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Isolation and Identification of *Pseudomonas protegens* from raw milk and its Molecular-Proteomic Analysis in Gelation of Sterilized Skim Milk

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ABSTRACT

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The present study investigated the molecular mechanisms and functional aspects of proteolytic gelation in autoclave-sterilized milk, focusing on *Pseudomonas protegens* and other psychrotrophic species. Raw milk collected from dairy farms in Isfahan, was screened for the presence of *Pseudomonas* species, and the strain *P. protegens* KSM1 was selected for detailed analysis. PCR results showed that none of the isolates carried the *AprX* gene of 890 bp, and ORF analysis confirmed the presence of only short ORFs (<171 bp). The genetic diversity of 30 isolates was evaluated using RAPD-PCR with the XD5 primer, revealing similarity coefficients ranging from 0.79 to 1.00 and divergence up to 43%; *P. aeruginosa* formed a compact cluster, whereas *P. protegens*, *P. koreensis*, *P. putida*, and *P. fluorescens* were distributed across separate branches. SDS-PAGE analysis indicated that inoculated samples experienced severe casein degradation, and the presence of a marker band at 45–50 kDa indicated bacterial alkaline protease activity. Proteolysis in inoculated samples increased significantly, particularly between days 3 and 10 ($p \leq 0.05$), while control samples showed minimal changes. These findings demonstrate that bacterial proteases are the main contributors to protein degradation during storage. Among the isolates, *P. protegens* KSM1 represents a suitable model for studying protein spoilage mechanisms and developing strategies to prevent gelation in UHT milk.

1- INTRODUCTION

Ultra-high-temperature (*UHT*) processing is designed to extend the shelf life of milk significantly and involves heating milk to very high temperatures (typically >135 °C for a few seconds) to inactivate vegetative microorganisms and spores [1]. Despite the severity of this thermal treatment, certain spoilage phenomena continue to challenge product stability, most notably protein gelation occurring during storage, commonly referred to as age gelation [2]. Gelation primarily results from the enzymatic degradation of caseins and is strongly influenced by the activity of residual proteolytic enzymes produced by psychrotrophic bacteria, which may remain active either before or after *UHT* treatment [3]. Consequently, psychrotrophic *Pseudomonas* species are of particular importance because of their ability to grow at refrigeration temperatures and spoil raw milk on farms or during transportation [4]. These bacteria secrete extracellular, heat-stable proteases, many of which can largely withstand *UHT* processing conditions [5]. Proteolytic enzymes secreted by *Pseudomonas* spp. Predominantly target α -, β -, and κ -caseins, leading to their hydrolysis and destabilization of the casein micelle structure. This destabilization reduces colloidal stability and ultimately facilitates gel formation over time [6]. Various *Pseudomonas* species have attracted considerable attention due to their production of serralsin-type proteases encoded by the *AprX* gene, which belongs to the family of zinc-dependent metalloproteases. To investigate the relationship between microbial genetics and functional spoilage outcomes, molecular typing techniques are often combined with assays evaluating proteolytic activity [7]. Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) is widely used to assess genetic diversity among raw milk isolates and can generate unique DNA profiles without requiring prior genomic information [8]. Establishing a clear link between molecular data and observable spoilage requires direct assessment of proteolysis in milk. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) is a reliable method for visualizing protein degradation patterns, as it separates

milk proteins based on molecular weight under denaturing conditions and enables the detection of casein breakdown and the formation of smaller peptides over time [9]. Comparing SDS-PAGE profiles of milk inoculated with characterized *Pseudomonas* isolates to those of non-inoculated control samples, while conducting storage experiments at different temperatures, allows evaluation of both the proteolytic potential of specific strains and the effect of environmental factors on spoilage kinetics [10]. Higher storage temperatures accelerate protease activity, whereas refrigeration slows microbial metabolism but does not completely inhibit heat-stable protease activity [11]. Integrating molecular and functional approaches provides a comprehensive understanding of the mechanisms underlying gelation in *UHT* milk and highlights the critical role of psychrotrophic bacteria in contamination occurring before or during processing [6]. Finally, this integrated view highlights the multifactorial nature of post-*UHT* spoilage since treatments effective in eliminating viable bacteria from *UHT* milk may leave certain bacterial proteases active. Hence, product shelf life is not only a result of microbial survival but also of the molecular stability of milk proteins when exposed to heat-stable enzymes [12]. The combined application of RAPD-PCR, targeted detection of *AprX*, and SDS-PAGE-based proteolysis analysis establishes a framework for linking genotype to phenotype, while also providing fundamental insights into microbial ecology in dairy systems and practical benefits for industrial milk quality control [13]. Accordingly, the aim of this study was to isolate and identify proteolytic *Pseudomonas* species from raw milk, assess their genetic diversity using molecular methods, and evaluate the role of proteases produced by *Pseudomonas protegens* KSM1 in inducing gelation of *UHT* milk during storage.

2- Materials and Methods

2-1 Milk Sampling

2-1-1 Collection and Preparation of Raw Milk Samples

A total of 30 raw milk samples were collected from dairy farms in Isfahan Province over eight months and transported to the laboratory under

refrigerated conditions (4 °C). For microbiological analysis, samples were serially diluted in Ringer's solution (Merck, Darmstadt, Germany) to facilitate the isolation and identification of proteolytic *Pseudomonas* species.

2-1-2 Microbiological Analysis

Diluted samples were spread-plated onto *Cetrimide Agar* (Ibresco, Spain) and incubated at 4 °C and 10 °C for up to five days, respectively. Two morphologically distinct colonies from each plate were selected and streaked onto *Blood agar* supplemented with 5% sheep blood. The isolates were then subjected to Gram staining and biochemical tests to achieve preliminary confirmation of *Pseudomonas* species [14].

2-2 Identification and Genetic Profiling of *Pseudomonas* Species

At this stage, bacterial isolates that were presumptively identified as *Pseudomonas* based on phenotypic characteristics and preliminary biochemical tests, were subjected to molecular analyses to confirm species identification and evaluate their genetic characteristics.

2-2-1 Assessment of Proteolytic Activity

Proteolytic activity was assessed through PCR-based detection of the *AprX* gene using the primers sm2F (5'-AAATCGATAGCTTCAGCCAT-3') and sm2R (5'-TTGAGGTTGATCTTCTGGTT-3'). PCR reactions were performed in a final volume of 25 µL, containing 12.5 µL of Master Mix (Amplicon), 5 µL of template DNA, 0.75 µL of each primer, and 6 µL of deionized water. Thermal cycling conditions consisted of an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 s, primer annealing at 55 °C for 45 s, and extension at 72 °C for 1 min, with a final

extension at 72 °C for 10 min. PCR products with an expected size of approximately 890 bp were analyzed by electrophoresis on 1% agarose gels and visualized using a Uvidoc GAS9500 system [4]. Sterile deionized water was used as a negative control in place of DNA template. Although a positive control was not available in this study, the specificity of the PCR reaction was confirmed by the presence of a band of the expected size in positive samples and the absence of any bands in the negative control.

2-2-2 16s rRNA Gene Amplification and Sequencing

PCR amplification was performed under the following conditions: an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 s, primer annealing at 57 °C for 45 s, and extension at 72 °C for 1 min, with a final extension step at 72 °C for 10 min. The primers 27F (TCAGCTCGTGTCTGAGAT) and 1492R (CGATCCGAACTGAGAACAG) were used. PCR products were sequenced using the forward primer on a SeqStudio Genetic Analyzer. Species identification of *Pseudomonas* isolates was achieved by comparing the obtained nucleotide sequences with those available in the NCBI GenBank database [15].

2-2-3 Genetic Profiling by RAPD-PCR and Cluster Analysis

RAPD-PCR was performed using the XD5 primer (5'-CTG GCG GCT G-3') in a reaction mixture containing 12.5 µL Master Mix (Ampliqon, Odense, Denmark), 1.5 µL primer, 5 µL DNA template, and 6 µL nuclease-free water. The thermal cycling program included an initial denaturation at 94 °C for 5 min, followed by 45 cycles of denaturation at 94 °C for 45 s, primer annealing at 55 °C for 1 min, and extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min. Amplification products were separated by electrophoresis on 1% agarose gels containing 0.8% Safe Stain

(Yekta Tajhiz Azma) at 80 V for 90 min. Banding patterns were recorded using BioNumerics version 6.5, similarity analyses were performed using ClusterVis software, and phylogenetic trees were constructed with GelQuest software [16].

2-3 SDS-PAGE Analysis of Milk Protein Profiles

To evaluate the production of proteolytic enzymes by *Pseudomonas* isolates recovered from raw milk and the heat resistance of these enzymes, skim milk samples were first heated at 110 °C for 10 min, then inoculated with *Pseudomonas* suspensions to achieve a final cell concentration of 10⁸ CFU/mL. The inoculated samples, along with non-inoculated control samples, were stored at 4 °C, 10 °C, and 25 °C for 24 h, then incubated at room temperature for 3, 7, or 10 days. A 50 µL aliquot of each sample was mixed with an equal volume of loading buffer, heated at 95 °C for 5 min, rapidly cooled, and subjected to electrophoresis. Electrophoresis was performed on polyacrylamide gel, consisting of a 4% stacking gel and a 12% resolving gel, with samples run at a constant voltage of 80 V on the stacking gel and 120–150 V on the resolving gel for 60–90 min. Following electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 and destained with a solution containing 40% methanol and 10% acetic acid. Gel images were captured using a Uvidoc GAS9500 system, and the intensities of α -, β -, and κ -casein bands, as well as low-molecular-weight fragments, were analyzed using Gel Analyser version 23.1. Data from storage days 3, 7, and 10 were analyzed using one-way analysis of variance (ANOVA), followed by LSD or Tukey post hoc tests [17,18].

2-4 Statistical Analysis and Software

Descriptive statistics, including mean, standard deviation, minimum, and maximum values, were calculated for protein band intensity data obtained from SDS-PAGE analysis, and one-way analysis of variance (ANOVA) was used to evaluate the effect of storage time (days 3, 7,

and 10) on protein degradation in inoculated and non-inoculated samples, with pairwise comparisons performed using LSD and Tukey HSD post hoc tests. Phylogenetic relationships among *Pseudomonas* isolates were analyzed based on RAPD-PCR results, with gel bands recorded and analyzed using BioNumerics software (version 6.5, Applied Maths, Belgium), similarity matrices calculated using the Jaccard index, and clustering performed using the Neighbor-Joining (NJ) method implemented in ClusterVis and GelQuest software. Protein band size and intensity in SDS-PAGE images were analyzed using Uvidoc GAS9500 and Gel Analyser version 23.1, and all numerical statistical analyses were conducted using SPSS Statistics software (version 26), with statistical significance set at $p \leq 0.05$ for all tests.

3- Results and Discussion

3-1 Identification of *Pseudomonas* Species in Raw Milk by Biochemical and Molecular Methods

Given the widespread distribution and high growth capacity of *Pseudomonas* species under refrigeration conditions, rapid and accurate identification of these bacteria plays a crucial role in spoilage control and quality assurance of dairy products. During the isolation stage, the use of selective Cetrimide agar eliminated many non-*Pseudomonas* bacteria and increased isolate purity, while the spread-plate technique enabled effective separation of individual colonies. Purified colonies were subjected to a series of biochemical tests, the results of which were fully consistent with the standard characteristics of the genus *Pseudomonas*. Oxidase- and catalase-positive reactions confirmed the strictly aerobic metabolism of the isolates. On triple sugar iron (TSI) agar, all isolates exhibited an alkaline/alkaline (K/K) reaction without gas production and no H₂S formation, indicating the absence of carbohydrate fermentation. Results from TSI and Hugh–Leifson OF media further confirmed that the isolates lacked fermentative pathways and relied entirely on oxidative metabolism. All isolates were citrate-positive, while methyl red (MR) and Voges–Proskauer (VP) tests were

negative—findings consistent with the non-fermentative and oxidative metabolic profile of *Pseudomonas* species. Gram staining revealed slender Gram-negative rods, in agreement with the known cell wall structure of *Pseudomonas* spp. [19,20]. Based on complete agreement between phenotypic and biochemical characteristics, 11 out of 30 isolates were selected for molecular confirmation and sequencing. A higher number of isolates grew at 10 °C compared to 4 °C, indicating the enhanced growth capacity of *Pseudomonas* at the upper range of refrigeration temperatures and underscoring the importance of strict temperature control during raw milk storage. Molecular amplification of the 16S rRNA gene produced products of approximately 1500 bp. Sequencing and alignment enabled accurate species-level identification and resolved ambiguities observed in biochemical analyses. Sequence alignment revealed that the analyzed isolate showed the highest similarity to *Pseudomonas protegens*, with an E-value of 0.0 and approximately 97% sequence coverage, closely matching strains CM-YJ10_15-44 and

H78. These findings indicate that the isolate most likely belongs to *P. protegens* KSM1 or a closely related strain. According to the results presented in Table 1, one of the bacterial species isolated from raw milk at 10°C in this study was *Pseudomonas protegens*. Based on the 16S rRNA gene sequence analysis, the isolate exhibited 97% sequence similarity with *P. protegens* strains CM-YJ10_15-44 and H78, supporting its identification as *Pseudomonas protegens*.

Table 1. *Pseudomonas* isolates recovered from raw milk and species identification results based on 16S rRNA gene sequencing

	Species	Strain	Query cover (%)	Sample Source (NCBI)	Gene Accession Number
1	<i>P. moravensis</i>	KR3M-28	97	Agricultural soil	MN752870.1
2	<i>P. putida</i>	M7	97	MTB-contaminated soil	KF358270.1
3	<i>P. protegens</i>	H78	99	Canola rhizosphere soil	CP013184.1
4	<i>P. koreensis</i>	N2	97	Rhizosphere	MN527329.1
5	<i>P. protegens</i>	CM-YJ10_15-44	97	Sterilized Dendrobium	MZ674076.1
6	<i>P. aeruginosa</i>	AO_15	97	Oil-contaminated soil	KX760140.1
7	<i>P. aeruginosa</i>	NBRAJG90	100	Water	EU734822.1
8	<i>Pseudomonas</i> sp.	S6-238	96	Plant tissue	JQ660196.1
9	<i>P. fluorescens</i>	16s	97	Seawater	EU543578.1
10	<i>Pseudomonas</i> sp.	KLEPS3	- ¹	Plant tissue	JQ910871.1
11	<i>Pseudomonas</i> sp.	1_29	-	Chemical fertilizers	MH475338.1

¹Because the portion of the sequence did not match the reference data effectively; it was not possible to provide the amount of overlap

3–2 Detection of the *AprX* Gene in Raw Milk Isolates

In this study, a PCR-based assay was performed to investigate the presence of the alkaline protease gene *AprX* in 30 *Pseudomonas* isolates

obtained from raw milk. None of the isolates produced the expected 890-bp amplicon. To further investigate this finding, the PCR product sequence corresponding to *Pseudomonas protegens* KSM1 was analyzed for open reading frames (ORFs) using CLC Genomics

Workbench version 8.5. The results are presented in Table 2. In molecular biology, the presence of a valid start codon is essential for the initiation of protein synthesis, ensuring the correct expression of the encoded protein [3]. In most *Pseudomonas* species, the canonical start codon is ATG; however, some strains may utilize alternative initiation codons such as GTG or TTG, both of which are translated as methionine during protein synthesis [9]. In the present study, ORF analysis was performed to identify the *AprX* gene in *P. protegens* KSM1; however, no complete and intact ORF corresponding to the approximately 890-bp *AprX* gene was detected. As shown in Table 2, the analyzed DNA fragment contained only short predicted ORFs, with the longest ORFs measuring 171 bp and 111 bp, while several additional ORFs were shorter than 100 bp. These findings indicate that the *AprX* gene was not present within the analyzed sequence. Several factors may explain this result. First, the

analyzed DNA fragment may not include the *AprX* gene, as this gene is located in specific regions of the *Pseudomonas* genome and may not have been encompassed by the selected sequence. Second, the sequencing data may have been incomplete or truncated, preventing recovery of the full-length gene and resulting in the prediction of only short ORFs. Third, sequencing or assembly errors, including frameshift mutations or premature stop codons, could disrupt a continuous coding sequence, dividing it into multiple small ORFs [21,22]. Nevertheless, the consistent detection of only short ORFs strongly suggests that the *AprX* gene is absent from the analyzed genomic region. Overall, both the ORF distribution pattern (data not shown) and the results summarized in Table 2 confirm that no intact coding sequence of approximately 890 bp corresponding to the *AprX* gene is present in the analyzed DNA fragment.

Table 2. Distribution and characteristics of predicted genes in the sequence: position, length, and start codon

Sequence ID	Start Codon	Strand	Length (bp)	End (bp)	Start (bp)
Group 1	AGA	+	99	99	1
	ATG	+	27	261	235
	ATG	+	93	438	346
	ATG	+	6	579	574
	ATG	+	27	816	790
	ATG	+	18	972	955
	ATG	+	78	1050	973
Group 2	GAG	+	12	13	2
	ATG	+	33	124	92
	ATG	+	24	223	200
	ATG	+	87	361	275
	ATG	+	96	763	668
	ATG	+	39	949	911
Group 3	AGG	+	33	35	3
	ATG	+	15	383	369
	ATG	+	90	719	630
	ATG	+	9	890	882
	ATG	+	63	1049	987
	AAC	–	6	1050	1045
	ATG	–	72	912	841
	ACT	–	171	1049	879
	ATG	–	18	608	591
	ATG	–	12	152	141
	CTA	–	111	1048	938
	ATG	–	9	841	833

Sequence 1 (Group 1) corresponds to the first region of the studied genome and includes several putative genes on the positive strand.

Sequence 2 (Group 2) represents the middle region of the genome, in which several short ORFs were identified on the

positive strand.

Sequence 3 (Group 3) covers the terminal region of the genome and contains predicted genes on both the positive and negative strands, some of which have atypical start codons. Each of these sequences contains multiple putative open reading frames (ORFs), identified through gene prediction analysis using **CLC Genomics Workbench version 8.5 (QIAGEN)**.

3-3 Genetic Diversity and Phylogenetic Relationships Based on RAPD-PCR

In the present study, the genetic diversity and phylogenetic relationships among *Pseudomonas* isolates were investigated using two complementary approaches, RAPD-PCR and 16S rRNA gene sequencing. RAPD-PCR products obtained from the 30 isolates were separated by electrophoresis on 1% agarose gel containing 0.8% nucleic acid stain at 80 V for 90 min, and the resulting banding patterns are shown in Figure 1. Based on the aligned 16S rRNA gene sequences, the data presented in Table 1, and comparative analysis of RAPD banding patterns using ClusterVis software, isolates in lanes 9 and 10 were identified as *P. putida*, lanes 15, 17, 18, and 19 as *P. fluorescens*, lanes 4 and 6 as *P. aeruginosa*, lanes 12 and 13 as *P. moraviensis*, lane 7 as *P. koreensis*, and lanes 3 and 20 as *Pseudomonas* sp. (Figure 2). The RAPD-based phylogenetic tree revealed significant genetic diversity among the isolates. The Jaccard-based similarity matrix showed similarity coefficients ranging from 0.79 to 1.00, indicating high genetic affinity among the isolates and, at the same time, appropriate genetic distinction among other isolates. Furthermore, the clustering pattern indicated significant genetic divergence (up to 43%) between some isolates. The *P. aeruginosa* isolates formed a compact cluster, indicating high genetic affinity and suggesting a common origin or similar environmental conditions. In contrast, *P. putida* and *P. fluorescens* were separated into distinct branches with a high genetic distance from the mentioned group. *P. protegens* (lane 4) together with *P. koreensis* (lanes 6 and 7) formed a common subcluster, suggesting phylogenetic affinity and possible overlap in metabolic functions, whereas *P. moraviensis* (lanes 12 and 13) was observed in an independent branch with the highest genetic differentiation (Figure 3). The similarity matrix calculated for the 30 isolates showed similarity coefficients ranging from 0.79 to 1.00, indicating high genetic affinity among some isolates and, at the same time, the presence of

genetic diversity within the studied population. Previous studies have demonstrated that RAPD possesses high discriminatory power for differentiating *P. aeruginosa* isolates and can serve as a valuable tool for assessing genetic diversity and molecular epidemiology. Campbell et al. (2000) identified 131 distinct genetic types among 200 *P. aeruginosa* isolates, highlighting the high intraspecific discriminatory capacity of RAPD [23]. Similarly, Hemmatzadeh and Haghkhah (2021) reported that RAPD, in combination with ERIC-PCR, distinguished isolates that could not be differentiated by ERIC-PCR alone, indicating RAPD's superior discriminatory performance in certain cases [24]. Furthermore, Abdel-Rhman and Rizk (2020) reported that RAPD showed favorable performance compared with other fingerprinting techniques, including REP-PCR and BOX-PCR, in terms of simplicity, rapidity, and intraspecific discriminatory power [25]. Collectively, these findings support the use of RAPD as a simple, cost-effective, and reliable molecular tool for evaluating genetic diversity, discriminating bacterial strains, and elucidating phylogenetic relationships both within and among *Pseudomonas* species [23–25].

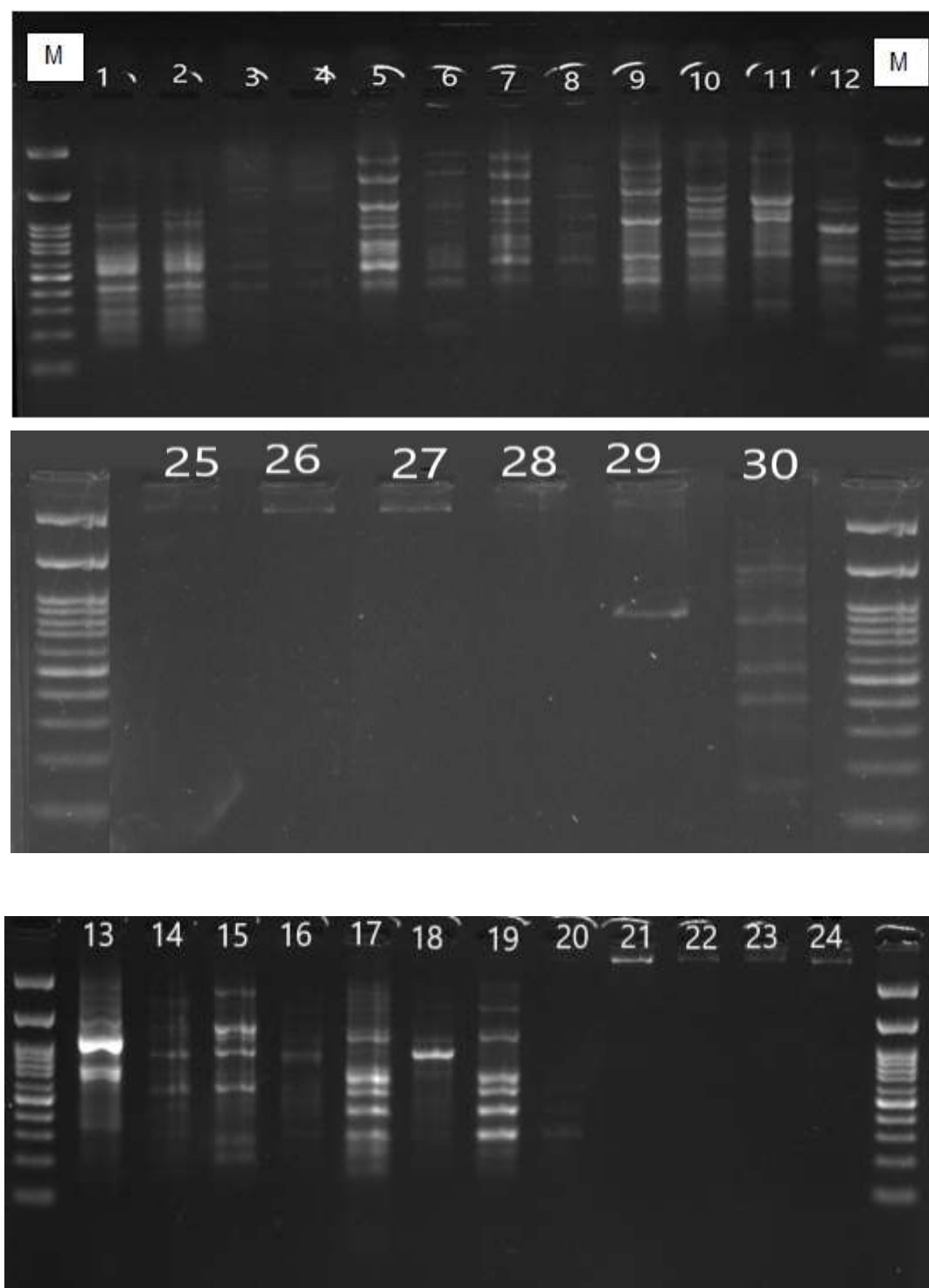


Fig 1. RAPD pattern of the XD5 gene fragment amplified by PCR in 30 *Pseudomonas* species.
M: 100 bp DNA ladder (molecular size standard); **Lanes 1–30:** XD5 gene fragments from the 30 *Pseudomonas* isolates in this study.

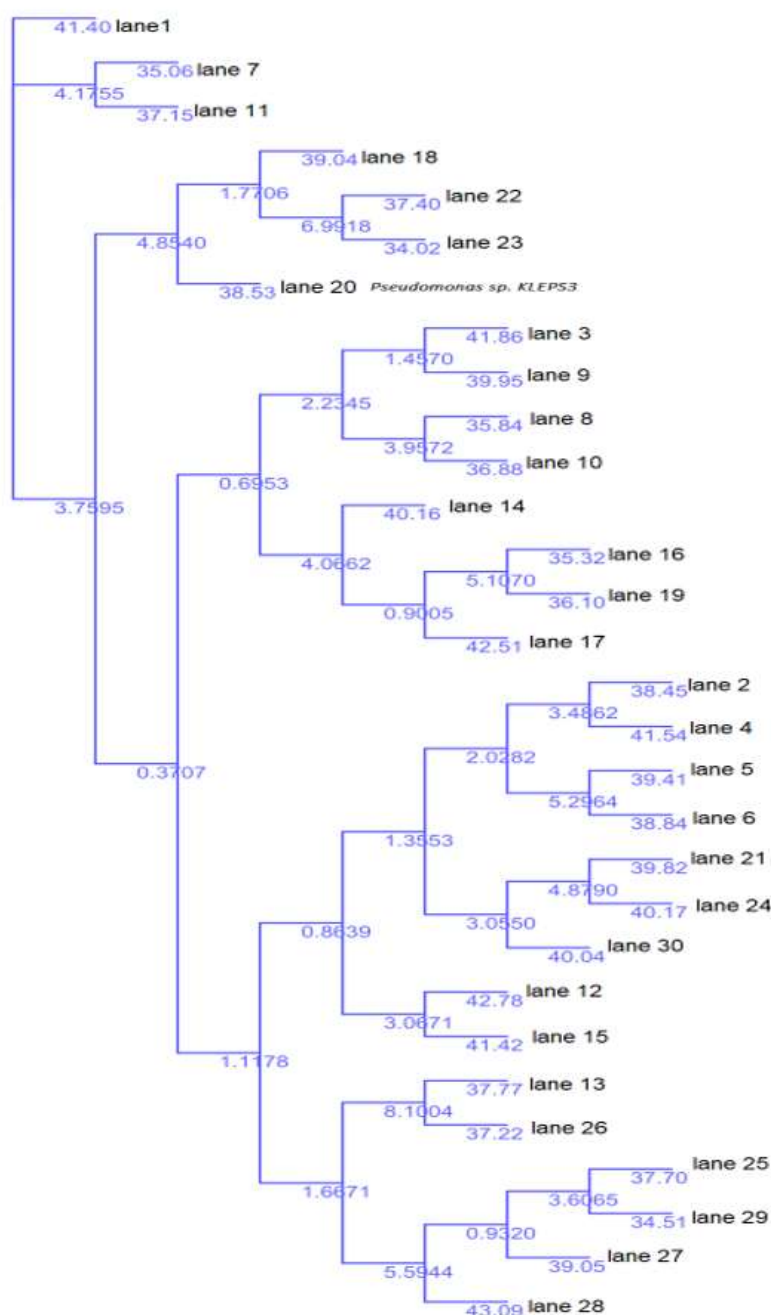


Fig 2. RAPD-PCR-based phylogenetic tree with bootstrap values and genetic distances for analyzing evolutionary relationships among 30 *Pseudomonas* isolates

3-4 SDS-PAGE Electrophoretic Profile of Skim Milk Inoculated with *Pseudomonas protegens* KSM1

The SDS-PAGE electrophoresis pattern in Fig 3 reveals significantly higher degradation of the α s-, β -, and κ -casein bands in the inoculated samples (A, B, and C) compared to non-inoculated ones (D, E and F). Thus, it can be seen that long storage at 25°C combined with the action of bacterial proteases caused pronounced proteolysis. A characteristic protein band at about 45-50 kDa is present in samples A, B, and C, which shows the presence of bacterial alkaline protease [7,14,26].

Meanwhile, in the case of non-inoculated samples, the 45-50 kDa bands appeared to be very faint and could be associated with naturally occurring proteins in milk rather than with any microbial enzyme activity. Most probably, they can be related to the native proteins in milk, such as bovine serum albumin (BSA), lactoferrin, IgG fragments or fragments of proteins denatured under the heat action

(110°C for 10 min) [27]. According to Fig. 4, the degree of protein degradation was maximal in sample A compared to samples B and C. Significant smearing and an increase of low molecular weight bands (10-20 kDa) were observed on days 7 and 10 while samples D, E and F revealed a stable electrophoretic pattern with slight protein degradation.

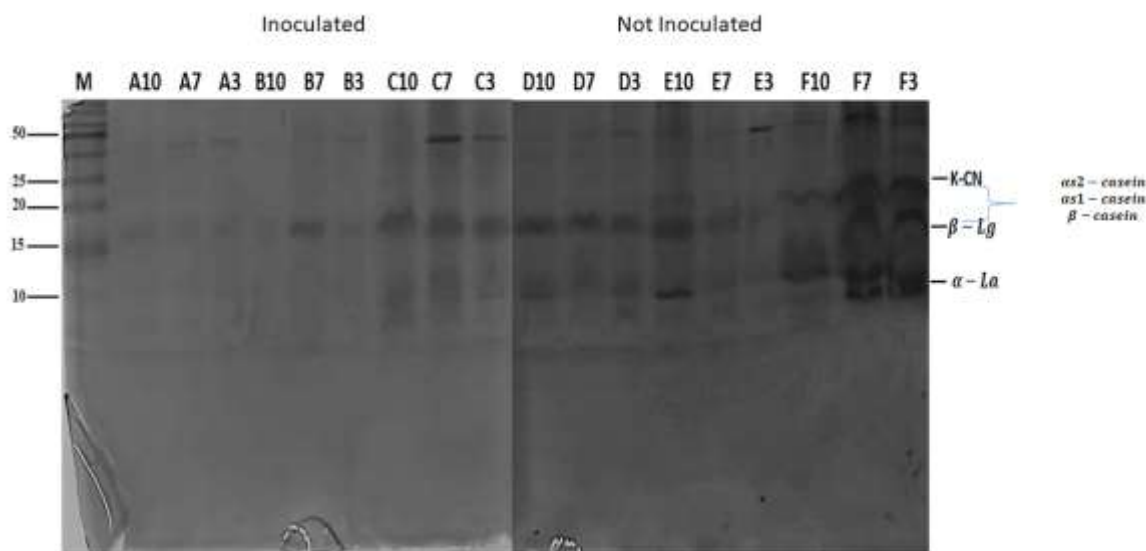


Fig. 3. SDS-PAGE electrophoresis patterns of inoculated and non-inoculated skim milk after thermal treatment and storage on days 3, 7, and 10, showing the degradation of α -, β -, and κ -casein bands in inoculated groups A, B, and C and non-inoculated groups D, E, and F.

A: Inoculated sample, initially stored at 4 °C; B: Inoculated sample, initially stored at 10 °C; C: Inoculated sample, initially stored at 25 °C; D: Non-inoculated sample, initially stored at 4 °C; E: Non-inoculated sample, initially stored at 10 °C; F: Non-inoculated sample, initially stored at 25 °C.

The data concerning the intensity of proteolysis in inoculated and non-inoculated samples is represented in Table 3 below. The comparison of these two groups has demonstrated that the presence of bacteria was the main cause of the observed differences in the degree of proteolysis. Moreover, the analysis conducted

on days 3, 7, and 10 has revealed that the intensity of protein degradation steadily grew during the process of storage in the inoculated samples. As for the non-inoculated samples, the intensity of proteolysis steadily decreased over time.

Table 3. Descriptive statistical summary of protein band intensities (BAND)

Group	Time (days)**	N	Mean $\pm$ SD	Minimum	Maximum
Inoculated	3	3	3.33 $\pm$ 0.58 ^{a*}	3	4
	7	3	3.67 $\pm$ 0.58 ^a	3	4
	10	3	4.67 $\pm$ 0.58 ^b	4	5
Total	—	9	3.89 $\pm$ 0.78	3	5
Not inoculated	3	3	4.67 $\pm$ 0.58 ^b	4	5
	7	3	3.67 $\pm$ 1.15 ^a	3	5
	10	3	3.33 $\pm$ 0.58 ^a	3	4
Total	—	9	3.89 $\pm$ 0.93	3	5

*Different letters indicate significant differences at ($p \leq 0.05$)

**the incubation days at 25°C

The post hoc analysis presented in Table 4 showed that, in the inoculated group, the extent of proteolysis changed over time. The greatest

mean difference was observed between days 3 and 10, which was statistically significant according to the LSD test ($p \leq 0.05$) and was at

the threshold of significance in the Tukey test, whereas the comparisons between days 3 and 7 and between days 7 and 10 were not statistically significant ($p > 0.05$). This pattern indicates a gradual increase in protein degradation with prolonged exposure to bacterial inoculation, although some intermediate changes did not reach statistical significance. In the non-inoculated group, none of the pairwise comparisons (days 3 vs. 7, days 3 vs. 10, and days 7 vs. 10) showed significant differences ($p > 0.05$), indicating that, in the absence of bacterial inoculation, the extent of proteolysis remained relatively stable throughout the

storage period, with only minor, non-significant fluctuations. Comparison of the two groups highlights the critical role of bacterial inoculation in promoting proteolysis, as only the inoculated samples exhibited a significant progressive increase in protein degradation over time, whereas the non-inoculated samples showed no appreciable changes. These findings demonstrate that bacterial proteases significantly enhance protein degradation during storage, while in the absence of bacterial activity, proteolysis remains limited and statistically unchanged over time.

Table 4. Post hoc test results – pairwise comparisons

Treatment	Comparison (Time)	Mean Difference	p-value (LSD)	p-value (Tukey)
Inoculated	Group 3 vs. Group 7 ^a	-0.33	0.506	0.768
	Group 3 vs. Group 10 ^b	-1.33	0.030	0.067
	Group 7 vs. Group 10 ^a	-1.00	0.078	0.165
Not inoculated	Group 3 vs. Group 7 ^a	1.00	0.184	0.355
	Group 3 vs. Group 10 ^a	1.33	0.092	0.193
	Group 7 vs. Group 10 ^a	0.33	0.635	0.874

Different letters (a, b) indicate significant differences at $p \leq 0.05$ (LSD test). Groups sharing the same letter are not significantly different

Overall, the gel electrophoresis and quantitative analyses consistently demonstrated that the greatest extent of protein degradation occurred in group A, which was initially stored at 4°C and subsequently transferred to 25°C. This pronounced proteolysis is likely attributable to the superior adaptation of the bacteria to cold conditions, followed by enhanced protease activity after transferring to the higher temperature. Group B, which was initially stored at 10°C, exhibited an intermediate level of protein degradation, whereas group C, maintained at 25°C from the beginning of storage, showed the lowest degree of proteolysis among the inoculated groups, suggesting that prior exposure to low temperatures (4°C and 10°C) stimulated bacterial protease production or activity, resulting in more extensive protein degradation after storage at 25°C. In contrast, the non-inoculated groups (D, E, and F) exhibited relatively stable electrophoretic patterns with only minor changes, primarily attributable to the activity of indigenous milk enzymes, such as plasmin, and heat-induced protein modifications. Comparison of all experimental groups showed that the inoculated samples exhibited a progressive increase in proteolysis over time, following the order $A > B > C$, whereas the non-inoculated samples displayed only slight or decreasing changes, reflecting the limited contribution of naturally occurring milk

enzymes. Similar to the results reported by Glück et al. (2016) [28], The present study demonstrated that the activity of heat-stable alkaline proteases produced by *Pseudomonas* spp., particularly *Pseudomonas protegens* KSM-1, plays a major role in the gelation of *UHT* milk during storage. Although none of the isolates were positive for the *AprX* gene using the commonly employed primer set, possibly because of sequence variation around the start codon, direct evidence of proteolysis obtained by SDS-PAGE confirmed the presence of proteolytic enzyme activity. Statistical analysis (ANOVA followed by post hoc tests) revealed a significant reduction ($p \leq 0.05$) in the intensity of the α s-, β -, and κ -casein bands in inoculated samples compared with the controls, with the greatest reduction observed after 10 days of storage. The appearance of low-molecular-weight protein fragments together with extensive smearing on SDS-PAGE further confirmed the strong activity of bacterial heat-stable alkaline proteases, even after *UHT* processing. These findings emphasize the importance of minimizing initial bacterial contamination and maintaining a strict cold chain to prevent proteolytic spoilage and preserve the quality of *UHT* milk during storage.

3-5 Conclusion

In this study, *Pseudomonas* isolates from raw milk were comprehensively isolated, identified, and evaluated for their proteolytic activity using biochemical, molecular, and electrophoretic approaches. Phenotypic characterization revealed features consistent with the genus *Pseudomonas*, including oxidase positivity, a non-fermentative metabolism, and a K/K reaction on TSI agar. Amplification and sequencing of the 16S rRNA gene enabled accurate species identification, and one isolate showed 97% sequence similarity to strains H78 and CM-YJ10_15-44 and was identified as *Pseudomonas protegens* KSM1. RAPD-PCR analysis confirmed clear genetic clustering and substantial genetic diversity among the isolates. SDS-PAGE analysis demonstrated markedly greater degradation of the α s-, β -, and κ -casein bands in inoculated samples than in the controls, while the appearance of an approximately 50 kDa band together with low-molecular-weight protein fragments confirmed the activity of heat-stable alkaline proteases produced by *Pseudomonas*. Statistical analysis using ANOVA and post hoc tests showed that increasing storage time significantly enhanced proteolysis in the inoculated samples, particularly between days 3 and 10, whereas no significant changes were observed in the non-inoculated controls. Comparison of the temperature treatments further revealed that the greatest protein degradation occurred in samples initially stored at 4°C and subsequently transferred to 25°C, indicating that cold stress stimulated bacterial protease activity following temperature elevation. Collectively, these findings demonstrate that *P. protegens* KSM1, through the production of heat-stable alkaline proteases, is a major contributor to age gelation and quality deterioration in *UHT* milk, highlighting the importance of minimizing initial contamination and maintaining an effective cold chain to prevent proteolytic spoilage.

Data Availability

All data generated or analyzed during this study are included in this published article and its tables. Therefore, all information necessary to evaluate, interpret, and support the findings of this study is available to readers.

Conflict of Interest

The authors declare that they have no competing interests or conflicts of interest regarding the publication of this article.

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Author Contributions

Meysam Salehi Sichani served as the principal investigator and was responsible for drafting the introduction and overseeing the overall conduct of the study. Morteza Khomeiri served as co-principal investigator and contributed to the study methodology, data analysis, and scientific supervision of the research. Soodabeh Rostami, Alireza Sadeghi Mahoonak, Ali Moayedi, and Majid Moghaddam contributed to the development and evaluation of the methodology, critically revised the manuscript for important intellectual content, and participated in finalizing the manuscript. All authors contributed to the revision of the manuscript, approved the final version for publication, and agreed to be accountable for all aspects of the work.

Artificial Intelligence (AI) Statement

All data, results, numerical values, tables, and findings presented in this manuscript were generated exclusively from the authors' experimental research. No research data, laboratory results, statistical analyses, or scientific conclusions were generated or interpreted using artificial intelligence (AI) tools. For the Persian version of the manuscript, AI was used solely for language editing, improving sentence structure, clarity, and readability. According to the **Undetectable AI** assessment, the AI-generated content score for

the Persian manuscript was **5%**. For the English version, AI tools were used primarily to translate the manuscript from Persian into English. According to the same assessment, the AI-generated content score for the English manuscript was **84%**. Nevertheless, the translated manuscript was thoroughly reviewed, edited, verified, and approved by the authors, who take full responsibility for the accuracy, originality, integrity, and scientific content of the manuscript.

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جداسازی و شناسایی *Pseudomonas protegens* از شیر خام و تحلیل مولکولی - پروتئومیکی آن در ژله‌ای شدن شیر پس چرخ استریل

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مطالعه حاضر، مکانیزم‌های مولکولی و عملکردی ژله‌ای شدن شیر استریل شده با اتوکلاو را با تمرکز بر فعالیت *Pseudomonas protegens* و سایر گونه‌های سایکروتروف بررسی کرد. شیر خام جمع‌آوری شده از دامداری‌های اصفهان از نظر حضور گونه‌های *Pseudomonas* غربالگری شد و سویه *P. protegens* KSM1 برای تحلیل دقیق انتخاب گردید. نتایج PCR نشان داد هیچ ایزوله‌ای حامل ژن *AprX* با اندازه ۸۹۰ جفت‌باز نیست و تحلیل ORF نیز تنها وجود ORFهای کوتاه‌تر از ۱۷۱ جفت‌باز را تأیید کرد. تنوع ژنتیکی ۳۰ ایزوله با RAPD-PCR و پرایمر XD5 ارزیابی شد و ضریب شباهت ۰/۷۹ تا ۱/۰۰ و واگرایی تا ۴۳٪ به‌دست آمد؛ به‌طوری‌که *P. aeruginosa* در یک خوشه منسجم قرار گرفت، اما *P. protegens*، *P. koreensis*، *P. putida* و *P. fluorescens* در شاخه‌های جداگانه توزیع شدند. بررسی SDS-PAGE نشان داد، نمونه‌های تلقیح‌شده تخریب شدید کازئین را تجربه کردند و وجود باند شاخص ۴۵-۵۰ کیلودالتن، بیانگر فعالیت آلکالین پروتئاز باکتریایی است. میزان پروتئولیز در نمونه‌های تلقیح‌شده به‌طور معنی‌داری به‌ویژه بین روزهای ۳ تا ۱۰ افزایش یافت ($p \leq 0.05$). در حالی که نمونه‌های شاهد تغییرات اندکی داشتند. این داده‌ها نشان می‌دهد پروتئازهای باکتریایی عامل اصلی تخریب پروتئین طی نگهداری هستند. در میان ایزوله‌ها، *P. protegens* KSM1 مدل مناسبی برای مطالعه مکانیسم‌های فساد پروتئینی و توسعه راهبردهای پیشگیری از ژله‌ای شدن شیر فرادما محسوب می‌شود.