Proteolysis of yogurts made from ultra-high-pressure homogenized milk during cold storage

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ABSTRACT

Proteolysis was investigated in yogurts made from milk that was ultra-high-pressure homogenized at 200 or 300 MPa and at 30 or 40°C and compared with those produced from heat-treated milk containing 3% skim milk powder. To evaluate changes in the protein fraction, samples were analyzed at d 1, 7, 14, 21, and 28 of storage for residual caseins, peptides, and total free amino acids. Results showed that yogurts from heat-treated milk and 300 MPa-treated milk presented similar levels of residual caseins, as well as similar profiles of soluble peptides and total free amino acids. On the contrary, greater amounts of hydrophobic peptides were detected in yogurts made from 200 MPa-treated milk at both 30 and 40°C, especially at the end of storage. In all treatments studied, caseins were hydrolyzed and hydrophobic peptides were increased during storage, as reflected by the increase in soluble nitrogen at the end of the storage.

Key words: ultra-high pressure homogenization, proteolysis, yogurt, capillary electrophoresis

INTRODUCTION

Ultra-high-pressure homogenization (UHPH) is a promising technology, based on the same principle as conventional homogenization but working at significantly greater pressures, and is currently being investigated for the manufacture of yogurt (Serra et al., 2007, 2008a,c). In a previous study (Serra et al., 2007) UHPH samples were compared with heat-treated milk both with and without skim milk powder (SMP) added, and it was concluded that yogurts from milk UHPH treated at 200 or 300 MPa had greater gel firmness, less syneresis, and lower titratable acidity than those obtained from conventionally treated milk with SMP. These results reflected the potential effect of UHPH as

a possible replacement of the SMP fortification step, leading to a continuous and potentially cheaper manufacturing process.

During milk fermentation the proteolytic system of starter cultures plays a key role, because starter cultures require an exogenous source of amino acids, which is provided by the proteolysis of casein. In general, hydrolysis of casein in yogurt is initiated by the cell-envelope proteinase (Courtin et al., 2002) from *Lactobacillus delbrueckii* ssp. *bulgaricus* (Law and Haandrikman, 1997), which yields oligopeptides that are subsequently incorporated into the microbial cells via specific peptide transport systems for further conversion into shorter peptides and amino acids by intracellular peptidases (Savijoki et al., 2006).

Proteolysis is a very important phenomenon during fermentation, because it will determine the survival of starter cultures, the formation of some flavor compounds and even some of the physical properties of the gels obtained (Tamime and Robinson, 2007). According to Courtin et al. (2002), the supply of these peptides from caseins by the lactobacilli proteinase B (PrtB) is of essential importance for the survival of *Streptococcus thermophilus*, because the peptides will act as assimilable nitrogen compounds when those of milk are exhausted. On the other hand, amino acids are important for the synthesis of flavor compounds such as acetaldehyde, which derives from threonine (Zourari et al., 1992), or diketones, the formation of which is regulated by the amount of branched-chain amino acids (BCAA) synthesized (Ott et al., 2000). Moreover, proteolysis in fermented milk is currently an important research topic because it is well known that proteolysis could lead to the formation of bioactive peptides, which are encrypted within the primary structure of milk proteins (Hayes et al., 2005).

The aim of this work was to compare the degree of proteolysis of yogurts obtained from milk UHPH-treated at 200 or 300 MPa with that of yogurts produced from heat-treated milk, as well as their evolution during cold storage, to evaluate the potential application of this emerging technology in the yogurt industry.

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MATERIALS AND METHODS

UHPH Treatment of Milk and Yogurt Production

Fresh raw bovine milk was obtained from a local dairy farm (S.A.T Can Badó, Santa Agnès, Barcelona, Spain). After standardization of fat content at 3.5%, the milk was kept under refrigeration for 24 h. For obtaining the UHPH milk base, milk was subjected to UHPH treatments at 200 or 300 MPa at 30 or 40°C, as previously explained (Serra et al., 2008d). The UHPH samples were compared with those obtained from milk heat-treated at 90°C for 90 s in a tubular heat-exchanger (ATI, Granollers, Barcelona, Spain), homogenized (Rannie, Copenhagen, Denmark) in one stage at 15 MPa, and fortified with 3% skim milk powder (54% lactose, 34.04% total protein and 8.02% ash; Reny Picot, Anleo-Navia, Asturias, Spain) (**HT+SMP** treatment). After UHPH or HT+SMP treatment, all samples were inoculated at the same time with commercial cultures of *Strep. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* (DVS YF-3331, Chr. Hansen, Horsholm, Denmark) and incubated at $43 \pm 2^\circ\text{C}$ for 3.5 h until pH 4.6 was reached (Serra et al., 2007). For the storage study, different samples of yogurts were prepared for each day of analysis. Two independent batches were performed for all conditions studied.

Yogurt Composition and Microbiological Analysis

Yogurts were analyzed in duplicate for total solids (FIL-IDF, 1991a), titratable acidity (FIL-IDF, 1991b), and pH. Selective enumeration of starter cultures was carried out using de Man, Rogosa, and Sharpe agar (Liochem Bacteriology Products, Italy) adjusted to pH 5.4 for *L. delbrueckii* ssp. *bulgaricus* counts, and M17 agar (Oxoid, Hampshire, UK) adjusted to pH 7.2 for *Strep. thermophilus* counts (FIL-IDF, 1997). Microbiological count data are expressed as $\log_{10}$ of colony-forming units per gram of yogurt. All determinations were made in duplicate for every experiment.

Separation of Yogurt Protein Fractions

The pH 4.6-insoluble fraction containing the isoelectric caseins was obtained by adding, if necessary, 1 *N* NaOH until pH 4.6 was reached, followed by centrifugation at $4,500 \times g$ and 5°C for 30 min. The casein pellets were washed 3 times with 1 *M* sodium acetate buffer (pH 4.6), and the remaining fat was eliminated by washing with dichloromethane-sodium acetate buffer (1:1, vol/vol). The final protein precipitate was then lyophilized. The pH 4.6-soluble fraction was filtered

through Whatman filter paper #1 and frozen at -30°C until day of analysis.

Separation of pH 4.6-Insoluble Fraction by Capillary Electrophoresis

Analysis of individual proteins was performed using an Agilent CE Instrument (Agilent Technologies, Böblingen, Germany) controlled by Chemstation Software (Agilent) and following the method described by Recio and Olieman (1996). Separations were carried out using a fused-silica capillary column of $0.6 \text{ m} \times 75 \mu\text{m}$ i.d. deactivated with OV-1701-OH (Beckman, Fullerton, CA) with an effective length of 50 cm at 45°C and applying a linear voltage gradient from 0 to 20 kV over 3 min. Detection of proteins was made at 214 nm and designation of peaks (α_{s2} -, α_{s1} -, κ -, and β -caseins) was carried out by comparing the electrophoretograms with those obtained by Recio et al. (1997). The area of each peak was integrated using Agilent ChemStation Operation software (Agilent). Results are expressed as integrated area peaks at d 1 and 28 of storage. All samples were analyzed in duplicate.

Peptides and Whey Protein Analysis

Peptides in the pH 4.6-soluble fraction were analyzed by reverse-phase HPLC using an automated HPLC system (HPLC P680, Dionex, Sunnyvale, CA) equipped with a UV detector (UVD170U, Dionex). Before analysis, samples were thawed at room temperature and filtered through $0.45\text{-}\mu\text{m}$ cellulose acetate filters (Tracer PVDF, Teknokroma, Sant Cugat del Vallès, Spain). Separation of peptides was carried out on a $250 \times 4.6\text{-mm}$ column packed with C18-bonded silica gel ($5\text{-}\mu\text{m}$ particle size and $300 \text{ \AA}$ pore size; Symmetry 300, Waters, Milford, MA) at a constant temperature of 40°C and detected at a wavelength of 220 nm. Eluent A was 0.1% trifluoroacetic acid-water, and eluent B was 0.1% trifluoroacetic acid-acetonitrile at a flow rate of 1 mL/min. A linear gradient from 0 to 100% B was followed by isocratic elution of 10 min. The area of all peaks including that of free amino acids (**FAA**) was integrated using Chromoleon Software (Dionex). The area of peptides eluted between 10 and 20 min (between Tyr and Trp) was considered the hydrophilic peptide area, whereas the area eluted after 20 min was considered the hydrophobic peptide portion. Results are expressed as units of chromatogram area.

Whey proteins (**WP**) were identified by the injection of standards (1 mg/mL) of α -LA, β -LG-A, and β -LG-B (Sigma Chemical Co., St. Louis, MO) and quantified using the response factor of the standards. All samples were analyzed in triplicate.

pH 4.6-Soluble Nitrogen and Total FAA

From the pH 4.6-soluble fraction, nitrogen content was determined following Dumas' method (FIL-IDF, 2002), and total FAA were determined by the cadmium-ninhydrin method described by Folkertsma and Fox (1992). All determinations were made in triplicate.

Statistical Analysis

An ANOVA was performed on all data using SPSS (SPSS Inc., Chicago, IL). Mean comparisons were carried out using the Student-Newman-Keuls test. For comparison of residual caseins at d 1 and 28 of storage, a Student *t*-test for the comparison of 2 samples was carried out. The level of significance was set at $P < 0.05$ for all tests. In addition, correlations between caseins, peptides, soluble WP, FAA, and soluble nitrogen were evaluated with the Pearson test.

RESULTS AND DISCUSSION

Residual Caseins

Caseinolytic activity in mixed cultures is mainly determined by *L. delbrueckii* ssp. *bulgaricus* because of its cell-wall-associated proteinase (Courtin et al., 2002) that is capable of hydrolyzing α_s -, κ -, and β -caseins. The optimal conditions for PrtB are 42°C and pH 5.5 (Law and Haandrikman, 1997), so it has maximum activity during the fermentation process, in particular during the exponential growth phase of *L. delbrueckii* ssp. *bulgaricus* as reported by Courtin et al. (2002).

Results of residual caseins in the pH 4.6-insoluble fraction after fermentation and at the end of storage are presented in Figure 1. In general, no statistical differences were found in the amount of caseins immediately after the fermentation process; only κ -casein levels were lower in yogurts from milk treated at 300 MPa at both inlet temperatures. Some authors (Sandra and Dalgleish, 2005; Serra et al., 2008c) have reported that at such high homogenization pressures the structure of casein micelles could be affected, which could lead to an increase in casein micelle fragments in the soluble fraction. However, other authors (Pereda et al., 2008) did not find statistical differences in the amount of insoluble casein just after UHPH treatment of milk under the same conditions as those in the present study. However, it is likely that shear forces during the UHPH treatment at 300 MPa induced conformational changes, especially in the micelle periphery, that could have made κ -casein more accessible to lactobacilli endoproteases during the fermentation step.

As shown in Figure 1, caseins were hydrolyzed during storage and so, levels at d 28 were significantly lower

than at d 1 in most of cases, especially for α_{s1} -, κ -, and β -caseins, which are preferential substrates for PrtB from lactobacilli (Tamime and Robinson, 2007). This decrease during storage was very low because of pH and temperature conditions and was similar between samples and types of casein. However, in general, in yogurts from milk UHPH-treated at 200 MPa, a greater decrease in caseins was observed during storage, especially in treatments carried out at 40°C. As described in a previous work (Serra et al., 2008b), at d 14 of storage, the viability of *L. delbrueckii* ssp. *bulgaricus* was sharply decreased in yogurts from milk UHPH-treated at 200 MPa at both inlet temperatures (Table 1). This loss of viability probably resulted in the liberation of many proteolytic enzymes into the medium because of cell lysis, which could explain the greater casein hydrolysis observed in samples from milk UHPH-treated at 200 MPa. According to Savijoki et al. (2006), lactic acid bacteria possess stress-inducible proteases that are capable of degrading casein, which could play an important role in dairy fermentations at the stage in which autolysis occurs.

Nevertheless, this decrease in caseins was not as pronounced in samples from milk UHPH-treated at 200 MPa at 30°C, although lactobacilli counts were decreased. This different behavior could be due to the high amounts of free fatty acids detected by Serra et al. (2008d) in yogurts from milk treated at 200 MPa at 30°C, because according to Tamime and Robinson (2007) proteolytic activity can be reduced by high levels of free fatty acids, especially those from capric and oleic acids.

pH 4.6-Soluble Fraction

Whey Protein Evolution. Values of soluble WP and their evolution during storage are shown in Table 2. Denaturation of WP by UHPH has been studied by other researchers (Desrumaux and Marcand, 2002; Zamora et al., 2007; Serra et al., 2008c); however, the changes in the content of these proteins in the soluble fraction after the fermentation process have not been already described. Starter cultures can hydrolyze both α -LA and β -LG, but to a limited extent (Bertrand-Harb et al., 2003); however, the interest for studying the WP-derived peptides after fermentation relies on the yielding of opioid peptides from β -LG (Hayes et al., 2007), and in the obtainment of nonallergenic products (Kleber et al., 2006). Under normal conditions, β -sheets in the β -LG structure hinder hydrolysis by starter cultures, but denaturation facilitates the accessibility of microbial proteases (Bertrand-Harb et al., 2003). Pescuma et al. (2000) noted that *L. delbrueckii* ssp. *bulgaricus* degrades β -LG releasing hydrophilic

Figure 1. Levels of residual α_{s2} -, α_{s1} -, β - and κ -caseins in the pH 4.6-insoluble fraction of yogurts from ultra-high-pressure homogenized milk (230 = 200 MPa at 30°C; 240 = 200 MPa at 40°C; 330 = 300 MPa at 30°C; 340 = 300 MPa at 40°C) and HT+SMP milk (treated at 90°C for 90 s, homogenized at 15 MPa, and enriched with 3% skim milk powder) at d 1 (white bars) and d 28 (gray bars) of storage. Results are expressed as integrated area peaks (mean $\pm$ SD). ^{a,b}Different letters indicate differences between d 1 and 28 in the same treatment. ^{A,B}Different letters indicate differences between treatments in the same day of storage.

and hydrophobic peptides, whereas *Strep. thermophilus* releases only hydrophilic peptides. In the present work, no changes were observed in the amount of soluble α -LA, either between treatments or during storage, indicating that the hydrolysis of this protein probably

took place during fermentation, especially at the end of the process, because an acidic pH is needed to make it susceptible to hydrolysis (Pescuma et al., 2000). On the other hand, no changes in the amount of soluble β -LG were detected in most of samples during storage.

Table 1. Compositional parameters and starter culture counts (at d 1, 14, and 28) in yogurts from milk during cold storage

Item	Treatment ¹				
	HT+SMP	200 MPa		300 MPa	
		30°C	40°C	30°C	40°C
Total solids ² (%)	13.37 $\pm$ 0.53 ^a	10.3 $\pm$ 0.46 ^b		11.01 $\pm$ 0.39 ^b	
pH	4.52 $\pm$ 0.06	4.54 $\pm$ 0.07	4.51 $\pm$ 0.01	4.5 $\pm$ 0.04	4.48 $\pm$ 0.01
Titrateable acidity ³	1.04 $\pm$ 0.03 ^a	0.83 $\pm$ 0.02 ^b	0.76 $\pm$ 0.06 ^c	0.82 $\pm$ 0.04 ^a	0.73 $\pm$ 0.05 ^c
Microbial counts ⁴					
<i>Streptococcus thermophilus</i>					
d 1	9.2 $\pm$ 0.21 ^a	9.01 $\pm$ 0.6 ^a	7.91 $\pm$ 1.02 ^a	8.8 $\pm$ 0.29 ^a	9.03 $\pm$ 0.35 ^a
d 14	8.95 $\pm$ 0.21 ^b	9.51 $\pm$ 0.52 ^a	7.48 $\pm$ 0.53 ^c	8.85 $\pm$ 0.06 ^b	8.65 $\pm$ 0.07 ^b
d 28	8.47 $\pm$ 0.63 ^a	8.5 $\pm$ 0.29 ^a	7.63 $\pm$ 0.41 ^a	8.46 $\pm$ 0.52 ^a	8.39 $\pm$ 0.15 ^a
<i>Lactobacillus delbrueckii</i> ssp. <i>bulgaricus</i>					
d 1	8.13 $\pm$ 0.31 ^a	7.77 $\pm$ 0.63 ^a	8.08 $\pm$ 0.42 ^a	7.91 $\pm$ 0.19 ^a	8.3 $\pm$ 0.43 ^a
d 14	7.84 $\pm$ 0.63 ^a	5.53 $\pm$ 0.4 ^b	5.62 $\pm$ 0.36 ^b	7.38 $\pm$ 1.62 ^a	8.01 $\pm$ 0.45 ^a
d 28	6.96 $\pm$ 2.49 ^a	2.47 $\pm$ 0.19 ^b	1.7 $\pm$ 0.16 ^c	5.09 $\pm$ 2.09 ^a	6.9 $\pm$ 0.30 ^a

^{a-c}Values are means $\pm$ SD; different superscript letters within the same parameter and day of storage significantly differ ($P < 0.05$).

¹HT+SMP = heat-treated milk (90°C for 90 s) homogenized 15 MPa and enriched with 3% skim milk powder (SMP); UHPH = ultra-high-pressure homogenized milk homogenized at 200 and 300 MPa and 30 or 40°C.

²No differences in total solids were observed between 30 and 40°C in the 200 and 300 MPa pressure treatments.

³Titrateable acidity expressed as g of lactic acid/100 g of yogurt.

⁴Microbial counts expressed at log₁₀ cfu/g of yogurt.

Table 2. Amount of whey proteins in the pH 4.6-soluble fraction of yogurts from milk during 28 d of cold storage

Item	Treatment ¹				
	HT+SMP	200 MPa		300 MPa	
		30°C	40°C	30°C	40°C
α-LA, mg/mL					
d 1	0.254 ^a	0.268 ^{ab}	0.228 ^c	0.246 ^{abc}	0.241 ^{bc}
d 14	0.245 ^{ab}	0.260 ^a	0.230 ^b	0.259 ^a	0.226 ^b
d 28	0.236 ^a	0.213 ^a	0.237 ^a	0.256 ^a	0.220 ^a
β-LG, mg/mL					
d 1	0.266 ^c	0.703 ^a	0.554 ^b	0.500 ^b	0.460 ^b
d 14	0.228 ^c	0.649 ^a	0.533 ^b	0.512 ^b	0.466 ^b
d 28	0.225 ^b	0.555 ^a	0.550 ^a	0.516 ^a	0.416 ^a

^{a-c}Values are means; different superscript letters within the same parameter and day of storage significantly differ ($P < 0.05$).

¹HT+SMP = heat-treated milk (90°C for 90 s) homogenized 15 MPa and enriched with 3% skim milk powder (SMP); UHPH = ultra-high-pressure homogenized milk homogenized at 200 and 300 MPa and 30 or 40°C.

A decrease in β -LG was observed during storage only in yogurts from milk UHPH-treated at 200 MPa and 30°C. As shown in Table 2, the amount of soluble β -LG at d 1 was greater in yogurts from milk UHPH-treated at 200 MPa and 30°C than in the rest of the samples. However, the amount of soluble β -LG in these samples decreased from d 14, indicating that the structure of β -LG could be affected by the UHPH treatment at 200 MPa, which would make the protein susceptible to hydrolysis by starter cultures. This late utilization of β -LG is in agreement with Bertrand-Harb et al. (2003) who noted that starter cultures require longer for the hydrolysis of β -LG compared with yogurt fermentation.

Peptide Profiles. Peptide profiles in the pH 4.6-soluble fraction were qualitatively similar between treatments. The supply of these peptides from caseins by the lactobacillus PrtB is essential for the survival of *Strep. thermophilus*, because they will act as assimilable nitrogen compounds when those of milk are exhausted (Courtin et al., 2002). Nevertheless, these peptides can be utilized by both *L. delbrueckii* ssp. *bulgaricus* and *Strep. thermophilus* by means of their aminopeptidases (Law and Haandrikman, 1997; Savijoki et al., 2006). As shown in Figure 2, levels of hydrophilic peptides at d 1 were similar between treatments, except those in yogurts from HT+SMP milk, which were greater albeit not significantly, probably because of the addition of SMP to the milk base. In general terms, hydrophilic peptides slightly decreased during storage in all samples because of their utilization by both starter cultures. Hydrophilic peptides in yogurts from milk UHPH-treated at 200 MPa at both inlet temperatures are in agreement with results obtained for casein hydrolysis. On the one hand, in yogurts from milk UHPH-treated at 200 MPa and

40°C, an increase in the level of these peptides was observed at d 14, probably because of the liberation of endoproteases as a result of lactobacilli autolysis, as explained in the previous section. On the other hand, this increase was not observed in yogurts from milk UHPH-treated at 200 MPa at 30°C, probably because of the high lipolysis developed in these samples (Serra et al., 2008d) interfering in the proteolytic activity.

However, in both yogurts from milk UHPH-treated at 200 MPa, levels of hydrophilic peptides significantly decreased at d 28 of storage. This fact, together with the utilization of these peptides by *Strep. thermophilus*, could be explained by assuming an important release of proteolytic enzymes capable of hydrolyzing these peptides, because of lactobacilli cell lysis. It should be mentioned that the proteolytic system of starter cultures is mainly composed of exoproteases located in the cytoplasm (Savijoki et al., 2006) that are capable of breaking large peptides into tri-, di-, or oligopeptides and FAA.

In the case of hydrophobic peptides, similar values were observed at the first day of storage, and a trend to increase was also observed in all treatments (Figure 2). This increase in hydrophobic peptides is in agreement with the significant increase observed in the ratio of hydrophobic:hydrophilic peptides in all treatments. These hydrophobic peptides are highly related to β -casein hydrolysis by *L. delbrueckii* ssp. *bulgaricus* (Law and Haandrikman, 1997), but also to α _{s1}-casein hydrolysis, which explains the significant correlation factors observed between residual α _{s1}- and β -casein and levels of hydrophobic peptides. The same correlation has been reported by other authors (Pereda et al., 2008) in milk UHPH-treated under the same conditions. As shown in Figure 2, this increase was more marked in treatments

Figure 2. Area counts of A) hydrophilic and B) hydrophobic peptides in the pH 4.6-soluble fraction of yogurts from ultra-high-pressure homogenized milk (230 = 200 MPa at 30°C; 240 = 200 MPa at 40°C; 330 = 300 MPa at 30°C; 340 = 300 MPa at 40°C) and HT+SMP milk (treated at 90°C for 90 s, homogenized at 15 MPa, and enriched with 3% skim milk powder) at d 1 (white bars), d 14 (gray bars), and d 28 (hatched bars) of storage. ^{a,b}Different letters indicate differences between d 1, 14, and 28 within the same treatment; error bars indicate standard deviation.

at 200 MPa, probably because of the massive release of endoproteases from d 14 as a consequence of the loss of viability of *L. delbrueckii* ssp. *bulgaricus*, as explained above.

High levels of hydrophobic peptides are generally not desired because they could affect the organoleptic characteristics of the product, because hydrophobic peptides are associated with bitter taste in yogurt (Schieber and Brückner, 2000; Tamime and Robinson, 2007). Nevertheless, most of the identified bioactive peptides released from caseins are known to be hydrophobic (Schieber and Brückner, 2000; Courtin and Rul, 2003; Tzvetkova et al., 2007).

pH 4.6-Soluble Nitrogen and FAA

Results of pH 4.6-soluble nitrogen and FAA are presented in Table 3. Levels of FAA have been widely used

by many authors for evaluating the proteolytic activity of yogurt cultures (Oberg et al., 1991; Abraham et al., 1993; Shihata and Shah, 2000; Donkor et al., 2006). However, it is noteworthy that levels of FAA are influenced by many factors. On one hand, the amount of FAA is conditioned by their release from soluble peptides and caseins, and on the other hand, by their synthesis de novo by starter cultures as in the case of BCAA. However, FAA can also be removed from the medium when used as intermediary products for obtaining flavor compounds such as aldehydes, acids, and esters (Savijoki et al., 2006) or when metabolized as assimilable source of nitrogen by starter cultures. In the present study, levels of FAA did not follow a clear trend, which was expected considering all the above-mentioned factors. In general, the FAA concentration was slightly greater in yogurts from HT+SMP milk, which could be explained by the addition of SMP. Within UHPH samples, both

Table 3. Amount of soluble nitrogen and free amino acids (means $\pm$ SD) in the pH 4.6-soluble fraction of yogurts from milk during 28 d of cold storage

Item	Treatment ¹				
	HT+SMP	UHPH at 200 MPa		UHPH at 300 MPa	
		30°C	40°C	30°C	40°C
Soluble N, % of total N					
d 1	0.088 $\pm$ 0.006 ^c	0.113 $\pm$ 0.005 ^a	0.104 $\pm$ 0.004 ^b	0.097 $\pm$ 0.004 ^b	0.099 $\pm$ 0.003 ^b
d 7	0.092 $\pm$ 0.004 ^d	0.122 $\pm$ 0.003 ^a	0.103 $\pm$ 0.002 ^b	0.101 $\pm$ 0.006 ^{bc}	0.097 $\pm$ 0.002 ^c
d 14	0.093 $\pm$ 0.004 ^c	0.122 $\pm$ 0.004 ^a	0.112 $\pm$ 0.003 ^b	0.107 $\pm$ 0.007 ^b	0.104 $\pm$ 0.003 ^b
d 21	0.094 $\pm$ 0.005 ^c	0.121 $\pm$ 0.012 ^a	0.114 $\pm$ 0.002 ^a	0.104 $\pm$ 0.009 ^b	0.104 $\pm$ 0.004 ^b
d 28	0.098 $\pm$ 0.007 ^c	0.127 $\pm$ 0.004 ^a	0.113 $\pm$ 0.004 ^b	0.103 $\pm$ 0.007 ^c	0.105 $\pm$ 0.002 ^c
Free AA, mg of Leu/mL of whey					
d 1	0.253 $\pm$ 0.078 ^a	0.122 $\pm$ 0.019 ^b	0.249 $\pm$ 0.038 ^a	0.117 $\pm$ 0.019 ^b	0.101 $\pm$ 0.082 ^b
d 7	0.313 $\pm$ 0.092 ^a	0.194 $\pm$ 0.031 ^b	0.213 $\pm$ 0.039 ^b	0.117 $\pm$ 0.043 ^c	0.278 $\pm$ 0.052 ^{ab}
d 14	0.275 $\pm$ 0.079 ^a	0.158 $\pm$ 0.027 ^b	0.209 $\pm$ 0.024 ^b	0.148 $\pm$ 0.026 ^b	0.204 $\pm$ 0.023 ^b
d 21	0.285 $\pm$ 0.057 ^a	0.215 $\pm$ 0.013 ^a	0.276 $\pm$ 0.043 ^a	0.102 $\pm$ 0.036 ^b	0.287 $\pm$ 0.067 ^a
d 28	0.270 $\pm$ 0.093 ^a	0.157 $\pm$ 0.008 ^b	0.319 $\pm$ 0.030 ^a	0.156 $\pm$ 0.026 ^b	0.303 $\pm$ 0.034 ^a

^{a-d}Different superscript letters within the same parameter and day of storage significantly differ ($P < 0.05$).

¹HT+SMP = heat-treated milk (90°C for 90 s) homogenized 15 MPa and enriched with 3% skim milk powder (SMP); UHPH = ultra-high-pressure homogenized milk homogenized at 200 and 300 MPa and 30 or 40°C.

treatments at 40°C presented slightly greater values of FAA compared with the same treatments at 30°C. In the case of yogurts from milk UHPH-treated at 200 MPa and 40°C, these greater levels could result from the proteolytic enzymes released, as explained above, which is also in accordance with the greater levels of hydrophobic peptides detected in these samples. On the contrary, in yogurts from milk UHPH-treated at 300 MPa at 40°C, these greater levels at the end of storage could be explained on the basis of synthesis de novo of BCAA, in agreement with the lower levels of diketones detected in these samples in a previous study (M. Serra, unpublished data), because BCAA regulates the synthesis of the latter.

Levels of soluble nitrogen in the pH 4.6-soluble fraction are mainly influenced by the amount of soluble WP and by the level of soluble peptides. In this sense, yogurts from HT+SMP had the lowest values of soluble nitrogen, whereas those from milk UHPH-treated at 200 MPa at 30°C had the greatest values, in agreement with the degree of denatured β -LG. In general, values of soluble nitrogen tended to increase in all treatments, in accordance with the increase in hydrophobic peptides in the pH 4.6-soluble fraction.

CONCLUSIONS

The present study indicated that proteolysis was very similar between all treatments studied. From these results, it could be concluded that the UHPH treatment of the milk, in particular at 300 MPa, resulted in yogurts with similar proteolytic profiles compared with those obtained following the conventional process

in industry. Greater concentrations of hydrophobic peptides were found only in yogurts made from milk homogenized at 200 MPa at both inlet temperatures; this increase in hydrophobic peptides could result in an undesirable bitter taste. One of the most relevant findings in the present work is that levels of hydrophobic peptides, which are of special interest for their potential biological activities, were found in similar concentrations between yogurts from milk treated at 300 MPa and in HT+SMP milk, even though no SMP was added to the samples undergoing UHPH treatment.

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