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Use of Response Surface Methodology to Evaluate some Variables Affecting the Growth and Acidification Characteristics of Yoghurt Cultures

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ABSTRACT

The fermentation temperature, fat and non-fat dry matter content of milk, inoculum size and the initial ratio of cocci/rods were varied according to a Central Composite Design with the aim of assessing the effects of these variables and their interactions on the acidification rate and the growth of Lactobacillus delbrueckii *subsp.* bulgaricus *ST56 and* Streptococcus thermophilus *DN21 strains used in yoghurt production.*

The technological and microbiological parameters selected to evaluate the fermentative behaviour, i.e. variation of pH and increase in the number of lactobacilli and streptococci, were analysed in order to develop predictive polynomial quadratic equations.

The models obtained emphasized the role attributable to a variable such as the initial ratio of cocci/rods and to its interactions with the other variables. A cocci/rods ratio of 1.5:1 was found to be optimum for all parameters evaluated.

An analysis of the response surfaces permitted the identification of the operational conditions needed to optimize the performances of the tested strains.

The methodology used in this research was very efficient and the model obtained was found to be valid and accurate for the evaluation of a complex biotechnological process such as yoghurt production. Copyright © 1996 Elsevier Science Limited

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INTRODUCTION

Yoghurt is a well-known fermented milk product which has gained great popularity throughout the world for its recognized organoleptic and health-promoting properties. A large variety of yoghurts, resulting from technologically diversified approaches, as well as biological characteristics of the bacterial strains used, is available on the market today.

Strains of thermophilic, obligate homofermentative lactic acid bacteria belonging to the species *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* constitute the specific mixture selected and used for yoghurt production (IDF, 1992). A protosymbiotic relationship between these bacteria has been shown clearly and some metabolic products responsible for their mutual stimulation have been identified (Driessen, 1981; Driessen *et al.*, 1982). However, the influence of biotechnological conditions on the associative growth of these specific bacteria has still not been investigated thoroughly.

In a recent study, Beal & Corrieu (1991) determined the initial and operating conditions which permitted optimization of the growth and the acidification characteristics of defined mixed cultures by using linear quadratic models as a function of pH, temperature and inoculum composition. Attempts to improve and to maximize the performance of yoghurt cultures, also adopting methodologies which permit the study of simultaneous effects of different variables of the fermentative process, are required increasingly both for practical and scientific purposes (Boudrant *et al.*, 1994).

The aim of this research was to evaluate the influence of the variation of some technological variables, as well as the compositional characteristics of the milk, i.e. fermentation temperature, fat percentage and non-fat dry matter in milk, inoculum size and the initial cocci/rods ratio, on the acidification rate and the growth of *Lb. delbrueckii* subsp. *bulgaricus* ST56 and *Str. thermophilus* DN21 strains used in mixed cultures. For this purpose, predictive polynomial quadratic equations and response surface methodology were used to describe the individual and interactive effects of the five variables at five levels, combined according to a Central Composite Design, on the fermentation process.

MATERIALS AND METHODS

Micro-organisms and culture media

Freeze-dried *Lb. delbrueckii* subsp. *bulgaricus* ST56 and *Str. thermophilus* DN21 cultures were obtained from the collection of the Dipartimento di Scienze e Tecnologie Agroalimentari, Ambientali e Microbiologiche of Campobasso (Italy). These strains have been used regularly as a starter culture in the industrial production of yoghurt because of their established protosymbiosis. The strains were grown and maintained in reconstituted (10%, w/w) skimmed milk (Difco) sterilized at 121°C for 5 min, with weekly transfers.

Experimental design

The basal medium used was commercial UHT skimmed milk containing 0.2% fat and 8.5% non-fat dry matter (Weight Watchers, Parmalat, Italy). The variables chosen for the experiments were: fermentation temperature, °C (*T*), percentage of milk fat (*F*) and non-fat dry matter (*DM*) added to the basal medium, inoculum (*I*) percentage and initial ratio of cocci/rods (*c*/*r*). They were varied (five levels) according to a Central Composite Design (CCD) (Box *et al.*, 1978). The composition of the various runs of the CCD is reported in Table 1. Two replicates of the 36 combinations of the CCD were prepared. To obtain the levels required by the CCD, suitable amounts of commercial UHT cream (Parmalat, Italy) and skimmed-milk powder (Difco) were added to the basal medium. The media were heat-treated at 121°C for 5 min and aliquots (50 mL) prepared for each of the 36 combinations. All dairy ingredients used in the study were free of inhibitory agents.

Active pure cultures of each strain were obtained by two consecutive daily transfers in basal medium using a 1% inoculum. The final cell density in the inoculum was 1×10^8 cells mL^{-1}, both for *Lb. delbrueckii* subsp. *bulgaricus* ST56 and *Str. thermophilus* DN21. The preparation of inocula was performed under well-standardized conditions following the accurate mixing of the pure cultures used. The initial (*c*/*r*) ratios were confirmed by microscopic observations of methylene blue-stained slides counting 60 fields of undiluted samples.

Enumeration of micro-organisms

Microbiological analyses were performed just after inoculation of the milk and after incubation for 3.5 h under the programmed conditions. Samples (1 mL) were mixed with 99 mL of sterile peptone diluent (0.1%, w/v) and homogenized using a Stomacher (Lab Blender 400, Seward Medical, London, UK) for 2 min. Blended samples were then serially diluted 10-fold and plated in duplicate on selective media following the methods suggested by IDF (1988). Lactobacilli were enumerated on MRS plates after incubation for 72 h at 37°C in anaerobic GasPak jars (Oxoid, Basingstoke, UK) in a H_2 plus CO_2 atmosphere. Streptococci were counted on M17 plates after incubation for 48 h at 37°C under aerobiosis. The selectivity of the growth conditions and media was checked by the microscopic appearance of the colonies.

Chemical analysis

The pH values were determined using a pH meter Mod 2001 (Crison, Barcelona, Spain).

Non-fat solids and fat content were determined following the methods of AOAC (1984).

Statistical procedures

The data were statistically analysed using the Statgraphics 4.0 (STSC Inc., Rockville, Maryland, USA) and SPSS for Windows 6.0 statistical packages.

The response surface was obtained from the final model permitting the variation (from −2 to +2) of two independent variables keeping the other independent variables constant at the 0 level; the isoresponse contour plot was obtained by calculating from the model the values of one factor when the second varied (from −2 to +2) with the constraint of a given value of the dependent variable.

TABLE 1
Runs with the Different Combinations of the Experimental Variables Used in the Central Composite Design

Run	*Fermentation temperature (°C) (T)*	*Non-fat dry matter added (%) (DM)*	*Milk fat added (%) (F)*	*Initial ratio of cocci/rods (c/r)*	*Inoculum size (%) (I)*
1	38	1	2	1	4
2	38	1	2	2	2
3	38	1	6	1	2
4	38	1	6	2	4
5	38	3	2	1	2
6	38	3	2	2	4
7	38	3	6	1	4
8	38	3	6	2	2
9	42	1	2	1	2
10	42	1	2	2	4
11	42	1	6	1	4
12	42	1	6	2	2
13	42	3	2	1	4
14	42	3	2	2	2
15	42	3	6	1	2
16	42	3	6	2	4
17	40	2	4	1.5	3
18	40	2	4	1.5	3
19	40	2	4	1.5	3
20	40	2	4	1.5	3
21	40	2	4	1.5	3
22	40	2	4	1.5	3
23	36	2	4	1.5	3
24	44	2	4	1.5	3
25	40	0	4	1.5	3
26	40	4	4	1.5	3
27	40	2	0	1.5	3
28	40	2	8	1.5	3
29	40	2	4	0.5	3
30	40	2	4	2.5	3
31	40	2	4	1.5	1
32	40	2	4	1.5	5
33	40	2	4	1.5	3
34	40	2	4	1.5	3
35	40	2	4	1.5	3
36	40	2	4	1.5	3

RESULTS

Thirty-six different fermentation runs were carried out using *Lb. delbrueckii* subsp. *bulgaricus* ST56 and *Str. thermophilus* DN21 strains and varying the levels of the variables under examination (i.e. fermentation temperature, % milk fat and % non-fat dry matter added to the skimmed milk, inoculum size and initial cocci/rods ratio) according to the CCD. The ranges of the values of the five variables considered included the minimum and maximum values used normally in the production of yoghurt having different compositional and organoleptic characteristics.

Three parameters were chosen to evaluate the behaviour of the fermentation; in particular, the variation in pH 3.5 h after inoculation was considered as a technological parameter and the increases in the numbers of lactobacilli and streptococci, after the same time, were chosen as microbiological indices. The results obtained for these parameters in the different runs of the CCD were modelled according to a polynomial quadratic equation in order to identify the variables that significantly affected the course of fermentation. In the final model, only the coefficients characterized by $P < 0.05$ were considered. In Table 2, the equations obtained for these three parameters and their correlation coefficients, P values and standard errors are reported. The values for the three indices underline the validity of the model used and its accuracy for describing the behaviour of the three parameters in relation to the five variables considered.

Decrease in pH

The variation in pH, with respect to the initial value of 6.8, 3.5 h after inoculation, was influenced by all the variables considered in the CCD. The (DM) variable was significant both in its linear term and the (c/r) variable in its quadratic term. The positive coefficient of the individual factor and the negative coefficient of the quadratic term indicates that it is possible to identify a level of (DM) optimum for pH decrease. The (c/r) ratio was significant also in its interaction with (DM), (F) and (T) but not with (I).

Response surfaces, derived from the relative polynomial equation, showing the effects of the significant interactions on pH variation, are represented in Fig. 1. In particular, Fig. 1(a) shows the effect of the (DM) $\times$ (c/r) interaction. Increasing variations in pH were obtained for decreasing values of the (c/r) ratio from 2.4 to 1.0 and when (DM) was added, these decreased from 4 to 0%; however, the highest variations in pH were obtained for the highest and lowest values of (c/r) and (DM), respectively. In other words, it is possible that, in order to maintain a high rate of pH decline, a reduction of (DM) in the milk should be compensated according to Fig. 1(a) by a reduction in the inoculum of the (c/r) ratio.

A positive effect of temperature on the pH variation can be observed in Fig. 1(b) relative to the (c/r) $\times$ (T) interaction. The figure permits the identification of the optimum values of (c/r) ranging from 1.75 to 1.5, depending on the temperature.

The response surface for the (c/r) $\times$ (I) interaction indicated that the changes in pH were higher for the higher values of (c/r) independently of the inoculum level [Fig. 1(c)].

According to the contour plots for the (c/r) $\times$ (F) interaction [Fig. 1(d)], there was a compensation between the two factors: in fact, the maximum pH variations can be attained either with minimum values of (c/r) and (F) or vice versa.

As shown in Fig. 1(e), the (DM) $\times$ (F) interaction had no effect on pH variations but, as indicated in Fig. 1(f), a diminution of (T) can be compensated by an (I) increase.

TABLE 2

Second-order Polynomial Models Relative to the Effects of the Five Variables and Their Interations on the Decrease of pH, the Increase in the Log Number of *Lb. delbrueckii* subsp. *bulgaricus* and the Increase in the Log Number of *Str. thermophilus* Concentrations. In the Final Model, Only Terms with $P < 0.05$ Were Accepted

Factors[a]	*pH Decrease*	*Increase of the log number of Lb. delbrueckii subsp. bulgaricus ST56*	*Increase of the log number of Str. thermophilus DN21*
(T)	—	−0.435	—
(DM)	−0.637	—	0.860
(F)	—	—	—
(c/r)	—	3.057	−7.921
(I)	—	4.620	4.763
$(T)^2$	—	0.011	—
$(DM)^2$	—	—	−0.186
$(F)^2$	—	—	−0.05
$(c/r)^2$	−0.408	−1.022	−1.297
$(I)^2$	—	0.189	—
$(T) \times (DM)$	—	—	—
$(T) \times (F)$	0.005	—	0.009
$(T) \times (c/r)$	0.029	—	0.257
$(T) \times (I)$	—	−0.143	−0.140
$(DM) \times (F)$	—	—	—
$(DM) \times (c/r)$	0.344	—	—
$(DM) \times (I)$	0.047	—	—
$(F) \times (I)$	—	—	—
$(F) \times (c/r)$	−0.128	—	—
R^2	0.946	0.915	0.979
S.D.	0.219	0.441	0.333
$P <$	0.0001	0.0001	0.0001

[a](T), temperature (°C); (DM), non-fat dry matter added (%); (F), milk fat added; (c/r), cocci/rods initial ratio; (I), inoculum size (%); R^2, coefficient of regression; S.D., standard deviation; P, significance level.

Variations in *Lb. delbrueckii* subsp. *bulgaricus* ST56 cell numbers

The equation for the increase in the number of lactobacilli in the yoghurt 3.5 h after inoculation is reported in Table 2. The variables (DM) and (F) were not significant and the growth was influenced solely by fermentation temperature, inoculum size and initial cocci/rods ratio.

The surface plots obtained from the equation showed (Fig. 2) that the number of lactobacilli reached maximum values in correspondence to a (c/r) ratio of about 1.5. This was evident in Fig. 2(a), relative to the $(c/r) \times (T)$ interaction in which, above and below this value, the *Lb. delbrueckii* subsp. *bulgaricus* ST56 final cell number decreased independently of temperature.

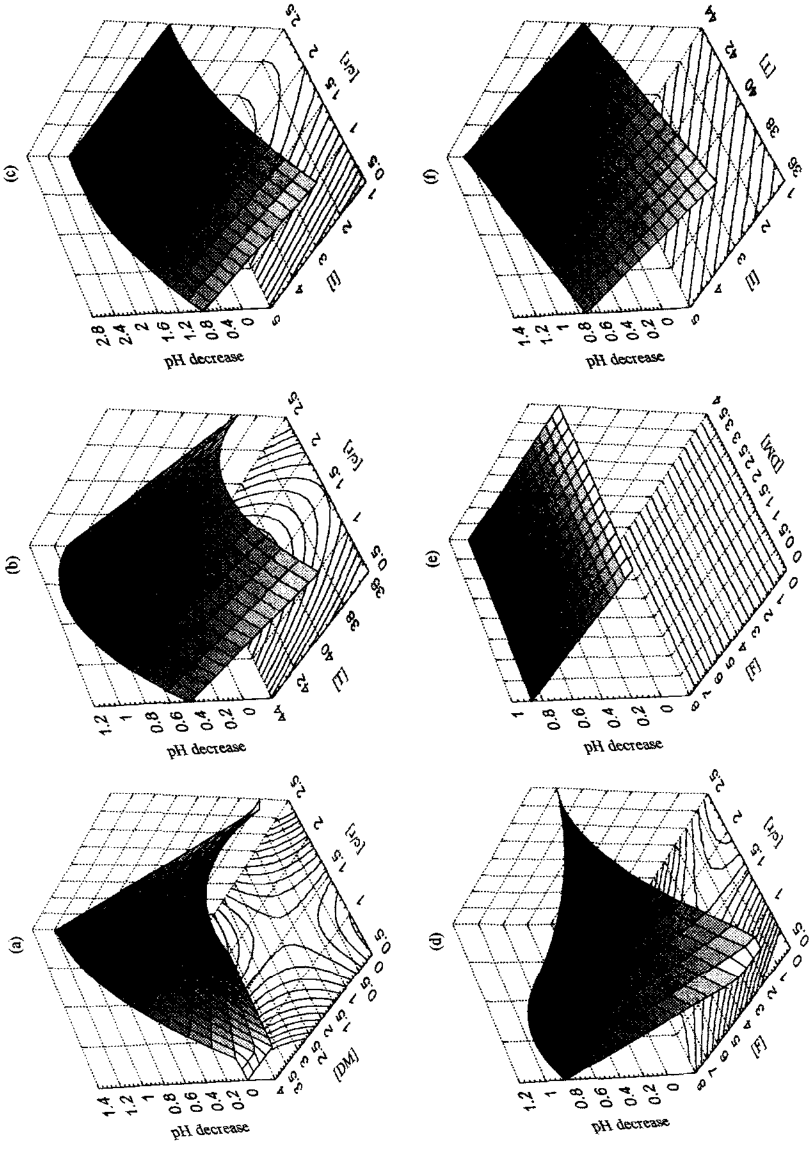

Fig. 1. Three-dimensional plots of various parameters relative to the pH decrease after 3.5 h of incubation; the units of the variables are the same as those in Table 1.

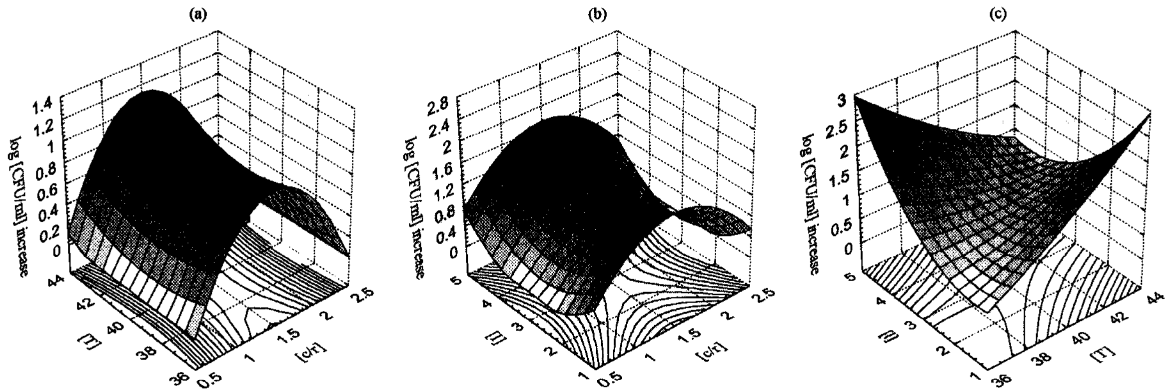

Fig. 2. Three-dimensional plots of various parameters relative to the increase in the log number of *Lb. delbrueckii* subsp. *bulgaricus* ST56 after 3.5 h of incubation; the units of the variables are the same as those in Table 1.

As indicated by the negative sign of the coefficient of the quadratic term of the (c/r), this factor affected positively the increase in ST56 log colony-forming units (CFU) mL^{-1} up to a threshold of 1.5, after which it depressed the cell increase [Fig. 2(b)]. Thus, a (c/r) ratio of 1.5 will guarantee the maximum increase in lactobacilli when the inoculum is kept constant.

The maximum values of the *Lb. delbrueckii* subsp. *bulgaricus* ST56 variation, as indicated in Fig. 2(c) for the $(T) \times (I)$ interaction, can be reached when the temperature is at its maximum and the % inoculum is at its minimum and vice versa. Between these two extremes, the minimum variation in *Lb. delbrueckii* subsp. *bulgaricus* ST56 can be obtained for lower values of temperature, decreasing the inoculum level.

Variations in *Str. thermophilus* DN21 cell numbers

The polynomial equation which describes the increase of the streptococci cell number after 3.5 h fermentation is reported in Table 2. In this case, in contrast to the equation for lactobacilli, all the variables considered showed a significant effect.

Even with regard to *Str. thermophilus* DN21 cell numbers, the (c/r) ratio variable played a relevant role; in fact, according to the contour plots for the $(c/r) \times (T)$ and $(c/r) \times (I)$ interactions [Fig. 3(b) and (c)], it is possible to obtain maximum values for the final number of *Str. thermophilus* DN21 cells when the values of (c/r) are approximately 1.5. Its optimum value has to be changed only slightly in relation to the values of (T) and (I).

Figure 3(a) and (d), for the $(c/r) \times (DM)$ and $(c/r) \times (F)$ interactions, permitted the identification of optimum values of (DM) (2.5) and (F) (3.5) and (c/r) (1.5) in order to obtain the maximum number of *Str. thermophilus* DN21 cells.

The sign of the coefficients of the linear and quadratic term of the factors (DM) and (F) indicates that it is possible to identify optimum values for both factors, as indicated in Fig. 3(e). With values of 2.2 and 3.5, respectively, for (DM) and (F), maintaining all the other factors at the coded level 0, the maximum growth of strain *Str. thermophilus* DN21 can be obtained.

Similarly, there was a very weak influence of temperature on the $(T) \times (F)$ interaction (not reported here). In fact, the maximum variations in *Str. thermophilus* DN21 were obtained when the fat level was between 3.5 and 4%, independent of temperature. Temperature played a more significant role in the (T) $\times$ (I) interaction: in fact, the two factors had compensatory effects on the *Str. thermophilus* DN21 log CFU increase [Fig. 3(f)].

DISCUSSION

The variables considered in this research are recognized to be among the most important for determining the quality and organoleptic characteristics of yoghurt. Moreover, the ranges chosen for each variable corresponded to those used generally in order to produce different types of yoghurt to satisfy consumer demand; in particular, the (DM) and (F) contents, which correspond to the 0 level of the CCD, reflect the composition of yoghurt prepared from a whole milk concentrated to 13–18% solids.

The use of a CCD is a valid method for analysing the individual and combined

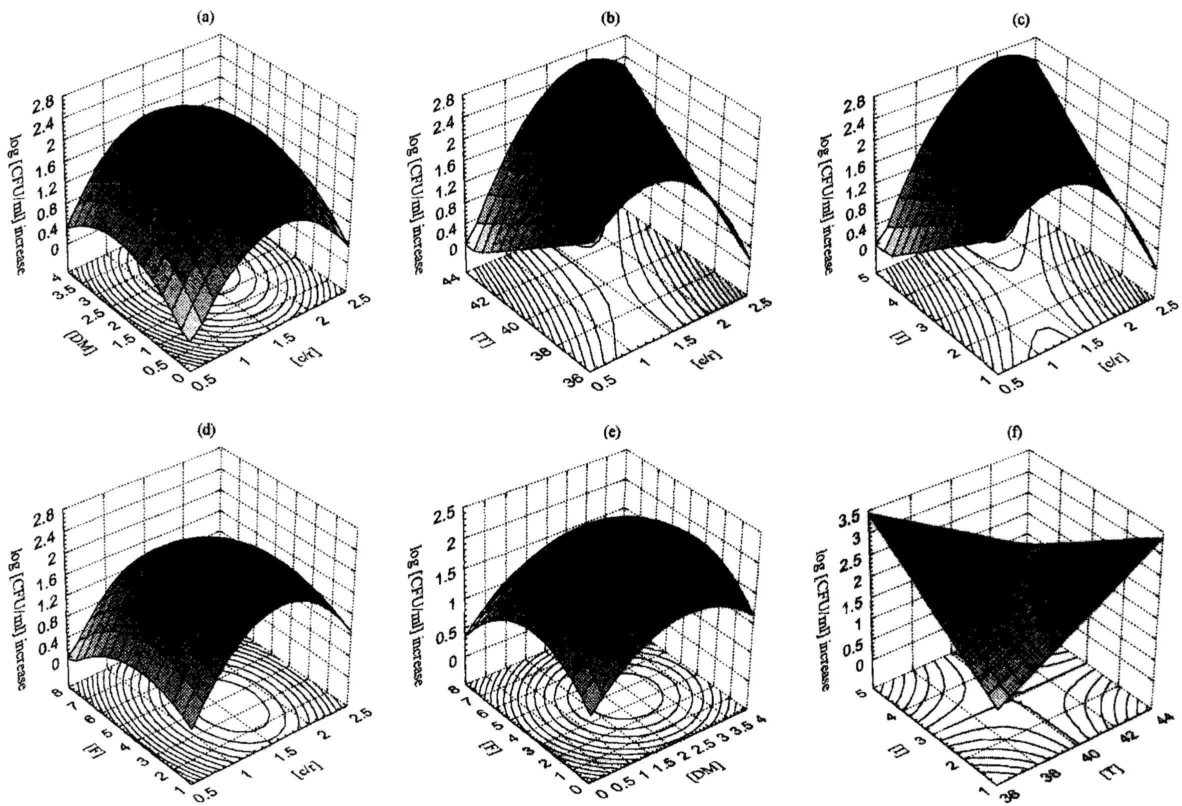

Fig. 3. Three-dimensional plots of various parameters relative to the increase in the log number of *Str. thermophilus* DN21 after 3.5 h of incubation; the units of the variables are the same as those in Table 1.

effects of the selected variables and for optimizing the technological and compositive characteristics of yoghurt production in order to obtain a final product with desired traits, as has been demonstrated for other food products (Abdullah *et al.*, 1994).

The results of this research indicate that the initial cocci/rods ratio, probably due to its influence on the proteolytic activity displayed by the starter, plays an important role in controlling the variation in final pH. In fact, this variable is able to counterbalance differences in the composition of the milk to be fermented (in particular non-fat dry matter and fat content). In yoghurt production, for example, the milk fat added to obtain a smoother body and an improved aroma might, therefore, influence the acidification process. A study of the effects of the modification of the non-fat dry solids of milk and of the homogenization process on the consistency, whey separation, microstructure and sensorial properties is in progress.

According to the best-fit equations obtained with the CCD, it was possible to conclude that an increase in the cocci/rods ratio in the inoculum must be combined with an increased level of dry matter in order to maximize pH variations, whereas a reduction in the percentage of *Str. thermophilus* in the inoculum is necessary, with the same aim, for a milk of low fat content. In other words, due to the interactions between the three variables, their individual optimum values changed in relation to the values of the others.

The insignificant effect of non-fat dry matter and fat content on the final number of *Lb. delbrueckii* subsp. *bulgaricus* suggests that the differences in the pH variation in relation to these two variables could be related to their effects on *Str. thermophilus* DN21. In particular, for this strain it was possible to identify the optimum values for the initial cocci/rods ratio of about 1.5, and above all, optimum values for added non-fat dry matter and fat content of about 2 and 4%, respectively. As mentioned above, these figures correspond to the composition of a whole yoghurt and these results may suggest that the best performance of strain DN21 can be obtained in slightly concentrated milk. Evidently, the positive or negative influence of the strain *Str. thermophilus* DN21 on the growth of *Lb. delbrueckii* subsp. *bulgaricus* depends on the values of the variables studied and their interactions.

Moreover, it should be emphasized that, according to the model, the best cocci/rods ratio, ranging from 1 to 2, confirms the results reported by others (Beal & Corrieu, 1991; Berkman *et al.*, 1990; Prevost & Divies, 1988). Thus, the results for the cocci/rods ratio can provide useful information for maximizing the increase of lactobacilli numbers. According to the model obtained, with an inoculum at 3%, non-fat dry matter 2%, milk fat at 4% levels and an initial cocci/rods ratio of 1.5, the effect of temperature on the development of lactobacilli is weak. Moreover, a high number of strain ST56 cells was reached using 5% inoculum at 40°C, under the above conditions. The model can be used to calculate the way in which the inoculum percentages can be modulated, when other factors have to be changed for practical and/or economic reasons.

Regarding the numerical variations of the *Lb. delbrueckii* subsp. *bulgaricus* ST56, the highest values were achieved at a high incubation temperature and low inoculum level or vice versa. These different operational conditions are applied usually in yoghurt production in countries with different food habits, such as the Mediterranean or Northern European areas. It can be observed also that yoghurt cultures, having a well-established protosymbiotic relationship, possess the necessary biological flexibility to maintain their collaborative activity even under very diversified biotechnological conditions.

In this research, by means of a methodology that allows the simultaneous variation of different technological factors, it has been possible to specify which variables can be modulated in order to obtain the potentially desirable characteristics for the yoghurt using the strains *Str. thermophilus* DN21 and *Lb. delbrueckii* subsp. *bulgaricus* ST56 at an initial ratio of 1.5:1.

Further improvements in the bioprocess for yoghurt and other fermented milks could be achieved by including more quantitative process data which validate the intrinsic assumption of the models (Lübbert & Simutis, 1994). This can be obtained by considering not only biological and process parameters but also microstructural features, which in turn depend both on the pH decrease and proteolytic activity of the starter, and the effect of the homogenization treatment on the fat droplet size (Jost, 1993).

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