



# Biochemical, microbiological, and structural evaluations to early detect age gelation of milk caused by proteolytic activity of *Pseudomonas fluorescens*

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## Abstract

Heat-stable peptidase AprX, released by *Pseudomonas* species in raw milk during cold storage, can cause gelation of UHT milk since it is able to split caseinomacropetides (CMP<sub>tot</sub>) from κ-casein, so inducing aggregation of casein micelles. Identifying raw milk susceptibility to gelation would allow UHT milk manufacturers to select appropriate processing conditions or give the milk a different destination. Two approaches, i.e., detection of free CMP<sub>tot</sub> and evidence of casein aggregates, were evaluated as possible indicators for early detecting milk destabilization. With this aim, microfiltered milk was inoculated with a *P. fluorescens* strain and incubated at either 4 or 25 °C. The presence of CMP<sub>tot</sub> was detected using capillary electrophoresis after 96 and 24 h at the two temperatures, respectively, when milk also became heat unstable and small flocks of protein appeared. Confocal laser scanning microscopy evidenced initial aggregates of casein micelles after 48 and 24 h at 4 and 25 °C, respectively. Keeping the milk at 25 °C/24 h could be a useful condition to accelerate milk destabilization. Despite the similar timing of instability detection, presence of CMP<sub>tot</sub> was the only trait specific for AprX activity.

**Keywords** Caseinomacropetide · UHT milk · Milk gelation · *Pseudomonas fluorescens* · Capillary zone electrophoresis · Confocal laser scanning microscopy

## Introduction

Raw milk is a highly perishable food that has to be heat treated to make it suitable for human consumption and to extend its shelf life. Pasteurization and ultra-high temperature (UHT) sterilization are processes in use to inactivate pathogens and reduce spoilage microorganisms count. UHT milk is by far the most popular milk type worldwide due to the advantages of distribution and storage at ambient temperature that grant it a priority access to countries reachable through long-distance transportation [1]. Unfortunately, UHT milk may suffer from spoiling phenomena, such as fat separation, sedimentation and gelation, that impair its storage stability and consumer acceptability [2–4]. UHT milk gelation, also known as “age gelation”, implies the formation

of a three-dimensional protein network that progressively occupies the whole pack volume or a large portion of it [4, 5].

Basically, two mechanisms have been proposed in the literature to explain the age gelation of milk: a “nonenzymatic” mechanism [6], that does not imply casein degradation, and another one that, in contrast, involves the proteolytic activities of indigenous or exogenous proteases toward casein micelles. The main native proteolytic system of milk is the plasmin system. Plasmin (EC 3.4.21.7) is primarily present in milk as its inactive zymogen, the plasminogen, and its activity is regulated through a series of specific activators and inhibitors. This complex enzyme system can partly remain active or reactivate in UHT milk, particularly when the direct process is adopted [4, 7]. A preheating step is usually adopted to overcome this problem [7, 8]. On the contrary, heat-resistant extracellular proteases from psychrotrophic bacteria still represent a threat for UHT milk stability since they can withstand the sterilization process [9]. Many psychrotrophic bacterial species, belonging to *Pseudomonas* sp., *Serratia* sp., *Chryseobacterium* sp., *Enterobacter* sp., or

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*Bacillus* sp. among others, are reported to be responsible for spoilage of refrigerated raw milk causing development of off-flavors, impairment of milk foaming properties and reduction of cheese yield [2]. An ample literature indicates *Pseudomonas* sp. to be responsible for age gelation of UHT milk, due to its ability to secrete a heat-resistant protease, namely AprX, related to the development of this defect [1, 10]. AprX is an alkaline metalloprotease having optimal temperatures for secretion and activity of 17.5–20 °C and 45 °C, respectively. The AprX production in milk increases in the late exponential or the early stationary phase of growth, when the cell density reaches  $10^7$ – $10^8$  cfu/mL and a rapid drop in oxygen tension, iron starvation and lack of easily metabolizable carbon sources take place [1].

The proteolytic activity of AprX was found to be more intense on  $\kappa$ -casein ( $\kappa$ -CN) than on the other casein fractions in studies using capillary electrophoresis [5], RP-HPLC [11] or SDS-PAGE [12]. A relative low number of peptides cleaved from  $\kappa$ -CN by AprX were identified by LC–MS and this may be due to the difficulty of detecting glycosylated peptides by mass spectrometry [11, 13].

Even though dedicated studies concluded that AprX does not have a strong specificity to selected amino acids [14], a preferred cleavage site at Phe105–Met106 bond of  $\kappa$ -CN may be due to the local conformation of the protein promoted by charged residues [1]. In a previous study, we evidenced that the AprX from *Pseudomonas fluorescens* can cleave  $\kappa$ -CN at bond Phe105–Met106 or nearby [5] and that this cleavage brings to the “rennet-like” milk gelation. This mechanism was easily detected by monitoring the release of the C-terminal fragments of  $\kappa$ -CN, i.e., the casein-macropptide (CMP) (f106–169) and the pseudo casein-macropptides (pseudo-CMPs) (f104–169 or f105–169), in the soluble fraction of milk. CMP and pseudo-CMPs were evaluated by capillary zone electrophoresis (CZE) and their presence was evidenced in milk also before the gelation onset [5]. This approach could be useful to milk industry for routing raw milk susceptible to gelation towards less vulnerable milk products, thus reducing food waste linked to UHT milk gelation. However, the best strategies to early predict the age gelation in UHT milk still have to be explored. This study aims at identifying suitable incubation conditions to anticipate the gelation onset and, at the same time, at studying the capability of capillary zone electrophoresis (CZE) and confocal laser scanning microscopy (CLSM) to identify, before UHT sterilization, milk susceptible to gelation. Milk samples, inoculated with a selected strain of *P. fluorescens* at three inoculation levels, were incubated and tested at defined sampling times for microbial count, heat stability, presence of free CMPs by CZE, and milk microstructure changes by CLSM. Two incubation temperatures were considered, i.e.,

4 and 25 °C, the former to monitoring changes occurring at usual conditions of milk storage before UHT sterilization, and the latter to evaluate conditions that accelerate the *P. fluorescens* growth and consequent phenomena and, thus, might be suitable for the early prediction of milk gelation. Microfiltered pasteurized milk was used as substrate to exclude interferences of other microbial species.

## Materials and methods

### Experimental design and incubation trials

Microfiltered pasteurized milk (4 L) was aseptically collected at a local commercial plant, immediately refrigerated at 4 °C and brought to the laboratory. 10-mL milk aliquots were aseptically transferred in sterile screw-cap PTFE tubes and inoculated with appropriate dilutions of a fresh culture of *P. fluorescens* strain RM3, previously isolated from raw milk, showing proteolytic activity and the presence of *aprX* gene [15]. Three final concentrations were selected and from here on are indicated as low ( $2 \times 10$  cfu/mL), medium ( $8.4 \times 10^2$  cfu/mL) and high ( $1.80 \times 10^3$  cfu/mL). Not-inoculated milk tubes were used as blank samples. Tubes were incubated at 4 °C or 25 °C and analyzed at fixed times. Specifically, samples incubated at 4 °C were sampled daily over a period of 10 days, while samples incubated at 25 °C were sampled every 8 h over a period of 72 h. At the end of each incubation time, the pH was measured by a Crison pH meter Basic 20, (Barcelona, Spain). Samples were evaluated for their visual appearance, stability to thermal test, microbial counts, accumulation of  $CMP_{tot}$  by CZE and changes in microstructure by CLSM. These last two approaches were tested to specifically identify milk susceptible to gelation.

### Visual appearance and heat stability test

Physical stability of milk samples during storage was evaluated both visually and with a heat stability test. At each sampling time, three inoculated tubes and three not-inoculated tubes (blank sample) were visually inspected for the presence of instability signs and a score from 1 to 4 was assigned as follows: score 1 indicates stable sample; score 2 indicates the presence of visible flocks; score 3 indicates gelled sample; and score 4 indicates contracted gel with whey separation. Score was assigned when the instability sign was observed in two out of three tubes at least. The heat stability of milk samples was tested by soaking tubes in a boiling water for 5 min. Samples were considered as unstable when flocculation (or coagulation) was observed.

## Microbial counts

Aliquots of each incubated milk sample were serially diluted in sterile saline solution (0.9% NaCl) and plated on Tryptic soy agar (TSA). After 2 days of incubation at 25 °C in aerobic conditions, microbial count was carried out. Data were reported as cfu/mL.

## Capillary zone electrophoresis (CZE) of caseinomacropeptide (CMP) and pseudo-CMPs

The presence of CMP and pseudo-CMPs was assessed by CZE as previously described [5]. Milk sample was adjusted to pH 4.6 using 2 N HCl to precipitate casein and then centrifuged at 3000g for 20 min at 10 °C. A volume of 500 µL of supernatant was added with 500 µL of urea buffer (pH 8.6) containing 0.5 mg/mL of tryptophan (Merck, Italy) as an internal standard. After 4 h at room temperature, the mix was filtrated through a 0.22 µm PVDF membrane filter (Merck, Italy) and then separated by P/ACE™ MDQ Plus CE system (Sciex, Italy) with UV detection at 214 nm. Separation was performed at 45 °C with a 50 cm coated capillary column (DB-WAX 126-7012, Agilent Technologies, Italy) using a linear gradient from 0 to 30 kV in 4 min followed by constant voltage at 30 kV for 56 min. Data of  $CMP_{tot}$  were normalized with the internal standard and expressed as corrected peak area counts. Analyses were carried out in duplicate. Pearson correlation coefficients ( $r$ ) were calculated considering a two-tailed significance level  $P < 0.05$ .

## Confocal laser scanning microscopy (CLSM)

CLSM was carried out at each sampling time to monitor microstructural changes of milk components. Milk samples were prepared according to [16] by staining with Fast Green FCF (Sigma-Aldrich, St Louis, USA) to observe the protein and Nile Red (Sigma-Aldrich) to observe the triacylglycerol core of fat globules. The stock solution of Fast Green was prepared at the concentration of 1 mg/mL in water (Millipore MilliQ), while the stock solution of Nile Red was prepared at the concentration of 1 mg/mL in water containing 0.8 mL/mL dimethyl sulfoxide (DMSO, Sigma-Aldrich). Both solutions were added 1:10 to milk at the time of staining. A sample volume of 8 µL was placed onto a microscopy slide and observed using an inverted confocal microscope (Nikon A1+, Minato, Japan). The excitation/emission wavelengths were set at 488 nm/520–590 nm for Nile Red and at 638 nm/660–740 nm for Fast Green FCF, respectively. In dedicated sample preparations, the Hoechst 34580 was

also added to milk at the final concentration of 12 µg/mL to stain bacteria cells. Hoechst 34580 was excited at a wavelength of 405 nm and the emission filter was set at 392–440 nm. Images were processed using the ImageJ software (NIH, USA).

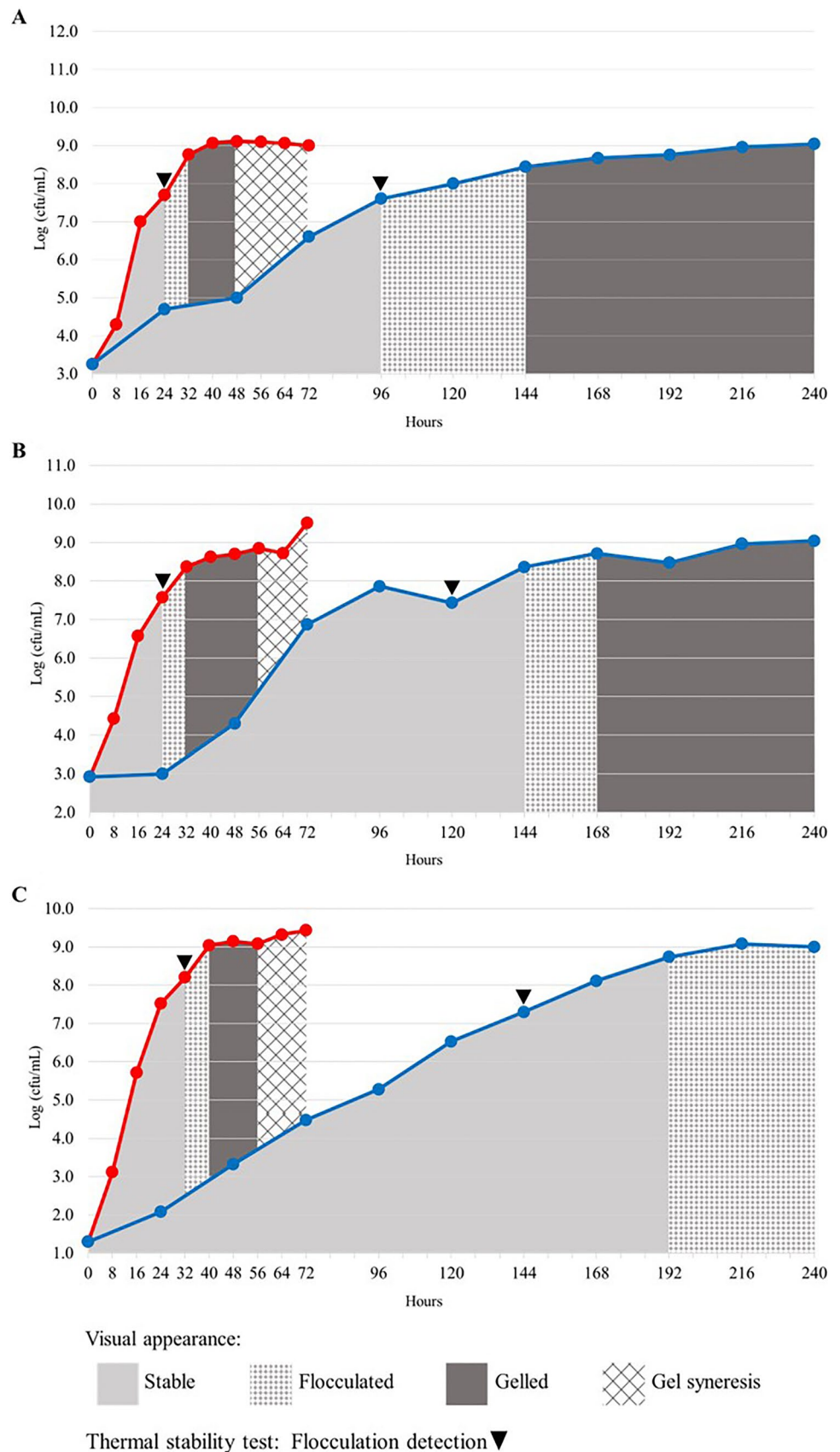
## Results and discussion

### Microbial growth and milk spoiling

Microfiltered pasteurized milk had proven to successfully replace raw milk in studies aiming at evaluating definite effects that should be traced back to growth of the inoculated microorganism [15]. Microfiltration allows milk to remain basically stable at ambient temperature for a sufficiently long time, as required for incubation trials, so excluding interferences from growth of microbial species other than the inoculated one. In this study, milk was inoculated with *P. fluorescens* RM3 at fairly low concentrations ( $< 1.80 \times 10^3$  cfu/mL) to test the feasibility of our approach for the detection of gelation susceptibility in good-quality milk [17]. Inoculated milk was then incubated at 4 °C to simulate the usual tank storage before UHT treatment, whereas incubation at 25 °C was taken as a reference condition since it promotes *P. fluorescens* growth, and thus the AprX activity, without accelerating a specific proteolysis, as previously evidenced [5]. Results from evaluation of microbial growth, visual appearance and heat stability tests are combined in Fig. 1. As expected, microbial growth was faster at 25 °C, compared to 4 °C, and reached 8 log after 32 h of incubation, no matter the inoculation level (Fig. 1). In contrast, microbial growth at 4 °C was dependent on the inoculation level. In fact, 8 log were reached in 5, 6 or 7 days for high, medium and low inoculation level, respectively, because microbial growth at 4 °C proceeded by about one log per day. The same growth rate was recently observed for *P. rhodesiae* and *P. synxantha* incubated at 7 °C [18]. No sample of inoculated milk showed significant acidification with respect to the respective control, with the final pH values ranging from 6.73 to 6.59, until 64 h of incubation at 25 °C (data not shown).

Depending on the tested conditions, instability signs detectable to naked eyes appeared according to the following order: formation of flocks, gelation and gel contraction with whey syneresis. Appearance changed much faster at 25 °C than at 4 °C (Fig. 1). Milk sample inoculated at low level and incubated at 4 °C remained stable (liquid) up to eight days (Fig. 1C). However, starting from the sixth day of incubation, it turned into gel as soon as it was brought to ambient temperature for performing the analyses, indicating that κ-CN cleavage had already taken place anyhow. This behavior is also observed as caused by

**Fig. 1** Growth of *Pseudomonas fluorescens* RM3 in milk samples inoculated at **A** high ( $1.80 \times 10^3$  cfu/mL), **B** medium ( $8.40 \times 10^2$  cfu/mL), and **C** low level ( $2.00 \times 10^1$  cfu/mL) and incubated at 25 (red line) or 4 °C (blue line). The visual appearance of samples and the outcome of the thermal stability test are shown



chymosin in the so-called cold-renneting [19]. The same phenomenon was observed with the inoculation at medium level (Fig. 1B), but not with the inoculation at high level (Fig. 1A). At 25 °C, gelation onset took place after 32 h of incubation for both high and medium inoculation levels, while it took place 8 h later for the low inoculation level. Gel persisted up to 24 h at 25 °C or 4 days at 4 °C. The increased aspecific proteolytic activity occurring at 25 °C was responsible for the gel contraction and syneresis, as already observed for proteolytic activity of other strains of *P. fluorescens* but also strains of *P. poae* and *Chryseobacterium joosteii* [5].

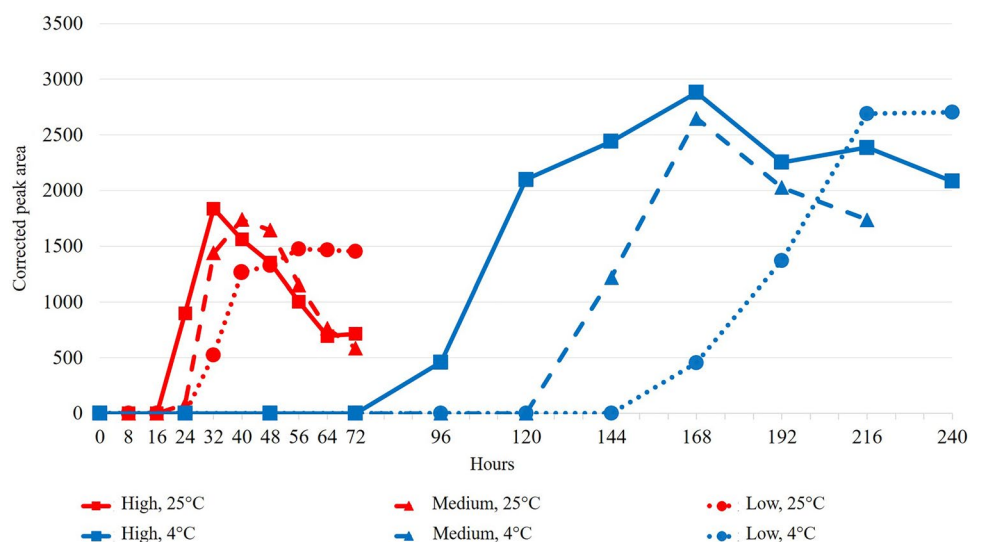
To accelerate milk destabilization, the incubated samples were subjected to a thermal treatment. Formation of flocks was taken as sign for loss of stability in judgement. Upon heating, occurrence of milk flocculation anticipated by one day for milk inoculated at medium level and by two days for milk inoculated at low level, when incubation was carried out at 4 °C. Differently, samples incubated at 25 °C were unstable to thermal test on the same day as flocculation appeared (Fig. 1). The thermal stability is considered a quality parameter of milk, being primarily related to its acidity, and it is generally tested at the receiving platforms of milk processing plants [20]. Since the pH value did not change in milk samples throughout the incubation period, regardless of the conditions, the instability observed upon heating for selected samples was related to the proteolytic activity of AprX that modified the surface properties of casein micelles.

### Specific proteolysis of k-casein and milk gelation

AprX from *P. fluorescens* has been reported to have a chymosin-like activity towards  $\kappa$ -CN [1, 21]. For this reason, the assessment of the presence of CMPs and pseudo-CMPs was proposed as a promising approach to monitor this activity

and to predict milk gelation [5]. The CZE analytical conditions allow to separate the CMP individuals that were previously identified by LC–MS and considered as a whole, CMP<sub>tot</sub> hereafter. The accumulation of CMP<sub>tot</sub> showed a positive correlation with the incubation period both at 4 and 25 °C:  $r=0.64$  and  $0.70$  ( $P<0.05$ ), respectively, until a degradation occurs. Differently, a negative correlation was observed with the incubation temperature ( $r=-0.97$ ;  $P<0.05$ ) (Fig. 2). It is known that CMP<sub>tot</sub> degradation is faster at high temperature [5]. It formed from 8 h to 3 days before the gelation onset and was detected more or less concomitantly with the appearance of very small flocks upon the heat stability test. Furthermore, it was detected when 7–8 log cycles counts were reached, in agreement with the evidence that *Pseudomonas* sp. in milk produce peptidases in the late exponential or in the early stationary growth phase [22]. Remarkably, by incubating milk at 25 °C, the presence of CMP<sub>tot</sub> was clearly evidenced within 24 or 32 h, with initial inoculum of  $1.80 \times 10^3$  or  $2.00 \times 10^1$  cfu/mL, respectively. These results indicate the very high sensitivity and fast response of this method in detecting the AprX activity towards  $\kappa$ -CN. Both these requisites are necessary to the dairy industry to predict milk stability prior UHT processing. Degradation of CMP<sub>tot</sub> was also fast at 25 °C; while, at 4 °C, it started after 9 days of incubation in milk with the lowest inoculation level. It is worth mentioning that no interfering peptides were detected in the incubated samples, regardless of the conditions, confirming that microfiltered pasteurized milk was a suitable medium for evaluating the proteolytic activity of the inoculated bacteria. Other authors adopted a severe heat treatment to inactivate native proteases that could interfere [13, 23].

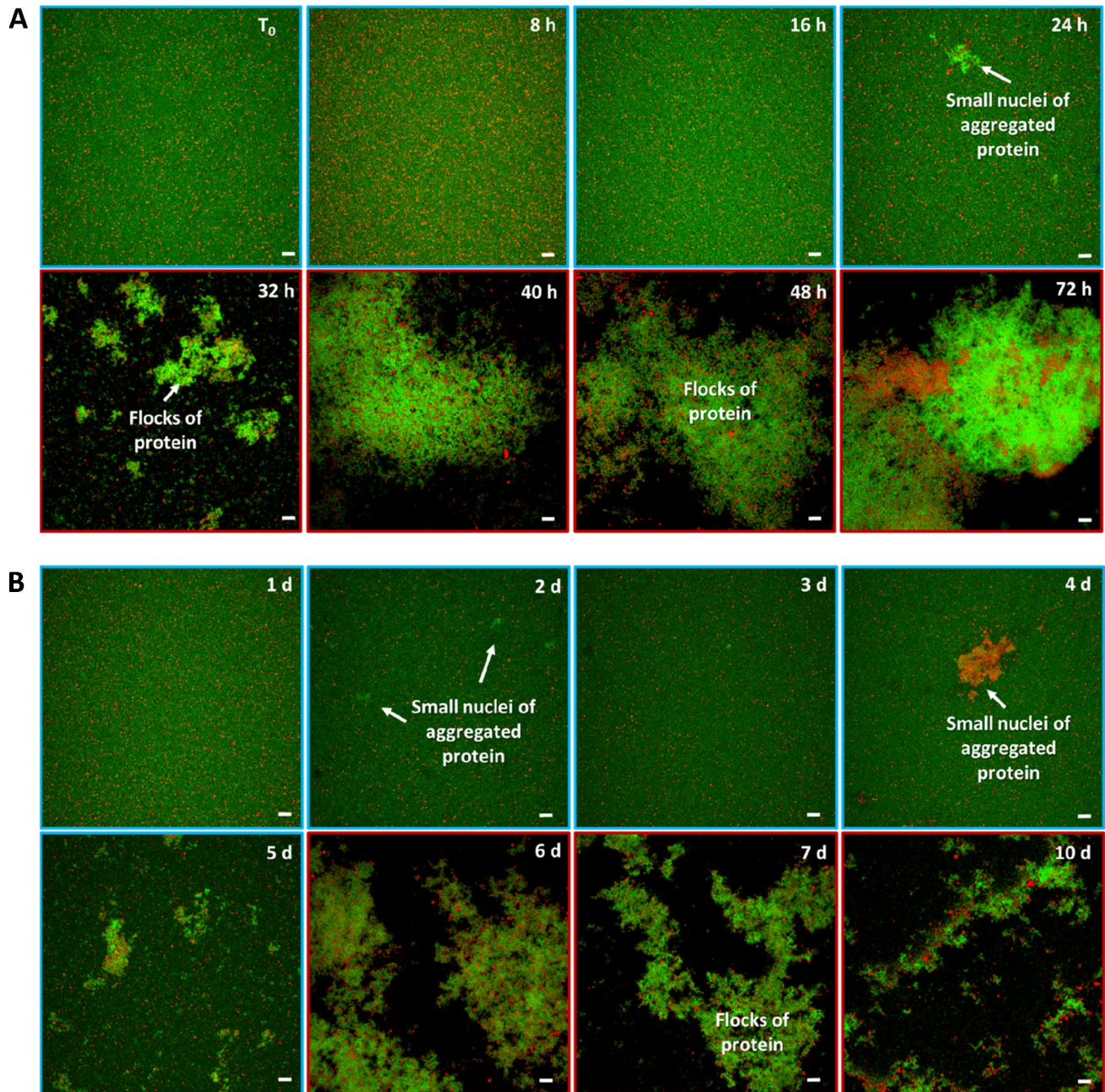
**Fig. 2** Caseinomacropeptides (corrected peak area) in milk inoculated at high ( $1.80 \times 10^3$  cfu/mL), medium ( $8.40 \times 10^2$  cfu/mL) and low ( $2.00 \times 10^1$  cfu/mL) level and incubated at 25 (red line) or 4 °C (blue line)



### Changes in microstructure of inoculated milk

Given the impact of AprX activity on milk destabilization, a better knowledge of spatial organization of casein within milk at the different sampling times was considered of central importance to improve understanding of this phenomenon. The microstructure of inoculated milk samples was monitored throughout the whole incubation period by CSLM, and changes were tracked using specific fluorescent

stains that allow fat and protein to be differentiated. Interestingly, milk microstructure evolved similarly at the two temperatures but the time at which significant changes occurred varied according to incubation conditions. This behavior is shown in Fig. 3A, B for milk samples inoculated at  $1.80 \times 10^3$  cfu/mL (high level) and incubated at 25 °C and 4 °C, respectively. Just after inoculation, milk ( $T_0$ ) was characterized by numerous, single fat globules (in red) almost regularly distributed within the homogeneous



**Fig. 3** Sequence of microstructural changes observed by CSLM during gelation of milk inoculated at high level ( $1.80 \times 10^3$  cfu/mL) and incubated at 25 (A) or 4 °C (B). Protein is green and fat is red. Blue

image frame indicates liquid sample, while red image frame indicates gelled sample. Bars are 10 µm in length

colloidal dispersion of protein (in green). At 25 °C, initial signs of instability were observed just after 24 h, when milk was still liquid, since small nuclei of aggregated casein appeared in the native background (Fig. 3A, 24 h). Eight hours later (32 h after inoculation), milk was gelled and its microstructure had changed considerably. Casein was mostly aggregated in porous flocks surrounded by serum phase (black area). As the incubation progressed, flocks increased in size and incorporated several fat globules. The increase in fluorescence intensity indicates that protein aggregation becomes more intense. At 4 °C, milk microstructure had a similar behavior, but the first small nuclei of aggregated protein appeared on the second day and persisted longer, one more day at least, before to evolve into large flocks that expel the serum phase out (from the sixth day forward) (Fig. 3B). As expected, interaction among casein micelles was delayed at low temperature. Recently, [24] observed slight aggregation among casein micelles in samples stored at 4 °C using field emission scanning electron microscopy.

The patterns of changes obtained with the medium and low levels of inoculum and observed by CLSM were equivalent to the high one (not shown). Again, the qualitative trends were comparable, with differences in timing mostly depending on temperature of incubation. In particular, in milk inoculated at low level and incubated at 4 °C, nuclei of aggregated protein appeared on the fourth day, i.e., well before visible flocks were produced by the thermal stability test.

Once the evolution of milk microstructure bringing to gelation was well understood, a matching with the visual appearance of the samples was made, regardless the inoculation level and the incubation temperature (Fig. 4). Interestingly, nuclei of aggregated protein were always observed when the milk was still liquid and stable (Fig. 4A–A'). Since CLSM allows to penetrate inside a sample and to collect images without disturbing its local structure, it successfully provided evidence of the very early signs of casein destabilization. The development of small aggregates further evolving into flocks is due to the interaction of casein micelles that became progressively unstable. The “rennet-like” milk gelation mechanism explains the destabilization of casein micelles as an effect of the specific cleavage of  $\kappa$ -CN at bonds 103–104, 104–105 and 105–106 carried out by extracellular heat-resistant peptidases produced by psychotropic bacteria, generally *Pseudomonas* sp. and *Serratia* sp. [5]. This mechanism is in line with the absence of  $\kappa$ -CN in the sediment that accumulates at the bottom of UHT milk packages during storage, as recently reported by [25].

### Bacterial cells and protein aggregates

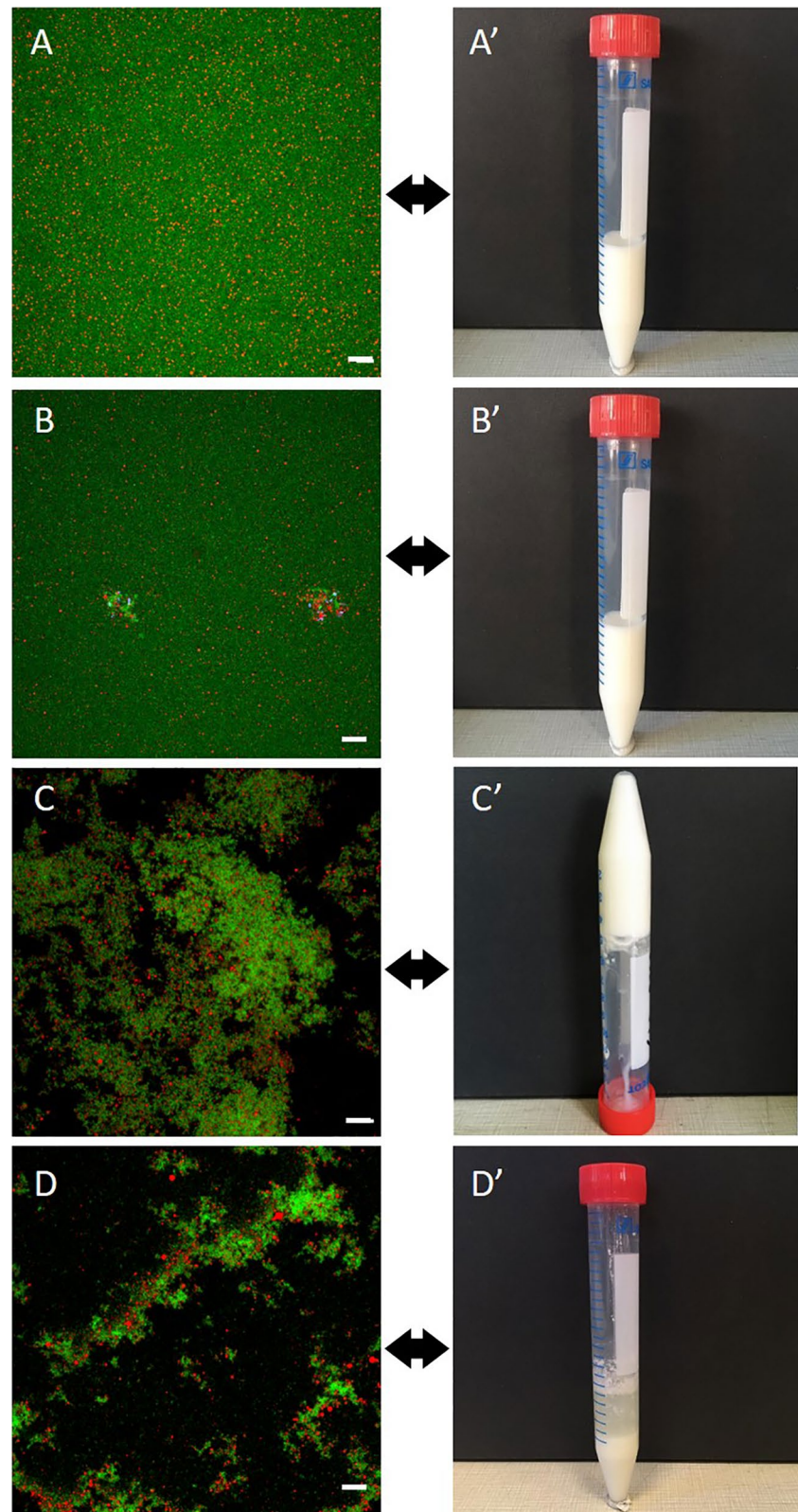
The role of bacteria in the mechanism of milk gelation was further investigated by CLSM. At this purpose, milk samples

were additionally treated with Hoechst 34580 to stain bacterial cells [26]. Thus, due to the presence of three fluorescent dyes, each having a separate emission condition, aggregated protein, fat globules and bacteria cells could be observed either in separated images, using the single channels, or in the merged image (Fig. 5). Interestingly, when milk samples began to spoil at microstructural level, bacteria were systematically located within the protein aggregates or very nearby, together with fat globules (Fig. 5D). This consistent co-localization suggests a specific relationship to exist between the presence of bacterial cells and occurrence of aggregated protein. Actually, it could be hypothesized that AprX released by bacterial cells exerts its specific activity towards the neighboring casein micelles which destabilize and, thus, aggregate. Alongside, the formation of strands of destabilized micelles promotes the entrapment of more bacterial cells that will continue to replicate within the whey pockets, so increasing AprX concentration and involvement of more micelles. This study showed that co-localized cells of *P. fluorescens* and aggregated casein micelles, with some entrapped fat globules, make up the initial nuclei of milk gelation. Such an early step in milk destabilization could be captured likely, thanks to the very low inoculation level and high sampling frequency we have adopted, in agreement with the assumption that the large availability of surrounding matrix promotes the bacterial cell activity [27]. Growth and localization of bacteria within fermented dairy foods are of huge importance in stirring phenomena related to their structure, spoilage and safety; hence, these aspects are increasingly investigated in products such as cheese and yogurt, i.e., in solid or highly viscous matrices [28]. Differently, to the authors' knowledge, no evidence of co-localization of *P. fluorescens* cells and aggregated casein micelles in liquid milk has been provided. It is known that, in Gram-negative bacteria, external cell structures such as flagella, pili or capsular/extra-capsular components. Are involved in the attachment to different substrates [29]. It would be worth of investigation a possible role of these structures also in the adhesion of cells to micelle surface.

### Comparison of the studied approaches

The efficacy of the different parameters considered in this study in predicting milk gelation is shown in Table 1. For each parameter, both the incubation time at which the earliest sign of spoiling was detected and the corresponding microbial count are compiled with respect to the time to gelation. All approaches allowed to detect prodromal signs of gelation before the defect onset, regardless of the inoculation level or the incubation temperature. By incubating milk at 25 °C, both bacterial growth and the resultant proteolytic activity were faster, so that detection of instability was achieved after around 24 h with all parameters. In milk

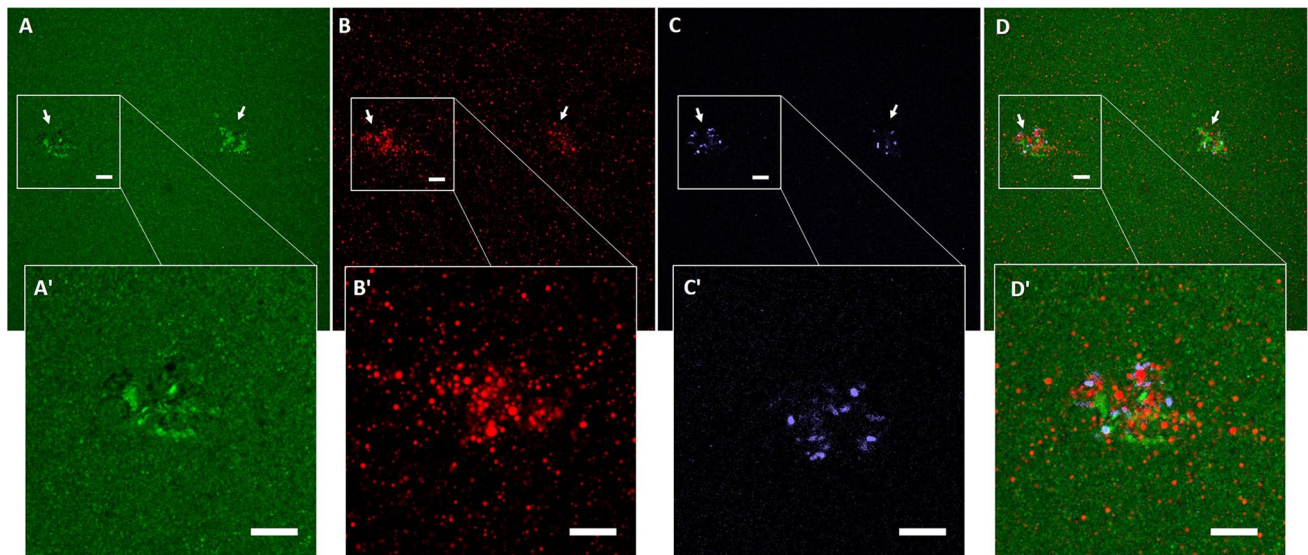
**Fig. 4** Comparison between microscopic (left) and visual appearance (right) of milk during gelation (low inoculation level, incubation at 25 °C). **A–A'** Native microstructure-liquid milk; **B–B'** small nuclei of aggregated protein-liquid milk; **C–C'** network of destabilized protein-gelled milk; **D–D'** contraction of protein network separation of whey. Bars in CLSM images are 10  $\mu\text{m}$  in length



incubated at 4 °C, development of instability signs took much longer and, also in this case, CLSM proved to provide

the earliest information. The slow progress of the destabilizing phenomena occurring at low temperature evidenced





**Fig. 5** CLSM of aggregated protein (arrows) observed in milk still liquid (low inoculation level, incubation at 25 °C for 24 h). Protein in **A**, **A'** is green, fat in **B**, **B'** is red, bacteria in **C**, **C'** are blue. Bacteria are co-localized with fat globules and protein within the nuclei of

aggregated protein (**D**). The framed nuclei of aggregated protein are enlarged in **A'–D'** for the respective channels. Bars in CLSM images are 10  $\mu\text{m}$  in length

**Table 1** Efficacy of the studied parameters in detecting susceptibility of milk to gelation when inoculated with three different levels of *Pseudomonas fluorescens* RM3 and incubated at either 25 or 4 °C

Inoculum level	Incubation temperature (°C)	Time to gelation	Visual flock appearance		Thermal stability test (flock detection)		Presence of $\text{CMP}_{\text{tot}}$		CLSM	
			Time	cfu/mL	Time	cfu/mL	Time	cfu/mL	Time	cfu/mL
High ( $1.80 \times 10^3$ cfu/mL)	25	32 h	24 h	$5 \times 10^7$	24 h	$5 \times 10^7$	24 h	$5 \times 10^7$	24 h	$5 \times 10^7$
	4	6 d	4 d	$4 \times 10^7$	4 d	$4 \times 10^7$	4 d	$4 \times 10^7$	2 d	$1 \times 10^5$
Medium ( $8.4 \times 10^2$ cfu/mL)	25	32 h	24 h	$3.7 \times 10^7$	24 h	$3.7 \times 10^7$	24 h	$3.7 \times 10^7$	24 h	$3.7 \times 10^7$
	4	7 d	6 d	$2.3 \times 10^8$	5 d	$2.7 \times 10^7$	6 d	$2.3 \times 10^8$	2 d	$2 \times 10^4$
Low ( $2 \times 10$ cfu/mL)	25	40 h	32 h	$1.6 \times 10^8$	32 h	$1.6 \times 10^8$	32 h	$1.6 \times 10^8$	24 h	$3.3 \times 10^7$
	4	> 10 d	8 d	$5.5 \times 10^8$	6 d	$2 \times 10^7$	7 d	$1.3 \times 10^8$	4 d	$1.9 \times 10^5$

Incubation time (days or hours) at the first instability sign and corresponding microbial count (cfu/mL) are compared

that their development trend dramatically depends on the inoculation level. Even though CLSM proved to be the best approach to predict milk gelation, it is far from being routinely applicable at industrial level for the in line control of milk. Differently, CZE was less powerful than CLSM; however, its detection limit can be improved by working on sample preparation procedure. Furthermore, it has the advantage of allowing large sets of samples to be analyzed automatically.

## Conclusions

The availability of analytical tools to choose the best destination for raw milk before processing is of primary interest to the dairy industry. In the present study, we have shown that the presence of  $\text{CMP}_{\text{tot}}$  could be detected in milk prior to heat-treatment when the AprX-positive strain of *P. fluorescens* was present at level as low as  $2.0 \times 10^1$  cfu/mL. The sample preparation, including a keeping period of 24 h at 25 °C followed by solubilization with a buffer solution, and the CZE separation are both solvent free, simple, and low cost. Furthermore, differently from the widely used heat-stability test, this approach is specific for

predicting long-term instability of milk due to the AprX activity leading to gelation and thus it is a good candidate for controlling milk before processing. Exploring the milk by CLSM proved that aggregation of destabilized casein micelles into small nuclei occurs even earlier than presence of  $\text{CMP}_{\text{tot}}$  at detectable levels.

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**Declarations**

**Compliance with Ethics Requirements**

**Conflict of interest** John Smith declares that he has no conflict of interest. Paula Taylor has received research grants from Company A. Mike Schultz has received a speaker honorarium from Company B and owns stock in Company C.

**Human or animal rights** This article does not contain any studies with human or animal subjects.

**Informed consent** Informed consent was obtained from all patients for which identifying information is included in this article.

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