of aldehydes, esters, and acids.

Pseudomonas has the ability to produce higher levels of aldehydes, with the most common aldehydes falling into hexanal, nonanal, and heptanal categories (Casaburi et al., 2015). With respect to the ethers, since there was no detection in other specimens except for *Pse. fragi* and *Pse. fluorescens*, high content arose in these two groups. As the most abundant category, esters also had notable content among *Pse. synxantha*, *Pse. fragi*, and *Psychrobacter maritimus*. *Pseudomonas* was known as a great producer in esters generation. In particular, *Pse. fragi* was responsible for fruity flavor, a smell which played an important role in the sensory quality (Casaburi et al., 2015; Ercolini et al., 2010).

The bacteria remarkably altered the composition of its VOCs; each one presented a distinct volatile profile. However, there were still some regular patterns. Microorganisms utilized different precursor compounds to generate their volatile metabolites (Olafsdottir et al., 2005). Four substances appeared in all 36 treatments including the medium control, namely, 2,4-di-*tert*-butylphenol (CAS: 96-76-4), 2,6-di-*tert*-butylquinone (CAS: 719-22-2), diisobutyl phthalate (CAS: 84-69-5), and 2,2,4-trimethyl-1,3-pentanediol diisobutyrate (CAS: 6846-50-0). In addition, the compounds of naphthalene, benzaldehyde, dibutyl phthalate, and isophorone were also detected 35 times. It is noteworthy that isophorone presented positive in all strains at a level higher than most of the other volatiles, yet negative in the control. We could speculate that the isophorone is the characteristic VOC of the cold bacteria.

Many researchers (Ercolini et al., 2010; Ercolini et al., 2011; Ferrocino et al., 2013) reported that the appearance of 2-ethyl-1-hexanol was related to *Pseudomonas* activity. *Pseudomonas* was also, to some extent, involved in the production of 3-methyl-1-butanol, ethanol, 2-heptanone, 2-nonanone, 2-undecanone, and ethyl tiglate (Parlapani et al., 2017; Wang et al., 2017), with generally agreed with our findings. We also discovered 2-heptanone, 2-nonanone, and ethyl tiglate only in *Pseudomonas*. Somewhat differently, the 3-methyl-1-butanol just occurred sporadically in *Pseudomonas*, but more in *Psychrobacter*. A number of researchers found that the presence of 2- and 3-methylbutanal in spoiled poultry and meat could originated from *Pseudomonas* (Argyri et al., 2015), while our results did not support this view. Although VOCs came from the metabolism of bacteria, this was found to vary depending on the environment and substrates utilized by the microbes.

4. Conclusions

This study is an overview of the distribution of cold-resistant bacteria surviving in various food end products in the cold chain, and it arrived at the corresponding dominant genera of each food category. As the most widely scattered genera, *Pseudomonas* and *Psychrobacter* stood out, and their growth experiments were performed at different conditions for the temperature sensitivity test. Furthermore, aimed at typical cases, we dissected the volatile organic compounds of representative psychrotrophs. A high content of isophorone appeared in all samples and may be considered a characteristic substance of the tested bacteria. This work could serve as a firm foundation for future research on the growth prediction of contaminated bacteria in food; simultaneously, it also provided a direction for the inhibitory or antiseptic control of destructive bacteria in food in the cold chain.

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