Evelin KORCZ[1,2], László VARGA[3], Zoltán KERÉNYI[1]

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Characterization of *Serratia* species and qualitative detection of *Serratia marcescens* in raw and pasteurized milk by an analytical method based on polymerase chain reaction

Keywords: nosocomial infection, *Serratia* species, *Serratia marcescens*, pathogen, prodigiosin, pigment, polymerase chain reaction (PCR), food diagnostics

1. SUMMARY

Serratia **species are opportunistic pathogenic microorganisms primarily known as nosocomial infectious agents, which can also cause food quality problems. The appearance of the extracellular pigment-producing *Serratia marcescens* in cow's milk causes its red discoloration, posing a challenge to the dairy industry and food certification laboratories. The detection of the bacterium by conventional procedures based on microbiological methods is time-consuming and labor-intensive, and in many cases does not lead to satisfactory results due to the competitive inhibitory effect of the accompanying microflora. Following the analysis of the relevant literature, the published endpoint PCR methods and the primers used for the detection of *S. marcescens* were evaluated in *in silico* and *in vitro* assays, and then the procedure was tested on farm milk samples. Using the method, a total of 60 raw and pasteurized milk samples were analyzed, more than half of which (i.e., 32) were identified as *S. marcescens* positive. The significance of our work is mainly represented by the application of the published test methods in food industry practice. Our results highlight to the importance of detecting this bacterial species.**

[1] Hungarian Dairy Research Institute Ltd, Mosonmagyaróvár
[2] Széchenyi István University, Wittmann Antal Multidisciplinary Doctoral School in Plant, Animal, and Food Sciences, Mosonmagyaróvár
[3] Széchenyi István University, Faculty of Agricultural and Food Sciences, Department of Food Science, Mosonmagyaróvár

KORCZ Evelin	ekorcz@mtki.hu	https://orcid.org/0000-0002-8435-8020
VARGA László	varga.laszlo@sze.hu	https://orcid.org/0000-0001-7431-852X
KERÉNYI Zoltán	kerenyiz68@gmail.com	https://orcid.org/0000-0001-8665-5216

2. Introduction and literature review

Nowadays, the impeccable quality and long shelf life of foods is a basic requirement of consumers. Accordingly, there is a growing demand for ever faster, more accurate and more reliable food diagnostic procedures. In this context, molecular diagnostic methods are gaining ground, for example in the rapid detection of pathogenic microorganisms. Polymerase chain reaction (PCR)-based diagnostic kits suitable for the identification of pathogenic microbes are produced by many manufacturers, and these are also used successfully in Hungarian food testing laboratories. These molecular biological tests are mainly suitable for the detection of microbes hose presence poses a high risk to public health (e.g., *Escherichia coli, Salmonella* Typhimurium*, Listeria* spp.). Less attention is paid to pathogens that are not required to be tested by law, such as *Serratia* species present in raw and pasteurized milk.

Serratia species are found in many places in our environment **[1]**. They are saprophytes or opportunistic pathogens **[2]**, facultative anaerobic, biofilm-forming organisms **[1, 3]**. *S. marcescens* grows particularly well in phosphorus-containing environments (e.g., soaps, shampoos) and is also resistant to certain disinfectants **[4, 5]**, so it can cause various nosocomial diseases **[6, 7, 8]**. Increasing antibiotic resistance of *S. marcescens* has also been reported in the literature **[8, 9, 10]**. The bacterium therefore survives and grows easily, so it may find its way into foods under inadequate hygienic conditions. Presumably, it can enter drinking milk as a result of violating hygiene rules, it can grow there and degrade the quality of food **[1, 11, 12]**. For some species, spoilage is indicated by a characteristic red hue.

In the case of the Hungarian dairy sector, accurate data are not available on the extent of the prevalence of *Serratia* species and *S. marcescens*, and on which species cause the infections and degrade milk quality. Nor is there a Hungarian survey on the extent of *Serratia* contamination of dairy farms. With the exception of a few publications, the available information on the exposure of the dairy industry to *Serratia* is also lacking at the international level. Such exceptions are a scientific article on the epidemic of mastitis caused by *S. marcescens* at Finnish diary farms **[1]**, and an older report discussing the role played by pigment-forming *Serratia* species in mastitis **[13]**.

The following *Serratia* species may be responsible for the red discoloration of milk: *S. marcescens*, *S. rubidaea, S. plymuthica* and *S. nematodiphila* (***Table 1***). According to their incidence, *S. marcescens* is of greater importance. Their characteristic pigment is the red prodigiosin, a water-insoluble secondary metabolite that is produced under specific environmental conditions **[14, 15, 16, 17]** (***Figure 1***). The typical red colonies appearing on the culture medium alone do not provide sufficient information to identify *Serratia*, as certain species of many other genera, not belonging to Enterobacteria, may also produce prodigiosin **[14, 18]**.

Table 1. Characterization of Serratia species and their pigment production [19–22]

Species	Charactereisation of pigment production	Source
Serratia aquatilis	Cream coloured	**[23]**
Serratia entomophila	No pigment production	**[24]**
Serratia ficaria	No pigment production	**[25, 26]**
Serratia fonticola	No pigment production	**[27]**
Serratia grimesii	No pigment production	**[28]**
Serratia inhibens	Pale pink	**[29]**
Serratia liquefaciens	No pigment production	**[28]**
Serratia marcescens	Reddis (prodigiosin) / No pigment production / Pink (pyrimine)	**[16, 30, 31]**
Serratia microhaemolytica	No pigment production	**[32]**

Serratia myotis	No pigment production	**[33]**
Serratia nematodiphila	Reddis (prodigiosin) / No pigment production	**[34]**
Serratia odorifera	No pigment production	**[35, 36]**
Serratia oryzae	No pigment production	**[37]**
Serratia plymuthica	Reddis (prodigiosin) / No pigment production	**[35, 38, 39]**
Serratia proteamaculans	No pigment production	**[28, 40, 41]**
Serratia quinivorans	No pigment production	**[28, 36, 42]**
Serratia rubidaea	Reddis (prodigiosin) / No pigment production	**[16, 43, 44]**
Serratia symbiotica	No pigment production	**[45]**
Serratia ureilytica	No pigment production	**[46]**
Serratia vespertilionis	No pigment production	**[33]**

Figure 1. Pure culture of Serratia marcescens on tryptone-soy agar (TSA) (30 °C, 48 h)

There is currently no ISO standard for the detection of *Serratia* species in foods. In their 2006 book chapter **[9]**, *Grimont* and *Grimont* discuss the characteristics of the genus *Serratia*, as well as aspects of their isolation and identification. However, identification by classical microbiological methods is rather cumbersome and often ineffective due to the inhibitory effect of the accompanying flora, despite the fact that the pink discoloration of the milk sample is clearly visible to the naked eye. Although culture media are available for the selective growth of the bacterium **[47]**, in practice their use does not provide a satisfactory solution. In addition, conventional methods are time and labor intensive.

There are commercially available rapid methods for the determination of *S. marcescens*, for example the miniaturized test kit from bioMérieux called Rapid ID 32 E, which satisfies the requirements of standard ISO 7218 **[48]**. However, a colony growing on a culture medium is required to perform the test. Diagnostic tests based on the PCR method, as mentioned before, could provide a solution to overcome the difficulties of detection. At present, however, only the Genesig product of Primerdesign can be mentioned as a molecular diagnostic kit for the detection of *S. marcescens* **[49]**.

The literature relevant for the food industry and, in particular, the dairy industry, is rather poor on the detection of *Serratia* species, including *S. marcescens*, by either endpoint PCR or real-time PCR methods. *Hejazi* et al. **[50]** carried out the serotyping of *S. marcescens* by the RAPD-PCR technique. Serological samples from patients in need of hospital care were used in their study. *Iwaya* et al. **[6]** also tested blood samples for *S. marcescens* strains using a real-time PCR method. *Zhu* et al. **[51]** performed molecular characterization of *S. marcescens* strains by RFLP and PCR methods, while *Joyner* et al. **[2]** detected *S. marcescens* strains in marine and other aquatic environmental samples (e.g., coral mucus, sponge pore water, sediment, sewage, wastewater and diluted wastewater) by real-time PCR. A study of *Bussalleu* and *Althouse*, published in 2018, reports a conventional endpoint PCR technique suitable for the identification of *S. marcescens* that effectively detects the presence of the microorganism in wild boar semen **[52]**.

Our goal was the set up a classical PCR method suitable for the detection of *S. marcescens* in milk. The significance of our work lies in the fact that PCR-based methods described in the literature and the primers used were analyzed, then the procedure deemed appropriate was adopted to food hygiene analytical practice. In our experiments, qualitative determination of the possible *S. marcescens* contamination underlying the discoloration of factory, raw and pasteurized milk samples was performed.

3. Materials and methods

3.1. In silico studies

Based on the literature, three primer pairs (***Table 2***) were selected, which were evaluated by computer modeling, by so-called *in silico* analysis, as well as *in vitro* experiments in order to find the most suitable one for subsequent PCR assays.

Table 2. Serratia marcescens-specific primer pairs used in this study

Name of oligo	Sequence	TM [°C]	Lenght of fragment	Source
Fpfs1	CCGGCATCGGCAAAGTCT	58,2	193 bp	**[53]**
Rpfs2	ATCTGGCCCGGCTCGTAGCC	65,5		
FluxS1	GCTGGAACACCTGTTCGC	58,2	102 bp	
RluxS2	ATGTAGAAACCGGTGCGG	56,0		
Serratia2-for	GGTGAGCTTAATACGTTCATCAA	57,1	107 bp	**[52]**
Serratia2-rev	AATTCCGATTAACGCTTGCAC	55,9		

In our *in silico* studies, the specificity of the a primer sequences was verified by comparison with a DNA database (NCBI BLAST) **[54]**. Comparison with the database allows for homology search (“blasting”). Following this, the suitability of the primers, i.e., whether a possible PCR reaction takes place with the selected genomes, was tested with a molecular biology software (SnapGene 5.1.5.) **[55]**. In the latter case, positive and negative control genomes were downloaded from the NCBI database, and then the SnapGene software was used, in an *in silico* way, to investigate whether the PCR reaction would take place with the primer pairs. The positive and negative controls used for reference purposes were whole chromosome genomes (***Table 3***).

Table 3. Genomes of bacterial strains used as positive and negative controls in in silico analyses and their reactions to primer pairs

Applied bacteria strain		Identifier of GenBank sequence	Result of amplification carried out with primer pairs		
			A.	**B.**	**C.**
Pozitív kontroll genomok	*Serratia marcescens* Db11	HG326223.1			
	Serratia marcescens WW4	CP003959.1			
	Serratia marcescens B3R3	CP013046.2			
	Serratia marcescens N4-5	CP031316.1			
	Serratia marcescens 1274	CP019927.2			
	Serratia marcescens AR_0099	CP027539.1			
	Serratia marcescens AS-1	AP019009.1			
	Serratia marcescens S2I7	CP021984.1			
	Serratia marcescens ATCC 274	AP021873.1			
	Serratia marcescens LY1	CP053918.1			
Negatív kontroll genomok	*Serratia grimesii* BXF1	LT883155.1			
	Serratia grimesii NCTC11543	NZ_UGYI01000001.1			
	Serratia liquefaciens ATCC 27592	NC_021741.1			
	Serratia liquefaciens FG3	CP033893.1			
	Serratia nematodiphila DH-S01	CP038662.1			
	Serratia nematodiphila DZ0503SBS1	NZ_JPUX01000001.1			
	Serratia rubidaea 1122	CP014474.1			
	Serratia rubidaea NCTC10848	NZ_LS483492.1			
	Escherichia coli 58-3	CP050036.1			
	Escherichia coli ATCC 8739	CP000946.1			
	Klebsiella pneumoniae Kp52.145	FO834906.1			
	Klebsiella pneumoniae N16-03892	CP047271.1			
	Pseudomonas aeruginosa UCBPP-PA14	CP000438.1			
	Pseudomonas putida T25-27	CP043576.1			

* Primerek: A. Fpfs1 és Rpfs2; B. FluxS1 és RluxS2; C. Serratia2-for és Serratia2-rev.

Jelmagyarázat:

	a primerpárral fragment amplifikálható
	a primerpárral nincs amplifikáció

3.2. In vitro experimental studies

To confirm the results of the *in silico studies, in vitro* were performed in which the selected primer pairs were tested in laboratory PCR analyses on genomic DNA samples of selected strains of bacteria (several *S. marcescens* strains were used as positive control and *Lactobacillus delbrueckii* subsp. *delbrueckii, Streptococcus thermophilus, Enterococcus faecalis* and *Micrococcus luteus* were used as negative controls). The microorganisms were bacterial strains belonging to the collection of MTKI Kft. and coming from factory environment, determined by genetic identification.

When putting together the components required for the PCR reaction, 5.2 µL of PCR grade sterile water, 10 µL of DreamTaq Green 2× PCR Master Mix (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 0.4 µL (10 pmol/µl) primer and 4 µL of isolated bacterial genomic DNA were used for each reaction. The negative control of the reactions was PCR grade sterile water. The program parameters of the PCR instrument (Mastercycler Nexus Gradient; Eppendorf International, Hamburg, Germany) were as follows: 95 °C for 1 minute, then for 40 cycles 95 °C for 15 seconds, 59.5 °C for 15 seconds, 72 °C for 10 seconds and, finally, 72 °C for 7 minutes **[52]**.

For size separation of the DNA segments formed during the PCR reaction, a 10 µL sample was analyzed on a 2% agarose gel [TBE buffer (Tris-borate-EDTA) (10×), Thermo Fisher Scientific; Agarose DNA Pure Grade, VWR International, Debrecen, Hungary; ECO Safe Nucleic Acid Staining Solution 20.000×, Pacific

Image Electronics, Torrance, California, USA]. The DNA size marker was the GeneRuler Low Range DNA Ladder (Thermo Fisher Scientific). Gel documentation was performed using the Gel Doc Universal Hood II gel documentation equipment and software (Bio-Rad, Hercules, California, USA).

3.3. analysis of raw and pasteurized milk samples

On the one hand, we used in our study factory raw and pasteurized milk samples in the case of which *S. marcescens* contamination was suspected due to their pink discoloration. On the other hand, factory raw and pasteurized milk samples that arrived at the laboratory together with the above samples but not exhibiting discoloration were also tested.

For the DNA digestion and purification process, the NucleoSpin Microbial DNA kit (Macherey-Nagel, Düren, Germany) was used according to the manufacturer's instructions. The reaction tubes containing the eluted DNA were stored in a freezer at -20 °C.

Next, the suitability of DNA isolation and the amplifiability of the samples were checked by 16S rDNS polymerase chain reaction, using primers 27f (5'-AGAGTTGATCMTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3'). The total volume of the PCR reaction for 1 sample was 5.6 µL of PCR grade sterile water, 10 µL DreamTaq Green 2× PCR Master Mix, 0.2 µL (10 pmol/µl) of the primers and 4 µL of isolated bacterial genomic DNA. The negative control of the reactions was PCR grade sterile water. The program parameters of the PCR instrument were as follows: 95 °C for 4 minutes, then for 40 cycles 95 °C for 20 seconds, 54 °C for 30 seconds, 72 °C for 1 minute and, finally, 72 °C for 5 minutes.

For the separation of the DNA segments formed during the PCR reaction, a 5 µL sample was analyzed on a 1% agarose gel. The DNA size marker was the GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific). The DNA sample tested was judged to be suitable for further PCR analysis if the length of the copies of the amplified DNA fragment was as expected (~1500 bp).

In the next step, samples were subjected to *S. marcescens*-specific PCR analysis and gel electrophoresis as described in subsection *IN VITRO EXPERIMENTAL STUDIES*. The results were evaluated on the basis of the presence/absence principle.

In order to check the suitability of the method, PCR results of the milk samples were compared with the few available API (bioMérieux, Budapest, Hungary) test results in a control test. The method was then used to detect the presence of *S. marcescens* in raw and pasteurized milks.

4. Results

In our *in silico* studies, when examining the homology of the primers, they showed similarity primarily to *S. marcescens* chromosome genomes. However, matches were also found in the case of *S. rubidaea* and *S. nematodiphila* strains and some non-*Serratia* species. These results were taken into account during the selection of reference genomes designed for our SnapGene software studies. The need for further investigation was justified by the fact that appropriate homology or the matching of the basis do not automatically mean that the PCR reaction will take place, because the direction of the primers, their melting temperature and the size of the PCR product formed are also critical, among other things.

In the SnapGene test, PCR reactions were predicted with the following parameters: our analyses were performed with at least 15 bases matching and the exclusion of single isolated mismatches. The minimum melting temperature was 50 °C and the maximum length of the fragment obtained as the result of the amplification was 3 kbp.

As shown in ***Table 3***, when matched with the *S. marcescens* genomes, the primer pair Serratia2-for and Serratia2-rev showed amplification in all cases. The PCR reaction generally resulted in six or seven amplicons on the 16S rDNA sections. The adhesion site of the Fpfs1–Rpfs2 and FluxS1–RluxS2 primer pairs is located outside the 16S rDNA in most *S. marcescens* strains, but in some cases they did not show *in silico* amplification, so their sensitivity did not prove to be adequate. In the negative control genomes, the completion of a PCR reaction was predicted by the primer pair Serratia2-for and Serratia2-rev in some cases for certain *S. rubidaea* and *S. nematodiphila* strains. Using primers Fpfs1–Rpfs2, the PCR reaction would take place in the case of a *S. nematodiphila* strain. Primers FluxS1–RluxS2 did not predict the occurrence of a reaction on any of the selected negative control genomes (***Table 3***).

In *S. marcescens* genomes selected as positive controls in *in vitro* experiments, all three primer pairs gave signals according to the expected fragment size, and none gave a signal on the negative controls. The analysis carried out with the primer pair Serratia2-for and Serratia2-rev is shown in ***Figure 2***. In the case of negative samples, the weak signals at around 50 bp are caused by the accumulation of the byproduct aspecific DNA fragments, primer dimers.

Based on the results of *in silico* analyses and *in vitro* studies, primers Serratia2-for and Serratia2-rev were considered to be suitable for further work, despite the fact that their specificity was not perfect. The decision was based on the probable frequency of occurrence of *S. marcescens* on the one hand and the importance of avoiding samples with false negative results on the other.

In order to check the suitability of the method that had been set up, factory milk samples were tested in a control study. Some of the milk samples (*n*=10) exhibited pink discoloration. Using our test method, nine samples were found to be positive for the microbe sought. We also had API test results for four of the samples. The four API-positive samples were also found to be positive in the PCR assay. The method was then used to detect *S. marcescens* in raw and pasteurized milks.

Some of the milk samples showed peach-pink discoloration (***Figure 3***), but it was not clear in many cases due to the pale or yellowish tint. A total of 60 samples were analyzed. Of these, 32 (53.3%) gave positive results and 28 (46.7%) gave negative results for the presence of *S. marcescens*.

Figure 4 shows the result of one of our assays, the separation by gel electrophoresis. It can be clearly seen that the positive control strain gave a positive signal, while the negative control sample gave a negative signal, and positive signals were obtained for three test samples. The weak signals appearing in the case of negative samples are again caused by the accumulation of primer dimers.

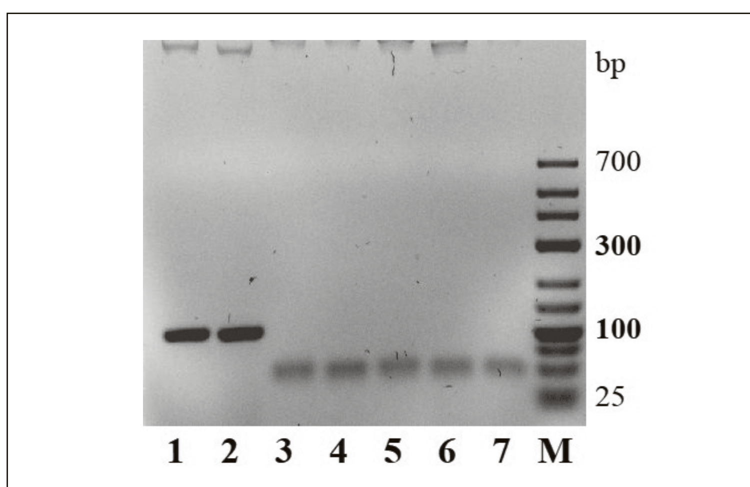

Figure 2. Results of PCR analysis with Serratia2-for and Serratia2-rev primers on the genome of selected bacterial strains. Lanes: 1. Serratia marcescens 551R; 2. Serratia marcescens 1911; 3. Lactobacillus delbrueckii subsp. delbrueckii 0801; 4. Streptococcus thermophilus 1102; 5. Enterococcus faecalis 1101; 6. Micrococcus luteus CLTB1; 7. Negative control (sterile water); M: Molecular weight marker

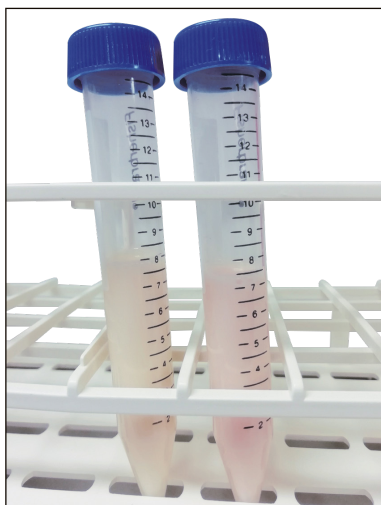

Figure 3. Milk samples. Left sample is netive and right sample is positive for Serratia marcescens, based on the result of PCR test

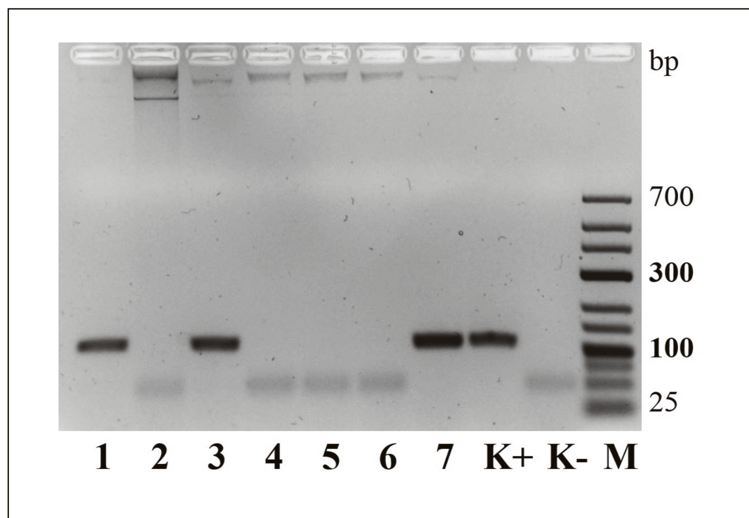

Figure 4. Gel electrophoresis image of Serratia marcescens-specific PCR assay. Lanes 1 to 7: Milk samples; K+: Positive control (genomic DNA from Serratia marcescens); K-: Negative control (sterile water); M: Molecular weight marker

5. Discussion

When evaluating our results, it is important to take into account that the PCR analysis is a method suitable for the amplification and detection of the target DNA in the sample, based on which it is not possible to determine whether the amplified *S. marcescens*-specific DNA comes from viable, dead or so-called VBNC cells. In the VBNC ("viable but not culturable") state, the cells are viable, metabolically active, but cannot be propagated by classical culture methods. This condition is reversible.

The objective of our work was to establish a classical PCR method for the detection of *S. marcescens*. Using the test procedure applied, qualitative determination of the *S. marcescens* contamination responsible for the discoloration of milk samples can be carried out.

Although the experiments presented here focused on the detection of pigment-producing *S. marcescens*, a future genus-level study could identify all 20 *Serratia* species (***Table 1***). The significance of the detection of other *Serratia* species is evidenced by the fact that, although the genus *Pseudomonas* is the main cause of the spoilage of chilled raw milk, the dangers of *Serratia* species in this respect are also known **[56]**. In addition to *Pseudomonas* strains, *Serratia* strains have also been identified in many cases as causes of milk spoilage. Members of the genus *Serratia* have been detected in dairy plants **[3, 12]**, in raw milk samples stored at 4° C **[56, 57, 58]** and in milk containers **[59]**. It was noted by *Grimont* and *Grimont* **[9]** already a decade and a half ago that raw milk lots can occasionally be contaminated with *Serratia* species, and the species most often occurring in diary products are *S. liquefaciens* and *S. grimesii*.

The presence of psychotrophic *Serratia* species (e.g., *S. liquefaciens*) in raw milk can cause spoilage even after heat treatment. *Baglinière* et al. found that the thermally stable Ser2 protease produced by *S. liquefaciens* may be a significant factor in the destabilization of UHT milk **[11, 60]**.

In conclusion, it can be stated that a genus-level study would be an interesting research project that would fill a gap, and which would allow the monitoring of raw milk in this respect, the wide detection of *Serratia* species. Presumably, the results would provide useful information not only to the stakeholders of the dairy economy and the dairy industry, but could also have an impact on Hungarian regulatory and monitoring practice.

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