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Modelling of the acidification process and rheological properties of milk fermented with a yogurt starter culture using response surface methodology

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Abstract

The simultaneous effects of fermentation temperature (FT, 36.7–43.4 °C), milk total solid level (TS, 11.3–14.7%, w/v) and total inoculum concentration (TI, 1.66–3.34 v/v) on the acidication process and the rheological properties of milk fermentation with *Lactobacillus delbrueckii* ssp *bulgaricus* Y 6.15 and *Streptococcus thermophilus* Y 4.10 were explored by the means of response surface methodology. Maximum storage modulus (G'_{max}), minimum loss tangent ($\tan\delta_{min}$), rate of gelation (I_E) and onset of gelation were the rheological parameters studied, whereas maximum acidification rate (V_m), time at which maximum acidification rate was observed (T_m) and time to reach the end of fermentation (T_e) characterized the kinetics of acidification during milk fermentation. TS strongly affected G'_{max} and $\tan\delta_{min}$; high TS resulted in a large increase in G'_{max} and decrease in $\tan\delta_{min}$. Increasing fermentation temperature gave a decrease in the onset of gelation, in T_m and T_e, and increase in the rate of gelation (I_E). V_m was mainly affected by the interaction TS×FT. Under conditions of relatively low FT (37–39 °C), high TS (around 14%) and high TI (3–3.5%), the gelation and acidification rates were at medium levels and fermentation took a longer time to finish, but the formed gels were firmer, showing higher G'_{max} and lower $\tan\delta_{min}$ values. Under all conditions examined, a high number of viable lactic acid bacteria in the final product was detected during storage (21 days, 4 °C) for both microorganisms used in the starter culture.

Keywords: Response surface methodology; Yogurt; Rheology; Modelling; Kinetics; Milk; Acidification; Lactic acid bacteria; Fermentation

1. Introduction

Yogurt is a product of milk fermentation with thermophilic homofermentative lactic acid bacteria *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. It has become a very popular staple food and its consumption is still increasing. The excellent sensory properties, the expanded variety and the health benefits yogurt has been credited with are the essential reasons for such popularity (Yukuchi, Goto, & Okonogi, 1992). The healthy properties of yogurt, to a great extent arise from the action of viable yogurt bacteria and their metabolites (Deeth & Tamime, 1981).

The textural characteristics of yogurt gel are of primary importance with reference to the quality of the final product. Different technological and milk compositional parameters have been reported as influencing the structural and biochemical characteristics of yogurt. Total solids content of milk has been shown to affect its firmness and viscosity (Becker & Puhan, 1989; Biliaderis, Khan, & Blank, 1992; Gastaldi, Lagaude, Marchesseau, & Tarodo De La Fuente, 1997; Tamime & Robinson, 1985). The susceptibility to syneresis (Harwalkar & Kalab, 1983; Hess, Roberts, & Ziegler, 1997) and the development of starter culture (Radke-Mitchell & Sandine, 1986; Tamime & Deeth, 1980) are also significant quality determinants of the fermented product. Fermentation temperature primarily affects the growth of yogurt bacteria (Radke-Mitchell & Sandine, 1986), and thereby the structure (Beal, Skokanova, Latrille, Martin, & Corrieu, 1999; Lucey, van Vliet,

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Grolle, Geurts, & Walstra, 1997b) and flavour of yogurt. Moreover, the amount of inoculum is important for a normal acidification process (Tamime & Robinson, 1985) and to ensure a desired level of final bacterial count at the end of fermentation and throughout the cold storage of the product.

In terms of rheology, yogurt is a viscoelastic and thixotropic (time-dependent) material (Benezech & Maingonnat, 1994; De Lorenzi, Pricl, & Torriano, 1995). Among the factors influencing the rheological properties of yogurt are: milk composition, dry matter content, amount of strains (inoculum) used, incubation temperature and storage time. Small deformation oscillatory testing provides useful information on the gel formation process. In a strain-controlled experiment, the sinusoidal oscillating stress is recorded as a response of an applied sinusoidal oscillating strain. Some of the parameters determined from this small deformation mechanical testing are: the elastic or storage modulus (G'), which is a measure of energy stored per oscillation cycle, the viscous or loss modulus (G''), which is a measure of the energy dissipated as heat per cycle and the loss tangent (tanδ), which is the ratio of the viscous to elastic modulus (Lucey & Singh, 1998). The pH measurement method, described by Spinnler and Corrieu (1989), is widely used in determination of the acidification kinetics during gel formation. Maximum acidification rate (V_m) and the time at which V_m is reached (T_m) are calculated from the pH curves obtained from a continuous record of pH during the fermentation process.

Torriani, Gardini, Guerzoni, and Dellaglio (1996) have recently studied the influence of variation of parameters such as fermentation temperature, fat and solid content, inoculum size and initial cocci/rods ratio on the fermentation process of yogurt by applying response surface methodology. The method was useful in modelling of the acidification rate and the growth of starter bacteria as a function of the above parameters and in considering optimal combinations of the factors for a better fermentation process. The objective of the present work was to model and evaluate the simultaneous effects of fermentation temperature, total solids level of milk and total inoculum level, on the rheological properties and acidification kinetics of milk fermentation with two starter bacteria *S. thermophilus* Y4. 10 and *L. bulgaricus* Y6. 15 isolated from traditional Greek yogurts.

2. Materials and methods

2.1. Materials

Starter bacteria of *S. thermophilus* Y 4.10 and *L. bulgaricus* Y6. 15 were obtained from the collection of the laboratory of Food Microbiology and Hygiene, Aristotle University of Thessaloniki, Greece; these strains have been isolated from traditional Greek yogurts. Skimmed milk (Regilait UCA, Sr. Martin, Belle-Roche, France) was purchased from a local retail market. The media used for microbiological analysis were from Oxoid Ltd (Barington, UK).

2.2. Methods

2.2.1. Culture propagation and maintenance

L. bulgaricus Y 6.15 and *S. thermophilus* Y 4.10 were stored at −75 °C in MRS broth + glycerol (70:30) and M 17 broth + glycerol, respectively. Each strain was subcultured twice in the respective media at 37 °C for 18 h. Finally, 0.2 ml of the prepared microbial suspension was inoculated in tubes with 7 ml of 10% (w/v) sterilized (121 °C, 10 min) reconstituted skim milk, mixed with a vortex and stored at −20 °C, without incubation, until required.

2.2.2. Preparation of cultures for experimental use

Strains stored at −20 °C were thawed completely and incubated for one night at 37 °C. Nine decimal dilutions in sterile peptone water (0.1% w/v) were prepared from the fresh cultures of each strain. Aliquots of 0.2 ml from each dilution (including the undiluted sample) were transferred into sterilized reconstituted skim milk (10% w/v) and incubated at 37 °C for 24 h. The last dilution of the tube series showing milk coagulation was employed as inoculum in yogurt manufacture (Hassan, Deschamps, & Richard, 1989).

2.2.3. Yogurt preparation

The predetermined amount of skim milk powder was dissolved in distilled water and aliquots of 100 ml were distributed in previously sterilized (121 °C, 15 min) glass bottles. Milk was heat-treated at 85 °C for 30 min by placing the bottles in a circulating water bath preset at 87 °C; the reported time (30 min) included the time required for milk to reach a temperature of about 85 °C (~5–6 min). The bottles were cooled to 4–5 °C by putting them into ice water. The starter cultures were added to the bottles under aseptic conditions to avoid contamination. The milk bottles were then incubated at the required temperature until a pH of 4.6 was reached. The bottles were fast cooled by immersing them in ice water and after that, stored in the refrigerator at 4 °C.

2.2.4. Rheological measurements

Gel development was monitored in a Physica MCR 300 Rheometer, using a concentric cylinder geometry and applying dynamic oscillatory measurements. The rheometer was preset to the desired fermentation temperature and a few drops of paraffin oil were added at the top of the mixture to prevent evaporation. Storage

modulus (G'), loss modulus (G'') and loss tangent (tanδ) were measured in 5 min intervals at a fixed frequency and strain of 1 Hz and 1%, respectively, throughout the time course of gel formation, until plateau values of G' were reached. The structure of the yogurt gel was characterized by the following rheological parameters:

Maximum storage modulus (G'_{max}) (Pa) was determined as the plateau value of G'.
Minimum loss tangent ($\tan\delta_{min}$) was the value of tanδ corresponding to G'_{max}.
Elasticity increment J_E [$(\mathrm{dlog}\ G'/\mathrm{d}t)_{max}$], as introduced by Bohm and Kulicke (1999), and defined by the slope of log $G'(t)$ at the turning point. This parameter is used as a measure of gelation rate (i.e. a high I_E value implies rapid gelation) and indicates the number of decades G' increases at maximum per unit time; its dimension is reciprocal time.
Onset of gelation was determined as the time (min) at which the G' value overcomes that of G''.

2.2.5. Microbiological analysis

Aliquots of about 10 ml yogurt were aseptically collected from the bottles in sterilized test tubes at the beginning and at the end of fermentation (i.e. '0 time' and when pH 4.6 was reached), and also after 7 and 21 days of storage at 4 °C. After mixing with a vortex, 1 ml of the sample was taken from the test tubes and decimally diluted in sterile peptone water. Enumeration of bacteria was carried out using the pour plate technique. *S. thermophilus* Y 4.10 was enumerated on M17 agar, adjusted to pH 7, whereas *L. bulgaricus* Y 6.15 was on MRS agar. Samples of 1 ml from three serial dilutions were transferred to Petri dishes, where melted-tempered media (at 45 °C) was poured. Anaerobic environment for *L. bulgaricus* Y 6.15 was created by pouring ~10 ml media onto the Petri dishes with already solidified media. Plates were incubated at 37 °C for 48 h. Plates containing 30–300 colonies were enumerated and recorded as colony forming units per ml of culture (cfu/ml).

2.2.6. Acidification activity measurements

The changes in pH during fermentation were monitored continuously by means of a glass electrode pH meter (Hanna Instruments, Padova, Italy) according to the method of Spinnler and Corrieu (1989). The electrode was standardized carefully by means of two buffers (pH 7.0 and 4.0), disinfected with 70% (v/v) alcoholic solution, rinsed with sterilized distilled water and placed in the milk bottle under aseptic conditions. The pH was automatically recorded at 15-min intervals. Maximum acidification rates (V_m) were calculated from the pH–time curves according to the equation $V_m = (\mathrm{dpH}/\mathrm{d}t)_{max}$ and expressed in absolute values. V_m, the time at which the maximum acidification rate was observed (T_m) and the time at which pH 4.6 was reached (T_e) were considered as responses which characterized the kinetics of the process.

2.2.7. Experimental design and statistical analysis

Twenty experiments were performed according to a second order central composite rotatable design with three variables and five levels of each variable (Khuri & Cornell, 1989). The independent variables were fermentation temperature (FT), total solids level (TS) and total inoculum level (TI). The experimental design in the coded and actual levels is shown in Table 1. The statistical analysis of the data was performed using the MINITAB Statistical Software, Release 13.1. Initially, the full term second order polynomial response surface models were fitted to each of the response variables, according to the following equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \varepsilon$$

where β_0, β_1,..., β_{23} represent the estimated regression coefficients, with β_0 being the constant term, β_1, β_2, β_3 represent the linear effects, β_{11}, β_{22}, β_{33} the quadratic effects and β_{12}, β_{13}, β_{23} the interaction effects;, ε is the random error, while the X_1, X_2, X_3 are the independent coded variables (FT, TS, TI, respectively).

The analysis was performed using coded units. Where possible, stepwise deletion of terms was applied to remove the statistically non-significant terms, so simplifying the model. However, when the exclusion of such terms from the model decreases R^2 (adjusted) and increases the estimator of the variance S. the term was included in the model. The statistically non-significant linear terms also remained in the model when the respective quadratic or interactive effects were statistically significant. Based on such considerations, besides the significant terms (at 0.05 level), statistically non-significant terms may also be included in the final models. The results are means of two replicates. All generated models, except those for I_E, V_m and T_e, adequately explain the variation of the responses with high R^2 values ($R^2 > 0.90$) and nonsignificant lack-of-fit. The models for I_E, V_m and T_e explained 82.3, 85.2 and 85.5% of the variability, respectively, and can be considered adequate, since the probability level of F was $P < 0.001$ (Thompson, 1982).

3. Results and discussion

3.1. General

Table 2 summarizes the estimated regression coefficients of the quadratic polynomial models for the response variables, along with the corresponding R^2 and F values.

Table 1
Experimental design and levels of factors in natural and coded values

Factors	Coded factor	Coded values				
		−1.682	−1	0	+1	+1.682
Fermentation temperature (°C)	FT	36.64	38	40	42	43.36
Total solid level (% w/v)	TS	11.32	12	13	14	14.68
Total inoculum level[a] (% v/v)	TI	1.66	2	2.5	3	3.34

Run	Factors		
	Fermentation temperature (°C)	Total solid level (% w./v)	Total inoculum level (% v/v)
1	38	12	2
2	38	12	3
3	38	14	2
4	38	14	3
5	42	12	2
6	42	12	3
7	42	14	2
8	42	14	3
9	36.64	13	2.5
10	43.36	13	2.5
11	40	11.32	2.5
12	40	14.68	2.5
13	40	13	1.66
14	40	13	3.34
15	40	13	2.5
16	40	13	2.5
17	40	13	2.5
18	40	13	2.5
19	40	13	2.5
20	40	13	2.5

[a] Total inoculum comprises both bacteria in ratio 1:1 (v/v) for each level.

Table 2
Regression coefficients of the second-order polynomial model for the response variables (analysis has been performed using coded units)

Factors[a]	G'_{max} (Pa)	$\tan\delta_{min}$	Onset of gelation (min)	I_E (min^{-1})	V_m (pHmU/min)	T_m (min)	T_e (min)
Constant	−257.67	0.253	199.86	−1.287	18.209	163.89	241.36
FT	−0.559	0.00191**	−27.44***	0.0708***	0.613*	−15.06***	−23.29***
TS	44.22***	−0.00350***	−5.14*	−0.0062	−0.881*	−2.95*	3.31
TI	8.976***	−0.00204***	−8.22***	−0.0080	−0.762**	−6.55***	1.42
$(FT)^2$	−4.138***	−0.00061	6.77***	–	−0.399	2.13	–
$(TS)^2$	2.226*	–	–	−0.0513**	0.339	−2.64*	−8.44*
$(TI)^2$	−3.607**	0.00168**	5.89***	−0.0577**	−0.231	4.25**	–
FT×TS	–	−0.00113	–	–	−1.237***	1.87	10.62*
FT×TI	−6.875***	0.00138*	–	−0.0655**	–	–	−9.88*
TS×TI	−6.625***	0.000879	–		0.357	4.87**	–
R^2	0.995	0.915	0.971	0.823	0.852	0.953	0.855
F	262.67	14.72	93.04	10.09	7.92	28.11	12.75
Probability of F	$P \leqslant 0.0001$	$P \leqslant 0.0001$	$P \leqslant 0.0001$	$P \leqslant 0.0001$	$P \leqslant 0.001$	$P \leqslant 0.0001$	$P \leqslant 0.0001$

[a] Factors: FT = fermentation temperature (°C); TS = total solid content (% w/v); TI = total inoculum level (v/v%).
* $P \leqslant 0.001$; ** $P \leqslant 0.01$; *** $P \leqslant 0.05$.

3.2. Rheological properties of milk gel

Small amplitude shear stress oscillatory testing was used in this study to follow the development of gel structure during fermentation. Storage modulus (G') and loss modulus (G'') were the parameters characterizing the viscoelastic character of the product; the relative contribution of each of these moduli in the viscoelastic character of the gel is expressed by the tangent of the phase shift angle (δ), known as loss tangent ($\tan\delta = G''/$

G'). The G' value characterizes the degree of solid-like character of the gel; on the other hand, tanδ is a better predictor of the viscoelastic properties of the cross-linked network as it includes the contribution of both the elastic (G') and the viscous (G'') components. The higher the G′ and the lower the tanδ values, the more solid-like is the character of the gel, and the firmer is the gel. The maximum or the plateau value of G' (G'_{max}) and the minimum value of tanδ (tanδ_{min}), corresponding to that of G'_{max}, were also recorded as responses and modelled versus FT, TS and TI to further rheologically characterize the acidified milk gel system.

TS was the main factor affecting the G'_{max} and tanδ_{min} values, as revealed by the respective regression coefficients (Table 2). It exerts a positive linear effect on G'_{max}, as depicted in Fig. 1a while, for tanδ_{min}, this effect was negative (Fig. 1c), as expected; i.e. G'_{max} increases and tanδ_{min} decreases by increasing the TS of milk, resulting in a firmer coagulum. The strong positive effect of TS in yogurt firmness is well known and has been confirmed by other investigations (Biliaderis et al., 1992; Gastaldi et al., 1997; Xu, Stanley, Goff, Davidson, & Le Maguer, 1992; Walstra, 1998). As an illustrative example, the G' and tanδ values as function of time for the tests 11 (11.32% w/v solids) and 12 (14.68% w/v solids), which differ only in the TS content, are depicted in Fig. 2. Faster gelation with higher G' and lower tanδ plateau values were observed for the latter experiment.

All factors examined, with their quadratic and interaction terms, significantly affected the G'_{max}, except the linear term of FT and the interaction term of FT–TS, as shown in Table 2. The positive linear effect of TI and the negative effect of FT–TI, suggest that the higher values of G'_{max} (around 310 Pa) are observed where low FT (37–39 °C) are combined with high TI (3–3.5%) and TS (around 14%). The lower tanδ_{min} values are also observed under similar conditions, as is supported by the positive sign of linear regression coefficients of FT and the negative signs of those of TS and TI (Table 2). In contrast, Arshad, Paulsson, and Dejmek (1993) found no dependence of tanδ values on gelation temperatures and the concentration of milk solids in gels made by acidification with glucono-δ-lactone. Previous studies have also indicated that lower gelation temperatures in acid milk gels made by acidification with glucono-δ-lactone (Arshad et al., 1993; Lucey, Tamehana, Singh, & Munro, 1998b; Lucey, van Vliet, Grolle, Geurts, & Waistra, 1997a) and by fermentation with a

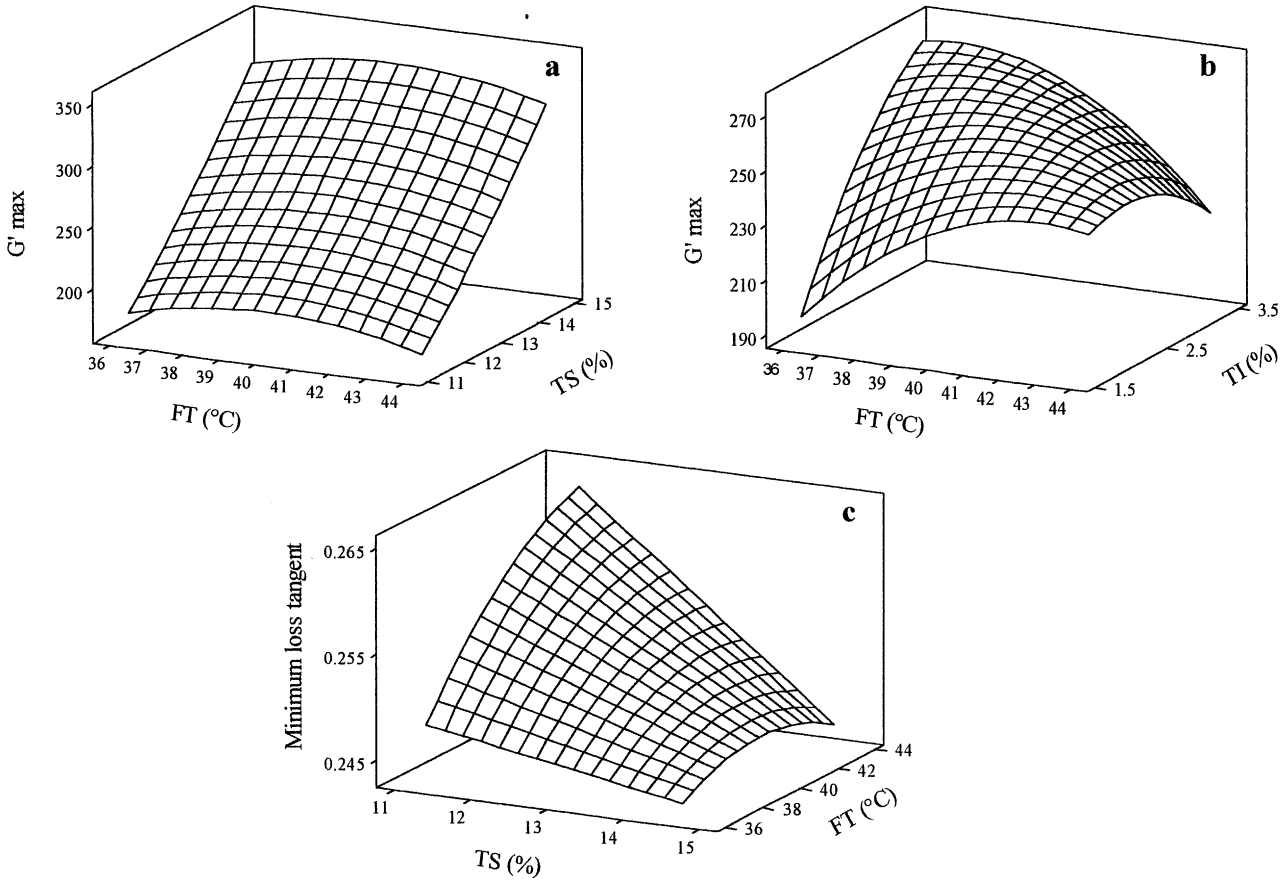

Fig. 1. Response surface plots for: maximum storage modulus (G'_{max}) as a function of fermentation temperature (FT)–total solid content (TS) at total inoculum level 2.5% v/v (a), and fermentation temperature (FT)–total inoculum level (TI) at total solid 13% w/v (b); minimum loss tangent as a function of total solid content (TS)–fermentation temperature (FT) at total inoculum level 2.5% v/v (c).

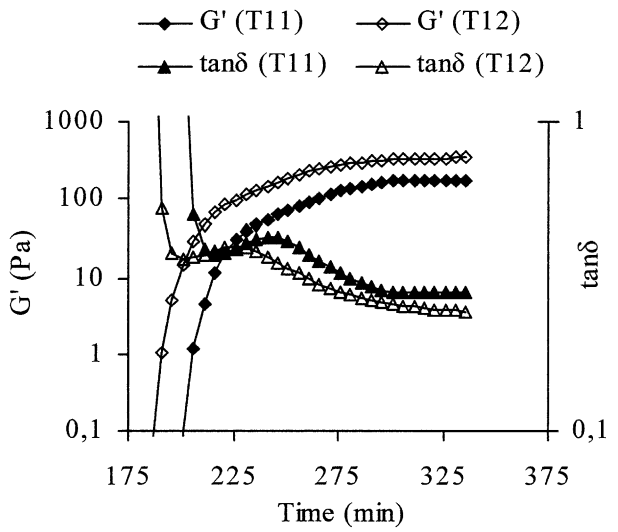

Fig. 2. Development of storage modulus (G') and loss tangent (tanδ) as a function of time for the tests 11 and 12; the respective levels of total solid, total inoculum and the fermentation temperature are specified in Table 1.

bacterial culture (Lucey et al., 1998b) result in higher values of G'. Walstra (1998) has shown the same effect of gelation temperature on the gel structure, whereas Haque, Richardson, and Morris (2001) reported the opposite, emphasizing that increasing fermentation temperature (and thus promoting hydrophobic association) gives stronger gels. Hydrophobic interactions play an important role in the aggregation of casein micelles (Horne, 1998), but other contributions should also be taken into account when one considers the effect of gelation temperature on the coagulum strength. According to Lucey et al. (1997a, 1997b), at low fermentation temperatures casein particles have a high voluminosity, which means that they can form larger/stronger intermolecular associations, with many protein molecules involved in these junctions. Particle rearrangement is weaker during gel formation at low temperatures and the resulting network is more continuous and rigid. At high temperatures, smaller particle voluminosity contributes to smaller area of interparticle contact and weaker junctions, which tend to rearrange locally and give a less homogeneous network. Similarly, Walstra (1998) supports the idea that milk gels formed at low fermentation temperatures have a finer structure, are very stable and show no short-term rearrangements of the casein particles. In all gelation profiles, as those illustrated in Fig. 2, a 'local maximum of loss tangent (tanδ)' was observed in the early stages of the process, implying a partial loosening of the casein network before the ultimate macrostructure is established. Such rheological responses for acid-coagulated milk gels were also observed for either glucono-δ-lactone (Lucey et al., 1998b) or microbially-acidified heat-treated milk samples (Biliaderis et al, 1992; Ronnegard & Dejmek, 1993). Lucey, Tamehana, Singh, and Munro (1998b) have recently suggested that the 'maximum in tanδ' reflects a transition from a milk gel initially dominated by denatured whey protein-induced interactions (denatured whey proteins associated with casein micelles) at relatively high pH to a network dominated by casein-casein interactions at low pH values, e.g. <5.0.

The elasticity increment $[I_E = (\mathrm{dlog}\ G'/\mathrm{d}t)_{max}]$ was introduced as a measure of gel development rate. This parameter was modelled versus the three independent variables, FT, TS and TI. The I_E was influenced by FT (linear term), TS and TI (quadratic effects) and interaction FT–TI (Table 2). The effect of TS and TI was minor compared with that of FT, as evidenced by the highest value of regression coefficient of the linear term of FT. The gelation rate increased with the increase of FT and rapid gelation might be observed when fermentation takes place at high FT (43–44 °C) (Fig. 3a). Because of the negative effect of interaction FT–TI and the negative quadratic effect of TI, faster gelation takes place by increasing FT and decreasing TI, whereas lower I_E values are obtained by decreasing or simultaneously increasing both factors and maintaining TS at middle level (Fig. 3a). The quadratic effect of TS and TI is negative; therefore, at high and low values of both factors, the rate of gelation is low. In conditions of low FT, high TS and TI, at which the highest G'_{max} values are attained, I_E is considerably low (around 1.1 min^{-1}). A slow gelation, taking place at low temperatures, allows time for large/strong junctions to develop, giving rise to a more rigid network with higher G' values (Lucey et al., 1997b).

The onset of gelation was arbitrarily chosen as the time (min) required for G' to cross-over G''. The onset of gelation mostly depends on the FT, as its linear as well as quadratic effects are significant (Table 2). Fig. 3b shows the predominant linear negative effect of FT as evidenced by the respective regression coefficient that has the highest absolute value among all other coefficients of the model. Furthermore, TI (linear and quadratic terms) and TS (only linear term) influenced the onset of gelation, but the effect of FT dominates, bringing about the earlier onset of gelation as FT increases (Arshad et al., 1993; Lucey et al., 1998b). This effect is illustrated once more when comparing the kinetics of G' for tests 9 and 10, where the lowest (36.64 °C) and the highest (43.36 °C) FT were employed (Fig. 4). Gelation also starts earlier when higher TS levels are employed (negative regression coefficient of TS, Table 2). This behaviour is in agreement with previous studies of Xu et al. (1992) and Biliaderis et al. (1992), while Arshad et al. (1993) did not find any effect of TS on the onset of gelation. The negative effect of inoculum concentration on the onset of gelation has been previously reported by Biliaderis et al. (1992).

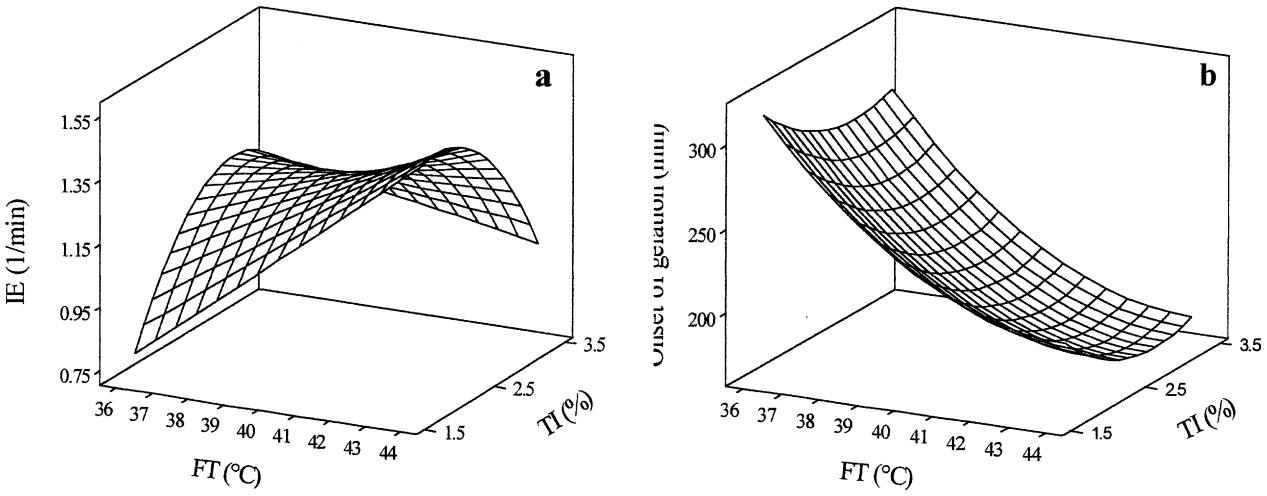

Fig. 3. Response surface plots for: elasticity increment (I_E) as a function of fermentation temperature (FT)–total inoculum level (TI) at total solid level 13% v/v (a); onset of gelation as a function of fermentation temperature (FT)–total inoculum level (TI) at total solid level 13% w/v (b).

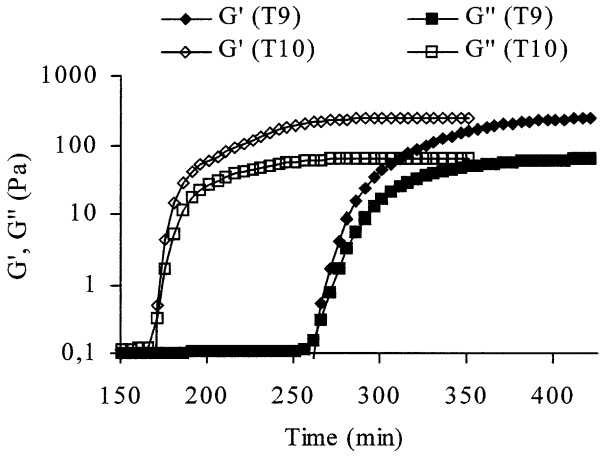

Fig. 4. Development of storage modulus (G') and loss modulus (G'') as a function of time for tests 9 and 10; the levels of total solids, total inoculum and the fermentation temperature are specified in Table 1.

3.3. Acidification kinetics and microbial characterization of milk gels

Viable count measurements for both bacteria were made at the beginning and at the end of fermentation. In all the tests, except test 14, the number of *S. thermophilus* dominated over that of *L. bulgaricus* at the end of fermentation, that is cocci reached higher numbers than rods under all conditions of the studied variables (Table 3). Similar results were reported by Amoroso, Manca de Nadra, and Oliver (1989), Birollo, Reinheimer, and Vinderola (2000), Kneifel, Jaros and Erhard (1993), Oliveira, Sodini, Remeuf, and Corrieu (2001), Radke-Mitchell and Sandine (1986), and Rajagopal and Sandine (1990). The acidification kinetics of milk gel were characterized by the maximum acidification rate (V_m), the time at which V_m was reached (T_m) (Spinnler & Corrieu, 1989) and the time necessary to reach pH 4.6, or the time to reach the end of fermentation (T_e) (Oliveira et al., 2001). V_m and T_m were calculated as described by Spinnler and Corrieu (1989). These three parameters were studied as a function of FT, TS and TI and the corresponding response surface plots were generated.

The V_m was significantly affected by the linear terms of all factors, as well as the interaction term of FT and TS, with the last one having the strongest effect, as is demonstrated by the respective regression coefficients (Table 2). As shown in Fig. 5a, the highest V_m values are observed when increasing FT and simultaneously decreasing TS and the lowest when FT and ST simultaneously decrease or increase. Moreover, TS exerts a linear negative effect on V_m, which is due to the buffering capacity of milk solids. Lower TS means lower buffering capacity, which in turn means a higher decrease in pH for the same amount of acid produced and vice-versa. On the other hand, higher TS favours increases of the numbers of both bacteria (Radke-Mitchell & Sandine, 1986; Tamime & Deeth, 1980); a higher TS means a richer medium of nutrients. Higher TS level also provides better protection to bacteria from lowering of the pH (Radke-Mitchell & Sandine, 1986; Tamime & Deeth, 1980). Furthermore, comparing the bacterial numbers, for example, in pairs of tests 1 and 3, 2 and 4, which differ mutually only in TS (Table 3), the bacterial number is higher in tests 3 and 4, which have higher TS than their respective counterparts. TI negatively affected V_m (Table 2), with higher TI resulting in lower V_m, confirming the recent findings of Champagne, Gardner, Soulignac and Innocent (2001). The highest V_m values could be achieved when fermentation was carried out at temperatures ranging from 42 to 44 °C, in combination with TI up to 2.5% (v/v) and low TS (around 11–12% w/v). However, a medium rate of acidification is preferable to a high one

Table 3
Log numbers of *L. bulgaricus* Y 6.15 and *S. thermophilus* Y 4.10 at the end of fermentation (N) and after 7 days (N7) and 21 days (N21) of storage at 4 °C (data are means of three replications)

Test	FT (°C)	TS (% w/v)	TI (% v/v)	*L. bulgaricus* Y 6.15			*S. thermophilus*		
				log*N*	log*N*7	log*N*21	log*N*	log*N*7	log*N*21
1	38	12	2	7.13 (±0.08)	6.68 (±0.67)	6.65 (±0.46)	8.16 (±0.06)	7.36 (±0.33)	7.66 (±0.5)
2	38	12	3	7.83 (±0.32)	7.45 (±0.53)	6.08 (±0.38)	8.30 (±0.29)	5.00 (±0.45)	8.39 (±0.35)
3	38	14	2	7.87 (±0.15)	6.11 (±0.44)	5.28 (±0.32)	8.54 (±0.08)	ng[a]	8.71 (±0.42)
4	38	14	3	8.32 (±0.28)	7.14 (±0.62)	6.58 (±0.43)	8.39 (±0.12)	8.25 (±0.24)	7.67 (±0.54)
5	42	12	2	7.63 (±0.32)	6.65 (±0.37)	6.62 (±0.25)	8.70 (±0.20)	8.49 (±0.36)	8.05 (±0.63)
6	42	12	3	7.63 (±0.09)	8.10 (±0.63)	5.94 (±0.22)	8.63 (±0.18)	8.46 (±0.27)	8.53 (±0.14)
7	42	14	2	7.84 (±0.31)	7.75 (±0.42)	5.99 (±0.33)	8.45 (±0.14)	8.27 (±0.43)	8.43 (±0.72)
8	42	14	3	8.32 (±0.45)	8.08 (±0.57)	7.89 (±0.52)	8.37 (±0.13)	7.98 (±0.49)	5.20 (±0.67)
9	36.64	13	2.5	7.78 (±0.21)	5.69 (±0.44)	6.28 (±0.34)	8.40 (±0.15)	8.41 (±0.34)	4.00 (±0.65)
10	43.36	13	2.5	7.78 (±0.45)	7.88 (±0.53)	6.80 (±0.41)	8.41 (±0.09)	8.45 (±0.29)	4.00 (±0.47)
11	40	11.32	2.5	7.20 (±0.23)	5.25 (±0.31)	5.35 (±0.25)	8.31 (±0.10)	7.49 (±0.50)	5.18 (±0.53)
12	40	14.68	2.5	7.57 (±0.20)	6.53 (±0.47)	5.02 (±0.22)	8.72 (±0.16)	8.76 (±0.25)	4.60 (±0.62)
13	40	13	1.66	7.01 (±0.63)	6.41 (±0.39)	5.15 (±0.36)	8.72 (±0.21)	8.36 (±0.19)	4.10 (±0.44)
14	40	13	3.34	7.82 (±0.02)	7.50 (±0.02)	5.73 (±0.48)	7.61 (±0.2)	8.37 (±0.45)	5.57 (±0.35)
15	40	13	2.5	7.82 (±0.33)	7.23 (±0.25)	5.74 (±0.68)	7.96 (±0.53)	7.72 (±0.88)	7.57 (±0.70)

[a] No growth.

because it gives a more regular acid production (Zanatta & Basso, 1992), resulting in more homogeneous structure of the coagulum, greater viscosity of the final product (Beal et al., 1999) and non-excessive acid production. Medium Vm values were observed at relatively low FT, high TS and medium TI values, all corresponding to the conditions at which a firmer coagulum (with higher G'_{max} and lower $\tan\delta_{min}$) is obtained.

The time at which maximum acidification rate is reached (T_m) was a function of the linear terms of all factors, the quadratic terms of TS and TI and the interaction TS–TI effects. However, FT had a prominent effect, as noticed from the respective regression coefficient of FT (Table 2) and Fig. 5b. As expected, this effect is negative; that is, increasing the FT, the maximum pH reduction takes place earlier, which is in agreement with the conclusions of Beal et al. (1999) and Lucey et al. (1998b). T_m was negatively related to the linear effect of TI, which agrees with the findings of Sebastiani, Gelsomino, and Walser (1998), who observed a general increase in T_m with lower inoculation percentages. Nevertheless, not only the effect of TI, but also that of TS, is rather complex, as both of them exert curvilinear and interactive effects on T_m (Table 2). The lowest T_m values were obtained in milks with high (14–15% w/v) TS combined with middle TI level (2.5% v/v) or low (11–12% w/v) TS combined with high TI (around 3.5%; Fig. 5c). This observation is rather suprising, considering that in conditions of high buffering capacity, i.e. in case of high TS, high TI is required to reach the V_m value early. However, the prevalent FT effect seems to compensate for the interactive effect of TS–TI and brings about the reduction of T_m in both circumstances.

The time to reach the end of fermentation (T_e) depends mostly on the FT, as the linear negative effect of FT was predominant and this is shown by the highest (in absolute value) regression coefficient of the linear term of FT (Table 2) and in Fig. 5d. Fermentation finishes earlier as incubation temperature increases, due to the increased metabolic activity of bacteria at higher temperatures (Haque et al., 2001). However, Beal et al. (1999) reported that, under conditions allowing longer acidification (e.g. low FT), a product of higher viscosity is obtained and that the textural properties of yogurt may

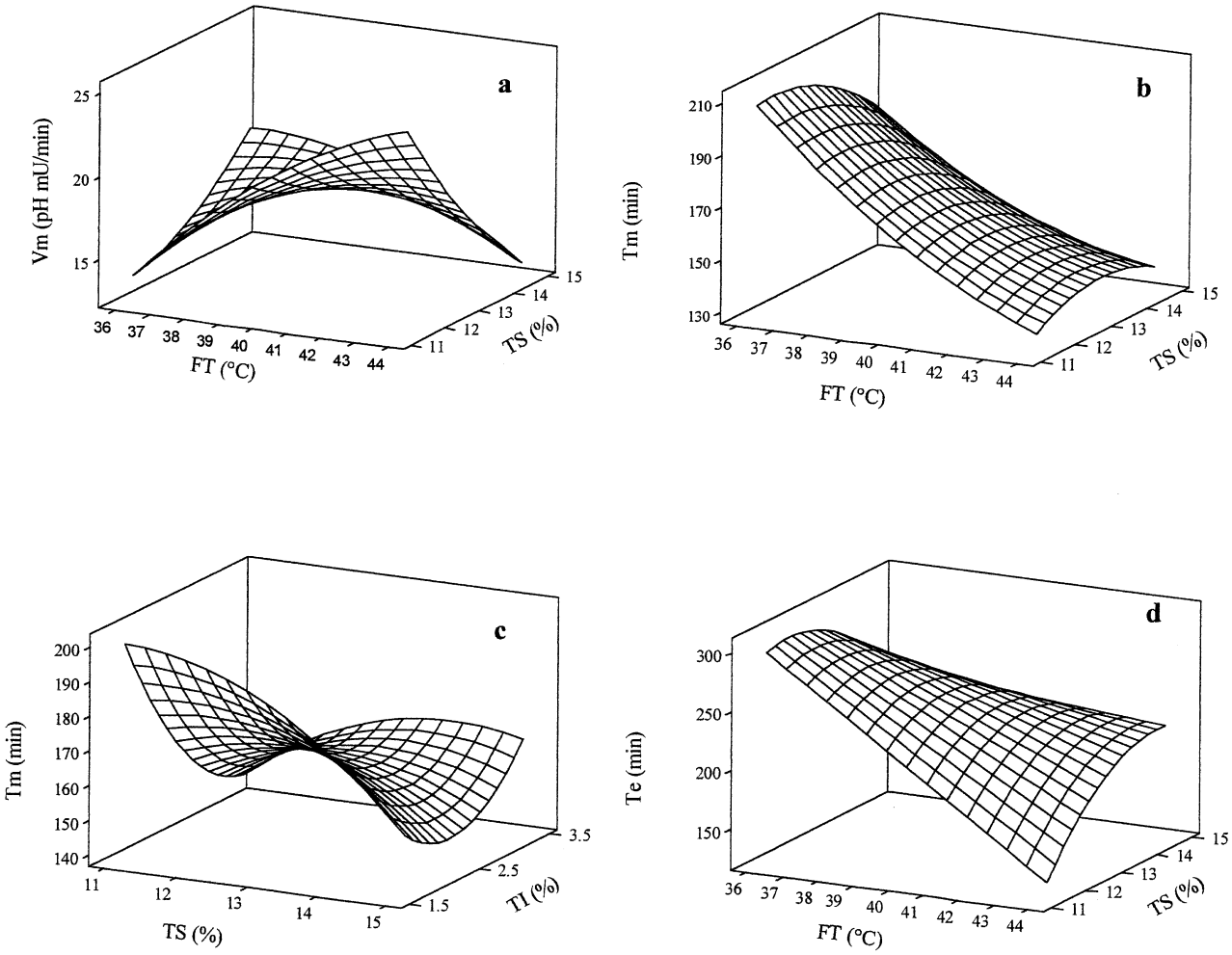

Fig. 5. Response surface plots for: maximum acidification rate (V_m) as a function of fermentation temperature (FT)–total solids level (TS) at total inoculum level 2.5% v/v (a); time at which maximum acidification rate occurred (T_m) as a function of fermentation temperature (FT)–total solids (TS) at inoculum level 2.5% v/v (b) and total solids (TS)–total inoculum level (TI) at fermentation temperature 40 °C (c); end fermentation time (T_e) as a function of fermentation temperature (FT)–total solids (TS) at total inoculum level 2.5% v/v (d).

be governed by the duration of fermentation. T_e showed a negative relationship with the quadratic term of TS and the interactive term FT–Tl, as well as a positive relationship with the interactive term FT–TS (Table 2).

The health beneficial effects of yogurt are related to the action of a high number of viable lactic acid bacteria in the product at the moment of consumption (Deeth & Tamime, 1981; IDF, 1988). In the present study, lactic acid bacteria, *S. thermophilus* Y 4.10 and *L. bulgaricus* Y 6.15, were enumerated at the end of fermentation and after 7 and 21 days of storage at 4 °C (Table 3). As expected, during storage, the counts of both bacteria declined gradually in all tests. Nevertheless, after 21 days of storage, both bacteria continued to give a high number of viable cells. In most of the tests, *S. thermophilus* Y 4.10 had the highest number (around 10^8 cells/ml), while for *L. bulgaricus* Y 6.15, this number ranged from 10^6 to 10^7 cells/ml; The prevalence of *S. thermophilus* over *L. bulgaricus* is in agreement with the results of Dave and Shah (1997) and Vinderola, Bailo, and Reinheimer (2000). However, the number of *S. thermophilus* Y4.10 for some tests (9, 10 and 12) was fairly low after 21 days (around 10^4 cells/ml). Although the microflora decreased during storage, in most of the tests, the reported value of total viable cells fell within the ranges determined by the legislation of different countries (10^6 cfu/ml in Switzerland and Italy; 10^7 cfu/g in Japan) (IDF, 1988).

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