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Original article

Fermentation by lactic bacteria at two temperatures of pre-heated reconstituted milk. II - Dynamic approach of the gel construction

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Abstract – The behaviour of heat-treated skim milk towards acidification by fermentation with lactic bacteria was studied at 30 °C and 42 °C. Rheological changes using dynamic oscillations and ^{1}H-NMR relaxation time, T2, were studied with time and pH. Front-face intrinsic fluorescence measurements studied with principal component analysis were performed during fermentation. The gel was formed at 42 °C at a shorter time and a higher pH (5.53 ± 0.01) than at 30 °C (5.24 ± 0.13). The maximum in tan δ was higher at 42 °C than at 30 °C, but the G' values at pH 4.6 were the same at the two temperatures. The changes in T2 with pH were not greatly different at the two temperatures, but the dT2/dpH curves showed some differences with the temperature. The changes in T2 were due to the changes in the particle structure caused by the fermentation and were correlated with calcium solubilisation. Front-face fluorescence can detect a first shift of the maximal intensity toward lower wavelengths and a second shift toward longer wavelengths. These shifts were separated by the gel point and were due to changes in the environment of tryptophan residues in the protein chains. The discussion of these results and the comparison with GDL-induced gels take into account the limitation of the transfer of the acid and the protons from the aqueous to the colloidal phase and the subsequent heterogeneity of the gel.

Milk / yoghurt / pH / ^{1}H-NMR / rheology / intrinsic fluorescence

Résumé – **Fermentation de lait reconstitué et chauffé par des bactéries lactiques à deux températures. II. Approche dynamique de la construction du gel.** Le comportement du lait écrémé traité thermiquement au cours de l'acidification par des bactéries lactiques est étudié à 30 et 42 °C. Les modifications rhéologiques par oscillations dynamiques et le temps de relaxation T2 en RMN du proton sont étudiés en fonction du temps et du pH. La fluorescence frontale intrinsèque étudiée conjointement avec l'analyse en composantes principales a été suivie au cours de la fermentation. La gélification du lait apparaît à un temps plus court et un pH plus élevé à 42 °C (5,53 ± 0,01) qu'à 30 °C (5,24 ± 0,13). Le pic de maximum de tan δ était plus marqué à 42 qu'à 30 °C, mais le G' à pH 4,6 avait la même valeur aux deux températures. Les changements de T2 au cours

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de l'acidification étaient peu différents aux deux températures étudiées, mais les courbes dT2/dpH montrent quelques différences entre 42 et 30 °C. Les changements de T2 sont dus à des changements de structure de la particule provoqués par la fermentation et sont corrélés à la solubilisation du calcium. La fluorescence frontale détecte un premier décalage des spectres vers des longueurs d'onde plus petites puis un décalage vers des longueurs d'onde plus grandes. Le point associé à l'inversion de l'environnement des tryptophanes des chaînes protéiques correspond au point de gel. Ces résultats et les différences avec des gels acides obtenus par l'addition de GDL sont discutés en terme de limitation des transferts d'acides et de protons de la phase aqueuse vers la phase colloïdale et de l'hétérogénéité du gel qui en découle.

Lait / yaourt / pH / RMN du proton / rhéologie / fluorescence intrinsèque

1. INTRODUCTION

Most of the basic studies on dairy acid gel, besides the course of rheological changes, are performed in static conditions: the state of milk proteins has been studied at a final equilibrium pH value [7, 8, 35]. These methods are consequently unable to describe the dynamic phenomena occurring during the gelation of milk. Non-destructive techniques such as NRM or fluorescence have recently received attention.

Front-face fluorescence spectroscopy is a powerful technique for dynamic studies of colloidal non-transparent systems such as dairy emulsions [9], acid or rennet gels [9, 14–17] or even cheeses [11, 18]. The fluorescence spectrum of a probe in the food product is related to its environment. This method might be regarded as a non-destructive, in situ, powerful and sensitive method, when associated with PCA, particularly recommended when studying a gelation under varying physicochemical environments such as acidification. During the acidification of unheated milk with GDL studied with intrinsic tryptophan (TRP), a shift of the maximum of spectra to lower wavelengths was evidenced, due to a more hydrophobic environment of TRP [17]. A first linear increase of the intensity at 320 nm versus pH was reported, followed by a second one with a higher slope.

^{1}H-NMR provides knowledge at a molecular/atomic level of the mechanisms underlying the macroscopic processes. Changes of T2 during acidification have been studied either by GDL addition [12, 31] or HCl addition at 4 °C [35]. During acidification, T2 reached a maximum around pH 5.2 and then decreased. Finally, as water had a slower mobility in acidified milk than in native milk, interactions between water and the proteins were reduced [32]. Nevertheless, it is impossible to describe this water in terms of bound and free water in milk under fermentation. Many fractions of protons take part in the general behaviour: the protons of free water and the protons of the water in interaction. Interactions can be with, first, the soluble or micellar casein, second, the soluble, denatured or "micellar" whey proteins, and third, the colloidal calcium phosphate (CCP) and the exchangeable bound protons. The changes in T2 during acidification of milk to pH 5.3 are mainly explained by the decrease in hydrating proton relaxation, either the relaxation rate or the relative population of this fraction of protons [12, 31]. As the increase in T2 runs parallel to the solubilisation of phosphorus from the casein micelle [31], T2 changes may be related directly to the changes in interactions between CCP and water or indirectly, because of the changes in the casein structure. A recent paper, using static measurements after 18 h equilibration at each pH value and dynamic conditions with GDL addition for which T2 was measured in situ, showed that T2 reached a plateau region near pH 5.0, in each mode,

with no subsequent decrease [32]. Nevertheless, in the dynamic mode, T2 was shifted toward lower pH values.

The rheological dynamic method is a non-destructive direct method that is able to follow the changes in the state of proteins. It is a relevant method to exhibit the great differences in the acid gelation of unheated and heat-treated milk. In unheated milk, the tan δ decreases at pH ≈ 4.8 and a weak gel is formed. With heat treatment, tan δ first decreases then increases to a local maximum value and finally decreases. According to Lucey et al. [25], this behaviour of tan δ was observed for a heat treatment at 85 °C for 30 min. A great increase in the final value of G' was observed for a heat treatment ≥ 80 °C for 30 min, at more than 70% denaturation of whey proteins. The heat treatment of milk at 80 °C for 30 min also leads to a higher pH at gelation, 5.25 instead of 4.80 in unheated milk [28]. The increase in the pH at gelation was observed when milk contained soluble and micelle-bound aggregates of denatured whey proteins [28]. Furthermore, micelle-bound aggregates of whey proteins are necessary to reach a value of G', at acid pH, close to that of heated milk [28]. Anyway, soluble aggregates of denatured whey proteins lead to higher final G' values than in unheated milk [28]. The maximum of tan δ was obtained with soluble or with micelle-bound aggregates of whey proteins, but was more pronounced with micelle-bound aggregates [28]. The maximum of tan δ can reflect a transition between a denatured whey protein gel to a network dominated by casein-casein interactions [28].

Most of the work devoted to the mechanisms of acid gel construction has been performed on unheated reconstituted skim milk in which acidification is provided by the addition of glucono-delta-lactone (GDL). The aim of the present study is to elucidate the built-up of acid gels made from heated skim milk fermented by lactic acid bacteria. The GDL gel and acid gel obtained by fermentation have different microstructures and rheological properties [27]. This can be due to different acidification rates and diffusion processes. Studies on acidification rates with GDL have been performed in previous papers by changing the temperature or the GDL concentration. The gelation time and the moduli decrease, the gelation pH increases and the elastic properties decrease slightly when the incubation temperature increases [5, 6]. When the concentration of GDL increases, tan δ and the pH at gelation increase [5] and G' slightly decreases [26]. According to Lucey et al. [27], a faster gelation, such as that performed at 40 °C compared with 20 °C, is commonly regarded as conditions leading to coarser networks [27]. Moreover, the effect of temperature on the gel is related to changes in hydrophobic interactions inside the particle and consequently to the ability of the particle inside the network to rearrange in more dense clusters, given lower G' values [26, 27]. Anyway, it must be borne in mind that, according to Ronnegard and Dejmek [36], decreasing the temperature of measurement from 50 to 10 °C leads to a high increase in G' and to an increased elasticity of a set yoghurt.

In that work, the study of acid gelation of heated milk was performed at low (30 °C) and high (42 °C) temperatures, for a low and a high rate of acid production. In the hypothesis of a diffusion-limited gel formation by fermentation, changing the rate of acid production will modify the diffusion processes. In a previous paper [23], the composition of the heated milk and static measurements during acidification were presented. Dynamic measurements such as rheological characterisations, front-face fluorescence of intrinsic tryptophan (TRP) and ^{1}H-NMR are studied in this one.

2. MATERIALS AND METHODS

2.1. Yoghurt manufacture

The skim milk was reconstituted to 11% solid content from a low-heat skim milk

powder and was heated at 90 °C for 10 min as described by Laligant et al. [23].

Milk was equilibrated at 30 °C or 42 °C for 1 h and was inoculated with non-ropy frozen concentrated cultures of *S. thermophilus* and *L. bulgaricus* at 10^7 total CFU·mL^{-1} each. The kinetics of acidification was registered from pH 6.5 to pH 4.20–4.00 using a Cinac system (Ysebaert, Frépillon, France) [38]. A part of the heated milk was stored at 4 °C then fermented one day after. Fermentation kinetics was not changed by this storage at 4 °C. Fermentation temperatures were 30 °C and 42 °C, considered, respectively as slow and rapid acidifications.

2.2. Dynamic characterisations

Each type of dynamic measurement started in parallel with the pH monitoring.

2.2.1. NMR measurements

^{1}H-NMR relaxation measurements were obtained on a low-field spectrometer (Bruker, Wissembourg, France, 20 MHz, 0.47 T) at 30 and 42 °C. NMR tubes of 5 mm diameter and 20 cm length were filled with 0.5 mL milk, immediately after inoculation. The spin-spin relaxation time, T2, was calculated from a Carr-Purcell-Meiboom-Gill (CPMG) sequence. The 90–180 pulse spacing was 1.5 ms and the recuperation delay was 10 s. Each point on the curves was the mean of 8 accumulations and the points were spaced every 5 min.

The exponential decay curve of 845 points was fitted to 1 or 2 exponential equations according to the least square method. When the residues were not well distributed, a 2-exponential equation was needed.

2.2.2. Rheological measurements

The elastic (G') and viscous (G") moduli and the loss tangent, tan δ, were followed as a function of time or pH on the AR1000 rheometer (TA instruments, Waters, St-Quentin-en-Yvelines, France), in the oscillatory mode with a coaxial cylinder geometry at a frequency of 1 Hz and a strain of 0.02. The temperature of the milk was 30 or 42 °C. Milk was placed in the cup and re-covered with a thin layer of paraffin. The gel time was defined as the point where G' > 1 Pa. Four experiments were performed at each temperature.

2.2.3. Front-face fluorescence spectroscopy

Fluorescence spectra were recorded using a Perkin Elmer LS50B spectrofluorimeter (St-Quentin-en-Yvelines, France), equipped with a thermostated front-face accessory. The incidence angle of the excitation radiation was set to 60°; this angle value ensures that reflected light, scattered radiation and depolarisation phenomena are minimised. Emission spectra of TRP (305–400 nm) were recorded every 5 min with the excitation wavelength set at 290 nm at 42 °C or 30 °C, with a resolution of 0.5 nm. The emission and excitation slits were set at 5 nm. Milk was placed in a 10 mm path-length optical quartz cell. In a preliminary experiment, we checked that the applied fluorescence did not modify the pH curve during fermentation. Fermentation kinetics was performed in duplicate for each temperature.

All spectra were corrected for instrumental distortions in excitation by using a rhodamine cell in the reference channel. In order to reduce scattering effects, the area under the emission peak was normalised according to the formula of Bertrand and Scotter [1].

Principal component analysis (PCA) was applied to the normalised data using SAS software, using all of the experiments, that is two experiments at each temperature.

3. RESULTS

3.1. NMR

The transversal relaxation time, T2, of the milk increased from 150 to 300 ms as

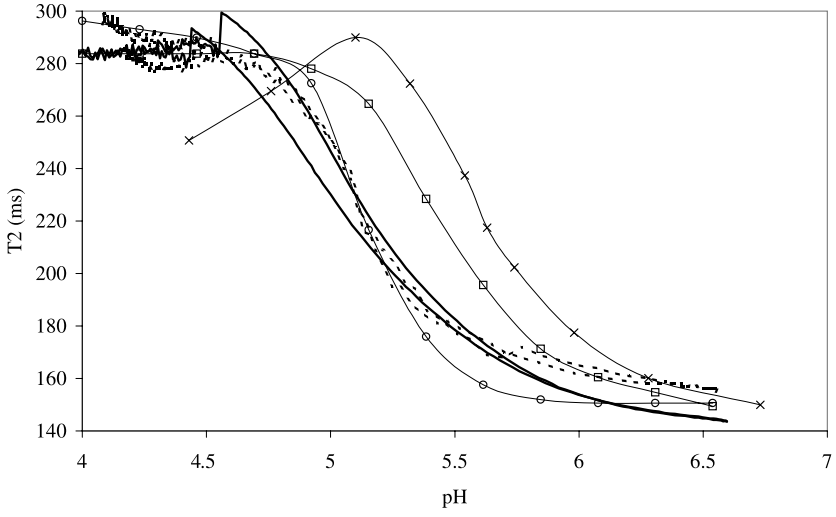

Figure 1. Protons-NMR transversal relaxation time, T2, versus pH values during acidification of heat-treated milk with a yoghurt starter, at 30 °C (—) and 42 °C (- - -). Two experiments at each temperature. Previous results from Mariette et al. [32] in a static way (□) and a dynamic way (○) and from Famelart et al. [12] in a static way (×).

the fermentation proceeded at 30 °C or at 42 °C and reached a plateau around pH 4.7 (Fig. 1). Some NMR data [12, 32] have been normalised to the same values as in the current study and are presented in Figure 1. T2 changes with acidification in the dynamic mode from Mariette et al. [32] are in good agreement with our results.

It should be noted that the reproducibility is better at 42 °C than at 30 °C. At 42 °C, at 215 min and pH 5.0, the relaxation curve can no longer be fitted by a monoexponential equation: a short T2 at 140–300 ms, and a long one, were obtained. The signal became noisy below pH 5 as reported by Roefs [34]. The presence of two populations of T2 indicated a proton heterogeneity, probably due to microsyneresis. At 30 °C, a single monoexponential behaviour was observed before 770 min and pH 4.5, beyond which a break in the record was noticeable. One parameter only was adjusted at 30 °C against 4 at 42 °C from a fixed number of data, resulting in a more regular pattern at 30 °C. As the sequence of measurement was not optimised for multicomponent analysis, only short T2 values were relevant and discussed.

T2 at pH 6.5 increased with the temperature, as expected from the increasing mobility of the hydrogen nuclei with temperature.

The calculated values of the first derivative of T2 against pH pointed out a small difference between 30 and 42 °C (Fig. 2). Depending on the pH zone (6.6–5.5, 5.5–5, 5–4.5), the dT2/dpH was greater (in negative values) at 30 or 42 °C. It seems that the dT2/dpH values did not change greatly from pH 6.6 to 5.5 at 42 °C, while they changed in a regular way from pH 6.6 to 5.0 at 30 °C. In fact, the difference between the initial T2 value and that at pH 5.0 was greater at 30 than at 42 °C (Fig. 1). The minimum of dT2/dpH was higher in absolute value at 42 °C than at 30 °C and the dT2/dpH value at pH 4.5 was around 0 at 42 °C, while it was between –50 and –100 ms at 30 °C. This means that T2 at 30 °C and pH 4.5 was still increasing while at 42 °C, it was almost constant.

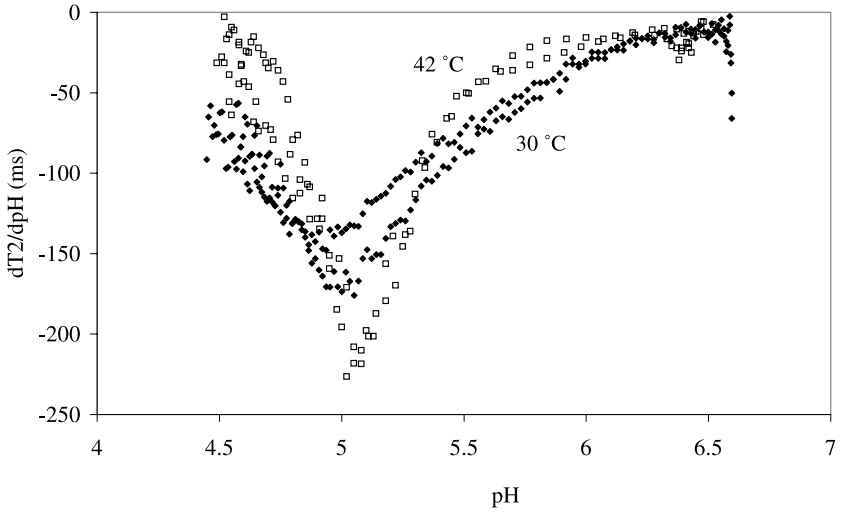

Figure 2. First derivative of T2 versus pH, dT2/dpH, versus pH values at 30 °C (◆) and at 42 °C (□). Two experiments at each temperature.

Table I. Kinetic parameters obtained by the dynamic rheological method during the fermentation of milk at 30 and 42 °C (values ± standard deviations).

Parameter	30 °C	42 °C
t_{gel}(in min)*	426 ± 29	149 ± 5
pH_{gel}	5.24 ± 0.13	5.53 ± 0.04
$min_{tan\ \delta}$ **	0.334 ± 0.006	0.339 ± 0.009
pH $min_{tan\ \delta}$ ***	5.16 ± 0.13	5.48 ± 0.04
$max_{tan\ \delta}$ **	0.350 ± 0.009	0.435 ± 0.003
pH $max_{tan\ \delta}$ ***	5.00 ± 0.10	5.02 ± 0.07
$G'_{pH\ 4.6}$ (in Pa)	414 ± 102	406 ± 30

* t_{gel} calculated from the decrease in tan δ; ** minimal and maximal values of tan δ; *** pH values at minimum and maximum values of tan δ.

3.2. Rheological characterisation

Changes in the dynamic moduli and tan δ versus time or pH at 30 and 42 °C are shown in Figure 3 and kinetic variables are presented in Table I. A shorter gel time and a higher pH at gelation at 42 °C than at 30 °C are observed. At 42 °C, a shoulder in the G' curve appeared, not visible at 30 °C. The pH at the minimum of the tan δ was higher at 42 °C than at 30 °C. The maximum in tan δ was found at pH 5.0 at both temperatures. Values of the minimum in tan δ appeared rather constant at 0.33 (δ = 18.3°) at both temperatures, while the "maximum in tan δ" appeared higher at 42 °C than at 30 °C. These results have been previously reported on heat-treated milk acidified by GDL [22, 25, 26] and with lactic bacteria [2, 27, 36, 40].

3.3. Front-face fluorescence

We obtained emission spectra for TRP with a maximum at 340–342 nm. The 8 spectral collections (4 at 30 °C, 4 at 42 °C), were represented toward principal components (PC) 1 and 2. An example for 2 collections at 30 and 42 °C is given in Figure 4. The other collections appeared similar, but for practical reasons, only two were represented. The spectral patterns are given in Figure 5. The first PC explained 95.2% of the total variance, and the second one

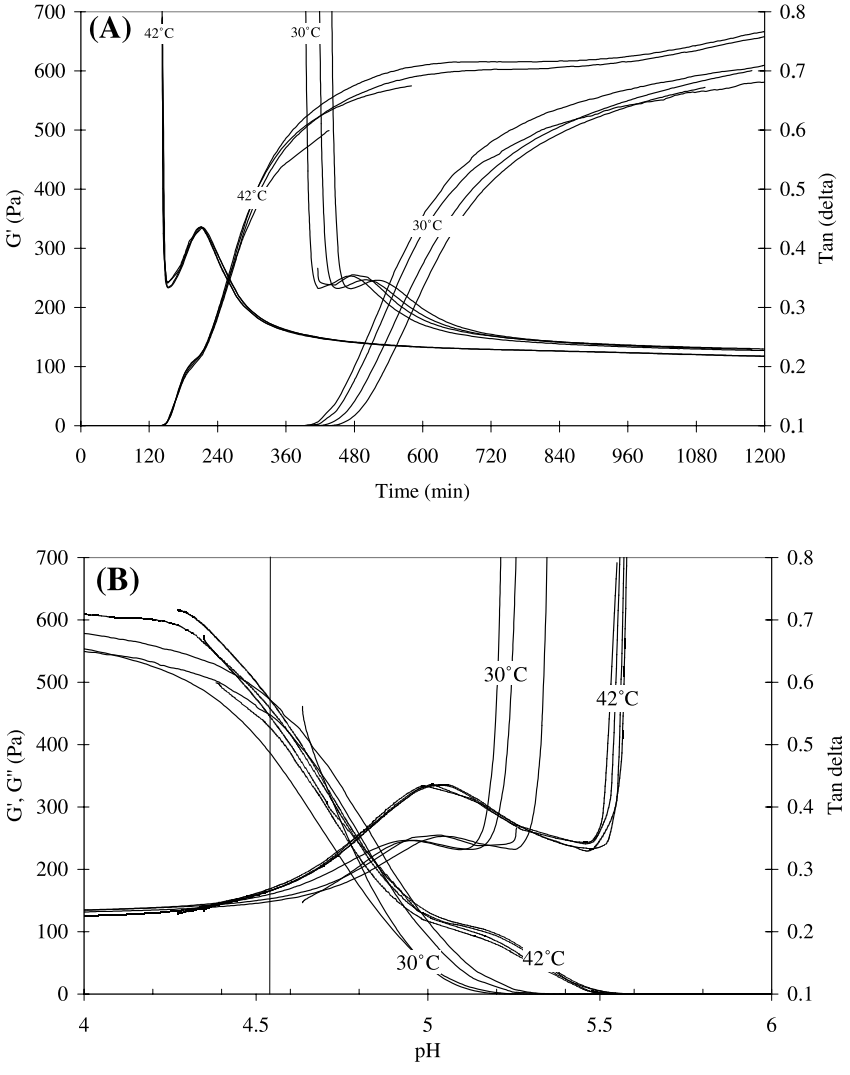

Figure 3. Rheological characterisation of milk under acidification with a yoghurt starter. The dynamic modulus, G' and tan δ are presented; (**A**) toward the time of acidification; and (**B**) toward the pH.

2.3%, which gives 97.5% in all. We contend that these two components are sufficient to map the data; they highly differentiated the samples regarding, first, the acidification time and second, the incubation temperature (Fig. 4).

The Eigenvectors for PC are similar to spectra and give structural information at the molecular TRP level. The spectral patterns for PC1 showed a minimum around 322 nm and a plateau from 370 to 400 nm, while for PC2 a maximum around 322 nm

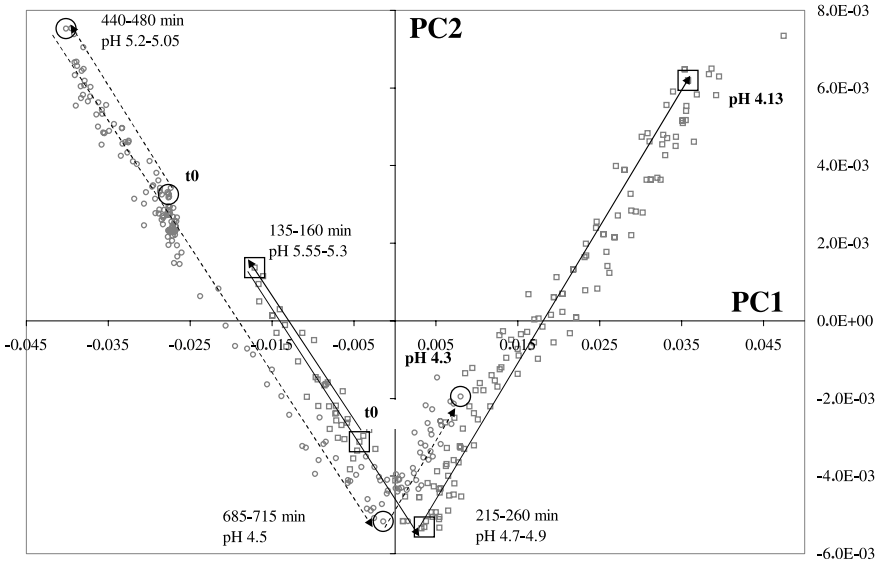

Figure 4. Principal component analysis map of the samples defined by the principal components 1 and 2 during acidification at 30 °C (· ○ ·) and 42 °C (—□—). Characteristic times and pH values on the curve are indicated, together with t0, the time when the starter has been added and the final pH value. Arrows indicate the direction of fermentation.

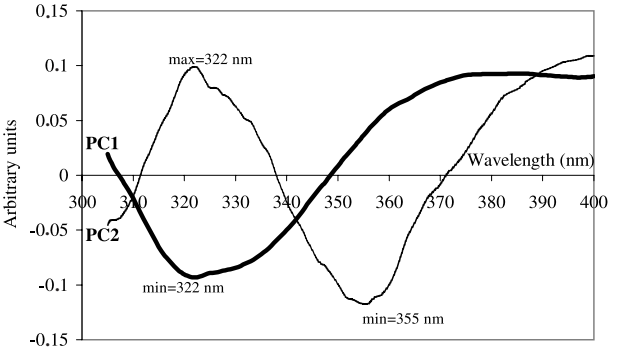

Figure 5. Spectral pattern of the first 2 principal components, PC1 and PC2, from front-face fluorescence spectra of intrinsic tryptophan. PC1 shows a minimum at 322 nm and PC2 a maximum at 322 nm and a minimum at 355 nm.

and a minimum at 355 nm were found. This result means that when successive spectra with time have increased positive scores on PC2, the intensity at 322 nm increased whereas that at 355 nm decreased with increasing time. As the emission spectra of TRP present a maximum around 340 nm in the present study, this would be explained by a displacement of spectra toward lower wavelengths. A thorough examination of a spectral collection during acidification showed that, in fact, before gelation, the spectra moved toward lower wavelengths. The maximal difference in wavelength was around 3 nm, which pointed up the small differences in the spectral

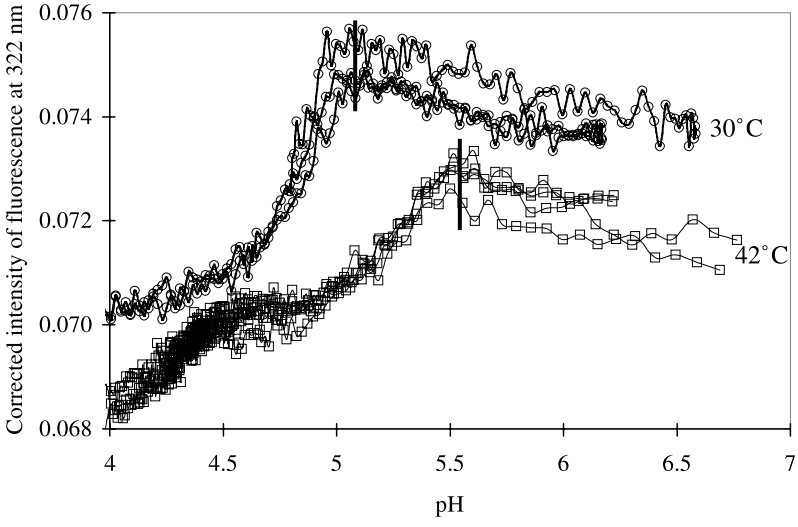

Figure 6. Corrected front-face fluorescence intensity at 322 nm versus the pH value along the acidification of heat-treated milk with a yoghurt starter at 30 °C (○) and 42 °C (□). The maximal intensity is shown with vertical bars.

changes. After the gel point, the spectra moved to higher wavelengths. The weak blue shift was probably due to a more hydrophobic environment of the TRP residues [15], while the red shift meant a more hydrophilic environment of the TRP.

The plateau for PC1 above 370 nm is explained by a broadening of the spectra with fermentation time. This broadening led to an intensity at 400 nm (the greater wavelength of the spectra) greater than 0. This is an artefact due to a signal not reverting to the baseline.

The factorial map in Figure 4 shows three phases: a first phase before the gel point, a second phase after the gel point and a third phase, from pH 4.5–4.9 to lower pH values. The scores on PC1 decreased during the first phase and increased in the two following phases. The scores on PC2 increased, decreased and re-increased from the first to the third phase.

The PC2 allowed discrimination between fermentation at 30 or 42 °C, because during the acidification down to pH 4.5–4.9 (the minimum value of PC2), the scores on the PC2 axis increased from samples at 42 °C to samples at 30 °C. At t0, the score on PC2 was much higher at 30 than at 42 °C (0.002 ± 0.001 versus -0.004 ± 0.001). This difference between 30 and 42 °C was maintained until a pH value of 4.5–4.9 was reached, when looking at the curves and even when comparing the co-ordinate on PC2 at the same pH value.

Additional knowledge given by PC2 compared with PC1 is that, when the pH reached values lower than 4.5–4.9, a new change took place, with a re-increase in hydrophobicity.

The changes in the corrected fluorescence intensity with pH for the 8 spectral collections at 322 nm are represented in Figure 6. This wavelength was selected because it discriminated the best between the samples of the spectral collection. Following a slight increase from t0 to the gel point, at pH 5.0–5.2 and 5.5–5.7, the intensity decreased until pH 4.5–4.6 and pH 4.7–4.9 were reached, where a plateau was observed, at 30 and 42 °C, respectively. The fluorescence is able to follow the acidification

process in situ, as the times detected are in accordance with those obtained by the other methods of this paper. The pH at gelation was higher at 42 °C than at 30 °C and the decrease in the fluorescence intensity at 322 nm following the gelation was much more pronounced at 30 than at 42 °C.

4. DISCUSSION

The effect of temperature between 30 and 42 °C on the acid gelation of milk cannot be related to a temperature-dependent solubilisation of casein [5]. Indeed, casein solubilisation is very close between 20 and 30 °C [7, 24, 37] and probably between 30 and 42 °C. From our point of view, the effect of temperature on the aggregation rate constant might explain the higher gelation pH at 42 than at 30 °C. In rennet gelation of milk, the Q_{10} of the aggregation reaction was estimated at 11–12 between 20 and 50 °C [4], meaning that the rate constant is divided by 10 when the temperature decreased by 10 °C. It is probably the same with acid gelation. At pH 5.3, no gelation is visible at 30 °C. Increasing the temperature from 30 to 42 °C would probably lead to gelation.

However, changing the temperature changes not only the rate of fermentation, but also some characteristics of the micelles, as observed for solvation data [23], the rate of aggregation and thermal motion. We chose to change the temperature rather than the inoculum level to obtain a low or high acidification rate because, as already shown by Mietton et al. [33], varying the inoculum level changes the lag time but has no effect on the acidification rate between pH 6 and 4.6. Changing the inoculum level only changes the acidification rate between pH 6.6 and 6.0, and mainly the duration of the lag phase. It has no effect on the acidification rate between pH 6 and 4.6, despite different total fermentation times. Anyway, it must be borne in mind that these results depend on the type of yoghurt strain or cultures, for example, the ropy or non-ropy characteristics, the method of inoculation and the optimum temperature of the strain used for yoghurt fermentation.

4.1. NMR

The great effect of pH on T2 values of skim milk under acid gelation confirms that pulse NMR measurement of hydrogen nuclei of water is a useful technique for monitoring in situ the protein-water interactions in such a complex system.

The changes in T2 during acidification of milk reflected the decrease in hydration of the casein structure. According to Roefs et al. [35], on acid gels formed in the cold, a decrease in T2 is obtained at pH under 5.2, that is explained by the increased gelation. In the current study, no decrease in T2 appeared at these pH values, as in the study of Mariette et al. [32]. A shift toward lower pH values of T2 curves versus pH was evidenced. Mariette and Marchal [30] and Mariette et al. [32] have already reported that the relaxation curve depends on the acidification process. The T2 curve of milk acidified by addition of GDL at 40 °C and measured during the course of the pH decrease (dynamic condition) is shifted toward acidic pH values compared with the T2 values of milk acidified by the same way and measured after a 24-h equilibrium (static condition). As a correlation between soluble phosphorus and T2 has been found in acidified milk [31], the dependence of the T2 curve on the rate of acidification was related to the mechanisms of solubilisation of the CCP [30]. At the final equilibrium (in the static condition), the diffusion of H^+ and CCP is complete and the concentration of CCP released from the casein micelle is maximum at a given pH, leading to a higher T2 value. In the previous paper of this series [23], we found a shift toward more acid values of the soluble concentrations of calcium with the bacterial acidification compared with either GDL or HCl addition. It is well known that the dissociation of minerals from milk under acidification is dependent on the rate of acidification [19].

This shift of the soluble concentration of calcium can be related to the shift of T2 in the present results.

Moreover, both temperatures used in this study gave similar changes in T2 (Fig. 1). When using the dT2/dpH, it was possible to show a slight difference between 30 and 42 °C (Fig. 2). Changes in the structural network organisation between pH 6.6 and 5.2 appeared more regular and more pronounced at 30 than at 42 °C. This means that the mobility of water or interactions between proteins and water changed from the beginning of the acidification at 30 °C, and not at 42 °C. T2 is not sensitive to gelation, but the time for the minimum value of dT2/dpH corresponded to the maximum of tan δ.

4.2. Rheology

The results are very similar to those obtained by Lucey et al. [27] when studying gelification either by GDL or lactic starters at 30 and 42 °C. The only difference is that the current values of G' are 200% of that of Lucey et al., but their heat treatment was 85 °C-30 min against 90 °C-10 min in the current study. Increasing the temperature leads to a decrease of 20% in G' at pH 4.6 [27], but we could not detect any G' difference. This rheological method is powerful, but even if mechanical spectra are performed as done by Lucey et al. [25, 29], it does not alone allow complete comprehension of acid gelation.

4.3. Front-face fluorescence spectroscopy

The maximum in the emission spectra of TRP was found at a relatively higher value than in previous papers, 340–342 nm against 330–333 nm, either in emulsions with dairy proteins, in processed milk or in soft cheeses [9, 10, 17, 18]. This shift of the maximum cannot be due to the heat treatment of milk. According to Dufour and Riaublanc [9], the spectra for heat-treated milk at 70 °C for 20 min are larger than those of unheated milk, but no shift was reported.

From the PCA, it appeared that at pH values lower than 4.5–4.9, a re-increase in hydrophobicity was observed. At these pH values, either with NMR or rheology, the changes resembled a plateau, while the fluorescence spectroscopy detected a real behaviour change.

The PC2 presented a maximum at 322 nm and a minimum at 355 nm. Such an opposition between 322 and 355 nm in the spectral pattern of PC2, due to the displacements of the TRP spectrum, has been widely observed [9, 10, 17]. In this paper, the factorial map shows 3 phases. During acidification of unheated milk with GDL, only a shift of the maximum of the spectra to lower wavelengths has been reported [9, 10, 17]. This can be explained, first, by the fact that acidification processes by GDL or fermentation are different and second, by the heat treatment of milk. The displacement toward higher wavelengths was not found by Herbert et al. [17], meaning that the second part of the reaction, the increase in the hydrophilic environment of TRP did not take place during the construction of acid GDL gel from unheated milk. It must be pointed out that the lower pH value studied by Herbert et al. [17] was at pH 4.5, the pH_I of casein.

The broadening of the emission peak meant that new populations of TRP appeared as the pH decreased, indicating an increased heterogeneity of the TRP. For example, TRP inside or outside the whey protein aggregates or TRP of casein chains or of native or denatured whey proteins can be in different environments. It is not possible to determine which protein takes part in this broadening, but using a milk model containing only a part of milk might allow verification of this.

This method is sensitive to the molecular state of a protein and probably to its heterogeneity. It detected the gel point and another point at pH 4.5–4.9. It has never been performed on fermented heat-treated milk. Unheated milk and heat-treated milk showed different results and different

rheological changes, so that we believe that this method can be really powerful. It seems to be the most informative method and the more simple as, in conclusion, only the fluorescence intensity at 322 nm is required. It is performed without any movement, contrary to rheological methods.

4.4. General discussion

To look at the diffusion mechanisms involved in the bacterial acid gelation of milk, we need to understand the complete process of acidification. We propose these successive steps: first, lactic acid is produced and immediately dissociated into lactate and protons. These protons are immediately bound by acids with a higher pK value: citric and phosphoric acids, for example. As the pH of the aqueous phase decreases, the CCP dissolves in the aqueous phase, since its solubility increases. This phenomenon is dependent on the pH of the aqueous phase, but does not require the diffusion of protons and acids inside the particle. In the second step, more or less superimposed with the first one, the protons and previously cited acids have to move through the micelle to reach the phosphoserine residues and dissociate the bound calcium. While in chemical acidification, the sources of acid production are numerous and well distributed, the production of acid from bacteria is confined to a few small areas. Indeed, bacterial cells grow in macro-colonies as demonstrated by Favrot [13], and milk under fermentation is composed of areas with colonies and areas without colonies, meaning that diffusion of acids is needed. The number of macro-colonies is probably very close to the number at inoculation, 10^6–10^7 per mL. In the case of acidification with GDL, the number of locations for acid production is close to 10^{23} per mL, so more uniformly distributed. We supposed that the aqueous phase surrounding a group of cells is at a very acidic pH. Consequently the first layer of casein around these void spaces, previously reported [3, 20, 21, 39], is at a rather low pH and CCP is already solubilised and the casein is gelled. We consider that this gel layer at the interface between the network and the aqueous phase is an additional barrier to the diffusion of the protons inside the casein and is responsible for an extreme pH gradient and an increased heterogeneity in the gel. The diffusion of acids is probably limited inside the colloid, despite its high porosity. For example, T2 measurement with ^{1}H-NMR of micellar casein is lower than for casein in a soluble form, as in a sodium caseinate [31]. The mobility of water is reduced in the micellar particle and a slow diffusion regime is met [30]. Increasing the temperature from 30 to 42 °C in GDL gels led to increased heterogeneity and decreased G' values [27]. At 42 °C, the milk will proceed in a state of limited diffusion due to the gelled state for a larger range of pH than at 30 °C. This can probably explain the differences observed between rheological changes of milk under acidification at 30 and 42 °C.

Lowering the temperature of fermentation can be a potential way of reducing the rate of acid production and the heterogeneity of acid gels, but it must be borne in mind that reducing the pH gradient by a lower temperature also reduced the diffusion rate of acids.

This study was conducted, firstly, with the use of lactic acid bacteria and secondly, with dynamic, non destructive methods such as rheology, NMR or fluorescence. Though this suitable approach, the complete understanding of the gel construction needs further investigation. We are aware that recent experimental methods sensitive to physicochemical heterogeneity are needed for a further comprehension of acid gelation, and that a better understanding of the molecular species formed by the heat treatment will probably help the research advances.

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