

VIABILITY OF *BIFIDOBACTERIUM LACTIS* AND *LACTOBACILLUS ACIDOPHILUS* IN MILK: SODIUM CHLORIDE CONCENTRATION AND STORAGE TEMPERATURE

A.M.P. GOMES, M.G.M. TEIXEIRA and F.X. MALCATA¹

*Escola Superior de Biotecnologia, Universidade Católica Portuguesa
Rua Dr. António Bernardino de Almeida, 4200 Porto, Portugal*

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ABSTRACT

The growth and viability of Bifidobacterium lactis and Lactobacillus acidophilus per se and for both strains as a coculture were studied in reconstituted skim milk so as to assess their dependence upon temperature (5, 10 and 15°C), NaCl concentration (0, 0.51 and 1.03 mol L⁻¹), and storage time (0-8 weeks). Cultures of B. lactis exhibited no significant loss of viability either with increasing NaCl concentration or increasing storage temperature within the ranges studied. On the other hand, viability of L. acidophilus decreased with increasing temperature and increasing NaCl concentration under similar conditions. When cocultured with L. acidophilus, B. lactis was significantly less tolerant to higher NaCl levels and higher temperatures than when in pure culture, although final numbers were still above the threshold required for commercial application. Coculturing with B. lactis had no detrimental effect on viability of L. acidophilus. A mechanistic model, which considers the behavior of the pure and mixed microbial populations is described by specific death rates which vary with temperature (following Arrhenius relationships) and NaCl levels (following simple inhibition kinetics) in the milk medium. Activation energies for the death rates of B. lactis and L. acidophilus were 14 kcal mol⁻¹ and 9-15 kcal mol⁻¹, respectively.

INTRODUCTION

Supplementation of milk with *Lactobacillus acidophilus* and/or *Bifidobacterium* spp. has been very popular in recent years because both of these species are resistant to intestinal bile salts (Rasic and Kurmann 1983). Moreover, these microorganisms are frequently associated with health-promoting and therapeutic properties which encompass anticarcinogenic activity, reduction of

¹Author for correspondence.

cholesterol, improvement of protein metabolism and antimicrobial activity (Marteau and Rambaud 1993; Modler *et al.* 1994). From a technological point of view, their ability to produce acid in milk and reduced acidification during post-processing storage enables them to be used in the industrial manufacture of various fermented dairy produces (Modler *et al.* 1990; Mital and Garg 1992). However, such dietary cultures will not play an effective biological role in fermented milk products unless they are present in sufficient viable numbers by the time of consumption. For this reason, changes in the population of viable bacteria during the expected shelf-life of the dairy product in question should be known to some extent and taken as a basis for selection criteria of such strains.

The use of the aforementioned probiotic species as an additive to cheese is not a new idea (El-Shafel 1994; Roy *et al.* 1995). However, the possibility of using a starter culture entirely composed of *B. lactis* and *L. acidophilus* for the manufacture of a Gouda-type cheese was not reported until recently by Gomes *et al.* (1995). These studies have shown that both species persist in cheese in moderately high numbers even after 9 weeks of refrigerated storage. Despite the fact that survival rates appeared promising, detailed work on improving the storage stability of the final probiotic product and, consequently, cheese quality as perceived by the final consumer is still in order; as asserted elsewhere (Kim 1988), the better the product capability of retaining strain viability, the higher its beneficial effect upon the final consumer.

Among several cheese processing factors, salt content and storage temperature are easily manipulated and are known to clearly affect the degree of survival and activity of *L. acidophilus* and bifidobacteria in a cheese matrix as single factors (Roy 1991; Guinee and Fox 1993). Storage temperature is also important when accelerated cheese ripening is sought (van den Berg and Exterkate 1993). To our knowledge, statistically reliable data pertaining to salt lethality and salt tolerance of both species are rather scarce. Likewise, there is relatively little information on survival under factors acting in combination and very few attempts have been made to mechanistically model the data acquired from such studies. The absence of such models greatly restricts our ability to predict the effect of varying processes and/or varying growth medium composition on survival of such strains, a matter of increasing relevance due to their biological role.

The aim of this research effort was, thus, to study the growth and viability of *B. lactis* and *L. acidophilus*, both independently and as a coculture, in reconstituted skim milk so as to assess their dependence upon temperature and salt concentration throughout an extended storage period and eventually model such dependence in mathematical terms from first principles. The data generated may then be used to accurately predict survival within the limits of temperature and NaCl levels used in the experiments to a known degree of statistical confidence, and so will be useful in efforts to produce a high quality cheese where the aforementioned strains will be incorporated to play the simultaneous roles of starter and probiotic vector.

MATERIALS AND METHODS

Bacterial Strains

Bifidobacterium lactis and *Lactobacillus acidophilus* strain Ki were obtained in the form of frozen starter concentrates from Coöperatieve Stremsel-en Kleurselfabriek (CSK, Leeuwarden, The Netherlands).

Growth Conditions

Reconstituted skim milk (Agros, Vila do Conde, Portugal) at the level of 100 g L⁻¹ total solids supplemented with 0.5 g L⁻¹ yeast extract (LabM, Bury, UK) was sterilized at 110°C for 10 min. When the pure (or the mixed culture) containing the *Bifidobacterium* strain was to be used, the milk was added with 0.5 g L⁻¹ cysteine-HCl, previously filter sterilized, to lower the redox potential. A fresh inoculum of each strain was then obtained from the corresponding sealed beaker of frozen concentrate (each culture was from a single batch and the contents of each beaker were melted at 20°C for 30 min prior to use) before each experiment and was transferred aseptically to the sterilized milk prepared as above at the ratio of 50 g L⁻¹ at 37°C. Once inoculated, the milk was immediately divided into 50-mL portions in glass flat-bottomed flasks which were entirely filled (to completely exclude oxygen) and stoppered. Incubation was at 37°C for 20 h. The required amount of dry-sterilized NaCl (Merck, Darmstadt, Germany) was added to the fully grown cultures, and these were stored in a thermostated incubator at the required temperatures. The range of salt concentrations was selected in an attempt to correctly characterize a semi-hard cheese ripened for a regular period of time; the range of temperatures was selected to cover both usual storage conditions and acceptable accelerated ripening conditions. One flask was randomly removed every week and assayed for number of viable bacteria and level of acid production.

Enumeration of Microorganisms

The fermented milk samples were decimally diluted in sterile peptone-saline solution, consisting of 1 g L⁻¹ peptone (Oxoid, Basingstoke, UK) and 8.5 g L⁻¹ NaCl (Merck), and the spread plate technique was used with various media.

Enumeration of *B. lactis* and *L. acidophilus* in the fermented milk samples was made after inoculation and incubation on MRS agar supplemented with 0.5 g L⁻¹ cysteine-HCl or TGV agar (Galesloot *et al.* 1961) in Petri dishes, respectively. For cocultures, each individual strain was counted on MRS agar supplemented with selective agents which included bile salts, sodium propionate, lithium chloride and sheep's blood (Gomes *et al.* 1995) or TGV agar supplemented with 20 g L⁻¹ NaCl (Merck), respectively. The plates were incubated at 40°C for 48 h in an anaerobic environment using BBL Gas Paks (Becton Dickinson, Cockeysville, MA).

Acidification

Acidification was monitored via measurement of pH according to the AOAC method using a Crison pH-meter with a combined pH electrode (Ingold, Urdorf, Switzerland).

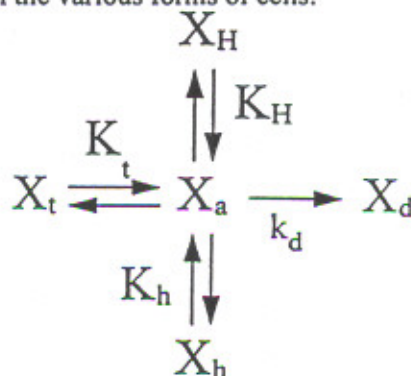
Statistical Design and Analysis

Three salt concentrations (0, 0.51 and 1.03 mol L⁻¹), three storage temperatures (5, 10 and 15°C), eight storage times (1, 2, 3, 4, 5, 6, 7 and 8 weeks) and two types of culture (pure culture and mixed culture) were combined in the form of a 3×3×8×2 full factorial design replicated twice to generate the full experimental layout. For simplicity of discussion, the type of culture was at times left out and hence the experiments could be interpreted as a set of two 3×3×8 full factorial design replicated twice.

The bacterial counts and the pH values were analyzed based on the ANOVA table. The estimation of the effects using this methodology is valid provided that the experimental errors are independent and normally distributed, and possess a constant variance (Box *et al.* 1978); since our data (in logarithmic form whenever viable counts were concerned) were consistent with these assumptions, a classical analysis of the original data was directly applied. To refine the analysis of significance, all possible pairwise comparisons were performed using a multiple *t*-statistic as reference according to the so-called Fisher's protected least significant difference test. All statistical analyses were done with the aid of the Statview™ package (Abacus Concepts, Berkeley CA).

Mathematical Analysis

Assume the following scheme in an attempt to describe mechanistic relationships between the various forms of cells:



where X_a denotes actively metabolizing cells, X_t denotes thermally-shocked vegetative cells, X_H denotes hypertonically-shocked vegetative cells, X_h denotes hypotonically-shocked vegetative cells and X_d denotes dead cells, and where K

denotes an equilibrium constant and k denotes a kinetic constant.

A mass balance to the whole living cell population, denoted by X , under the assumption that the system can be considered well mixed (i.e. no preferential distribution of cells throughout the milk medium exists and the time scales associated with transport of nutrients are very small compared with the time scale associated with cell death) reads

$$\frac{d[X]}{dt} = -k_d[X_a] \quad (1)$$

where the brackets denote mole concentration, t denotes batch time and X is accounted for by both active and vegetative cells according to

$$[X] = [X_a] + [X_H] + [X_i] + [X_h] \quad (2)$$

Each vegetative species may in turn be expressed as a function of the actively metabolizing species by the following equations:

$$[X_H] = K_H\{[S]\}[X_a] \quad (3)$$

$$[X_i] = K_i\{T\}[X_a] \quad (4)$$

$$[X_h] = K_h\{[S]\}[X_a] \quad (5)$$

where S denotes sodium chloride and T absolute temperature. Combination of Eq. (1)-(5) yields

$$\frac{d[X]}{dt} = -\frac{k_d\{T\}[X]}{1 + K_H\{[S]\} + K_i\{T\} + K_h\{[S]\}} \quad (6)$$

The simplest functionalities indicated in Eq. (6) that bear a physicochemical meaning are given by

$$K_H = \alpha_H[S] \quad (7)$$

where α_H is a constant,

$$K_i = \exp\left\{\frac{\Delta s}{R} - \frac{\Delta h}{RT_0}\right\} \exp\left\{-\frac{\Delta h}{R} \left(\frac{1}{T} - \frac{1}{T_0}\right)\right\} \quad (8)$$

where R is the ideal gas constant, Δs a standard entropy of cell thermal inactivation, Δh a standard enthalpy of cell thermal inactivation and T_0 the median temperature of the experimental range selected,

$$K_h = \frac{\alpha_h}{[S]} \quad (9)$$

where α_h is a constant, and

$$k_d = \beta \frac{k_B}{h} \exp\left\{-\frac{E_{act}}{RT_0}\right\} \exp\left\{-\frac{E_{act}}{R} \left(\frac{1}{T} - \frac{1}{T_0}\right)\right\} \quad (10)$$

where β is a proportionality constant associated with cell death, k_B Boltzmann's constant, h Planck's constant and E_{act} an activation energy of cell death. Combination of Eq. (6) - (10) followed by integration finally gives

$$\log\left\{\frac{[X]}{[X_0]}\right\} = -\frac{\frac{\beta k_B}{h \ln\{10\}} \exp\left\{-\frac{E_{act}}{RT_0}\right\} \exp\left\{-\frac{E_{act}}{R} \left(\frac{1}{T} - \frac{1}{T_0}\right)\right\} t}{1 + \alpha_H [S] + \exp\left\{\frac{\Delta s}{R} - \frac{\Delta h}{RT_0}\right\} \exp\left\{-\frac{\Delta h}{R} \left(\frac{1}{T} - \frac{1}{T_0}\right)\right\} + \frac{\alpha_h}{[S]}} \quad (11)$$

where $[X]_0$ denotes the initial biomass concentration. The (six) statistically independent parameters to be estimated via nonlinear fitting to the experimental data sets, in the form of $\log\{[X]/[X]_0\}$ vs t , generated at various values of $[S]$ and T are, thus, β , E_{act} , α_H , Δs , Δh and α_h .

The model given by Eq. (1) follows the original proposal by Chick (1908). The overall temperature dependence of loss of cell viability depicted in Eq. (11) is similar to the one suggested in Bailey and Ollis (1986) for cell growth, which is in turn analogous to the model proposed, in more mechanistically detailed terms, for

enzyme action and inactivation. The model parameters were fitted to the experimental data sets using nonlinear regression via the software package GREG (Caracotsios *et al.* 1985).

RESULTS

Experimental Results

The initial numbers of *B. lactis* and *L. acidophilus* varied between $10^9 - 10^{10}$ cfu mL⁻¹ and $10^8 - 10^9$ cfu mL⁻¹, respectively, when cultured individually. *B. lactis* numbers were slightly lower ($10^8 - 10^9$ cfu mL⁻¹) when cocultured with *L. acidophilus*, whereas numbers remained similar to pure cultures as far as *L. acidophilus* was concerned. The viable cell numbers during refrigerated storage decreased gradually in all sets of experiments as storage time elapsed (Table 1).

TABLE 1.
MEAN VALUES OF VIABLE NUMBERS FOR *BIFIDOBACTERIUM LACTIS* AND
LACTOBACILLUS ACIDOPHILUS AS SINGLE CULTURES AND AS A COCULTURE IN MILK
AT VARIOUS SODIUM CHLORIDE CONCENTRATIONS DURING REFRIGERATED STORAGE
AT VARIOUS TEMPERATURES

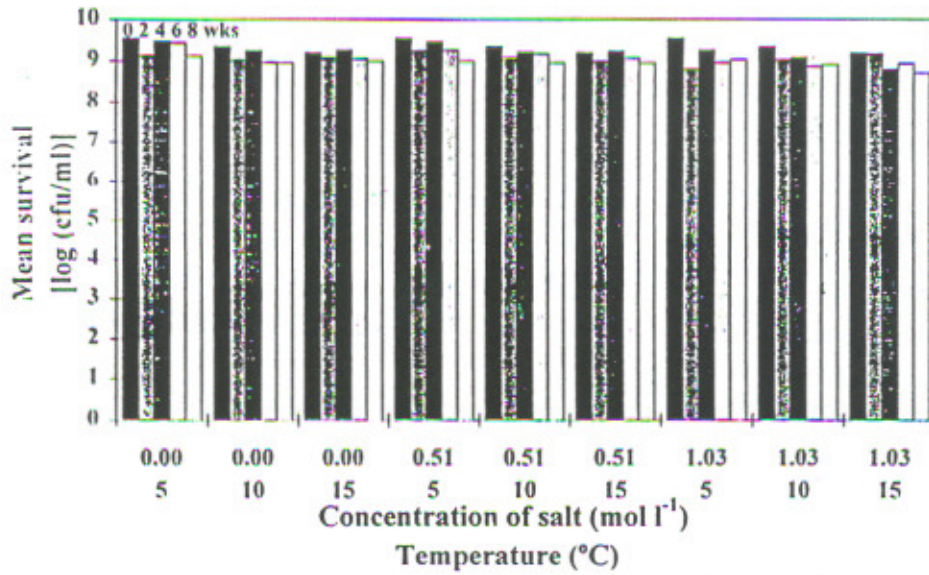
Storage conditions	Culture type	<i>Bifidobacterium lactis</i> [log (cfu ml ⁻¹)]		<i>Lactobacillus acidophilus</i> [log (cfu ml ⁻¹)]	
		Initial	Reduction ¹	Initial	Reduction
5°C, 0.00 mol l ⁻¹ NaCl	Single	9.55	0.43	8.96	4.69
5°C, 0.53 mol l ⁻¹ NaCl	Single	9.55	0.56	8.96	4.80
5°C, 1.03 mol l ⁻¹ NaCl	Single	9.55	0.51	8.96	5.45
10°C, 0.00 mol l ⁻¹ NaCl	Single	9.34	0.38	9.04	4.05
10°C, 0.53 mol l ⁻¹ NaCl	Single	9.34	0.38	9.04	3.11
10°C, 1.03 mol l ⁻¹ NaCl	Single	9.34	0.43	9.04	4.19
15°C, 0.00 mol l ⁻¹ NaCl	Single	9.20	0.20	9.29	5.22
15°C, 0.53 mol l ⁻¹ NaCl	Single	9.20	0.24	9.29	5.78
15°C, 1.03 mol l ⁻¹ NaCl	Single	9.20	0.48	9.29	6.29
5°C, 0.00 mol l ⁻¹ NaCl	Mixed	8.60	0.43	9.58	1.73
5°C, 0.53 mol l ⁻¹ NaCl	Mixed	8.60	2.27	9.58	3.45
5°C, 1.03 mol l ⁻¹ NaCl	Mixed	8.60	3.60	9.58	4.63
10°C, 0.00 mol l ⁻¹ NaCl	Mixed	8.17	2.09	8.46	1.40
10°C, 0.53 mol l ⁻¹ NaCl	Mixed	8.17	3.17	8.46	1.94
10°C, 1.03 mol l ⁻¹ NaCl	Mixed	8.17	3.67	8.46	3.01
15°C, 0.00 mol l ⁻¹ NaCl	Mixed	8.58	4.24	8.93	5.04
15°C, 0.53 mol l ⁻¹ NaCl	Mixed	8.58	4.33	8.93	5.31
15°C, 1.03 mol l ⁻¹ NaCl	Mixed	8.58	4.43	8.93	5.71

¹ Initial [mean log (cfu ml⁻¹)] - Final [mean log (cfu ml⁻¹)] after 8 weeks of storage.

However, this decrease took place at different rates depending on the storage temperature and salt concentration. All three manipulated variables under study, viz. storage time (t), storage temperature (T) and salt concentration (S), significantly ($P < 0.0001$) affected the viability of *L. acidophilus*, and during 8 weeks of storage the pure *L. acidophilus* culture decreased its viability by three to six log cycles down to $10^3 - 10^6$ cfu mL⁻¹. The most significant variable that accounts for this behavior was t ($F=1022.5$), followed by S ($F=132.5$) and, to a lesser extent, T ($F=64.5$). Viable counts of the *B. lactis* counterpart showed no significant decrease over the same time period, as apparent in Fig. 1 (where only the even time groups have been plotted as histograms for easier trend perception; the omitted odd time groups follow exactly the trends shown), although effects statistically significant at the 5% level could be detected for t and T. The degree of importance of these variables on viable numbers of *B. lactis* was comparable to that observed with *L. acidophilus*. All second order interactions had a statistically significant effect on the viability of *L. acidophilus* at the 5% level, but only the interactions t*T and t*S were associated with a statistically significant effect on *B. lactis* viability. The results of Fisher's PLSD tests showed that there were clear statistical differences between the viable numbers of *L. acidophilus* at 5C and 15C ($P < 0.0001$) on the one hand, and at 10C and 15C ($P < 0.0001$) on the other, but similarity was observed at 5C and 10C ($P=0.584$). Salt concentrations of 0 and 0.51 mol L⁻¹ led to statistically similar effects ($P=0.883$), and significant differences were only noticeable in changes from either 0 or 0.51 mol L⁻¹ up to 1.03 mol L⁻¹ salt concentration ($P < 0.0001$ for both pairwise comparisons). In terms of the viable numbers of *B. lactis*, clear statistical differences between those and 5C and 10C ($P=0.0001$) on the one hand, and at 5C and 15C ($P < 0.0001$) on the other, were observed but similarity existed at 10C and 15C ($P=0.591$). As happened with the observations encompassing the pure culture of *L. acidophilus*, salt concentrations of 0 and 0.51 mol L⁻¹ were associated with statistically similar effects ($P=0.706$), and significant differences were only noticeable in changes from either 0 or 0.51 mol L⁻¹ up to 1.03 mol L⁻¹ salt concentration ($P=0.015$ and $P=0.038$, respectively).

In a mixed culture environment, trends in *L. acidophilus* numbers (Fig. 2) were apparent and similar to those previously discussed pertaining to the pure culture counterpart, but in this instance *B. lactis* numbers decreased up to three log cycles down to $10^4 - 10^5$ cfu mL⁻¹ (in the presence of *L. acidophilus*). All four manipulated variables (i.e. t, T, S and culture type C) showed statistically significant ($P < 0.0001$) effects on the viable numbers of both *L. acidophilus* and *B. lactis*. Moreover, the importance of the variables chosen was similar for both strains, i.e. smaller for T and S and higher for t and C. The culture effect was particularly high ($F=5238.0$ and $F=5012.7$ for *B. lactis* and *L. acidophilus*, respectively). Interestingly, the degree of importance associated with these results for variables t, S and T with respect to the viability of *L. acidophilus* followed a similar trend of those obtained in pure culture ($F=1444.0$, $F=309.2$ and $F=182.2$ for t, S and T, respectively, in decreasing order of significance). It is also worth

a)



b)

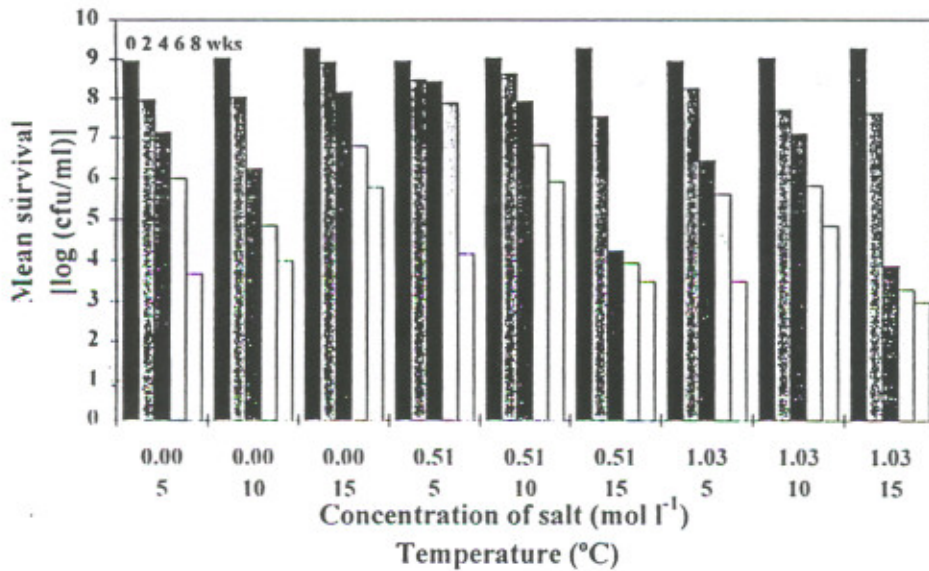
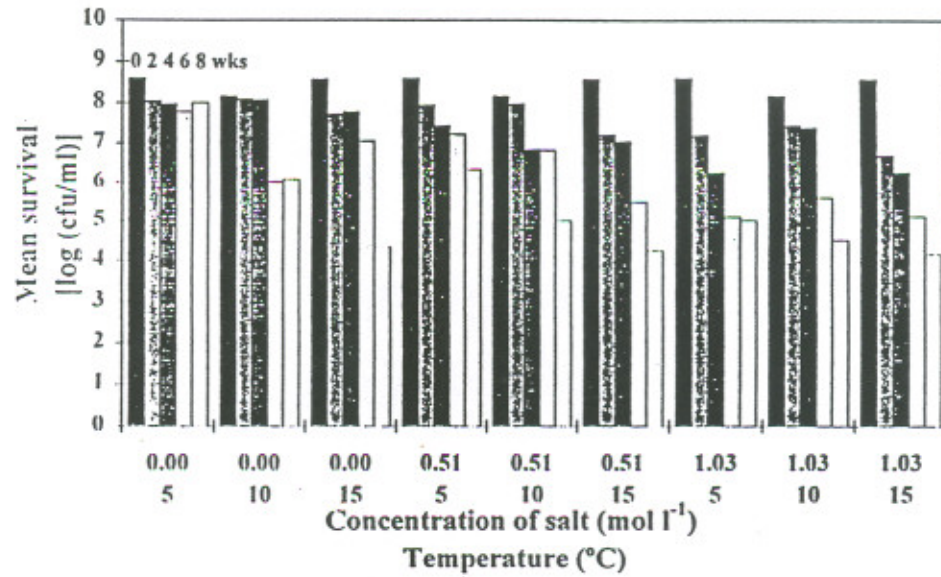


FIG. 1. MEAN SURVIVAL OF (a) *BIFIDOBACTERIUM LACTIS* AND (b) *LACTOBACILLUS ACIDOPHILUS* IN SINGLE MILK CULTURES STORED FOR 8 WEEKS AS A FUNCTION OF SALT CONCENTRATION AND STORAGE TEMPERATURE

Data plotted are average values of duplicate experiments. Standard errors of the mean for *Bifidobacterium lactis* and *Lactobacillus acidophilus* are 0.02 and 0.14, respectively.

a)



b)

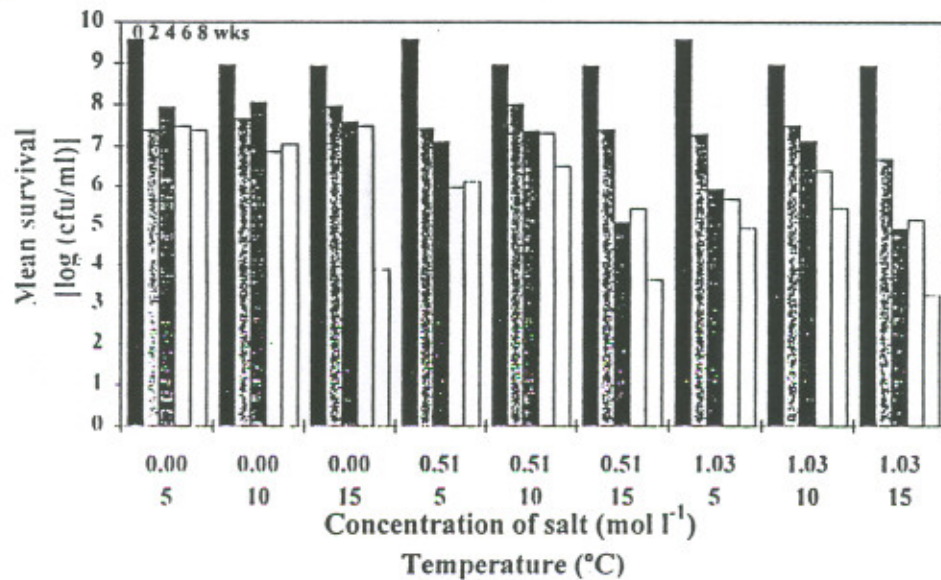


FIG. 2. MEAN SURVIVAL OF (a) *BIFIDOBACTERIUM LACTIS* AND (b) *LACTOBACILLUS ACIDOPHILUS* IN MIXED MILK CULTURES STORED FOR 8 WEEKS AS A FUNCTION OF SALT CONCENTRATION AND STORAGE TEMPERATURE

Data plotted are average values of duplicate experiments. Standard errors of the mean for *Bifidobacterium lactis* and *Lactobacillus acidophilus* are 0.10 and 0.11, respectively.

noting that the order of magnitude of the salt effect ($F=358.6$) on the viable numbers of *B. lactis* increased 100-fold when compared to that of the pure culture counterpart ($F=3.6$). All second order interactions had statistically significant effects on viable numbers of both *L. acidophilus* and *B. lactis*. The results of Fisher's PLSD tests indicated that, in mixed culture, all changes in either salt concentration or storage temperature yielded statistically significant differences for the viable numbers of both *B. lactis* and *L. acidophilus* ($P<0.001$ and $P<0.0001$, respectively). It is remarkable that changes from a single culture of *B. lactis* to a mixed culture in the presence of *L. acidophilus* led to clear statistical differences ($P<0.0001$) in terms of viability.

The pH value in milk was monitored during the 8 weeks of storage. In all instances the pH values of the inoculated milk remained stable during this period, showing variations below 0.2 unit as depicted in Table 2. However, changes of the set values for the variables affected the pH to a significant degree; for a combined stress treatment, the pH values drop usually 0.3 unit when going from individual to mixed cultures of *B. lactis*. As opposed to their effects on the viable numbers of either *B. lactis* or *L. acidophilus* in pure or mixed culture media, the most significant ($P<0.0001$) variable that accounts for pH value was S ($F=610.6$, $F=552.7$ and $F=1808.7$ for *B. lactis* pure culture, *L. acidophilus* pure culture and mixed culture, respectively), followed by T ($F=545.9$, $F=123.2$ and $F=431.7$, respectively) and, to a lesser extent, t ($F=25.2$, $F=32.0$ and $F=155.7$, respectively). All second order interactions had a statistically significant effect on pH at the 5% level for all instances. The triple interactions, although statistically significant at this level in some instances, had values well below those of the independent effects.

Modeling Results

In attempts to relate the survival response of the strains selected to several extrinsic factors, viz. controlled storage conditions of temperature and salt concentration, a mechanistic model was produced and its parameters fitted to the experimental data obtained as detailed above. Nonlinear regression analysis should be applied to the data themselves because standard deviations between replicates plotted versus the corresponding replicate averages (data not shown) did not indicate biased trends. Table 3 summarizes the estimates for the six independent parameters, β , E_{act} , α_{th} , Δs , Δh and α_b , for both independent and mixed cultures of *B. lactis* and *L. acidophilus*. Inspection of Table 3 indicates that most (but not all) parameters in the postulated model were significant at the 5% level. It is also noteworthy that the salt dependence parameters violate, in some instances, physico-chemical constraints and should thus be regarded solely as statistical estimates, without a strong physico-chemical meaning; additional data points in wider ranges of salt concentration might have overcome this shortcoming.

TABLE 2.
MEAN VALUES AND RANGE OF pH OF MILK MEDIUM INCUBATED WITH
BIFIDOBACTERIUM LACTIS AND *LACTOBACILLUS ACIDOPHILUS* AS SINGLE CULTURES
AND AS A COCULTURE AT VARIOUS SODIUM CHLORIDE CONCENTRATIONS DURING
REFRIGERATED STORAGE AT VARIOUS TEMPERATURES

Storage conditions	Culture type	<i>Bifidobacterium lactis</i>		<i>Lactobacillus acidophilus</i>	
		pH mean	pH range	pH mean	pH range
5°C, 0.00 mol l ⁻¹ NaCl	Single	4.06	3.98 - 4.12	3.64	3.55 - 3.73
5°C, 0.53 mol l ⁻¹ NaCl	Single	3.99	3.98 - 4.01	3.54	3.47 - 3.59
5°C, 1.03 mol l ⁻¹ NaCl	Single	3.95	3.93 - 3.98	3.48	3.45 - 3.65
10°C, 0.00 mol l ⁻¹ NaCl	Single	4.16	4.12 - 4.20	3.71	3.66 - 3.75
10°C, 0.53 mol l ⁻¹ NaCl	Single	4.10	4.05 - 4.14	3.64	3.62 - 3.66
10°C, 1.03 mol l ⁻¹ NaCl	Single	4.05	4.00 - 4.12	3.57	3.55 - 3.66
15°C, 0.00 mol l ⁻¹ NaCl	Single	4.08	3.99 - 4.15	3.62	3.60 - 3.64
15°C, 0.53 mol l ⁻¹ NaCl	Single	4.04	3.99 - 4.13	3.60	3.51 - 3.64
15°C, 1.03 mol l ⁻¹ NaCl	Single	4.00	3.95 - 4.06	3.58	3.50 - 3.63
5°C, 0.00 mol l ⁻¹ NaCl	Mixed	3.81	3.75 - 3.87	3.81	3.75 - 3.87
5°C, 0.53 mol l ⁻¹ NaCl	Mixed	3.75	3.71 - 3.85	3.75	3.71 - 3.85
5°C, 1.03 mol l ⁻¹ NaCl	Mixed	3.70	3.63 - 3.80	3.70	3.63 - 3.80
10°C, 0.00 mol l ⁻¹ NaCl	Mixed	3.79	3.72 - 3.87	3.79	3.72 - 3.87
10°C, 0.53 mol l ⁻¹ NaCl	Mixed	3.70	3.66 - 3.75	3.70	3.66 - 3.75
10°C, 1.03 mol l ⁻¹ NaCl	Mixed	3.65	3.59 - 3.72	3.65	3.59 - 3.72
15°C, 0.00 mol l ⁻¹ NaCl	Mixed	3.75	3.67 - 3.88	3.75	3.67 - 3.88
15°C, 0.53 mol l ⁻¹ NaCl	Mixed	3.66	3.56 - 3.76	3.66	3.56 - 3.76
15°C, 1.03 mol l ⁻¹ NaCl	Mixed	3.61	3.54 - 3.70	3.61	3.54 - 3.70

In general, the proposed 6-parameter model given by Eq. (11) gave a reasonably good description of the microbial reduction observed under the experimental conditions applied, as apparent from inspection of Fig. 3 and 4. Similar trends were obtained for the experimental death curves estimated at the remaining temperatures (not shown). The predicted loss of viability of *B. lactis* in a mixed culture with *L. acidophilus* is significantly higher than as an independent culture (Fig. 3). In addition, it appears that such loss in a coculture environment is strongly dependent upon the storage temperature and, to a much lesser extent, upon NaCl concentration (as apparent from inspection of Tables 3 and 4), and variations in the NaCl content at 5C lead to a sharper decline in viable numbers than similar variations at 10C and 15C; e.g. at 5C the loss of viability after 8 weeks of storage ($\log(X/X_0)$) of *B. lactis* in the presence of 1.03 mol L⁻¹ NaCl is 5-fold higher than that in the absence of NaCl, whereas at 15C the loss of viability was only 1.5-fold higher. The opposite effect was observed in pure cultures of *B. lactis*,

although less pronounced. With respect to survival of *L. acidophilus*, the ratio of the rate of decrease in viable numbers to the rate of increase of NaCl levels remains essentially constant over the whole experimental salt range for both types of cultures considered irrespective of temperature (Fig.4); an increased sensitivity of *L. acidophilus* to NaCl concentration is, however, apparent in a coculture environment. Surprisingly, addition of 0.51 mol L⁻¹ of NaCl to the pure culture of *L. acidophilus* inhibited the decline in viable numbers during subsequent refrigerated storage. Similar effects were not observed for *L. acidophilus* in the presence of *B. lactis*.

TABLE 3.
ESTIMATES OF PARAMETERS IN THE MECHANISTIC MODEL FITTED BY NONLINEAR REGRESSION TO THE NUMBER OF VIABLE *BIFIDOBACTERIUM LACTIS* AND *LACTOBACILLUS ACIDOPHILUS* IN EITHER PURE CULTURE OR COCULTURE AS A FUNCTION OF STORAGE TEMPERATURE (K), SALT CONCENTRATION, (MOL L⁻¹) AND STORAGE TIME (S)

Microbial strain	Parameter	Pure	Coculture
<i>Bifidobacterium lactis</i>	β (s ⁻¹)	6.6996x10 ⁻⁷	2.7544x10 ⁻³
	E_{act} (cal mol ⁻¹)	-1.0866x10 ⁵	1.3641x10 ⁵
	α_H (l mol ⁻¹)	-3.9864x10 ⁻¹	0.0000
	Δs (cal mol ⁻¹ K ⁻¹)	-6.8507x10 ²	3.3177x10 ²
	Δh (cal mol ⁻¹)	-1.9051x10 ⁵	7.8002x10 ⁴
	α_h (mol l ⁻¹)	-2.1900x10 ⁻⁵	4.5660x10 ⁻²
<i>Lactobacillus acidophilus</i>	β (s ⁻¹)	1.1966x10 ⁻⁵	1.4120x10 ⁻⁵
	E_{act} (cal mol ⁻¹)	1.5460x10 ⁴	8.6311x10 ³
	α_H (l mol ⁻¹)	-1.0929x10 ⁰	-7.9596x10 ⁻¹
	Δs (cal mol ⁻¹ K ⁻¹)	4.4723x10 ⁰	4.9500x10 ⁰
	Δh (cal mol ⁻¹)	-9.2487x10 ⁰	4.0589x10 ¹
	α_h (mol l ⁻¹)	0.0000	1.3328x10 ⁻⁴

Although the inactivation of refrigerated bacteria is normally fastest just below their minimum growth temperature, the opposite effect was observed with single cultures of *B. lactis* where survival was better at 15C than at 5 or 10C, irrespective of the NaCl level (Fig. 3). Further confirmation of this observation was obtained from inspection of the calculated values of k_d and K_t (Table 4). The death rate of pure *B. lactis* tended to a minimum at 15C; when in coculture with *L. acidophilus*, k_d showed an approximately linear increase with temperature and was two to four orders of magnitude higher than its pure culture counterpart. With respect to *L. acidophilus*, the slopes of the predicted death curves were somewhat similar between single and mixed cultures, and lower variations of k_d from 5 to 15C were noticeable (Table 4). Nevertheless, k_d exhibited a 25% increase when *L. acidophilus* was cocultured with *B. lactis*, a competitive microorganism. Similar trends were observed for the estimated values of K_t for both *B. lactis* and *L. acidophilus*.

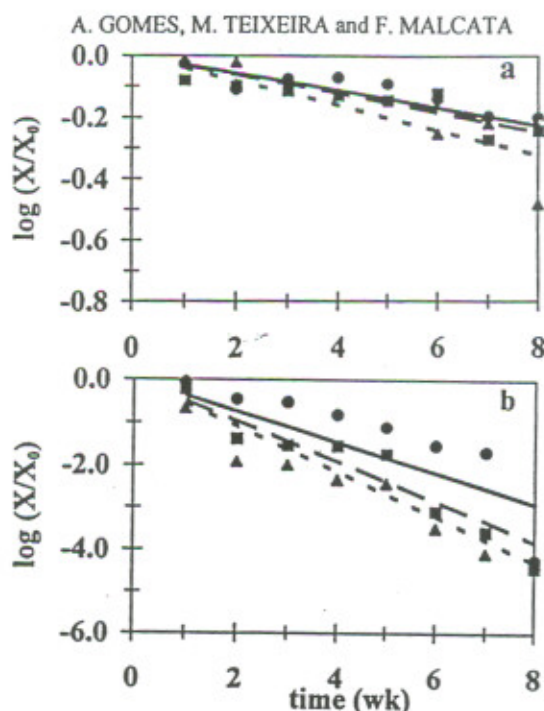


FIG. 3. PLOT OF THE VIABLE NUMBERS OF *BIFIDOBACTERIUM LACTIS* IN (a) SINGLE MILK CULTURES AND (b) MIXED MILK CULTURES AT 15C AS A FUNCTION OF STORAGE TIME (WEEKS)

Experimental values (● - 0 mol L⁻¹ NaCl, ■ - 0.51 mol L⁻¹ NaCl, ▲ - 1.03 mol L⁻¹ NaCl) and theoretical fit (— 0 mol L⁻¹ NaCl, - - - 0.51 mol L⁻¹ NaCl, - · - · 1.03 mol L⁻¹ NaCl).

The overall activation energy (E_{act}), often described as the temperature characteristic of the microorganism, was 14 kcal mol⁻¹ and 9-15 kcal mol⁻¹ for *B. lactis* and *L. acidophilus*, respectively (Table 3). If one neglects the estimated value of E_{act} for the *B. lactis* pure culture (the negative numerical value of the estimate was actually associated with a confidence interval encompassing the null hypothesis), significant differences were detected between the mixed culture when compared with the pure culture counterpart. The higher E_{act} , the more sensitive death rate is on temperature; therefore, the higher E_{act} observed for pure cultures of *L. acidophilus* (15 kcal mol⁻¹) than for cocultures (9 kcal mol⁻¹) of the same strain indicates that coculturing reduces the heat lability of *L. acidophilus*.

DISCUSSION

Pure cultures of *B. lactis* exhibited no statistically significant loss of viability in milk stored at temperatures ranging from 5 to 15C when sodium chloride concentration was increased from 0 to 1.03 mol L⁻¹. Such observation may be partly accounted for by inhibition of metabolic activity at low temperatures (which was further confirmed by minor pH changes, which are a result of low acidolytic action). In addition, stable pH values also ensure uniform quality of

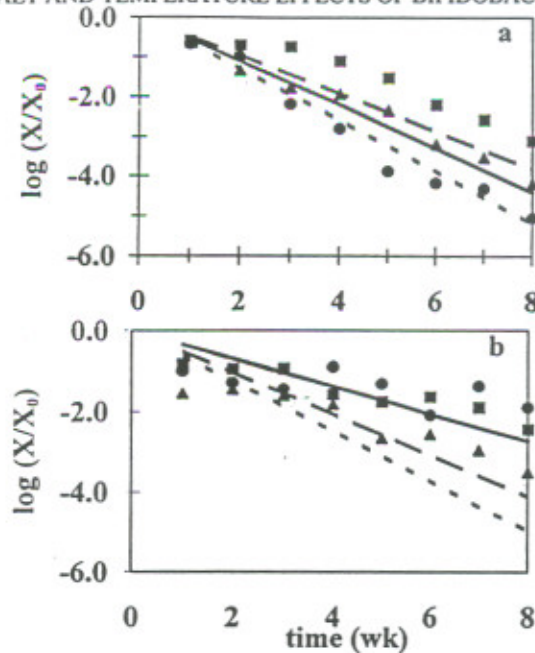


FIG. 4. PLOT OF THE VIABLE NUMBERS OF *LACTOBACILLUS ACIDOPHILUS* IN (a) SINGLE MILK CULTURES AND (b) MIXED MILK CULTURES AT 10°C AS A FUNCTION OF STORAGE TIME (WEEKS)

Experimental values (● - 0 mol L⁻¹ NaCl, ■ - 0.51 mol L⁻¹ NaCl, ▲ - 1.03 mol L⁻¹ NaCl) and theoretical fit (--- 0 mol L⁻¹ NaCl, ---- 0.51 mol L⁻¹ NaCl, ···· 1.03 mol L⁻¹ NaCl).

TABLE 4.
VALUES OF LUMPED PARAMETERS ESTIMATED FROM THE MECHANISTIC MODEL FOR PURE CULTURES AND COCULTURE OF *BIFIDOBACTERIUM LACTIS* AND *LACTOBACILLUS ACIDOPHILUS* AT DIFFERENT STORAGE TEMPERATURES (K)

Microbial strain	Temperature	k_d		K_T	
		Pure	Coculture	Pure	Coculture
<i>Bifidobacterium lactis</i>	278	1.5374×10^{-6}	9.7096×10^{-4}	1.0219×10^0	4.7188×10^2
	283	6.6996×10^{-7}	2.7544×10^{-3}	2.3821×10^{-1}	8.5661×10^2
	288	3.0050×10^{-7}	7.5357×10^{-3}	5.8409×10^{-2}	1.5231×10^3
<i>Lactobacillus acidophilus</i>	278	1.0632×10^{-5}	1.3218×10^{-5}	1.7193×10^0	1.7821×10^0
	283	1.1966×10^{-5}	1.4120×10^{-5}	1.7192×10^0	1.7827×10^0
	288	1.3412×10^{-5}	1.5048×10^{-5}	1.7191×10^0	1.7832×10^0

milk during refrigerated storage. Survival of *L. acidophilus* decreased with increasing storage temperature and with increasing sodium chloride levels above 0.51 mol L⁻¹ (an assertion correctly predicted by the postulated model and which is in agreement with the ANOVA results discussed previously). It has become clear that *L. acidophilus* was more susceptible to higher sodium chloride

concentrations than *B. lactis*, a result which is consistent with those obtained by Gomes *et al.* (1995) in previous studies pertaining to survival of these strains in a semi-hard cheese matrix. Among several possible explanations for this observation, some authors (Irvine and Price 1961; Fernández-Salguero *et al.* 1986) have suggested that the reductions in water activity effected by the increase in sodium chloride concentration could account for the decreased resistance and survival of cells during storage. Proof for this assertion has been provided for lactobacilli involved in food fermentations (Montañó *et al.* 1993) and may easily be extended to encompass the results of our research effort. The independent salt effect is more relevant for *L. acidophilus* than for *B. lactis* probably because the former, like many other enteric bacteria, is sensitive to osmotic stress brought about by high salt concentrations (Jorgensen *et al.* 1994). It should be noted, however, that a certain salinity of the storage medium (0.51 mol L⁻¹) is advocated for maximum survival of *L. acidophilus* during refrigerated storage, and that the increased sensitivity to cold and lower sodium chloride concentrations may be partially attributed to membrane destabilization (Mackey 1984); this observation suggests that the definition of temperature influence on survival cannot be fully effective unless the sodium chloride concentration is taken into account, thus providing a justification for the usefulness of the mechanistic model in the form postulated.

The effect of the culture medium on survival of refrigerated microorganisms has not been systematically studied to date; however, greater protection against metabolic injury of *Streptococcus lactis* was observed when milk solids were added to the refrigerated medium (Moss and Speck 1963). In a study focused on storage stability of freeze-dried cultures of bifidobacteria, Blanchette *et al.* (1995) concluded that the presence of milk proteins and lactose provides a protective coating for the cells during long-term storage. This suggests that milk and its nutrients may have a protective effect on *B. lactis*, although no experimental validation of this assumption has been carried out. On the other hand, such milk components as free fatty acids or certain metabolites may have a direct or indirect (enhanced bactericidal effect pertinent to chilling) detrimental effect on survival of *L. acidophilus* as happens with other bacteria (Gyllenberg 1954).

B. lactis was significantly less tolerant to higher sodium chloride levels and higher temperatures when cocultured with *L. acidophilus* than when cultured alone. In addition to salt and temperature effects, other environmental factors such as pH, initial inoculum levels and competing microorganisms may also influence the viability of *B. lactis*. Previous experiments have shown that coculturing *B. lactis* with such a proteolytic species as *L. acidophilus* resulted in release of nitrogen compounds sufficiently simple to serve as nitrogen sources and hence permit growth in milk (data not shown), hence suggesting commensalistic growth (Stadhouders and Hup 1975). Nevertheless, this postulated commensalism implies a drawback for the less proteolytic strain as it requires a competitive advantage over the more proteolytic strain to remain in the food matrix. It is thus plausible

that the interactions between the two stress factors under study (i.e. salt concentration and storage temperature) and the competition by *L. acidophilus* possibly decrease such an advantage; the large magnitude of the effect of coculture versus pure culture on the viable numbers of *B. lactis* irrespective of the other manipulated variables reinforces this statement. A similar statistical effect of pH was also observed, and the slightly increased acidity of the coculture milk medium, when compared to that of a pure culture of *B. lactis*, may partially explain the decreased survival since most bifidobacteria are susceptible to high acidities; the stress treatments applied in this study produce injured microorganisms which are more freely permeable to hydrogen and hydroxyl ions, and so more sensitive to acid and alkali. It must, however, be taken into consideration that *B. lactis* (previously referred to as *Bifidobacterium* sp. Bo) shows no significant reduction in counts during storage of yoghurt at 5 to 7°C and at pH 4.0. to 3.8 (Klaver *et al.* 1993). Secondly, the effect of pH on survival parameters is temperature- and a_w -dependent, and hence the latter need to be specified and confirmed. More recently, Lankaputhra *et al.* (1996) have indicated a synergistic inhibition of some *Bifidobacterium* strains by acid and hydrogen peroxide during cold storage for 6 weeks. Therefore, the possible production of hydrogen peroxide by *L. acidophilus* is further support for the decreased survival of *B. lactis*: Collins and Koichiro (1980) have reported that *L. acidophilus* was able to produce hydrogen peroxide in 10% reconstituted skim milk and hence cause inhibition of growth of certain strains. Studies on the effects of nutrient media on growth of *L. acidophilus* by Fernandes *et al.* (1988) have led to the observation that all 10 selected strains produced traces of hydrogen peroxide; accumulation of hydrogen peroxide coupled with the inability to synthesize catalase (Rasic and Kurmann 1983) may have a direct contribution to the toxic effect of hydrogen peroxide on *B. lactis*. Nevertheless, detailed studies must still be conducted encompassing deliberate addition of oxygen scavengers catalase itself to the mixed culture for confirmation of these assumptions. The claim that the added stress of counting the bacterial strains on selective agar media exaggerates the difference in viable counts between pure and mixed cultures (because injured, yet surviving bacteria may not show) is doubtful in view of results reported in previous studies where the recoveries (90% or higher) for *B. lactis*, from both individual and mixed cultures, on the selective medium used here were comparable to that on MRS agar (data not shown).

It is noteworthy that, despite the lower viability of *B. lactis* in a coculture with *L. acidophilus*, final numbers in several of our experiments were still above the minimum number suggested for commercial application: Nahaisi (1986) and Kim (1988) have proposed that the final product should contain not less than one million viable cells per gram (or milliliter) at the time of consumption for full probiotic functionality to be displayed.

Since studies on refrigerated storage and salt tolerance of *L. acidophilus* and bifidobacteria, as well as models to predict such behavior, are still lacking, critical assessment of our work is difficult: Valdez and Biori (1993) have reported a ten-

fold reduction in viable counts of *L. acidophilus* after 21 days of storage at 5C in 10% skim milk, whereas Gagné *et al.* (1993) have demonstrated maintenance of a high degree of viability of *Bifidobacterium* cells during storage at 4C for 12 weeks. In comparison to these available data the losses in viability of *B. lactis* and *L. acidophilus* observed under some of the stress conditions described in this work were of a lower order of magnitude. With respect to the mechanistic model considered, it certainly meets the requirements of simplicity and physicochemical meaning; the model postulated is able to fit the experimental data accurately. Estimates of activation energies are also within the range usually found in the literature, viz. 15-20 kcal mol⁻¹ (Fu *et al.* 1991; Willocx *et al.* 1993). The greatest advantage of such a model is its utility in efforts to interpret and rationalize the dynamics of cell population survival under a variety of combinations of experimental conditions; this is of particular value when predictions of the evolution of *B. lactis* and *L. acidophilus* viable numbers in a complex cheese matrix are ultimately sought, as in the present instance. The microscopic food structure and other factors not included in our model should not be overlooked in more detailed modeling studies since they may contribute to whether or not an organism will survive and grow.

With the set of results obtained, one can conclude that *B. lactis* is able to survive in adverse conditions of salt concentration and storage temperature whereas *L. acidophilus* is rather susceptible to such stress conditions. Therefore, to achieve the desired goal of manufacturing a cheese with true 'probiotic' properties using such probiotic strains as the sole starter culture, careful adaptation of the selected cheesemaking technology is required to maintain appreciable viability and activity of both strains.

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