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Original article

Effect of selected bacteria of the genus *Pseudomonas* on the quality of raw cow's milk

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Abstract

Pseudomonas spp. are a psychrotrophic species associated with milk spoilage caused by its enzymatic activities. The aim of this study was to identify *Pseudomonas* spp. in raw cow's milk and to investigate their associated enzymatic properties and the ability to produce pyoverdine pigment. For microbiological analysis, 2 ml of milk samples was taken in sterile sample boxes. Milk sampling was carried out according to the principles of STN EN ISO 707. By identification using the PCR method, of n=320 samples of raw cow milk a total of 73 isolates of *Pseudomonas* spp. were identified as *P. putida* (34.25%); *P. fragi* (13.70%); *P. lundensis* (9.59%) and *Pseudomonas* spp. (42.47%). Proteolytic activity determined at a temperature of 7°C was positive from n=20 selected isolates of *Pseudomonas* spp. (60%) isolates and a temperature of 25°C was positively detected (85%). Lipolytic activity determined at a temperature of 7°C was confirmed in 35% of isolates and a temperature of 25°C it was confirmed in 70% isolates. Pyoverdine pigment production was detected in 65% of isolates. The results reveal the enzymatic activity of *Pseudomonas* spp. present in raw cow's milk and its spoilage potential at different temperatures in relation to pigment production.

Keywords: enzymatic activity, food safety, microorganisms, milk quality, milk contamination, pigment production, *Pseudomonas*, raw milk, spoilage

Introduction

Representatives of the genus *Pseudomonas* belong to Gram-negative, aerobic bacteria. In addition, some species can grow anaerobically, using nitrate as an electron acceptor. Most representatives are positive for oxidase and catalase except for *Pseudomonas oryzihabitans* and *Pseudomonas luteola*. *Pseudomonas* spp. have high genetic diversity and metabolic ability, allowing them to survive in different environments and grow at an ambient temperature of around 4°C (Pilipčinec et al. 2018, Kumar et al. 2019, Navrhus et al. 2021).

Psychrotrophic microorganisms can grow and multiply in raw refrigerated milk which makes them predominant microflora in in the cold chain system of raw milk (Li et al. 2023). However, raw milk is usually kept in a refrigeration temperature range (0-10°C; REG. CE 853/2004) until it is processed, which is insufficiently low to prevent psychrotrophic microorganisms from growing at low temperatures (Bellasi et al. 2021). The ability of the microorganisms to function in different types of environments depends on the strategies directly associated with cell metabolism, stress factors, and physicochemical constrain. *Pseudomonas* belongs to a group of microorganisms which are predominant in cold environments with a wide range of biotechnological applications (Chauhan et al. 2023).

Spoilage of milk by representatives of the genus *Pseudomonas* leads to a change in sensory properties (off-flavor), mainly due to the production of volatile substances and amino acid metabolites. In addition, they can produce heat-resistant proteolytic and lipolytic enzymes that significantly reduce the quality and shelf life of protein foods (e.g., milk and fresh dairy products) and cause significant losses for the food industry. Spoiled foods, especially dairy products, show color changes due to the biosynthesis of pigments produced by species of *Pseudomonads* (Carrascosa et al. 2015, Pilipčinec et al. 2018, Quintieri et al. 2019). Production of proteases and lipases that can degrade milk proteins and fats, results in bitterness, rancidity, and gelation, which has a significant negative impact on the quality of milk and dairy products (Atia et al. 2023).

The main *Pseudomonas* species present in the dairy chain are *P. fluorescens*, *P. aeruginosa*, *P. putida, P. fragi P. lurida, P. cedrina, P. psychrophila and P. gessardii* (Du et al. 2023). They can cause an unpleasant odour or taste in pasteurised milk and other dairy products (Whitfield et al. 2000). Consequently, detecting *Pseudomonas* spp. or its antecedent activity in milk is critical to preventing quality defects in the dairy products and minimizing food spoilage (De Jonghe e al. 2011).

Microbial proteases cleave milk protein fractions with varying intensity. Of the casein fractions, κ-casein decomposes the fastest. Cleavage of κ-casein and its degradation results in milk coagulation. Rapid cleavage also occurs with β-casein, while degradation of α-casein fractions occurs only minimally. Whey proteins are resistant to proteases, as indicated by their minimal degradation (Alves et al. 2018, Glantz et al. 2020).

The lipases produced by microorganisms, in contrast to milk lipases, belong to heat-resistant enzymes. Most microorganisms produce lipases in the later lag phase and early stationary phase. The result of this event is that the number of microorganisms and the concentration of lipases are not directly related (Kováčová et al. 2021). Production of lipases by Gram-negative psychrotrophic bacteria causes hydrolysis of milk fat and lecithin and releases free fatty acids. Through the action of lipases, triacylglycerols are split into diacylglycerols and monoacylglycerols, eventually free fatty acids. An increased concentration of free fatty acids with a short chain (C4 - C8) causes a change in the taste of dairy products. The soapy, impure to bitter taste and smell is caused by the high amount of free medium chain fatty acids (C10 - C12) (Samaržija et al. 2012, Kováčová et al. 2021).

The production of proteolytic and lipolytic enzymes resistant to high temperatures, thus causing milk deterioration, premature coagulation, and odours, even in processed dairy products, cause not only food but also economic losses (Dogan et al. 2003).

The presence of psychrotrophic microorganisms can cause potential sensory changes in milk. *Pseudomonas* species produce a wide diversity of siderophores. Some of pseudomonads produce the class of fluorescent pyoverdines as a major siderophore (Grosse et al. 2023). Color changes in milk can be localized on the surface, in the entire volume of milk or at the bottom in the form of sediment. Color changes most often occur during long-term storage. *Pseudomonas* representatives that produce pigments include *Pseudomonas syncyanea*, which causes a blue coloration, and *Pseudomonas fluorescens*, which produces a brown coloration of milk due to oxidation of tyrosine (Burdová 2001, Ribeiro junior et al. 2018).

The nature of pigmentation remains an important factor among the diagnostic features of *Pseudomonads*. Pigments can be water-soluble and diffusible into the medium, or they can be associated with cells. *Pseudomonads* can produce diffuse pigments with a short wavelength (254 nm) that fluoresce in ultraviolet light. Psychrotrophic bacteria *Pseudomonas* spp. produce one or more pigments. The most common pigments produced by *Pseudomonas* species are pyoverdine and pyocyanin. Pyoverdine can also be a tool for identifying *Pseudomonas* spp., since each genomic group is characterised by a specific pyoverdine (DeBritto et al. 2020, Kothari et al. 2022).

The aim of this study was to detect the presence of the genus *Pseudomonas* occurring in samples of raw cow's milk collected from four farms with the same management strategy, and to analyse the potential spoilage of raw cow's milk due to the proteolytic and lipolytic activity of the genus *Pseudomonas* associated with pyoverdine production.

Materials and Methods

Collection of milk samples

Raw cow's milk samples were collected from four production farms (PF1, PF2, PF3 and PF4) located in the Slovak Republic in the regions: Zemplín (PF1), Abov (PF2, PF3) and Spiš (PF4). The selected production farms focus on the breeding of Slovak Spotted Cattle. An experimental group of dairy cows was created in each production farm and examined throughout the experimental period (autumn 2020 / winter 2020 / spring 2021 / summer 2021). An important point in the selection of farms was the control of the uniformity of the microbiological quality of milk produced in PFs, whose milk is used for further processing in the same dairy plant located in their vicinity. The selection of cows from which raw milk samples were taken was carried out by random selection. A total of 320 samples of raw milk were collected during the study period. The cows were milked twice daily, and samples were collected from morning milking. The lactating cows were raised in a free stall barn and in a holding pen. Thorough the study period constant managerial conditions (operators, similar batches of feed, milking frequency, and working routine) were maintained on all farms.

Milk sampling was carried out according to the principles of STN EN ISO 707 (2011). After performing the basic toilet of the udder, samples of milk were taken for microbiological analysis (2 ml) in sterile sample boxes. Subsequently, the milk samples were transferred to the Department of Food Hygiene, Technology and Safety at UVLF in Košice, Slovak republic. Raw cow's milk samples were stored at 6° C during transport and until analysis.

Isolation and identification of *Pseudomonas* spp.

Isolation of members of the genus *Pseudomonas*

Pseudomonas Agar Base (Hi-Media, India) was used for cultivation and isolation of selected microorganisms of the genus *Pseudomonas*, which is used for the selective isolation of *Pseudomonas* spp. Pseudomonas agar was supplemented with penicillin (100.000 IU/ l, Dr. Ehrenstorfer GmbH, Augsburg, Germany) and pimaricin (0.01 g/l, Dr. Ehrenstorfer GmbH), for the selective isolation of *Pseudomonas* spp.

Tenfold dilutions according to STN EN ISO 6887-5 (2020) were prepared from the samples of raw cow's milk. Subsequently, 0.1 ml of selected tenfold dilutions were inoculated onto the surface of the selective diagnostic medium Pseudomonas Agar Base. Incubation took place for 48 hours at a temperature of 25°C (ISO/TS 11059, 2009). After the respective incubation, 5 identical solitary colonies were taken from each plate, and were subsequently purified and subjected to identification. The isolated strains were stored in brain-heart infusion broth (BHI broth; Hi-Media, India) with 50% glycerol at -80°C until DNA isolation was performed.

Identification of the genus *Pseudomonas*

Isolates of representatives of *Pseudomonas* spp. were submitted to identification based on their phenotypic characteristics and subsequent identification using the PCR method. Phenotype identification was performed using a 24-hour culture of isolates from the surface of blood agar. Identification according to phenotypic characteristics was performed using Gram stain, oxidase assay, catalase assay and glucose fermentation assay (Scatamburlo et al. 2015).

Isolation of DNA

Isolates of *Pseudomonas* spp. obtained from raw cow's milk samples were used for DNA isolation, a 24-hour bacterial culture previously cultured on blood agar (Merck, Germany). Bacterial culture of the isolate was resuspended in 1.5 ml of saline placed in an Eppendorf tube. Subsequently, centrifugation at 10,000 rpm for 10 minutes at a temperature of 25°C was used. After removal of the supernatant, 200 µL of Chelex 20% (Sigma-Aldrich, Germany) was added to the sediment. Subsequently, the test tube was mixed using a Vortex and incubated at 95°C for 10 minutes. After incubation, the contents of the tube were centrifuged again at 10,000 rpm for 3 minutes at 4°C. The subsequently obtained supernatant contained isolated DNA, which was used as a template in PCR reactions.

PCR identification

To confirm the *Pseudomonas* genus and individual species, the isolated DNA was used in individual PCR reactions. The primers used are listed in Table 1. The reaction mixture in a volume of 20 µl, used for the identification of genus and species, contained 1 µl of genomic DNA, 10 pmol. L^{-1} of each primer and HotFirepol® Mastermix (Amplia s.r.o., Bratislava, Slovakia). The PCR protocol was as follows: initial denaturation at 95°C for 12 minutes, 25 cycles consisting of denaturation at 95°C for 20 seconds, annealing for 1 minute and elongation at 72°C for 2 minutes. The last cycle was followed by a final elongation at 72°C for 10 minutes. Amplification was terminated by cooling to 6°C.

The amplified PCR products (in the amount of 5 µl) were separated on a 2% agarose gel using Gold View

Table 1. Primers used in identification using the PCR method.

	Primer	Sequence (5‘ - 3‘)	TA (°C)	PS	Reference
GENUS					
Pseudomonas spp.	PA-GS-F	GACGGGTGAGTAATGCCTA	57	618 bp	(Moon et al. 2008)
	PA-GS-R	CACTGGTGTTCCTTCCTATA			
SPECIES					
P. aeruginosa	DG10-03F	GGGGGATCTTCGGACCTCA	55	956 bp	(Martin et al. 2018)
	DG10-04R	TCCTTAGAGTGCCCACCCG			
P. putida	P734	CAACTCGGGCGTTGGCATTCTGCT	62	744 bp	(Meng et al. 2017)
	P1455r	CAAGATCGCCTGGGTACGACGGTT			
P. fluorescens	16SPSEfluF	TGCATTCAAAACTGACTG	55	850 bp	(Meyer 2000)
	16SPSER	AATCACACCGTGGTAACCG			
P. fragi	Pfrag-F	CGTCAGCACCGAAAAAGCC	61	397 bp	(Moon et al. 2008) modified in this study
	Pfrag-R	TGCACCGTGATGGCCATGACCC			
P. lundensis	Plund-F	TGTGGCGATTGCAGGCATT	61	310 bp	(Moon et al. 2008)
	Plund-R	TACCAACATGCGCAAAATGT			

TA – annealing temperature of individual primers in the reaction, PS – product size.

dye (Ecoli s.r.o., SR) for 1 hour at 120 V. Subsequently, individual PCR fragments were visualized under UV light using a Mini reader Bis Pro® (DNR Bio-Imaging Systems Ltd., Israel). After visualization, the amplified products were sequenced. Sequencing of PCR products was performed using the Sanger method (GATC Biotech AG, Konstanz, Germany). The obtained strains were entered into the Gen-Bank - EMBL database for comparison with the sequences available in the nucleotide database of the National Center for Biotechnology Information (NCBI).

The following reference strains were used as positive controls for this PCR: *Pseudomonas aeruginosa* CCM 1960T, *Pseudomonas fluorescens* CCM 4795, *Pseudomonas fragi* CCM 1974T, *Pseudomonas putida* CCM 7156T, *Pseudomonas lundensis* CCM 3503T (Czech Collection of Microorganisms, Brno, Czech Republic).

Enzymatic activity detection

Proteolytic activity

The proteolytic activity of the isolates was determined using Pseudomonas Agar Base (Hi-Media, India) supplemented with 10% sterile skim milk (Hi-Media, India).

Confirmed identified isolates of *Pseudomonas* spp. were subjected to investigation and proof of proteolytic activity using the plate method. A 24-hour suspension was used for the determination. Culture was propagated in BHI at 37°C. The bacterial suspension was adjusted to a turbidity of 0.5 McFarland. suspension 100 µL was then inoculated onto the surface of the medium. The plates were incubated at 7°C and 25°C for 5 days. We used *P. fluorescens* (CCM 4795) as a positive control. A posi-tive result was demonstrated by the formation of a clearing zone around the colonies. We determined the proteolytic activity of the isolates using the plate method twice (Scatamburlo et al. 2015, Meng et al. 2017).

Lipolytic activity

Tributyrin Agar Base w/o Tributyrin (Hi-Media, India) was used to detect lipolytic microorganisms. Confirmed identified isolates of *Pseudomonas* spp. underwent confirmation of lipolytic activity using the plate method. A suspension of the culture multiplied in BHI at 37°C for 24 hours was used for the determination. The bacterial suspension was then adjusted to a turbidity of 0.5 McFarland. We inoculated a suspension of 100 µL onto the surface of Tributyrin agar medium enriched with Tributyrin and incubated plates at 37°C for 7 days. A positive result was demonstrated by the formation of a clearing zone around the colonies. We determined the lipolytic activity of the isolates using the plate method twice (Capodifoglio et al. 2016).

Production of pigments

Determination of pyoverdine production was carried out using Pseudomonas agar for Florescein (Hi-Media, India). Determination of pyocyanin production was carried out using Pseudomonas agar for pyocyanin (Hi-Media, India) The prepared preserves were revived in BHI agar and the isolates were subse-

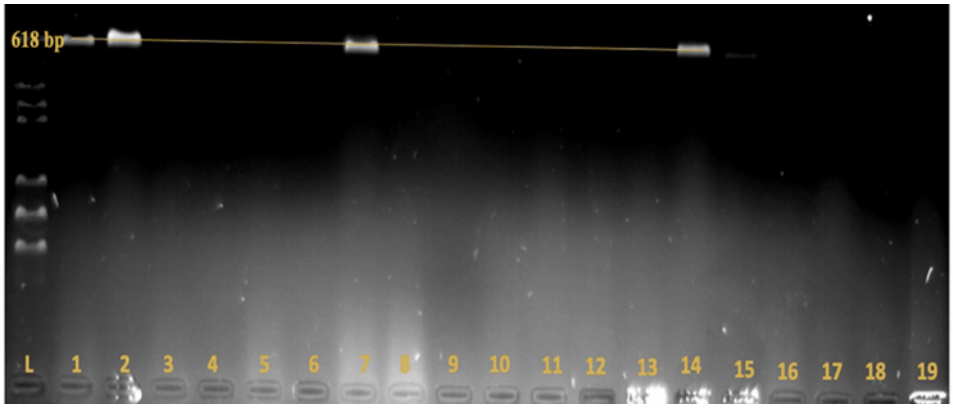

Fig. 1. Detection of 16S rDNA gene (618 bp) specific for *Pseudomonas* spp. in isolates originating from raw cow's milk. L. – standard; lanes: 1 – positive control of *Pseudomonas* spp. (CCM); 2, 7, 14 – positive genus-confirmed isolates of *Pseudomonas* spp

Table 2. Quantity (%) of *Pseudomonas* spp. isolated from samples of raw cow's milk from production farms (PF1. PF2, PF3, PF4) during seasons.

	PF1	PF2	PF3	PF4
Autumn	0 (0%)	1 (5.88%)	3 (14.29%)	4 (11.43%)
Winter	0 (0%)	2 (11.76%)	2 (9.52%)	4 (11.43%)
Spring	0 (0%)	6 (35.29%)	7 (33.33%)	12 (34.29%)
Summer	0 (0%)	8 (47.06%)	9 (42.86%)	15 (42.86%)
Σ	0 (0%)[a]	17 (23.29%)	21 (28.77%)	35 (47.95%) [b]

[a, b] values in line with different labels are statistically different ($p \leq 0.05$), Σ – total count of isolates

quently incubated for 24 hours at a temperature of 37°C. The prepared bacterial suspension was diluted according to McFarland's 0.5 degree turbidity standard ($1x10^8$ KTJ.ml^{-1}) and 0.1 ml of the suspension was then inoculated onto the surface of the pre-dried media. Incubation lasted 48 hours. at 25°C (Kwan and Skura 1985, Xu et al. 2005, Vinckx et al. 2010).

Statistical analysis

One-way analysis of variance (ANOVA) and statistics software GraphPad Prism 8.3.0.538 (GraphPad Software, San Diego, CA, USA) was used as a statistical analysis method. ANOVA and the Tukey test for multiple comparison was set at a confidence interval of 95%.

Results

Identification of *Pseudomonas* spp.

A total of 151 isolates were obtained by microbiological examination of raw cow's milk samples from a total of 320 samples of raw cow's milk collected during the entire experimental period (autumn 2020 to summer 2021). Total 73 isolates were tested positive by PCR based on their phenotypic characteristics.

The presence of the 16S rDNA gene, which is typical for the genus *Pseudomonas* spp., was confirmed using the PCR method in 73 isolates of a selected group of *Pseudomonas* spp. (Fig. 1).

Individual milk samples taken from PF1 were negative to the presence of a selected group of *Pseudomonas* spp. during the entire experimental period (0%). The presence (23.29%; 17 isolates) of *Pseudomonas* spp. was detected in milk samples taken from PF2. In PF3, the presence (28.77%; 21 isolates) of *Pseudomonas* spp. was detected. The highest amound of microorganisms of the group *Pseudomonas* spp. was detected in PF4 (47.95%; 35 isolates). Representation of isolates of *Pseudomonas* spp. in individual production farms is shown in Table 2.

Although throught the experimental period constant managerial conditions (operators, similar batches of feed, milking frequency, and working routine) were maintained on all farms, the results show statistically significant defferences ($p \leq 0.05$) in the presence of genus *Pseudomonas* detected at PF1 and PF4.

Species identification of isolated and confirmed strains of *Pseudomonas* spp. was performed using the PCR method. Based on species identification, the following three species of selected microorganisms of the genus *Pseudomonas* were determined. From a total of 73 confirmed strains, the following species were identified: *Pseudomonas putida* (34.25%; 25 isolates); *Pseudomonas fragi* (13.70%; 10 isolates); *Pseudomonas lundensis* (9.59%; 7 isolates). 42.47% (31 isolates) of *Pseudomonas* spp. remained unconfirmed (Table 3).

Table 3. Species representation of isolates of *Pseudomonas* spp. isolated from samples of raw cow's milk from production farms (PF1. PF2, PF3, PF4).

	PF1	PF2	PF3	PF4
P. putida	0 (0%)	6 (35.29%)	8 (38.1%)	11 (31.43%)
P. fragi	0 (0%)	3 (17.67%)	3 (14.29%)	4 (11.43%)
P. lundensis	0 (0%)	(0%)	1 (4.76%)	6 (17.14%)
Pseudomonas spp.	0 (0%)	8 (47.06%)	9 (42.86%)	14 (40%)

Table 4. Enzymatic activity of strains of *Pseudomonas* spp. isolated from raw cow's milk.

Isolate	Species	Proteolytic activity		Lipolytic activity	
		7 °C	25 °C	7 °C	25 °C
PP1	*P. putida*	-	+	-	-
PP2	*P. putida*	+	+	+	+
PP3	*P. putida*	-	-	-	-
PP4	*P. putida*	-	+	-	+
PP5	*P. putida*	+	+	+	+
PF1	*P. fragi*	+	+	-	-
PF2	*P. fragi*	+	+	-	+
PF3	*P. fragi*	-	+	-	+
PF4	*P. fragi*	+	+	+	+
PF5	*P. fragi*	+	+	-	-
PL1	*P. lundensis*	+	+	+	+
PL2	*P. lundensis*	+	+	-	+
PL3	*P. lundensis*	-	-	-	-
PL4	*P. lundensis*	+	+	-	+
PL5	*P. lundensis*	+	+	+	+
PS1	*Pseudomonas* spp.	-	-	-	+
PS2	*Pseudomonas* spp.	-	+	-	-
PS3	*Pseudomonas* spp.	+	+	+	+
PS4	*Pseudomonas* spp.	+	+	+	+
PS5	*Pseudomonas* spp.	-	+	-	+
ΣP		12 (60%)	17 (85%)	7 (35%)	14 (70%)
ΣN		8 (40%)	3 (15%)	13 (65%)	6 (30%)

ΣP – number of all positive strains, ΣN – number of all negative strains

The presence of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* species was not detected in any of the collected samples of raw cow's milk (0%).

P. putida was confirmed as the dominant species of bacteria of the genus *Pseudomonas* in this study. However, although the representation of *P. putida* was dominant, we did not establish a statistically significant difference ($p \geq 0.05$) between the species.

Proteolytic activity

From the total number of 73 confirmed isolates, n=20 isolates (n=5 *Pseudomonas putida*; n=5 *Pseudomonas fragi*; n=5 *Pseudomonas lundensis*; n=5 *Pseudomonas* spp.) were randomly selected for determination of enzymatic activity. From the total number (n=20) of isolates of *Pseudomonas* spp. 12 isolates were positive for proteolytic activity determined at an incubation temperature of 7°C, of which *Pseudomonas putida* (PP2; PP5) was represented (40%; 2 isolates); *Pseudomonas fragi* (PF1; PF2; PF4; PF5) (80%; 4 isolates); *Pseudomonas lundensis* (PL1; PL2; PL4; PL5) (80%; 4 isolates) and *Pseudomonas* spp. (PS3; PS4) (40%; 2 isolates) (Table 4). The results of this study showed proteolytic activity determined at an incubation temperature of 25°C in a total of 85% (17 isolates): *Pseudomonas putida* (PP1; PP2; PP4; PP5) (80%; 4 isolates); *Pseudomonas fragi* (PF1; PF2; PF3;PF4;PF5) (100%; 5 isolates); *Pseudomonas lundensis* (PL1; PL2; PL4; PL5) (80%; 4 isolates) and *Pseudomonas* spp. (PS1; PS3; PS4; PS5) (80%; 4 isolates).

Table 5. Pigment production of strains of *Pseudomonas* spp. isolated from raw cow's milk.

Isolate	Species	Pyoverdine	Pyocyanin
PP1	*P. putida*	+	-
PP2	*P. putida*	+	-
PP3	*P. putida*	+	-
PP4	*P. putida*	+	-
PP5	*P. putida*	+	-
PF1	*P. fragi*	-	-
PF2	*P. fragi*	-	-
PF3	*P. fragi*	-	-
PF4	*P. fragi*	-	-
PF5	*P. fragi*	-	-
PL1	*P. lundensis*	+	-
PL2	*P. lundensis*	+	-
PL3	*P. lundensis*	+	-
PL4	*P. lundensis*	+	-
PL5	*P. lundensis*	+	-
PS1	*Pseudomonas* spp.	+	-
PS2	*Pseudomonas* spp.	-	-
PS3	*Pseudomonas* spp.	-	-
PS4	*Pseudomonas* spp.	+	-
PS5	*Pseudomonas* spp.	+	-
ΣP		13 (65%)	0 (0%)
ΣN		7 (35%)	20 (100%)

ΣP – number of all positive strains, ΣN – number of all negative strains

Lipolytic activity

The activity of lipolytic enzymes of the isolates was significantly lower than the activity of proteolytic enzymes. At the incubation temperature of 7°C, the presence of lipolytic activity was determined in 35% (7 isolates) (Table 4). Of these, there were: *Pseudomonas putida* (PP2; PP5) (40%; 2 isolates); *Pseudomonas fragi* (PF4) (20%; 1 isolate); *Pseudomonas lundensis* (PL1; PL5) (40%; 2 isolates) and *Pseudomonas* spp. (PS3; PS4) (40%; 2 isolates). Lipolytic activity at a temperature of 25°C was detected in 70% (14 isolates), of which: *Pseudomonas putida* (PP2; PP4; PP5) (60%; 3 isolates); *Pseudomonas fragi* (PF2; PF3; PF4) (60%; 3 isolates); *Pseudomonas lundensis* (PL1; PL2; PL4; PL5) (80%; 4 isolates) and *Pseudomonas* spp. (PS1; PS3; PS4; PS5) (80%; 4 isolates).

Pigment production

To determine the ability to produce pigments, the same group of isolates n=20 isolates (n=5 *Pseudomonas putida*; n=5 *Pseudomonas fragi*; n=5 *Pseudomonas lundensis*; n=5 *Pseudomonas* spp.) was used to determine the enzymatic activity (Table 5). Production of pyoverdine pigment was confirmed in *Pseudomonas putida* (PP1; PP2; PP3; PP4; PP5); *Pseudomonas lundensis* (PL1; PL2; PL3; PL4; PL5) and *Pseudomonas* spp. (PS1 and PS5), representing 65% (13 isolates) of all tested isolates.

Pseudomonas putida and *Pseudomonas lundensis* species showed 100% positivity for pyoverdine production, while we did not confirm pyoverdine production in *Pseudomonas fragi* species (0% positive isolates). Isolates of *Pseudomonas* spp. showed pyoverdine pigment production in 60% (3 isolates).

The formation of pyocyanin pigment produced by the isolates (n=20) was not confirmed in any of the *Pseudomonas* species isolate.

Discussion

The contamination of milk and milk products by psychrotrophic microorganisms results in significant losses for the food industry, especially for the dairy industry. Bacteria from *Pseudomonas* species have been identified in many studies as the predominant psy-chrotrophic bacteria associated with milk. *Pseudomonas* spp. are one of the most important bacterial groups in the dairy industry (Oliveira et al. 2015, Meng et al. 2017).

The genus forms a significant part of the microbiota of raw milk and is considered the main cause of spoilage of milk and dairy products (Marchand et al. 2009, Vithanage et al. 2016). Among the most frequently isolated species in milk are *Pseudomonas* spp. and in dairy products it is *P. fluorescens*, *P. fragi* and *P. lundensis* (Meng et al. 2017).

This is also confirmed by the results of our study, where a total of (n=73) isolates were represented by secies *P. fragi* (13.70%); *P. lundensis* (9.59%).

A study by Du et al. (2022), reported a total of 116 *Pseudomonas* strains of raw milk collected from Inner Mongolia, Heilongjiang, Gansu, Henan, Anhui, Jiangsu, and Chongqing. The predominant species were identified as *P. fluorescens*, *P. veronii*, *P. psychrophila*, *P. lundensis*, *P. lactis*, *P. azotoformans*, *P. granadensis*, *P. lurida*, *P. rhizosphaerae*, *P. rhodesiae* and *P. extremorientalis*.

Based on PCR sequencing, Meng et al. (2017) confirmed that 42.7% (n=61) of the isolates from milk samples were *P. fluorescens*. Marchand et al. (2009) and Caldera et al. (2016) reported in their study that the most common species isolated from milk were *P. fragi*, *P. lundensis* as well as *P. gessardii*, *P. proteolytica*, *P. brenneri*, *P. rhodesiae*, and *P. peli*.

Although *Pseudomonas* spp. are among the ubiquitous bacteria that are often isolated from raw milk, our findings indicate that the species representation of bacteria of the genus *Pseudomonas* spp. present in milk vary considerably from study to study. These differences may result from regional and environmental differences (Kelly and Wilson 2016, Martin et al. 2018).

According to current valid legislation in many countries, raw milk must be stored at a temperature below 7°C (FDA 2013, REG. CE 853/2004). Bacteria isolated from raw milk can show enzymatic activity such as protease, lipase, and phospholipase C. Psychrotrophic bacteria is often proteolytic and lipolytic in nature, which is perhaps the most common cause of milk and milk products spoilage (Scatamburlo et al. 2015, Vithanage et al. 2016).

Milk quality is not only affected by the amount of psychrotrophic bacteria; it is also affected by the properties of their heat resistant proteolytic and lipolytic enzymes. Therefore, the growth of psychrotrophic bacteria in raw, chilled milk can also seriously change the quality of the milk after UHT treatment with ensuing storage. Even a low residual activity of psychrotrophic bacteria microbial enzymes after UHT treatment can lead to quality problems (Stoeckel et al. 2016).

Caldera et al. (2016) reported that 46.8% (15/32) of *Pseudomonas* isolates from milk and milk products after incubation at 5°C for 5 days were enzymatically active.

The results of this study confirmed the findings of other studies demonstrating, in isolates of *Pseudomonas* spp. the prevalence of proteolytic and lipolytic enzymes. Among the tested isolates, *P. fragi* and *P. lundensis* species showed the highest proteolytic activity at 7°C. The predominant species in lipolytic activity production were confirmed as *P. putida* and *P. lundensis.*

Marchand et al. (2009), in a study of raw cow's milk in Belgium, reported the occurrence of *P. fragi* (33.9%) and *P. lundensis* (19.4%) isolates as dominant psychrotolerant species producing proteolytic enzymes. The results of their study support the results of this study, where *Pseudomonas fragi* and *Pseudomonas lundensis* species were also detected as dominant representatives of the genus *Pseudomonas* in the production of proteolytic enzymes.

The quality of milk is affected not only by the number of psychrotrophic bacteria. An important factor is the resistance of proteolytic and lipolytic enzymes to thermal processes. Psychrotrophic bacteria produce, during the exponential growth phase, as well as in the early stationary phase, heat-resistant proteolytic and lipolytic enzymes that, during storage, negatively affect the quality attributes of milk and milk derived products, including milk powders (Stevenson et al. 2003).

Bacterial species belonging to the genus *Pseudomonas* are well known for their ability to produce siderophores, which represent one of the important virulence factors (Ortiz-Castro et al. 2014). Pyoverdine, pyocyanin, pyorubin, pyomelanin, and indigoidin are the main known pigments produced by various *Pseudomonas* spp. associated with milk discoloration disorders and dairy products, especially cheese (Meyer 2000, Moon at al. 2008, Quintieri et al. 2019).

The results of this study confirm pyoverdine production (65%, 13 isolates), while representatives of the species *Pseudomonas putida* and *Pseudomonas lundensis* showed 100% positivity for pyoverdine production. In unclassified isolates of the genus *Pseudomonas* spp. pyoverdine pigment production was detected in 60% (3 isolates). However, none of the representatives of the *Pseudomonas fragi* species showed pyoverdine production (0% of positive isolates).

Of all the spoilage defects observed in cold-storage dairy products, the most studied defect in the last decade is certainly color changes. Many studies have confirmed cases of anomalous discoloration of Mozzarella cheese caused by *P. putida* contamination (reddish discoloration), a biovar *P. fluorescens* IV and *P. libanensis* (blue coloration), *P. gessardii* (yellow and purple spots) and *P. fluorescens* (greenish and fluorescent coloration), due to the production of different pigments (pyoverdine, pyocyanin, pyorubin and pyomelanin) (Franzeti

and Scarpellini 2007, Palleroni 2015, Quintieri et al. 2019).

Tančin et al. (2020) state that some of the most important information for dairy practice could be the results of experimental work carried out on production farms at regular time intervals during a certain experimental period, focusing mainly on the microbiological quality of milk. After processing, these data could be used to improve the management of dairy farming in individual production farms.

The results of this study confirm that despite constant management conditions (operators, similar feed batches, frequency of milking and working regime), differences in the representation of individual species were detected, which can be attributed to regional and environmental differences.

Conclusions

The aim of this study was to provide further knowledge of the diversity of *Pseudomonas* strains using 16S rDNA by PCR method in raw cow's milk from four production farms in Slovakia. A total of 73 isolates were identified as *Pseudomonas* spp. of which the main species was *Pseudomonas putida.* This study shows high proteolytic activity of *Pseudomonas fragi* and lipolytic activity of *Pseudomonas lundensis*. *Pseudomonas putida* and *Pseudomonas lundensis* were found to have positive pigment production of pyoverdine in all tested strains. The result of the present study provides some fundamental information on the spoilage potential of raw cow's milk caused by *Pseudomonas* bacteria in an environment with reduced temperature. Proteolytic and lipolytic activity from extracellular thermoresistant enzymes connected with pyoverdine production from strains of *Pseudomonas* is likely to depend on the properties of individual strains of *Pseudomonas* spp., which can be used for the dairy industry to enable the selection of raw milk for technological processing.

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