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Behaviour of *Listeria monocytogenes* under combined chilling processes

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J.-M. MEMBRÉ, T. ROSS AND T. McMEEKIN. 1999. The behaviour of *Listeria monocytogenes* under chilling processes was investigated. Growth kinetics were measured at 7 °C in TSBYE culture medium as a function of pH (7·2 and 6·2), pre-incubation temperatures (4 or 7 °C), cooling (0·05 or 0·1 °C min^{-1}) and freezing (0 and – 5 °C) treatments. Growth curves generated were fitted by Gompertz and Baranyi functions. The Baranyi function gave better parameter estimation values than the Gompertz equation which over-estimated the specific growth rate values. *Listeria monocytogenes* grew at 7 °C without a lag phase, except when the sub-culture was performed at 37 °C, whereas the specific growth rate was affected by the chilling processes. In fact, *L. monocytogenes* grew slightly faster at 7 °C when a 4 °C pre-incubation treatment was applied than with a 7 °C pre-incubation treatment. These results suggest that to mimic the processes of contamination in industry, predictive microbiology studies with *L. monocytogenes* should be performed with organisms cultured at low temperatures.

INTRODUCTION

Listeria monocytogenes, a food-borne pathogenic bacterium, has been involved in several outbreaks. As *L. monocytogenes* can grow at refrigeration temperatures, it is important to control its growth during food processes such as cooling, freezing or storage in cool rooms. For 15 years, the predictive modelling approach has been developed in order to determine microbial growth as a function of temperature, pH and water activity (McMeekin *et al*. 1993). Concerning the temperature effect on *L. monocytogenes*, models generally take the whole range of temperatures permitting growth into account, e.g. from 0–35 °C, and the studies are performed with bacteria sub-cultured in optimal conditions (Bajard *et al*. 1996; Murphy *et al*. 1996; McClure *et al*. 1997).

A few papers have dealt specifically with the effects of refrigeration temperature on *L. monocytogenes* in relation to pre-incubation conditions. Incubation of cultures at low temperatures (from 0 to 20 °C) before inoculation into a new medium caused a marked reduction in the lag time compared with cultures previously incubated at 30 or 37 °C (Walker *et al*. 1990; Buchanan and Klawitter 1991). Likewise, Gay and Cerf (1997) established that survival of *L. monocytogenes* was dependent on pre-incubation temperatures, with cells pre-incubated at 2 or 6 °C having a higher resistance than those pre-incubated at 30 °C.

To extend the knowledge of the effect of chilling processes, the growth behaviour of *L. monocytogenes* under a combination of sub-optimal conditions such as storage at refrigeration temperatures, and during chilling and freezing periods, was investigated. To reproduce industrial contamination patterns, and to take possible *L. monocytogenes* adaptation into account, the study was performed with bacteria pre-incubated at low temperatures.

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MATERIALS AND METHODS

Organism and culture medium

The strain used in this study was *L. monocytogenes* L5/22, a wild-type isolated at the University of Tasmania, Australia, from cold smoked salmon. The synthetic culture medium was Tryptone Soya Broth (Oxoid) supplemented with 0·6% Yeast Extract (TSBYE) and adjusted at pH 7·2 or 6·2 by addition of lactic acid. Experiments were carried out in 250 ml flasks containing 100 ml medium.

Temperature profile

The chilling process was studied in a full factorial experimental design with four factors at two levels, combined with

two levels of pH (6·2 and 7·2). From a TSBYE + 1·5% agar plate, stored at 4 °C, three colonies were transferred into TSBYE medium and incubated at 37 °C for 20 h. This primary culture was used to inoculate secondary cultures at each of the experimental conditions. First, cultures in 100 ml flasks (culture 1) were incubated at a constant temperature, T1, of 4 or 7 °C (Fig. 1) for 3 or 5 d, respectively. Then, the temperature was decreased (rate of 0·05 or 0·1 °C min^{-1}) to reach the freezing temperature T_f (0 or −5 °C). After having been maintained at the T_f value for 20 h, the temperature was increased (rate of 0·05 or 0·1 °C min^{-1}) up to 7 °C. The time when the temperature reached 7 °C was considered as the initial time, T_{init} = 0 h, for microbial growth kinetics. Moreover, at T_{init}, a fresh culture in TSBYE, called 'culture 2', was inoculated with bacteria from culture 1, and its growth kinetics also determined (Fig. 1). The inoculum was adjusted to obtain about 10^3 cfu ml^{-1} at T_{init}.

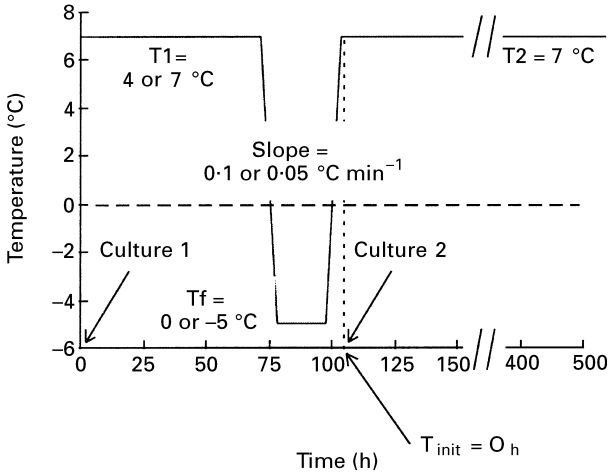

Fig. 1 Temperature profile applied during the chilling/freezing pre-treatment process

In addition, three controls were performed. The first was done with TSBYE media inoculated from the culture at 37 °C and incubated directly at 7 °C. The second and third were carried out with media inoculated at temperature T1 of 4 or 7 °C for 6 or 4 d, and then incubated at 7 °C without the cooling and freezing treatments. The controls were carried out in TSBYE culture medium at pH 6·2 and 7·2.

All cultures were incubated with shaking (50 rev min^{-1}) in a Ratek water-bath (Model SWB20 with a precision of ± 0·1 °C, Ratek Instruments, Victoria, Australia). Biomass was measured by counting cell colonies on Oxford Listeria Selective Agar (CM 856 supplemented with SR 140, Oxoid) up to 16 d. Duplicate growth curves were generated for each experimental treatment.

Curve fitting

From the factorial design, 55 growth curves (2^5 treatments, two replicates per treatment, nine randomly missing data) and 22 from the controls (six for the first control corresponding to two pH levels and three replicates; eight for the second and third controls corresponding to pH 6·2 or 7·2, culture 1 or 2, and two replicates) were obtained. The change in natural logarithm of biomass (cfu ml^{-1}) *vs* time was described with the sigmoidal curve proposed by Gompertz and re-parameterized by Zwietering *et al.* (1990), and also with the logistic curve modified by Baranyi (Baranyi *et al.* 1993; Kalathenos *et al.* 1995). Inoculum size ln(No), lag phase L, specific growth rate μ_{max}, and maximal population density $\ln(N_{max})$, were calculated. Non-linear regression was computed using S-plus software (AT & T Bell Laboratories, Murray Hill, NJ, USA), with the parameters of the regression estimated by the maximum likelihood method.

Statistical analysis

Analysis of variance was computed with the General Linear Model procedure of SAS software (SAS Institute Inc., Cary, NC, USA). Boxplot diagrams (Tukey 1990) were performed with S-plus software (AT & T Bell Laboratories).

RESULTS AND DISCUSSION

Growth curve modelling

Kinetics were fitted by the Gompertz and Baranyi functions. Concerning the specific growth rate parameter, μ_{max}, a difference between the two models was observed (Fig. 2). The

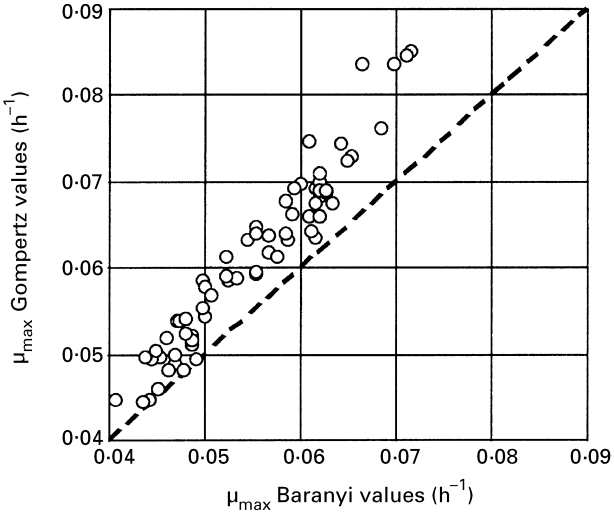

Fig. 2 Comparison between the specific growth rate μ_{max}, estimated by the Gompertz and Baranyi equations

Gompertz model systematically over-estimated μ_{max}. The Gompertz-estimated growth rate was used to predict the increase in ln(biomass) *vs* time curve, and the resulting plot is shown as Fig. 3. Over-estimation of μ_{max} by the Gompertz function has been reported previously (Whiting and Cygnarowicz-Provost 1992; Ross 1993; Dalgaard *et al.* 1994), while the Baranyi model has been employed more recently and has given satisfactory results (McClure *et al.* 1997; Membré and Kubaczka 1998). Consequently, in this paper, the following parameter estimates are derived from the Baranyi equation.

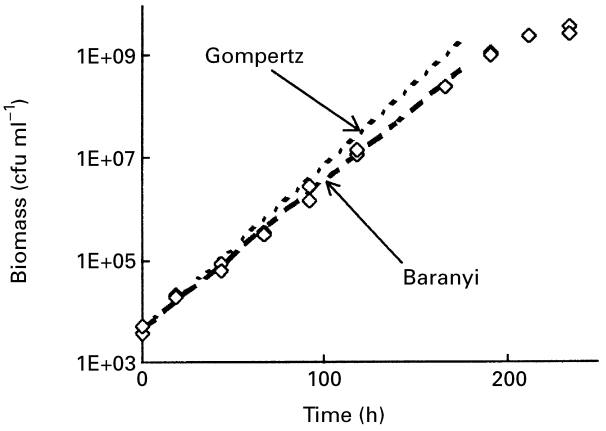

Fig. 3 Illustration of the effect of the discrepancy between the specific growth rate estimation given by Gompertz and Baranyi functions. Both μ_{max} estimates were used to predict the increase in microbial numbers over time, based on the observed initial count and assuming exponential growth. Observations (◇)

Chilling process effects on Lag phase

Whatever the combination of experimental conditions in the chilling/freezing process, no lag phase was ever observed, neither with culture 1 nor culture 2 conditions. Culture 1 corresponded to 4- or 6-day-old cultures, having undergone sub-optimal treatments, whereas culture 2 corresponded to bacteria provided from culture 1 and inoculated in fresh media at T_{init} (Fig. 1). In both cases, after the preliminary stage at the storage temperature of T1 (4 or 7 °C), bacteria seemed adapted to the temperature of 7 °C, and were able to grow without a lag period. Moreover, the chilling/freezing treatment did not induce a lag phase response. We interpret this to indicate that those temperatures did not greatly affect the physiology of the organism.

On the other hand, with the control flasks that were inoculated from culture at 37 °C and incubated directly at 7 °C, a lag period of approximately 31 h (data not shown) was observed. This observation accords with those of Walker *et al.* (1990) and Buchanan and Klawitter (1991).

Chilling process and pH effects on specific growth rate

The specific growth rate, μ_{max}, at 7 °C changed as a function of pH and sub-optimal temperature pre-treatment. Decreasing pH from 7·2 to 6·2 led to a reduction in μ_{max} of 23% (Fig. 4). The pH value of 6·2 was chosen because it corresponds to a wide variety of foods such as minced beef, cheese, egg yolk, smoked salmon and vegetables. Our results confirm the preservative effect of acidity in foods.

More surprising is the effect of the pre-treatment temperature on μ_{max}. In fact, μ_{max} values were higher when a pre-incubation temperature of 4 °C was applied than with a pre-incubation temperature of 7 °C (Fig. 4). This slight difference was confirmed by analysis of variance (Table 1). Pre-incubation temperatures and pH affected μ_{max} ($P < 0{\cdot}05$) while the cooling and freezing periods did not have a significant influence. Likewise, no difference between culture 1 and culture 2 specific growth rates was observed. An effect of the pre-incubation temperature T1 was noticed with the 2^5-experimental design and with the control data set. In the latter case, sub-culture was performed at 4 or 7 °C for 6 or 4 d, respectively, and cultures were then incubated at 7 °C without cooling/freezing stages. In Fig. 5, growth curves obtained at pH 7·2 and 6·2 are presented; μ_{max} estimates and 95% confidence interval values are reported in Table 2. The minimal growth temperature of the *L. monocytogenes* strain

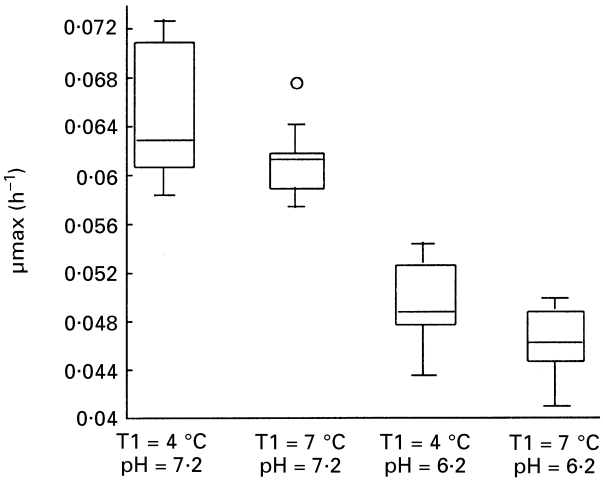

Fig. 4 Boxplot diagram of the specific growth rate response studied in a 2^5-factorial experimental design with two replicates (9 missing data). The box contains 50% of the data, the horizontal line within the box corresponds to the median value. The vertical lines are an index of the data variability

Table 1 Analysis of variance of the specific growth rate response studied in a 2^5-factorial experimental design with two replicates (9 missing data)

Source	DF	Sum of squares	F Value	Pr > F
T1	1	0·00019603	15·38	0·0003
PH	1	0·00301999	236·96	0·0001
T1*PH	1	0·00000014	0·01	0·9184
Model	3	0·00318895	83·41	0·0001
Error	51	0·00064999		

The factors Tf, Slope, Culture 1 or 2, being insignificant, were not kept in the final ANOVA.

Table 2 Effect of the pre-incubation temperature and pH on the specific growth rate, μ_{max}, for the control curve kinetics (Fig. 5)

		μ_{max} (h^{-1}) at 7 °C		
		estimate	lower limit	upper limit
pH = 7·2	T1 = 4 °C	0·067	0·064	0·069
	T1 = 7 °C	0·058	0·056	0·060
pH = 6·2	T1 = 4 °C	0·052	0·049	0·055
	T1 = 7 °C	0·046	0·043	0·048

Estimated values and 95% confidence interval limits.

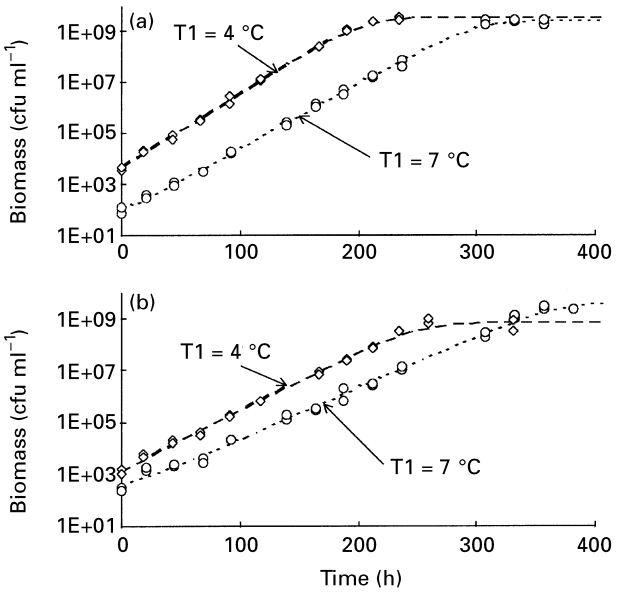

Fig. 5 Effect of the pre-incubation temperature on the specific growth rate μ_{max}, at 7 °C, for the control kinetics (a) pH = 7·2; (b) pH = 6·2

L5/22, isolated from smoked salmon, has been determined as 3 °C (Soontranon 1998). The T1 level of 4 °C, just 1 °C above the minimal growth temperature, was chosen because it corresponded to the temperature in refrigerated rooms. This temperature seems to play an activator role in the growth phase at 7 °C.

In the literature, no influence of temperature history on the specific growth rate was observed by Buchanan and Klawitter (1991) when a high temperature pre-treatment (30 °C) was performed compared with a 4 °C pre-treatment. Conversely, Walker *et al.* (1990) reported the same tendency as in this study, i.e. for *L. monocytogenes* cultured at 7·5 °C in chicken broth or UHT milk, μ_{max} values were higher when the preculture was at 4 °C than at 30 °C.

This study has demonstrated that *L. monocytogenes*, a psychrotrophic organism, can be adapted sufficiently at refrigerated temperatures to grow instantaneously at the time of contamination. In addition, it has demonstrated a slight temperature history effect on the growth rate of *L. monocytogenes*. It is concluded that to mimic the process of industrial contamination of refrigeration products, predictive microbiology studies with *L. monocytogenes* should be performed with bacteria sub-cultured at low temperatures.

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