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Monitoring the effect of high pressure and transglutaminase treatment of milk on the evolution of flavour compounds during lactic acid fermentation using PTR-ToF-MS

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ABSTRACT

In this study, the effects of thermal or high hydrostatic pressure (HHP) treatment of a milk base in the absence or presence of a transglutaminase (TGase) protein cross-linking step on the flavour development of yoghurt were investigated. The presence of several tentatively identified volatile flavour compounds (VOCs), both during the enzymatic treatment and the lactic acid fermentation of the milk base, were monitored using a proton transfer reaction time-of-flight mass spectrometer (PTR-ToF-MS). The formation of the major flavour compounds (acetaldehyde, diacetyl, acetoin, and 2-butanone) followed a sigmoidal trend described by the modified Gompertz model. The HHP treatment of milk increased significantly the volatile compound formation rate whereas it did not affect the duration of the lag phase of formation, with the exception of acetaldehyde and diacetyl formation. On the contrary, the TGase cross-linking of milk did not significantly modify the formation rate of the volatile compounds but shortened the duration of the lag phase of their formation.

1. Introduction

Transglutaminase (TGase, EC 2.3.2.13) is a transferase that catalyses the reaction between the γ-carboxyamide groups of peptide bound glutamyl residues (acyl donor) and several primary amines including ε-amino group of lysine, leading to protein cross-linking through the formation of both inter- and intramolecular isopeptide bonds (Motoki & Seguro, 1998; Özrenk, 2006). Non-globular milk proteins such as α-, β- and κ-caseins, and to a lesser extent whey proteins (α-lactalbumin, β-lactoglobulin) are favourable substrates for TGase, rendering possible the modification of several functional and structural characteristics of dairy products (Bönisch, Huss, Lauber, & Kulozik, 2007; Schorsch, Carrie, Clark, & Norton, 2000; Sharma, Zakora, & Qvist, 2002). The functionality of TGase in dairy systems, such as yoghurt gels, can lead to modification of protein solubility, hydration ability, water-holding capacity, emulsifying and rheological properties, enhancement of gelation in absence of thermal treatment, and changing of the elasticity, strength and microstructure of dairy gels. Also it can positively affect the nutritive value and quality characteristics of low fat or solid non-fat (SNF) unfortified products, and decrease the availability of amino acids, e.g., lysine and lipids or lipid-soluble materials which are responsible for deteriorative chemical reactions (Bönisch et al., 2007; Dickinson, 1997; Færgemand, Otte, & Qvist, 1998; Færgemand, Sørensen, Jørgensen, Budolfsen, & Qvist, 1999; Jacob, Nöbel, Jaros, & Rohm, 2011; Jaros, Jacob, Otto, & Rohm, 2010; Lauber, Henle, & Klostermeyer, 2000; Lorenzen, Neve, Mautner, & Schlimme, 2002; Myllärinen, Buchert, & Autio, 2007; Ozer, Kirmaci, Oztekin, Hayaloglu, & Atamer, 2007; Özrenk, 2006).

High hydrostatic pressure (HHP) processing is an alternative method of modifying milk proteins, towards improving the yoghurt-making properties of milk which are related to the formation of a tight and compact structure. The HHP treatment of milk also enables the inactivation of its pathogenic and spoilage microflora, while minimally affecting its endogenous enzymes and its quality characteristics and nutritional value (Cheftel, 1995; Moatsou et al., 2008; Trujillo, Capellas, Saldo, Gervilla, & Guamis, 2002; Trujillo et al., 2000). With regard to the functionality of milk proteins and in contrast with the application of TGase in milk, it is shown that HHP treatment can cause changes in both caseins and whey

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proteins (Dumay, Lambert, Funtenberger, & Cheftel, 1996; Huppertz, Smiddy, Upadhyay, & Kelly, 2006; Iametti et al., 1997; Law et al., 1998; Lopez-Fandino, de la Fuente, Ramos, & Olano, 1998; Schmidt & Kooper, 1997). Previous studies on HHP implementation in yoghurt manufacture have shown that HHP-treated milk exhibits higher rate of acidification and coagulates at higher pH values; as a result, yoghurt prepared from HHP-treated milk exhibits lower amounts of whey separation and increased gel strength compared to those made from non-pressurised milk (Ferragut, Martinez, Trujillo, & Guamis, 2000; Harte, Amonte, Luedecke, Swanson, & Barbosa-Cánovas, 2002; Harte, Luedecke, Swanson, & Barbosa-Cánovas, 2003; Needs, Stenning, Gill, Ferragut, & Rich, 2000; Needs et al., 2000). HHP may also show a remarkable synergy with enzymatic cross-linking (TGase) of milk base proteins in order to achieve products with improved textural and sensorial attributes, without the need of addition of external protein sources or stabilisers (Anema, Lauber, Lee, Henle, & Klostermeyer, 2005; Tsevdou & Taoukis, 2012).

Flavour is among the most important parameters affecting the perceived quality of fermented milks, being influenced by compositional and processing factors (Tamime & Robinson, 2007). Although more than 60 volatile compounds have been reported to contribute to the formation of the flavour profile of yoghurt, acetaldehyde, diacetyl (2,3-butanedione), 2-propanone, acetoin (3-hydroxy-2-butanone), 2-butanone, ethanol, and 2,3-pentanedione are considered as the key components for the development of the typical yoghurt aroma (Kneifel, Ulberth, Erhard, & Jaros, 1992; Ott, Fay, & Chaintreau, 1997; Ott, Germond, Baumgartner, & Chaintreau, 1999). Milk base fortification (e.g., addition of external protein source, stabilisers etc.) can induce important changes in the release of endogenous volatile organic compounds (VOCs), due to kinetic and thermodynamic factors such as air/product partition coefficients and diffusion, lipophilic–hydrophobic character of flavour compounds, matrix–VOCs interactions, etc. (Déléris, Lauverjat, Tréléa, & Souchon, 2007; Guichard, 2002; Soukoulis et al., 2010; Tamime & Robinson, 2007). Moreover, it is well established that individual processing steps, such as homogenisation, heat treatment, incubation and cooling may also change flavour development and release (Tamime & Robinson, 2007). For example, it has been previously reported that severe milk heat treatments may lead to important changes in evolution profiles of the major endogenous flavour compounds during fermentation and the development of off-flavours as well (Labropoulos, Palmer, & Tao, 1982; Vazquez-Landaverde, Torres, Velazquez, & Qian, 2005). Recently, Serra, Trujillo, Guamis, and Ferraga (2009) reported that the application of high pressure homogenisation of milk instead of the conventional thermal treatment may affect the flavour quality of the final products proportionally to the pressure conditions. However, there is still a lack of knowledge on the potential differences and advantages, concerning the evolution and release of flavour, which could be achieved by the application of high pressure and enzymatic treatment of fermented milk products.

Proton transfer time-of-flight mass spectrometry (PTR-ToF-MS) is a novel direct injection mass spectrometric technique that allows the rapid monitoring of VOCs, based on the hydronium ion (H_3O^+) transfer reaction (Jordan et al., 2009). PTR-ToF-MS allows the non-invasive real time detection of VOCs with higher proton affinities than that of water (e.g., carbonyl compounds, carboxylic acids, alcohols, esters, sulphur and nitrogen compounds, ammonia) at the low pptv level. Therefore, PTR-ToF-MS can be considered as an efficient technique for the measurement of flavour release studies in complex food systems or in matrices undergoing time-dependent transformations, as in the case of dairy gels formation (Fabris et al., 2010; Lauverjat, de Loubens, Déléris, Tréléa, & Souchon, 2009; Soukoulis et al., 2010) or of food mastication (Aprea, Biasioli, Gasperi, Märk, & van Ruth, 2006).

The effects of HHP and TGase milk base pre-treatment on the physicochemical, textural, rheological and structural properties of yoghurt have been extensively studied over the last years. However, to the best of our knowledge there are no studies dealing with the impact of TGase and HHP on flavour development during the lactic acid fermentation of milks. For the purposes of the present study, PTR-ToF-MS was implemented for the non-destructive monitoring of the endogenous flavour compounds changes of thermally or HHP-treated milk in the presence or absence of a TGase milk base pretreatment step.

2. Materials and methods

2.1. Milk treatment

Homogenised milk (protein content of 3.0% and standardised fat content of 4.0%) was provided directly from the plant of a dairy company. Milk was either subjected to thermal (85 °C for 30 min) or HHP (600 MPa at 55 °C for 10 min) treatment. For thermal treatment, milk was put into beakers of 2000 mL capacity, preheated in a microwave and, placed in a water-bath maintained at the desirable temperature for the appropriate time. Samples were then stored at 4 °C overnight until use.

HHP treatment was performed using a laboratory-scale HHP system with a maximum operating pressure of 1000 MPa (Food Pressure Unit FPU 1.01, Resato International BV, Roden, Netherlands), consisting of an HHP unit with a pressure intensifier, an HHP vessel of 1.5 L volume and a multi-vessel system consisting of six vessels of 42 mL capacity each. All HHP vessels were 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). Milk samples (750 mL) 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. The pressure of the vessel was released after a preset time interval (10 min pressurisation 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.

2.2. Enzymatic cross-linking with transglutaminase

For the on-line monitoring of ammonia evolution during transglutaminase treatment, thermally and HHP-treated milks were heated to 42 °C, inoculated with TGase at a concentration of 2.2 U/g protein (ACTIVA YG, Ajinomoto, 100 U/g transglutaminase activity), shared into 120-mL glass vials (30 mL of milk sample) and, incubated in a water-bath maintained at the aforementioned temperature (42.8–43.1 °C) for 180 min. Duplicate samples were taken every 30 min and zero time was set 30 min after the inoculation of the enzyme, in order to achieve a desirable headspace for the samples.

For the monitoring of volatile aroma compounds during lactic acid fermentation, thermally and HHP-treated milks were heated to 42 °C, inoculated with TGase at a concentration of 2.2 U/g protein (ACTIVA YG, Ajinomoto, 100 U/g transglutaminase activity) and, incubated in a water-bath maintained at the aforementioned temperature for 180 min. 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). Afterwards, milk was

heated in a microwave at 80 °C for 1 min to inactivate the enzyme and cooled down to the fermentation temperature.

2.3. Preparation of fermented milk samples

Milk was heated to fermentation temperature (43 ± 0.2 °C) and inoculated with commercial starter culture. The starter culture was prepared as a 1:5 (w/v) dilution of freeze-dried YC-X11 culture (Chr. Hansen, Athens, Greece) in commercial UHT skim milk and maintained in cold storage until inoculation. The inoculated milk was divided into sterile glass vials sealed with PTFE/silicone septa (Supelco, Bellefonte, PA) and, closed with sterile plastic caps. Incubation at 42 °C was followed until pH reached 4.75. Samples were taken every 15 min, transferred to a refrigerator at 4 °C and stored until testing by PTR-ToF-MS.

2.4. pH measurement and acidification kinetics determination

The pH of yoghurts during the fermentation process was continuously monitored using a glass electrode pH-meter (Inolab pH/ION, lev.2, Weilheim, Germany). pH measurements were taken every 5 min until the fermentation end point (pH 4.75). pH values were plotted against time, and the parameters describing the kinetics of the fermentation process were determined by fitting the data to the modified Gompertz equation (Eq. (1)) as previously proposed by De Brabandere and De Baerdemaeker (1999);

$$pH = pH_0 + (pH_\infty - pH_0)\exp\left\{-\exp\left[\frac{\mu e}{(pH_\infty - pH_0)}(\lambda - t)\right] + 1\right\} \quad (1)$$

where: pH_∞ and pH_0 = the final (end point) and initial pH values respectively, μ = the maximal acidification (pH drop) rate expressed in pH/min and λ = the duration of lag phase (min).

2.5. Proton transfer time of flight mass spectrometry

2.5.1. On-line monitoring of the ammonia evolution during transglutaminase treatment

For the determination of ammonia formation during transglutaminase treatment of milks, two vials (40 mL) for each batch (thermal or HHP-treated) were put in 120-mL glass vials (Supelco), flushed with air, sealed with PTFE/silicone septa (Supelco) and incubated as described in section 2.2. Direct injections into the PTR-TOF-MS drift tube of the headspace formed were implemented every 30 min. The initial point for the headspace measurements was set 30 min after the initialisation of the transglutaminase treatment, in order to collect a significant amount of volatile compounds according to pre-test measurements.

2.5.2. Monitoring of the volatile flavour compounds during lactic acid fermentation

A procedure similar to that described in Soukoulis et al. (2010) was followed for the measurement of the VOCs headspace during the fermentation process. Two samples were removed from a water-bath which was held at fermentation temperature (43 ± 0.2 °C) at the following times: 0, 20, 40, 60, 75, 90, 105, 120, 135, 150, 165, 180, 200, 220 and 240 min, and rapidly cooled to 4 °C using an ice-bath in order to slow down the fermentation. Cooled, acidified milk samples were then placed in 1000-mL glass jars supplied with two PTFE/silicone septa and held at 28 °C for 45 min to allow equilibration and collect the volatile compounds headspace. VOCs measurement was carried out for 30 s in the PTR-ToF-MS. These conditions are adequate for carrying out a semi-static measurement of semi-solid systems such as dairy gels without alterations induced by the metabolic activity of the starter culture (Soukoulis et al., 2010, 2012).

2.5.3. Acquisition of PTR-ToF-MS spectral data

Measurements were carried out using a commercial PTR-ToF-MS 8000 instrument from Ionicon Analytik GmbH (Innsbruck, Austria) in its standard configuration (V mode). The sampling time per channel of ToF acquisition is 0.1 ns, amounting to 350,000 channels for a mass spectrum range from m/z 10–400, and the ionisation conditions in the reaction chamber were maintained at 600 V for drift voltage and 2.25 mbar for drift pressure. Thus, the instrument was operated at an E/N value of 125 Td. Internal calibration of the ToF data and peak extraction was performed according to the procedure described in detail by Cappellin et al. (2011). Throughout this study, VOCs concentration is expressed in ppbv and is been calculated according to the formula described in Lindinger, Hansel, and Jordan (1998):

$$[VOC] = \frac{1}{\tau}\frac{[VOCH^+]}{k[H_3O^+]} \quad (2)$$

where $[VOCH^+]$ and $[H_3O^+]$ are the measured ion count rates corresponding to the protonated VOC ions and to the primary ion H_3O^+; k is the reaction rate coefficient between the VOC and H_3O^+ ($k_R = 2 \times 10^{-9}$ cm^3/s) and t_R ($t_R = 105$ μs) is the residence time of the primary ions in the drift tube of the PTR-MS.

2.6. Statistical analysis

Two-way ANOVA followed by Duncan's means post hoc comparison test was applied on selected tentatively identified mass peak data of both enzymatic and lactic acid fermentation process to evaluate the significance of the main factors (HHP vs. thermal milk pre-treatment, untreated vs. TGase-treated milk) and their interaction as well. One-way repeated measurements ANOVA was applied on the entire spectral dataset acquired from the headspace analysis of TGase milk treatment, in order to investigate the time dependence of the VOCs concentration in the headspace. Principal components analysis (PCA) was applied on the standardised data at the low-mid spectral region (m/z 15–180) after removing the peak mass ions exhibiting non-significant time dependence. All statistical analyses have been performed using Statistica® release 8 software (StatSoft Inc., Tulsa, OK).

3. Results and discussion

3.1. Monitoring of ammonia evolution during the transglutaminase treatment

Fig. 1a and b display the average spectra of thermally or HHP-treated milks in the low mass region (e.g., m/z 17–105) as they were influenced by TGase. As it can been seen, a great number of peaks corresponding to the protonated VOCs and their fragments was acquired during the monitoring of the enzymatic milk treatment. The depletion or emission of several VOCs as a function of the implemented processing practices is well illustrated in Fig. 1a and b. Ammonia is the main volatile compound emitted during the TGase milk protein cross-linking process and its formation is related with the activity of the enzyme and the protein substrate as well (Özrenk, 2006). Ammonia concentration in the headspace above milk samples depends on incubation time ($p < 0.05$), as illustrated on Fig. 2. Moreover, ammonia levels were significantly higher ($p < 0.001$) in the headspace of HHP-treated milks suggesting a synergistic effect between HHP and TGase treatments. Sharma et al. (2002) have also reported a slight increase in ammonia production in HHP-treated samples. This effect was attributed to the

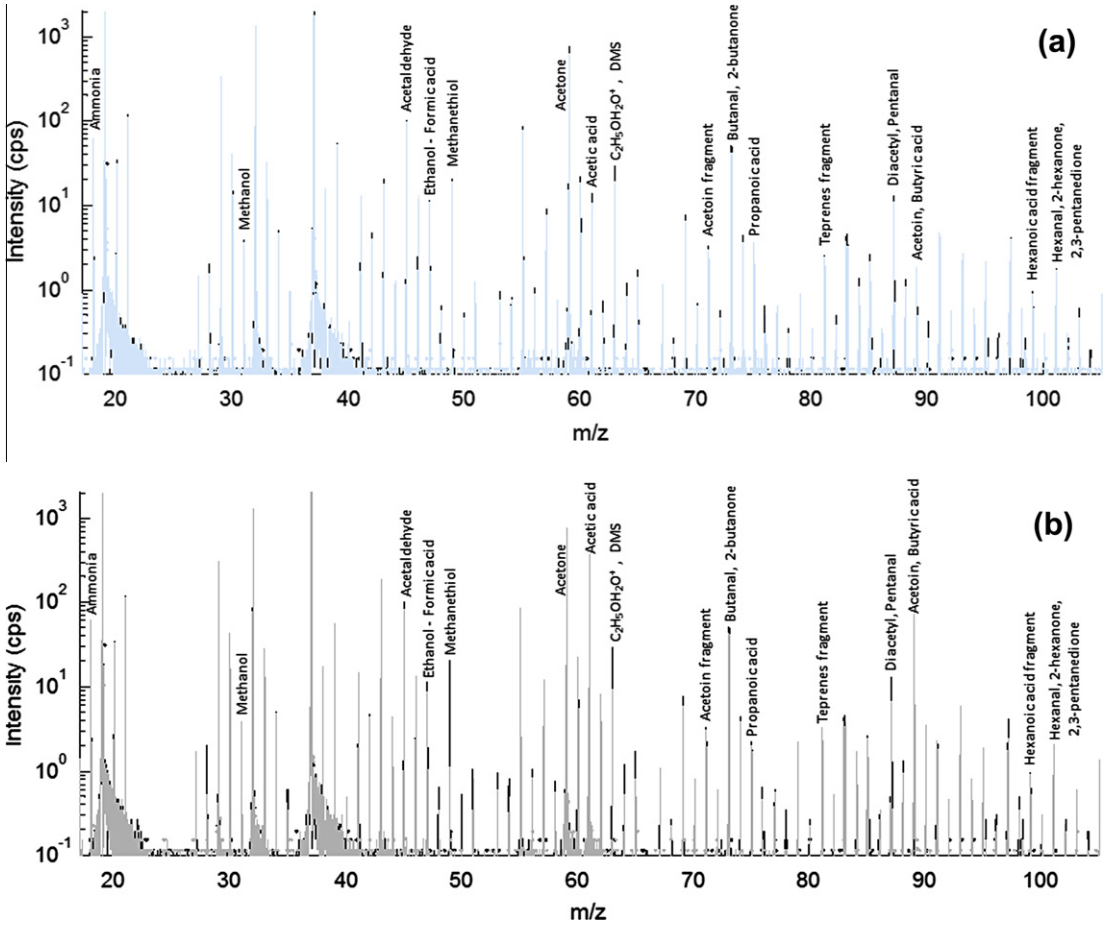

Fig. 1. Illustration of the average spectra in the low – intermediate spectral mass region (*m/z* 17–105), as function: (a) of the duration of transglutaminase (TGase) treatment (black = after 30 min, cyan = after 180 min) and (b) of the milk pre-treatment (black = thermal treatment, gray = high hydrostatic pressure (HHP) treatment). The peaks correspond to either protonated volatile organic compounds or their fragments. Ammonia is detected in the protonated form (NH_4^+) at *m/z* 18.0331.

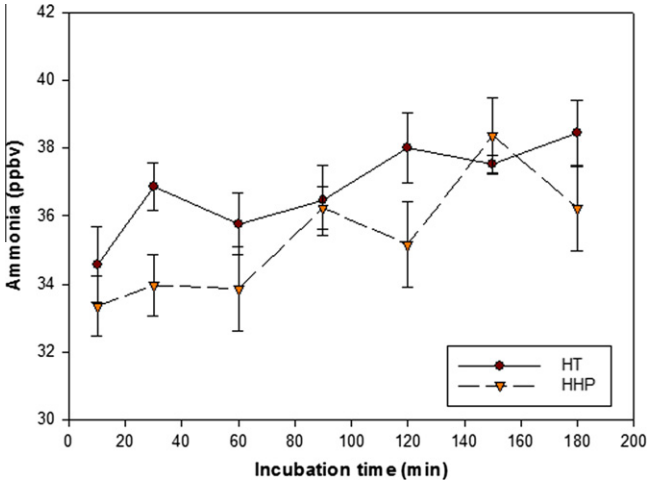

Fig. 2. Effect of thermal and high hydrostatic pressure (HHP) milk pre-treatment on the emission of ammonia during transglutaminase (TGase) treatment of milk at 42 °C for 180 min.

exposure of some reactive sites due to the occurrence of protein unfolding. Moreover, Huppertz et al. (2006) reported that the application of HHP on milk can lead to the rupture of casein micelles due to the solubilisation of micellar calcium phosphate (MCP), as well as the partial denaturation of the whey proteins (Ye, Anema, & Singh, 2004). On the contrary, depending on the severity, thermal treatment can only induce the denaturation of whey proteins without affecting the colloidal properties of micellar casein. Thus, the HHP-treated milk seems to be a more favourable substrate for intra- and inter-molecular cross-linking of proteins and, as a consequence, for ammonia emission than the thermal treatment (Anema et al., 2005; Tsevdou et al., 2012).

Besides ammonia release, there are no existing literature data dealing with the impact of TGase induced protein cross-linking on the release of endogenous flavour volatile compounds in milk systems. Exploiting the PTR-ToF-MS feasibility, we have also monitored a remarkable number of different VOCs present in milk, such as ketones, aldehydes, carboxylic acids, terpenes, and sulfur and nitrogen compounds during the TGase treatment. According to our data, the enzymatic treatment time did not influence the concentration of the most abundant identified compounds, such as acetaldehyde, 2-propanone, ethanol, 2-butanone/butanal, diacetyl, acetoin/butyric acid, hexanoic acid and DMS. Nevertheless, the type of the pre-treatment method significantly influenced the flavour profile of milks with HHP-treated milks being characterised by significantly ($p < 0.05$) lowest concentrations of sulfur compounds (methanethiol, dimethylsulfide, dimethyldisulfide), ethanol, and diacetyl. Methanethiol is generally formed by the thermal decomposition of methionine and acts as precursor for the generation of other volatile sulfur compounds such as DMS and DMDS (Vazquez-Landaverde, Torres, & Qian, 2006). Heat-treated milks were characterised by the significantly lowest signals for the masses corresponding to acetoin/butyric acid (*m/z* 89.059), acetic acid (*m/z* 61.029), hexanoic acid (*m/z* 117.010) and terpenes (*m/z* 81.0).

3.2. Effects of the milk treatment on the acidification kinetics

The values for the lag phase duration (λ) and maximal acidification rate (μ) were calculated using the modified Gompertz model and found to be equal to: (a) 50.2 ± 2.0 min and −0.0144 ± 0.001 pH/min for thermally-treated milk, (b) 46.5 ± 3.0 min and −0.0171 ± 0.001 pH/min for HHP-treated milk and, (c) 55.2 ± 2.4 min and −0.0164 ± 0.000 pH/min for TGase-treated milk regardless of other pre-treatment. The lag phase duration of the lactic acid fermentation is related to many parameters, such as incubation conditions, milk base formulation (total solids, SNF content, sugars, etc.) presence of lactic acid bacteria stimulators/inhibitors, etc. (Tamime & Robinson, 2007). The HHP pre-treatment of milk resulted in significant ($p < 0.001$) reduction of the lag phase duration and increase in the maximal acidification rate. However, the effects of HHP pre-treatment were minimised in the case of TGase-treated milks. Generally, the lag phase duration is associated with the production of proteolytic enzymes from *Lactobacillus delbrueckii* subsp. *bulgaricus*, triggering the release of free amino acids, which act as activators of *Streptococcus thermophilus* (Tamime & Robinson, 2007). Our results suggest that the solubilisation of micellar casein phosphate and the release of free amino acids, caused by the applied pressure, favour the adaptation of the starter cultures to milk substrate, leading to the reduction of the lag phase duration (Huppertz et al., 2006). The TGase treatment of milks led to a slight yet significant ($p < 0.05$) increase in the lag phase duration (12–16.6% for HT and HHP-treated milks respectively), probably due to the reduced availability of proteins for the proteolytic action of *Lactobacillus bulgaricus*. Moreover, TGase had no significant effect on the maximal acidification rate, although it led to a small increase in the incubation time, suggesting that other factors such as the milk composition and incubation conditions are pronouncedly affecting the acidification phenomena. Similar results have been also reported by Yüksel and Erdem (2005) and Lorenzen et al. (2002).

3.3. Monitoring of endogenous flavour compounds formation during lactic acid fermentation

In Fig. 3 the profiles of ammonia evolution during the fermentation process are displayed as a function of the applied milk pre-treatment. No particular trend was observed on the ammonia evolution during the milk fermentation of all samples and the incubation time had no significant impact on it. However, two points are of particular importance: (a) the TGase treated milks were characterised by reduced amounts of ammonia, and (b) in the case of thermally-treated milk samples, a rapid increase in ammonia was observed during the first 40 min. Ammonia formation during milk fermentation is rather limited, taking place as a result of the breakdown of proteins to oligopeptides or free amino acids (Tamime & Robinson, 2007). Both the duration and the ammonia emission rate in the case of control milk samples was found to be well correlated with lag phase duration data (steeper rate and shorter time in the case of HHP-treated milks). Thus, the observed behaviour may be associated with the *L. bulgaricus* proteolytic activity during the lag phase. In addition, the TGase-induced protein cross-linking seems to hinder the proteolytic activity of *L. bulgaricus*, leading to non-significant changes of the ammonia levels during the initial stages of the fermentation.

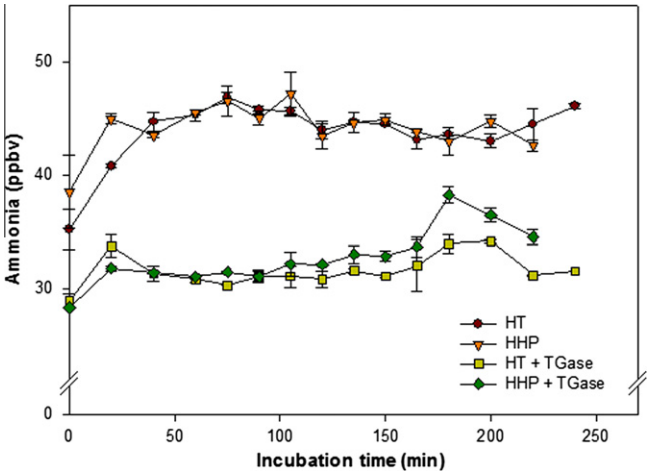

Fig. 3. Effect of thermal, high hydrostatic pressure (HHP) and transglutaminase (TGase) milk pre-treatment on the emission of ammonia during lactic acid fermentation.

Generally, the data on the continuous or semi-continuous monitoring of the volatiles formation during milk fermentation are very limited, and in many cases, destructive chromatographic techniques have been used (Beshkova, Simova, Frengova, & Simov, 1998). In Fig. 4a–f, the evolution profiles of the most important volatile compounds contributing to the development of a balanced yoghurt flavour are displayed. As was expected, acetaldehyde was the most abundant volatile compound followed by diacetyl, acetoin and 2-butanone. Moreover, other compounds such as 2-propanone, ethanol, and methanol (data not shown) were also present in considerable amounts ranging from 40 to 85 ppbv throughout the fermentation process. The temporal evolution of these major volatile compounds was found to follow a sigmoidal trend. The sigmoidal pattern of the evolution curves is mainly attributed to three consecutive fermentation stages; (a) VOCs formation lag phase, (b) logarithmic increase in VOCs and, (c) reaching a VOCs concentration plateau (Fig. 4a–f). A similar trend in the case of acetaldehyde production has been also observed by Hamdan, Kunsman, and Deane (1971) by means of the 3-methyl-2-benzothiazalone test. Considering the biochemical (bacterial growth), physicochemical (lactic acid production, viscosity and texture development) and colloidal (MCP solubilisation, formation of casein-whey protein linkages, precipitation of casein) changes, we propose that the sigmoidal description of the VOCs formation is related both to the metabolic activity of the lactic acid bacteria and the progressive impact of the gel matrix (at least in the pH 5.2–4.7 region). In order to quantify the information provided by evolution curves in Fig. 4a–f, the experimental data were fitted to the modified Gompertz model, which has been previously successfully applied for the description of pH decrease and viscosity development by De Brabandere and De Baerdemaeker (1999) and Soukoulis, Panagiotidis, Koureli, and Tzia (2007), modified as follows:

$$[VOC] = [VOC]_0 + ([VOC]_\infty - [VOC]_0) \times \exp\left\{-\exp\left[\frac{\mu e}{([VOC]_\infty - [VOC]_0)}(\lambda - t)\right] + 1\right\} \quad (3)$$

where: $[VOC]_\infty$ and $[VOC_0]$ = the final (end point) and initial pH values respectively, μ = the maximal VOC formation rate expressed in ppbv/min and λ = the duration of lag phase (min).

In Table 1, the experimental values of the lag phase duration and the maximal VOC formation rate for acetaldehyde, diacetyl, acetoin and 2-butanone are listed. The data were adequately fitted by the modified Gompertz model (r^2 = 0.905–0.981) with acetaldehyde being the best fitted flavour compound.

The duration of lag phase was longest in the case of acetaldehyde and acetoin formation whereas diacetyl and 2-butanone evolution revealed a shortest lag phase (Table 1). The results seem to be in accordance with the biochemical pathway describing their synthesis; e.g., acetoin is formed by the decomposition of diacetyl

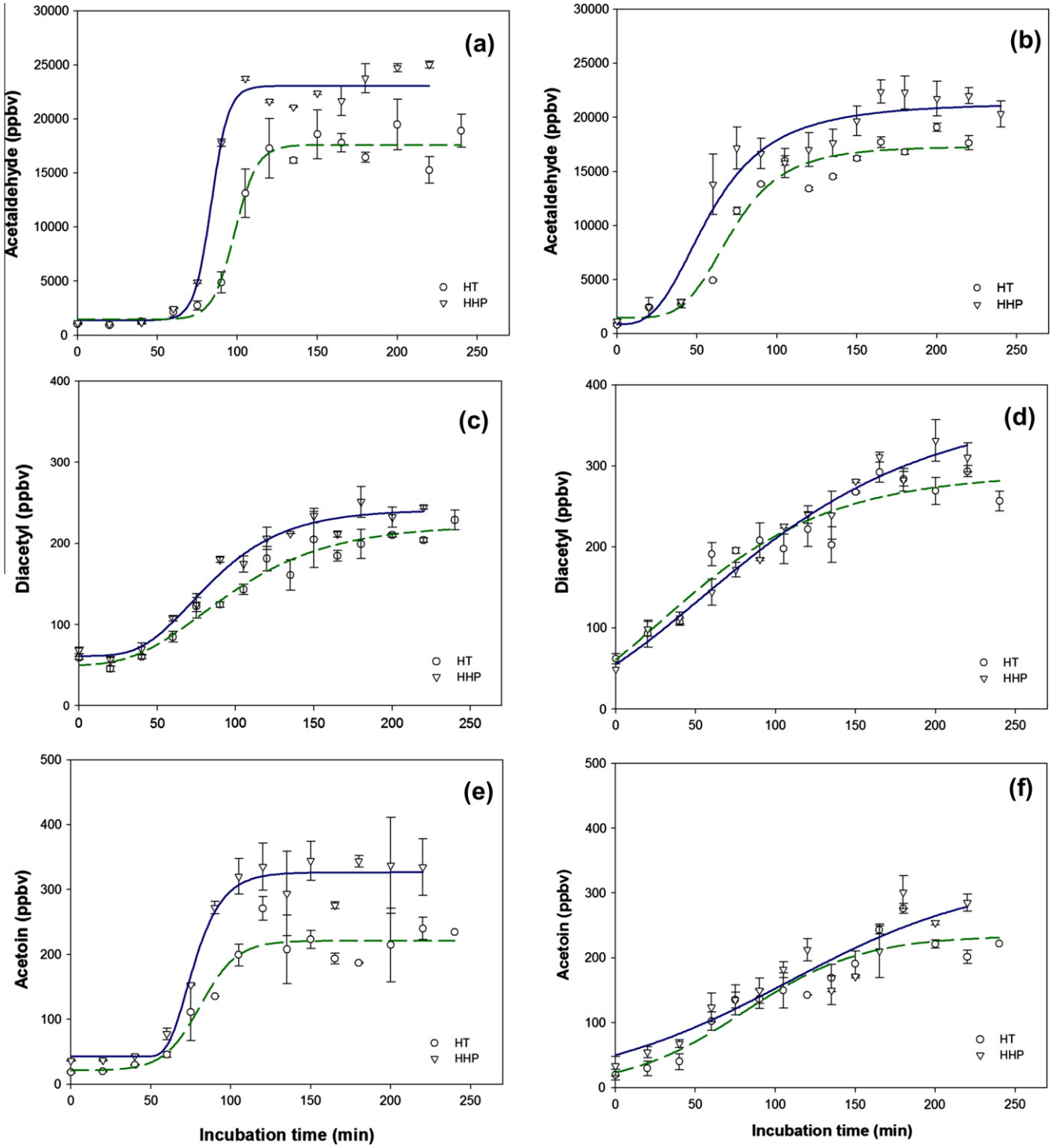

Fig. 4. Effect of thermal and high hydrostatic pressure (HHP) milk pre-treatment on the acetaldehyde, diacetyl and acetoin formation during lactic acid fermentation. a, c and e: without a subsequent transglutaminase (TGase) treatment, b, d and f: with a subsequent transglutaminase (TGase) treatment at 42 °C for 180 min.

(Ott, Germond, & Chaintreau, 2000). Moreover, according to ANOVA results for diacetyl, acetoin and 2-butanone, the λ parameter was not significant ($p > 0.05$) in the case of TGase-treated milks and could be considered as equal to zero in Eq. 3. The rate of VOCs was found to be significantly lowest ($p < 0.001$) in the control ones, following the kinetics of the acidification process. In the literature, there are no existing data dealing with the possible effects of TGase milk treatment on the synthesis of volatile compounds. Generally, pyruvate is considered to be the main precursor for the formation of lactic acid, acetolactate and acetyl-CoA with the last two being the precursors of diacetyl and acetaldehyde respectively (Tamime & Robinson, 2007; Zourari, Accolas, & Desmazeaud, 1992). According to our results, formic acid, which is an intermediate product of pyruvate breakdown to acetyl-CoA, was present in substantially highest concentrations in the initial stages of the fermentation and was progressively reduced. Thus, our results indicate that the transformation of pyruvate to acetolactate and acetyl-CoA instead of lactic acid is favoured in TGase-treated milks, which is also

Table 1
Effect of milk treatment on the formation kinetics of several endogenous flavour compounds during lactic acid fermentation.

Milk treatment	Lag phase duration λ (min)	Maximal flavour formation rate μ (pbbv/min)	R^2
Acetaldehyde			
Thermal	79.0 ± 4.1d	25.7 ± 5.1c	0.981
HHP	69.9 ± 3.3c	50.4 ± 4.7d	0.971
Thermal + TGase	31.4 ± 3.6b	13.1 ± 1.9a	0.961
HHP + TGase	22.6 ± 4.7a	16.2 ± 2.8b	0.946
Diacetyl			
Thermal	29.5 ± 7.7a	1.44 ± 0.16a	0.963
HHP	36.2 ± 5.3b	2.11 ± 0.22c	0.978
Thermal + TGase	ns	1.76 ± 0.11b	0.931
HHP + TGase	ns	1.60 ± 0.09ab	0.924
Acetoin			
Thermal	56.1 ± 6.6a	4.63 ± 1.20b	0.935
HHP	60.7 ± 5.3a	8.96 ± 2.41c	0.951
Thermal + TGase	ns	1.34 ± 0.10a	0.904
HHP + TGase	ns	1.56 ± 0.14b	0.952
2-Butanone			
Thermal	26.6 ± 8.6a	1.29 ± 0.24a	0.931
HHP	20.9 ± 2.1a	1.37 ± 0.18a	0.935
Thermal + TGase	ns	1.06 ± 0.07a	0.896
HHP + TGase	ns	1.29 ± 0.09b	0.883

Different letter between the rows indicates significant difference ($p < 0.05$) among the tested samples according to Duncan's mean values post hoc comparison test.

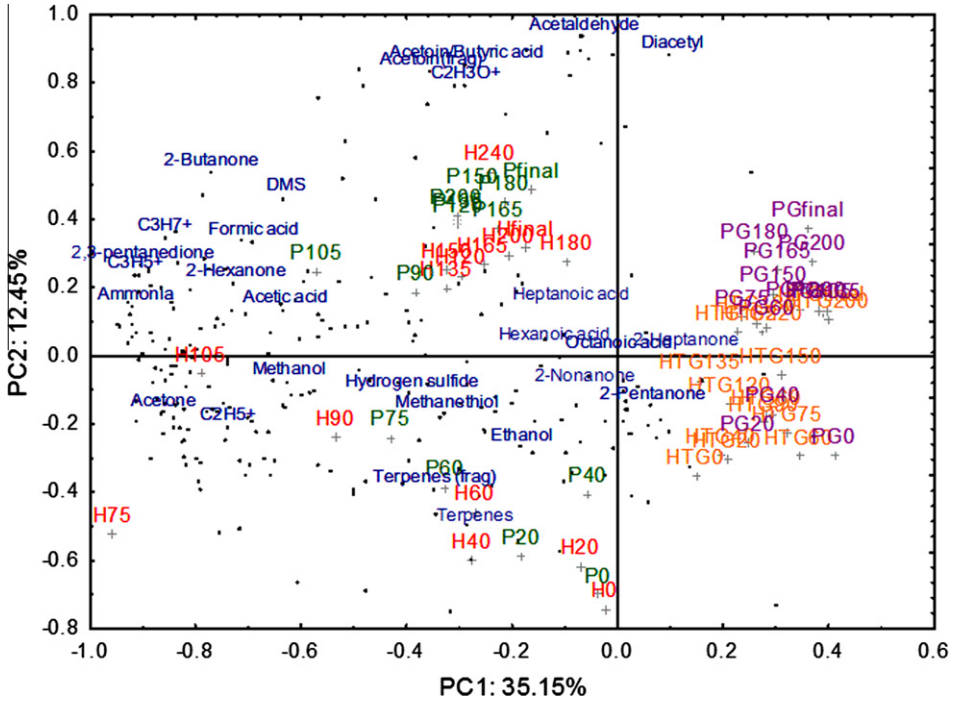

Fig. 5. Principal components analysis biplot for the investigation of the biochemical changes in the formation of the several tentatively identified flavour volatile compounds during lactic acid fermentation of thermally (H) or high-hydrostatic-pressure-treated milk (P) subjected to transglutaminase-induced (HTG and PG) protein cross-linking. The numbers following each letter refer to the time of sampling during lactic acid fermentation.

reflected on the λ values data i.e. lower lactic acid production is related with increased lag phase duration. Moreover, TGase incorporation reduced the level of all the major volatile compounds released in the headspace of the fermented milks. This observation is in accordance with the findings of Ozer et al. (2007) who reported a significant decrease in the acetaldehyde amounts found in non-fat set yoghurts due to slower formation rate during incubation. In recent studies, it has been shown that the impact of the matrix also seems to play an important role on the release of endogenous flavour compounds (Decourcelle, Lubbers, Vallet, Rondeau, & Guichard, 2004; Déléris et al., 2007; Soukoulis et al., 2012). Generally, the TGase yoghurt gels are characterised by improved viscosities and firmness values (Anema et al., 2005; Færgemand, Otte, & Qvist, 1997; Lauber et al., 2000; Tsevdou et al., 2012), forming a physical barrier on the release of flavour compounds.

The HHP treatment of the milk had also significant effects on both the lag phase duration and maximal VOCs formation rate ($p < 0.05$). Regardless of the flavour volatile compound, the application of HHP almost doubled their formation rate during the incubation process. Thus, the colloidal changes induced by HHP seem to accelerate the medium adaptive and metabolic activity of the lactic acid bacteria leading to faster flavour development.

3.4. Principal components analysis

In order, to evaluate the effect of the applied milk pre-treatments on the changes of the flavour compounds formation during the different fermentation steps, the spectral data were subjected to principal components analysis (Fig. 5). Principal components 1 and 2 accounted together for almost the 48% of the total variance explained. Acetone, 2-butanone, methanol, ammonia, formic acid, acetic acid, DMS and 2,3-pentanedione were correlated with PC1 whereas acetoin/butyric acid, diacetyl, acetaldehyde, terpenes and ethanol were associated with PC2. C_4–C_9 2-ketones and C_6–C_8 carboxylic acids were not correlated with the first two axes, and consequently were not affected by TGase treatment (first axis) or incubation time (second axis). Considering the temporal changes of the flavour compounds concentration during the fermentation, it can be seen that the lag phase is mainly related with higher ethanol and terpenes amounts, whereas acetaldehyde, acetoin/butyric acid are the predominant flavour compounds present in the headspace above milk after 90–135 min of incubation, depending on the milk pretreatment applied. The concentration of the major flavour compounds (acetaldehyde, diacetyl, acetoin/butyric acid) was higher in the headspace of HHP-treated milks compared to that of HT-treated milk samples. According to Fig. 5, it is clearly demonstrated that the TGase treatment led to remarkable reduction of the lag phase and the time required for maximising most of the flavour compounds formed during the fermentation. However, the concentration of the volatile compounds in the headspace of TGase-untreated samples was higher compared to TGase-treated samples, suggesting that the changes in the protein/peptidic material availability to the lactic acid bacteria and the gel matrix impact (stronger and more dense protein entanglements in the TGase treated yoghurts) are the main drivers of the flavour profile of the samples.

4. Conclusions

It was demonstrated that the protein cross-linking induced during TGase milk pre-treatment did not alter significantly the flavour compounds composition and content, apart from the ammonia emitted. High-pressure treatment of the milk base caused a significant reduction of the lag phase duration and an increase in the acidification rate. However, the changes between thermally and HHP-treated milks were limited in the presence of TGase treatment. Regardless of the milk pre-treatment method, the formation of the major flavour compounds (acetaldehyde, diacetyl, acetoin, and 2-butanone) followed a sigmoidal trend. The application of HHP led to a significant increase in the formation rate of the volatile compounds whereas no significant changes were observed in the duration of lag phase. TGase treatment of milk induced a significant reduction of the formation rate of acetaldehyde and acetoin and shortened pronouncedly the duration of the lag phase for VOCs formation. The effects of HHP and TGase treatment on the availability of the protein/peptidic material and the changes in the colloidal and structural characteristics of the formed gels are among the factors that affected the flavour profile of the yoghurt samples.

In conclusion, it would be of particular interest to perform a further investigation of the evolution and release of flavour compounds in other fermented milk products, in which during their production procedure a breakdown of the formed gels occurs, e.g., in stirred yoghurts. In that case, because of the destruction of the barrier on the release of flavour compounds, alterations in the mechanism and release kinetics could be observed and differences in sensorial characteristics could be identified.

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