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Predictive model of the effect of CO_2, pH, temperature and NaCl on the growth of *Listeria monocytogenes*

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Abstract

The growth responses of *L. monocytogenes* as affected by CO_2 concentration (0–100% v/v, balance nitrogen), NaCl concentration (0.5–8.0% w/v), pH (4.5–7.0) and temperature (4–20°C) were studied in laboratory medium. Growth curves were fitted using the model of Baranyi and Roberts, and specific growth rates derived from the curve fit were modelled. Predictions for specific growth rate, doubling time and time to a 1000-fold increase could be made for any combination of conditions within the matrix. Predictions of growth from the model were compared with published data and this showed the model to be suitable for predicting growth of *L. monocytogenes* in a range of foods packaged under a modified atmosphere.

Keywords: *Listeria monocytogenes*; Predictive modelling; Food safety; CO_2; NaCl; pH; Temperature

1. Introduction

Listeria monocytogenes is an important foodborne pathogen that causes listeriosis. The disease has a high mortality rate (ca. 30%), particularly for pregnant women and other susceptible groups. Outbreaks of listeriosis have been associated with milk (Fleming et al., 1995), cheese (James et al., 1985; Bille and Glauser, 1988; Goulet et al., 1995), vegetables and salads (Schlech et al., 1983) and meat products (Anon., 1993; Jacquet et al., 1995). *L. monocytogenes* is a facultative anaerobe capable of growth at temperatures as low as −1.5°C (Hudson et al., 1994).

Modified atmosphere packaging (MAP) is used to extend the shelf life of a food product, by maintaining product quality and appearance and by preventing growth of aerobic spoilage microorganisms. Gases used in MAP include: O_2, at low concentrations, to maintain the redness of meat, to allow the continued respiration of fruit and vegetables and to avoid anaerobic conditions for white fish; N_2, primarily to replace O_2 thereby restricting growth of aerobic spoilage organisms; CO_2, at concentrations of 25–100% to inhibit growth of spoilage microorganisms (Parry, 1993; Rönner, 1994). Most MAP

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foods are stored at refrigeration temperatures, therefore *L. monocytogenes* is of particular concern because of its ability to grow in low O_2 or anaerobic conditions and its ability to grow at low temperatures. It has also been suggested that *L. monocytogenes* may be more tolerant of adverse conditions when grown in an O_2-restricted environment (Buchanan and Klawitter, 1990).

The inhibitory effect of elevated CO_2 concentrations on growth and metabolism of microorganisms has been reviewed (Dixon and Kell, 1989). Growth of *L. monocytogenes* on turkey roll slices at 4 and 10°C was prevented by 70% CO_2 (Farber and Daley, 1994), although this concentration did not prevent growth on cabbage at 5°C (Kallander et al., 1991). The presence of 80% CO_2 prevented growth of *L. monocytogenes* on frankfurters at 4, 7 and 10°C (Krämer and Baumgart, 1992) and on catfish fillets at 2°C (Silva and White, 1994), but did not prevent growth on chicken nuggets or chicken loaf at 3, 7 and 11°C (Ingham et al., 1990; Marshall et al., 1991, 1992). Although often inhibitory, there are some reports of growth of *L. monocytogenes* in foods packed under 100% CO_2 (Hart et al., 1991; Hudson et al., 1994). Growth on chicken breast occurred at 15°C but not 6°C (Hart et al., 1991), and growth on beef occurred at 3°C, but not at −1.5°C under 100% CO_2. However, growth did occur at −1.5°C under vacuum (Hudson et al., 1994). The effect of CO_2 on growth of *L. monocytogenes* on meat has been reviewed by García de Fernando et al. (1995).

To prevent growth of *L. monocytogenes* in MAP foods, it is essential to have a thorough understanding of the effect of environmental factors on growth. An important step in quantifying the effect of environmental factors has been the development of mathematical models to predict the effect of a combination of factors on growth in foods. Predictive modelling allows interpolation between data points, and so it is possible to predict responses for conditions not tested. This increases the value of generated data. The range of the model is limited by the conditions under which growth was observed experimentally and predictions should not be made outside these boundaries. This area, the interpolation region, is defined by Baranyi et al. (1996). Models can be used to predict the effects of combinations of preservative factors on growth in many foods. This can be particularly useful in ensuring the safety of new or modified food formulations. A number of models of the effect of preservative factors on the rate of growth of *L. monocytogenes* have been published (Buchanan and Phillips, 1990; Cole et al., 1990; Duh and Schaffner, 1993; Wijtzes et al., 1993; Bennik et al., 1995; George et al., 1996; Farber et al., 1996). Buchanan and Phillips (1990) published models that predicted the effect of aerobic or anaerobic (nitrogen) atmosphere on growth of *L. monocytogenes*. The model of Bennik et al. (1995) included CO_2, in combination with either 1.5% or 21% O_2, as a controlling factor. Farber et al. (1996) examined the effect of a range of CO_2 concentrations from 10–90%, balance N_2, combined with temperature and pH.

The aim of this study was to develop a mathematical model of the combined effect of CO_2, temperature, pH and NaCl concentration on the growth of *L. monocytogenes*. Oxygen was excluded to determine the effect of CO_2 alone, and the balance was N_2. Growth was in laboratory media so that conditions other than those under test were optimal. The pH was adjusted independently of CO_2 concentration. The model was validated by comparing predictions for growth with the growth data used to develop the model and it was determined whether predictions from the model provided a good description of growth of *L. monocytogenes* in foods.

2. Materials and methods

2.1. Strain

L. monocytogenes F6861 (serotype 4b) was isolated from Mexican-style cheese involved in a listeriosis outbreak in USA in 1985 (James et al., 1985). It was originally obtained from Dr. R. Weaver, CDC, Atlanta, USA and maintained freeze-dried and on tryptose agar slopes (TA; Difco) held at 4°C.

2.2. Media

Tryptone soya broth (TSB, Oxoid CM129) with 3 g/l yeast extract (Oxoid L21) and 10 g/l additional glucose (TSYGB) (George and Lund, 1992), with up to 8.0% NaCl (total, w/v) was bubbled through with the test gas mixture until the pH stabilised and the

medium was assumed to be saturated with gas. A standard time of 45 min was shown to be adequate and was used for all media. The pH was then adjusted with 1 or 5 M KOH or 1 M HCl to the desired value. When the pH was stable, 100 ml aliquots of the medium were pipetted under gas into 125 ml vials (Pierce and Warriner, Chester, UK) whilst flushing out with the gas mixture. The vials were closed with butyl rubber septa (Wheaton, Millville, NJ, USA) and aluminium seals (The West Co. (UK) Ltd., St. Austell, UK), and autoclaved. Media were equilibrated at the intended incubation temperature for each experiment before inoculation.

2.3. Gas mixture and composition of headspace

Gas mixtures included a range of CO_2 concentrations from 0 to 100% (v/v), the balance being N_2. They were obtained as prepared mixtures from BOC Special Gases (London, UK) and were supplied with a certificate of analysis. The gas composition of the head space of uninoculated vials was measured by gas chromatography after storage at room temperature for a minimum of two weeks. The samples were analysed on a stainless steel column 0.6 m × 4 mm i.d. packed with 80–100 mesh Poropak Q (Waters Associates) joined in series with a second stainless steel column 2.1 m x 4 mm packed with 40–60 Molecular sieve 5A (BDH). The columns were operated at 80°C, with helium as the carrier gas (flow rate 40 cm^3/min). The composition was measured using a gas chromatogram fitted with a thermal conductivity detector (Pye Unicam PU 4500) connected to a computing integrator (Pye Unicam PU 4811) that recorded and analysed the peaks. The CO_2 concentration was always within 5% of the target concentration demonstrating that the headspace composition was not greatly affected by autoclaving or storage.

2.4. Experimental procedure

Inocula were preconditioned for temperature as described previously (George et al., 1996). Cultures were diluted in PSDF (ICMSF, 1978) and 1 ml was injected into each test vial to give 10^2–10^3 cells/ml. Vials containing the test medium equilibrated to temperature were inoculated, mixed and samples were removed by syringe, to determine the initial inoculum concentration and pH. The pH was measured by transferring a sample into a tube flushed out with the test gas mixture and maintaining a headspace of the gas mixture whilst the reading was taken. This avoided a pH change due to loss of CO_2. Test vials were incubated in waterbaths to maintain the required temperature which was monitored daily. Samples were taken at appropriate intervals to determine viable counts by dilution in PSDF and plating 100 μl volumes onto triplicate TA plates. Whilst sampling, care was taken to ensure that the headspace was replaced with the test gas mixture via a sterile needle (0.45 × 10 mm) to maintain a constant CO_2 concentration until growth commenced. Plates were incubated at 35°C for 48 h. The mean of three plate counts was used to calculate the number of colony forming units/ml. A minimum of 10 samples were taken per growth curve.

2.5. Experimental design

The effect of 115 combinations of factors was tested. These included:

- pH: 4.5–7.0
- Temperature: 4, 8, 12, 20°C
- NaCl: 0.5, 2.0, 4.0, 6.0, 8.0% w/v
- CO_2: 0, 25, 50, 60, 80, 100% v/v, balance nitrogen

Growth was not observed in 50 days in 16 sets of conditions tested. A test resulting in no growth cannot be modelled using this technique, leaving 99 growth curves for use in model construction (Fig. 1).

2.6. Modelling

The first stage was to fit a sigmoid function to the growth data. The method used was that described by Baranyi and Roberts (1994), which derives the parameters specific growth rate (μ), h_0, the product of μ and lag time, and y_{max}, the maximum population density. The second stage modelled μ as a function of the controlling factors temperature, pH, NaCl concentration and CO_2 concentration, while h_0 and y_{max} were taken as constant. A quadratic response surface was used, which was represented by a polynomial of the form:

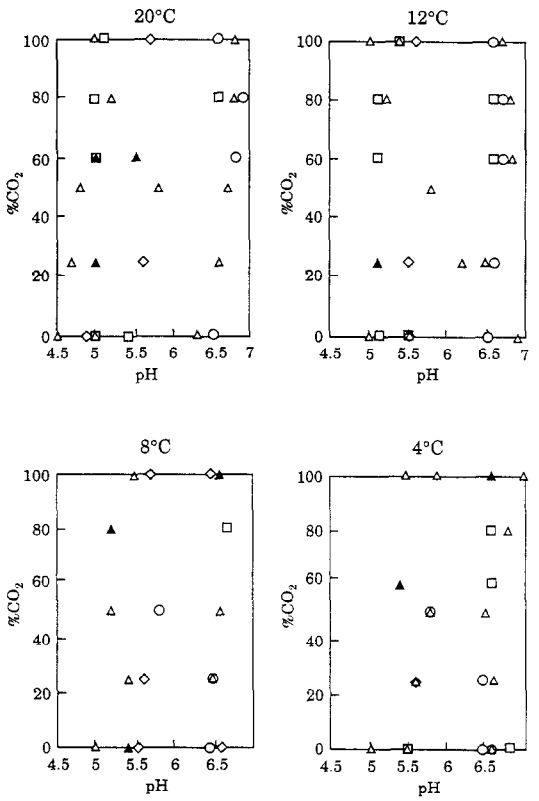

Fig. 1. Experimental plan showing combinations of temperature, pH, CO_2 concentrations and NaCl concentrations used in the model: △, 0.5% NaCl; ▲, 2% NaCl; □, 4% NaCl; ◇, 6% NaCl; ○, 8% NaCl.

$$\ln \mu = c_1 + c_2T + c_3P + c_4S + c_5G + c_6TP + c_7TS + c_8TG + c_9PS + c_{10}PG + c_{11}SG + c_{12}T^2 + c_{13}P^2 + c_{14}S^2 + c_{15}G^2.$$

Where $\ln \mu$ is the natural logarithm of the specific growth rate, T, P, S and G are temperature (°C), pH, NaCl concentration (% w/v) and CO_2 concentration (% v/v) respectively, and c_1–c_{15} are the coefficients to be estimated. The natural logarithm, as a link function, was used to damp the variance of μ. To determine whether predictions from the model were reliable as a description of growth in foods, predictions were compared with doubling times and times to a 1000-fold increase (t_{1000}) in cell numbers reported in the literature. Doubling time, t_D, was calculated from μ; $t_D = \frac{\ln 2}{\mu}$. For time to 1000-fold increase, $\ln h_0$ was taken as constant and t_{1000} was calculated as;

$$t_{1000} = \frac{(\ln 1000 + h_0)}{\mu}.$$

3. Results

When temperature, pH and NaCl were optimal an inhibitory effect was observed when CO_2 was present at 80% or higher. When these conditions were suboptimal, 25% CO_2 restricted growth. At 4°C, pH 6.5, 80% CO_2, growth was observed in the presence of 4.0% NaCl but not 8.0% NaCl. At 4°C, pH 6.5, 100% CO_2, growth was observed in the presence of 2.0% NaCl but not 4.0% NaCl demonstrating that the sensitivity of *L. monocytogenes* to NaCl increased with increasing CO_2 concentrations. Growth was observed at 20°C, pH 4.5, 0.5% NaCl in the absence of CO_2 but not in the presence of 50% CO_2. Examples of the effect of CO_2 concentration on growth of *L. monocytogenes* are shown in Figs. 2 and 3. At 12°C, pH 6.6–6.7, 8.0% NaCl, the time to a 1000-fold increase was less than 4 d with 25% CO_2, 13.7 d with 60% CO_2, 20.8 d with 80% CO_2 and 33.3 d with 100% CO_2 (Fig. 2). With 0% CO_2 the time for a 1000-fold increase at 4°C, pH 5.5–5.6, 0.5% NaCl was approximately 14.6 d (Fig. 3). Under 25% CO_2 a 1000-fold increase was achieved in 22.0

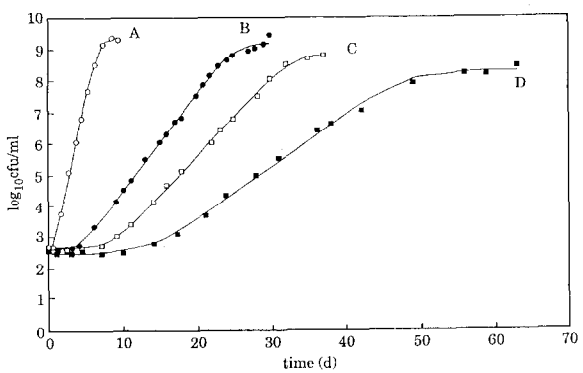

Fig. 2. Effect of CO_2 concentration at 12°C, pH 6.6–6.7 and 8% NaCl on growth of *Listeria monocytogenes*.

A = 25%; B = 60%; C = 80%; D = 100%. Points are actual counts and growth curves were fitted using the model of Baranyi and Roberts (1994).

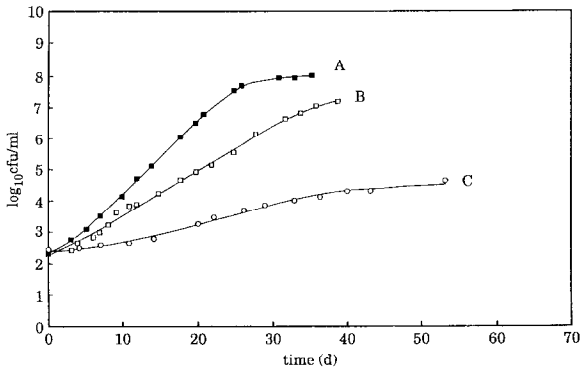

Fig. 3. Effect of CO_2 concentration at 4°C, pH 5.5–5.6 and 0.5% NaCl on growth of *Listeria monocytogenes*.

A = 0%; B = 25%; C = 100%. Points are actual counts and growth curves were fitted using the model of Baranyi and Roberts (1994).

d, but under 100% CO_2 *L. monocytogenes* achieved only a 10-fold increase in the same time.

Growth curves fitted (stage 1) with the model of Baranyi and Roberts (1994) provided a good description of the experimental growth data (Figs. 2 and 3) and of the inhibitory effect of an atmosphere containing CO_2 on growth of *L. monocytogenes*. The second stage of modelling, fitting a quadratic response surface to the quantities derived in stage 1 showed a good fit; the percentage variance accounted for by the model was 92% and the value of the RMSE (residual mean square error) for $\ln \mu$ was 0.29. The RMSE provides a measure of the goodness of fit of a model to the data used to produce it. It can be used to assess the performance of the model as follows:

The RMSE estimates that

$$\ln \mu(\text{observed}) - \ln \mu(\text{predicted}) \approx 0.29$$

If this equation is transformed into multiplicative error estimation we obtain the following:

$$\ln (\mu(\text{observed})/\mu(\text{predicted})) \approx 0.29$$

$$(\mu(\text{observed}) - \mu(\text{predicted}))/\mu(\text{predicted})$$
$$\approx \exp(0.29) - 1 \approx 0.34$$

This expresses that, in the interpolation region, the error of the prediction is about 34% of the prediction itself. Note that the same is true for the doubling time since that is equal to $t_D = \frac{\ln 2}{\mu}$.

The fitted specific growth rates (from the second stage of modelling) compare well with those measured (from the first stage of modelling) (Table 1, Fig. 4). The model was used to derive doubling

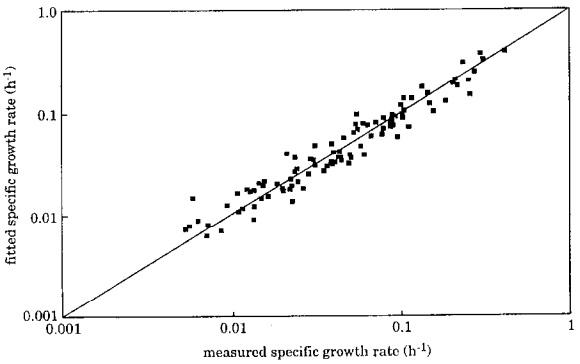

Fig. 4. Comparison of measured specific growth rates with fitted specific growth rates from the model for *Listeria monocytogenes*.

Table 1
Comparison of measured specific growth rates (μ) with fitted specific growth rates from the model for *Listeria monocytogenes*

Temp (°C)	pH	NaCl (%w/v)	CO_2 (%v/v)	Specific growth rate (h^{-1})	
				Measured[a]	Fitted[b]
20	6.8	0.5	100	0.215	0.214
20	5.0	4.0	0	0.107	0.108
12	6.7	8.0	80	0.024	0.027
12	5.0	0.5	100	0.025	0.021
8	5.2	0.5	50	0.023	0.023
8	5.8	8.0	50	0.032	0.031
4	5.6	6.0	25	0.017	0.015
4	6.6	4.0	60	0.011	0.011

[a] Specific growth rate (μ) generated in the first stage of modelling.
[b] Specific growth rate (μ) fitted in the second stage of modelling.

Table 2
Comparison of observed and predicted growth data for *Listeria monocytogenes*

Ref[a]	Substrate	Temp. (°C)	pH	NaCl (%w/v)	Atmosphere (%v/v $CO_2/N_2/O_2$)	Doubling time (h)		Time to 1000-fold increase (d)		Strain	Estimated variables
						observed	predicted	observed	predicted		
1	Raw asparagus	15	5.9	0.5	6/79/15	7.2	3.3	3.3	1.9	LCDC 81-861	pH, NaCl
	Raw broccoli	15	6.5	0.5	10/79/11	11.1	2.9	6.5	1.6	Scott A	pH, NaCl
	Raw cauliflower	4	6.4	0.5	3/79/18	54.2	26.2	–		Scott A	pH, NaCl
2	Lettuce	10	6.2	0.5	0/97/3	10.8	6.3	10	3.5	LCDC 81-861	pH, NaCl
3	Cottage cheese	7	5.1	3.8	35/52/13	243	43.5		–	cocktail	NaCl
4	Raw beef	10	6.0	0.6	100/0/0	16.5	16.1	6.5	9.0	ATCC 19111	NaCl
	Raw beef	5	6.0	0.6	vacuum-pack	26.5	19.2	14	10.7	cocktail	NaCl
5	Corned beef	15	6.3	3.2	vacuum-pack	4.5	3.8	–	–	Murray B	–
	Ham	5	6.6	3.2	vacuum-pack	33	23.9	–	–	Murray B	–
6	Raw chicken	15	5.8	0.8	30/56/14	8.0	3.9	–	–	NCTC 11994	pH, NaCl
	Raw chicken	15	5.8	0.8	30/70/0	5.9	3.9	–	–	NCTC 11994	pH, NaCl
	Raw chicken	15	5.8	0.8	100/0/0	12	6.7	–	–	NCTC 11994	pH, NaCl
7	Cold-smoked salmon	10	6.1	3.0	vacuum-pack	8.4	7.9	4.2	4.4	cocktail	pH, NaCl
	Cold-smoked salmon	5	6.1	3.0	vacuum-pack	19.3	21.6	20.8	12.0	cocktail	pH, NaCl
8	Cooked beef	5	5.8	0.5	vacuum-pack	22.6	21.9	13.7	12.2	cocktail	pH, NaCl
	Cooked beef	10	5.8	0.5	vacuum-pack	8.5	7.8	4.8	4.3	cocktail	pH, NaCl
9	Raw pork	4	5.8	0.6	vacuum-pack	169	28.0	–	–	Scott A	NaCl
	Raw pork	4	5.8	0.6	20/80/0	112	31.7	–	–	Scott A	NaCl
	Raw pork	4	5.8	0.6	40/60/0	126	37.8	–	–	Scott A	NaCl
10	Chicken nuggets	11	6.3	0.5	76/13.3/10.7	11	8.8	4.5	4.9	Scott A	pH, NaCl
	Chicken nuggets	11	6.3	0.5	80/20/0	11	9.2	4.5	5.1	Scott A	pH, NaCl

[a] References: 1. Berrang et al., 1989; 2. Beuchat and Brackett, 1990; 3. Chen and Hotchkiss, 1993; 4. Gill and Reichel, 1989; 5. Grau and Vanderlinde, 1992; 6. Hart et al., 1991; 7. Hudson and Mott, 1993a; 8. Hudson and Mott, 1993b; Manu-tawiah et al., 1993; 10. Marshall et al., 1992.

times and time to a 1000-fold increase in cell numbers and compared with growth data from the literature (Table 2, Figs. 5 and 6).

4. Discussion

The extent of the inhibitory effect of CO_2 on the doubling time of *L. monocytogenes* can be predicted from the model. For example, at 8°C, pH 6.5 and 0.5% NaCl predicted doubling times were 9.1 h with 0% CO_2, 10.8 h with 30% CO_2, 12.9 h with 50% CO_2, 16.1 h with 70% CO_2 and 21.2 h with 90% CO_2. These doubling times compare well with those predicted by the models of Bennik et al. (1995) and Farber et al. (1996). The model can also be used to predict the combined effect of CO_2 concentration and temperature on time to a 1000-fold increase (Fig. 7). At pH 6.0 and 0.5% NaCl predicted times to a 1000-fold increase with 0% CO_2 (100% N_2) and 100% CO_2 were; 1.0 d and 1.5 d at 20°C, 2.7 d and 5.7 d at 12°C, 5.5 d and 14.3 d at 8°C, 8.5 d and 24.0 d at 6°C, and 13.5 d and 42 d at 4°C (Fig. 7). Elevated CO_2 concentrations were, therefore, particularly inhibitory to *L. monocytogenes* at refrigeration temperatures. This may be at least partially attributed to the greater solubility of CO_2 at lower temperatures (Dawson et al., 1986).

The measured specific growth rates and the fitted specific growth rates derived from the overall model were similar, indicating that the model provides a good description of the data used to generate it (Fig. 4). The reliability of the model for predicting growth in foods was tested by comparing predictions with observations of growth in foods reported in the literature. A total of 94 data sets from 27 references were used. Fig. 5 shows a comparison of predicted doubling time with doubling times observed in foods. Most points fall close to or below the line of unity, indicating that the model predicts doubling times similar to or slightly faster than those reported in the published studies. One of the points from the

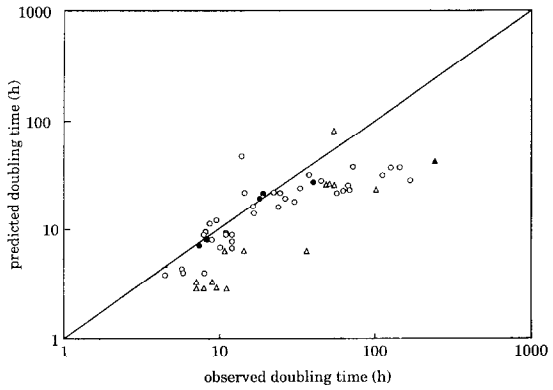

Fig. 5. Comparison of doubling times from observed (published) data with predictions from the model for *Listeria monocytogenes*.

Data taken from experiments on meat and poultry (○); fish(●); vegetables (△); and dairy products (▲). References include those used in Table 2 and the following: Barbosa et al., 1995 Brunskill et al., 1991; Fang and Lin, 1994; Glass and Doyle, 1989; Grau and Vanderlinde, 1990; Hudson and Avery, 1993; Ingham et al., 1990; Kallander et al., 1991; Mano et al., 1995; Marshall et al., 1991; Peterson et al., 1993; Rørvik et al., 1991; Sheridan et al., 1995; Wederquist et al., 1994; Wimpfheimer et al., 1990; Zeitoun and Debevere, 1991.

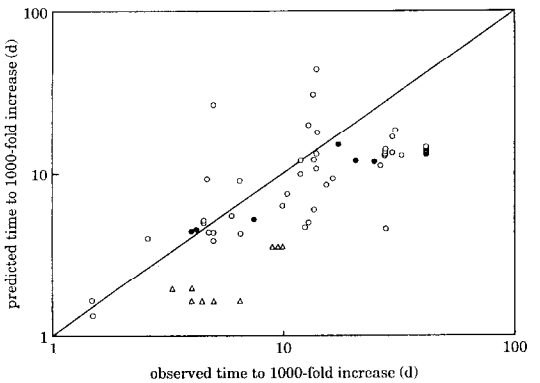

Fig. 6. Comparison of time to a 1000-fold increase from observed (published) data with predictions from the model for *Listeria monocytogenes*. Symbols and references as in Fig. 5.

literature, obtained in meat incubated at 7°C, fell well above the line of unity (Marshall et al, 1991). In this study, the modified atmosphere contained 10.7% O_2, the presence of which increases the growth rate of *L. monocytogenes* (George and Lund, 1992). When the meat was stored at 11°C, the observed doubling time was similar to that predicted. In the same report, growth in a modified atmosphere lacking O_2 was slower than predicted by the model. The

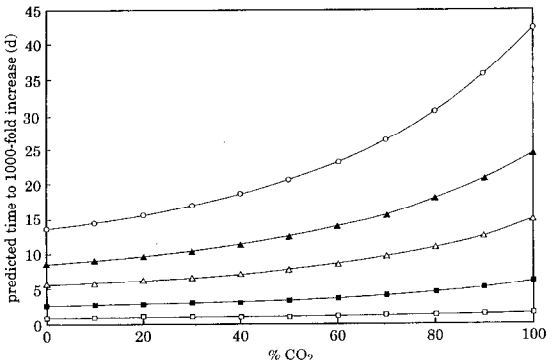

Fig. 7. Effect of temperature and CO_2 concentration on predicted time to a 1000-fold increase of *Listeria monocytogenes* at pH 6.0 and 0.5% NaCl. □, 20°C; ■, 12°C; △, 8°C; ▲, 6°C; ○, 4°C.

predicted time to a 1000-fold increase, taking account of the lag phase, was also compared with observations from the literature (Fig. 6). Most of the points are below the line of unity, demonstrating that our model provides a good description of the effect of a combination of environmental factors, including modified atmosphere, on time to a 1000-fold increase. Points above the line of unity were from Fang and Lin (1994), Ingham et al. (1990), Wimpfheimer et al. (1990) and Zeitoun and Debevere (1991). In all of these studies O_2 was included in the modified atmosphere, typically at 5–10%. Interestingly, when O_2 was omitted, predictions from our model were comparable with the results reported by these workers. In the case of some of the published data in which O_2 was included in the modified atmosphere, growth was slower than that predicted by our model, typically in foods which may not be nutritionally optimal for *L. monocytogenes* e.g. vegetables. Examples are shown in Table 2.

These comparisons demonstrate that the model can be used to predict growth of strains of *L. monocytogenes* in a wide range of MAP foods. The presence of O_2 in the atmosphere increases the rate of growth of *L. monocytogenes* compared with when O_2 is excluded and growth may be faster than predicted by this model. Our model can be used to predict growth within the range of 0–100% CO_2, 4°–20°C, pH 4.5–7.0 and 0.5–8.0% NaCl and complements the models of Bennik et al. (1995) that described the effect of 0–50% CO_2, at 8°C, and pH 6.7–7.2, and includes two concentrations of O_2, 1.5% and 21%,

and Farber et al. (1996) that described the effect of 10–90% CO_2, at 4, 7 and 10°C and pH 5.5 and 6.5.

Our model can be used as a rapid and reliable guide to manufacturers of MAP foods as to how changes in the composition of a modified atmosphere, pH, NaCl and storage temperature affect the ability of a food to support growth of *L. monocytogenes*. The model can thus contribute to the safety of a range of foods and reduce the amount of expensive and time-consuming challenge testing needed.

Acknowledgments

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