Spoilage potential of *Pseudomonas* species isolated from goat milk

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ABSTRACT

Pseudomonas spp. are usually associated with spoilage microflora of dairy products due to their proteolytic potential. This is of particular concern for protein-based products, such as goat milk cheeses and fermented milks. Therefore, the goal of the present study was to characterize the proteolytic activity of *Pseudomonas* spp. isolated from goat milk. Goat milk samples (n = 61) were obtained directly from bulk tanks on dairy goat farms (n = 12), and subjected to a modified International Organization for Standardization (ISO) protocol to determine the number and proteolytic activity of *Pseudomonas* spp. Isolates (n = 82) were obtained, identified by PCR, and subjected to pulsed-field gel electrophoresis with *Xba*I macro-restriction. Then, the isolates were subjected to PCR to detect the alkaline protease gene (*apr*), and phenotypic tests were performed to check proteolytic activity at 7°C, 25°C, and 35°C. Mean *Pseudomonas* spp. counts ranged from 2.9 to 4.8 log cfu/mL, and proteolytic *Pseudomonas* spp. counts ranged from 1.9 to 4.6 log cfu/mL. All isolates were confirmed to be *Pseudomonas* spp., and 41 were identified as *Pseudomonas fluorescens*, which clustered into 5 groups sharing approximately 82% similarity. Thirty-six isolates (46.9%) were positive for the *apr* gene; and 57 (69.5%) isolates presented proteolytic activity at 7°C, 82 (100%) at 25°C, and 64 (78%) at 35°C. The isolates were distributed ubiquitously in the goat farms, and no relationship among isolates was observed when the goat farms, presence of *apr*, pulsotypes, and proteolytic activity were taken into account. We demonstrated proteolytic activity of *Pseudomonas* spp. present in goat milk by phenotypic and genotypic tests and indicated their spoilage potential at distinct temperatures. Based on these findings and the ubiquity of *Pseudomonas* spp. in goat farm environments, proper

monitoring and control of *Pseudomonas* spp. during production are critical.

Key words: *Pseudomonas*, spoilage, proteolysis, goat milk

INTRODUCTION

Goat milk and derivative products are considered high-quality products by the dairy industry because of their biochemical and organoleptic characteristics. Among consumers, goat milk is considered a healthy food (Haenlein, 2004), and the characteristics of its components allow production of highly priced dairy products, such as cheeses, yogurts, and a variety of other fermented products (Pandya and Ghodke, 2007; Scintu and Piredda, 2007).

As with any other food of animal origin, goat milk is susceptible to microbial contamination during production, which can jeopardize its quality and safety (Chambers, 2002). In this context, keeping the product at low temperatures during production is important for preventing bacterial growth during storage and transport and avoiding the development of most spoilage microorganisms (Quigley et al., 2013). Refrigeration is usually adopted for this purpose, but must be applied considering adequate conditions of the equipment, temperature, and storage period. Otherwise, microbial counts, especially those of the psychrotrophic microbiota, can increase dramatically, thus resulting in poor quality goat milk and its dairy products (Sorhaug and Stepaniak, 1997; Yamazi et al., 2013).

Pseudomonas spp. is a relevant psychrotrophic genus responsible for spoilage of raw milk, including goat milk (Dufour et al., 2008; Arslan et al., 2011; Chen et al., 2011). *Pseudomonas* spp. have high genetic diversity and metabolic versatility, allowing them to survive in different environments, such as soil, water, and air. These characteristics allow them to survive on the utensils and equipment used in the dairy production chain, such as pipelines, bulk tanks, milking machines, and animal production environment (Simões et al., 2010). *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, and *Pseudomonas putida* are the main *Pseudomonas*

Received August 15, 2014.

Accepted November 2, 2014.

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species found in the dairy chain and are responsible for the production of proteolytic enzymes that are resistant to high temperatures and cause spoilage, even in processed dairy products (Rajmohan et al., 2002; Marchand et al., 2009). The metalloprotease AprX is one of the enzymes produced by *Pseudomonas* spp., and it is of particular interest to the dairy industry due to its specific spoilage activity in casein, which results in significant modifications to the physico-chemical and organoleptic properties of raw milk (Dufour et al., 2008).

The goal of the present study was to characterize the proteolytic potential and genetic diversity of *Pseudomonas* spp. present in the spoilage microbiota of raw goat milk, and to report information to support strategies to control their contamination and growth.

MATERIALS AND METHODS

Goat Milk Samples and *Pseudomonas* spp. Counts

Raw milk goat samples ($n = 61$) were collected directly from bulk tanks on 12 dairy goat farms located in Minas Gerais State, Brazil. At least 3 samples were obtained from each farm from different milking procedures. All samples were refrigerated during transport to the laboratory, where they were diluted 10-fold in 0.85% NaCl (wt/vol; Wehr and Frank, 2004) and subjected to microbial analysis to determine the counts of *Pseudomonas* spp. and proteolytic *Pseudomonas* spp. using a modified protocol of ISO/TS 11.059 (ISO, 2009).

Aliquots (0.1 mL) of selected dilutions were plated in duplicate onto the surface of *Pseudomonas* agar (Oxoid Ltd., Basingstoke, UK) supplemented with penicillin (100,000 IU/L, Sigma Aldrich, St. Louis, MO), pimarcin (0.01 g/L, Sigma), and sterile skim milk [10%, vol/vol, previously prepared with Molico skim powder milk (Nestlé, São Paulo, Brazil) diluted in sterile water]. The plates were incubated at 25°C for 72 h, and then all colonies were counted based on presence (proteolytic *Pseudomonas* spp.) or absence (*Pseudomonas* spp.) of a proteolytic halo.

From each sample, one plate that had 30 to 300 colonies was selected, and 10% of the colonies were streaked onto *Pseudomonas* agar plates (Oxoid), incubated at 25°C for 24 h, and subjected to Gram staining, assessment of oxidase production, and a glucose fermentation test (Downes and Ito, 2001). *Pseudomonas* spp. were preliminarily identified as gram-negative rods, oxidase positive, and able to use glucose, according ISO (2009). Based on these results, *Pseudomonas* spp. and proteolytic *Pseudomonas* spp. counts were expressed as colony forming units per milliliter for each sample and converted to $\log_{10}$ for data presentation.

Pseudomonas spp. Identification and Fingerprinting

Isolates that were preliminarily identified as *Pseudomonas* spp. ($n = 82$) were subjected to molecular analysis to confirm their identification and genetic profiles. Polymerase chain reaction was conducted to confirm the isolates as belonging to the genus *Pseudomonas* and to identify the species *P. fluorescens*. Single colonies from each isolate were transferred to trypticase soy broth (Oxoid) and incubated at 25°C for 24 h. The aliquots were subjected to DNA extraction using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI). The concentration of DNA was estimated by spectrophotometry (NanoDrop Lite, Thermo Scientific, Wilmington, DE).

Pseudomonas spp. were confirmed by checking a genus-specific region of the 16S DNA region, as previously described by Spilker et al. (2004), and using the primers PA-GS-F (GACGGGTGAGTAATGCCTA) and PA-GS-R (CACTGGTGTTCCTTCCTATA). The PCR reactions consisted of 12.5 μ L of Go Taq Green Master Mix 2 $\times$ (Promega), 1 μ L of extracted DNA (~50–100 ng/ μ L), 10 pmol of each primer, and ultra-pure water (Promega) to bring the final volume to 25 μ L. Amplification conditions were 1 cycle at 95°C for 2 min, 25 cycles of 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. The PCR products were electrophoresed in a 1.5% (wt/vol) agarose gel in 0.5 $\times$ Tris-borate EDTA, stained in a GelRed bath (Biotium Inc., Hayward, CA), and visualized using a transilluminator LPIX (Locus Biotecnologia, São Paulo, Brazil). Amplicons of 618 bp were considered typical for *Pseudomonas* genus.

A region from DNA 16S specific for *P. fluorescens* was investigated using the primers 16SPSEfluF (TG-CATTCAAACACTGACTG) and 16SPSER (AATCA-CACCGTGGTAACCG; Scarpellini et al., 2004). The PCR reactions consisted of 12.5 μ L of Go Taq Green Master Mix 2 $\times$ (Promega), 1 μ L of DNA (~50–100 ng/ μ L), 10 pmol of each primer, and ultra-pure water (Promega) to bring the final volume to 25 μ L. Amplification conditions were 1 cycle of 94°C for 2 min, 30 cycles of 94°C for 1 min, 60°C for 30 s, and 72°C for 1 min, and a final extension step at 72°C for 7 min. The PCR products were electrophoresed in a 1.5% (wt/vol) agarose gel in 0.5 $\times$ Tris-borate EDTA, stained in a GelRed bath (Biotium), and visualized using a transilluminator LPIX (Locus). Amplicons of approximately 800 bp were considered typical for *P. fluorescens*.

Genetic profiles of the isolates were obtained by pulsed-field gel electrophoresis (PFGE). Isolated colonies from each *Pseudomonas* spp. isolate were transferred to trypticase soy broth (Oxoid) and incubated at 25°C to obtain a culture with an optical density of 1 (λ

= 590 nm). Then, 400- μ L aliquots of each culture were transferred to micro-tubes and 5 μ L of lysozyme (20 mg/mL, Sigma) and 5 μ L of proteinase K (1 mg/mL, Sigma) were added, followed by incubation at 37°C for 15 min. Plugs were prepared with agarose and digested with 5 U of *Xba*I (Promega) at 37°C for 7 h. Macro-restriction products were separated in 1.5% (wt/vol) agarose gels in 0.5 $\times$ Tris-borate EDTA, by PFGE in a CHEF-DRIII (Bio-Rad Laboratories, Hercules, CA) using the following parameters: 17 h, 6 V/cm gradient, 120° angle, and 2- to 20-s interpoled pulses, at 12°C. The gels were stained in a GelRed bath (Biotium) and visualized using a transilluminator LPIX (Locus). Profiles were analyzed using BioNumerics 6.6 (Applied Maths, Ghent, Belgium) with 5% as the tolerance for the Dice similarity of bands. The unweighted pair group method with arithmetic mean (UPGMA) was used for clustering and dendrogram construction.

Proteolytic Potential

The PCR was performed on the DNA obtained previously from all isolates to detect a specific region of the alkaline protease gene (*apr*) that is responsible for AprX production by *Pseudomonas* spp. using the primers FP *apr* I (TAYGGBTTCAAYTCCAAYAC) and RF *apr* II (VGCGATSGAMACRTTRCC), according to the protocol described by Bach et al. (2001). Amplification reactions contained 12.5 μ L of Go Taq Green Master Mix 2 $\times$ (Promega), 1 μ L of DNA (~50–100 ng/ μ L), 10 pmol of each primer, and ultra-pure water (Promega) to bring the final volume to 25 μ L. Amplification conditions were 1 cycle of 95°C for 5 min, 30 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 20 s, and a final extension step at 72°C for 10 min. The PCR products were electrophoresed in a 1.5% (wt/vol) agarose gel in 0.5 $\times$ Tris-borate EDTA, stained in a GelRed bath (Biotium), and visualized using a transilluminator LPIX (Locus). Amplicons of 194 bp were considered typical for *apr*.

The proteolytic activity of isolates was verified by inoculating 1- μ L aliquots of cultures onto the surface of trypticase soy agar (Oxoid) supplemented with sterile skim milk (10%, vol/vol, prepared as previously described). The plates were incubated at 7°C for 10 d, 25°C for 3 d, or 35°C for 2 d. Then, 8 mL of 1% (vol/vol) HCl was added to the plates for 1 min and discarded. The presence of halos in the inoculated area was a positive result for the proteolytic activity of the isolates (Downes and Ito, 2001).

RESULTS AND DISCUSSION

Table 1 shows the mean values and standard deviations for counts of presumptive *Pseudomonas* spp. and

proteolytic *Pseudomonas* spp. obtained from goat milk samples from each dairy farm included in the study, as well as mean percentages of the proteolytic colonies based on the total *Pseudomonas* spp. counts by farm. Mean *Pseudomonas* spp. counts ranged from 3.0 to 4.8 log cfu/mL, whereas mean proteolytic *Pseudomonas* spp. counts ranged from 1.9 to 4.6 log cfu/mL. We detected no direct relationship between the presence or number of proteolytic *Pseudomonas* spp. and the total number of *Pseudomonas* spp. Milk samples from farm 11 had relatively high mean *Pseudomonas* spp. counts (4.7 log cfu/mL) but only 6.4% were proteolytic, whereas farm 10 presented a relatively low mean count (3.0 log cfu/mL), with 50.3% being proteolytic (Table 1).

Even though *Pseudomonas* species are known to be common components of raw goat milk microbiota (Quigley et al., 2013), there is a lack of information regarding their typical counts in this product. Because goat milk production chains are similar to cow milk production chains, they share the main sources of contamination by different microbial groups, including *Pseudomonas* spp. Mean counts of *Pseudomonas* spp. vary in cow milk from 5.0 log cfu/mL just after milking to 5.2 log cfu/mL after storage in a bulk tank (Fagundes et al., 2006), which is similar to the highest mean values of *Pseudomonas* spp. obtained in the present study from raw goat milk from bulk tanks (Table 1). *Pseudomonas* spp. counts varied from 1.0 to 6.6 log cfu/mL in samples of cow milk obtained from bulk tanks from different dairy farms (Pinto et al., 2006), demonstrating a large variation in the contamination of milk by this group. Based on the obtained data, variation in *Pseudomonas* spp. counts by goat farm can be observed (Table 1). These results also indicated the relevant participation of proteolytic strains in the *Pseudomonas* spp. microbiota of goat milk (Table 1): in 37 goat milk samples (60.1%), proteolytic colonies represented more than 50% of the total *Pseudomonas* spp. counts (data not shown). Once *Pseudomonas* spp. can produce spoilage enzymes, their presence at such levels in goat milk is a concern (Marchand et al., 2009; Chen et al., 2011).

All isolates (n = 82) were confirmed as belonging to the *Pseudomonas* genus, with 41 being identified as *P. fluorescens* (Figure 1). After enzymatic digestion with *Xba*I, isolates were clustered into 5 groups sharing approximately 82% similarity (Figure 1). Based on PFGE analysis, the random distribution of *Pseudomonas* spp. strains in different dairy goat farms included in the study was identified. Specifically, identical strains were isolated from different farms, and one farm had *Pseudomonas* spp. strains with different pulsotypes. These results demonstrate the ubiquitous nature of *Pseudomonas* spp. in dairy goat farms, highlighting their rel-

Table 1. Mean values and standard deviations of presumptive *Pseudomonas* spp. and proteolytic *Pseudomonas* spp. in goat milk samples obtained from bulk tanks of 12 dairy goat farms located in Minas Gerais state, Brazil, and mean percentages of proteolytic colonies considering the total *Pseudomonas* counts

Farm	No. of samples	<i>Pseudomonas</i> spp. (log cfu/mL)		Mean percentage of proteolytic colonies
		Total	Proteolytic	
1	4	3.9 ± 0.7	3.4 ± 0.4	40.4
2	4	4.3 ± 0.5	3.6 ± 0.8	36.1
3	10	4.0 ± 0.7	3.7 ± 1.1	58.0
4	3	4.5 ± 0.4	4.2 ± 0.6	58.0
5	9	3.9 ± 0.7	3.6 ± 0.8	61.1
6	5	3.1 ± 0.9	1.9 ± 0.2	9.2
7	5	3.0 ± 0.3	2.7 ± 0.7	65.9
8	5	4.8 ± 1.1	4.6 ± 1.3	72.1
9	5	3.4 ± 1.0	3.2 ± 0.9	77.7
10	5	3.0 ± 0.8	2.7 ± 1.0	50.3
11	3	4.7 ± 1.2	2.9 ± 0.2	6.4
12	3	4.1 ± 0.7	3.6 ± 0.3	42.1
Mean	61	3.9 ± 0.9	3.4 ± 1.0	51.0

evance as natural contaminants of goat milk (Quigley et al., 2013). In a similar study conducted with cow milk, *P. fluorescens* and *P. putida* were the main *Pseudomonas* species identified from different samples and points during production. Additionally, high genetic diversity is observed among isolates by ribotyping, demonstrating diversity in the origin of contamination within the environment (Dogan and Boor, 2003). Isolates clustered by PFGE did not show an association with the identified species (*P. fluorescens*) or to *apr* and proteolytic activity (data not shown), thus demonstrating the diversity of *Pseudomonas* spp. in the dairy goat farms included in the present study.

Table 2 shows the frequencies of isolates that harbored *apr*, as well as the results for proteolytic activity after incubation at different temperatures. The *apr* gene was detected in 32 isolates, and proteolytic activity was observed at all tested temperatures (at different frequencies). Even *apr*-negative isolates had proteolytic activity, indicating that proteases other than AprX were produced. Among *Pseudomonas* species, *apr* is usually studied to assess the proteolytic and spoilage potential of isolates (Bach et al., 2001; Marchand et al., 2009). Martins et al. (2005) extracted total DNA from samples of raw milk and studied *apr* to assess the spoilage potential of the microbiota due to the relevance of AprX in casein degradation during storage. In addition to AprX being considered the most important proteolytic enzyme associated with milk spoilage, other proteases can be produced by *Pseudomonas* spp., as described here (Table 2) and in other studies (Rajmohan et al., 2002; Chen et al., 2003; Hantsis-Zacharov and Halpern, 2007; Marchand et al., 2009; Samarzija et al., 2012). This highlights the relevance of controlling contamination by this genus in dairy product production.

Independent of the presence of the *apr* gene, the obtained data demonstrate protease production by the tested isolates at different temperatures (Table 2). This is a relevant finding because it confirms the ability of *Pseudomonas* spp. to produce such enzymes under the psychrotrophic conditions usually observed in the dairy production chain, even for goat milk. The Brazilian Ministry of Agriculture recommends a temperature of 7°C as an alternative to refrigeration at 4°C for storing milk in dairy farms in immersion tanks (Brasil, 2011). This procedure involves submerging milk cans in tanks filled with cold water, keeping them at an average temperature of 7°C (Perin et al., 2012). Even though this procedure has been indicated for the storage of raw milk produced by cows, it is widely used on dairy goat farms, because the current Brazilian legislation for goat milk does not specify temperatures or equipment for this purpose (Brasil, 2000). Based on our results, proteolytic *Pseudomonas* spp. were able to produce proteases at 7°C, jeopardizing the quality of goat milk during storage (Table 2). At this temperature, 90.3% of the isolates that harbored the *apr* gene also showed proteolytic activity, whereas 56.0% of isolates negative for *apr* showed proteolysis. Even though it is not possible to determine a statistical difference between such frequencies, this result indicates that the presence of *apr* can be associated with proteolysis at 7°C. The isolates were also able to produce proteases at 25°C and 35°C (Table 2), demonstrating their spoilage activity under transport and processing conditions, and highlighting the need for contamination control in the early steps of production.

The present study demonstrated the ubiquity of *Pseudomonas* spp. in raw goat milk and their ability to produce proteolytic enzymes at different temperatures,

Figure 1. Dendrogram obtained by *Xba*I macro-restriction and pulsed-field gel electrophoresis of *Pseudomonas* isolates obtained from goat milk. Pulsotype was obtained considering 5% as the tolerance for the Dice similarity of bands, and the unweighted pair group method with arithmetic mean (UPGMA) was used for clustering and dendrogram construction. Id. = isolate code; identification indicates genus and species by PCR; farm = goat farm from which the isolate was obtained. Numerals I to V indicate clusters of isolates that shared approximately 82% similarity.

Table 2. Frequencies of *Pseudomonas* spp. isolates obtained from goat milk that carried the alkaline protease gene (*apr*) gene and proteolytic activity after incubation at different temperatures

<i>apr</i> gene	No. of samples	Incubation temperature		
		7°C	25°C	35°C
Positive	32	29	32	21
Negative	50	28	50	43

thus emphasizing the relevance of this genus as potential spoilage organism in the dairy chain. These findings indicate the need for proper control of contamination and growth of *Pseudomonas* spp. during production and storage of goat milk.

ACKNOWLEDGMENTS

This research was supported by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brasília, DF, Brazil), CNPq (Conselho Nacional de Pesquisa Científica, Brasília, DF, Brazil), and FAPEMIG (Fundação de Amparo a Pesquisa do Estado de Minas Gerais, Belo Horizonte, MG).

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