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Transglutaminase treatment of thermally and high pressure processed milk: Effects on the properties and storage stability of set yoghurt

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ABSTRACT

The objectives of this work were to study the effect on the quality parameters of set yoghurt made from high pressure (HP) and transglutaminase (TGase) treated milk (separately or in combination), and to determine the shelf life of this product. Yoghurt samples made from HP— in combination with TGase-treated milk exhibited the highest values of firmness, lowest values of whey separation and similar values of acidity with the other samples. All yoghurt samples made from HP-treated milk, (with or without subsequent TGase treatment), exhibited a creamier perception than the ones from thermally-treated milk. Yoghurt samples prepared by the conventional procedure were judged as unacceptable after the fifth week of storage due to an intense syneresis and separation of the coagulum from the cup. Overall, HP and TGase treatment of milk can be a useful tool for the dairy industry to achieve products of improved structure and desirable sensorial characteristics.

Industrial relevance: In order to achieve a tight and compact structure of yoghurt, solid fortification and/or addition of stabilizers is needed. Transglutaminase and high pressure treatment of milk (when applied individually or in combination) can be alternative treatments of milk to produce yoghurt with improved textural and sensorial characteristics, without dependence on costly solid fortification.

1. Introduction

The texture of yoghurt is determined by the composition of the milk base and the method which is used for its production. Any alteration related to the above parameters can lead to a significant modification to the structure and texture of the final product (Lankes, Ozer, & Robinson, 1998). Many techniques have been introduced in order to improve the body and structure of yoghurt, mainly involving modification of milk composition by fat replacement with starches, gelatin, gums or carrageenan (Keogh & O'Kennedy, 1998; Teles & Flôres, 2007).

High pressure (HP) processing has been proposed as an alternative method of modifying the functionality of milk components, such as caseins and whey proteins, while it can lead to significant inactivation of pathogenic and spoilage microflora of milk, with limited effect on indigenous milk enzymes and its quality characteristics and nutritional value (Huppertz, Smiddy, Upadhyay, & Kelly, 2006; Law et al., 1998; Lopez-Fandino, de la Fuente, Ramos, & Olano, 1998; Trujillo et al., 2008; Trujillo, Capellas, Saldo, Gervilla, & Guamis, 2002; Moatsou et al., 2000). It has been demonstrated that HP treatment of the milk base used for the production of yoghurt, alters the quality parameters of the final product (Huppertz et al., 2006; Hemar, Liu, Meunier, & Woonton, 2010; Lanciotti, Vannini, Pittia, & Guerzoni, 2004). The rate of acidification of HP-treated milk is higher than of thermally treated milk. Moreover, HP-treated milk coagulates at higher pH value and yoghurt prepared from HP-treated milk exhibits lower amounts of whey separation and increased gel strength (Ferragut, Martinez, Trujillo, & Guamis, 2000; Harte, Amonte, Luedecke, Swanson, & Barbosa-Cánovas, 2002; Harte, Luedecke, Swanson, & Barbosa-Cánovas, 2003; Needs, Capellas, et al., 2000; Needs, Stenning, Gill, Ferragut, & Rich, 2000).

Transglutaminase (TGase) is an enzyme able to introduce covalent cross-links in proteinaceous systems by catalyzing acyl transfer reactions between the g-carboxyamide group of peptide or protein bound glutamine (acyl donor) and primary amines (acyl acceptor) including the e-amino group of lysine residues. When the e-amino group of protein bound lysine reacts as an acyl acceptor, intra-molecular and/or intermolecular cross-links (isopeptide bonds) are formed, resulting in the polymerization of proteins (Dickinson, 1997). Among milk proteins, caseins can be easily cross-linked by TGase due to their flexible and, little or no secondary structure, while whey proteins are not efficiently cross-linked due to their globular compact structure (de Jong & Koppelman, 2002; Lorenzen, 2002; O'Connell & de Kruif, 2003; Sharma, Zakora, & Qvist, 2002). Although, κ-casein was found to be the most susceptible to cross-linking in unheated milk followed by β-casein (Sharma, Lorenzen, & Qvist, 2001), it was shown that heat pre-treatment of milk enhances protein cross-linking by TGase treatment (Kulozik, Tolkach, Bulca, & Hinrichs, 2003; Rodriguez-Nogales, 2006a, 2006b) and, the used concentration of TGase is an important factor in the physicochemical characteristics and textural attributes of the

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final product (Kuraishi, Yamazaki, & Susa, 2001; Ozer, Kirmaci, Oztekin, Hayaloglu, & Atamer, 2007).

Thermal pre-treatment of milk can enhance protein cross-linking by TGase (Kulozik et al., 2003; Rodriguez-Nogales, 2006a, 2006b). TGase treatment of milk can be applied prior or during the fermentation of the milk. In the first case the enzyme should be subsequently inactivated, usually by a thermal treatment step (Kuraishi et al., 2001) or by chemical inhibition, i.e. with N-Ethylmaleimide (NEM) (Jacob, Noebel, Jaros, & Rohm, 2011). The utilization of TGase in dairy products results in altered firmness, viscosity, water holding capacity, stability, fermentation capacity and mechanical properties of the gel (Boenisch, Huss, Lauber & Kulozik, 2007; Cancino, Fuentes, Kulozik, & Boenisch, 2006; Farnsworth, Li, Hendricks, & Guo, 2006; Guyot & Kulozik, 2010; Jaros, Partschefeld, Henle, & Rohm, 2006; Lorenzen, Neve, Mautner, & Schlimme, 2002; Ozrenk, 2006).

The combined treatment of milk with HP and TGase applied individually or simultaneously before producing gels acidified with glucono-δ-lactone (GDL) has been studied by Anema, Lauber, Lee, Henle, and Klostermeyer (2005). It was shown that the combined treatment of TGase under HP conditions resulted in gels with lower gelation time, higher gelation pH and higher storage modulus (G′) values than those obtained by HP or TGase treatment individually, suggesting an increased level of cross-linking of the milk proteins when milk is treated with TGase under HP compared with treatment under atmospheric pressure conditions.

In yoghurt production practice in order to achieve a final gel of increased firmness, demanded by the current consumer trends, milk powders, including non-fat dry milk or milk protein concentrates, are blended with the milk during the standardization procedure. The total solids content of milk can also be increased by evaporation under vacuum and membrane processing and by adding to the milk base stabilizers, like pectin or gelatin, to enhance yoghurt texture, and prevent wheying-off (Tamime & Robinson, 1999). However, the use of stabilizers, can lead to over-stabilization (gelatinous texture) or under-stabilization (weak texture and whey separation), while in some countries regulations do not allow the use of stabilizers for plain (unsweetened) yoghurt (Lee & Lucey, 2010).

The replacement of milk fortification with protein concentrates or stabilizers with HP and/or TGase treatment of milk and milk proteins to achieve products with improved structure needs further exploration in view of a potential commercial application. The objectives of this study were a) to evaluate the effect of HP or TGase treatment or the combined treatment of milk on the quality attributes of set yoghurt at different levels of added protein, and b) to investigate the effect of the above treatments of milk on the shelf life of set yoghurt during refrigerated storage, to determine whether such a product could be produced and be commercially viable.

2. Materials and methods

2.1. Sample preparation

Homogenized milk of different levels of protein content (3.00%, or standardized to 3.45 and 3.70%) was provided directly from the plant of a leading dairy company. The standardization of the protein content of milk from the initial level of 3.00% was achieved by adding external protein, with a ratio of 50:50 caseins to whey proteins. After standardization of milk proteins, the casein:whey protein (CWP) ratio for each milk was equal to 80:20, 76.5:23.5 and 74:26 for milks of 3.00, 3.40 and 3.75% protein content, respectively. The fat content of milk was standardized to 4.0%. Both protein and fat content standardization were performed by the dairy company. Homogenization was carried out in a two stage industrial homogenizer at 150/50 bar. Milk was either subjected to thermal (85 °C for 30 min) or HP (600 MPa/55 °C for 10 min) treatment.

2.2. HP treatment

HP treatments were performed using a laboratory-scale HP system with a maximum operating pressure of 1000 MPa (Food Pressure Unit FPU 1.01, Resato International BV, Roden, Netherlands), consisting of an HP unit with a pressure intensifier, an HP vessel of 1.5 L volume and a multi-vessel system consisting of six vessels of 42 mL capacity each. All HP vessels are surrounded by a water circulating jacket connected to a temperature control system. The pressure-transmitting fluid used was polyglycol ISO viscosity class VC 15 (Resato International BV, Netherlands). Milk samples of 750 mL volume were put into multilayer (PP, foil, PE) packaging and placed in the 1.5 L chamber for processing. The desired value of pressure was set and, after pressure build-up (approximately 20 MPa s^{-1}), the pressure vessel was isolated; this point defined the zero time of the process. Pressure of the vessel was released after a preset time interval (10 min pressurization time) by opening the pressure valve (release time <3 s). The initial temperature increase during pressure build-up (about 3 °C per 100 MPa) was taken into consideration in order to achieve the desired operating temperature. Pressure and temperature were constantly monitored (intervals of 1 s) and recorded during the process. All samples were pressurized at 600 MPa and 55 °C for a process time of 10 min. After processing, samples were kept to overnight storage at 4 °C until use.

2.3. Enzymic cross-linking with transglutaminase (TGase)

Thermally and HP-treated milk was heated to 42 °C, inoculated with TGase at a concentration of 2.2 U/g protein (ACTIVA YG, Ajinomoto, 100 U/g transglutaminase activity). Incubation of milk with the enzyme was carried out into 2 L sterile beakers, which were placed into a water-bath of controlled temperature (43 ± 0.2 °C) for 180 min. The milk was then rapidly heated at 80 °C for 1 min in order to inactivate the enzyme and cooled down to the fermentation temperature. Samples not treated with TGase were also incubated at 42 °C for 180 min, then heated at 80 °C for 1 min and cooled down to the fermentation temperature.

2.4. Yoghurt preparation

Milk was heated to fermentation temperature and inoculated with commercial starter culture. The starter culture was prepared as a 1:5 (w/v) dilution of freeze-dried YC-X11 culture (Christian Hansen, Denmark) in commercial UHT skim milk and maintained in cool storage until inoculation. The inoculated milk was divided into UV-sterile plastic cups and sealed with foil. Incubation at 42 °C was followed until pH reached 4.75. After fermentation, yoghurts were transferred to a refrigerator at 4.8–5.1 °C and stored until testing. For comparison of the applied treatments in milk, tests were performed three days after the production of the samples. For shelf life study, samples were tested in weekly intervals and as zero time it was considered the day after the production of the yoghurts.

2.5. Fermentation time

A 0.001 precision pH meter (AMEL 338, AMEL Instruments, Italy) was used for pH measurements during fermentation procedure. Fermentation time is defined as the time needed for milk coagulum to reach pH value of 4.75, which is a common pH value of commercial set yoghurt and was recommended by the Greek dairy industry.

2.6. Study on the quality attributes of yoghurt

2.6.1. Microbiological analysis

Yoghurt sample of 10 g was transferred to a sterile stomacher bag with 90 mL sterilized Ringer solution (1.15525, Merck, Germany) and was homogenized for 60 s with a Stomacher (BagMixer® interscience,

France). Samples of 10-fold serial dilutions of yoghurt homogenates were spread on the surface of the appropriate growth media in Petri dishes for enumeration of different microorganisms. Yeast and mold viable count was enumerated on RBC Agar (1.00467, Merck, Germany) after incubation at 25 °C for 72 h under aerobic conditions. *Streptococcus thermophilus* was enumerated on M17 Agar (1.15108, Merck, Germany) after incubation at 37 °C for 24 h under aerobic conditions. For *Lactobacillus bulgaricus* enumeration the pour-plate method on De Man-Rogosa-Sharpe Agar with modified pH value at 4.58 (1.10660, Merck, Germany) was used, followed by incubation at 45 °C for 72 h in anaerobic jars with an Anaerocult A catalyst (1.16387/1.13829, Merck, Germany). Two replicates of at least three appropriate dilutions were enumerated.

2.6.2. Physicochemical analysis

The acidity of yoghurts was measured using a pH meter (WTW 522, Germany) and, by titration of a 1:1 mixture of yoghurt/ionized water with 0.1 N NaOH using phenolphthalein as an indicator and expressed as % lactic acid. Syneresis of yoghurt was expressed as the grams of separated whey of 100 g of sample after incubation at 4 °C for 3 h. An amount of 100 g was transferred to a funnel with Whatman paper #1 placed on a conical flask. The flask then stored at 4 °C and the amount of eliminated serum was weighted after 3 h of storage.

2.6.3. Texture Profile Analysis

Texture analysis was performed using a TA-XT Plus texture analyzer with a load cell of 5 kg (Stable Micro Systems Ltd., UK). Samples were transferred from 5 °C to a refrigerator of 10 °C until testing. Samples were subjected to Double Compression test to construct the Texture Profile Analysis (TPA) graphs, using a clear acrylic cylinder probe TA3/1000 of 25.4 mm diameter and 35 mm long (Brookfield Viscometers Ltd., Harlow Essex, UK). The following parameters have been set; pre- and post-test speed at 10 mm/s; test speed at 4 mm/s; distance at 13 mm; temperature at 10 °C; and force at 4 g. Force–time (g–s) curves were recorded and analyzed using the Texture Exponent 32 Application (Stable Micro Systems Ltd, UK) and texture analysis parameters (firmness, cohesiveness, adhesiveness, index of elasticity and gumminess) were calculated.

2.6.4. Sensory evaluation

Sensory evaluation was based on the recognition of the relative importance of selected quality attributes such as the overall appearance, taste and odor, mouthfeel and textural attributes. The panel group consisted of eight trained panelists. Samples were presented to the panelists in plastic-covered coded containers (200 mL). Four samples were presented to the panel group at each session and the sessions were repeated five times. Rating was assigned separately for each parameter on a 1 to 9 descriptive hedonic scale (9 being the highest quality score and 1 the lowest). A score of 5 was taken as the average score for minimum sensory acceptability.

2.7. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE analysis was performed in milk samples of 3.00% protein content in all treatments of milk. For determination of the extension of intermolecular cross-linking, milk samples were analyzed by SDS-PAGE. Prior to SDS-PAGE analysis, milk samples were defatted by centrifugation at 2000× *g* for 20 min at 4 °C, and the subnatant was collected and analyzed. Defatted milk samples were diluted 2× or 5× with SDS buffer (2% SDS, 25 g L^{-1} Tris pH = 6.8, glycerol, 2-Mercaptoethanol, 0.1% bromophenol blue). The separation of the protein bands was carried out on a 12% precast gel (Mini-PROTEAN TGX, BioRad Lab., US) using Mini-PROTEAN system (Mini-PROTEAN TGX, BioRad Lab., US). Gels were run at 35 mA and then stained using 4 g L^{-1} Coomassie Brilliant Blue G in a 5:4:1 mixture of de-ionized water, methanol and acetic acid. The relative intensity of the stained bands was determined by scanning the gel (HP Scanjet 4670, China).

2.8. Scanning Electron Microscopy (SEM)

SEM was performed for samples prepared with milk of 3.00% protein content in all treatments of milk. Samples were freeze-dried using a laboratory scale freeze-drying unit (Alpha 1-4LDplus, CHRIST, Germany) and then gold-palladium coated in vacuum using a sputtering device (Polaron 5100). Microstructures of yoghurts were examined with a FEI Quanta 200 (FEI Company, Netherlands) scanning electron microscope using a large-field detector (LFD) and operating at 25 kV and at ×2000 magnification.

2.9. Statistical analysis

Three-way analysis of variance (ANOVA) was applied for the determination of the main effects of the investigated factors (protein content, milk processing, enzyme treatment) and their interactions on the experimental data. Duncan's post hoc means comparison test was used to separate means of data when significant differences ($p<0.05$) were observed. One way analysis of variance (ANOVA) at a significance level of 95% was used for the analysis of all quality attributes in the shelf life study of yoghurt samples (STATISTICA® 7.0, StatSoft Inc., Tulsa, USA).

3. Results & discussion

3.1. Fermentation time of milk

Milk subjected to treatment with TGase exhibited reduced but not significantly different fermentation times compared to the ones of thermally treated milk (approximately 210 and 230 min, respectively), which is in agreement with previous studies reporting that the pre-treatment of milk with TGase had minor effect on the fermentation time of milk (Boenisch, Huss, Weitl & Kulozik, 2007; Færgemand et al., 1999; Lorenzen et al., 2002). However, when HP treatment was applied in milk, fermentation times exhibited a significant decrease ($p<0.05$) of 30 to 40 min than those of thermally treated milk, regardless of the protein content of milk. It is known that pressure and heat cause changes in the serum environment of milk affecting the activity of the enzymes involved in lactose hydrolysis and thus, in altering the acidification rate in cultured dairy products (Anema et al., 2005; Needs, Stenning, et al., 2000). Our results indicate that these changes may be more enhanced in the case of HP-treated milk than in thermally treated milk. Results showed that the addition of external protein had minor affect on fermentation time, indicating that the buffering capacity of the milk base is not altered by protein fortification (Boenisch, Huss, Weitl, et al., 2007).

3.2. Viability of thermophilic bacteria

The viable counts of the starter culture remained above the desirable limits (total count > 7 logCFU/g) during refrigerated storage. The viability of *L. bulgaricus* was found to be slightly dependent on the applied treatment of milk ($p<0.05$). Both microorganisms of the starter culture found to have greater viability in samples made of HP- or TGase-treated milk than in the samples made of thermally treated milk. This observation is in agreement with previous studies which reported that TGase treatment of milk enhances the viability of thermophilic and probiotic bacteria (Farnsworth et al., 2006). With regard to yeasts and molds, no growth (<2 logCFU/g) was observed in all tested samples during the 7 weeks of storage period.

3.3. Physicochemical properties of the coagulum

The changes in pH values and titratable acidity (TA) for all tested samples during refrigerated storage are illustrated in Fig. 1. The pH value of 4.75 at the end of fermentation time decreased to values ranging from 4.59 to 4.38, after overnight cool storage (Day 0). This decrease was attributed to the continued fermentation during overnight cooling until the temperature of the product reached 5 °C. It was observed that samples made from TGase-treated milk exhibited higher ($p<0.01$) changes in pH value, compared to samples from TGase-untreated milk. Samples made from HP-treated milk exhibited similar final pH values with the ones made from TGase-treated milk. It is reported that TGase treatment of milk affects the acidity of the final product, but after refrigerated storage post-acidification is reduced for samples made from TGase-treated milk compared to TGase-untreated ones (Farnsworth et al., 2006; Ozer et al., 2007). Similar results, where post-acidification was delayed after storage, were observed in the samples made from HP-treated milk. The drop in pH values was similar in yoghurts made of TGase-treated, HP-treated or combined HP-TGase-treated milk during the storage period, with a final pH value (end of shelf life) of 4.23.

The initial TA of milk (0.11–0.12%) increased to 0.79–0.80% after overnight cool storage and to 0.84–0.86% after one week at 5 °C in all tested yoghurts. This increase might be attributed to the residual fermentation changes. Yoghurt samples made from TGase-untreated milk exhibited greater increase in TA than those made from TGase-treated milk, with final TA of TGase-untreated samples in the range of 0.90–0.93% and of 0.85–0.86% for TGase-treated samples. This is attributed to a slow degree of post-acidification, due to a possible delay in bacterial multiplication and thus, to slower acidity development as it has been also observed previously (Lorenzen et al., 2002; Ozer et al., 2007).

With regard to whey separation, an increase in the protein content of the milk decreased the percentages of the separated serum. When milk was treated either with TGase or HP, the percentages of the separated serum significantly decreased ($p<0.001$) compared to those obtained from samples made from thermally treated milk (Fig. 2, zero time data). The smallest amount of whey separation (highest improvement) was observed in samples made from combined HP-TGase-treated milk. A decrease of whey separation from 31% to 17% was observed between samples made from thermally treated milk and those made from HP-TGase treated milk respectively.

It is has been reported that TGase treatment of milk under high pressure conditions increased the rate and degree of cross-linking of whey protein and intermolecular cross-linking between whey protein and casein (Anema et al., 2005). HP treatment of milk causes significant dissociation of micelles and denaturation of whey εproteins depending on the applied conditions. When pressure is released caseins tend to re-associate and, for applied pressures above 450 MPa and temperatures 70 or 90 °C, this re-association seems to lead to smaller micelles than the original ones (Udabage et al., 2010). The aforementioned mechanism combined with a following protein cross-linking by the TGase treatment could account for the formation of a tighter protein network with smaller casein micelles and thus with smaller water permeability than the one achieved by applying each treatment of milk separately. Our results suggest that HP and TGase treatment of milk, applied sequentially, are capable to have a synergistic effect on the improvement of water holding capacity of yoghurt gels.

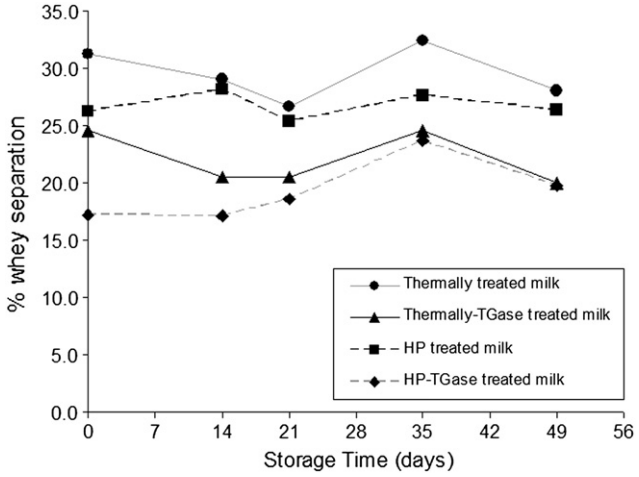

Fig. 2. Changes in whey separation during the storage period for samples with 3.00% protein content made from different treated milk (Day 0 is considered after overnight storage at 5 °C).

No significant changes ($p>0.5$) on whey separation were observed during the storage period for each tested yoghurt sample (Fig. 2). Yoghurts made from thermally or HP-treated milk followed by TGase treatment exhibited lower percentages of syneresis compared to those from yoghurts made from thermally or HP-treated milk without subsequent TGase treatment during the storage period. Yoghurts made from HP-TGase-treated milk exhibited the lower percentages of syneresis, while those made from thermally treated milk had the highest percentages, which is in agreement with previous studies (Boenisch, Huss, Lauber, et al., 2007; Farnsworth et al., 2006; Guyot & Kulozik, 2010; Kuraishi et al., 2001; Lanciotti et al., 2004; Lorenzen et al., 2002; Needs, Stenning, et al., 2000). Moreover,

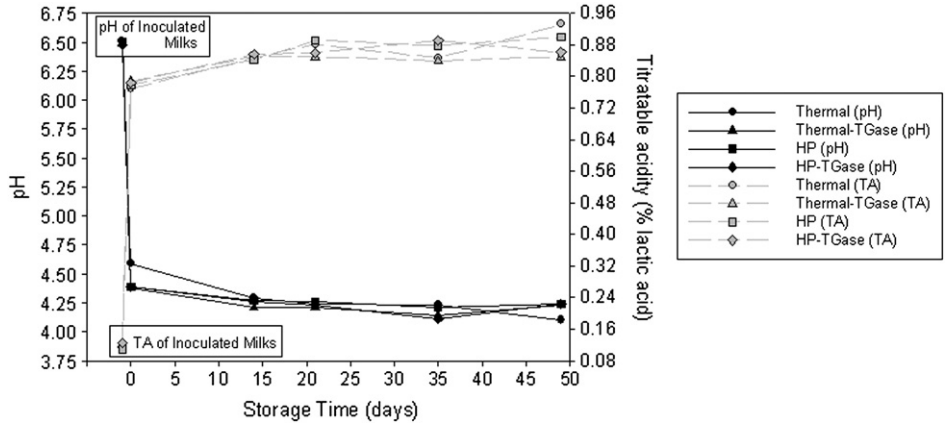

Fig. 1. Changes in titratable acidity (TA) and pH during the storage period of 7 weeks for samples with 3.00% protein content made from different treated milk (the values of inoculated milks are referred to measurements after the inoculation with starter culture; Day 0 is considered after overnight storage at 5 °C).

yoghurts made from thermally treated milk revealed whey separation on the surface of the coagulum and detachment from the cup, especially after the fifth week of storage, which resulted in their rejection during the sensory evaluation.

3.4. Texture analysis of yoghurt samples

Firmness was found to be significantly dependent on all tested conditions; thermal or HP processing ($p<0.001$), enzyme treatment of milk ($p<0.001$) and, to a lesser degree on protein content of milk ($p<0.01$) especially when milk of high protein content (3.75%) was used. Samples made from HP-treated milk exhibited an increase in firmness compared to samples made from thermally treated milk of about 35–40 units (Fig. 3a). The relative increase in firmness in samples made from HP-treated milk can be explained by whey protein denaturation and casein micelles alteration under HP processing, resulting in small particles which can be formed into clumps and chains (Harte et al., 2003; Needs, Stenning, et al., 2000). Previous studies reported that HP treatment of milk solely may not be capable to promote to a great extent the gel properties of yoghurt, since α-lactoalbumin is more pressure resistant and only denaturation of β-lactoglobulin is present, and thus simultaneous or subsequent thermal treatment of milk is necessary to be applied in order to achieve a final product of improved structure (Harte et al., 2003; Trujillo et al., 2002). Our results indicate that HP treatment of milk under moderately elevated temperatures (50–60 °C) could be capable to achieve the desirable level of protein denaturation and thus, to lead to products with significantly increased firmness, while no addition of external protein sources or stabilizers is needed.

Similar behavior was observed when milk was treated with TGase, with the relevant benefit being more pronounced in the milk of low protein content for which the casein ratio was higher than in the other tested milks (CWP ratio of 80:20 compared to 76.5:23.5 and 74:26 for milks with 3.40 and 3.75% protein content respectively). Caseins are reported to be the most efficient substrate for TGase reaction (de Jong & Koppelman, 2002; Sharma et al., 2002; O'Connell & de Kruif, 2003; Şanlı, Sezgin, Deveci, Şenel, & Benli, 2011). Samples made from HP and subsequent TGase-treated milk exhibited the highest increase in firmness compared to those made from either thermally treated milk or from individually applied HP or TGase treatment in milk, regardless the level of the protein content of milk. As mentioned before, it has been reported that TGase treatment of milk under high pressure resulted in acid gels with improved properties (Anema et al., 2005). In this study, results from texture analysis of the samples indicate synergistic phenomena occurring when HP and TGase treatment of milk are applied individually and in sequence, improving the yoghurt-making properties of treated milk.

For all tested samples, the values of firmness increased during the 7 weeks of refrigerated storage (Fig. 3b) and thus, these values were also dependent on the storage period ($p \ll 0.05$). Although a high percentage of whey separation was observed at the fifth week of storage, this was not followed by a decrease of the values of firmness.

Adhesiveness was found to be significantly affected from the applied treatment of milk ($p<0.004$) and the addition of external protein ($p<0.04$). Samples made from HP-treated milk exhibited higher values compared to those made from thermally treated or TGase-treated milk (Table 1). These findings could be a consequence of the different effects of HP and TGase treatment of milk on the gel properties of the final product. The denaturation of proteins due to the applied pressure can result in compact and homogenous micelles and when pressure is combined with a subsequent thermal treatment, a creamy gel could be obtained (Harte et al., 2003). A similar mechanism may be present when pressure is applied under moderately elevated temperatures (50–60 °C) as in the present study. On the other hand TGase treatment of low protein content milk, and increased casein ratio (see casein:whey protein ratios as discussed in 2.1), may result in gels with too firm

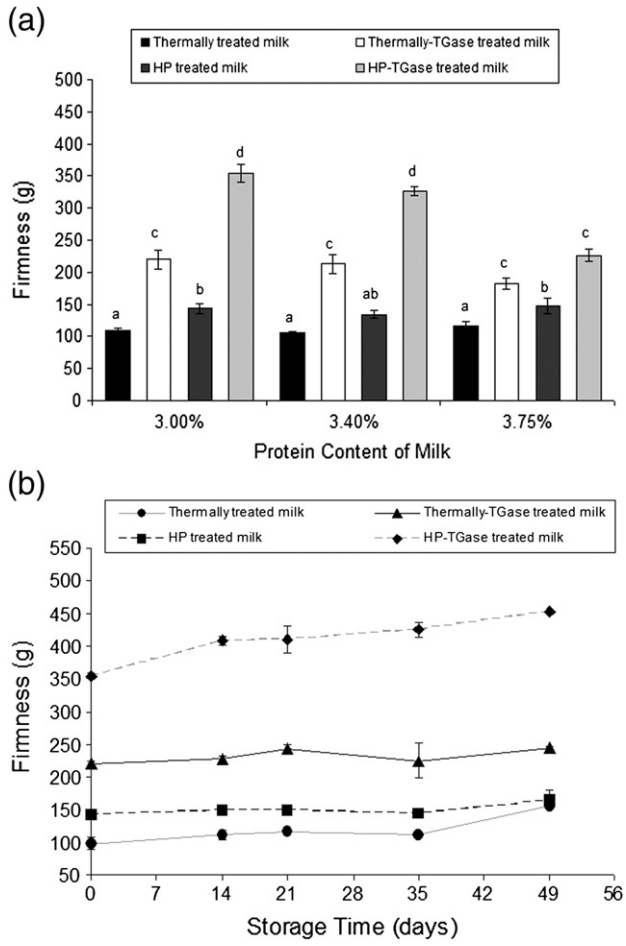

Fig. 3. (a) Effect of protein content of milk and milk treatment on the firmness of set-style yoghurt and (b) Instrumental firmness of yoghurt samples with 3.00% protein content made from different treated milk during the 7 weeks of storage (Day 0 is considered after overnight storage at 5 °C). (Mean values of four measurements ± standard deviation. Different letters between the columns indicate significant difference ($p<0.05$) among the yoghurt samples according to Duncan's mean values comparison test).

Table 1
Textural attributes of yoghurt samples of different protein content and various treatments of milk.

3.00% protein	Adhesiveness (g · s)	Cohesiveness[a]
Thermally-treated milk	105.6 ± 9.2b	0.50 ± 0.01a
Thermally-TGase-treated milk	83.5 ± 3.3a	0.50 ± 0.04a
HP-treated milk	176.8 ± 8.3e	0.53 ± 0.02a
HP-TGase-treated milk	262.4 ± 11.9 g	0.45 ± 0.06a
3.40% protein	**Adhesiveness (g · s)**	**Cohesiveness[a]**
Thermally-treated milk	116.9 ± 3.5c	0.50 ± 0.02a
Thermally-TGase-treated milk	114.5 ± 14.7bc	0.47 ± 0.04a
HP-treated milk	179.7 ± 16.2de	0.50 ± 0.04a
HP-TGase-treated milk	193.2 ± 17.8de	0.47 ± 0.04a
3.75% protein	**Adhesiveness (g · s)**	**Cohesiveness[a]**
Thermally-treated milk	122.5 ± 9.5bc	0.51 ± 0.01a
Thermally-TGase-treated milk	159.6 ± 2.7d	0.51 ± 0.01a
HP-treated milk	193.7 ± 6.7e	0.54 ± 0.02b
HP-TGase-treated milk	212.7 ± 5.4f	0.51 ± 0.02a

Values are mean ± standard deviations of results from four separate experiments. Different letters between the rows indicate significant difference ($p<0.05$) among the yoghurt samples according to Duncan's mean values comparison test.
[a] Dimensionless.

structures since thermal treatment may not cause sufficient whey protein denaturation in order to act as protective colloid for caseins and, as a result the covering surface of micellar caseins with denaturated whey proteins reduces the surface hydrophobicity and very firm and coarse structures are formed (Boenisch, Huss, Weitl, et al., 2007).

With regard to the effect of storage period on adhesiveness, no significant changes were observed during storage for yoghurt samples prepared from TGase-treated milk. The highest values of adhesiveness were observed and were maintained for yoghurts made of HP-TGase treated milk, which is in agreement with previous studies showing that HP treatment of milk increases the value of adhesiveness and the final product becomes distinctively smooth and creamy (Færgemand et al., 1999).

Concerning the cohesiveness of the prepared samples, no significant differences were observed between the tested samples regardless of the treatment of the milk and the levels of protein content (Table 1). The cohesiveness of each tested sample was maintained almost constant, with no statistical differences ($p > 0.5$), during refrigerated storage. Yoghurt samples made from thermally treated milk exhibited the lowest values of this attribute during refrigerated storage (average value of adhesiveness was 0.52) compared to other samples (average values of adhesiveness were in the range of 0.54–0.58). This is evidence that both HP and TGase treatment of milk are capable to increase the gel strength of the coagulum resulting in an improved and cohesive final product.

The index of elasticity of the prepared samples was not affected by the treatment of milk. It was found to be equal to 0.94 ± 0.01 and maintained constant during the 7 weeks of storage for all tested samples.

Overall, the presented data demonstrate that different treatments of milk significantly affected the textural attributes (mainly firmness and adhesiveness) of the final yoghurt and that, HP and TGase treatment of milk could act synergistically on the structural improvement of the coagulum.

3.5. Microstructure of the coagulum

SEM micrographs (×2000) of yoghurt made from milk of low protein level (3.00%) and in combination of the tested technologies are illustrated in Fig. 4. In general, yoghurt gels made from TGase-treated milk were more compact (tighter) compared to those made from thermally-treated milk. This was evident by the finer-meshed with less porosity network correlated to improved gel strength (Lauber, Henle, & Klostermeyer, 2000; Lorenzen et al., 2002; Farnsworth et al.,

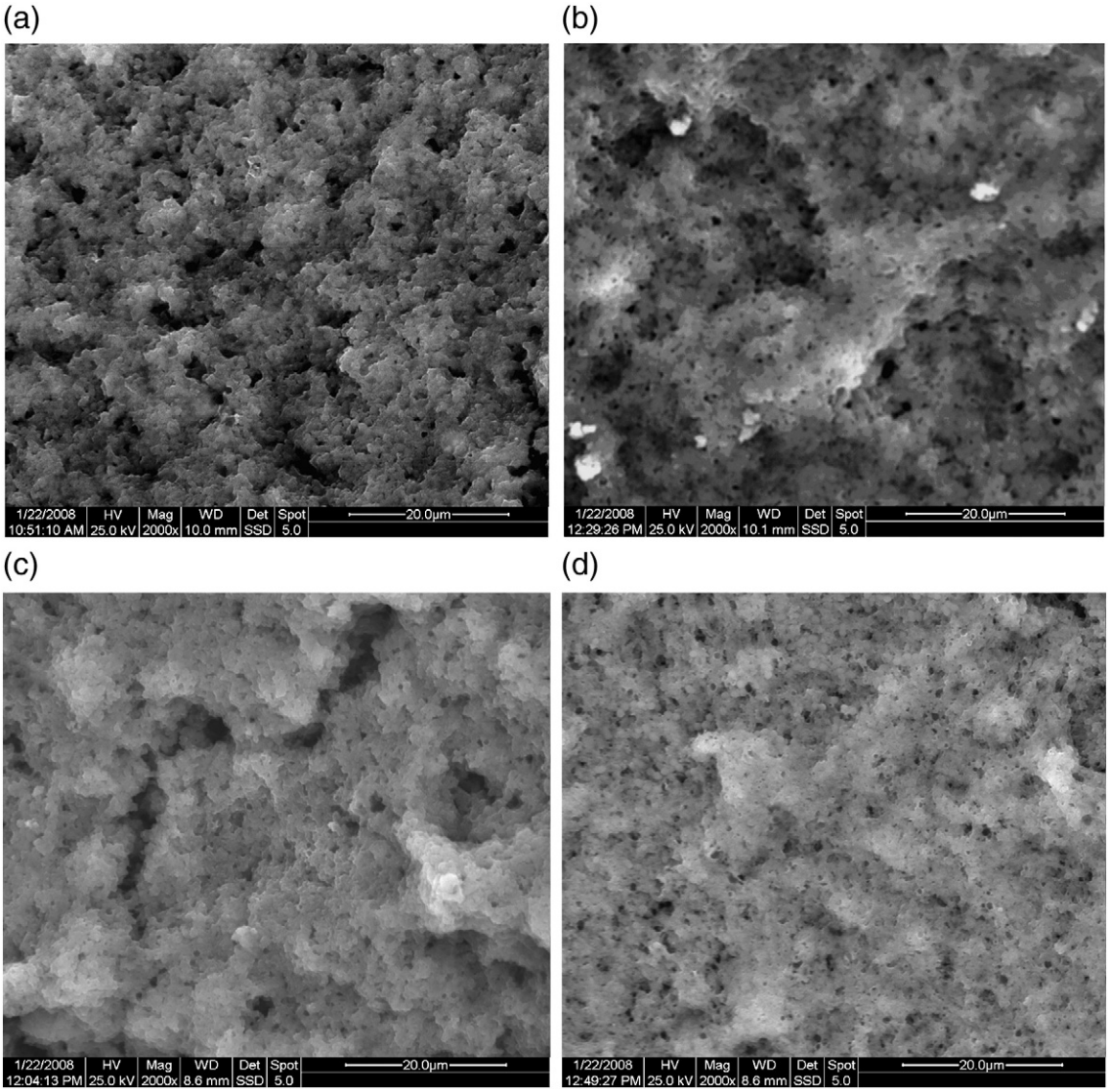

Fig. 4. Scanning electron micrographs (×2000) of set-style yoghurt with 3.00% protein content produced from thermally treated milk (a), thermally-TGase-treated milk (b), HP treated milk (c) and, HP-TGase-treated milk (d).

2006; Şanlı et al., 2011). Similar improvement of the protein network was observed for samples prepared with HP-treated milk, which is in agreement with the findings of previous studies (Harte et al., 2003; Penna, Gurram, & Barbosa-Cánovas, 2007). It has been reported that TGase treatment of milk with added protein can lead to an improved protein network in the coagulum. It is shown that TGase treatment of milk can lead to similar results in milk with no added protein. Similar results were observed in the case where the milk was HP-treated. Samples made from combined HP-TGase treated milk exhibited an even tighter gel compared to all tested samples with more continuous protein network and smaller spaces compared to either TGase-treated or HP-treated samples. The observations from SEM analysis support the hypothesis of the synergistic effect of the sequential HP and TGase treatment on the yoghurt-making properties of milk.

3.6. Protein denaturation and cross-linking

The SDS-PAGE electropherograms are helpful to evidence the difference between the milk bases (Fig. 5). As it can be seen in line 3, raw milk samples revealed all the expected bands of milk proteins; whey proteins as well as caseins are presented (bands of 25–30 kDa and 14–18 kDa, respectively). In thermal-treated samples (line 5), caseins are presented to a smaller extent compared to untreated samples whereas whey proteins have been denaturated and thus, their bands are not present. Similar results are exhibited for HP-treated samples (line 14), where the bands of caseins are even finer compared to thermal treated milk samples and, again whey proteins are not present. As it can be seen in line 8 and 17, TGase treatment of milk, regardless of other treatments, seems to lead in small or no residual caseins in the milk base, indicating that inter- and intramicellar bonds between caseins are created. It should be noticed that in order to identify any residual casein in TGase-treated samples (lines 8 and 17), milk samples were diluted 2 times in SDS-PAGE buffer instead of 5 times dilution of raw milk, thermal-treated and HP-treated milk samples. The TGase has a molecular weight of 37 kDa and it is not visible in any of the TGase-treated samples, indicating that thermal treatment at 80 °C for 1 min was sufficient for the denaturation of the enzyme.

3.7. Sensory evaluation of yoghurt samples

All samples were judged as acceptable by the sensory panel after the overnight cool storage. Yoghurts prepared with thermally treated milk exhibited higher perceived sourness than those prepared with TGase-treated milk. Samples made from HP-treated milk, with or without subsequent TGase treatment, exhibited a buttery flavor and creamy mouthfeel which was judged as desirable by the panelists. Yoghurts made from combined thermally-TGase treated milk of low protein level exhibited less sourness than those made from similar treated milk of high protein level, and got similar overall scores to those made from HP-treated milk of high protein level.

With regard to textural attributes, when thermally treated milk with high protein level was used, the produced yoghurt gels were more viscous, but still smooth, than those made from milk of low protein level (3.00%). Similar results were observed in the case where HP-treated milk was used. When the milk was treated with the TGase, regardless of other treatments of milk, it was observed that when milk of low protein level was used the produced yoghurt gels were firmer than those prepared with milk of high protein level. This can be explained by the fact that the external protein used to achieve high protein concentration of the milk was a CWP ratio of 50:50, that resulted in a CWP ratio of 76.5:23.5 and 74:26 for milks of 3.40 and 3.75% protein content respectively, leading to lower level of caseins of these milks and consequently to lower the concentration of the required substrate for TGase reaction. The main reason for the use of this kind of external protein concentrate was a recommendation by the dairy industry that provided us the raw material, since high ratios of caseins usually lead to unacceptable bitter taste of the final product and are not usually preferred for solid fortification in the preparation of set yoghurt gels.

In summary, the highest overall sensory scores were observed for yoghurt made from HP-treated milk of low protein level or combined thermally-TGase treated milk of low protein level. Yoghurt samples made from combined HP-TGase-treated milk of low protein level exhibited the most desirable firmness and good scores in most sensorial attributes, although they exhibited a slightly easy break down in the mouth.

The sensory scores of all tested samples in the fourth week of storage are illustrated in Fig. 6a–b. No statistically significant differences were observed between the treatments with the exception of yoghurts made of thermally treated milk, which from the beginning of the shelf life test got the lowest overall scores between all tested yoghurts. All samples were judged as acceptable during the whole storage period except for ones made from thermally treated milk which got unacceptable scores after the fifth week of storage. This was attributed to the shrunken appearance with free whey and weak and ropy structure.

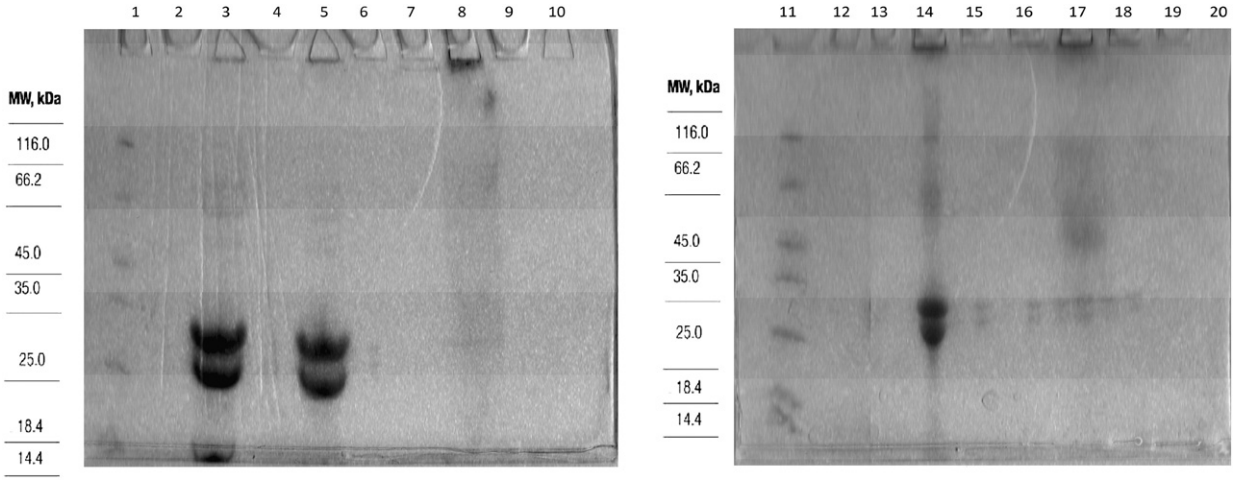

Fig. 5. SDS-PAGE electrophoregrams of molecular weight marker (line 1 and 11); raw milk, 5× diluted (line 3); thermally-treated milk, 5× diluted (line 5); thermally-TGase-treated milk, 2× diluted (line 8); HP-treated milk, 5× diluted (line 14); and HP-TGase-treated milk, 2× diluted (line 17). Milk samples were defatted and had a protein content of 3.00% (CWP ratio 80:20).

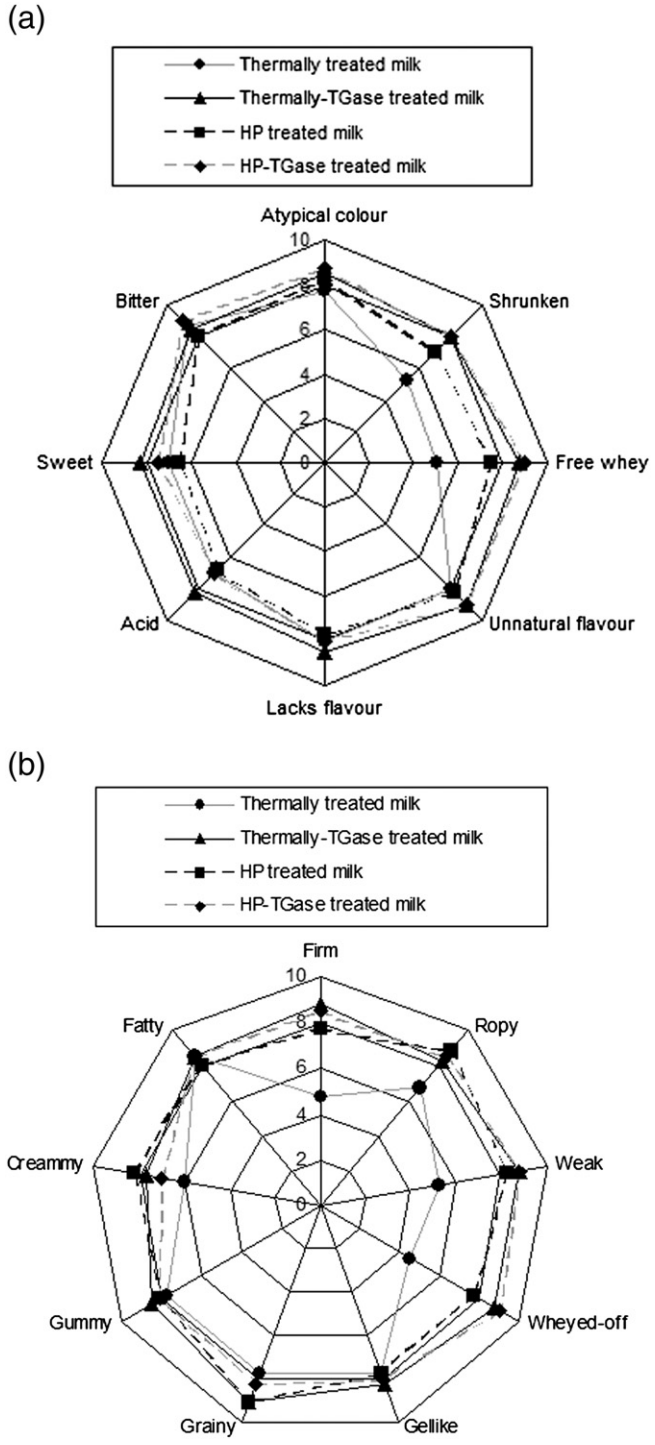

Fig. 6. Radar chart of sensory scores of appearance, taste and flavor attributes (a) and of body and textural attributes (b) of yoghurts with 3.00% protein content in the 4th week of storage.

4. Conclusions

Due to the increased cost of solid fortification for the production of yoghurts of denser structure demanded by the market, the application of HP or TGase treatment of milk during the production of yoghurt is being investigated as an alternative. Until now these processes has been studied separately in set or stirred yoghurt. The combination of those technologies has been studied only in model systems such as gels chemically acidified. In this study, the combination of the above technologies in milk treatment is used for the production of a fermented product such as set yoghurt. Results indicate that both the above technologies reduce the fermentation time and seem to have a synergistic effect on the quality parameters of yoghurt, such as on whey separation, textural attributes and post-acidification. Moreover, sensorial characteristics of these products remained in acceptable limits during an extended period of storage.

The use of external protein fortification results in an additional cost of approximately 0.09 to 0.42€/kg of product (based on an average cost of protein source given by the dairy industry) when producing set yoghurt of up to 10% protein content. The introduction of the above alternative technologies in the dairy industry would include an additional cost of 0.07€/kg of product (average cost of TGase by the enzyme supplier) or of 0.07–0.15€/kg of product (average operating cost provided by manufacturers of industrial high pressure units), for TGase-treated or HP-treated milk respectively, for the processes proposed in the present study. HP or TGase treatment of milk, each separately but even more effectively sequentially applied, can be a useful tool for the dairy industry to achieve acidified products of improved structure, acceptable sensorial characteristics and prolonged shelf life. Products could thus be produced and be commercially viable, without the need of solid fortification with external protein sources.

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