Identification and proteolytic activity quantification of *Pseudomonas* spp. isolated from different raw milks at storage temperatures

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ABSTRACT

Commercial milk products worldwide come not only from cows, but also from goats, buffaloes, camels, and yaks. Milk from non-bovine animals is important culturally and economically. *Pseudomonas* spp. are frequently linked to milk spoilage under storage temperatures. The objectives of this study were to identify *Pseudomonas* spp. isolated from goat, buffalo, camel, and yak milks, and to measure proteolytic activity of *Pseudomonas* spp. under different storage temperatures. Raw milk samples of goat (n = 50), buffalo (n = 25), camel (n = 25), and yak (n = 25) were collected from 5 provinces in China. *Pseudomonas* spp. were analyzed by *Pseudomonas*-specific 16S, universal 16S rRNA, and *rpoB* gene sequence analyses. Proteolytic activity on milk agar, quantification via the trinitrobenzenesulfonic acid assay at 2°C, 4°C, 7°C, 10°C and 25°C, as well as alkaline peptidase gene (*aprX*) identification were performed to ascertain the proteolytic activity of these isolates. *Pseudomonas* spp. were found in 46 samples out of total 125 samples. A total of 67 *Pseudomonas* spp. were identified. Of *Pseudomonas* isolates, we obtained extracellular peptidase activity in 7 (10.4%) at 2°C, 17 (25.4%) at 4°C, 24 (35.8%) at 7°C, 39 (58.2%) at 10°C, and 41 (61.2%) at 25°C. The results revealed that a wide diversity of *Pseudomonas* spp. were present in different non-bovine raw milks, with the ability to produce peptidases at storage temperatures. However, proteolytic activity varied widely among the peptidase-positive isolates. A majority of isolates from yak milk had high proteolytic activity.

Key words: non-bovine raw milk, *Pseudomonas* spp., proteolytic activity, spoilage

INTRODUCTION

The largest proportion of commercial milk products is derived from cows. However, milks from other ruminants (e.g., goats, buffaloes, camels, and yaks) are also important culturally and economically (Quigley et al., 2013). Milk from different animals varies in its nutritional value, presence of bioactive vesicles, and potential therapeutic properties (Guo et al., 2014; Baddela et al., 2016; Kumar et al., 2016). The microbial populations of goat, buffalo, camel, and yak milk play significant roles in fermentation, milk spoilage, and health promotion (Quigley et al., 2013).

Psychrotrophic bacteria, which cause spoilage, have the ability to grow at refrigerated storage temperatures (1°C to 4°C; Quigley et al., 2013; Li et al., 2016). Psychrotrophic microorganisms comprise the main microbial population in refrigerated raw milk (Xin et al., 2017). Among psychrotrophic microorganisms, *Pseudomonas* has been identified as the major genus in milk, leading to poor quality of raw milk (Marchand et al., 2009; Baur et al., 2015a; de Oliveira et al., 2015). *Pseudomonas* spp. produce several extracellular enzymes (e.g., peptidases and lipases). Although *Pseudomonas* bacteria are sensitive to pasteurization and UHT, their heat-stable enzymes could contribute to coagulation and reduced quality of milk and dairy products (Rajmohan et al., 2002; Dufour et al., 2008; Marchand et al., 2008).

Peptidases secreted by *Pseudomonas* spp. are primarily active on casein, leading to alterations in the physico-chemical and organoleptic properties of raw milk (Dufour et al., 2008). These peptidases belong mainly to the class of metallopeptidases (EC 3.4.24; Scatamburlo et al., 2015; Caldera et al., 2016). The heat-resistant peptidase alkaline metallopeptidase (AprX), which belongs to the serralyisin family, is widespread among *Pseudomonas* spp. It is responsible for the spoilage of milk because of its high protein-degrading activity. The spoilage effects of *Pseudomonas* spp. vary dramatically

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Table 1. Primer sequences used in the study

Primer	Sequence (5' to 3')	Amplified region	Reference
PA-GS-F	GACGGGTGAGTAATGCCTA	16S gene signature for <i>Pseudomonas</i>	Spilker et al. (2004)
PA-GS-R	CACTGGTGTTCCCTTCCTATA		
27F	AGAGTTTGATCCTGGCTCAG	16S rRNA gene (universal primer)	Scarpellini et al. (2004)
1492R	CTACGGCTACCTTGTACGA		
PSF	AGTTCATGGACCAGAACAACC	<i>rpoB</i>	Decimo et al. (2014)
PTR	CCTTGACGGTGAACCTCGTTTC	<i>aprX</i> gene of <i>Pseudomonas</i>	Sajben et al. (2011)
SM2F	AAATCGATAGCTTCAGCCAT		Caldera et al. (2016)
SM3R	TTGAGGTTGATCTTCTGGTT		

depending on strains and growth conditions (Chessa et al., 2000; Chabeaud et al., 2001; Nicodème et al., 2005).

There are differences in nutrient composition and microbiota among milks; however, optimal storage conditions are similar for non-bovine and bovine raw milk. Therefore, the aims of this study were to (1) identify *Pseudomonas* spp. from goat, buffalo, camel, and yak milks obtained from 5 provinces in China; and (2) evaluate the proteolytic activity of these isolates. This study contributes to our understanding of the occurrence of the most important *Pseudomonas* species and their spoilage peptidase activity in goat, buffalo, camel, and yak raw milks.

MATERIALS AND METHODS

Sample Collection and *Pseudomonas* spp. Identification

We collected 125 raw milk samples from dairy herds in China, including 25 milk samples of goat from Shaanxi province (in spring), 25 milk samples of goat from Shandong province (in spring), 25 milk samples of buffalo from Guangxi Zhuang Autonomous Region (in spring), 25 milk samples of camel from Xinjiang Uygur Autonomous Region (in spring), and 25 milk samples of yak from Sichuan province (in summer). All samples were collected in bulk tanks, transferred into sterile plastic bottles (Corning Inc., Corning, NY), and transported to the laboratory at 4°C within 4 h.

The isolation and identification of *Pseudomonas* spp. were performed as reported by Scatamburlo et al. (2015). Briefly, all raw milk samples were diluted 10-fold in 0.85% NaCl (wt/vol) and homogenized. Aliquots (1 mL) of selected dilutions were placed onto *Pseudomonas* agar (Oxoid Ltd., Basingstoke, UK), which is composed of 16.0 g/L gelatin peptone, 10.0 g/L casein hydrolysate, 10.0 g/L potassium sulfate, 1.4 g/L magnesium chloride, and 11.0 g/L agar, and the pH was adjusted to 7.1 ± 0.2 at 25°C. The *Pseudomonas* agar was supplemented with penicillin (100,000 IU/L, Dr. Ehrenstorfer GmbH, Augsburg, Germany)

and pimarcin (0.01 g/L, Dr. Ehrenstorfer GmbH) to selectively isolate *Pseudomonas* spp. The plates were incubated at 25°C for 48 h. Five to 8 colonies from each plate were streaked onto new *Pseudomonas* agar plates (Oxoid Ltd.) and incubated at 25°C for 48 h.

Isolates of *Pseudomonas* spp. were identified by PCR thermal cyclers (Bio-Rad S1000; Bio-Rad, Hercules, CA) and 16S rRNA and *rpoB* sequencing. First, DNA of each isolate was extracted using the InstaGene Matrix DNA extraction kit (Bio-Rad) following the manufacturer's instructions. The PCR reaction mixtures (25 µL) consisted of 12.5 µL of EmeraldAmp Max PCR Master Mix (Takara, Dalian, China), 9.5 µL of deionized water (Takara), 1 µL of each primer (100 µM; Sangon Biotech Shanghai Co. Ltd., Shanghai, China; Table 1), and approximately 50 ng of bacterial genomic DNA. A negative control (a sample without genomic DNA) and a positive control (DNA of *P. fluorescens* CICC 21620; China Center of Industrial Culture Collection, Beijing, China) were included in all PCR assays.

We identified *Pseudomonas* spp. by evaluating a genus-specific region of 16S rDNA. The amplification conditions consisted of 1 cycle at 95°C for 2 min; followed by 25 cycles at 94°C for 20 s, 54°C for 20 s, and 72°C for 40 s; and a final extension step at 72°C for 1 min (Scatamburlo et al., 2015). Amplicons of 618 bp were considered indicative of *Pseudomonas*.

The 16S rRNA gene was then amplified under the following conditions, 1 cycle at 94°C for 4 min; 30 cycles at 94°C for 1 min, 57°C for 30 s, and 72°C for 1.5 min; and 1 cycle at 72°C for 10 min. Because the 16S rRNA gene is known for its low resolution for discriminating species within *Pseudomonas*, comparison of partial *rpoB* sequences was also performed as a basis for phylogenetic analyses (Ercolini et al., 2007; Decimo et al., 2014). The thermal program consisted of 1 cycle at 94°C for 3 min; 30 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 10 min.

Finally, the derived amplicons were sequenced (Sangon Biotech). All available sequence data of *Pseudomonas* spp. were analyzed using BLAST against the National Center for Biotechnology Information data-

base (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). For universal 16S rRNA and *rpoB*, the degree of homology was >98%.

Proteolytic Activity

Aliquots (100 μ L) of selected dilutions were plated onto *Pseudomonas* agar (as described before) previously supplemented with UHT milk (10%, vol/vol; Granarolo S.p.A., Bologna, Italy). The plates were incubated at 2°C, 4°C, 7°C, 10°C, or 25°C for 7 d and observed daily. The presence of a proteolytic halo was considered a positive result (Scatamburlo et al., 2015).

Quantification of Proteolytic Activity

The quantification of proteolytic activity was performed as reported by Caldera et al. (2016), with slight modifications. Based on the proteolytic activity results obtained from milk agar, *Pseudomonas* isolates were incubated in 5 mL of UHT milk (Granarolo S.p.A.) at 2°C, 4°C, 7°C, 10°C, or 25°C for 5 d. The milks were subsequently centrifuged at $6,000 \times g$ at 4°C for 20 min (Hitachi CR22GIII, Hitachi, Japan). The supernatant was transferred into a new tube (Corning Inc.) with added bronopol (Beijing Chemical Factory, Beijing, China) and sodium azide (Beijing Chemical Factory) at final concentrations of 0.025% and 0.01%, respectively, to prevent bacterial growth. The supernatant (1 mL) was added to 9 mL of fresh UHT milk (Granarolo S.p.A.) and incubated at 37°C for 14 d. The bacterial cell pellets were diluted and plated on agar plates to calculate bacterial count.

Afterward, we used the trinitrobenzenesulfonic acid (TNBS) method to quantify free α -amino groups, which can indicate protein hydrolysis (Polychroniadou, 1988; Marchand et al., 2009). Released α -amino groups were reacted with TNBS reagent (Sigma-Aldrich, Bornem, Belgium) at pH 9.2 in the dark for 100 min, and the intensity of the yellow–orange color was measured at 420 nm (Varioskan Flash Multimode Reader, ThermoFisher Scientific, Waltham, MA). The levels of proteolytic enzymes in raw milk were determined from the absorption ratios. Bacterial strains were considered active when the value of the proteolytic activity was more than 2 μ mol of glycine equivalents per mL. The standard curve was generated using glycine (Sigma-Aldrich).

aprX Analysis

The *aprX* gene of *Pseudomonas* isolates was amplified using the primers shown in Table 1. The amplification program consisted of 1 cycle at 94°C for 3 min; 30

cycles at 95°C for 15 s, 60°C for 15 s, and 72°C for 30 s; and 1 cycle at 72°C for 8 min. The PCR products were electrophoresed in a 1.5% (wt/vol) agarose gel in 0.5 $\times$ Tris-borate EDTA, stained with SYBR Safe DNA Gel Stain (ThermoFisher Scientific), and visualized using VersaDoc (Bio-Rad).

RESULTS

Molecular Classification and Identification

Identification was based on universal 16S rRNA and *rpoB* gene sequences. To minimize the chances of colonies being from the same strain, several colonies of different morphologies were selected from each sample. *Pseudomonas* spp. were found in 46 samples of 125 samples originating from 125 separate dairy farms; 67 colonies were identified as *Pseudomonas* spp. (Tables 2, 3, 4, and 5).

Species allocation was based on the 16S rRNA gene sequences and phylogenetic clustering of *rpoB* gene sequence data. Several isolates were close to a particular species because they clustered together with a type strain (~99%). We did not obtain any *rpoB* sequences for 6 strains. Therefore, these strains were referred to as *Pseudomonas* sp. and named as *Pseudomonas* sp. 1 to 6 (Tables 2–5 and Supplementary Table S1; <https://doi.org/10.3168/jds.2017-13617>).

Ten goat milk isolates were identified as *P. fluorescens* and 7 were *P. putida*, respectively. The remaining 14 goat milk isolates belonged to *P. fragi*, *P. moraviensis*, *P. cedrina*, *P. rhodesiae*, *P. protegens*, *P. lurida*, *P. reactans*, *P. chlororaphis*, and *P. poae*. The 8 buffalo milk isolates were equally distributed among *P. graminis*, *P. koreensis*, and *P. fluorescens*. Six of 9 isolates from camel milk were identified as *P. fluorescens* and *P. putida*. In yak milk, *P. fluorescens* was the dominant species (31.6% of 19 isolates), followed by undefined *Pseudomonas* sp. (26.3%), *P. fragi* (15.8%), and other strains (e.g., *P. putida*, *P. veronii*, *P. reinekei*, *P. cedrina*, and *P. koreensis*).

Proteolytic Activity of the Isolates

Tables 2 to 5 show the detailed phenotypic results. All 67 isolates grew between 4°C and 25°C; however, 2 isolates did not grow at 2°C. We obtained proteolytic activity in all isolates at all test temperatures (Table 6). Approximately 60% (39/67 and 41/67, respectively) of the isolates had extracellular peptidase activity on milk agar at 10°C and 25°C after 7 d of incubation. Approximately 30% (17/67 and 24/67, respectively) of the isolates had extracellular peptidase activity at 4°C and 7°C. At 2°C, 7 isolates showed extracellular peptidase

Table 2. The putative identification and phenotypical spoilage characterization of tested isolates (goat milk) from Shandong and Shaanxi provinces

Isolate identification	Growth					Extracellular peptidase activity on milk agar ¹									
	2°C	4°C	7°C	10°C	25°C	2°C	4°C	7°C	10°C	25°C					
From Shandong province															
<i>Pseudomonas putida</i>	+	+	+	+	+	–	–	–	–	–					
<i>Pseudomonas fluorescens</i>	+	+	+	+	+	–	+	(4)	+	(3)	+	(1)			
<i>Pseudomonas fluorescens</i>	+	+	+	+	+	–	–	+	(7)	+	(3)	–			
<i>Pseudomonas fragi</i>	+	+	+	+	+	–	–	+	(5)	+	(3)	+	(3)		
<i>Pseudomonas fluorescens</i>	+	+	+	+	+	–	+	(4)	+	(4)	+	(2)	+	(1)	
<i>Pseudomonas moraviensis</i>	+	+	+	+	+	–	+	(7)	+	(4)	+	(3)	+	(2)	
<i>Pseudomonas putida</i>	+	+	+	+	+	–	–	–	–	–	–	–	–		
<i>Pseudomonas putida</i>	+	+	+	+	+	–	–	–	–	–	–	–	–		
<i>Pseudomonas cedrina</i>	+	+	+	+	+	–	–	+	(5)	+	(4)	+	(5)		
<i>Pseudomonas putida</i>	–	+	+	+	+	–	–	–	+	(5)	+	(5)			
<i>Pseudomonas</i> sp. 1	+	+	+	+	+	–	–	–	–	–	+	(1)			
<i>Pseudomonas fluorescens</i>	+	+	+	+	+	–	–	–	+	(3)	+	(3)			
From Shaanxi province															
<i>Pseudomonas putida</i>	+	+	+	+	+	–	+	(7)	+	(5)	+	(3)	+	(2)	
<i>Pseudomonas fluorescens</i>	+	+	+	+	+	–	–	–	+	(4)	+	(3)			
<i>Pseudomonas putida</i>	+	+	+	+	+	–	–	–	–	–	–	–			
<i>Pseudomonas rhodesiae</i>	+	+	+	+	+	–	–	+	(4)	+	(2)	+	(1)		
<i>Pseudomonas fragi</i>	+	+	+	+	+	+	(4)	+	(2)	+	(2)	+	(2)	+	(1)
<i>Pseudomonas fragi</i>	+	+	+	+	+	–	–	–	–	–	–	–			
<i>Pseudomonas fragi</i>	+	+	+	+	+	–	–	–	–	–	–	–			
<i>Pseudomonas fluorescens</i>	+	+	+	+	+	–	–	–	–	–	–	–			
<i>Pseudomonas putida</i>	+	+	+	+	+	–	–	–	–	–	+	(1)			
<i>Pseudomonas protegens</i>	+	+	+	+	+	–	–	–	–	+	(3)	+	(3)		
<i>Pseudomonas fluorescens</i>	+	+	+	+	+	–	–	–	–	+	(5)	+	(5)		
<i>Pseudomonas fluorescens</i>	+	+	+	+	+	–	–	–	–	–	–	+	(1)		
<i>Pseudomonas fragi</i>	+	+	+	+	+	–	–	–	–	–	–	–			
<i>Pseudomonas lurida</i>	+	+	+	+	+	–	+	(3)	+	(3)	+	(3)	+	(2)	
<i>Pseudomonas reactans</i>	+	+	+	+	+	–	–	+	(6)	+	(6)	+	(5)		
<i>Pseudomonas chlororaphis</i>	–	+	+	+	+	–	–	–	–	+	(4)	+	(5)		
<i>Pseudomonas fluorescens</i>	+	+	+	+	+	–	–	–	–	–	–	–			
<i>Pseudomonas fluorescens</i>	+	+	+	+	+	–	–	–	+	(5)	+	(5)			
<i>Pseudomonas poae</i>	+	+	+	+	+	+	(6)	+	(5)	+	(3)	+	(2)	+	(1)

¹Numbers in parentheses indicate the incubation days required to observe proteolytic activities.

activity, and 4 of 7 isolates had peptidase activity after 6 and 7 d of incubation. No extracellular peptidase activity was observed on the first day between 2°C and 10°C.

When focusing on isolates from different matrices (milk types), we observed several matrix-dependent findings. Extracellular peptidase activity at 25°C was

observed in 75.0% (6/8) of buffalo milk isolates, 67.7% (21/31) of goat milk isolates, 57.9% (11/19) of yak milk isolates, and 33.3% (3/9) of camel milk isolates. Similar results were obtained at 7°C and 10°C. At 4°C, we observed extracellular peptidase activity in 37.5% (3/8) of buffalo milk isolates, 26.3% (4/19) of yak milk isolates, 22.6% (7/31) of goat milk isolates, and 22.2%

Table 3. The putative identification and phenotypical spoilage characterization of tested buffalo milk isolates from Guangxi

Isolate identification	Growth					Extracellular peptidase activity on milk agar ¹				
	2°C	4°C	7°C	10°C	25°C	2°C	4°C	7°C	10°C	25°C
<i>Pseudomonas graminis</i>	+	+	+	+	+	–	–	–	–	+ (5)
<i>Pseudomonas koreensis</i>	+	+	+	+	+	–	+ (7)	+ (4)	+ (2)	+ (1)
<i>Pseudomonas fluorescens</i>	+	+	+	+	+	–	–	–	+ (2)	+ (1)
<i>Pseudomonas graminis</i>	+	+	+	+	+	–	–	–	–	–
<i>Pseudomonas koreensis</i>	+	+	+	+	+	–	–	+ (4)	+ (3)	+ (1)
<i>Pseudomonas graminis</i>	+	+	+	+	+	–	–	–	–	–
<i>Pseudomonas fragi</i>	+	+	+	+	+	+ (5)	+ (2)	+ (2)	+ (2)	+ (1)
<i>Pseudomonas fluorescens</i>	+	+	+	+	+	–	+ (7)	+ (6)	+ (3)	+ (1)

¹Numbers in parentheses indicate the incubation days required to observe proteolytic activities.

Table 4. The putative identification and phenotypical spoilage characterization of tested camel milk isolates from Xinjiang

Isolate identification	Growth					Extracellular peptidase activity on milk agar ¹				
	2°C	4°C	7°C	10°C	25°C	2°C	4°C	7°C	10°C	25°C
<i>Pseudomonas putida</i>	+	+	+	+	+	–	–	–	–	–
<i>Pseudomonas putida</i>	+	+	+	+	+	–	–	–	–	–
<i>Pseudomonas fluorescens</i>	+	+	+	+	+	–	+	(3)	+	(1)
<i>Pseudomonas psychrophila</i>	+	+	+	+	+	–	–	–	–	–
<i>Pseudomonas fragi</i>	+	+	+	+	+	–	–	–	+	(3)
<i>Pseudomonas fluorescens</i>	+	+	+	+	+	–	–	–	+	(4)
<i>Pseudomonas fluorescens</i>	+	+	+	+	+	–	+	(5)	+	(3)
<i>Pseudomonas fragi</i>	+	+	+	+	+	–	–	–	–	–
<i>Pseudomonas putida</i>	+	+	+	+	+	–	–	–	–	–

¹Numbers in parentheses indicate the incubation days required to observe proteolytic activities.

(2/9) camel milk isolates. However, camel milk isolates had no peptidase activity at 2°C. Therefore, buffalo milk had the most isolates with peptidase activity on milk agar at different storage temperatures, followed by isolates from goat milk and yak milk. Few isolates from camel milk had extracellular peptidase activity.

Quantification of Proteolytic Activity

We quantified the total proteolytic activity of all isolates in UHT milk (Figure 1). The strains were considered to have proteolytic activity when proteolytic activity was >2 µmol of glycine equivalent per mL. The highest percentage of proteolytic activity isolates was obtained at 25°C (56.7%, n = 38) and 10°C (53.7%, n = 36). At 7°C, 4°C, and 2°C, the percentage of proteolytic activity isolates was 23.9% (n = 16), 14.9% (n = 10), and 3.0% (n = 2), respectively.

Among the isolates from each milk matrix, different levels of proteolytic activity were detected, which could be attributed to different behavior of the isolated species. The isolates from yak milk had high activities, as did isolates from goat and camel milks.

Analysis of *aprX*

We amplified the *aprX* gene from all isolates via PCR. An amplicon of the expected size (±850 bp) was obtained from 47 isolates. No amplification product was obtained in the remaining 20 isolates.

DISCUSSION

Psychrotrophs play a major role in the spoilage of refrigerated milk and milk products. Therefore, it is important to learn about the psychrotrophic bacteria

Table 5. The putative identification and phenotypical spoilage characterization of tested yak milk isolates from Sichuan province

Isolate identification	Growth					Extracellular peptidase activity on milk agar ¹								
	2°C	4°C	7°C	10°C	25°C	2°C	4°C	7°C	10°C	25°C				
<i>Pseudomonas putida</i>	+	+	+	+	+	–	–	–	–	–				
<i>Pseudomonas fragi</i>	+	+	+	+	+	–	–	–	–	–				
<i>Pseudomonas</i> sp. 2	+	+	+	+	+	–	–	–	–	–				
<i>Pseudomonas</i> sp. 3	+	+	+	+	+	–	–	–	–	–				
<i>Pseudomonas</i> sp. 4	+	+	+	+	+	–	–	–	–	–				
<i>Pseudomonas</i> sp. 5	+	+	+	+	+	+	(5)	+	(4)	+	(2)			
<i>Pseudomonas veronii</i>	+	+	+	+	+	–	–	–	+	(4)	+	(6)		
<i>Pseudomonas fluorescens</i>	+	+	+	+	+	–	–	–	–	–				
<i>Pseudomonas fluorescens</i>	+	+	+	+	+	–	–	–	–	–				
<i>Pseudomonas reinekei</i>	+	+	+	+	+	+	(7)	+	(4)	+	(3)	+	(1)	
<i>Pseudomonas fluorescens</i>	+	+	+	+	+	–	–	–	+	(4)	+	(2)		
<i>Pseudomonas cedrina</i>	+	+	+	+	+	+	(7)	+	(7)	+	(4)	+	(6)	
<i>Pseudomonas fragi</i>	+	+	+	+	+	–	–	–	+	(4)	+	(3)		
<i>Pseudomonas fluorescens</i>	+	+	+	+	+	–	+	(4)	+	(4)	+	(2)	+	(1)
<i>Pseudomonas fragi</i>	+	+	+	+	+	–	–	–	+	(3)	+	(2)		
<i>Pseudomonas fluorescens</i>	+	+	+	+	+	–	–	–	+	(4)	+	(3)		
<i>Pseudomonas fluorescens</i>	+	+	+	+	+	+	(6)	+	(3)	+	(3)	+	(2)	
<i>Pseudomonas koreensis</i>	+	+	+	+	+	–	–	+	(5)	+	(3)	+	(2)	
<i>Pseudomonas</i> sp. 6	+	+	+	+	+	–	–	–	–	–				

¹Numbers in parentheses indicate the incubation days required to observe proteolytic activities.

Table 6. *Pseudomonas* spp. isolates¹ with observed proteolytic activity² at different temperatures

Day	Incubation temperature				
	2°C	4°C	7°C	10°C	25°C
1	0	0	0	0	19
2	0	2	3	10	7
3	0	2	5	16	7
4	1	6	8	8	0
5	2	2	5	3	7
6	2	0	2	2	1
7	2	5	1	0	0
Total	7	17	24	39	41

¹The total number of *Pseudomonas* spp. isolates obtained from raw milk was 67.

²As determined during growth on milk agar. The number of new isolates exhibiting extracellular proteolytic activity on a given day of incubation on milk agar is shown.

present in bovine and non-bovine raw milks. In this study, we isolated *Pseudomonas* spp. in goat, buffalo, camel, and yak milks from 5 provinces; identified bacterial isolates to the species level; evaluated the proteolytic activity of *Pseudomonas* spp., and analyzed the presence of *aprX* as an indicator of proteolysis.

Following genomic confirmation of the genus level, the isolates were assigned at the species level. The identification of isolates was primarily based on 16S rRNA and *rpoB* sequences. In general, we obtained a large diversity of *Pseudomonas* species in the raw milk samples from different sources. *Pseudomonas fluorescens* was the predominant strain in goat milk, and it has been recognized as an important agent of raw goat milk spoilage. Scatamburlo et al. (2015) identified 82 *Pseudomonas* isolates clustered into 5 groups from 61 raw goat samples; 50% (n = 42) of the isolates were identified as *P. fluorescens*, consistent with our results (Table 2). More *Pseudomonas* isolates were obtained

Figure 1. Proteolytic activity quantification of isolates obtained from goat, buffalo, camel, and yak milk samples stored for 5 d at 2°C, 4°C, 7°C, 10°C, or 25°C. The x-axis represents proteolytic activity (μmol of glycine equivalents per mL), and the y-axis represents bacterial count (10⁶ cfu/mL). The species names of isolates with very low or no activity are not shown.

from Shaanxi province than from Shandong province, reflecting a different farm environment. Raw buffalo milk contains a diverse microbial population. At the genus level, *Streptococcus* (14.2%), *Lactococcus* (8.2%), and *Pseudomonas* (4.9%) were the 3 dominant populations in raw buffalo milk after 2 h of storage. After 72 h of storage, *Pseudomonas* was the dominant genus, accounting for more than 51% of total bacteria (Li et al., 2016). However, we identified 8 *Pseudomonas* isolates from 25 raw buffalo milk samples, which indicates that *Pseudomonas* spp. comprise a small proportion of the microflora buffalo when grazing, in the parlor, or during the milking process (Table 3).

Several peptidase-producing psychrotrophic bacteria can be isolated from raw camel milk. Zahran and Al-Saleh (1997) identified *Pseudomonas* as the predominant peptidase-producing psychrotrophic bacteria in raw camel milk. However, among 25 raw camel milk samples, we obtained only 9 *Pseudomonas* isolates (Table 4). Among these 9 *Pseudomonas* isolates, *P. fluorescens* and *P. putida* were predominant. Similar results were reported by Zahran and Al-Saleh (1997).

Yak is an animal that is typically associated with difficult climates, and approximately 92% of the world yak population is located in China. Yak milk is produced during the summer. Most studies have focused on the presence of lactic acid bacteria, yeast, and coliforms in yak milk (Watanabe et al., 2008; Zhang et al., 2008; Yu et al., 2011). Our findings revealed that *Pseudomonas* spp. represent an important bacteria component of raw yak milk. More than 7 *Pseudomonas* spp. were identified in this study (Table 5), indicating a large diversity of *Pseudomonas* spp. in yak milk. Moreover, the count of isolated *Pseudomonas* strains was higher in yak milk ($n = 19$) than in buffalo ($n = 8$) or camel milk ($n = 9$), which may be due to the collection of yak milk in summer. Even though the biochemical and organoleptic characteristics of raw milk in different animal species differ widely, our findings indicate that *Pseudomonas* spp. are ubiquitous in milk.

The results showed that most isolates could grow at storage temperatures, except for 2 isolates from goat milk that were inhibited at 2°C (Tables 2–5). Munsch-Alatossava and Alatossava (2006) reported that *Pseudomonas* strains isolated from farms failed to grow at 4°C or 7°C, which suggests that the growth of *Pseudomonas* isolates is temperature-dependent (Wilcox et al., 1993; Munsch-Alatossava and Alatossava, 2006).

Several *Pseudomonas* species produce extracellular peptidases that can affect different kinds of milk. At 10°C and 25°C, most goat milk isolates ($\geq 60\%$) in the current study exhibited proteolytic activity, which is an important spoilage feature (Scatamburlo et al., 2015). As temperatures decreased from 7°C to 2°C, less than

40% of isolates from goat milk exhibited proteolytic activity. Although buffalo milk had the lowest number of strains, more than half of the isolates were proteolytically active at 7°C, 10°C, and 25°C. These results are consistent with the findings of Caldera et al. (2015), who reported that comparable levels of blue spoilage in mozzarella cheese were observed after 2 d at 25°C, 7 d at 12°C, and 17 d at 4°C, respectively. However, the association between storage temperature and storage time for other milks, including camel milk, is unclear; in camel milk, a minor fraction of the isolates ($\leq 45\%$ from 2°C to 25°C) exhibited proteolytic activity. Fewer studies have been performed on yak milk, but the findings obtained here for extracellular peptidase activity were in agreement with those of cow milk (Wiedmann et al., 2000). Detectable extracellular peptidase activity is very important for assessing dairy product quality. Proteolytic enzymes degrade milk proteins, leading to the production of noxious end products and significant economic losses for the food industry (Dogan and Boor, 2003; Arslan et al., 2011).

Proteolytic activity was confirmed by quantitative analysis and reported as glycine equivalents per milliliter. Certain types of cheeses are produced using raw milk not subjected to any heat-processing conditions. Therefore, we quantified the native proteolytic activity, not just heat-resistant proteolytic activity. Goat and yak milk isolates had higher peptidase activity than buffalo and camel milk isolates at 2°C, 4°C and 7°C. However, we obtained more isolates with peptidase activity from buffalo milk than from goat or yak milk at 10°C and 25°C. A small percentage of camel milk isolates showed proteolytic activity. This is similar to the study of Glück et al. (2016), who evaluated the thermostability of peptidases from 231 microorganisms isolated from raw milk and reported that only 38 *Pseudomonas* isolates showed proteolytic activity after cultivation at 6°C. In the study of Caldera et al. (2016), 33% (3/9) of raw cow milk and pasteurized milk isolates and 52% (12/23) of milk product (soft cheese and mozzarella) isolates had measurable proteolytic activity. Because our milk samples were from raw milk of different animal species and theirs were mainly from produced products, it is difficult to compare the results. However, Marchand et al. (2017) concluded that good quality cow milk (raw or processed) should have a mean TNBS value of approximately 1.01 ± 0.04 μmol of glycine equivalents per mL. Also, they concluded that there was no clear correlation between the onset of off-flavors and the rate of protein hydrolysis in UHT-processed milk by different *Pseudomonas* peptidase groups.

Among the isolated species, we detected key differences in proteolytic activity. The highest proteolytic activity was detected in *P. fluorescens* in yak and camel

milk. However, the proteolytic activity of all *P. fluorescens* varied considerably. We observed a large heterogeneity of *P. fragi* proteolytic activity, in agreement with the results reported by Caldera et al. (2016).

According to laws and local standards, raw goat milk, buffalo milk, and yak milk should be stored below 7°C (China Dairy Industry Association, 2012; The Guangxi Zhuang Autonomous Region Health and Family Planning Commission, 2014; Scatamburlo et al., 2015). Many studies have found that *Pseudomonas* spp. from cow milk produce peptidases at 4°C and 7°C. In this study, we identified several *Pseudomonas* isolates from non-bovine milk that produced peptidases at different storage temperatures. This contributes to a very complex microbial ecology of raw milk, especially goat and yak milks, and creates challenges in the prevention of milk spoilage (Ercolini et al., 2009). Moreover, Baur et al. (2015b) and Glück et al. (2016) found that several peptidases secreted from *Pseudomonas* spp. withstood UHT treatments in milk and showed considerable proteolytic activity under storage conditions of UHT milk. Therefore, the storage temperatures for raw milk from goat and yak should be lower and storage time should be limited to a short period.

The match between the presence of *aprX* and proteolytic activity on milk agar was convincing, confirming the role of *AprX* in milk degradation (Dufour et al., 2008; Caldera et al., 2016). However, 3 *aprX*-positive isolates did not exhibit proteolytic activity at any storage temperature, which indicated that *AprX* was not active during growth. In *Pseudomonas*, *AprX* is usually involved in nutrient utilization and extracellular protein degradation, resulting in it being an important bacterium in the spoilage of milk and dairy products (Zhang et al., 2009). Therefore, production of *AprX* highlights the importance of controlling *Pseudomonas* contamination by potentially using this enzyme as a key indicator of proteolysis.

In conclusion, our findings demonstrate that different raw milk types harbor a wide diversity of *Pseudomonas* spp. Most isolates are able to grow at storage temperatures and produce peptidases that affect milk quality. Our results highlighted the importance of *Pseudomonas* species as broadly adaptable spoilage organisms, of which *P. fluorescens* is the most common. Therefore, to control *Pseudomonas* spp. growth and prevent milk spoilage during the storage and transportation, correct storage temperatures and time should be established for raw milks originated from different animal species.

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