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Original article

Fermentation by lactic acid bacteria at two temperatures of pre-heated reconstituted milk. I - Behaviour of proteins and minerals

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Abstract – The pH-induced physico chemical changes in a reconstituted heated (90 °C - 10 min) skim milk were studied during fermentation by lactic acid bacteria at two temperatures (30 °C and 42 °C). Different variables were examined during acidification: the water content of ultracentrifugation pellets (150000 *g* for 70 min at 30 or 42 °C), the partition of proteins into pelleted and unpelleted fractions (analysed by RP-HPLC on supernatants) and the dissociation of colloidal salts (determined by atomic absorption spectrophotometry on the ultrafiltrates). The proportion of unpelleted protein was found to be pH-dependent with a gradual linear decrease during acidification. All the caseins pelleted below pH 5.5. As the pH was reduced, calcium was progressively released from the micelles. In contradiction with a number of previous findings, fermentation induced a shift toward acid pH values of the salt dissociation compared with a glucono-delta-lactone acidification. The partition of casein between the pellet and the supernatant due to acidification by fermentation was temperature-dependent, as was the solvation of the pellet. Both were greater at 30 °C compared with 42 °C. The temperature had no effect on the salt distribution. The results are discussed in terms of transfer phenomena between the colloidal and the solvent fractions and diffusion-limited processes.

Milk / yoghurt / pH / mineral / micellar solvation / casein

Résumé – **Fermentation du lait reconstitué traité thermiquement par des bactéries lactiques à deux températures. I - Protéines et minéraux.** L'effet du pH sur les évolutions physico-chimiques de lait écrémé reconstitué et traité thermiquement à 90 °C, 10 min a été étudié au cours d'une acidification par fermentation lactique à deux températures (30 °C et 42 °C). La quantité d'eau a été évaluée par séchage des culots d'ultracentrifugation (150000 *g* pendant 70 min à 30 ou 42 °C). La distribution des protéines entre le culot et le surnageant a été analysée par chromatographie RP-HPLC sur les surnageants avec ou sans dithiothreitol. La dissociation des sels colloïdaux a été déterminée par spectrométrie d'absorption atomique dans les ultrafiltrats de lait. La proportion de protéines dans le surnageant était dépendante du pH avec une diminution graduelle au cours de

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l'acidification. La majorité des caséines était culottée vers pH 5,5. Au cours de l'acidification, il y a une dissociation progressive du calcium colloïdal. La température est sans effet sur les équilibres minéraux. Comparée à l'acidification par la glucono-delta-lactone, la fermentation induit un décalage vers des pH plus acides de la dissociation des sels colloïdaux. La répartition de la caséine entre le culot et le surnageant, tout comme la teneur en eau des culots, est fonction de la température de fermentation et augmente à 30 °C par rapport à 42 °C. La baisse de la température accroît les concentrations de caséines non centrifugées, particulièrement autour de pH 6,0. Ces résultats sont discutés en terme de phénomènes de transfert entre le solvant et la fraction colloïdale et de limitation par la diffusion.

Lait / yaourt / pH / minéraux / hydratation / caséine

1. INTRODUCTION

The physicochemical changes in proteins during acidification of unheated skim milk, performed either by organic/mineral acid or glucono-delta-lactone (GDL) addition to milk have been studied for a very long time. As the pH decreases, the solubility of calcium phosphate increases, leading to its dissociation from the micelle, and the negative charge of ionised groups decreases, leading to the dissociation of calcium from phosphoserine residues [13, 19, 20, 29]. A small proportion of casein dissociates from the micelle, with a maximum at pH 5.5, and reassociates at lower pH [12, 19, 29]. The micelle diameter decreases until pH 5.0 and then sharply increases as the gelation proceeds [4, 8]. The ζ-potential, the voluminosity and the solvation of the casein micelle decrease, often with a maximum around pH 5.5 (or 5.1 for the potential) [3, 12, 13]. Around pH 4.6, if the acidification has been performed quiescently, a viscoelastic gel is produced, as in yoghurt manufacture.

In fact, the quality of yoghurt is strongly dependent on the heat treatment of milk prior to acidification. Heat treatment is given for hygienic considerations, but mostly for textural or fundamental rheological point of view. It is well known that heating milk at temperatures ≥ 80 °C leads firstly to a shorter time of gelation and to an increased pH value at gelation, and secondly to a great increase in the storage modulus [14]. The shape of the tan δ versus time or versus pH in acidified heated milk is very different from the one obtained from unheated milk, with a "maximum in tan δ" between pH 5.3 and 5.0, while no maximum is found with unheated milk [21–23].

It is well known that heat treatment of milk induces many changes in proteins. After a heat treatment at 80 °C for 5 min, a denaturation of 74% of β-lactoglobulin (β-LG) and of 58% of α-lactalbumin (α-LA) is observed. Only 58% and 40% of these denatured proteins associate with casein micelles, respectively [29]. The behaviour of α-LA and β-LG are dependent upon the time of heating, the temperature, the pH and the amount of proteins [5, 25]. After heating at 85 °C for 10 min, a marked increase in the concentration of κ-casein (κ-CN) in the supernatant has been reported (from 15 in unheated to 23% of total κ-CN in heated milk) [18], but it should be noted that increasing the pH value to 7.1 leads to a greater concentration of supernatant casein in the milk heated to 70–80 °C [2]. Above 80 °C, at pH 7.1, the proportion of κ-CN in the supernatant increases, while that of α_S-CN (α_{S1}+ α_{S2}) decreases [2].

Moreover, the role of micro-organisms is not limited to acid production. Indeed, micro-organisms can play a role, firstly with their enzymatic potential and secondly because they produce acid only in the solvent permeate. This can lead to heterogeneity in the acid production and to a pH gradient. The acid production by micro-organisms proceeds very differently from

what happens with GDL, as GDL is soluble in milk and can diffuse quickly in the solvent permeate and through the casein micelle. Bacteria generally grow in large colonies in milk and are weakly mobile, so that the local pH value around the bacteria is more acidic than in the bulk [11]. Micro-organisms are probably rapidly blocked into a microscopic gel area, so that the acid production is more and more confined within small areas. With micro-organisms, lactic acid is produced merely in the solvent permeate.

The aim of this series of articles is a better understanding of the construction of yoghurt, from materials close to the manufacture, i.e. with heat-treated milk acidified by lactic acid starters. In this paper, the static point of view is presented: the partition of cations and proteins between the solvent and the colloidal fractions and the water content of the pelleted proteins. The dynamic point of view will be presented in the next article, including direct rheological and physicochemical measurements during acidification.

Table I. Chemical composition of the reconstituted milk at 11% total solids used for the manufacture of yoghurt, before the heat treatment. The percents of proteins were calculated from the HPLC analysis.

	Unheated milk ($g \cdot L^{-1}$)
Dry matter	110.0
Ashes	9.39
Total nitrogen (TN) $*_{(1)}$	38.97
Non-casein nitrogen (NCN)$*_{(2)}$	7.72
Non-protein nitrogen (NPN)$*_{(3)}$	2.11
Total proteins (TN-NCN)*	36.86
Whey proteins (NCN-NPN)*	5.61
– α-lactalbumin **	1.0
– β-lactoglobulin **	3.4
Total casein (TN-NCN)*	31.25
– κ-casein **	3.0
– α_{S1}-casein **	12.6
– α_{S2}-casein **	2.5
– β-casein **	12.2

* From Nitrogen determination; ** from HPLC determination; (1) using 6.38 as converting factor; (2) using 6.25 as converting factor; (3) using 6.19 as converting factor.

2. MATERIALS AND METHODS

2.1. Milk

Milk was reconstituted at 11% solid content (casein 33.3 $g \cdot kg^{-1}$) with the same batch of a low-heat skim milk powder (WPNI = 8.0) (Ingredia, Arras, France) in MilliQ water. The composition of the reconstituted milk is presented in Table I. After 1 h equilibrium at room temperature, the milk was heat-treated in a spiral stainless steel tube of 6 mm internal diameter immersed in a thermostatically controlled water bath. The flow rate of milk through the system was approximately 3.5 $L \cdot min^{-1}$ and the residence volume of the loop was 180 mL. Milk samples (0.5 L or 1 L) were held at 90 °C for 10 min. Immediately after heating, the milk was quickly cooled by immersion in ice-cold water with a gentle motion. The heating profile was monitored with a thermocouple placed inside the bottle. Warm-up time of ~1–2 min was taken into account for the calculation of the heating time. After heating, there was a slight drop in pH (0.05 unit).

Each batch of milk was divided into equal portions of 100 g in capped sterile glass vials filled to the top to prevent air incorporation and stored at 4 °C.

2.2. Yoghurt gels

Set yoghurts were produced with a mixed culture of non-ropy *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* provided by Danone and inoculated in milk at 10^7 $CFU \cdot mL^{-1}$ each.

Fermentation temperatures were 30 °C and 42 °C, considered, respectively, as a slow and a rapid acidification. The milk sample (100 g) was equilibrated for 1 h at the fermentation temperature in a water

bath before inoculation with the culture-diluted ferments.

Inoculated milk was quiescently incubated at the fermentation temperature until pH 4.0 was achieved. The kinetics of acidification were registered for 20 h using a Cinac system (Ysebaert, Frépillon, France) [32]. Fermentation at each temperature was carried out in duplicate, the day of the heat treatment and the following day. Changes in physical properties as a function of pH are mean values.

Three different preparations of milk at 30 and 42 °C were realised so that 6 kinetics of acidification were obtained.

2.3. Acidification by GDL

Unheated and heated milk samples with 0.2 $g \cdot kg^{-1}$ Na azide added were transferred into 10 vials of 50 mL and stored at 30 °C. GDL (Merck Eurolab, Fontenay-sous-Bois, France) was added to each sample at final concentrations ranging from 0.56 to 22 $g \cdot L^{-1}$ to reach pH values from 6.64 to 4.05 in 17 h at 30 °C. Acidified milk samples were then stirred and the analysis of minerals in the permeates obtained, as described in the 2.4.2.1 section, at each final pH value, was performed.

2.4. Analyses

2.4.1. Unheated milk

Total solids content was obtained at 103 °C for 7 h using an air-oven by drying duplicate samples under atmospheric conditions [15]. Milk ash was determined according to the method described by the AOAC [1]. The nitrogen content was determined from N analysis by the Kjeldahl method using a Kjeltec digestion system (Técator, Foss France, Nanterre, France). Fractionation of total nitrogen (NT) in the milk was according to the Rowland procedure for non-casein nitrogen (NCN) and for non-protein nitrogen (NPN) [28]. The converting factors were 6.38, 6.25 and 6.19, respectively, for NT, NCN and NPN. The whey protein and casein concentrations were determined as NCN-NPN and TN-NCN. Individual proteins were analysed using HPLC.

2.4.2. Acidified milk

Acidified milk samples were examined after fractionation in terms of pelleted casein and diffusible salts. Water content of the pellet materials was also determined. The acidification was stopped by addition of 2.05 $g \cdot L^{-1}$ sodium azide at specific pH values, i.e. at pH 6.0, 5.5, 5.0 and 4.6. Immediately after the addition of azide, the milk was gently stirred at room temperature and fractionated. The initial pH represents the observations taken immediately after the inoculation of the milk followed by azide addition.

2.4.2.1. Fractionation of acidified milk

The milk permeate was obtained by ultrafiltration on membranes of 25 $kg \cdot mol^{-1}$ molecular mass cut-off (Centriflo CF 25, Amicon, Grace, Épernon, France) for the determination of diffusible salts. Ultrafiltration in the centrifuge was carried out at the appropriate temperature (42 °C or 30 °C) at 750 *g* for 45 min.

The sedimentation of proteins was performed by centrifugation at 150000 *g* for 70 min at 42 °C or 30 °C. The separation was carried out using a L8-55 ultracentrifuge with a 50.2 Ti rotor (Beckman Instruments France S.A., Gagny, France). Supernatants and pellets were separated by gently pouring out the supernatant and were analysed for the unpelleted and the pelleted fractions.

2.4.2.2. Analyses of acidified milk

Total (Ca_T, Mg_T, Na_T, K_T) and diffusible cation concentrations were determined by atomic absorption spectrometry using Varian equipment (Les Ulis, France) on milk and milk permeate, respectively, according to Le Graet and Brulé [20]. Duplicate measurements on 2 different

samples of milk were performed at each temperature.

Casein analysis was performed on milk and on the supernatant by reversed-phase high-performance liquid chromatography (RP-HPLC) on 2 different samples of milk.

The determination of the water content was done on the pellet on 2 different samples. Drained pellets were weighed and dried at 103 °C for 7 h. Water content of the pellet was expressed as grams of water per gram of dry pellet. As in the literature, when the water contents of the pellet are expressed per gram of proteins, the current values will be underestimated. The pellets were mainly composed of casein micelles, plus some denatured whey proteins and minerals and bacterial cells. A bacteria cell represents 10^{-12} g and 2×10^{-13} g, on a moisture and dry basis respectively. During fermentation, bacteria grow from 10^6 to 10^9 CFU·mL^{-1}. The weight of bacteria in the pellet only changes the 1000th of the water content and was neglected.

2.4.3. RP-HPLC

The protein composition of unheated and heat-treated milk and in corresponding supernatants was determined by RP-HPLC on a Waters 600 delivery system, a 481 variable wavelength LC spectrophotometer and a 740 data module (Waters, Milford, MA, USA). Elution was performed in duplicate on a C_4 Vydac 214 TP 54 column (Touzart & Matignon, Vitry-sur-Seine, France) (15 cm × 4.6 mm i.d., 5 µm particle size), maintained at 40 °C.

This method was adapted from [16] and [36]. The solvent A was 0.1% (V/V) trifluoroacetic acid (TFA) in water and solvent B, 0.096% (V/V) TFA in 80% (V/V) acetonitrile. The initial mobile phase was 46% solvent B. After equilibration of the column with 46% B, samples (50 or 200 µL) were loaded onto the column. Proteins were eluted at a flow rate of 1 mL·min^{-1}, using a linear gradient of solvent B as follows: 0–15 min, 46–61%; 15–17 min, 61–100%; 17–22 min 100%; 22–24 min, 100–46%; 24–34 min, 46%. The elutes were quantified for protein by measuring the absorbance at 280 nm.

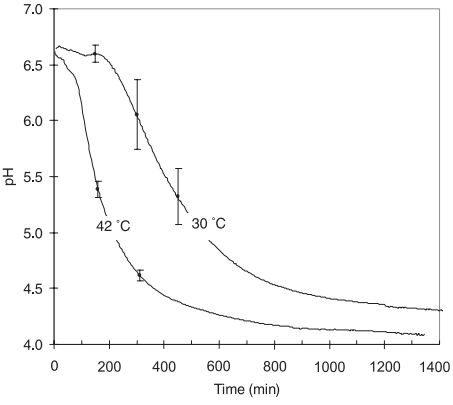

Figure 1. Kinetics of milk acidification with lactic acid bacteria at 30 and 42 °C.

The method enabled the evaluation of concentrations with a coefficient of variation of 5%.

The milk and supernatant were respectively diluted 1/10 and 1/2 in 8 mol·L^{-1} urea buffer, 1 mol·L^{-1} Tris/HCl, Na-Citrate 13 g·L^{-1}, pH 7.0. This buffer allowed reduction of the proteins in the presence of dithiothreitol (DTT). Under reducing conditions, milk samples with ~10 mg protein·mL^{-1} were treated with 10 mmol·L^{-1} DTT and incubated for 1 h at 37 °C. Samples were diluted 1/2 in solvent A and then filtered through a 0.45 µm filter prior to injection. Thus, milk samples and supernatants were diluted twenty-fold and four-fold, respectively.

3. RESULTS AND DISCUSSION

3.1. Kinetics of milk acidification during fermentation

The pH kinetics are shown in Figure 1. The confidence intervals were greater at 30 than at 42 °C. The maximal rate of acidification at 42 °C was twice that at 30 °C, say 0.9 and 0.4 pH unit·h^{-1}, respectively.

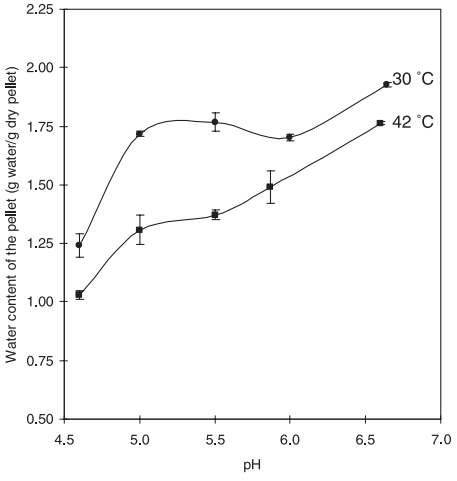

Figure 2. Water content of the ultracentrifugation pellet (150000 *g* for 70 min at 30 or 42 °C) expressed in g of water/g dry pellet upon acidification of heated skim milk by fermentation at 30 °C (●) or 42 °C (■). Means are plotted with 95% confidence interval indicated by vertical bars for 2 determinations.

The lag times and the times needed to reach pH values such as 5.5, 5.0 or 4.6 were reduced as the temperature increased. At 42 °C, the final pH at 20–24 h was close to 4.0–4.2. It slightly increased to 4.3 at 30 °C. Fermentation at 30 °C produced slower rates of acid development and of gel formation, for the same final pH value.

3.2. Water content of the pellet

Figure 2 shows the relationship between the pH and the water content of the pellet. At initial pH (6.7) and 30 °C, the water content of the pellet was 1.9 g H_2O per g. As predicted by equations of thermodynamics, the water sorption by proteins decreases with increasing temperature [17]. But, as in Snoeren et al. [30], the centrifugation force was not corrected between 30 and 42 °C for the differences in viscosity. The water content of the pellet decreased during acidification. There was a plateau near pH 5.0–5.5, more pronounced at 30 than at 42 °C. Previous studies on acidification obtained with GDL or HCl at 20 or 30 °C reported the same pattern for micelle solvation versus pH curves, a maximum or a shoulder, around pH 5.4 [30, 31, 33, 35]. However, Vreeman [37] did not observe this behaviour.

The water content of the pellet during acidification remained higher at 30 °C than at 42 °C. The difference between the water content at 30 and 42 °C was not constant over the total range of pH. It was minimum near pH 6.0 (0.20 g water/g dry pellet) and became maximal between pH 5.5 and 5.0 with a value of 0.50 g water/g dry pellet.

The same changes in water content of the pellet fraction as a function of pH appeared in heated milk compared with unheated: as the net negative charge of casein was reduced, the affinity for water decreased, but around pH 5.0–5.5, the micelle swelled, or some casein left the micelle and was replaced by water.

3.3. Diffusible salts

Figure 3 shows the extent of transfer of Ca from the micelle to the serum as a function of the pH. The changes in diffusible Mg were similar to Ca. The dissociation of colloidal Ca with the pH decrease showed two distinct stages: first, above pH 6.0 with a slight increase in concentration (10.3% of Ca_T), then below pH 6.0, with an extensive increase (57.6% of Ca_T). At pH 4.6, calcium is almost entirely diffusible at 42 °C and at 30 °C. Le Graet and Brulé [20] suggest that calcium is entirely diffusible at pH 3.5.

At initial pH, potassium and sodium were present almost entirely as free ions in milk. Nevertheless, their levels seemed to increase very slightly as pH decreased (results not shown).

The concentration of diffusible Ca shown in Figure 3 is lower compared with that described in the literature for unheated reconstituted milk, under acidification either by HCl or GDL addition [3, 7, 10, 12, 18, 20, 35]. Acidification by GDL of unheated or heated milk released calcium differently compared with the fermented

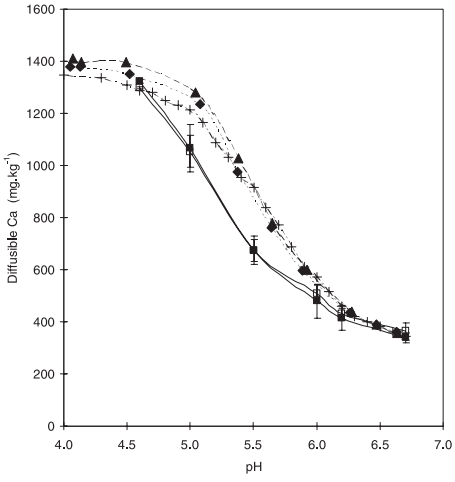

Figure 3. Concentration of diffusible calcium upon acidification by addition of glucono delta lactone at 30 °C in unheated milk (▲), in milk heat-treated at 90 °C for 10 min (◆) or by fermentation at 30 °C (□) or 42 °C (■) in milk heat-treated at 90 °C for 10 min. Diffusible salts are determined in the permeates obtained by ultrafiltration on Centriflo CF25. For each temperature, values are the means of 4 assays. Ninety-five % confidence intervals are indicated by vertical bars. Cross symbols are values from a curve published by Le Graet et al. [17] at room temperature.

sample (Fig. 3). From the initial pH down to pH 6.0, the dissociation of colloidal calcium was completely superimposable with that in the fermented sample. Then, between pH 6.0 and 5.0, acidification by GDL led to a greater dissociation. Experiments at 30 °C and 42 °C showed a similar salt partition with acidification. Heat treatment of milk from 70 to 90 °C for 5 min or 85 °C for 10 min has little effect on the dissociation of calcium and Pi from the micelle [18, 29].

These results cannot be due to a different affinity of lactic acid for the calcium than gluconic acid, as the pK of these acids are not so different (pK of lactic acid = 3.8; pK of gluconic acid = 3.6, [24]). Anyway, dissociation of colloidal calcium with HCl is rather close to that obtained with gluconic acid and that of lactic acid is different, and the affinities of lactic and gluconic acid for calcium are probably closely related and are very different from that obtained with a strong acid such as HCl.

These results led to the conclusion that fermentation induced a shift towards lower pH values of the concentration of diffusible Ca. As a matter of fact, Le Graet et al. [20] have used HCl at 30 °C and studied only the final state, which means when chemical equilibrium is complete. Law and Leaver [19] tested the effect of the rate of acidification in the presence of HCl or GDL on the dissociation of colloidal calcium phosphate (CCP) at 20 °C: the extent of dissociation of CCP was dependent on the pH of milk, but was not significantly affected by the time that was required to reach the pH or by the mode of acidification.

The mode of acidification may also modify the salt dissociation. It has been proved that the production of lactic acid by bacteria led to low local pH values. Growth of *Lactococcus lactis* inoculated at low level (10^2 CFU·mL^{-1}) shows evidence, through visual observation of the colour of a pH indicator, that the acids diffuse rather slowly in liquid milk around bacteria grouped in macro-colonies [11]. Thus, the micelle interface is probably at a pH value susceptible to aggregation or gelation. At the same time, the inner part is still at high pH and highly mineralised, because of the high buffering capacity of the phosphate groups. The micelle can present a pH gradient from the inner to the outer part. It may even be possible that the layer of gelled whey proteins and caseins on the particle surface of heated milk acidified by lactic acid bacteria could hinder the dissociation of colloidal salts.

Protons produced during acidification are immediately bound by phosphate and citrate, whose pK are higher than the pK of lactic acid. The transfer of these acids inside the casein micelle can be hindered by steric or electrostatic tensions, as these acids are negatively charged. It is not only H^+ that must diffuse, but H^+ together with

these acids. These phenomena, which are dependent on diffusion processes, can lead to a rather different kinetics and behaviour, when considering the protein environment.

The fact that, between pH 6.6 and 6.0, the quantity of diffusible calcium was equivalent between fermented milk and chemically acidified milk (Fig. 3) can be explained as follows: in this pH range, the dissociation of colloidal calcium phosphate is only induced by the acidification of the solvent phase and, consequently, is due to the increase in the solubility of CCP. No diffusion limitation occurred in this transfer. Under pH 6.0, the dissociation of the calcium from phosphoserine takes place only if citric and phosphoric acids and H^+ can diffuse into the micelle, and we assumed this step to be dependent on the mode of acidification.

3.4. Changes in protein status

3.4.1. Initial heat-treated milk (results not shown)

The amount of proteins in the supernatant was 15% of total proteins in unheated milk and decreased to 5% in heat-treated milk. They were whey proteins along with a small quantity of casein: 3 and 5% of total casein in unheated and heat-treated milk are present in this fraction, respectively. Heating at 90 °C for 10 min produced total denaturation of β-LG, whereas 12% of residual α-LA was found in the heat-treated milk. The HPLC profile of the supernatant showed a new broad peak, eluted behind β-LG, which was dissociated into 4 peaks in the presence of DTT. As this peak was DTT-sensitive, it probably contained whey protein aggregates. As a matter of fact, heat treatments higher than 70 °C provoke an extensive denaturation of α-LA and β-LG [29]. Heating at 80 °C for 5 min induces the association of 58% of total β-LG with casein micelles, while 74% of β-LG is denatured [29].

Due to the heat treatment, an increase of 13% in the pH 4.6-insoluble protein content (NT-NCN) and a decrease of 75% in the concentration of whey proteins (NCN-NPN) were found in milk, expressed as a percentage of that in unheated milk.

The increase in unpelleted casein by heating was mainly due to an enrichment of the supernatant in κ-CN. The ratio of unpelleted κ-CN to total κ-CN was 6–7% in unheated milk and increased to 30–40% in heated milk, as previously reported [2, 18, 34].

3.4.2. Acidified milk

3.4.2.1. Whey proteins

The denatured whey protein present in the unpelleted fraction, accounting for about 1.3 $g \cdot L^{-1}$ of protein nitrogen and supposed to constitute the broad peak in HPLC of the unpelleted fraction of heated milk treated with DTT (Fig. 5B) decreased rapidly as the pH was lowered. This result is in accordance with the isoelectric pH value of 5.3 for β-LG, which is the major whey protein. At 30 °C, in heated milk (85 °C - 10 min), the serum concentrations of denatured whey proteins become negligible below pH 5.5 [18].

3.4.2.2. Casein

Fermentation of heated milk at 30 °C or 42 °C resulted in a regular and linear decrease in the concentration of casein in the supernatant as the pH decreased from 6.7 to 5.5 (Fig. 4). Unpelleted casein disappeared completely between pH 5.5 and 5.0 at both temperatures (Fig. 5A), but not at pH values currently expected, such as 4.1, 5.3, 4.5 and 4.1 which are values of isoelectrical pH for α_{S1}, α_{S2}, β and κ-CN, respectively [9]. In fact, due to the complexation of whey proteins with κ-CN, presumptions can be made that the casein micelle behaved during acidification as the outer layer of whey proteins. The isoelectric pH of α-LA and β-LG is 5.1 and 5.3, respectively [27].

Law [18] observed similar trends at 30 °C in milk heat-treated at 85 °C for

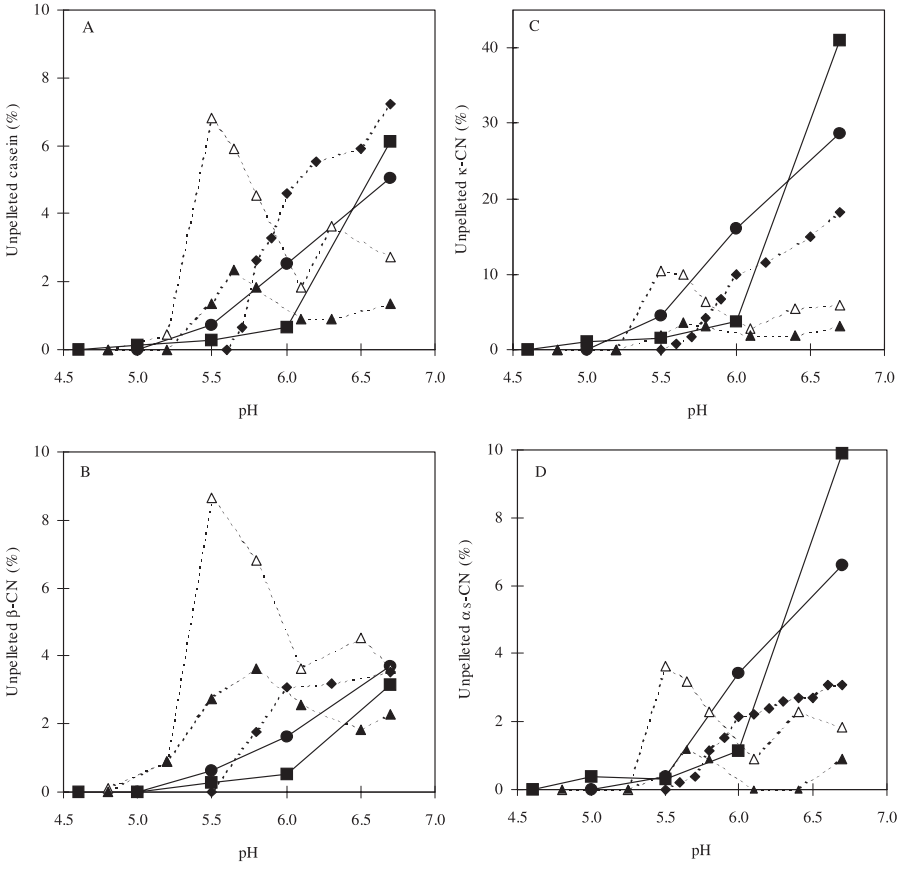

Figure 4. Changes in the amounts of unpelleted caseins as a function of pH determined with RP-HPLC. (A) total casein; (B) β-CN; (C) κ-CN; (D) α_S-CN (α_{S1}+α_{S2}). The acidification was carried out at 42 °C (■) or at 30 °C (●). The κ-CN and α_S-CN were analysed with DTT, while β-CN was analysed without DTT from the supernatant of ultracentrifugation performed at the fermentation temperature. Mean of 2 replicates. Values from curves in published papers: Law [15] on milk heated at 85 °C for 10 min and acidified by GDL at 30 °C (◆); Singh et al. [24] on unheated milk (△) or milk heated at 90 °C for 5 min (▲) and acidified by GDL at 22 °C.

10 min, after acidification with GDL. Many studies reported a dissociation of casein during acidification around pH 5.5 [6, 26, 29], with a maximum dissociation being dependent on various factors, such as the temperature of acidification or the heat treatment.

There is no difference in the casein content in the supernatant during acidification performed either by GDL or HCl [6]. This can be explained by the long time which is necessary for the pH-adjustment, which allowed numerous equilibria to take place.

All the caseins were affected by acidification (Fig. 4). At pH 6.6 and 42 °C, unpelleted casein appeared 1% higher than at 30 °C, but this led to rather small differences in concentrations, lower than 0.3 $g \cdot L^{-1}$ in the case of total casein. At 30 °C, a slightly higher concentration of unpelleted κ–CN and α_{S2}-CN was detectable around pH 6.2 (results not shown).

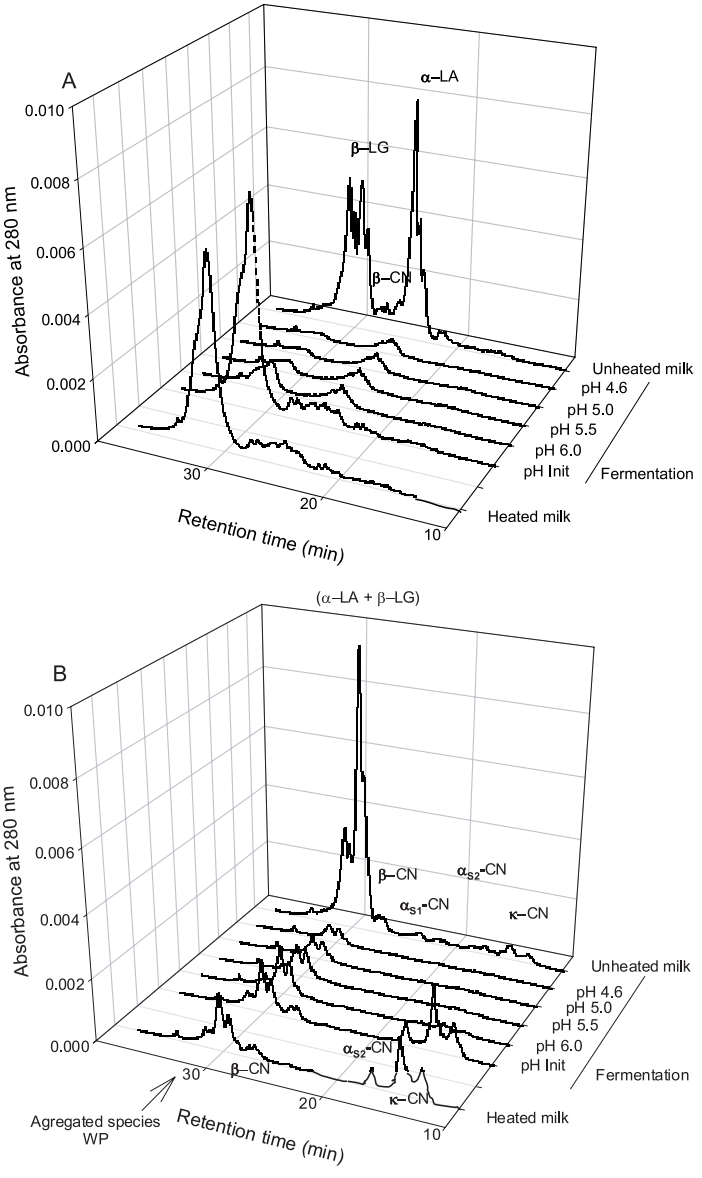

Figure 5. Changes in RP-HPLC elution profile patterns of supernatants for fermented milk as a function of pH. Fermentation was performed at 42 °C and supernatants were treated in urea buffer without DTT (A) or with DTT (B). Caseins: κ, α_{S1}, $\alpha_{S2,}$ β. Whey protein: α-LA, β-LG.

A delay of insolubilisation of these caseins during fermentation at 30 °C can be expected. According to Law [18] and Singh et al. [29], the concentration of casein in the supernatant decreases with an increased temperature of heat treatment and decreases with an increased temperature of acidification. On heat-treated milk, acidified with

GDL at 30 °C, a progressive decrease of the unpelleted casein with acidification was reported by Law [18], as in the present paper.

It is probable that the pelleted casein behaved like the colloidal salts and that its dissociation was shifted toward lower pH values. The comparison of the concentration of unpelleted casein during acidification obtained with GDL or bacteria was not performed. As the shift in salt dissociation was observed at pH lower than 6, the comparison will probably be difficult, because of the low levels of unpelleted casein at these pH values and the high standard deviation for the determination of casein concentration compared with that of minerals.

4. CONCLUSIONS

This static approach on fermentation of heated milk confirmed that the partition of casein in the pellet fraction has been shifted toward higher pH values than for unheated milk. The casein behaved like a complex governed by whey protein properties and it can explain the new texture properties of acid gels.

The comparison of calcium partition into diffusible and colloidal fractions between milk acidified by HCl (or GDL) and fermented milk has pointed out the importance of transfer phenomena between the solvent and the colloidal fractions during acidification, together with the calcium exchangeability inside the casein micelle. These phenomena are probably the slowest, despite the high hydration and porosity of the casein micelle.

It has been suggested that some differences between the outside and the inside of the micelle particle can arise during fermentation. We assumed that, while the outside can be at a pH where protein-protein interactions can progress and a gel can be formed, the inside is still in a mineralised state. These hypotheses pointed out the possibility for the micelle to present a decreasing pH gradient from the core to the surface with considerable technological consequences.

As transfer phenomena are suspected to be important, we studied the effect of the temperature at 30 and 42 °C. When the fermentation was carried out at 30 °C rather than at 42 °C, more casein was present in the unpelleted fraction, while the pelleted casein was more hydrated, but the salt dissociation was similar at both temperatures. We expect that these physicochemical changes of casein will result in modified rheological properties of yoghurt.

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