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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, buffalos, 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 serralysin 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 *Pseudomonas*	Spilker et al. (2004)
PA-GS-R	CACTGGTGTTCCTTCCTATA		
27F	AGAGTTTGATCCTGGCTCAG	16S rRNA gene (universal primer)	Scarpellini et al. (2004)
1492R	CTACGGCTACCTTGTTACGA		
PSF	AGTTCATGGACCAGAACAACC	*rpoB*	Decimo et al. (2014)
PTR	CCTTGACGGTGAACTCGTTTC		Sajben et al. (2011)
SM2F	AAATCGATAGCTTCAGCCAT	*aprX* gene of *Pseudomonas*	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 pimaricin (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 μ*M*; 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-

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