Spoilage potential of psychrotrophic bacteria isolated from raw milk and the thermo-stability of their enzymes[*]

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Abstract: The storage and transportation of raw milk at low temperatures promote the growth of psychrotrophic bacteria and the production of thermo-stable enzymes, which pose great threats to the quality and shelf-life of dairy products. Though many studies have been carried out on the spoilage potential of psychrotrophic bacteria and the thermo-stabilities of the enzymes they produce, further detailed studies are needed to devise an effective strategy to avoid dairy spoilage. The purpose of this study was to explore the spoilage potential of psychrotrophic bacteria from Chinese raw milk samples at both room temperature (28 °C) and refrigerated temperature (7 °C). Species of *Yersinia*, *Pseudomonas*, *Serratia*, and *Chryseobacterium* showed high proteolytic activity. The highest proteolytic activity was shown by *Yersinia intermedia* followed by *Pseudomonas fluorescens* (d). Lipolytic activity was high in isolates of *Acinetobacter*, and the highest in *Acinetobacter guillouiae*. Certain isolates showed positive β-galactosidase and phospholipase activity. Strains belonging to the same species sometimes showed markedly different phenotypic characteristics. Proteases and lipases produced by psychrotrophic bacteria retained activity after heat treatment at 70, 80, or 90 °C, and proteases appeared to be more heat-stable than lipases. For these reasons, thermo-stable spoilage enzymes produced by a high number of psychrotrophic bacterial isolates from raw milk are of major concern to the dairy industry. The results of this study provide valuable data about the spoilage potential of bacterial strains in raw milk and the thermal resistance of the enzymes they produce.

Key words: Spoilage enzyme; Psychrotrophic bacteria; Raw milk; Thermo-stability
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1 Introduction

Raw milk serves as an ideal medium for the growth of bacteria due to its high nutritional value (Champagne et al., 1994). A cold chain system is commonly used for controlling the growth of bacteria in raw milk during storage and transportation. Psychrotrophic bacteria are ubiquitous organisms that have the ability to grow at 7 °C or below, regardless of their higher optimal growth temperature (Sørhaug and Stepaniak, 1997). These bacteria usually account for less than 10% of the total microflora of raw milk, but invariably become predominant during the transportation and prolonged storage of raw milk at low temperatures (Sørhaug and Stepaniak, 1997).

Raw milk can be contaminated with psychrotrophic bacteria from a variety of sources including air, water, soil, and milking equipment (Vacheyrou et al., 2011). Among psychrotrophic bacteria, genera of *Pseudomonas*, *Acinetobacter*, *Flavobacterium*, *Chryseobacterium*, and *Serratia* are the most frequently isolated from raw milk (Vithanage et al., 2016; Yuan et al., 2017).

Increasing global demand for dairy products requires dairy manufacturers to produce products with high quality and prolonged shelf-life. The dairy industry is facing constraints related to maintaining high quality and avoiding losses as a result of microbial spoilage. Mesophilic and thermophilic spore forming bacteria have the potential to contaminate processed dairy products, which may then fail to comply with specifications for spore content (Sadiq et al., 2016). However, psychrotrophic bacteria present more serious challenges to the dairy industry. Raw milk is contaminated with heat-stable enzymes produced by a broad spectrum of psychrotrophic bacteria at low temperatures, which can survive all successive processing conditions and remain active in processed dairy products (Vithanage et al., 2016). For example, the decimal reduction time (*D*-value) of proteases produced by *Pseudomonas* spp. was 416.67 min at 75 °C and 250 min at 85 °C, indicating that these enzymes are difficult to inactivate by normal thermal processing techniques (Machado et al., 2016). Consequently, these heat-stable enzymes may lead to unacceptable biochemical changes, a decrease in nutritional value, and reduced shelf-life of dairy products (Stoeckel et al., 2016). Lipases catalyze the hydrolysis of triglycerides which cause rancid, butyric, or soapy flavors and also may lead to a reduction in milk foaming properties (Chen et al., 2003; Bekker et al., 2016). Proteases hydrolyze casein fractions and produce defects described as bitter off-flavors and result in age gelation (Stoeckel et al., 2016). Phospholipases degrade the integrity of the milk fat globule membrane, facilitating more lipolysis by milk's natural lipases (Lilbaek et al., 2007). β-Galactosidases catalyze the hydrolysis of β-1,4-galactosidic bonds in lactose of milk (Deeth et al., 2002; Chen et al., 2009). Therefore, spoilage caused by psychrotrophic bacteria and their enzymes is a major concern in the dairy industry.

In our previous study (Yuan et al., 2017), a diverse range of psychrotrophic bacteria were found in Chinese raw milk, but their ability to produce spoilage enzymes or the thermo-stability patterns of these enzymes were not studied. Thus, the objective of this study was to explore the enzymatic characteristics of all bacterial isolates in Chinese raw milk at both room temperature (28 °C) and refrigerated temperature (7 °C), and to evaluate the thermo-resistance of spoilage enzymes, thereby enhancing knowledge of the spoilage potential of psychrotrophic bacteria.

2 Materials and methods

2.1 Bacterial strains

Four-hundred and eighty strains previously isolated from Chinese raw milk samples were studied (Yuan et al., 2017). These strains, representing 24 genera, 74 species, and 85 different random amplified polymorphic DNA (RAPD)-based genetic groups, were maintained in nutrient broth containing 20% glycerol (Sinopharm Chemical Reagent Co., Ltd., China) at −80 °C.

2.2 Screening for enzyme production

All strains were checked for their abilities to produce protease, lipase, β-galactosidase, and phospholipase on the following screening media: plate count agar supplemented with 6% (0.06 g/ml) skimmed milk powder (Difco, USA) for protease; tributyrin agar (Sigma-Aldrich, USA) for lipase; nutrient agar plate spread with 35 μl 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Sigma-Aldrich, USA) and 20 μl isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma-Aldrich, USA) for β-galactosidase; and nutrient agar plate supplemented with 8% (v/v) egg yolk emulsion (Oxoid, UK) for phospholipase. Each test was performed at 7 °C for 7 d and 28 °C for 2 d.

2.3 Quantitative assessment of total proteolytic activity

Strains that showed positive protease production on screening medium were selected to quantify their total proteolytic activity. Strains were first grown in nutrient broth for 36 h on a shaking incubator at 150 r/min. They were then diluted in 50 ml commercial ultra-heat-treated (UHT) milk to a final concentration of 1×10^4 colony-forming unit (CFU)/ml, corresponding to the psychrotrophic bacterial cell density found in our previous study (Yuan et al., 2017), and incubated on a shaking incubator at 120 r/min under two different conditions: 7 °C for 7 d and 28 °C for 2 d.

Samples were centrifuged at 12000*g* for 10 min at 4 °C after the incubation. Proteolytic activity was measured using azocasein (Sigma, USA) as a substrate following the protocol described by Santos et al.

(1996) with some modifications. To initialize the reaction, 100 µl cell-free supernatant was mixed with 500 µl phosphate buffer saline (PBS; 50 mmol/L, pH 7.2, Sangon Biotech Co., Ltd., China). Then, 100 µl azocasein (1.5% (15 g/L), dissolved in 50 mmol/L PBS) was added. The mixture was incubated at 37 °C for 60 min and the reaction was stopped by adding 500 µl of 20% (0.2 g/ml) trichloroacetic acid (Aladdin, China). After centrifugation at 12 000g for 5 min at 4 °C, absorbance at 366 nm was measured using a spectrometer-based absorbance microplate reader (Thermo Fisher Multiskan FC, USA). A control was included, in which 100 µl of sterile PBS was used in place of supernatant. The proteolytic activity is expressed as the increase of absorption at 366 nm per hour and milliliter (ΔA h^{-1}·ml^{-1}). Each assay was performed in triplicate and with three replicates.

2.4 Quantitative assessment of total lipolytic activity

Strains that showed positive lipase production on screening medium were selected to quantify the total lipolytic activity. Strains were grown in nutrient broth for 36 h on a shaking incubator at 150 r/min, and then diluted in 50 ml UHT milk to a concentration of 1×10^4 CFU/ml. The milk samples were then incubated on a shaking incubator at 120 r/min under two different conditions: 7 °C for 7 d and 28 °C for 2 d.

After incubation, samples were centrifuged at 15 000g for 20 min at 4 °C, and the supernatants were filtered using 0.45-µm cellulose acetate filter units to avoid the interference of milk proteins (Abdou, 2003; Chen et al., 2003). Lipolytic activity was measured using p-nitrophenol palmitate (p-NPP; Sigma, USA) as a substrate following the method described by Teh et al. (2013) with slight modifications. To initialize the reaction, 100 µl of cell-free supernatant was added to 900 µl substrate solution containing 3 mg of p-NPP dissolved in 1 ml isopropanol (Aladdin, China), 9 ml of 50 mmol/L Tris-HCl (pH 8) solution containing 40 mg of Triton X-100 (Aladdin, China), and 1 mg of arabic gum (Aladdin, China). The mixture was incubated at 37 °C for 20 min and the reaction was stopped by adding 500 µl 95% ethanol (Sinopharm Chemical Reagent Co., Ltd., China). After centrifugation at 15 000g for 5 min at 4 °C, absorbance at 410 nm was measured using a spectrometer-based absorbance microplate reader. A control was included,

in which 100 µl sterile PBS was used in place of the supernatant. The lipolytic activity was expressed as the amount of p-nitrophenol (µmol) released per minute per milliliter (µmol/(min·ml)) of milk sample under the assay conditions described above. Each assay was performed in triplicate and with three replicates.

2.5 Heat resistance of proteases and lipases

Strains that proved to be positive for protease or lipase production were selected to determine the thermo-resistance of their enzymes. Strains were grown in nutrient broth for 36 h on a shaking incubator at 150 r/min and then diluted in 50 ml UHT milk to a concentration of 1×10^4 CFU/ml, followed by incubation on a shaking incubator at 120 r/min and 7 °C for 7 d or 28 °C for 2 d. Samples were heat-treated in a water bath at 70 °C for 15 min. After heat treatment, samples were cooled immediately in an ice bath followed by the addition of 0.01% (0.1 g/L) sodium azide (Sinopharm Chemical Reagent Co., Ltd., China) and 0.025% (0.25 g/L) bronopol (Aladdin, China) to inhibit bacterial growth (Machado et al., 2016). Subsequently, milk samples were incubated at 37 °C for 5 d. Proteolytic and lipolytic activity was measured as described above.

Strains shown to produce heat-stable enzymes in the tests described above were selected for further thermal inactivation trials. Milk samples divided into aliquots of 5 ml in sterile tubes were heat-treated under the following time-temperature conditions. For protease, three different temperature-time combinations were used: 70 °C for 180 min, 80 °C for 150 min, and 90 °C for 100 min. For lipases, different temperature-time combinations were used: 70 °C for 80 min, 80 °C for 70 min, and 90 °C for 60 min. After each heat treatment, samples were cooled immediately in an ice bath followed by the addition of 0.01% (0.1 g/L) sodium azide and 0.025% (0.25 g/L) bronopol. Subsequently, milk samples were incubated at 37 °C for 5 d. Log of percentage residual activity (R) was plotted against heating time and expressed as the thermo-stability. The rate constant k for first-order inactivation, activation energy (E_a), half-life ($t_{1/2}$), D-value, change in enthalpy ($\Delta H^{\#}$), change in entropy ($\Delta S^{\#}$), and change in free energy ($\Delta G^{\#}$) were calculated according to Olusesan et al. (2011).

3 Results

3.1 Screening for enzyme production

A total of 423 isolates incubated at 28 °C showed the ability to produce at least one type of enzyme, while 166, 142, and 32 isolates produced two, three, and four different enzymes, respectively (Fig. 1). Isolates of *Pseudomonas*, *Acinetobacter*, *Flavobacterium*, *Chryseobacterium*, *Serratia*, and *Aeromonas* were not only the most predominant in raw milk samples, but also proved to be high enzyme producers. Among the *Pseudomonas* isolates, 235 showed proteolytic activity and 164 showed lipolytic activity (Fig. 2a). Also, 153 isolates showed phospholipase activity and 17 showed β-galactosidase activity. Species of *Acinetobacter* exhibited high lipolytic (63 out of 64 strains) but weak proteolytic (14 out of 64) and phospholipase (22 out of 64) activity, and none of them produced β-galactosidase. Of the 29 *Flavobacterium* isolates, 27, 10, 11, and 21 isolates showed proteolytic, lipolytic, β-galactosidase, and phospholipase activity, respectively. Isolates belonging to *Serratia* displayed a strong tendency to produce proteases (15 out of 15 strains), lipases (14 out of 15), and phospholipases (15 out of 15), but a weak ability to produce β-galactosidases (1 out of 15). Species belonging to *Aeromonas* produced all four types of enzymes, indicating their high spoilage potential. *Sphingobacterium* and *Rahnella* produced only β-galactosidases, and *Janthinobacterium* showed negative protease and lipase production.

Similar results were obtained at 7 °C, but slightly fewer isolates belonging to each genus showed spoilage potential. A total of 403 isolates showed the ability to produce at least one type of enzyme, while
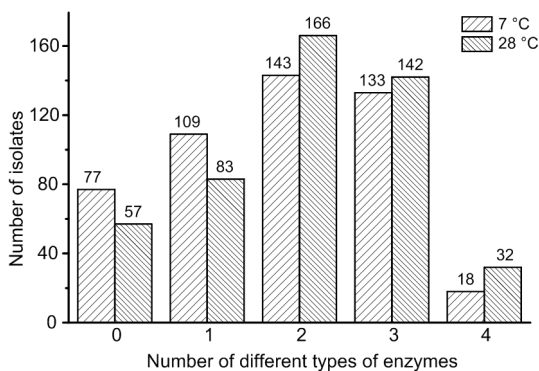
143, 133, and 18 isolates produced two, three, and four different enzymes, respectively (Fig. 1). Among the *Pseudomonas* isolates, 221, 156, 17, and 134 isolates showed proteolytic, lipolytic, β-galactosidase, and phospholipase activity (Fig. 2b), respectively. Species of *Acinetobacter* exhibited high lipolytic (56 out of 64 strains), but weak proteolytic (10 out of 64) and phospholipase (22 out of 64) activity. Among *Flavobacterium* isolates, 24, 9, 8, and 19 isolates showed proteolytic, lipolytic, β-galactosidase, and phospholipase activity, respectively. The abilities of isolates belonging to *Serratia*, *Aeromonas*, *Sphingobacterium*, and *Rahnella* to produce each enzyme were the same as those observed at 28 °C.

3.2 Quantitative assessment of total proteolytic activity

The proteolytic activity of the predominant psychrotrophic bacteria is shown in Fig. 3. The range of proteolysis was 1.23–13.02 $h^{-1} \cdot ml^{-1}$ at 28 °C and
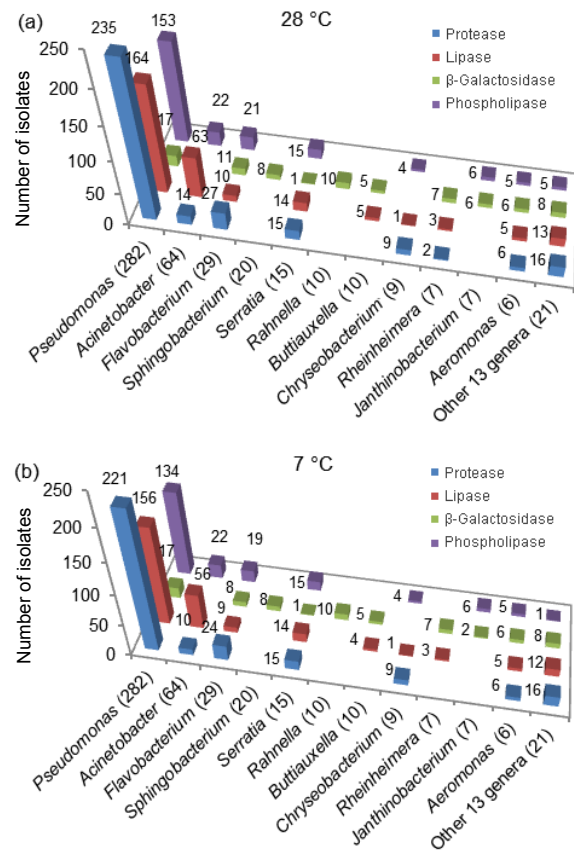

Fig. 2 Abilities of isolates to produce protease, lipase, β-galactosidase, and phospholipase at 28 °C (a) and 7 °C (b)
The number in the bracket implies the total number of strains for each genus

Fig. 1 Number of isolates that produce different types of enzymes at 7 and 28 °C

$0.42-5.72\ h^{-1}\cdot ml^{-1}$ at 7 °C. At 28 °C, *Yersinia intermedia* showed the highest proteolytic activity followed by *Pseudomonas fluorescens* (d), *Chryseobacterium oncorhynchi*, *Serratia grimesii*, and *Pseudomonas gessardii*. Species of *Acinetobacter* showed low proteolytic activity, and the lowest proteolytic activity was shown by *Acinetobacter johnsonii* (a). Isolates belonging to *Serratia* showed high proteolytic activity with a mean value of $8.51\ h^{-1}\cdot ml^{-1}$.

The proteolytic activity of isolates incubated at 7 °C was lower than that at 28 °C. The highest protease activity was shown by *Y. intermedia*, followed by *P. fluorescens* (d), *C. oncorhynchi*, and *Serratia liquefaciens*. *Rheinheimera chironomi* showed the lowest protease activity followed by *A. johnsonii*.

3.3 Quantitative assessment of total lipolytic activity

The lipolytic activity of the predominant psychrotrophic bacteria is shown in Fig. 4. The range of lipolytic activity was 0.20–13.09 μmol/(min·ml) at 28 °C and 0.11–7.54 μmol/(min·ml) at 7 °C. At 28 °C, *Acinetobacter guillouiae* showed the highest lipolytic

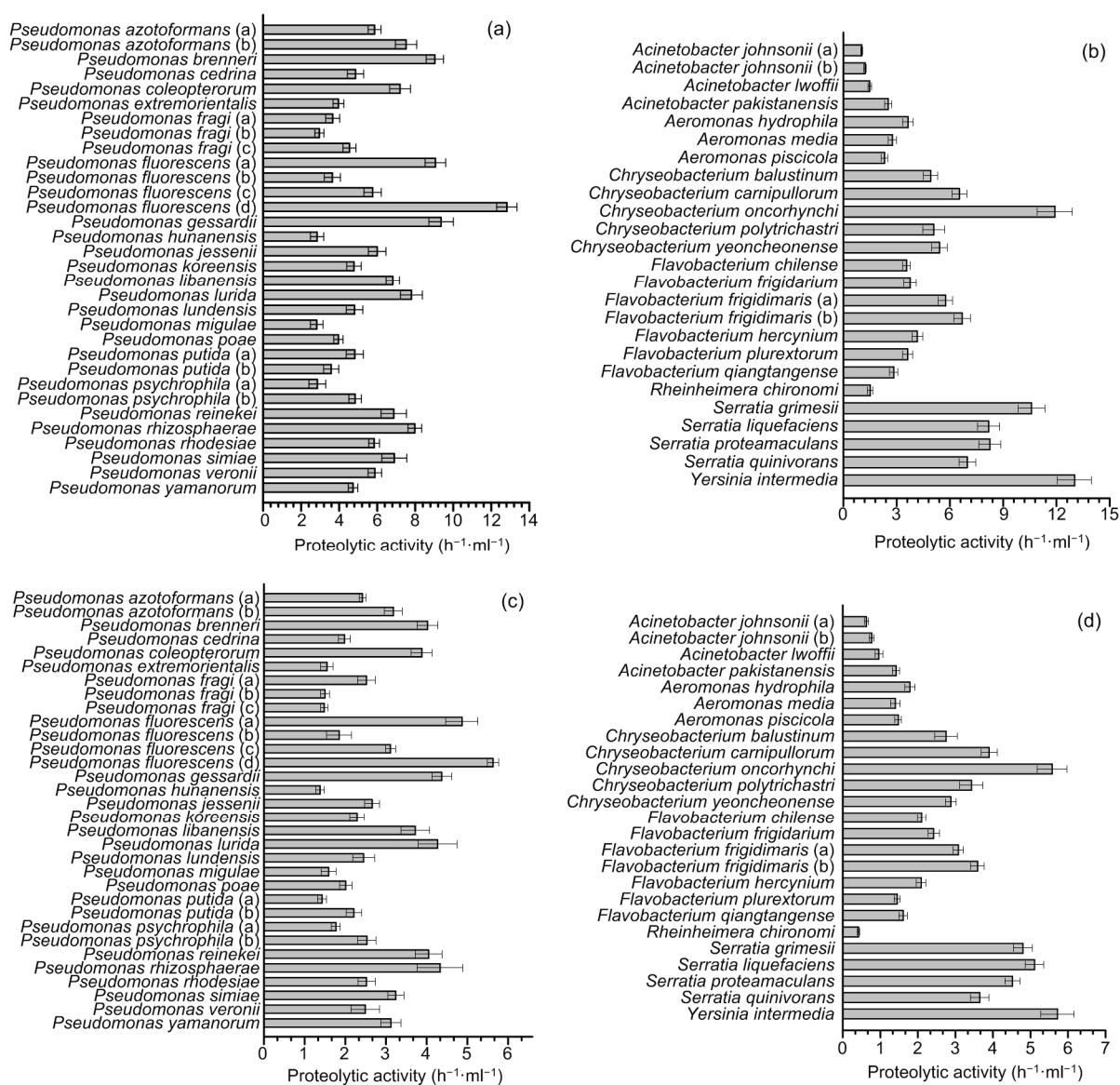**Fig. 3 Proteolytic activity of psychrotrophic bacteria isolated from raw milk**
(a) Isolates belonging to *Pseudomonas* incubated at 28 °C; (b) Isolates belonging to other predominated species incubated at 28 °C; (c) Isolates belonging to *Pseudomonas* incubated at 7 °C; (d) Isolates belonging to other predominated species incubated at 7 °C. Data are expressed as mean±standard deviation (*n*=3)

activity followed by *Pseudomonas coleopterorum*, *Acinetobacter pakistanensis*, and *A. johnsonii* (b). The lowest lipase activity was shown by *Pseudomonas poae*. Isolates belonging to *Acinetobacter* showed relatively high lipase activity with a mean value of 7.15 μmol/(min·ml).

The lipolytic activity of each isolate incubated at 7 °C was also lower than that of corresponding bacteria incubated at 28 °C. The highest lipolytic activity was shown by *A. guillouiae* followed by *P. coleopterorum*, *A. johnsonii* (b), and *Pseudomonas extremorientalis*. *P. poae* showed the lowest lipolytic activity followed by *Flavobacterium frigidarium*.

3.4 Heat resistance of proteases and lipases

Milk samples incubated at 7 °C were heat-treated at 70 °C for 15 min. As shown in Fig. 5, 49 isolates showed a residual proteolytic activity of <30%. The highest proportion of isolates exhibited a residual activity from 30% to 70%. Eighty-four isolates had a residual activity of ≥70%. For lipases, residual activity was <30% for 77 isolates, ≥70% for 48 isolates, and from 30% to 70% for 137 isolates.

Residual activity was compared after the incubation of samples at 28 °C. Fifty-four isolates showed <30%, 91 showed ≥70%, and 179 showed from 30% to 70% residual activity. For lipases, 82 isolates

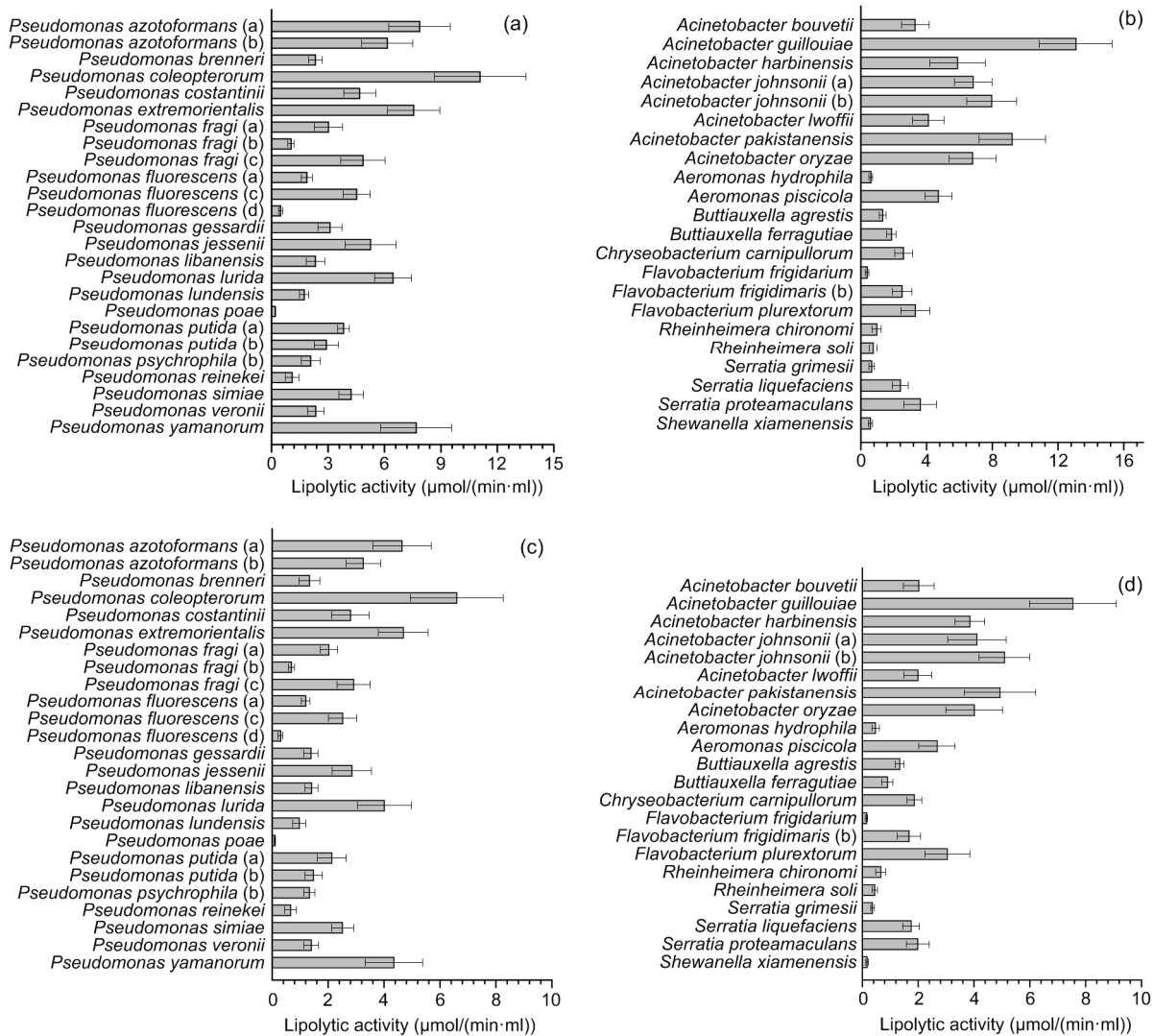**Fig. 4 Lipolytic activity of psychrotrophic bacteria isolated from raw milk**
(a) Isolates belonging to *Pseudomonas* incubated at 28 °C; (b) Isolates belonging to other predominated species incubated at 28 °C; (c) Isolates belonging to *Pseudomonas* incubated at 7 °C; (d) Isolates belonging to other predominated species incubated at 7 °C. Data are expressed as mean±standard deviation (*n*=3)
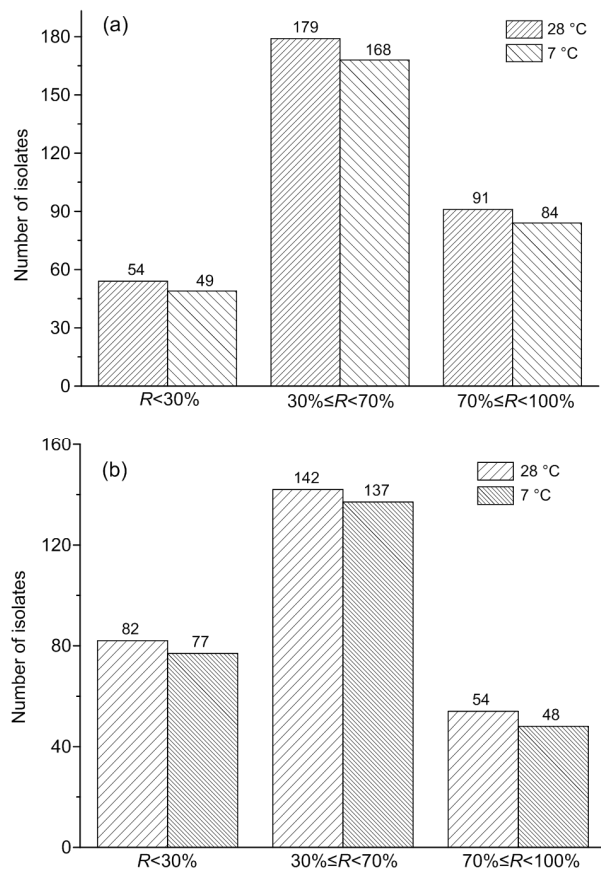
Fig. 5 Distribution of isolates based on their residual activity (R) of proteases (a) and lipases (b) after the heat treatment at 70 °C for 15 min

showed <30%, 54 showed ≥70%, and 142 showed from 30% to 70% residual activity.

Strains that showed high (≥70%) residual proteolytic activity (*P. fluorescens* (d), *Pseudomonas azotoformans* (a), *S. grimesii*, *Chryseobacterium polytrichastri*, and *Flavobacterium frigidimaris* (b)) or lipolytic activity (*A. johnsonii* (b), *A. pakistanensis*, *P. fluorescens* (c), and *P. azotoformans* (a)) were selected for evaluating kinetic parameters of thermal inactivation. An increase in enzyme inactivation correlated well with temperature and heating time. *D*-values and $t_{1/2}$ decreased with increasing temperature (Table 1). At 7 °C, the *D*-values of proteases produced by these five isolates ranged from 116.34 to 220.51 min at 70 °C, 87.40 to 133.59 min at 80 °C, and 59.83 to 77.13 min at 90 °C. E_a ranged from 31.33 to 53.72 kJ/mol, and $\Delta S^{\#}$ from −171.23 to −111.14 J/(mol·K) at 70 °C, −164.48 to −104.29 J/(mol·K) at 80 °C, and −157.55 to −97.65 J/(mol·K) at 90 °C.

No significant differences in these kinetic parameters were observed when samples were incubated at 28 °C. Proteases produced by *S. grimesii* proved to be the most resistant, both at 28 and 7 °C.

Lipases showed less heat resistance than proteases (Table 2). At 7 °C, *D*-values of lipases produced by the four isolates ranged from 44.31 to 72.74 min at 70 °C, 35.41 to 57.44 min at 80 °C, and 28.98 to 40.69 min at 90 °C. E_a ranged from 21.03 to 28.20 kJ/mol, and $\Delta S^{\#}$ from −194.04 to −176.81 J/(mol·K) at 70 °C, −186.89 to −170.17 J/(mol·K) at 80 °C, and −180.62 to −163.27 J/(mol·K) at 90 °C. Similarly, no significant differences in these kinetic parameters were observed when samples were incubated at 28 °C. Lipase produced by *A. pakistanensis* showed the highest heat resistance, both at 28 and 7 °C.

4 Discussion

Heat-stable enzymes produced by psychrotrophic bacteria cannot be completely inactivated by thermal processing techniques and pose threats to the quality and shelf-life of dairy products. Some studies have reported results of screening for enzymes produced by psychrotrophic bacteria isolated from raw milk (von Neubeck et al., 2015; Vithanage et al., 2016). However, quantitative assessments and the thermo-stabilities of proteases and lipases have rarely been reported. The objective of this work was to explore the spoilage potential of psychrotrophic bacteria and the thermo-stabilities of proteases and lipases they produced.

A wide variety of psychrotrophic bacteria can produce spoilage enzymes during late log or stationary growth phases (Rajmohan et al., 2002). Most isolates in this study had enzymatic activity at low temperatures. *Pseudomonas* is reported to be the most frequently isolated genus in raw milk, probably because of its short generation time (von Neubeck et al., 2015; Yuan et al., 2017). The spoilage potential of *Pseudomonas* has been extensively studied and those bacteria tend to produce proteases combined with lipases (Capodifoglio et al., 2016; Vithanage et al., 2016).

In this study, most *Pseudomonas* species, such as *P. fluorescens* (strains b and d), *P. gessardii*, *P. lurida*, and *P. brenneri*, showed high protease activity,

Table 1 Thermodynamic parameters of proteases produced by representative isolates cultured at 28 and 7 °C

Cultured temp (°C)	Isolate	Temp		1/temp ($\times 10^{-3}$ K^{-1})	k (min^{-1})	D (min)	$t_{1/2}$ (min)	E_a (kJ/mol)	$\Delta H^{\#}$ (kJ/mol)	$\Delta S^{\#}$ (J/(mol·K))	$\Delta G^{\#}$ (kJ/mol)
		(°C)	(K)								
28	*Pseudomonas fluorescens* (d)	70	343	2.92	0.013	171.17	50.48	48.33	45.48	−124.86	88.24
		80	353	2.83	0.021	107.82	30.91		45.40	−117.95	87.08
		90	363	2.75	0.035	65.79	18.81		45.32	−111.38	85.82
	Pseudomonas azotoformans (a)	70	343	2.92	0.012	177.80	52.78	49.44	46.59	−122.23	88.45
		80	353	2.83	0.021	111.17	30.71		46.51	−114.81	87.08
		90	363	2.75	0.033	69.40	18.67		46.43	−108.74	85.97
	Serratia grimesii	70	343	2.92	0.010	225.75	66.66	53.72	50.87	−111.14	88.93
		80	353	2.83	0.017	135.19	39.30		50.79	−104.29	87.64
		90	363	2.75	0.030	77.59	22.03		50.71	−97.65	86.22
	Chryseobacterium polytrichastri	70	343	2.92	0.019	122.27	33.66	33.64	30.79	−164.89	87.26
		80	353	2.83	0.026	88.22	22.18		30.71	−157.94	86.52
		90	363	2.75	0.038	59.87	15.56		30.63	−151.20	85.61
	Flavobacterium frigidimaris (b)	70	343	2.92	0.017	133.05	37.16	36.72	33.35	−158.26	87.55
		80	353	2.83	0.026	90.28	23.61		33.27	−149.79	86.52
		90	363	2.75	0.036	63.42	16.44		33.19	−144.54	85.75
7	*Pseudomonas fluorescens* (d)	70	343	2.92	0.014	166.37	47.73	44.92	42.07	−134.26	88.05
		80	353	2.83	0.022	106.67	30.59		41.99	−127.24	86.95
		90	363	2.75	0.035	65.21	18.23		41.91	−120.75	85.82
	Pseudomonas azotoformans (a)	70	343	2.92	0.013	176.21	51.21	46.90	44.05	−129.03	88.24
		80	353	2.83	0.021	110.53	30.07		43.97	−122.00	87.08
		90	363	2.75	0.034	68.41	18.05		43.89	−115.53	85.90
	Serratia grimesii	70	343	2.92	0.010	220.51	58.46	53.72	50.87	−111.14	88.93
		80	353	2.83	0.017	133.59	37.70		50.79	−104.29	87.64
		90	363	2.75	0.030	77.13	21.58		50.71	−97.65	86.22
	Chryseobacterium polytrichastri	70	343	2.92	0.020	116.34	32.00	31.33	28.48	−171.23	87.12
		80	353	2.83	0.026	87.40	21.36		28.40	−164.48	86.52
		90	363	2.75	0.038	59.83	15.24		28.32	−157.55	85.61
	Flavobacterium frigidimaris (b)	70	343	2.92	0.018	130.05	35.46	34.23	31.38	−163.58	87.40
		80	353	2.83	0.026	90.02	22.71		31.30	−156.27	86.52
		90	363	2.75	0.036	63.53	16.24		31.22	−149.96	85.75

while *P. azotoformans* (a), *P. coleopterorum*, *P. extremorientalis*, *P. fragi* (c), *P. fluorescens* (c), and *P. lurida* showed high lipase activity. *P. fluorescens*, *P. lurida*, and *P. simiae* can produce all four of the different enzymes screened in this work, indicating their great spoilage potential. The *aprX* gene coding for the protease is located at the beginning of a polycistronic operon with the *lipA* gene that encodes the lipase (Woods et al., 2001). Thus, most isolates belonging to *Pseudomonas* have the ability to produce proteases and lipases due to the simultaneous expression of both genes. However, expression of enzymes can be tightly regulated, which may explain the absence of one or both enzymes in some isolates. In this work, some isolates belonging to *P. fluorescens* and *P. fragi*

did not show proteolytic or lipolytic activity. This phenotypic variation within the same species is consistent with previous findings (Wiedmann et al., 2000; Caldera et al., 2016). For example, only 36% of isolates belonging to *P. fragi* from different foods showed proteolytic activity (Caldera et al., 2016). For this reason, in the future, whole genome sequencing of these isolates will be carried out to differentiate their spoilage potential.

Acinetobacter is considered to be the predominant bacterial genus in raw milk (von Neubeck et al., 2015; Yuan et al., 2017). This genus is ubiquitous in nature and characterized by the tendency to tolerate dry conditions and multidrugs (Gurung et al., 2013). In the current work, all isolates belonging to *Acinetobacter*

Table 2 Thermodynamic parameters of lipases produced by representative isolates cultured at 28 and 7 °C

Cultured temp (°C)	Isolate	Temp		1/temp ($\times 10^{-3}$ K^{-1})	k (min^{-1})	D (min)	$t_{1/2}$ (min)	E_a (kJ/mol)	$\Delta H^{\#}$ (kJ/mol)	$\Delta S^{\#}$ (J/(mol·K))	$\Delta G^{\#}$ (kJ/mol)
		(°C)	(K)								
28	*Acinetobacter*	70	343	2.92	0.033	70.17	21.56	27.24	24.39	−179.38	85.82
	johnsonii (b)	80	353	2.83	0.041	56.64	15.46		24.31	−172.69	85.33
		90	363	2.75	0.058	39.41	10.60		24.23	−165.77	84.51
	Acinetobacter	70	343	2.92	0.031	75.43	23.96	28.73	25.45	−176.75	85.98
	pakistanensis	80	353	2.83	0.040	58.23	16.32		25.37	−169.88	85.40
		90	363	2.75	0.056	41.06	11.40		25.29	−163.10	84.60
	Pseudomonas	70	343	2.92	0.051	45.55	13.44	21.51	18.66	−192.81	84.69
	fluorescens (c)	80	353	2.83	0.064	36.13	10.10		18.58	−185.65	84.18
		90	363	2.75	0.079	29.07	8.11		18.50	−169.62	83.74
	Pseudomonas	70	343	2.92	0.041	56.42	16.87	25.94	23.09	−181.51	85.25
	azotoformans (a)	80	353	2.83	0.055	41.59	11.67		23.01	−174.21	84.57
		90	363	2.75	0.070	32.74	8.60		22.94	−167.97	84.02
7	*Acinetobacter*	70	343	2.92	0.033	69.92	21.31	27.24	24.39	−179.38	85.82
	johnsonii (b)	80	353	2.83	0.041	56.13	15.20		24.31	−172.69	85.33
		90	363	2.75	0.058	39.55	10.62		24.23	−165.77	84.51
	Acinetobacter	70	343	2.92	0.032	72.74	21.66	28.20	25.35	−176.81	85.90
	pakistanensis	80	353	2.83	0.040	57.44	15.77		25.27	−170.17	85.40
		90	363	2.75	0.057	40.69	11.28		25.19	−163.27	84.56
	Pseudomonas	70	343	2.92	0.052	44.31	12.78	21.03	18.18	−194.06	84.64
	fluorescens (c)	80	353	2.83	0.065	35.41	9.67		18.10	−186.89	84.14
		90	363	2.75	0.079	28.98	7.77		18.02	−180.62	83.70
	Pseudomonas	70	343	2.92	0.042	55.34	16.23	25.46	22.61	−182.73	85.19
	azotoformans (a)	80	353	2.83	0.056	41.19	11.53		22.53	−175.43	84.52
		90	363	2.75	0.071	32.60	8.47		22.45	−169.21	83.98

showed high lipolytic activity and low proteolytic activity, which is in agreement with previous studies (von Neubeck et al., 2015; Vithanage et al., 2016). However, a high number of *Acinetobacter* isolates with proteolytic activity were found in Mozzarella cheese. Such isolates can carry out the hydrolysis of α-casein and spoil the cheese (Baruzzi et al., 2012). This might be explained by horizontal gene transfer from proteolytic species or phenotypic variation among isolates. *Chryseobacterium* also appears as a dominant member of raw milk microbiota (Yuan et al., 2017) and some species show a greater spoilage ability than *P. fluorescens* based on their proteolytic and lipolytic activity (Bekker et al., 2015, 2016). In this study, species of *Chryseobacterium* showed more proteolytic than lipolytic activity, and *C. oncorhynchi* showed higher proteolytic activity than many isolates of *Pseudomonas*. To our knowledge, few studies have reported the spoilage potential of *Flavobacterium*. The results of our study suggest that it has high proteolytic but weak lipolytic activity. Species of *Serratia*

are also characterized as predominant milk spoilers due to their ability to produce heat-stable proteases and lipases (Machado et al., 2016). In this study, *Serratia* was shown to have high spoilage potential based on its ability to produce protease, lipase, β-galactosidase, and phospholipase. However, isolates belonging to other predominant genera (*Sphingobacterium*, *Rahnella*, *Buttiauxella*, and *Janthinobacterium*) lacked the ability to produce both proteases and lipases. Thus, the population of psychrotrophic bacteria plays a key role in the determination of the quality of dairy products.

The productions of phospholipases and β-galactosidases have been less frequently reported than those of proteases and lipases, since proteases and lipases pose more serious threats to the quality and shelf-life of dairy products. Phospholipase activity was found among the isolates belonging to the genera *Pseudomonas*, *Acinetobacter*, *Flavobacterium*, *Serratia*, *Chryseobacterium*, *Janthinobacterium*, and *Aeromonas*, consistent with their ability to cause

sweet curdling defects or bitter cream in milk as a result of fat globule aggregation (Titball, 1993; Vithanage et al., 2016). β-Galactosidase is an important indicator of lactose fermentation in dairy products, and may catalyze the hydrolysis of β-1,4-galactosidic bonds in lactose (Chen et al., 2009). The production of β-galactosidase by psychrotrophic bacteria has seldom been reported. In this study, β-galactosidases were produced by isolates of the genera *Pseudomonas*, *Flavobacterium*, *Sphingo-bacterium*, *Rahnella*, *Buttiauxella*, *Rheinheimera*, *Sphingobacterium*, and *Aeromonas*.

The production of enzymes is a temperature-dependent process (Buchon et al., 2000). As reported by Decimo et al. (2014), enzymatic activity is highly influenced by the incubation temperature as results obtained at 30 °C differ from those obtained at 7 °C. In our work, more isolates showed positive results for each enzymatic activity, and activity of proteases and lipases was higher at 28 °C than at 7 °C, indicating that the storage and transportation of raw milk at low temperatures could be effective to some extent in controlling the spoilage of raw milk.

Considering the potential effects of enzymes on dairy products, appropriate practices should be adopted to minimize the quality losses, by either limiting the growth of psychrotrophic bacteria or inactivation of their enzymes by thermal processing. Adequate addition of CO_2 to raw milk has been shown to reduce proteolysis and lipolysis by limiting microbial growth and the production of microbial proteases (Ma et al., 2003). Sophisticated dairy farm management systems, such as Good Manufacturing Practice (GMP) and Hazard Analysis and Critical Control Point (HACCP), are also effective ways to limit the initial number of psychrotrophic bacteria in raw milk (Cusato et al., 2014). However, the effects of these practices on the heat stability of enzymes are unknown. The heat stability of enzymes originating from psychrotrophs is a limiting factor in maintaining the quality and shelf-life of dairy products. Thus, better knowledge of their heat stability is needed.

Heat treatments adopted by the dairy industry are insufficient to completely inhibit the activity of enzymes produced by psychrotrophic bacteria (Glück et al., 2016; Vithanage et al., 2016). Reducing the activity and limiting the secretion of heat-stable enzymes are scientific challenges. In this work, prote-

ases produced by a large number of predominant isolates still remained active after heat treatment at 70 °C for 15 min, and some even showed residual activity of ≥70%. Kinetic parameters for the thermal inactivation of *P. fluorescens* (d), *P. azotoformans* (a), and *Serratia quinivorans* highlight the high potential of these enzymes to cause dairy spoilage. In previous studies, the thermo-stability of proteases produced by *Pseudomonas* or *Serratia* has been extensively reported. The *D*-values of proteases produced by *S. liquefaciens* and *Pseudomonas* sp. were 96.15 and 80.00 min, respectively, at 90 °C (Machado et al., 2016). The *D*-values of proteases produced by *Pseudomonas proteolytica* were 6.3, 4.0, and 1.3 min at 120, 130, and 140 °C, respectively (Stoeckel et al., 2016). The residual activity of protease produced by *P. fluorescens* was 97.2% after heat treatment at 80 °C for 30 min (Zhang and Lv, 2014). The purified protease Ser2 secreted by *S. liquefaciens* was highly heat-stable in skimmed, semi-skimmed, and whole milk at 140 °C, with *D*-values of 2.4, 3.9, and 4.5 min, respectively, and the presence of milk fat increased the heat-stability of the protease (Baglinière et al., 2017). The heat inactivation of the purified protease from *Chryseobacterium indologenes* did not follow first-order kinetics, but showed biphasic inactivation curves, and this protease is much more heat-labile than other psychrotrophic proteases (Venter et al., 1999). In our study, the kinetic parameters for inactivation of the protease secreted by *C. polytrichastri* suggested that this protease is also heat-resistant. This may be because the crude enzyme is more heat-stable than the purified enzyme, and the different methods for estimating heat resistance should also be taken into account. Kinetic thermal inactivation of proteases produced by species belonging to *Flavobacterium* has not been reported previously, and our results suggest that the protease produced by *F. frigidimaris* (b) is thermo-stable.

Many studies have determined the heat stability of proteases, but the heat stability of lipases is less well known. It has been reported that lipases produced by various *Pseudomonas* isolates showed residual activity ranging from 55% to 100% after heat treatment at 63 °C for 30 min (Law et al., 1976). The *D*-value of lipase produced by *P. fluorescens* was 23.5 min after heat treatment at 100 °C (Andersson et al., 1979). Vithanage et al. (2016) reported that

more than 30% of strains belonging to *Pseudomonas* isolated from raw milk showed 50% to 75% residual lipase activity after heat treatment at 142 °C for 4 s. In this study, many isolates showed high residual lipase activity after heat treatment at 70 °C for 15 min. Kinetic parameters showed that lipases produced by *A. johnsonii* (b), *A. pakistanensis*, *P. fluorescens* (c), and *P. azotoformans* (a) are heat-resistant and can survive after pasteurization. Surprisingly, lipases were less heat-resistant than proteases. For example, the *D*-value of the most heat-stable lipase produced by *A. pakistanensis* was 41.06 min at 90 °C, which is much smaller than that of the most heat-stable protease produced by *S. grimesii*. Moreover, enzymes are more heat-stable in synthetic milk salt solutions than in phosphate buffer due to the protective effect of milk components in the milk system (Baur et al., 2015).

The inactivation of enzymes fits first-order kinetics, and kinetic parameters indicate that while a higher temperature and longer heat treatment period may result in a higher reduction of enzymatic activity, the destruction and inactivation of milk constituents will be increased. Results for the Gibbs free energy ($G^{\#}$), enthalpy ($H^{\#}$), and entropy ($S^{\#}$) of thermal inactivation of enzymes have rarely been reported. Hydrophobic interactions, hydrogen bonds, disulfide bridges, and electrostatic interactions are the forces that stabilize a protein and are determined by $\Delta G^{\#}$, while $\Delta H^{\#}$ and $\Delta S^{\#}$ provide a measure of structural disorder upon protein folding associated with the stability of an enzyme. High levels of $\Delta G^{\#}$ and $\Delta H^{\#}$, and low values of $\Delta S^{\#}$ indicate the structural stability of enzymes in this study. It is difficult to compare the results of heat stability of enzymes provided by different laboratories due to different time-temperature combinations of heat treatments and because heat resistance varies at the species or strain level. The thermo-stability patterns exhibited by proteases and lipases demonstrate the importance of exploring the combination of optimal temperature and time for heat treatments in the dairy industry.

The cold-chain of storage and transportation reduces the production and activity of spoilage enzymes. However, no significant difference in thermo-stability was observed between low and high incubation temperatures in this study. This can be explained by the fact that the thermo-stability of enzymes depends on the genetics of the specific isolates tested.

It is important to stress that this work focused only on enzymes produced by planktonic cells. The amount of proteolysis and lipolysis produced is higher in biofilms than in planktonic cultures, and the thermo-stability of enzymes can be enhanced by the protective matrix of EPS (Teh et al., 2012, 2013). This suggests that biofilms formed by spoilage psychrotrophs may pose more serious threats to the dairy industry. To our knowledge, few studies have reported any possible link between spoilage enzymes and biofilm formation by psychrotrophic bacteria. This will be taken into consideration in the future research.

5 Conclusions

The production of heat-resistant spoilage enzymes presents a formidable challenge to today's dairy industry. A high number of predominant bacteria isolated from Chinese dairy milk can produce spoilage enzymes. Species of *Pseudomonas*, *Serratia*, and *Chryseobacterium* are highly proteolytic, while isolates of *Acinetobacter* and *Pseudomonas* can produce highly active lipases. Isolates belonging to certain genera show positive β-galactosidase and phospholipase activity. Most enzymes are heat-resistant as they can survive after different heat treatments applied in the manufacture of dairy products. There is a vast amount of scientific knowledge about the ability of psychrotrophic bacteria to produce spoilage enzymes, but no effective strategy has yet been presented to control this problem. Thus, the heat stability and molecular characteristics of enzymes and the development of novel screening methods should be addressed in the future.

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中文概要

题　目：原料奶中嗜冷菌产腐败酶的能力及腐败酶的热稳定性研究

目　的：探究从原料奶样品中分离到的 480 株嗜冷菌（鉴定为 24 个属、74 个种，并根据随机扩增多态性 DNA（RAPD）图谱特征聚类为 85 组）在室温和低温下产腐败酶的能力，以及这些腐败酶的耐热性。

创新点：系统地分析和比较不同种类的嗜冷菌在室温和低温下产腐败酶的能力，并探究这些腐败酶的耐热性，为控制乳制品和其它低温储藏食品的腐败变质提供理论基础。

方　法：采用不同的筛选培养基评估 480 株嗜冷菌在室温（28 ℃）和低温（7 ℃）产蛋白酶、脂肪酶、磷脂酶及 β-半乳糖苷酶的能力；根据初筛的结果分别以偶氮酪蛋白和对硝基苯酚棕榈酸酯为底物定量测定蛋白酶和脂肪酶的酶活力；挑选酶活力高的蛋白酶和脂肪酶，分别测定经 70、80 和 90 ℃ 条件下处理一段时间后的残留酶活计算出热稳定性，并根据热动力学公式计算相关的热力学参数。

结　论：*Pseudomonas*、*Serratia* 及 *Chryseobacterium* 产蛋白酶的能力强，而 *Yersinia intermedia* 产蛋白酶的活力最高；*Acinetobacter* 产脂肪酶的能力强，其中 *Acinetobacter guillouiae* 产脂肪酶的活力最高；而一些特定的属能产生 β-半乳糖苷酶及磷脂酶。细菌产腐败酶的能力在种水平上表现出差异性（如 *Pseudomonas fluorescens* 和 *Pseudomonas fragi*）。大量的蛋白酶及脂肪酶即使经高温热处理后仍有酶活残留，且所得的热灭活动力学的参数证实这些腐败酶经常规方法灭菌后仍对乳制品的品质及货架期有潜在的危害。

关键词：腐败酶；嗜冷菌；原料奶；热稳定性