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The quality of low lactose milk is affected by the side proteolytic activity of the lactase used in the production process

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ABSTRACT

Lactose intolerance syndrome can be efficiently tackled consuming low lactose products. Lactase is the key tool to manufacture low lactose milk (LLM): its addition during milk processing can be done "in batch", i.e. before thermal treatment, or directly "in pack" after sterilization. In this paper data on sensory properties, Maillard Reaction products (MRPs) and free amino acids formation were obtained on six commercial Italian LLMs over six months storage. They showed that the side proteolytic activity of lactase caused the release of amino acids with a significant higher MRPs and off-flavors formation in four out of five samples produced by adding the enzyme in the pack after thermal treatment. We concluded that the in pack addition of lactase after milk sterilization can have negative sensorial and nutritional consequences mainly related to the enzyme side proteolytic activity especially for prolonged storage time.

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1. Introduction

Lactose (*O*-β-D-galactopyranosyl-(1 → 4)-D-glucopyranose) is present at high concentration (4–7%) in mammals milk. The presence of disaccharides in the newborn foods is due to the need to reduce the osmotic effect, allowing more carbohydrate to be included in milk (Belitz, Grosch, & Schieberle, 2009). Lactose requires a specific enzyme to be metabolized: the β-1,4-galactosidase (lactase-phlorizin hydrolase or lactase). The enzyme lactase is anchored to the surface of the "brush border" of the human epithelial cells and it splits lactose in two monosaccharides, galactose and glucose, which are absorbed in the small intestine (Fox & McSweeney, 1998). Lactase is always present in the newborn, but its activity naturally decreases after weaning, with the only exceptions of infants suffering from congenital hypolactasia, an extremely rare condition (Heyman, 2006). In Caucasians a specific mutation, favored by the high consumption of milk in livestock-based diet, allowed the persistence of lactase also in adults. However, these genetic modifications are not common in other geographical area and around 70% of the world population has non-persistence lactase, in particular in some Asian countries this rate increases up to 100% (Swallow, 2003). In particular, in northern, in central and southern Italy 49%, 82% and 46% of the population have lactase persistence, while in Sardinian this value drops to 15% (Itan, Jones, Ingram, Swallow, & Thomas, 2010).

As several nutritional and genetic factors influence tolerance, not all lactose intolerances result in clinical symptoms, and lactose deficiency remains undiagnosed (Lomer, Parkes, & Sanderson, 2008). Undigested lactose is fermented in the gut with production of short chain fatty acid, methane, carbon dioxide and hydrogen that increase the abdominal pressure and intestinal transit (He et al., 2006). Lactose-intolerant people avoid milk and dairy products with high concentration of lactose to control symptoms, however lactose-free products and low lactose milks (LLMs) represent a simple solution to keep the nutrients intake related to milk and dairy products consumption eliminating intestinal discomfort.

The availability of commercial lactase preparations favors the development of different technological approaches to manufacture LLM. The factors supervising the enzymatic hydrolysis are: the lactose concentration of the products, the pH, the presence of some activators (mono and bivalent ions), the time and the temperature of the enzyme incubation. Commercially available lactases have an optimal temperature range between 35 °C and 65 °C (Mahoney, 1997) and the growth of undesired bacteria during lactase incubation must be avoided. The resulting LLMs are characterized by an increase of the cryoscopic value from 0.454 to 0.650 °C and an intense sweet taste since the sweetness of lactose is significantly lower than that of glucose and galactose. Some manufacturers adopted a combined process of micro and ultrafiltration in order to reduce lactose concentration to 1,6% before the lactose hydrolysis and avoid the sweetness (Dunker, McCloskey, & Gomez, 2007; Rehman, Farkye, Considine, Schaffner, & Drake, 2003).

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The simplest method to produce LLM milk is the "in batch" addition of lactase before the UHT treatment step: lactose hydrolysis can be done by single-use batch system; recovery systems (enzyme re-use) or using immobilized enzymes (Mahoney, 1997).

Recently, thanks to the use of aseptic packaging technology, the sterile addition of the lactase in each milk box becomes possible (in pack addition process). This process reduces the operational costs as it requires a lower quantity of the enzyme and it saves the holding time necessary for the batch hydrolysis. Moreover it allows to reach lower lactose concentrations (Harju, Kallioinen, & Tossavainen, 2012). In Fig. 1, a typical flow chart of the LLMs production is reported: both processes share common features, but the main difference relies on the step of lactase addition: hereinafter the "in pack" addition will be indicated as process A and the "in batch" addition as process B. It is important to bear in mind that LLM obtained by process A contained active lactase during the shelf life, as the enzyme is added after sterilization while LLM obtained by process B have no lactase activity because the enzyme is inactivated during the sterilization. Consequently, in milk obtained by process A the lactose hydrolysis continued during the shelf life while in samples obtained by process B the hydrolysis is interrupted at the level reached at the end of the batch incubation before sterilization.

The presence of the active lactase in the final pack could have some drawbacks as the high concentration of glucose and galactose renders the product less stable compared with conventional UHT milk. In fact, it is well known that commercial lactases have enzymatic side activities that could generate other reactive intermediates (Evangelisti, Calcagno, Nardi, & Zunin, 1999; Tossavainen & Kallioinen, 2007). In particular, commercial lactase preparations could have both arylsulfatase activity, that catalyzes the degradation of milk alkyl phenols generating off flavor volatiles, (Dekker, Edens, De Swaaf, & Van Dijk, 2015; Stressler, Leisibach, Lutz-Wahl, Kuhn, & Fischer, 2016) and also proteolytic activity that releases peptides and free amino acids generating nonenzymic browning and off flavors during the shelf life.

Two recent papers by a Danish group deeply investigated the effects of the addition of lactase after the thermal treatment and compared the chemical profile of the LLM to conventional UHT milk over nine months storage (Jansson, Clausen, et al., 2014; Jansson, Jensen, et al., 2014). Results showed that LLMs are characterized by an higher concentration of free amino acids and furosine, higher reaction rates for volatiles formation and in general more favorable conditions for the Maillard reaction (MR) than conventional UHT milk. Moreover, these papers highlighted that β-casein and αs1-casein were significantly hydrolyzed after approximately 90 days of storage in the LLM.

In this framework, it is possible that the LLMs obtained with the two technologies have different chemical composition and sensorial profiles due to the presence of higher concentration of free amino acids (Van Boekel, 1998; Nursten, 2005). To verify these hypotheses a survey of six LLMs present on the Italian market was performed and the influence of the different LLM production technologies on the residual lactase activity, hence on quality during the shelf life was investigated. Reactants (free amino acids, total lysine and lactose), initial stage products (free Amadori products and furosine), intermediate products (CML and CEL) and off-flavor volatiles were monitored during six months of storage. Results of chemical analysis were combined with sensory analysis to confirm the link between the formation of MRPs and the presence of sensory defects as a consequence of higher concentration of amino acids.

2. Materials and methods

2.1. Chemicals

Acetonitrile, methanol and water for liquid chromatography–high resolution mass spectrometry (LC-HRMS) and liquid chromatography–tandem mass spectrometry (LC-MS/MS) were obtained from Merck (Darmstadt, Germany). The ion-pairing agent perfluoropentanoic acid (NFPA), the amino acids standards, acetic acid, hydrochloric acid (37%), the analytical standard [4,4,5,5-*d4*]-L-lysine hydrochloride (*d4*-lysine), D(+)-lactose monohydrate and D(+)-melezitose were purchased from Sigma-Aldrich (Saint-Louis, MO). *N*-(1-deoxy-D-fructos-1-yl)-L-phenylalanine, *N*-(1-deoxy-D-fructos-1-yl)-L-leucine, *N*ε-(carboxyethyl)-L-lysine and its internal standard *N*ε-(carboxy[$^2H_4$]ethyl)-L-lysine (*d4*-CEL) were obtained from Toronto Research Chemicals (Toronto, Canada). Analytical standards *N*ε-(2-furoylmethyl)-L-lysine (furosine), *N*ε-(carboxymethyl)-L-lysine (CML) and their respective deuterated standard *N*ε-(carboxy[$^2H_2$]methyl)-L-lysine (*d2*-CML) and *N*ε-(2-furoyl)-methyl-L-[4,4,5,5-[$^2H_4$]lysine HCl salt (*d4*-furosine) were obtained from Polypeptide laboratories (Strasbourg, France). *N*-(1-deoxy-D-fructos-1-yl)-L-asparagine, *N*-(1-deoxy-

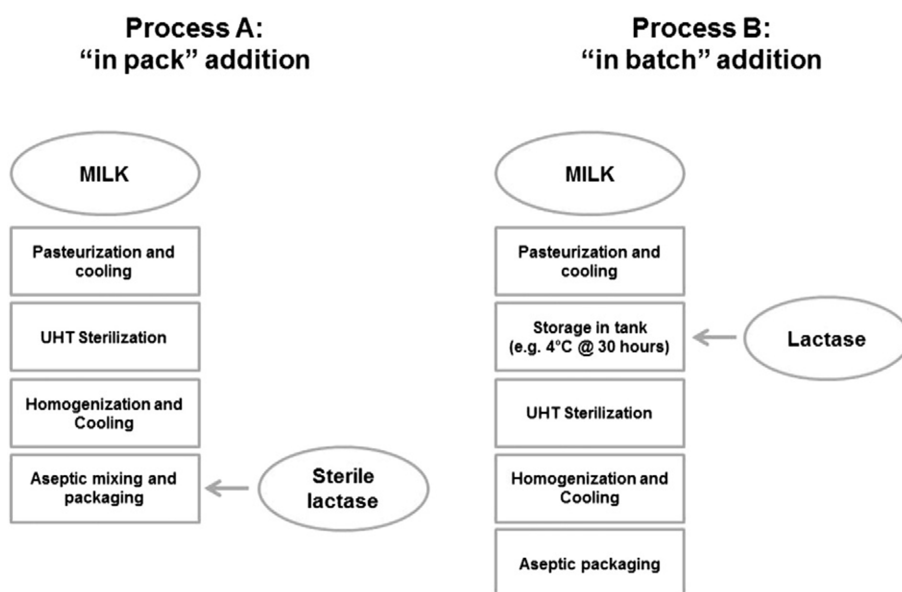


Fig. 1. Sketch of the two technological processes used for the production of low lactose milk (LLMs). Process A: addition of the enzyme in the pack after the thermal treatment; Process B: addition in batch of the lactase before the thermal treatment. Using Process A the enzyme is active during the shelf life, while using process B the lactase in not active during the shelf life as revealed by lactose addition.

Table 1
Analysis at each point of the sampling. The sensory analysis was performed at the beginning of the storage time and after three months, at the best before consumption date. APs (free Amadori products), MRPs (bound Maillard reaction end products).

Days of storage	Free amino acids	APs	MRPs	Sensory analysis	Volatiles
30	X	X	X	X	X
60	X	X	X		X
90	X	X	X	X	X
120	X	X	X		X
150	X	X	X		X
180	X	X	X		X

D-fructos-1-yl)-L-aspartic acid, *N*-(1-deoxy-D-fructos-1-yl)-L-lysine, *N*-(1-deoxy-D-fructos-1-yl)-L-histidine were synthesized according to Troise, Fiore, Roviello, et al. (2015) and Troise, Fiore, Wiltafsky, et al. (2015). The calibration solution (see "Free MR markers: Amino acids and Amadori products (LC-HRMS)" section) was obtained from Thermo Fisher Scientific (Bremen, Germany).

2.2. Sampling and shelf-life

A sampling of six commercial brands of low lactose milk (LLM) having close production day were obtained from Italian supermarket in November 2014. The analysis plan is reported in Table 1. The samples were stored at room temperature (22 ± 3 °C) up to 180 days. The time zero (T0) of the shelf life study was established four weeks after the production date.

2.3. Residual lactase activity and lactose determination

Residual lactase activity in LLM was determined by adding 5% of lactose monohydrate (4.75% anhydrous lactose) to LLM and measuring residual lactose after incubation at 72 h at 6 °C. Lactose analysis was performed according to ISO Guidelines (ISO 22662:2007(IDF 198:2007), 2007). Aliquots of 3 mL of test sample were prepared into a 10 mL volumetric flask. Two milliliters of D(+)-melezitose internal standard solution and 1.2 mL of chemical reagent (Biggs-Szijarto solution) were added in order to precipitate fat and protein fractions of milk. The solution was gently mixed at room temperature and the procedure was repeated three times. The contents were filtered by paper filter, then the filtrate was purified through a 0.45 μm nylon filter. Finally 20 μL was analyzed by HPLC using a cationic exchange column Aminex HPX-87P (Bio-Rad, US) 300 × 7.8 mm and detected by a differential refractometer detector. The chromatographic conditions were as follows: the mobile phase was water, the internal detector temperature was at 35 °C, the guard column temperature was 20 °C, the column temperature was at 85 °C; the flow rate was 0.6 mL/min in isocratic mode. The run time was of 15 min, the retention time of D(+)-melezitose was 9 min and retention time of lactose was 11 min.

2.4. Free MR markers: amino acids and Amadori products (LC-HRMS)

Free amino acids and Amadori products (APs) were monitored according to Troise, Fiore, Roviello, et al. (2015) and Troise, Fiore, Wiltafsky, et al. (2015) with minor modifications. For the chromatographic separation of APs from their respective amino acids, the mobile phases consisted of 5 mM NFPA in water (solvent A) and 5 mM NFPA in acetonitrile (solvent B) by using the same gradient as previously described. The flow rate was set to 200 μL/min and the injection volume was 5 μL. The separation of APs was achieved through a thermostated (30 °C) Kinetex 2.6 μm core-shell C-18 (100 × 2.1 mm, Phenomenex, Torrance, CA). The Accela 1250 U-HPLC system (Thermo Fisher Scientific, Bremen, Germany) was directly interfaced to an Exactive Orbitrap high-resolution mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The analytes were detected through a heated electrospray interface (HESI) operating in the positive mode and scanning the ions in the *m*/*z* range 60–400; the resolving power was set to 50,000 full width at half maximum (FWHM, *m*/*z* 200) resulting in a scan time of 1 s. All the parameters were optimized according to the procedure previously described. Before interday analysis the instrument was externally calibrated by infusion of a solution that consisted of caffeine, Met-Arg-Phe-Ala (MRFA), fluorinated phosphazines Ultramark 1621, and acetic acid in a mixture of acetonitrile/methanol/water (2:1:1, v/v/v) (Thermo Fisher, Bremen, Germany). The mass range tolerance was set to 5 ppm and the exact mass of diisooctyl phthalate ($[M + H]^+$: 391.28429) was used as lock mass for the recalibration of the instrument during the analysis.

2.5. Bound MR markers: CML, CEL, furosine and total lysine (LC-MS/MS)

Lysine and its derivatives furosine, CML, CEL along with their respective internal standards were analyzed considering previous papers and introducing some modifications (Delatour et al., 2009; Troise, Fiore, Wiltafsky, & Fogliano, 2015). Briefly, 100 μL of milk was accurately mixed with 4 mL of hydrochloric acid (6 M). The mixture was saturated by nitrogen (15 min at 2 bar) and hydrolyzed in an air forced circulating oven (Memmert, Schwabach, Germany) for 20 h at 110 °C. The mixture was filtrated by polyvinylidene fluoride filters (PVDF, 0.22 Millipore, Billerica, MA) and 200 μL was dried under nitrogen flow. The samples were reconstituted in 190 μL of water and 10 μL of internal standard mix (*d4*-lysine, *d4*-furosine, *d2*-CML and *d4*-CEL) was added in order to obtain a final concentration of 200 ng/mg of samples for each internal standard. Samples were loaded onto equilibrated Oasis HLB 30 mg cartridges (Waters, Wexford, Ireland) and eluted according to the method previously described. Finally, 5 μL was injected onto the LC-MS/MS system. Separation of furosine, CML, CEL, lysine and their internal standards was achieved on a reversed-phase core shell column (Kinetex C18, 2.6 μm, 2.1 × 100 mm, Phenomenex, Torrance, CA) using the following mobile phases: A, 5 mM NFPA and B, acetonitrile 5 mM NFPA. The compounds were eluted at 200 μL/min through the following gradient of solvent B (t in [min]/[%B]): (0/10), (2/10), (5/70), (7/70), (9/90), (10/90). Positive electrospray ionization was used for detection and the source parameters were selected as follows: spray voltage 5.0 kV; capillary temperature 350 °C, dwell time 100 ms, cad gas and curtain gas were set to 45 and 5 (arbitrary units). The chromatographic profile was recorded in MRM mode and the characteristic transitions were monitored in order to improve selectivity using an API 3000 triple quadrupole (ABSciex, Carlsbad, CA). The tandem mass spectrometry set up was optimized according to our previous paper (Troise, Fiore, Wiltafsky, & Fogliano, 2015).

2.6. Volatile compounds (GC–MS)

Frozen low lactose milk samples were thawed and incubated at 60 °C for 1 min. Afterward, volatile compounds in the headspace were extracted at 60 °C for 5 min with a 75 μm Carboxen–PDMS-SPME fiber (Supelco, Bellefonte, PA) using TriPlus autosampler (Thermo Scientific, Bremen, Germany). Chromatography grade water was analyzed as

Table 2
Concentration of lactose after the addition of 5% of lactose monohydrate (4.75% of anhydrous lactose) and incubation at 6 °C for 72 h. In samples LL1 to LLM5, lactase was still active (Process A), while enzyme was inactivated in samples LLM6 (process B).

Products	Lactose (g/100 g)	Process type
LLM1	1.40 ± 0.25	A
LLM2	1.71 ± 0.32	A
LLM3	1.42 ± 0.09	A
LLM4	1.68 ± 0.21	A
LLM5	1.23 ± 0.15	A
LLM6	4.77 ± 0.42	B

blank sample. The SPME fiber was desorbed for 10 min in the GC injection port. The GC–MS analysis was performed using Trace GC Ultra connected with DSQ II mass spectrometer (Thermo Scientific, Bremen, Germany). The Stabilwax-DA-Crossband-Carbowax-polyethylene-glycol column with 30 m length, 0.32 mm internal diameter, and 1 μm film thickness (Restek, Bellefonte, PA) was used. The oven temperature was maintained at 40 °C for 3 min, then increased at 15 °C/min to 220 °C and maintained for 1 min. The carrier gas was helium fed with a constant flow rate at 1.5 mL/min. The MS ion source was maintained at 225 °C with full scan. Electron impact mode was at 70 eV with the mass range 33–250 *m*/*z*. This procedure was modified based on Hettinga, van Valenberg, Lam, and van Hooijdonk (2008). Volatile metabolites were identified using AMDIS software (NIST, Gaithersburg, MD, USA) referred to NIST/EPA/NIH database and library provided by Hettinga et al. (2008). Peaks from column bleed and SPME fiber were corrected using the blank sample. Specific retention time and *m*/*z* model were used for automated peak integration in Xcalibur software package (Thermo Scientific, Bremen, Germany).

2.7. Sensory analysis

Sensory analysis for the evaluation of LLM samples throughout product shelf life were developed by 15 panelists (staff from the Parmalat R&D Center in Collecchio, Italy) using QDA methodology according to ISO Guidelines (ISO 13299:2003) (2003); (Chapman, Rosenberry, Bandler, & Boor, 1998; Phillips, Mcgiff, Barbano, & Lawless, 1995). Panelist training and evaluation sessions were accomplished during ten working sessions. The descriptive terms used for analysis were the followings: white intensity, aroma intensity, mouthfeel, sweet, cooked, milky, stale, irregular aftertaste. Attributes were quantified with an visual analogue scale (VAS) from 1 to 9; where 1 = attribute not detected and 9 = attribute extremely strong. After the terminology development phase, the 15 panelists were specifically trained in the evaluation of LLM. Training consisted of evaluating milk samples varying in fat content, degree of freshness, with and without lactose reduction, by use of the descriptive terms developed to describe and quantify color, aroma, flavor, and aftertaste characteristics on a scale from 1 to 9. At each testing period, containers were mixed by inversion, then, in dim light, 60 mL of sample was poured into 148 mL plastic cups with three-digit codes, capped with the a plastic lid; finally the samples was presented to the panelists. In each session, the performances were checked by using a blind reply of the previous samples. The 15 panelists performed independent observations on randomized samples of milk.

2.8. Statistical analysis

Each sample was analyzed four times from independent incubation sets and the results were reported as mg/100 g of protein for lysine, furosine, CEL and CML, while the intensity of the signals of free APs, amino acids and volatiles was reported as area counts. Evolution of the bound and free markers was recorded by using Prism (GraphPad, La Jolla, CA), the Tukey test ($\alpha = 0.05$) for bound MRPs and for the sum of APs were performed by using XLStat (Addinsoft, New York, NY). The amino acids sequence in β-casein (Accession Number: AAA30431) and α-s1-Casein (NP_851372, NCBI) were monitored by using *seqtool* in Matlab (Mathworks, Natick, MA).

3. Results and discussion

3.1. Lactose determination and lactase addition

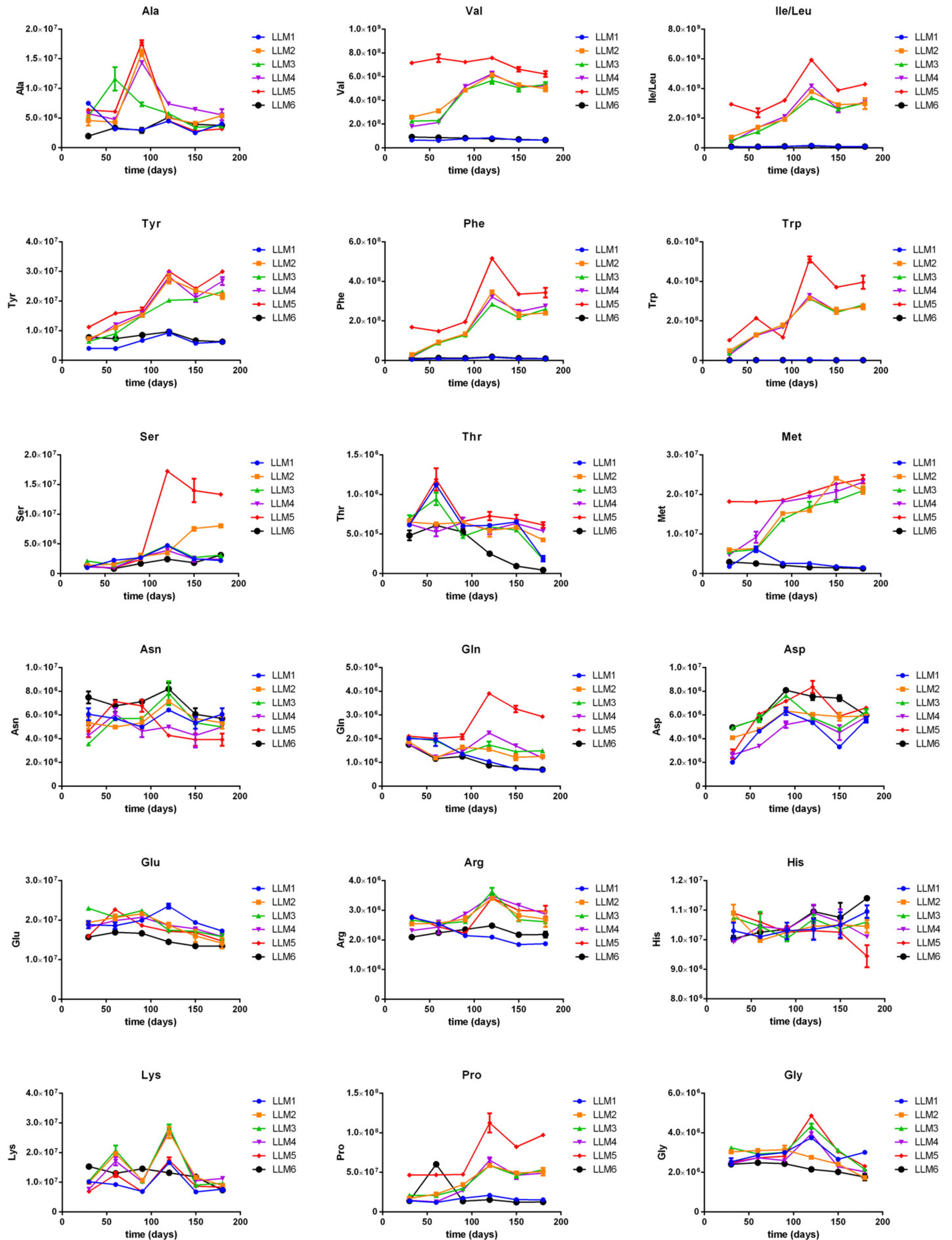
The lactase activity in LLM samples during storage was assessed by measuring the final concentration of lactose after the addition of 5% of lactose monohydrate. Results are reported in Table 2: the residual concentration of lactose ranged from 1.40 ± 0.25 to 1.71 ± 0.32 g/100 g of milk in the samples from LLM1 to LLM5, and was 4.77 ± 0.42 g/100 g of milk in sample LLM6. These results indicated that in the five samples from LLM1 to LLM5 there was a clear hydrolysis after the addition of extra lactose, therefore the enzyme was still active while the sample LLM6 showed no residual lactase activity. These findings demonstrated that samples from LLM1 to LLM5 were obtained using the process A with the enzyme aseptically added in each pack after thermal treatment; while the sample LLM6 were produced by process B with the enzyme added in batch before thermal treatment (see Fig. 1). This implies in all samples, but LLM6, the desired lactose hydrolysis continued during the storage. On the other hand also the lactase side activities, such as proteolysis, can continue.

3.2. Free amino acids and free Amadori products

Free amino acids impact in the six LLMs was monitored by LC-HRMS over 180 days of storage (Troise, Fiore, Roviello, Monti, & Fogliano, 2015). In Fig. 2 the area counts of 18 free amino acids were reported. Cys was not detectable with the method used and Leu and Ile were quantified together, since the separation of the two isomers was not efficient. Data indicated that in samples LLM2, LLM3, LLM4 and LLM5 the area counts of Val, Trp, Phe, Ile/Leu clearly increased during storage being up to ten times higher than in LLM1 and LLM6. In general, while the four samples LMM2, LLM3, LLM4 LLM5 were characterized by an increase of almost all amino acids, the opposite trend was visible in the samples LLM1 and LLM6 characterized by a decrease of many amino acids.

The release of free amino acids is likely due to the side proteolytic activity of lactase and the hydrolytic cleavage was evident for aliphatic and aromatic amino acids. These results confirmed previous evidences reported by Jansson and coworkers: the concentration of Ile increased up to 3.56 mg/L after nine months of storage as a consequence of the proteolytic activity (Jansson, Clausen, et al., 2014). Previous findings highlighted that β-casein and αs1-casein, were significantly hydrolyzed after approximately 90 days of storage in LLM. By analyzing the amino acids sequence it can be noted that the most representative amino acids were Pro, Leu, Val (15.2%, 12.1% and 9.4%) and Glu, Leu, Pro (11.7%, 10.3%, 7,9%) for β-casein and αs1-casein, respectively (Jansson, Jensen, et al., 2014). This suggested that the high levels of Ile/Leu and Pro reached after 120 days of storage is the consequence of an extensive proteolysis on β-casein and αs1-casein. Along with Ile/Leu the proteolysis resulted in the increase of Val, Phe, Trp and Tyr. All the free amino acids can play an active role in the further development of MR considering the high concentration of glucose and galactose present in LLM.

The first step of the reaction between these monosaccharides and the free amino acids is the formation of free Amadori products (APs) and their trends were reported in Fig. 3. The total APs increased during the first 120 days, then decreased during the last part of the incubation. Fru-Ser Fru-Glu, Fru-Trp, Fru-Ala, Fru-Gln, Fru-Val and Fru-Lys provided the highest signals. As expected the formation of free APs was correlated to the amount of free amino acids released by the lactase side proteolytic activity. LLM6 showed the lowest total free APs signals however also LLM1 was characterized by values of free APs generally lower than the other samples, even if some compounds, such as Fru-Lys and Fru-Pro showed levels higher than the other samples. This is the first paper using the trends of free APs as tool to monitor the quality of milk during the storage. APs followed a typical second order kinetic: they increased during the first 90 days then they decreased likely because their conversion into volatiles and other end products became the dominant phenomenon (Martins & van Boekel, 2005). Considering the relative abundance and the trends of formation, some of the APs can be investigated as potential markers of the MR development beside the conventional markers bound to proteins, as also highlighted in another recent paper from our group (Troise, Buonanno, Fiore, Monti, & Fogliano, 2016).



Ala
2.0×10^7
1.5×10^7
1.0×10^7
5.0×10^6
0
0 50 100 150 200
time (days)
LLM1
LLM2
LLM3
LLM4
LLM5
LLM6
Val
1.0×10^9
8.0×10^8
6.0×10^8
4.0×10^8
2.0×10^8
Ile/Leu
8.0×10^9
6.0×10^9
4.0×10^9
2.0×10^9
Tyr
4.0×10^7
3.0×10^7
2.0×10^7
1.0×10^7
Phe
6.0×10^8
4.0×10^8
2.0×10^8
Trp
6.0×10^8
4.0×10^8
2.0×10^8
Ser
2.0×10^7
1.5×10^7
1.0×10^7
5.0×10^6
Thr
1.5×10^6
1.0×10^6
5.0×10^5
Met
3.0×10^7
2.0×10^7
1.0×10^7
Asn
1.0×10^7
8.0×10^6
6.0×10^6
4.0×10^6
2.0×10^6
Gln
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4.0×10^6
3.0×10^6
2.0×10^6
1.0×10^6
Asp
1.0×10^7
8.0×10^6
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4.0×10^6
2.0×10^6
Glu
3.0×10^7
2.0×10^7
1.0×10^7
Arg
4.0×10^6
3.0×10^6
2.0×10^6
1.0×10^6
His
1.2×10^7
1.1×10^7
1.0×10^7
9.0×10^6
8.0×10^6
Lys
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2.0×10^6

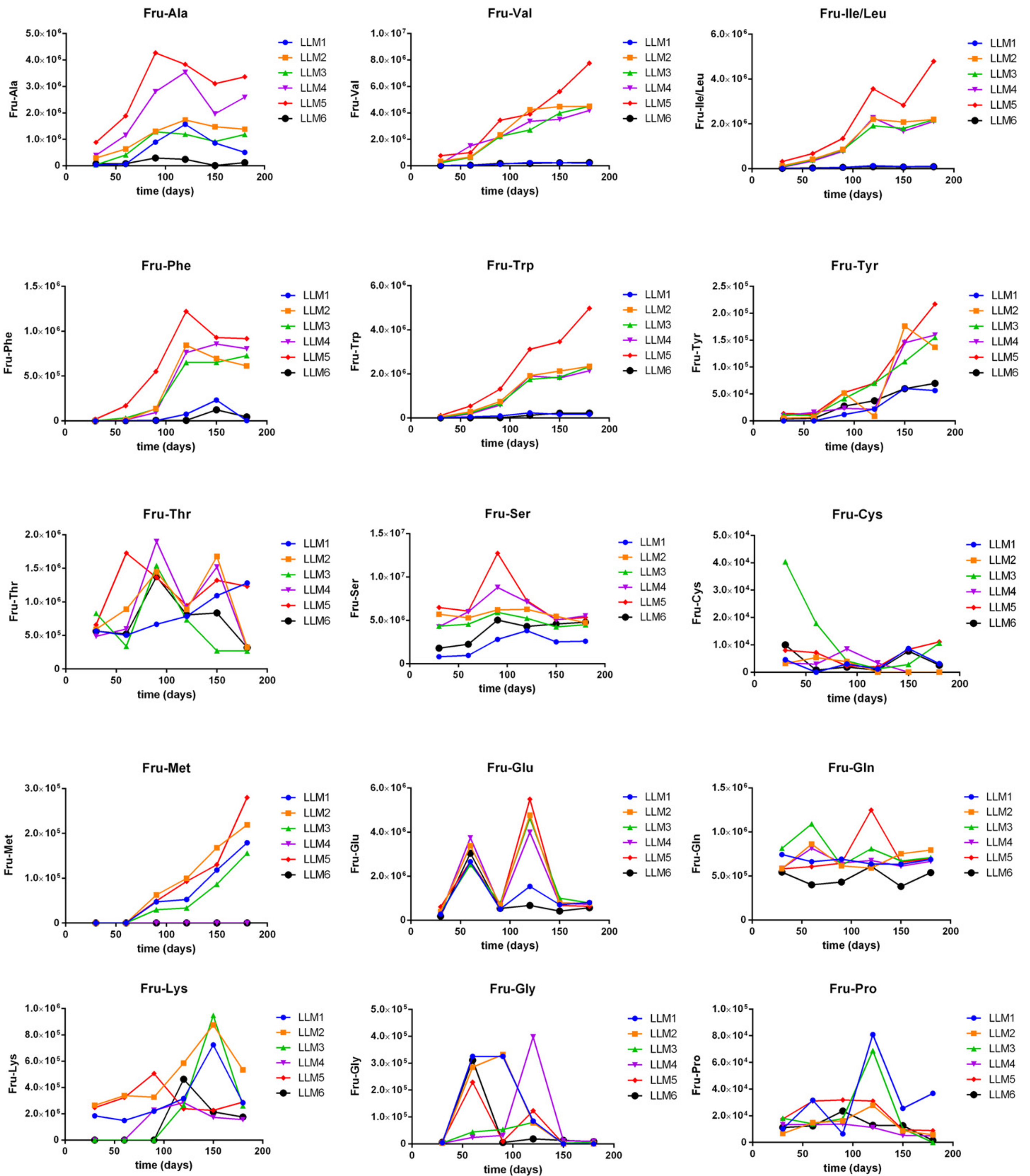

Fig. 3. Area counts of free Amadori products (APs) during 180 days the storage (n = 4).

The overall information coming from the determination of free amino acids and free APs were summarized in Fig. 4. In the top panel the total area counts of free amino acids obtained by summing the value of the single compounds was reported. As expected in the four samples LLM2, LLM3, LLM4 and LLM5 the amino acids were significantly higher than in other two (LLM1 and LLM6): after 120 days of storage the relative abundance of amino acids in the former group of samples was ten times higher than the values in the latter.

Fig. 2. Area counts of free amino acids in milk samples during 180 days of storage (n = 4).

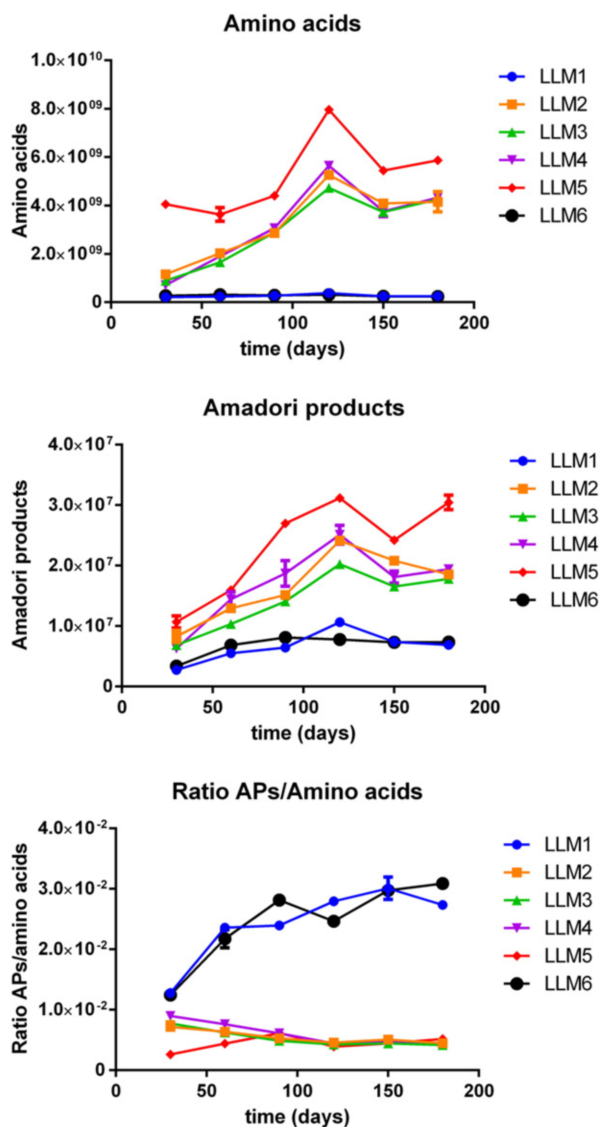

Fig. 4. Total free amino acids (top panel), total free APs (central panel) and ratio between the two. The free amino acids and free APs were calculated by summing the area counts of the compounds reported in Figs. 1 and 2, while the ratio was calculated according to Eq. (1).

Interestingly, a slight difference was also detected between LLM1 and LLM6 (not visible in the Fig. 4 because the scale is to wide). The levels of the free amino acids for LLM6 were about 12% lower at the end of the storage: the slight decrease was due to the conversion into the corresponding APs. Conversely, the levels of free amino acids in sample LLM1 increased of around 9% at the end of the storage. Also at 120 days the concentration of all amino acids was 15% lower in LLM6 than LLM1. This scenario indicated that although very small respect to the other samples obtained by process A, a residual side proteolytic activity was present also in LLM1.

The central panel of Fig. 4 reported the sum of free APs. In this case the profiles for the six samples were quite similar: the free APs increased during the first three months of incubation, they reached a plateau around the fourth month of incubation, then they rapidly decreased at the end of the storage, with the exception of the APs in LLM5 that increased during the last 30 days. LLM5 had the highest levels throughout the incubation, revealing that a higher concentration of precursors resulted in the increase of free intermediates. The sum of APs in LLM1 and LLM6 was significantly lower than the other samples because a lower concentration of free amino acids is present in these samples ($\alpha < 0.05$). This trend indicated that upon prolonged storage time the formation of advanced MRPs and volatiles via Strecker degradation became the most important phenomenon. In this respect the monitoring of free APs by LC-HRMS has the potential to be used as an important parameter to foresee the quality decay of food products.

The bottom panel reported the ratio between the sum of free APs and the sum of free amino acids at each point of the sampling according to the following formula:

$$\text{Ratio} = \text{Sum of APs (area counts)} / \text{Sum of amino acids (area counts)} \quad (1)$$

The ratio for the sample LLM2, LLM3, LLM4 and LLM5 constantly decreased during storage, thus indicating that the release of free amino acids due to the lactase proteolytic activity, was faster than the free amino acid glycation by glucose and galactose. Conversely, the ratio for samples LLM1 and LLM6 increased during the first 50 days of storage. This was due to the limited release of free amino acids in these samples while the amount of reducing sugars was the same in all samples. As pointed out above a difference is detectable between LLM1 and LLM6 in the last part of the storage time. In fact, while in LLM6 the ratio keeps on increasing, in LLM1 it reached a plateau and during the last 30 days it decreased.

The trends of free amino acids, free APs and the ratio between free APs and amino acids revealed that at time zero (i.e. 4 weeks after the UHT treatment), the differences among free markers were still not large: it is only upon prolonged storage that the presence of active lactase led to the release of amino acids and consequent formation of a significant amount of free APs amount. This is in line with what is well known for the proteins bound MRPs.

3.3. Bound Maillard reaction products and total bound lysine

The concentration of total lysine and the formation of bound MRPs, such as CML, CEL and furosine during storage are shown in Fig. 5. In this case the concentrations of bound MRPs was similar among the six LLMs being only in furosine in LLM5 significantly higher than in the other samples. Furosine is the most used marker of the thermal damage in milk (Erbersdobler & Somoza, 2007). As expected, furosine steadily increased over the storage in all samples and LLM5 had the highest concentration of furosine ($\alpha < 0.05$). Interestingly, LLM5 was the only sample showing a decrease of furosine concentration in the last 30 days (from 1275.5 ± 84.7 to 1056.1 ± 84.7 mg/100 g protein). It was hypothesized that when a 30% of lysine derivatization is reached the conversion of APs into intermediates products (cross link products, volatiles, CML and CEL) become more relevant than its neo formation (Burvall, Asp, Bosson, San José, & Dahlqvist, 1978; Ferrer et al., 2003). The concentration of furosine at the various time was not significantly different among the other five samples.

The different behavior between free and bound markers of the MR was not surprising. The post-translational modifications on proteins (bound MRPs) are not expected to be considerably influenced by the proteolytic activity of lactase. The main drivers of furosine formation are proteins and carbohydrates type and concentration (which are the same in all samples) and intensity of thermal treatments, whose thermal impact was not different according to the formation of bound MRPs. The only exception was represented by LLM5 and it is possible that this sample was subjected to a higher thermal load during processing.

The concentration of CML in the six samples ranged from 2.062 ± 0.206 (LLM3 after 30 days) to 19.711 ± 1.479 (LLM5 after 150 days) mg/100 g of protein. In particular after 90 days CML ranged from 7.517 ± 0.466 to 9.830 ± 0.358 mg/100 g of protein. The concentration of both CML and CEL were in line with other quoted papers (Delatour et al., 2009; Hull, Woodside, Ames, & Cuskelly, 2012; Pischetsrieder & Henle, 2012; Troise, Dathan, Fiore, Roviello, Di Fiore, Caira, et al., 2014; Troise, Fiore, Wiltafsky, & Fogliano, 2015). Interestingly all the six

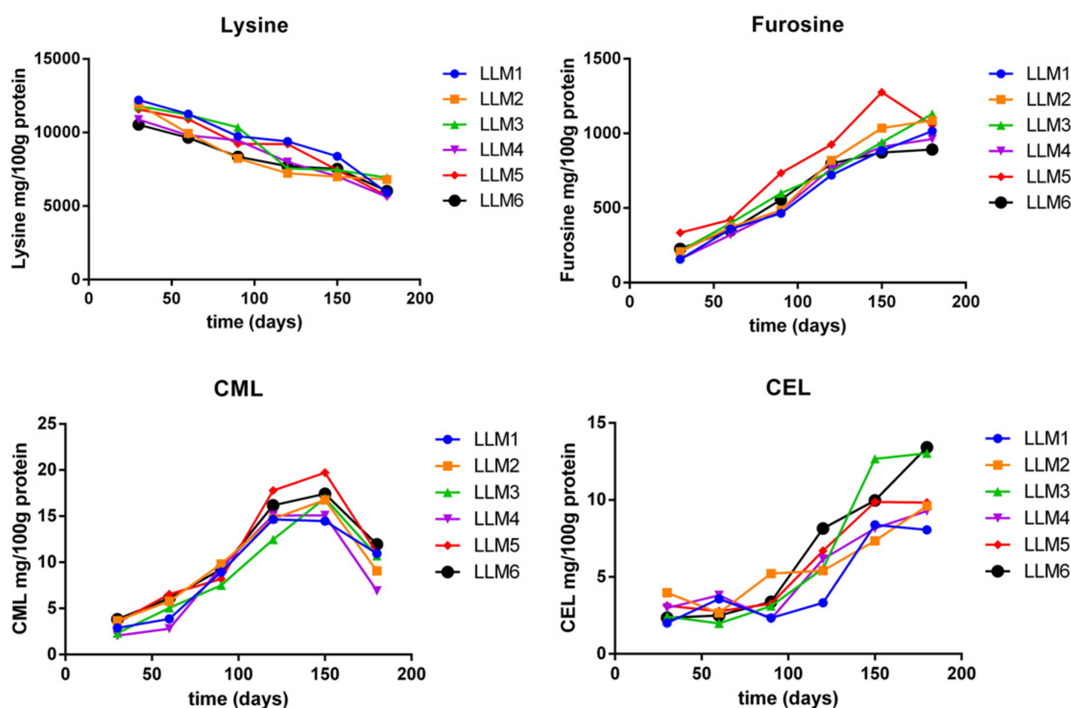

Fig. 5. Concentration of the Maillard reaction products (MRPs) and total lysine during 180 days of storage (n = 4). Protein bound furosine, CML, CEL and total lysine were determined after acidic hydrolysis. Results are expressed in mg/100 g of protein.

samples showed a similar trend: CML constantly increased during the first 150 days, but it decreased during the last 30 days of incubation. CEL followed a similar profile to CML during the first 150 days of incubation but it keep on increasing up to the last storage time.

The concentration of these two markers measured at each time point was the result of neo-formation and conversion in advanced Maillard products. The different behavior between CML and CEL can be explained considering their different formation pathways in presence of monosaccharides glucose and galactose. In the case of glucose as reducing sugars, the formation of bound Fru-Lys is the prerequisite for the formation of both CML and CEL (Ahmed, Frye, Degenhardt, Thorpe, & Baynes, 1997; Ahmed, Thorpe, & Baynes, 1986). However, beside the further fragmentation via oxidative cleavage of the Amadori compounds (Hodge pathway) also the oxidative glycosylation mediated by α-dicarbonyls, the reverse aldol reaction of the sugar moiety, hydration and dehydration reactions upon the Cannizzaro rearrangement, are possible formation pathways (Kasper & Schieberle, 2005). In this respect it is worth to notice that Nguyen, van der Fels-Klerx, and van Boekel (2016) recently showed that while the oxidation mediated by methylglyoxal significantly contributed to the formation of CEL, the contribution of glyoxal to the formation of CML was negligible.

Total lysine is one of the most important parameters to assess the nutritional quality of milk proteins during the storage. At the beginning of storage the concentration of total lysine was higher than 10% of total protein for all the samples, then it constantly decreased. After 180 days, total lysine was reduced to 43%, 41%, 52%, 51%, 43% and 48% of the initial concentration for LLM3, LLM1, LLM2, LLM5, LLM6 and LLM4, respectively. Interestingly, already after 90 days (which is the best before date of the LLM samples) the reduction of total lysine was remarkable being 31%, 12%, 29%, 20%, 21% and 13% for LLM3, LLM1, LLM2, LLM5, LLM6 and LLM4, respectively.

The results here obtained on free lysine reduction were of the same order of magnitude than those found in previous published papers (Troise, Fiore, Colantuono, Kokkinidou, Peterson, & Fogliano, 2014; Troise, Fiore, Wiltafsky, & Fogliano, 2015). The strong reduction of lysine can be explained by the presence of glucose and galactose instead of lactose of conventional UHT milk. These monosaccharides are much faster than lactose in promoting the formation of bound MRPs this blocking the ε-amino group of lysine residues (Jansson, Clausen, et al., 2014; Mendoza, Olano, & Villamiel, 2005). This evidence calls for a further attention to the processing conditions with the aim of improving LLM nutritional quality.

3.4. Sensorial analysis

Sensory profiles of LLMs were determined at the beginning of the study (30 days after the production date) and after 90 days of storage at room temperature (23 ± 3 °C) and the results were reported in Fig. 6. Eight attributes were selected and they were divided into two categories: positive attributes (white color, aroma intensity, mouthfeel, sweet flavor, milky taste) put on the right of the plot and negative attributes (cooked taste, irregular aftertaste and stale aftertaste) put on the left of the spider plot. As expected, for all samples the surface of the positive attributes was higher at the beginning of the storage than at the end. The opposite was true for the negative attributes whose values increased during the storage. Results of the sensory test indicated that in the samples LLM2–LLM5 the negative attributes are well perceived at the end of the storage time with values often higher than 5. In particular for LLM4 irregular aftertaste, cooked taste and stale aftertaste were 5.2, 5.9 and 5.3, respectively. LLM1 sample despite having active lactase did not develop significant defects during storage: irregular aftertaste, cooked taste and stale aftertaste were almost not perceived with score of 1.4, 2.6 and 1.9, respectively. As expected the absence of residual enzymatic activity guaranteed a positive performance also to the LLM6: stale aftertaste, cooked taste and irregular aftertaste were slightly perceived and evaluated as 3.1, 3.2 and 2.8, respectively.

3.5. Volatiles compounds

LLM volatiles were analyzed by using GC–MS in order to tentatively identify the markers of the off-flavor and find a correlation with the results of the sensory analysis. In Fig. 7 the volatile profiles of 15 compounds identified by using an internal library and matching the spectra with AMDIS (NIST) were reported. The compounds were

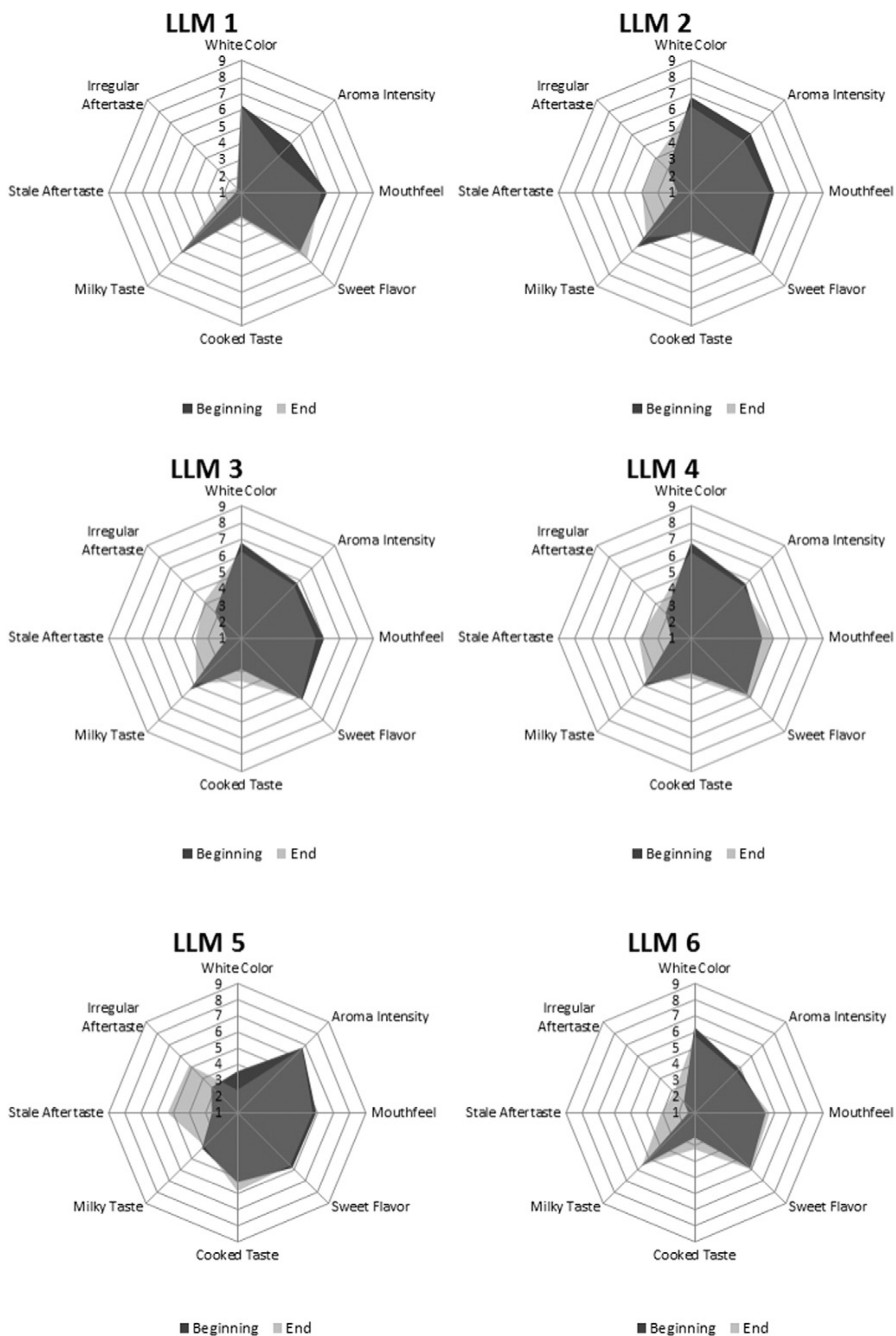

Fig. 6. Sensory profiles of LLMs at the beginning of the study (four weeks after the production date) and after 90 days of storage at room temperature (23 ± 3 °C). Intensities of individual attributes were performed by Visual Analogue Scale (VAS) from zero point (center or not detected) to 9 (most intense attributes).

grouped according to their functional groups or to the chemical structures: aldehydes, ketones, furan, acetic acid and sulphur derived compounds (Jansson, Clausen, et al., 2014). Benzaldehyde, methional, 5-methylfurfural and 2-methylbutanal were present in all samples and as expected, both LLM1 and LLM6 develop lower levels of these molecules during the storage. The results were particularly evident for methional and 2-methylbutanal after 180 days. The LLM5 showed extremely high values of 5-methylfurfural after 60 and 90 days thus confirming the indication obtained for furosine of a higher thermal load during sterilization respect to the other samples. Hexanal also showed an interesting trend: in samples LLM1 and LLM6 it decreased throughout the storage, while it increased in the other samples as a consequence of the lipid oxidation. The formation of ketones seemed to be related to the lactase side proteolytic activity combined to the sugars autoxidation. They steadily increased during storage in sample LLM2-LLM5 while LLM1 and LLM6 exhibited the lowest values for acetone, hydroxyacetone and 2-heptanone and also the concentration of *o*-aminoacetophenone in LLM1 and LLM6 decreased over the storage.

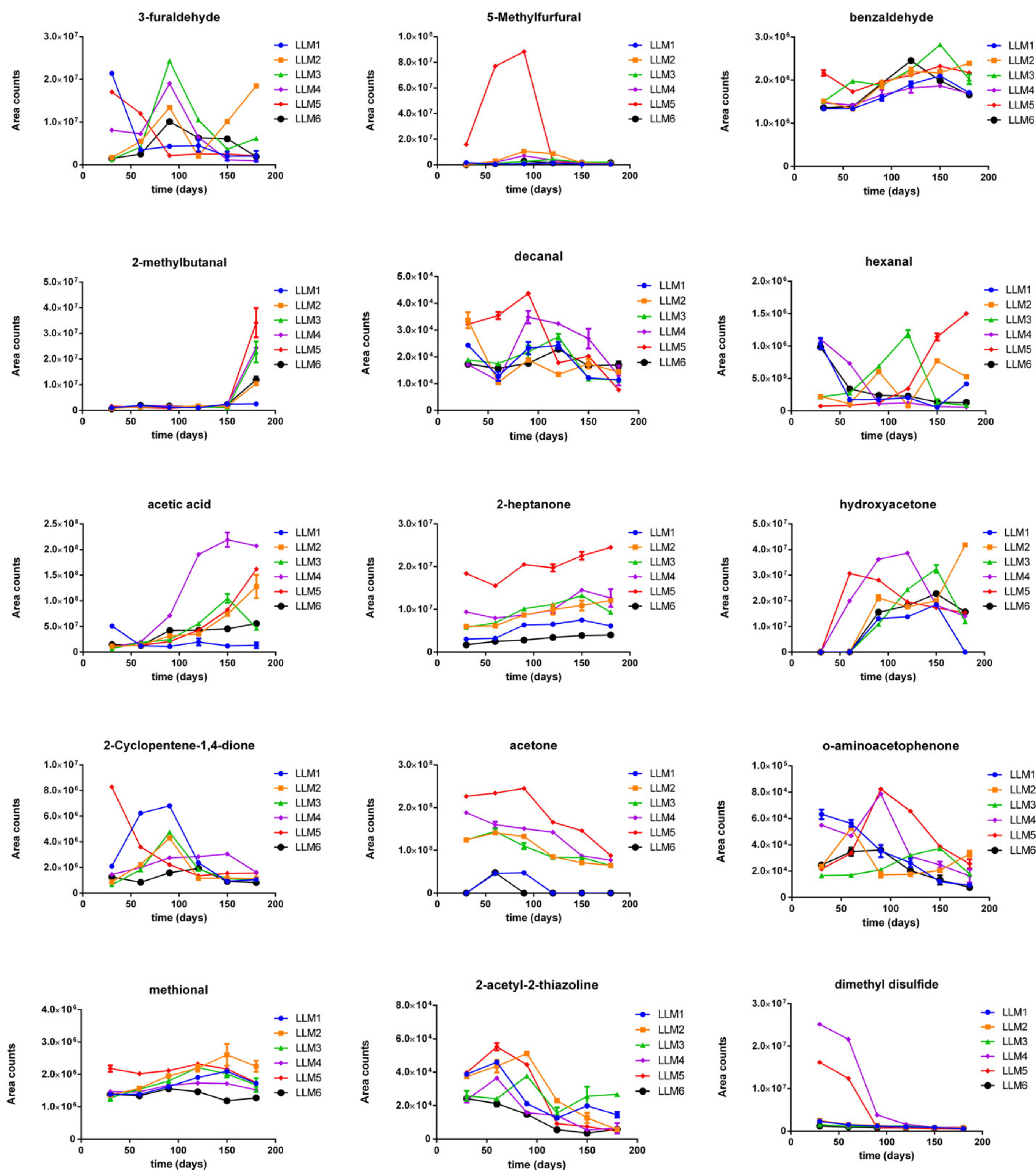

Fig. 7. Volatiles profiles monitored by using AMDIS (NIST) and Xcalibur (Thermo Fisher, Bremen). The values were reported as area counts.

Finally four sulphur derived compounds were monitored and in all case LLM6 showed the lowest concentration of these compounds during the storage.

The combined measure of volatile and free APs could be useful to unravel the pathways leading to the off flavor formation starting from the free amino acids. A good example was provided by the trend of free Ile/Leu, the formation of the intermediate Fru-Ile/Leu and finally the Strecker degradation led to the formation of 2-methylbutanal (Cremer, Vollenbroeker, & Eichner, 2000; Davidek, Clety, Aubin, & Blank, 2002). Looking at data in samples LLM2, LLM3, LLM4 and LLM5 the increase of free Ile/Leu concentration in the first four months is followed by the increase in the levels of the two APs and of the 2-methylbutanal at the end of the storage.

Along with C2/C4 and C3/C3 cleavages to produce glycolaldehyde, tetrose, and C3-reactive sugar derivatives such as acetol, glyceraldehyde, and pyruvaldehyde, the Strecker degradation on APs should

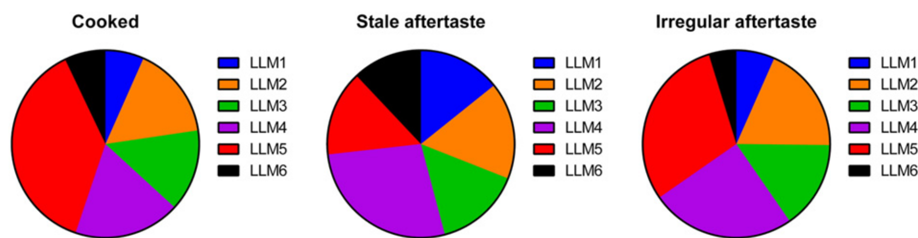

Fig. 8. Cluster of the sensory attributes and the volatile compounds tentatively identified in the six LLM samples. Stale aftertaste included sulphur containing compounds, cooked taste included oxygen containing aldehydes and ketones with the exception of acetone and irregular aftertaste was derived by the sum of acetone, acetic acid, hexanal and decanal.

have a significant role in these systems (Mottram, 2007). In this respect, it can be hypothesized that other odor active markers, such as 2-acetyl-2-thiazoline, 5-methylfurfural and 3-furaldehyde were formed as a direct consequence of the degradation of the APs. Beside the formation of volatiles and off flavor from Strecker aldehyde formation, also other pathways, such as sugar fragmentation can be monitored. As shown in Fig. 7, acetic acid formation parallel the development of MR and the trend of furosine. The adoption of acetic acid as marker of MR development and in particular of the 2,3-enolisation has been previously put forward (Brands & van Boekel, 2001). It derives primarily from C2/C3 β-cleavage of the 1-deoxyosone isomerization product 1-deoxy-2,4-hexodiulose (Smuda & Glomb, 2013).

In Fig. 8 a summary of the obtained volatiles data was presented following a methodology described by previous papers (Jensen et al., 2015; Mottram, 2007). The sum of volatile compounds was combined to the three negative sensory attributes previously described: cooked taste, stale aftertaste and irregular aftertaste. Sulphur derived compounds contributed to stale aftertaste, typical markers of lipid oxidation, acetic acid, acetone, decanal and hexanal were associated to irregular aftertaste, while the attribute “cooked taste” resulted from the sum of oxygen containing aldehyde and ketones with the exception of acetone (Bendall, 2001; Mottram, 2007).

Regarding sulphur containing compounds in presence of the active lactase a clear release of Met and the consequent formation of Fru-Met is observed. Fru-Met could be a precursor of methional, through the Strecker degradation and in fact methional increased in the LLM2-LLM5 samples up to the fifth month (Pfeifer & Kroh, 2010; Yaylayan & Keyhani, 2001). Hexanal and decanal were included in the study even if they were not directly linked to the MR or at the least they arose from the interplay between MR and lipid oxidation (Zamora & Hidalgo, 2005). Anyway, both aldehydes can be considered as strong odor active compounds and they are commonly used as an indicator for the characterization of off-flavors resulting from lipid peroxidation of linoleic acid (hexanal) and oleic acid (decanal) (Vazquez-Landaverde, Velazquez, Torres, & Qian, 2005). The trends reported in Fig. 7 were well in line with those reported by Jansson and coworkers for low lactose milk during storage (Jansson, Clausen, et al., 2014).

The concentration of volatiles associated to negative sensory attributes are very high in samples LLM2, LLM3 LLM4 and particularly in LLM5 which performed very badly in all sensory panel. The contribution of volatiles compounds provided by LLM1 and LLM6 are rather limited thus confirming the evidence on free amino acids and sensory test.

4. Conclusions

The category of lactose free products and in particular LLM is increasing its popularity as it answers an important consumer request. Because of the chemical composition, LLM production deserves a special attention as a significant decrease of sensory and nutritional quality during the shelf life might occur. Data of this work showed that the addition of lactase “in pack” after milk sterilization (see Process A of Fig. 1) can have negative sensorial and nutritional consequences mainly related to the residual side proteolytic activity showed by commercial lactase preparation. The significant release of free amino acids is the first step leading to off flavor development. Anyway, as shown by the quality parameters of one of the sample obtained with Process A (LLM1), the “in pack” process does not always result in bad quality performance during storage. It is likely that using highly pure lactase preparations having reduced side proteolytic activity the problem can be effectively tackled. We concluded that the quality of lactase is the key point to keep a satisfactory LLM quality during the shelf life when the in pack addition process is used, while this is not the case when the in batch process is applied. In other words while with “in batch” technology the quality of the final product during the shelf life is quite independent from the purity of lactase the “in pack” addition process could result in a good product only if a high pure protease-free lactase is used. This phenomenon can be negligible in the weeks immediately after the production, however it can be clearly perceived also by untrained consumers after two months of storage. Last but not least, data proved that MRPs and in particular the APs of free amino acids are suitable markers to monitor the quality of this product category. The fast detection of free APs ensured by High Resolution MS could allow to use these markers to estimate the extent of MR avoiding the acid protein hydrolysis step which is a prerequisite for the detection of bound MRPs.

Abbreviations

MR	Maillard reaction
APs	Amadori products
MRPs	Maillard reaction end products
HRMS	high resolution mass spectrometry
MS/MS	tandem mass spectrometry
LC	liquid chromatography
GC–MS	gas chromatography mass spectrometry
CML	*N*ε-(carboxymethyl)-L-lysine
CEL	*N*ε-(carboxyethyl)-L-lysine
furosine	*N*ε-(2-furoylmethyl)-L-lysine

Conflict of interest

E. B., R.D. and M.T. are employee of Parmalat which is a producer of low lactose milk.

Chemical compounds studied in this article

β-lactose (PubChem CID: 6134); L-Threonine (PubChem CID: 6288); L-Serine (PubChem CID: 5951); L-Lysine (PubChem CID: 5962); L-Histidine (PubChem CID: 60366274); L-Arginine (PubChem CID: 6322); L-Asparagine (PubChem CID: 6267); L-Glutamine (PubChem CID: 5961); L-Glycine (PubChem CID: 750); L-Alanine (PubChem CID: 5950); L-Proline (PubChem CID: 6036; L-Valine (PubChem CID: 6287); L-Methionine (PubChem CID: 6137); L-Tyrosine (PubChem CID: 6057); L-Isoleucine (PubChem CID: 6306); L-Leucine (PubChem CID: 6106); L-Phenylalanine (PubChem CID: 6140); L-Tryptophan (PubChem CID: 6305); L-Glutamic acid (PubChem CID: 611); L-Aspartic acid (PubChem CID: 5960); (*N*ε-(2-furoylmethyl)-L-lysine (furosine) (PubChem CID: 123889), *N*ε-(carboxymethyl)-L-lysine (CML) (PubChem CID: 123800); *N*-

(1-deoxy-D-fructos-1-yl)-L-leucine (PubChem CID: 71316981); *N*-(1-deoxy-D-fructos-1-yl)-L-phenylalanine (PubChem CID: 71316982); *N*-(1-deoxy-D-fructos-1-yl)-L-lysine (PubChem CID: 123708); *N*-(1-deoxy-D-fructos-1-yl)-L-glycine (PubChem CID: 3081391); *N*-(1-deoxy-D-fructos-1-yl)-L-valine (PubChem CID: 71777427); *N*-(1-deoxy-D-fructos-1-yl)-L-tryptophan (PubChem CID: 159983); *N*-(1-deoxy-D-fructos-1-yl)-L-asparagine (PubChem CID: 71316980); *N*-(1-deoxy-D-fructos-1-yl)-L-glutamic acid (PubChem CID: 56971968); *N*-(1-deoxy-D-fructos-1-yl)-L-proline (PubChem CID: 71316983).

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