Johannes Annetta Hogenboom[1*], Veronica Rosi[1], Lucia Monti[2]

Effect of processing and storage conditions on the evolution of the proteose peptone content in pasteurized and extended shelf- life milk

Effetto delle condizioni di produzione e di conservazione sull'accumulo di proteoso-peptoni in latte pastorizzato e latte ESL

[1] Department of Food, Environmental and Nutritional Sciences, State University of Milan, Via Celoria 2, 20133 Milan, Italy
[2] CREA - Centro di Ricerca Zootecnia e Acquacoltura,Via Antonio Lombardo 11, 26900 Lodi, Italy

***Corresponding author:**
Johannes Annetta Hogenboom
Department of Food, Environmental and Nutritional Sciences, State University of Milan, Via Celoria 2, 20133 Milan, Italy
Tel.: +39 02 5031 6663, E-mail: john.hogenboom@unimi.it

Received: Jul 29, 2020
Accepted: Nov 09, 2020
DOI: 10.36138/STLC.06.2020.01

Abstract

Accumulation of proteose peptones (PP) deriving from β-casein degradation by plasmin was studied during storage of pasteurized and extended shelf-life (ESL) milk, with the aim of identifying the optimum conditions to preserve milk freshness. Two lots of pasteurized milk were obtained from the same raw milk, heat treated 3h or 48h after milking and stored at 6 and 8°C. Initial PP content was higher in samples pasteurized after 48h and the difference remained during storage. Plasmin activity persisted until the end of observations and was more intense in samples stored at 8°C. After 12 days, in all but one of the samples stored at 8°C, PP exceeded 900 mg/L, suggested threshold limit for pasteurized milk, never reached in samples stored at 6°C. Two lots of ESL milk were produced as well and stored at 8°C: PP content did not increase during storage. Besides the storage at 6°C rather than at 8°C, an early heat treatment is a crucial condition to maintain pasteurized milk freshness.

Keywords:

- Pasteurized milk
- Proteose peptones
- Plasmin
- Milk freshness
- Storage

Riassunto

L'accumulo di proteoso peptoni (PP), prodotti di degradazione della β-caseina da parte della plasmina, è stato studiato per identificare le condizioni ottimali per preservare la freschezza del latte. Due lotti di latte crudo sono stati pastorizzati dopo 3 e 48 ore dalla mungitura e conservati a 6 e 8°C. L'attività della plasmina si è mantenuta in tutti i campioni, ma il contenuto in PP è risultato più alto nei campioni trattati dopo 48 ore e in quelli conservati a 8°C. Dopo 12 giorni, in tutti i campioni tenuti a 8°C, eccetto in uno, i PP hanno superato i 900 mg/l, considerato il valore soglia per valutare la freschezza del latte, mai raggiunto nei campioni conservati a 6°C. In aggiunta, dallo stesso latte crudo sono stati prodotti due lotti di latte ELS, stoccati a 8°C: il contenuto in PP non è aumentato durante la conservazione. Quindi, oltre alla conservazione a 6°C piuttosto che a 8°C, un trattamento termico precoce si dimostra determinante per mantenere la freschezza del latte pastorizzato.

Parole chiave:

- Latte pastorizzato
- Proteoso peptoni
- Plasmina
- Freschezza del latte
- Conservazione

INTRODUCTION

Due to increasing international exchanges of raw and finished food products, the possibility of evaluating freshness of milk has become an important issue, especially for countries like Italy which heavily depend on import for their milk supply (1). Milk is considered "fresh" as long as it retains its original composition and organoleptic characteristics, compatibly with the heat-treatment it received. During storage, pasteurized milk undergoes a series of reactions indicated as "milk ageing", due to the presence of several heat-resistant enzymes of different origin (endogenous, microbial, somatic cells), mainly lipases and proteases (2, 3).

The most important protease found in pasteurized milk is plasmin, an endogenous enzyme, part of a bovine blood enzyme system which also comprises plasminogen (its inactive precursor), a plasminogen activator and inhibitors of plasmin and of plasminogen activator (4). These enzymes may filtrate into the milk through the mammary gland, in higher quantity in case of udder infections, like mastitis, due to a major permeability of the mammary epithelium (5, 6).

Plasmin is particularly heat-resistant (7) and its activity in milk has been shown to increase after pasteurization (8), due to the inactivation of plasmin inhibitors or because of a partial denaturation of the casein micelle structure caused by heat-treatment. In milk, plasmin is associated with casein micelles which constitute its substrates, especially αs- and β-casein. In particular, the latter casein fraction is degraded into to γ-caseins and proteose-peptones (PP) (9, 10). During a study on pasteurized milk characteristics, coordinated by the Italian Ministry of Health and the Ministry of Agriculture and Forestry, PP resulted the most suitable parameter for the evaluation of milk ageing and an early marker for proteolysis (11): organoleptic analyses suggested an upper threshold value of 900 mg/L for PP, as milk having much higher levels presented sensorial defects.

In the present work the accumulation of PP in pasteurized milk was studied, to verify which combination of processing and storage conditions are best suited to preserve its freshness. Furthermore, a study was carried out on PP accumulation in ESL milk in order to check whether this parameter is suitable as a maker of freshness for this type of milk, too.

MATERIALS AND METHODS

Milk samples

A total of 39 milk samples were analyzed, including: 4 raw, 24 pasteurized [fresh pasteurized, according to Italian legislation (12)] and 11 extended shelf-life (ESL) milk samples.

Pasteurized milk samples:

- First trial: after milking, raw milk was kept at 6°C for 3 (C1) and 48h (C2), before being pasteurized at 75°C for 15 s and homogenized at 180 bar. Pasteurized milk P1 and P2 were both stored at 6 and 8°C and sampled before and after heat treatment, and at 5, 7 and 12 days of storage.
- Second trial: The procedure was repeated on raw milk C3 and C4, obtaining pasteurized milk P3 (treated 3h after milking and sampled at 0, 3, 6, 9 and 13 days) and P4 (treated 48h after milking and sampled at 0, 4, 7, 11 and 14 days). All samples were stored at 8°C.

Extended shelf-life (ESL) milk:

Two lots of ESL milk were produced from the same raw milk used for producing P3 and P4, with a treatment at 120°C for 4 seconds and homogenization at 180 bar. Samples E1 (treated 3h after milking and sampled at 0, 3, 6, 9, 13 and 16 days) and E2 (treated 48h after milking and sampled at 0, 4, 7, 11 and 14 days) were obtained and stored at 8°C.

RP-HPLC determination of proteose peptones

RP-HPLC was performed on the whey obtained by acidification of milk to pH 4.6, according to De Noni et al. (2007) (11). Analyses were carried out using an Alliance 2695 pump system (Waters, Milford, MA, USA) combined with a 996-diode array detector (Waters) and with a PLRP-S column (4.6mm i.d. x 150 mm, 5 µm 300Å (Polymer Laboratories, Shropshire, UK) kept at 40°C. In the chromatographic conditions applied, PP elute as a series of 4-5 peaks between 10 and 13 min, whereas small peptides (SP) elute between 4 and 10 min and α-lactalbumin (α-LA) elutes as a well separated single peak at 19 min **(Fig. 1)**. As no commercial PP standard is available, and considering the complexity of an in-house preparation, pure α-LA (Sigma, St. Louis, MO, USA) was used as an external standard for quantification of PP.

Capillary zone electrophoresis (CZE) determination of proteose peptones

CZE according to D'Incecco et al. (2018) (13) was applied to the same acid milk whey samples to check PP trend. A Beckman P/ACE system MDQ equipment (Beckman Coulter, Fullerton, CA) with UV detection at 214 nm was adopted. A coated capillary column (DB-WAX, J & W Agilent Technologies Inc., Santa Clara, CA) was cut to 500 mm effective length and low-flame window burning was used to remove the polyimide coating. The slit opening was set at 100 × 800 µm. In the electropherogram **(Fig. 2)** α-LA and β-lactoglobulin mi-

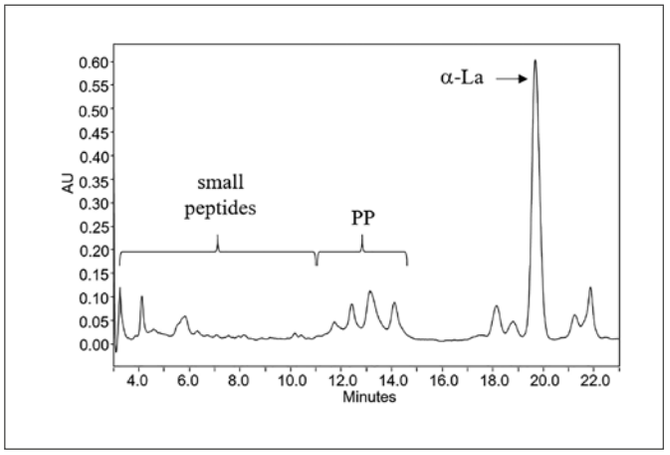

Figure 1. RP-HPLC profile of acid whey from a pasteurized milk sample after 12 days storage at 6°C.

Figura 1 Tracciato RP-HPLC di un siero acido ottenuto da un latte pastorizzato dopo 12 giorni di conservazione a 6oC.

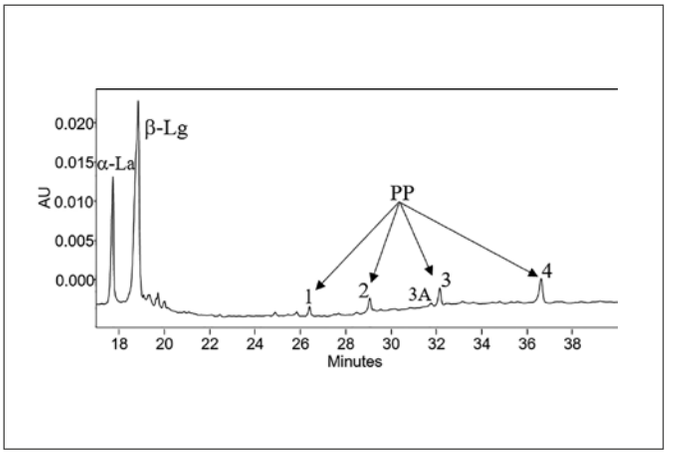

Figure 2. CZE electropherogram of acid whey from a pasteurized milk sample at the end of shelf life.

Figura 2. Elettroferogramma di un siero acido ottenuto da un latte pastorizzato al termine della shelf life.

grate at 17 and 18 min respectively, while PP separate into 4 peaks between 25 and 35 min. PP content was expressed as percentage ratio between sum of PP areas and α-LA area.

RESULTS

HPLC analysis was performed on all samples to follow PP release in raw and treated milk. During the first trial **(Fig. 3)**, PP content (expressed as α-LA) of raw milk C1, heat treated 3h after milking, decreased during processing from 334 mg/L to 296 mg/L (P1). Successively, in the samples stored at 8°C, PP content steadily increased, reaching a value of 601 mg/L after 7 days of storage and 877 mg/L after 12 days, thrice the content observed in the freshly pasteurized sample. In those stored at 6°C, PP content reached 459 mg/L after 7 days, whereas the final PP content was equal to 596 mg/L, twice the initial value. Raw milk C2, prior to pasteurization 48h after milking, presented a PP content of 434 mg/L which dropped down to 362 mg/L during heat treatment. During storage PP incremented up to 885 mg/L after 7 days and up to 1153 mg/L after 12 days at 8°C; in the samples kept at 6°C the observed values were 664 mg/L and 856 mg/L respectively. Also, in this case, final amounts approximately equaled respectively three and two times the initial value.

During the second trial **(Fig. 4A)**, PP content of raw milk C3, heat treated 3h after milking, decreased during processing from 324 mg/L to 285 mg/L (P3) then raised up to 878 mg/L after 6 days and up to 1482 mg/L after 13 days of storage at 8°C, over five times the value

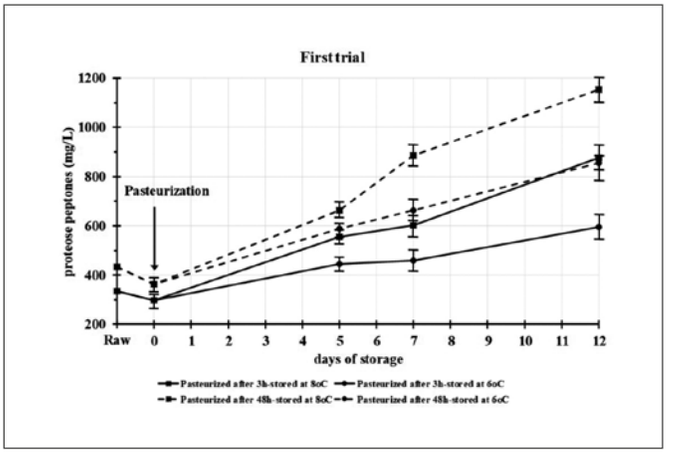

Figure 3. First trial: evolution of proteose peptone content in milk samples (P1, P2) pasteurized 3h or 48h after milking, and stored at 8°C or 6°C. Data obtained by RP-HPLC.

Figura 3. Prima campionatura: accumulo di proteoso-peptoni nei campioni di latte (P1, P2) pastorizzati 3 ore o 48 ore dopo la mungitura, e conservati a 6oC o 8oC. Dati ottenuti con RP-HPLC.

of the freshly processed milk. In raw milk C4, PP content decreased during processing 48h after milking, from 432 to 425 mg/L, a minimum variation. During successive storage at 8°C PP incremented up to 928 mg/L at 7 days storage and was triplicated up to 1318 mg/l (P4) after 14 days. CZE data confirmed these results: percentage ratio between PP areas and α-LA showed the same trend as HPLC data for both productions, stored 3h and 48h after milking **(Fig 4B)**. In the ESL samples, produced during the second trial from raw milk

DOI: 10.36138/STLC.06.2020.01

samples C3 and C4 **(Fig. 4A)**, PP contents determined immediately after the heat treatment in E1 and E2 were 147 and 290 mg/L, respectively. During successive storage at 8°C these values remained substantially constant, reaching 211 mg/L in E1 after 16 days, and 283 mg/L in E2 after 14 days.

DISCUSSION

Raw milk sample C2, pasteurized after 48h at 6°C, showed a higher initial PP content (434 mg/L) than C1 milk (334 mg/L), heat treated after 3h. The observed increase is most probably due to proteolytic activity by the microbial population during the period elapsed between milking and pasteurization. In both samples PP decreased after heat treatment, while during the subsequent storage period their content in P1 and P2 increased until the end of the observation period, at a higher rate in the samples stored at 8°C than in those kept at 6°C. This proves that both storage time and temperature affect plasmin activity. Also, the accumulation of PP was more rapid in the samples pasteurized after 48h (P2) than in those pasteurized after 3h (P1) and stored at the same temperature. This acceleration is probably caused by heat resistant microbial proteases accumulated in milk during the time interval between milking and pasteurization, and that remain active after pasteurization.

Results on pasteurized samples of the second trial partially confirmed the previous observations. As for trial 1, the PP content immediately before heat treatment was major in raw milk C4 pasteurized after 48h (432 mg/L) than in C3 pasteurized after 3h (324 mg/L). Also in this case, PP dropped during heat treatment and increased constantly during storage at 8°C. Curiously, however, during the first 3 days of storage, the accumulation of PP was significantly more rapid in samples P3 than in samples P4. As a result, after 4 days storage the contents of PP determined in the milk samples pasteurized 3h after milking exceeded those found in samples pasteurized after 48h, and this gap remained until the end of the observations. The very rapid increase of PP in sample P3 after 3 days is probably due to storage conditions (involuntarily) not corresponding to the protocol during this period; in fact, small differences in temperature may cause significant variations in PP accumulation.

Apart from sample P1, after 12 days the PP content of all pasteurized samples stored at 8°C exceeded the value of 900 mg/L, suggested upper threshold limit for milk to be considered as fresh. On the contrary, PP content of samples stored at 6°C remained well below this limit, even if pasteurized after 48h. This finding was recently confirmed in a study (14) where PP content reached the mentioned threshold level in pasteurized milk after 13 days of storage at 5-6°C.

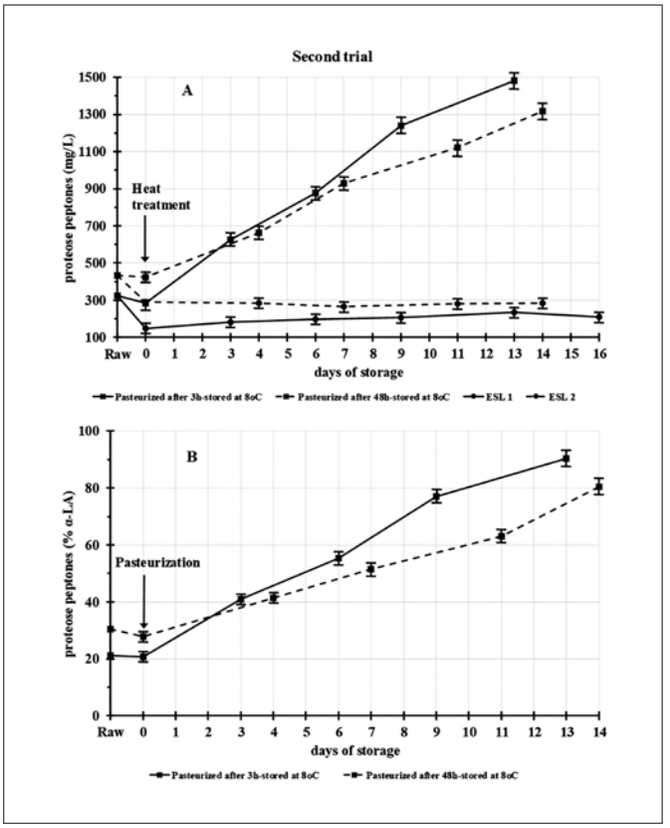

Figure 4. Second trial: evolution of proteose peptone content in pasteurized (P3, P4) (HPLC and CZE data) and ESL (E1, E2) milk samples. Samples were heat treated 3h or 48h after milking and stored at 8°C. Data obtained by RP-HPLC (A) and by CZE (B).

Figura 4. Seconda campionatura: accumulo di proteoso-peptoni nei campioni di latte pastorizzato (P3, P4) (dati HPLC e CZE) e ESL (E1, E2). I campioni sono stati sottoposti a trattamento termico 3 ore o 48 ore dopo la mungitura, e conservati a 8oC. Dati ottenuti con RP-HPLC (A) e con CZE (B).

In ESL milk, the evolution of PP during storage was very different from what observed for pasteurized milk. In fact, during the heat treatment PP content decreased by 55% (E1) and 33% (E2) whereas during pasteurization the loss was lower than 20%. Furthermore, only limited variation of PP occurred during storage, most probably due to thermal inactivation of plasmin. In fact, as no increase of small peptides (SP) was observed in ESL milk chromatograms (not shown), PP formation and successive degradation into SP by heat-resistant microbial proteases was excluded.

In all samples PP decreased during the heat treatment, proportionally to the severity of the treatment. It is not clear whether the observed reduction was due to thermal denaturation of PP, although generally classified as heat-resistant molecules, or to their interaction with casein upon heating (15, 16).

DOI: 10.36138/STLC.06.2020.01

CONCLUSIONS

Plasmin appears to be active throughout storage of pasteurized milk, thus altering its compositional characteristics and shortening freshness of this product. The formation of PP through β-casein degradation proved an adequate measure of milk ageing. This research has demonstrated that plasmin activity is affected by the time interval elapsed between milking and application of the heat treatment and by storage time and temperature. PP increase of about 100 mg/l if pasteurization is performed 2 days after milking instead of 3h. Also, PP content raises with lengthening of storage and accelerates with increasing storage temperatures. According to our data the optimum conditions to preserve freshness of pasteurized milk are a minimum time lapse between milking and pasteurization and storage at 6°C rather than 8°C.

On the contrary, PP are not suitable as a marker of milk ageing for ESL milk. In this product plasmin appears to be completely inactivated and no significant variation of PP content is observed.

CONFLICT OF INTEREST DISCLOSURE

All authors declare that they have no conflicts of interest inherent the present paper.

REFERENCES

1. Ferraro LA. I numeri della filiera del latte. Sci Tecn Latt-Cas. 2020; 70 (1): 68-71.

2. Barbano DM, Ma Y, Santos MV. Influence of Raw Milk Quality on Fluid Milk Shelf Life. J. Dairy Sci. 2006; 89(E. Suppl.): E15-E19.

3. Meunier-Goddik L, Sandra S. Liquid milk products: Pasteurized milk. In: Encyclopedia of Dairy sciences. Elsevier Science Publishing Co Inc, San Diego, US. 2011; 274-280.

4. Ismail B, Nielsen SS. *Invited review*: Plasmin protease in milk: Current knowledge and relevance to dairy industry. J. Dairy Sci. 2010; 93(11): 4999-5009.

5. Saeman AI, Verdi RJ, Galton DM, Barbano DM Effect of mastitis on proteolytic activity in bovine milk. J Dairy Sci. 1988; 71(2): 505-512.

6. Hogenboom, JA, Pellegrino L, Sandrucci A, Rosi V, D'Incecco P. Invited review: Hygienic quality, composition, and technological performance of raw milk obtained by robotic milking of cows. J. Dairy Sci. 2019; 102(9): 7640-7654.

7. Metwalli AAM, De Jongh HHJ, Van Boekel MAJS. Heat inactivation of bovine plasmin. Int. Dairy J. 1998; 8(1): 47-56.

8. Lu R, Stevenson CD, Guck SE, Pillsbury LA, Ismail B, Hayes KD. Effect of various heat treatments on plasminogen activation in bovine milk during refrigerated storage. Int. J. Food Sci. Tech. 2009; 44 (4): 681-687.

9. Andrews AT, Alichanidis E. Proteolysis of caseins and the proteose-peptone fraction of bovine milk. J. Dairy Res. 1983; 50(3): 275-290.

10. Bastian D, Brown RJ. Plasmin in milk and dairy products: an update. Int. Dairy J. 1996; 6(5): 435-457.

11. De Noni I, Pellegrino L, Cattaneo S, Resmini P. HPLC of proteose peptones for evaluating ageing of packaged pasteurized milk. Int Dairy J. 2007; 17(1): 12-19.

12. Italian Law 169, of May, 3, 1989, "Discipline on treatment and commercialization of bovine milk".

13. D'Incecco P, Rosi V, Cabassi G, Hogenboom JA, Pellegrino L. Microfiltration and ultra-high- pressure homogenization for extending the shelf-storage stability of UHT milk. Food Res. Int. 2018; 107, 477-485.

14. Limbo S, Pellegrino L, D'incecco P, Gobbi S, Rosi V, Fracassetti D. Storage of pasteurized milk in clear PET bottles combined with light exposure on a retail display case: a possible strategy to define the shelf life and support a recyclable packaging. Food Chem. 2020; 329, 127116.

15. Innocente, N., Biasutti, M., & Blecker, C. HPLC profile and dynamic surface properties of the proteose–peptone fraction from bovine milk and from whey protein concentrate. Int. Dairy J. 2011; 21(4), 222-228.

16. O'Mahony JA, Fox PF. Milk Proteins: Introduction and Historical Aspects. In PLH McSweeney & PF Fox (Eds.), Advanced dairy chemistry (Proteins: Basic aspects 4th ed., Vol. 1A). New York, NY: Springer. 2013; 43-85.

DOI: 10.36138/STLC.06.2020.01