Bacterial proteolysis of casein leading to UHT milk gelation: an applicative study

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Abstract

Heat-stable peptidases released in refrigerated raw milk by psychrotrophic bacteria are responsible for UHT milk gelation. K-casein-derived caseinomacropeptides, identified by mass spectrometry, were constantly detected in gelled milk by capillary electrophoresis. Strains of *Pseudomonas fluorescens*, *Ps. poae* and *Chryseobacterium joostei*, selected among *aprX*-positive strains from raw milk, were incubated in milk up to 6 days at 4 °C before sterilization (98 °C/4 min). Samples were then stored at 25 or 40 °C, visually observed for gelation, and analysed for presence of caseinomacropeptides throughout 90 days of storage. Depending on cold pre-incubation time, caseinomacropeptides accumulated well before gelation onset in milk stored at 25 °C. Caseinomacropeptides were successively degraded, especially in milk stored at 40 °C, due to extensive proteolysis, and an abundant sediment developed instead of a gel. The caseinomacropeptides are here presented as an early indicator of UHT milk gelation and a mechanism explaining this phenomenon is proposed.

Keywords: milk gelation, caseinomacropeptides, AprX, psychrotrophic bacteria, capillary zone electrophoresis

1. Introduction

The premature deterioration of consumption milk remains a major problem for the dairy industry that increasingly points at providing high-quality products to gain and maintain consumer loyalty. In this context, finding out effective measures to prevent the entry of spoilage microorganisms, control their growth and predict their enzymatic activities represents a priority challenge. Microbial community of raw milk is complex and variable depending on several factors including cattle health status, feed, milking equipment and procedures, environmental conditions (Bava et al. 2011; Vithanage et al. 2016). In addition, the refrigeration conditions raw milk is stored until processing further define the composition of microbial population by creating selective conditions for the growth and prevalence of psychrotrophic bacteria. Psychrotrophic bacteria are able to growth at low temperatures, although the optimal growth temperature is above 15-20 °C. *Pseudomonas* is the main genus responsible for spoilage of refrigerated raw milk. Nevertheless, presence of other Gram-negative bacteria belonging to *Serratia liquefaciens, S. marcescens, Klebsiella oxytoca, Hafnia alvei, H. paralvei, Enterobacter aerogenes, Chryseobacterium joostei, Stenotrophomonas* spp., *Burkholderia* spp., along with Gram-positives such as *Bacillus cereus, B. licheniformis, B. subtilis, Paenibacillus polymixa, Anoxybacillus* spp., was reported as well (Mcphee and Griffiths, 2011; von Neubeck et al., 2015; Brasca et al., 2017; Machado et al., 2017). Many of these bacteria are able to produce extracellular peptidases and lipases that are highly heat-stable (withstanding UHT temperatures) and can seriously impair technological performances of milk and cause sensory deterioration of the dairy products (Decimo et al. 2014; Glück et al. 2016; Baglinière et al., 2017). It is sufficient to keep raw milk at 6 °C for 48 h to observe an increase of two logs in the psychrotrophic bacterial load (Stoeckel et al. 2016a), allowing the production of peptidases that

generally occurs in the late exponential growth phase (Stevenson et al., 2003; Alves et al., 2018). Proteolytic activity in milk has been related to development of off-flavours and visually detectable alterations (sediment formation, gelation or coagulation), to decreased milk foaming properties, reduced cheese yield, and shortening of the shelf-life (Stoeckel et al. 2016b; D'Incecco et al., 2018). Extracellular thermostable peptidases are alkaline metallo-peptidases with molecular mass between 40-50 kDa and belong to the family of serralysin peptidases. In particular, AprX peptidases from *Pseudomonas* species isolated from raw milk have been extensively studied and, although the protein is highly conserved within species, differences in optimum pH and temperature as well as in thermal stability were observed among species (Marchand et al., 2009; Matéos et al., 2015). According to Machado et al. (2017), occurrence of peptidases from *Ps. fluorescence* group is likely overestimated by current literature due to cases of misidentification of this species. The *aprX* gene, which encodes this protein, is rather heterogeneous within *Pseudomonas spp*. and its expression and regulation are very complex (Marchand et al., 2009; Caldera et al., 2016).

Proteolytic activity of AprX from strains of *Ps. fluorescens* was studied in model solutions of single casein fractions (Recio et al., 2000a; Matéos et al., 2015; Stuknyté et al., 2016). This approach allowed identification of numerous derived peptides and their assignment to the parent casein. However, the kinetics of proteolysis was always very fast and the single intact fractions (β-, α_s-, k-casein) disappeared within 1-4 hours of hydrolysis, depending on the study, with most of primarily released peptides being subsequently further cleaved. Although these studies provided relevant information on the enzyme specificity, actual capability of AprX to degrade casein is more effectively assessed in milk, where casein fractions are associated into large micelles. According to this consideration, studies aiming to elucidate mechanisms leading to destabilization of UHT milk were mostly carried out by adding milk with either the cells of selected *Pseudomonas* strains (Baglinière et al., 2012; Matéos et al., 2015) or thermostable proteases purified from their culture broth (Alves et al., 2018; Zhang et al., 2018). Beside a non-specific proteolysis of casein, largely differing in terms of extent among studies, the preferential cleavage of k-casein (k-CN) was a rather

77 common finding (Machado et al., 2017; Zhang et al., 2018). In particular, k-casein cleavage at
78 bonds 103-104, 104-105 and 105-106 was observed (Matéos et al., 2015), suggesting that AprX
79 from *Pseudomonas* could have chymosin-like activity. Chymosin specifically cleaves the Phe_{105}-
80 Met_{106} bond of k-CN and releases the C-terminal casein-macropeptide (CMP), the hydrophilic
81 "brush" protruding from the surface of the micelles and stabilizing them against interactions. The
82 hydrophobic para-k-casein remains at the surface of the micelles that progressively aggregate to
83 form a three-dimensional network appearing like a continuous gel.
84 We repeatedly observed a typical HPLC pattern of soluble peptides in gelled UHT milks of
85 different origin. Based on this observation, the hypothesis of this work was that all gelled samples
86 contain peptides deriving from the specific action of AprX. The aim of this work was first to assess
87 the presence of CMP or pseudo-CMPs in gelled UHT milk, supporting the role of the chymosin-like
88 proteolysis. We developed an analytical method using the capillary electrophoresis for evaluating
89 these peptides with high reliability. Then a protocol to simulate the industrial manufacturing and
90 storage conditions of UHT milk was set up as a suitable tool for laboratory-scale studies. By using
91 this protocol, the accumulation of CMP or pseudo-CMPs and gel formation were monitored over 90
92 days of storage in sterilized milk obtained from milk intentionally inoculated with *aprX*-positive
93 bacterial strains. Selected strains were: *Pseudomonas fluorescens* LPF3, *Pseudomonas poae* LP5
94 and *Chryseobacterium joostei* LPR1, all isolated from local raw milk. The feasibility of using our
95 approach for early diagnosis of UHT milk gelation was studied.
96
97 **2. Materials and methods**
98 2.1 Milk samples
99 Twelve commercial samples of UHT milk occasionally recalled from the market due to gelation
100 problems were obtained from four manufacturers of Northern Italy between 2015 and 2017. When
101 analysed, milk samples were not more than 3-month old from manufacturing date. At the processing

site of one of the manufacturers, six separate samples (100 mL) of raw bulk milk were aseptically collected from the storage tank (4±1 °C) on different days, were brought to the laboratory under refrigerated conditions (4 °C) and used within 24 h for bacterial strain isolation. For the trials of milk inoculation and storage, partly-skimmed (1.5 g fat/100 mL) microfiltered pasteurized milk (25 L) was aseptically collected just after manufacturing at an industrial plant and brought to the laboratory under refrigerated conditions (4 °C).

2.2 Bacterial strain isolation and identification

Fourteen psychrotrophic strains were isolated from the six samples of raw milk. Samples were serially diluted in quarter-strength Ringer's solution (Scharlau Microbiology, Barcelona, Spain), inoculated into Penicillin-Pimaricin (PP) (Biolife, Milan, Italy) agar supplemented with PP *Pseudomonas* supplement (Biolife) and incubated aerobically at 30 °C for 24-48 h. The colonies with different morphologies were isolated and cultured in Brain Heart Infusion (BHI) broth (Scharlau Microbiology) and purified by streaking repeatedly on PP agar. The 14 isolates were cultivated routinely overnight at 30 °C in BHI broth and preserved in litmus milk at -18 °C.

Genomic DNA was extracted from overnight cultures using the Microlysis kit (Aurogene Rome, Italy) following the manufacturer's instructions. Strain identification was performed by partial 16S rRNA gene and *rpoB* gene sequencing according to McCabe et al. (1995) and Sajben et al. (2011). The obtained PCR products were sent to Macrogen Europe (Amsterdam, the Netherlands) for sequencing and sequences were analyzed with NCBI BLAST search (http://www.ncbi.nlm.nih.gov/BLAST).

2.3 Detection of the *aprX* gene and proteolytic activity of the strains

The 14 strains were screened for the presence of the *aprX* gene as reported by Marchand et al. (2009). Proteolytic activity was evaluated according to Hull (1947) and Pinto et al. (2014). Briefly, strains were inoculated (1%) in reconstituted sterile non-fat dry milk (10%, w/v) (Sacco srl,

128 Cadorago, Italy) and incubated at 10 and 30 °C for 7 days. After incubation, the samples were
129 analyzed by measuring the absorbance at a wavelength of 650 nm. Results were expressed as mg
130 tyrosine released/5 mL milk.
131
132 2.4 Milk storage trials
133 2.4.1 Preparation of inocula
134 Three strains, one from each species, harboring the *aprX* gene and differing in proteolytic activity
135 were selected for the milk inoculation. The inocula were prepared as described by Stoeckel et al.
136 (2016a). Each strain was incubated in BHI broth at 30 °C and refreshed two times. The cell
137 suspension was then centrifuged (3,000 rpm, 10 min) and the pellet was resuspended in partially
138 skimmed UHT milk and incubated at 2 °C for 3 days to allow the bacteria to adapt to the milk
139 medium and to cold conditions. The final cell count was ~10^8 CFU/mL for all of the three strains.
140
141 2.4.2 Cold incubation, sterilization and storage of inoculated milk
142 For each strain, 4 mL of an appropriate dilution of the adapted culture was aseptically inoculated in
143 4 L of microfiltered pasteurized milk in order to obtain a final concentration of 10^3-10^4 CFU/mL.
144 Inoculated milk was kept in a sealed bottle in the dark at 4 °C. Aliquots were aseptically collected
145 just after the preparation and, thereafter, daily until 6 days, for counting (PP agar incubated
146 aerobically at 30 °C for 48 h), for casein and peptide analysis, and for further processing. A blank
147 sample consisting of 1-L non-inoculated milk was processed the same way. On the day of sampling,
148 samples were aseptically filled into 10-mL sterile high-density polyethylene tubes (15 tubes per
149 sample) and sealed with screw cap. Tubes were immediately heated at 97-98 °C for 4 min (with
150 additional 4 min heating time) in a water bath and one tube was tested for sterility. Tubes were
151 randomly divided into two sets that were stored in an upright position in the dark at 25 and 40 °C,
152 respectively, and visually inspected daily by gentle inversion for gelation or sedimentation onset.

153 Two tubes from each set were analyzed in duplicate after 1 week, 3 weeks, and 3 months of storage
154 or at gelation.
155
156 2.4.3 Protein and peptide analyses
157 Intact milk proteins in milk samples were analysed by capillary zone electrophoresis (CZE) as
158 previously described (D'Incecco et al., 2018). For sample preparation, 400 µL milk were added
159 with 800 µL of 10 mol/L urea buffer (pH 8.6) and kept at room temperature for 4 hours. Then the
160 sample was diluted 1:5 with the same buffer and filtered (0.22 µm PVDF membrane filter)
161 (Millipore, Italy) prior to CZE analysis.
162 The soluble milk proteins and peptides were analysed by both HPLC and CZE, adopting the same
163 sample preparation conditions. The milk sample was acidified to pH 4.6 using 2N HCl to precipitate
164 casein and then centrifuged at 3,000 *g* for 20 minutes at 10 °C. The supernatant was filtered through
165 a 0.22 µm filter before analysis. Conditions for HPLC analysis were those of the ISO Standard
166 13875:2005 with the minor modifications described by Pellegrino et al. (2015). The HPLC
167 equipment was an Alliance 2695 coupled with a DAD 2996 detector (Waters, Milford, MA, USA)
168 set at 205 nm and a Polymer PLRP-S column (250x4.6 mm, 300 Å pore size, 5 µm particle size)
169 (Varian Medical System, Milan, Italy) was used. Chromatographic data were processed using
170 Empower2 software (Waters). The same equipment and capillary described above were used for
171 CZE but the operating conditions were optimized for CMPs analysis as follows. An aliquot of 750
172 µL of the filtered supernatant was added with 700 µL of urea buffer (pH 8.6) and 50 µL of
173 tryptophan (5 mg/mL water) (Sigma Aldrich, Italy) as an internal standard. The mix was kept at
174 room temperature for 4 hours, then filtered through a 0.22 µm filter and separated by CZE at 45 °C
175 using a linear gradient from 0 to 30 KV in 4 min followed by constant voltage at 30 KV for 56 min.
176 Data of CMPs were expressed as corrected peak area counts.
177
178 2.5 Identification of CMP and pseudo-CMPs by LC-HR-MS/MS analysis

Four main peaks eluting at retention time 7, 7.5, 8.2 and 10 min respectively were collected from the HPLC eluate of repeated injections of the pH 4.6-soluble fraction of a gelled UHT milk sample. The collected fractions were neutralized using ammonia and lyophilized. Mass spectrometry analysis was performed using a Q Exactive Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA), online coupled with an Ultimate 3000 ultra-high performance liquid chromatography instrument (Thermo Scientific). Samples were resuspended in 0.1% (v/v) formic acid solution, loaded through a 5mm long, 300 µm id pre-column (LC Packings, USA) and separated by an EASY-Spray™ PepMap C_{18} column (2 µm, 15 cm x 75 µm) 3 µm particles, 100 Å pore size (Thermo Scientific). Eluent A was 0.1% formic acid (v/v) in in Milli-Q water; eluent B was 0.1% formic acid (v/v) in acetonitrile. The column was equilibrated at 5% B. Peptides were separated applying a 4–40% gradient of B over 60 min. The flow rate was 0.3 µL/min. The mass spectrometer operated in data-dependent mode and all MS1 spectra were acquired in the positive ionization mode with an *m/*z scan range of 350 to 1600. A resolving power of 70,000 full width at half maximum (FWHM), an automatic gain control (AGC) target of $1x10^6$ ions and a maximum ion injection time (IT) of 256 ms were set to generate precursor spectra. MS/MS fragmentation spectra were obtained at a resolving power of 17,500 FWHM. In order to prevent repeated fragmentation of the most abundant ions, a dynamic exclusion of 10s was applied. Ions with one or more than six charges were excluded. Spectra were processed using the Xcalibur Software 3.1 version (Thermo Scientific). Mass spectra were processed using the Proteome Discoverer 2.1 software (Thermo Scientific), restricting the search to *Bos taurus* extracted from the NCBI (downloaded on March 2017) and with no cleavage specificity. Identification was carried out on the basis of peptide accurate MW. LC-HR-MS/MS analyses were run in duplicate.

2.6 Statistical treatment of data

Statistical evaluation of pH values was performed using the T-test Window 2010, Excel (Microsoft, Redmond, USA). The level of significance was 95%.

205

206 **3. Results**

207 3.1 Optimization of the analytical and experimental conditions

208 Twelve commercial packages of UHT milk with destabilization signs were collected from
209 manufacturers over two years. The samples were different in origin, processing conditions, fat
210 content and age but, although sterile and normal in pH value, at the opening all showed a rennet-
211 like gel involving the whole product or, in a few cases, separated at the bottom of the package with
212 a clear liquid phase on top. Initially, the pH 4.6-soluble fraction of these samples was analysed by
213 HPLC. Interestingly, besides the peaks of whey proteins, we systematically detected the presence of
214 few major peaks, eluting between 7 and 11 min in the HPLC chromatogram (Fig. 1, pattern b), that
215 we never detected in stable UHT milk samples from previous studies (Fig. 1, pattern a). This
216 suggested that a rather similar proteolytic pathway should bring UHT milk to gelation, regardless
217 the microbial species responsible.

218 Based on the HPLC-MS analysis, four peaks eluting at 7, 7.5, 8.2 and 10 min respectively proved to
219 contain fragments of k-CN, i.e. (f 105-169) from the genetic variants A and B and having either
220 single or double phosphorylation, and the canonical CMP (f 106-169) from the two variants, singly
221 phosphorylated only. The peak assignment is detailed in Table S1.

222 The identification of these peptides is consistent with previous findings reporting that, when
223 incubated with pure k-CN, AprX from different strains of *Ps. fluorescens* can cleave the peptide
224 bonds 104-105 and 105-106. These cleavages respectively generate the so-called pseudo-CMP and
225 pseudo-para-k-CN fragments, beside the true CMP and para-k-CN as also generated by the specific
226 action of chymosin (Baglinière et al., 2012; Stuknyté et al., 2016). In contrast with findings reported
227 by Recio et al., (2000a), we did not detect fragments (f 104-169), (f 107-169) and (f 108-169) in our
228 milk samples. The HPLC analysis of CMP in rennet whey samples was previously reported (Thoma

et al., 2006; Pellegrino et al., 2015), with patterns comparable to those obtained here for gelled UHT milk.

The four peaks became hardly distinguishable when an extensive proteolysis in milk gave rise to a more complex HPLC pattern (Figure 1, pattern c). Such a pattern was observed for UHT milk samples in which a compact sediment was present instead of a rennet-like gel. Consequently, we discontinued using the HPLC for milk analyses, although it was a unique approach for peak identification by MS, and preferred the CZE, which provides reliable and accurate separation of milk proteins (Heck et al., 2008).

Considering the definite presence of different CMPs in all gelled UHT milks, an attempt was made to evaluate the degradation of k-CN as a possible analytical approach not suffering from interferences of small peptides. Indeed, all the CZE patterns of gelled UHT milks showed the k-CN degradation, and the presence of one or two peaks corresponding to para-k-CN and pseudo-para-k-CN fractions (Fig. 2, pattern b). These patterns also showed that no other casein fractions were degraded. This allowed to exclude a residual plasmin activity and, most importantly, confirmed that a chymosin-like cleavage of k-CN occurred in gelled milk. However, the obtained CZE patterns were not satisfactory for a quantitative study since the tween peaks of β-lactoglobulin migrated very close to those of para-k-CN and pseudo-para-k-CN fragments making the identification of these last unreliable. Other authors reported the same difficulties when analysing milk added with rennet whey solids (Recio et al., 2000b). To overcome these problems, we decided to develop novel CZE conditions intended to separate the target CMPs present in the pH 4.6-soluble fraction of milk. These fragments migrate as three distinct peaks in the last part of the CZE pattern, which is free of interferences (Fig. 3). A limitation of using CZE as an analytical technique is the difficulty of adopting MS detection to achieve direct peak identification. Therefore, the CMP peaks were identified by analysing the same fractions that were collected from the HPLC of a gelled UHT milk and tested by HPLC-MS. The A and B genetic variants eluted as single peaks for both CMP and pseudo-CMPs, as already observed by Recio et al., (2000a). Proteose peptones peaks were

identified in a previous work (D'Incecco et al., 2018) and, like other peptides, migrated in the first part of the pattern causing no interference with the peaks of CMPs. Tryptophan was added to the samples as an internal standard to correct the peak area of target peptides for the instrumental error in the injected volume.

The CZE of UHT milk inoculated with different counts of *Ps. fluorescens* were reported in previous papers (Van Riel & Olieman, 1995; Recio et al., 2000a, b). However, these papers were focused on the identification of rennet whey solids in adulterated milk and consequently no relation was established between the presence of these fragments and occurrence of milk gelation. Nevertheless, the peak assignments reported for CMP and pseudo-CMPs peaks by these authors were the same as we found in commercial UHT milk samples where gelation occurred. The sum of corrected peak area counts of CMP and pseudo-CMPs (f 105-169) peaks, hereafter named CMP_{tot}, was thus considered in the present study. Formation of CMP_{tot} was monitored in milk samples inoculated with selected bacterial strains, as it is discussed further, in a set of trials that we designed to best simulate the conditions milk undergoes at the industrial manufacturing plant before the sterilization treatment and during the successive shelf life. Contrary to previous studies using UHT milk (Datta & Deeth, 2003) or sterile reconstituted milk (Alves et al., 2018) as a substrate, we used pasteurized milk in order to make proteases released by the studied strains to act on casein micelles in a nearly native state. Furthermore, interference of proteolytic activity from contaminating bacterial species was avoided since these were preliminarily removed by milk microfiltration, as previously done by other authors (Baglinière et al., 2012; Matéos et al., 2015). Thus, the microfiltered pasteurized (MP) milk inoculated with the target strains could be stored at refrigeration conditions for some days before the thermal processing, as it usually occurs at industrial plants for UHT milk processing.

3.2 Bacterial strain selection

Fourteen isolates were identified by partial 16S rDNA gene sequencing and *rpoB* gene as *Pseudomonas fluorescens* (11 strains), *Chryseobacterium joostei* (2 strains), and *Ps. poae* (1 strain)

(Table 1). These findings were in agreement with those of Vithanage et al., (2016), that considered these species as part of culturable psychrotrophic microbiota in refrigerate raw milk. The *aprX* gene was widespread amongst the *Pseudomonas* strains, as only four strains out of the 11 tested did not harbor this gene (Table 1).

All the strains exhibited proteolytic activity at both 10 and 30 °C, although to a different extent. At 30 °C, all the *aprX* positive strains were able to hydrolyze casein in the range 1.0 to 2.0 mg tyrosine 5 mL^{-1} milk and *Ps. fluorescens* LPF3 exhibited the highest proteolytic activity. At lower temperature (10 °C) four strains out of the eight characterized by the highest activity at 30 °C showed a decreased proteolytic activity, while *Ps. poae* LP5 showed values comparable with those obtained at 30 °C ($1.0 < OD_{650} < 2.0$). *C. joostei* LPR1 and LPR2 showed a similar behavior, a higher proteolytic activity being observed at 30 °C. For each species, the strain possessing the highest proteolytic activity was selected for the subsequent experiments: *C. joostei* LPR1, *Ps. fluorescens* LPF3, *Ps. poae* LP5.

3.3 Proteolysis and gelation in experimental milk samples

Three batches of MP milk were inoculated (final concentration 10^3 -10^4 CFU/mL) with LPF3, LP5 and LPR1 strains, respectively, and incubated at 6 °C for up to 6 days. Each day, an aliquot of incubated milk was sterilized (97-98 °C/4 min) in sealed tubes and further stored at 25 and 40 °C, the latter representing storage temperature abuse with respect to room temperature. The evaluation of CMP_{tot} by CE was carried out in milk just before sterilization and after 1 week, 3 weeks and 3 months of storage or at the gelling/instability onset when it happened at an intermediate time. The results of this trial are compiled in Table 2. Concerning gelation, only samples entirely gelled were referred to as gelled, depending on gel stability at the inversion of the tube (Fig. S1). Other instability signs were the formation of a compact and robust sediment at the bottom of the tube and, in a few cases, the flocculation of milk during the sterilization (Fig. S1).

306 *P. fluorescens* LPF3 exhibited a faster growth rate than *P. poe* LP5, nevertheless both strains
307 approximately reached 10^8 cfu/mL after 6 days of incubation.
308 Differently, *C. joostei* LPR1 grew much more slowly since the beginning of incubation. Indeed,
309 after 6 days of cold incubation LPR1 reached counts comparable to those reached by the other two
310 strains after 1-2 days. In all of the samples, pH values were within the range 6.5-6.8 and were not
311 significantly different ($P > 0.05$) from that of the control (not inoculated) milk, indicating that no
312 milk acidification had happened during the cold storage.
313 Production of CMP_{tot} was markedly different among strains (Table 2), according to the differences
314 observed in the total proteolytic activity (as tyrosine equivalents) (Table 1). In this respect, LP5
315 proved to be the most active strain as CMP_{tot} presence was detected even in milk samples that were
316 previously kept at low temperature for one or two days only. The strain LPR1 only produced small
317 amounts of CMP_{tot} after 3 months of storage at 25 °C in the samples that were previously incubated
318 at low temperature for 5 days, consistently with the slow growth observed. Milk storage at 40 °C
319 dramatically anticipated the release of CMP_{tot}, also with the lowest bacterial counts. As expected,
320 the proteolytic activity of AprX against k-CN was faster at higher temperature and the released
321 CMP_{tot} accumulated. Optimum temperature for AprX from different strains of *Ps. fluorescens*
322 isolated from milk was reported to be 37-40 °C (Matéos et al., 2015; Alves et al., 2018). However,
323 when storage at 40 °C was prolonged, the degradation of CMP_{tot} took place as well.
324 Gelation of sterilized milk occurred earlier when the prior cold incubation period was increased,
325 consistently with CMP_{tot} accumulation, but again large variations were recorded depending on the
326 strain. Milk inoculated with LP5 and stored at 25 °C gelled after 24 days when the cold incubation
327 was of 4 days and after only 5 days when the cold incubation was of 6 days. Differently, in milk
328 inoculated with LPF3 and incubated at low temperature for 4 and 5 days, a firm gel formed after 21
329 and 2 days of storage at 25 °C, respectively. Both strains cold incubated for 3 days did not show any
330 gelation but a compact sediment was clearly observable after 90 days of storage. When milk storage
331 was conducted at 40 °C, gelation occurred much earlier, when degradation CMP_{tot} had already taken

place. Flocculation occurred upon heat treatment in milk inoculated with LPF3 and cold incubated for 6 days, thus no further storage was carried out for these samples. Due to the very low amounts of CMP_{tot} produced even under the most favourable conditions (cold incubation for 5 or 6 days and subsequent storage for 3 months), no instability signs were observed in the samples inoculated with strain LPR1.

4. Discussion

In our trials, presence of CMP_{tot} was observed in all the gelled milks well before the gelation sets. The differences in proteolytic capacity and/or the specific activity among species may be attributed to the heterogeneity of the *aprX* gene, as observed for *Pseudomonas* (Machado et al., 2017). Observation of slow proteolytic activity of *C. joostei* can be explained by the low cell counts reached in our conditions (up to 10^5 UFC/mL in 6 days) since it has been evidenced that psychrotrophic bacteria start producing peptidases in the exponential growth phase. In a study conducted on a single strain (UFSBC 256^T) inoculated with cell count of approximately 10^6 CFU/mL, Bekker et al (2015) found proteolytic activity comparable to that of *Ps. fluorescence* type strain ATCC 13525.

It has been highlighted that, at high storage temperature, the CMP_{tot} degradation occurs together with formation. In fact, the amount of CMP_{tot} was lower at gelling onset at 40 °C than at 25 °C. Therefore, the amount of CMP_{tot} itself was not directly related to gel presence during the whole storage period. Nevertheless, when the amount of CMP_{tot} decreased or disappeared after 90 days of storage, an abundant sediment was observed, instead of a gel (Table 2). Considering the long storage, a parallel non-specific proteolysis, also involving the other casein fractions, brought milk to destabilize as a soft sediment and not as a gel. This is in agreement with our hypothesis that gelation is caused by slow interaction of casein micelles whose surface becomes progressively hydrophobic due to the release of CMP. The setting of a compact gel obviously requires a high number of destabilized but sufficiently intact micelles. Malmgren et al. (2017) observed gelation to occur in

358 commercial UHT milk after 6 months of storage at 22 °C while a sediment developed consequent to
359 intense proteolysis when milk stored at 40 °C.
360 Overall, with respect to the studied strains, our data indicated a negative correlation between
361 bacterial counts in raw milk and time to gelation after the sterilization, since less days elapsed
362 before gelation onset when initial bacterial counts were higher. In fact, *Pseudomonas* spp. in
363 refrigerated milk produce peptidases in the late exponential, or early stationary, growth phase
364 (Stevenson et al., 2003; Alves et al., 2018). Stoeckel et al. (2016a) worked with three *Pseudomonas*
365 strains (*Ps. weihenstephanensis, Ps. proteolytica* and *Pseusomonas* R35698 W15a isolated from
366 raw milk) individually incubated in milk at 6 °C for 4 and 5 days before thermal treatment and
367 observed a complete milk gelation only after 4 months of subsequent storage at 20 °C. The related
368 degree of proteolysis in milk samples was measured as the amount of pH 4.6-soluble peptides
369 released during storage using the fluorescamine assay. Therefore, like in other similar studies
370 (Gaucher et al., 2011; Rauh et al., 2014), it was not possible to go deeper into the mechanism
371 leading to milk gelation. Baglinière et al. (2012) observed no gelation in milk inoculated with nine
372 strains of *Ps. fluorescens* and incubated at 4 °C for 3 days before thermal treatment and subsequent
373 storage at 20 °C up to 90 days. These authors identified many released peptides by HPLC-MS but,
374 since they did not work with gelled samples, no relation between presence of specific peptides and
375 gelation could be established. Based on the number of released peptides, they showed the casein
376 degradation to be β- > αs1- > k- > αs2-CN fractions, whereas more studies reported that AprX in
377 milk preferentially hydrolyses k- > β- > αs-CNs (Datta & Deeth, 2003; Zhang et al., 2018).
378 Although conducted under not always comparable experimental conditions, many studies showed
379 that both type and amount of AprX produced by *Ps. fluorescens* are strain-dependent, with different
380 response (enzyme expression) of strains to growth conditions (Marchand et al., 2009; Decimo et al.,
381 2014; Caldera et al., 2016). In addition, the activity of AprX in UHT milk is regulated, both
382 qualitatively and quantitatively, by storage temperature. Consequently, milk gelation may take so
383 long time that is not observed during the studied storage period or may not settle at high storage

384 temperature, when an intense proteolytic activity takes place and destabilization evolves into a
385 sediment accumulation. The number and complexity of these aspects suggested us checking for the
386 release of CMP or pseudo-CMPs rather than for the quantification of AprX activity or the total
387 proteolysis extent for predicting UHT milk stability. Due to the selectivity of the analytical
388 conditions, we were able to detected CMP_{tot} in milk well before its gelation, in some cases even
389 before the sterilization treatment (not shown). Similarly, Matéos et al., (2015) observed
390 accumulation of these peptides in milk during storage at 6 °C before UHT treatment. This confirms
391 that these peptides can be useful markers for predicting the propensity of a milk to gel.
392 Different mechanisms have been proposed to explain UHT milk gelation, either enzymatic or non-
393 enzymatic (McMahon, 1996; Datta and Deeth, 2001, Machado 2017, Anema 2018). Recently,
394 Machado et al., (2017) reported that AprX peptidases may hydrolyse either hydrophobic or
395 hydrophilic areas of casein micelles thus causing their aggregation and sedimentation in UHT milk.
396 In contrast, Anema (2018) proposed the interactions to occur *via* hydrophobic bonding between
397 para-k-casein either on micelles or in serum phase. Zhang et al., (2018) observed that, in UHT milk
398 intentionally added with AprX purified from *Ps. fluorescens*, the onset of gelation goes together
399 with an increase in particle size distribution above that expected for casein micelles and the specific
400 hydrolysis of k-CN. Based on the results of the present study and considering the most recent
401 literature, we hypothesized a "rennet-like" milk gelation mechanism (Fig. 4), in which the heat-
402 stable bacterial peptidases cleave k-CN at the peptide bond 105-106 or in its proximity, depending
403 on the species and strains, and releases the soluble CMP_{tot}. Consequently, the un-solvated micelles
404 slowly aggregate creating a gel that may occupy the whole milk volume when a critical number of
405 casein micelle is involved. Gelation onset is dependent on this event as the first necessary step and
406 our diagnostic approach relies on the detection of the soluble product (CMP_{tot}) originated from this
407 step. Although gelled UHT milks typically contain higher amounts of pseudo-CMPs than CMP, this
408 does not seem to affect the phenomenon. In contrast, broad casein proteolysis plays against
409 gelation. Therefore, a parallel non-specific proteolytic activity that may occur during prolonged

storage or when storage temperature is high impairs gel establishment and brings to its solubilisation and the formation of a sediment.

5. Conclusions

The heat-stable AprX peptidase produced by psychrotrophic species has a chymosin-like activity as it cleaves CMP and pseudo-CMPs from k-CN and promotes micelle destabilization. Therefore, presence of AprX in milk is a challenge for UHT milk manufacturers. This study has shown that the presence of CMP_{tot} in milk represents a phenotypical character of strains in terms of their capability of producing AprX peptidases. Due to the large variation in peptidase expression and activity among bacterial species and strains, we have here proposed to evaluate the presence of CMP_{tot} as a useful indicator of milk susceptibility to gelation, irrespective of the responsible species. The modern CZE equipment allows the implementation of this control for routine evaluation of raw milk before processing. Based on the evidences collected during this study, a model supporting the specific hydrolysis of k-CN as the first necessary step for milk gelation onset is here presented.

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Table 1. *aprX* gene detection and proteolytic activity of the 14 bacterial strains isolated from raw bulk milk collected from storage tank at the processing site.

Species	Strain	*aprX* gene	Proteolytic activity	
			10 °C	30 °C
Chryseobacterium joostei	LPR1	+	+	++
	LPR2	+	+	++
Pseudomonas fluorescens	LPF3	+	++	+++
	LPF39	+	++	++
	LR1	+	++	++
	LR2	+	+	++
	F1	-	+	+
	F2	+	++	++
	L2	-	+	+
	R3	+	++	++
	R4	+	++	++
	R5	-	+	+
	T1	-	+	+
Pseudomonas poae	LP5	+	++	++

−: no proteolytic activity, +: < 1.0 tyrosine (mg/5 mL milk); ++:1.0 < tyrosine (mg/5 mL milk) < 2.0; +++: > 2 tyrosine (mg/5 mL milk)

Table 2. Values of bacterial count (TBC) and CMP_{tot} (corrected peak area) in milk inoculated with (a) *Pseudomonas poae* LP5, (b) *Pseudomonas fluorescens* LPF3, and (c) *Chryseobacterium joostei* LPR1, incubated up to 6 days before thermal treatment and stored at 25 or 40 °C. Values are the mean of two different trials ± standard deviations.

* = sediment at the bottom of the tube.

G = gelled sample.

NS= not stable to heat treatment.

Table 2a

Pseudomonas poae LP5					
Incubation at 4°C (days)	TBC (CFU/mL)	Storage at 25°C (days)	$CMPtot_{25}$	Storage at 40°C (days)	$CMPtot_{40}$
1	2.70E+05	0	0	0	0
		7	0	7	0
		21	0	21	38 ± 15
		90	15 ± 4	90	16 ± 7
2	3.70E+06	0	0	0	0
		7	0	7	0
		21	0	21	41 ± 13
		90	23 ± 6	90	16 ± 3
3	2.28E+07	0	0	0	0
		7	0	7	16 ± 5
		21	18 ± 4	21	64 ± 18
		90	562* ± 83	90	0*
4	2.56E+07	0	0	0	0
		7	117 ± 33	7	201 ± 56
		21	254 ± 38	21	269 ± 61
		24	916 ± 104 **G**	42	472 ± 111 **G**
5	5.25E+07	0	0	0	0
		7	267 ± 67	7	50 ± 23 **G**
		21	1093 ± 190 **G**		
6	1.29E+08	0	60 ± 19	0	60 ± 23
		5	629 ± 88 **G**	4	290 ± 75 **G**

Table 2b

Pseudomonas fluorescens LPF3					
Incubation at 4°C (days)	TBC (CFU/mL)	Storage at 25°C (days)	$CMPtot_{25}$	Storage at 40°C (days)	$CMPtot_{40}$
1	1.47E+05	0	0	0	0
		7	0	7	0
		21	0	21	0
		90	0	90	12 ± 5
2	3.71E+06	0	0	0	0
		7	0	7	0
		21	0	21	0
		90	0	90	19 ± 5
3	4.43E+07	0	0	0	0
		7	106 ± 48	7	136 ± 44
		21	190 ± 63	21	158 ± 61
		90	493* ± 204	90	0*
4	5.48E+07	0	0	0	0
		7	328 ± 120	8	102 ± 42 **G**
		21	218 ± 81 **G**		
5	1.25E+08	0	100 ± 34	0	100 ± 23
		2	36 ± 8 **G**	1	87 ± 10 **G**
6	2.66E+08	0	150 ± 43 **NS**	0	150 ± 26 **NS**

649 **Table 2c**

Chryseobacterium joostei LPR1					
Incubation at 4°C (days)	TBC (CFU/mL)	Storage at 25°C (days)	$CMPtot_{25}$	Storage at 40°C (days)	$CMPtot_{40}$
1	1.15E+04	0	0	0	0
		7	0	7	0
		21	0	21	0
		90	0	90	0
2	9.50E+03	0	0	0	0
		7	0	7	0
		21	0	21	0
		90	0	90	0
3	4.19E+04	0	0	0	0
		7	0	7	0
		21	0	21	0
		90	0	90	0
4	2.27E+05	0	0	0	0
		7	0	7	0
		21	0	21	0
		90	0	90	0
5	1.31E+05	0	0	0	0
		7	0	7	0
		21	0	21	0
		90	113 ± 27	90	85 ± 15
6	4.15E+05	0	0	0	0
		7	0	7	0
		21	62 ± 7	21	60 ± 26
		90	113 ± 15	90	0

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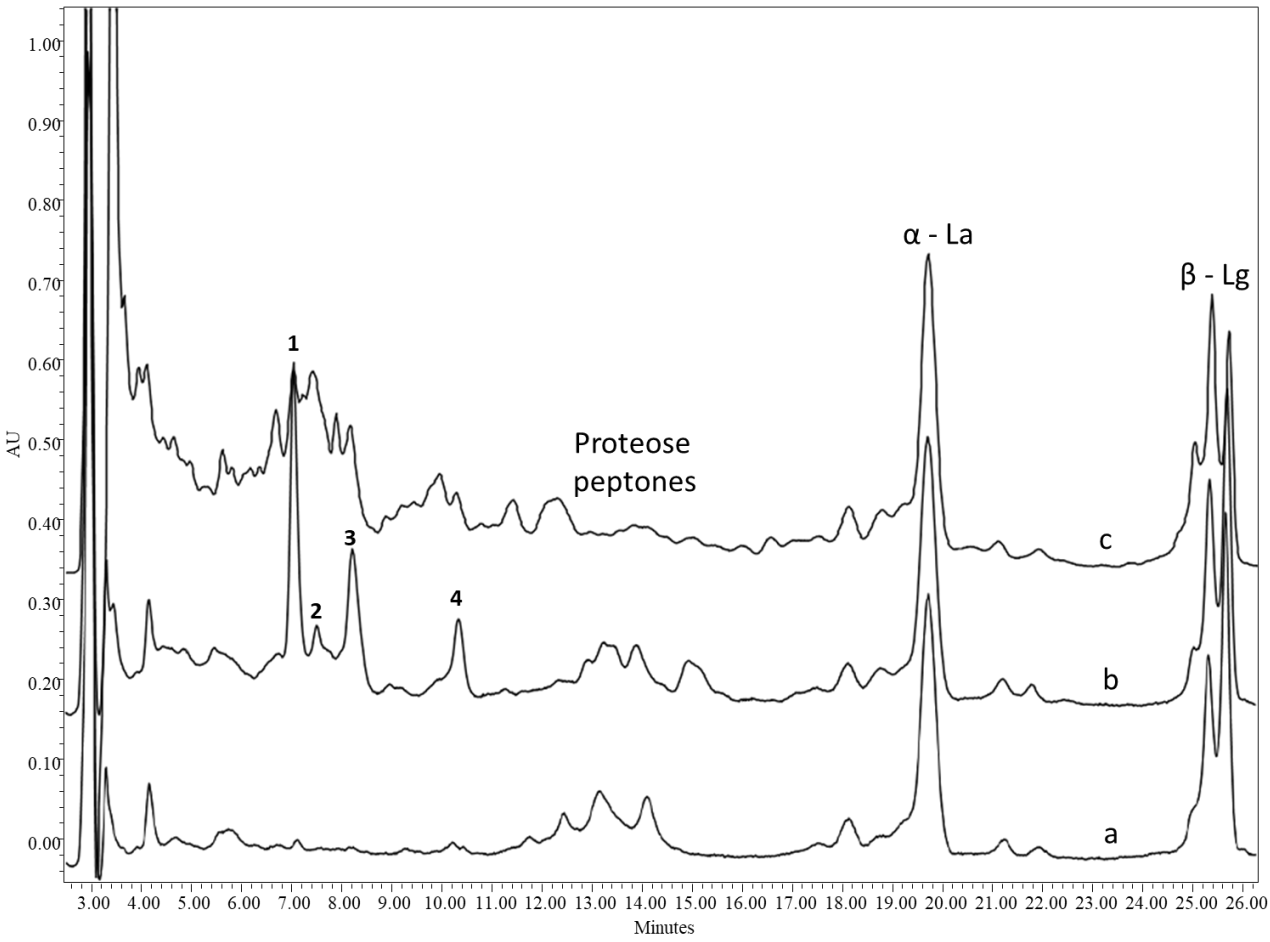
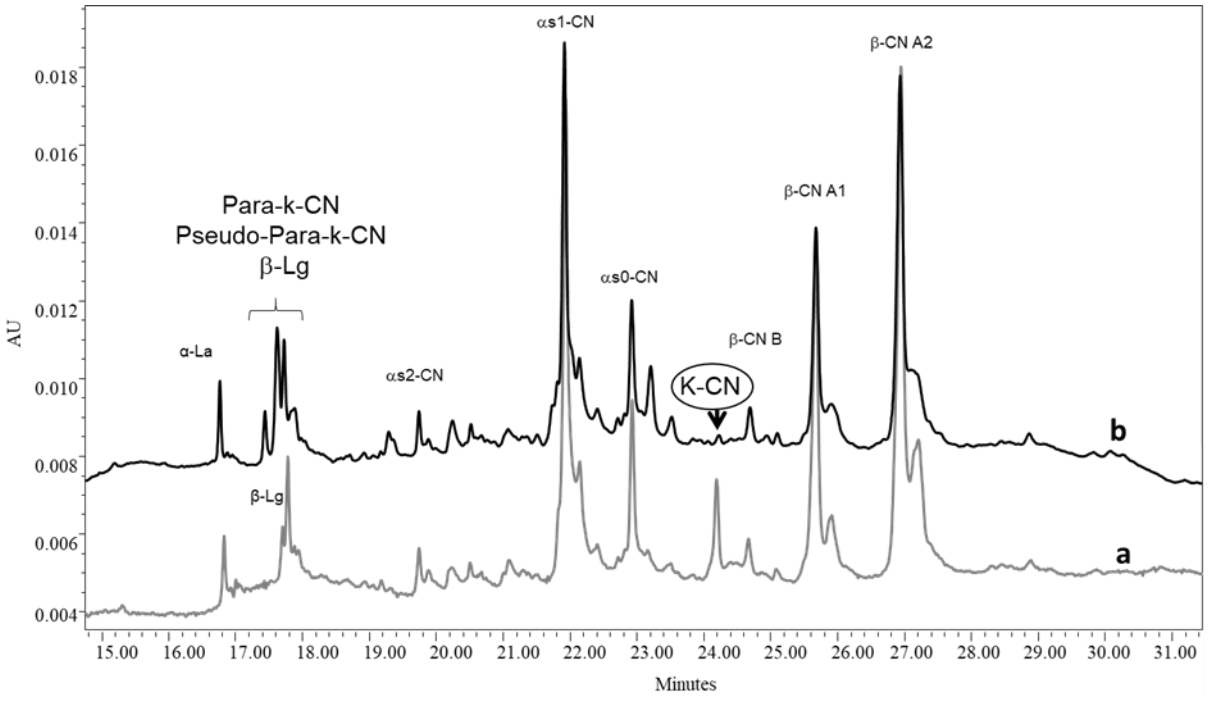


Fig. 1. HPLC patterns of the pH 4.6-soluble fraction of commercial samples of (a) UHT milk, (b) gelled UHT milk, and (c) UHT milk with extensive proteolysis. Peaks in gelled UHT milk were identified by LC-MS as: peak 1 = k-CN A f (105-169) 2P and k-CN B f (105-169) 2P fragments; peak 2 = k-CN A f (106-169) 1P fragment; peak 3 = k-CN A f (105-169) 1P fragment; peak 4 = k-CN B f (106-169) 1P and k-CN B f (105-169) 1P fragments.



665

666 **Fig. 2.** CZE patterns of commercial samples of (a) UHT milk and (b) gelled UHT milk.

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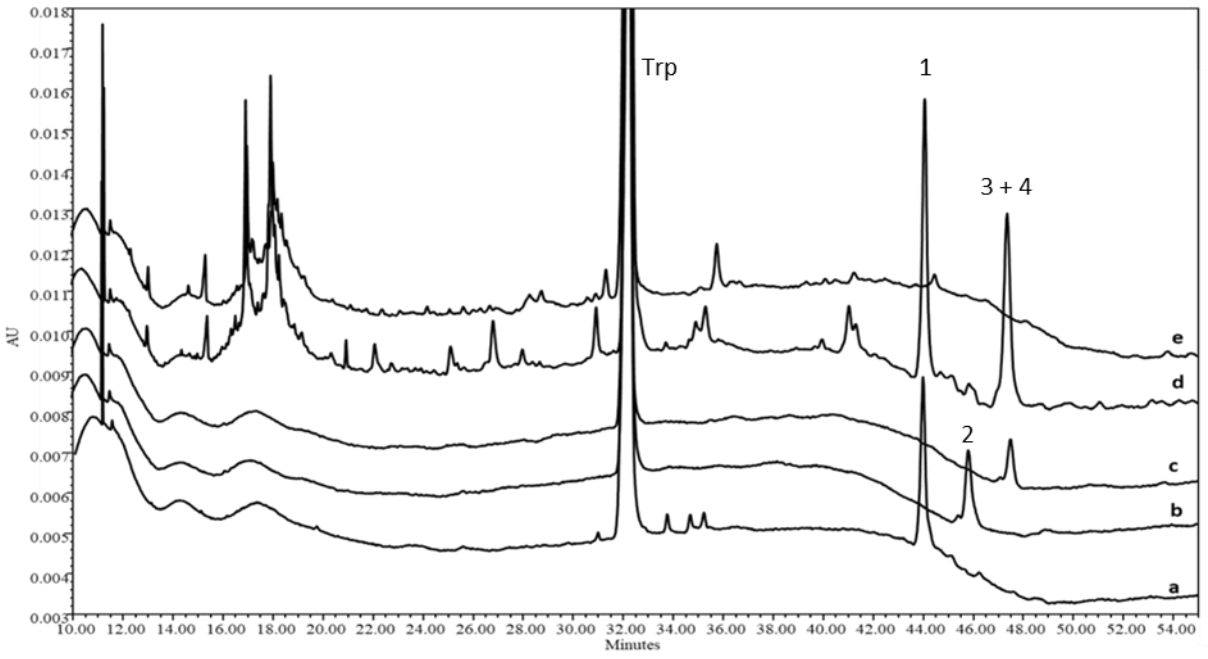
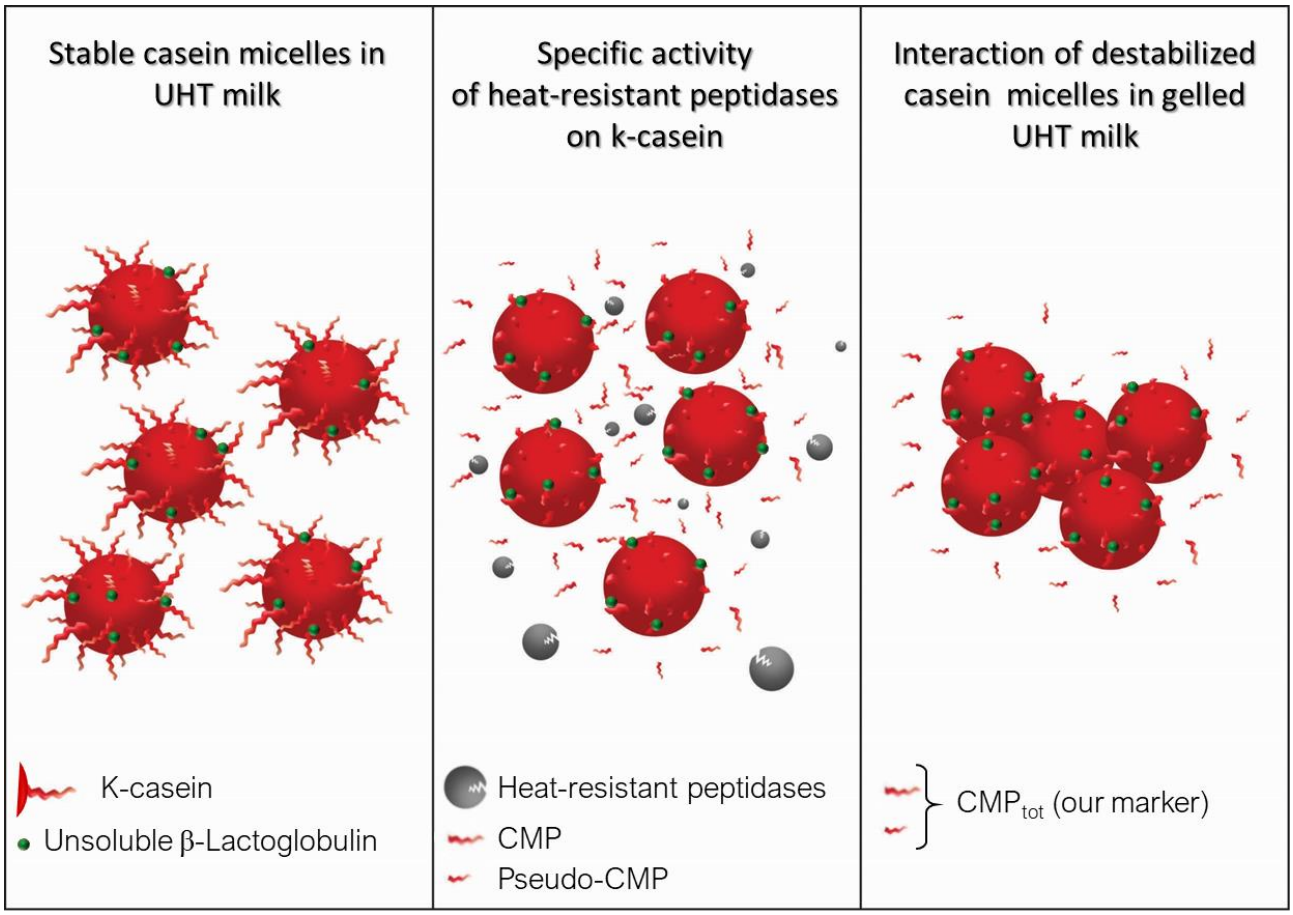


668

669 **Fig. 3.** CZE patterns of (a) peak 1, (b) peak 2, (c) peaks 3 and 4 collected from HPLC in Fig.1 and
670 pH 4.6 soluble fraction of commercial samples of (d) gelled UHT milk and (e) regular UHT milk.
671 Tryptophan (Trp) was used as internal standard.



672

673 **Fig. 4**. The "rennet-like" milk gelation mechanism. According to the proposed mechanism, UHT
674 milk gelation is due to specific k-casein proteolysis by extracellular heat-resistant peptidases
675 produced by psychotropic bacteria. The k-casein cleavage causes release of the soluble peptides
676 CMP and pseudo-CMP in the water phase of milk while the no-longer stable micelles aggregate.

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678

679 **Fig. S1.** Milk inoculated with *Pseudomonas fluorescens* LPF3 showing the typical instability signs.

680 Tubes were turned upside down just before taking the picture.

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682