Food Microbiology, 1995, **12**, 509–519

ORIGINAL ARTICLE

Growth of psychrotrophic foodborne pathogens in a solid surface model system under the influence of carbon dioxide and oxygen

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A solid surface model system was developed to study the effect of gas atmosphere composition on the growth of Aeromonas hydrophila, Yersinia enterocolitica, Listeria monocytogenes, *and a cold tolerant strain of* Bacillus cereus. *The organisms were incubated on an agar surface at 8 °C under either 1·5 or 21% O_2, combined with 0, 5, 20 or 50% CO_2. The remainder of each atmosphere was made up to 100% with N_2. Growth was evaluated on the basis of three parameters, namely maximum specific growth rate, maximum population density and lag time. These parameters were derived from growth data by fitting with a modified Gompertz equation. In all instances the maximum specific growth rate decreased significantly with increasing CO_2 concentration. A strong reduction in the maximum population density was noted only for* B. cereus *at the highest level of CO_2. The O_2 concentrations tested did not significantly affect maximum specific growth rates nor maximum population densities in any case. Prolonged lag times were observed only for* Y. enterocolitica *under 50% CO_2/21% O_2/29% N_2.*

The results indicate that the model system may be a suitable means of estimating the growth of bacteria on minimally processed produce, packaged under modified atmospheres. Extrapolation of our results to modified atmosphere packaged (MAP) fruits and vegetables using typical O_2 concentrations of 1–5% and CO_2 concentrations of 5–10%, suggest that growth of the above pathogens may occur at 8 °C, thereby imposing a safety hazard for these products.

Received:
7 September 1995

Introduction

Modified atmosphere packaging (MAP) in combination with refrigeration is increasingly used to extend the storage life of minimally processed fresh (MPF) fruits and vegetables. The low O_2 concentrations employed in MAP (1–5%) have been found to reduce oxidative processes as well as produce respiration and ripening (Kader 1980, Kader et al. 1989, Gill and Molin 1993). The relatively high CO_2 concentrations prevailing (5–10%) also suppress respiration of produce, but more importantly seem to inhibit a number of common food spoilage micro-organisms (Daniels et al. 1985, Farber 1991).

The microbiology of MPF fruits and veg-

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0740-0020/95/060509+11 $12.00/0

etables has been extensively reviewed (Nguyen-The and Carlin 1994). Hazardous situations may arise with products that are organoleptically acceptable. Such products may contain relatively low numbers of spoilage organisms, but they may harbour cold tolerant pathogens (Hintlian and Hotchkiss 1986, Nguyen-The and Carlin 1994). Fortunately, outbreaks of foodborne diseases with MAP vegetables are scarce (Sizmur and Walker 1988). This potential risk may however increase, since consumption of these products has risen due to recent trends towards healthy and fresh foods.

Of particular concern is the growth and survival of psychrotrophic pathogens such as *Aeromonas hydrophila, Yersinia enterocolitica* and *Listeria monocytogenes* on MAP foods stored at refrigeration temperatures (Berrang et al. 1989, Beuchat and Brackett 1990, Kallander et al. 1991, Toan and Beutling 1992). A number of studies have indicated that MAP may select for such psychrotrophic pathogens (Hintlian and Hotchkiss 1986, Brackett 1994).

The presence of O_2 is required for optimal storage conditions of MPF fruits and vegetables (Kader 1980), since anoxic conditions cause anaerobic metabolism of the product, thereby leading to the formation of off-odours. While minimum O_2 concentrations (1–2%) prevent the growth of obligatory anaerobes such as *Clostridium botulinum*, growth of obligatory aerobes is not inhibited (Clark and Burki 1972, Enfors and Molin 1980).

Typical CO_2 concentrations for MAP fruits and vegetables are in the range of 5–10% (Kader 1980). The antimicrobial properties of CO_2 vary depending on the organism, age and load of the initial bacterial population, gas concentration, temperature of incubation, and type of food (Ogrydziak and Brown 1982, Dixon and Kell 1989, Hudson et al. 1994). Gram negative bacteria, particularly aerobes such as pseudomonads, are relatively sensitive to CO_2, whereas other bacteria, e.g. lactic acid bacteria, are quite resistant (Enfors et al. 1979, Enfors and Molin 1980). The mechanism by which CO_2 exerts its antimicrobial activity is unclear. The general effects of CO_2 are related to reduction of the intra- and extracellular pH and interference with cellular metabolism (Dixon and Kell 1989).

Since many studies on the antimicrobial effects of CO_2 are performed with packaged products, it is difficult to ascertain if the effects are due to the gas atmosphere, variations in food compositions, presence of endogenous micro-organisms, or combinations of these and other factors. In addition, CO_2 concentrations are not always stable as a result of gas diffusion through the packaging material, the respiratory activity of the product inside the package or dissolving and binding of CO_2 in the food matrix (Hintlian and Hotchkiss 1986, Zagory and Kader 1988, Gill 1988). To avoid these variations, studies have been undertaken to determine the effect of CO_2 on the growth of pure bacterial cultures in laboratory media. In many studies however, broths are commonly used (King and Nagel 1967, 1975, Gill and Tan 1979, 1980, Enfors and Molin 1980, Eklund and Jarmund 1983, Molin 1983, Baker et al. 1986, Rowe 1988, Hao and Brackett 1993). Only a few studies have described the behaviour of pure cultures under modified atmospheres on solid surfaces of laboratory media (Golden et al. 1989, Eyles et al. 1993). Considering that many foods are mainly contaminated at the surface, the latter approach is probably a more accurate reflection of the practical situation.

The aim of the present study was to set up a model test system to investigate the growth of pure bacterial cultures on a solid surface under constant gas phase conditions, and to design an objective assessment of relevant growth parameters. The system was used to study the growth of psychrotrophic foodborne pathogens on MPF fruits and vegetables stored under modified atmospheres.

Materials and Methods

Micro-organisms

Two Gram negative and two Gram positive psychrotrophic foodborne pathogens were studied. *Aeromonas hydrophila* DSM 31087 and *Yersinia enterocolitica* DSM 4780 were obtained from the German Collection

of Microorganisms and Cell Cultures (Braunschweig, Germany). *Listeria monocytogenes* Scott A was obtained from the Culture Collection of the Department of Food Science of the Agricultural University of Wageningen (The Netherlands). A psychrotrophic strain of *Bacillus cereus* F4626/90 (=IFR-NL94-25), isolated from milk by Dr R. Gilbert (PHLS, Colindale, UK), was kindly provided by Dr M. Peck (IFRN, Norwich, UK).

Media and cultural conditions

All cultures were stored at −80°C in brain heart infusion broth (BHI, OXOID, UK) supplemented with 20% glycerol. Bacteria were cultivated at 30°C in BHI broth for 24 h, and subsequently subcultured for another 16 h, using 0·1% inocula. Cultures in stationary phase were diluted in 0·85% NaCl supplemented with 0·1% (w/v) peptone, to give approximately $5{\cdot}5\times10^6$ colony forming units (cfu) per ml. Samples (50 µl) of diluted culture were surface spread onto 60 mm diameter Petri dishes, containing 9 ml of brain heart infusion agar (BHIA). Initial populations of bacteria were about 10^4 cfu cm^{-2}. Unless stated otherwise, the medium was buffered at pH 7·2 with phosphate buffer (0·1 M), using equimolar amounts of sodium and potassium phosphate (Na/K P_i). A number of experiments were also performed using buffered BHIA adjusted to pH 6·7. Bacterial growth on the agar surface was examined under eight modified gas atmosphere compositions, i.e. 1·5 or 21% O_2, combined with 0, 5, 20, or 50% CO_2. The remainder of each atmosphere was made up to 100% with N_2.

Buffered media were used to minimize acidification caused by CO_2 dissolving in the medium. The effect of buffer strength upon growth was examined at 30°C in BHI broth without additional phosphate and with final Na/K P_i concentrations of 0·05, 0·10, 0·15, 0·20, and 0·25 M (pH 7·2).

Assessment of pH reduction by dissolved CO_2

Acidification of buffered BHIA plates (pH 7·2) by CO_2 was examined in a gas flow-through system over 10 days incubation at 8°C. Uninoculated buffered plates (0·05, 0·10, 0·15 and 0·20 M of Na/K P_i) were incubated in atmospheres containing 0, 1·0, 5·0, 10·0, 25·0, 50·0, and 100·0% CO_2 made up to 100% with N_2. On day 0, 1, 3, 6 and 10, duplicate samples were removed from the system and the pH was measured instantly at 8°C, using a flat surface pH electrode (Phoenix, USA).

Experimental design and storage of samples

Plates were incubated in a series of 1 l flasks, connected via silicon rubber tubings for each gasphase composition. Flasks were placed in a climatized room at 8°C and flushed continuously (flow rate=200 ml min^{-1}) with the desired gas atmosphere. Mass flow controllers (5850 TR series, Brooks Instrument b.v., The Netherlands) were used to mix the N_2, O_2 and CO_2. The incoming gas was humidified by passage through a 500 ml gas wash bottle. Concentrations of O_2 and CO_2 in the outcoming gas were measured and controlled at 2 h intervals by an O_2 and CO_2 analyser (Servomex, analysers series 1400). Agar plates were stored in the modified atmospheres at 8°C for up to 13 days. On day 2, 4, 7, 9 and 13, the last flask of a series was disconnected from the flow-through system. This enabled plates to be removed individually, without disturbing the gas conditions of the remaining plates.

Microbial analysis

Viable bacterial counts were determined by analysing samples of the agar medium in duplicate as follows. Agar samples were aseptically transferred from Petri dishes into stomacher bags and homogenized for 1 min with 41 ml of 0·85% NaCl using a stomacher (Seward, UK). Serial dilutions of each homogenized sample were made in 0·85% NaCl supplemented with 0·1% peptone and plated on BHIA in duplicate. Plates were incubated at 30°C for 24 h and the cfu cm^{-2} values were determined. The pH of the homogenate from each agar sample was recorded.

Data handling

Bacterial growth curves were generated for each gas phase composition by fitting the data to the Gompertz equation, as modified by Zwietering et al. (1990) to include microbiologically significant parameters (Equation 1).

$$ln\left(\frac{N}{N_0}\right) = A \cdot \exp\left\{-\exp\left[\frac{\mu_m \cdot e}{A}(\lambda - t) + 1\right]\right\} \quad (1)$$

N is the number of micro-organisms at time t, N_0 is the number of micro-organisms at t=0, A (=ln(N_∞/N_0)) is the asymptotic level of micro-organisms, μ_m is the maximum specific growth rate achieved, e=exp(1), and λ is the lag time.

Bacterial counts were fitted to Equation 1 by a non-linear regression program, using a Marquardt algorithm. This program estimates the maximum specific growth rate (μ_m), the final level of micro-organisms (A= ln(N_∞/N_0)), the lag time (λ) and their 95% confidence intervals (Zwietering et al. 1990). Confidence intervals were based on the variance–covariance matrix of the parameters, calculated with the Jacobian matrix. Estimated μ_m values were subjected to linear regression using Equation 2.

$$y = b_0 + b_1 x \quad (2)$$

with regression coefficients b_0 (intercept) and b_1 (x coefficient), y representing the estimated growth parameter, and x the CO_2 concentration (%). 95% Confidence intervals of the linear regression parameters were also determined.

Results

Acidification of the medium by CO_2 and gas analyses

The pH of the buffered BHIA (pH 7·2) was influenced by the concentration of CO_2 in the gas atmosphere. Equilibrium medium pH values of 7·2, 7·2, 7·0, 6·9, 6·8, 6·7 and 6·6 were observed in atmospheres containing 0, 1, 5, 10, 25, 50 or 100% CO_2 respectively, at 8°C. Due to permanent gas flushing of the incubators, the CO_2 concentrations in the gas phase were not influenced by CO_2 dissolving in the medium.

The pathogen experiments were performed under a maximum CO_2 concentration of 50%. As the medium pH decreased from 7·2 to 6·7 under these conditions, growth was also monitored on BHIA buffered at pH 6·7 in the absence of CO_2, as a control for acidification of the medium.

General growth characteristics

The phosphate concentration (0·1 M) employed did not inhibit growth of the pathogens at 30°C (data not shown). In some instances, growth of *A. hydrophila*, *Y. enterocolitica* and *B. cereus* on agar surfaces at 8°C resulted in a slight alkalinization of the medium during the course of the 13-day incubation period (Table 1). At the maximum population density, the pH was found to increase by a maximum of 0·9 units. In contrast, growth of *L. monocytogenes* caused acidification of the medium, with a maximum pH drop of 0·5 units at maximum population density. In general, the final pH of the medium depended on the maximum population density.

An example of growth of *Y. enterocolitica* on the surface of BHIA (pH 6·7 and 7·2) under 1·5% O_2 and 0, 5, 20, or 50% CO_2 is shown in Fig. 1. The growth curves were obtained using the modified Gompertz equation (Equation 1).

Lag times

Except for *Y. enterocolitica* under 50% CO_2 and 21% O_2 (but not under 50% CO_2 and 1·5% O_2), no significant lag times were found for the four pathogens grown on BHIA medium (pH 6·7 or 7·2) under the different gas phase compositions. Furthermore, viable counts of *B. cereus* gradually decreased under 50% CO_2 and 1·5% or 21% O_2 (data not shown).

Maximum specific growth rates

Maximum specific growth rates (μ_m) and their 95% confidence intervals, derived from the growth curves, are presented as a func-

Table 1. Medium pH (initial and final after 13 days of incubation) and estimated maximum population densities (PD) of *A. hydrophila, Y. enterocolitica, L. monocytogenes* and *B. cereus* on BHIA incubated at 8°C under 1·5% O_2 or 21% O_2. Measured initial population densities are also presented.

Micro-organism	% CO_2	Medium pH			PD (log cfu cm^{-2})		
		Initial	1·5% O_2 Final	21% O_2 Final	Initial	1·5% O_2 Maximum	21% O_2 Maximum
A. hydrophila	0	6·7	6·9	7·1	3·73	9·70	9·87
	0	7·2	7·4	7·5	3·73	9·56	9·75
	5	7·2	7·3	7·3	3·73	9·48	9·72
	20	7·2	7·1	7·1	3·73	9·31	9·44
	50	7·2	6·8	6·6	3·73	9·18	8·80
Y. enterocolitica	0	6·7	7·0	7·3	4·12	9·64	9·86
	0	7·2	7·8	7·8	4·12	9·51	9·82
	5	7·2	7·4	7·4	4·12	9·56	9·68
	20	7·2	7·2	7·3	4·12	9·31	9·56
	50	7·2	6·8	6·6	4·12	8·85	9·55[a]
L. monocytogenes	0	6·7	6·3	6·2	4·81	9·13	8·70
	0	7·2	6·8	6·8	4·81	9·21	9·14
	5	7·2	6·8	6·8	4·81	9·25	9·25
	20	7·2	6·8	6·8	4·81	9·04	9·11
	50	7·2	6·7	6·7	4·81	8·93	8·57
B. cereus	0	6·7	7·6	8·0	4·10	7·82	7·96
	0	7·2	7·8	8·1	4·10	8·22	8·12
	5	7·2	7·2	7·4	4·10	8·17	8·03
	20	7·2	6·8	6·9	4·10	7·28	7·54
	50	7·2	6·7	6·7	4·10	3·60[b]	3·88[b]

[a]estimation based on growth under 20% CO_2.
[b]measured instead of estimated values.

tion of the CO_2 concentration in Fig. 2. The maximum specific growth rates for *A. hydrophila, Y. enterocolitica* and *L. monocytogenes* under 1·5 or 21% O_2 combined with CO_2 were almost halved as compared to growth under 0 and 5% CO_2 (Figs 2(a), (b) and (c)). In the case of *B. cereus*, the maximum specific growth rates were reduced to about half of the maximum under 20% CO_2. The effect of CO_2 on the growth of this organism was even more pronounced under 50% CO_2 since incubation at 8°C for 13 days resulted in a decline of viable counts (Table 1).

The reduction of maximum specific growth rates at increasing CO_2 concentrations was substantiated by linear regression analysis (Equation 2) for the two O_2 concentrations. The regression lines are represented in Fig. 2. A significant reduction of the maximum specific growth rates as a function of the CO_2 concentration was found for all four pathogens, as indicated by negative values of both the estimates and the 95% confidence intervals of b_1 (x coefficient of regression) under 1·5% and 21% O_2 (Table 2). No significant differences between the maximum specific growth rates under 1·5% and 21% O_2 were found, since estimated values of regression coefficients b_0 (intercept) and b_1 under 21% O_2 were situated within the 95% confidence intervals of the 1·5% O_2 regression coefficients in all cases (Table 2).

The maximum specific growth rates of *A. hydrophila, Y. enterocolitica* and *L. monocytogenes* were generally lower at pH 6·7 than at pH 7·2 under 1·5% or 21% O_2 and 0% CO_2. However, the maximum specific growth rates at pH 6·7 were higher than the μ_m values under 50% CO_2 (Figs 2(a), (b) and (c) plus inserts). The observed decrease in the maximum specific growth rates obtained under elevated CO_2 concentrations may therefore be attributed to both acidification of the medium and a direct inhibitory effect of CO_2 on the

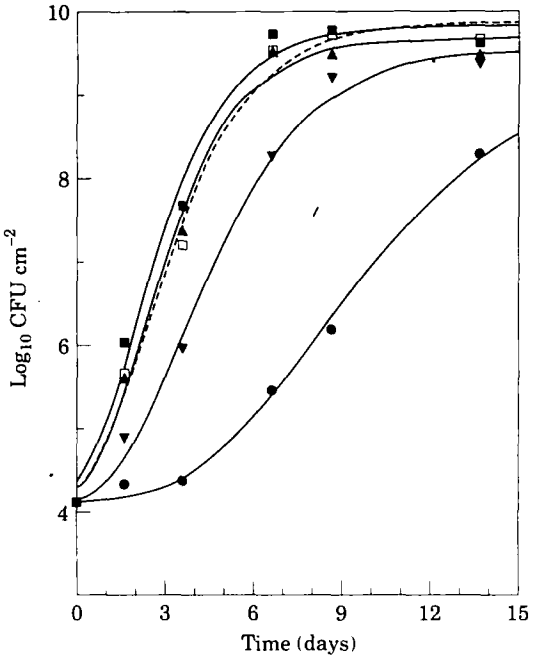

Figure 1. Growth of *Y. enterocolitica* (Log_{10} cfu cm^{-2}) on the surface of buffered BHIA (pH 7·2) stored under 1·5% O_2 and (■) 0% CO_2, (▲) 5% CO_2, (▼) 20% CO_2 or (●) 50% CO_2 and on buffered BHIA (pH 6·7) stored under 1·5% O_2 and 0% CO_2 (- -□- -). Data are fitted by the modified Gompertz equation (Equation 1).

growing cells. Maximum specific growth rates of *B. cereus* on pH 6·7 medium were similar to those on pH 7·2 medium under 0% CO_2, while growth was completely absent under 50% CO_2 (Fig. 2(d) plus insert). The observed reduction under 50% CO_2 is due to a direct inhibitory effect of CO_2.

Maximum population densities

The estimated maximum population densities of *A. hydrophila, Y. enterocolitica* and *L. monocytogenes* on BHIA medium (pH 7·2) were slightly reduced under 50% CO_2 compared to 0% CO_2 (maximum one log unit) under both 1·5% and 21% O_2 (Table 1). Incubation of *B. cereus* under 20% CO_2 resulted in a reduction in the maximum population density, compared to 0 and 5% CO_2 (<1 log unit) for both O_2 concentrations (Table 1), while no growth was observed under 50% CO_2. The 95% confidence intervals of the estimated maximum population densities were less than one log unit (data not shown).

Since the estimated maximum population densities found on pH 6·7 and pH 7·2 medium were similar for each pathogen under 1·5% or 21% O_2 and 0% CO_2 the observed reduction of the maximum population densities under 50% CO_2 cannot be attributed to acidification of the medium (Table 1).

DISCUSSION

The surface model system developed enabled us to study the growth of pure cultures of micro-organisms under different O_2 and CO_2 concentrations. The growth data obtained were evaluated using the modified Gompertz equation which allowed objective assessment of the maximum specific growth rate, maximum population density and lag time. Although a lag time was only observed for *Y. enterocolitica* under 50% CO_2 and 21% O_2, the modified Gompertz equation was chosen as the more versatile curve fit compared to the logistic equation, since the latter formula cannot fit growth curves that have a lag phase. Maximum population densities and specific growth rates estimated by both types of models were in comparable ranges for both curve fits (data for logistic curve fits not shown).

The main effect of CO_2 on the growth parameters of *A. hydrophila, Y. enterocolitica, L. monocytogenes* and *B. cereus* was observed for the maximum specific growth rates. These rates significantly decreased with increasing concentrations of CO_2 for all pathogens. Within the range of CO_2 concentrations tested, our data showed a linear relationship between the maximum specific growth rate and CO_2.

Although elevated CO_2 levels reduced the maximum specific growth rates of all pathogens, maximum population densities for *A. hydrophila, Y. enterocolitica, L. monocytogenes* were not noticeably affected by CO_2 concentrations up to 50%. The maximum population densities of *B. cereus* were slightly reduced under 20% CO_2 compared to 0% CO_2, and growth of this organism was completely absent under the highest CO_2 concentration

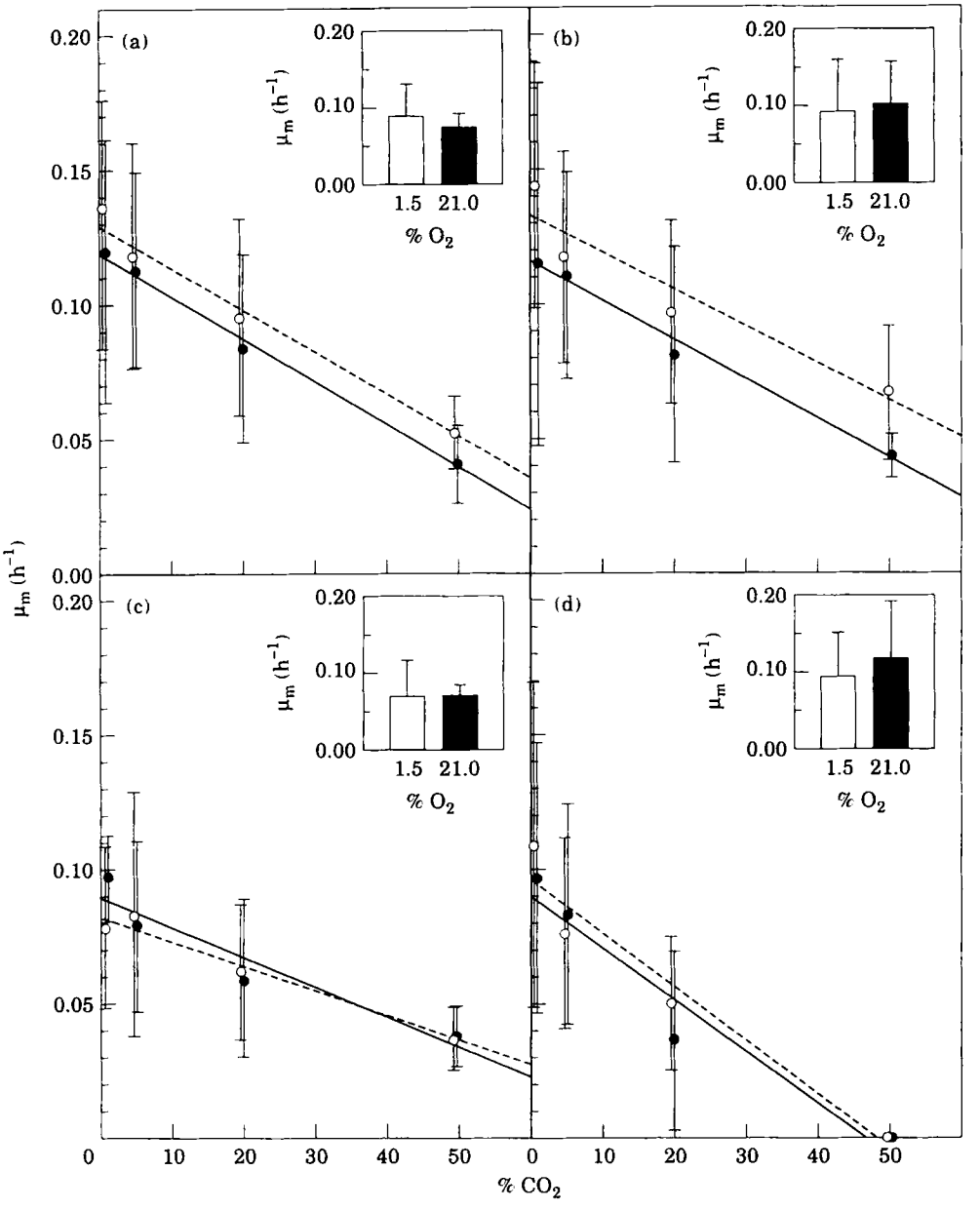

Figure 2. Influence of CO_2 concentration on the maximum specific growth rates (μ_m) of (a) *A. hydrophila*, (b) *Y. enterocolitica*, (c) *L. monocytogenes*, and (d) *B. cereus* on BHIA (pH 7·2) stored under (○) 1·5% O_2 and (●) 21% O_2. Lines represent linear regression under 1·5% O_2 (- -) or 21% O_2 (—). Inserts show μ_m on BHIA (pH 6·7) under 1·5% O_2 (white bar) or 21% O_2 (black bar) and 0% CO_2. 95% Confidence intervals of the estimated μ_m are indicated by error bars.

tested (50%). These results are in agreement with previous reports on the high sensitivity of *B. cereus* towards the antimicrobial effect of CO_2 (Enfors and Molin 1980, Enfors and Molin 1981, Molin 1983).

Extension of the lag phase is often considered to be an important consequence of increased CO_2 concentrations on bacterial growth (Farber 1991), although a number of studies disagree with this finding (Enfors and Molin 1981, Hintlian and Hotchkiss 1987, Eyles et al. 1993). In our studies, no

Table 2. Linear regression parameter values (b_0 and b_1) and their 95% confidence intervals for the effect of CO_2 on the estimated maximum specific growth rate (μ_m) values of *A. hydrophila, Y. enterocolitica, L. monocytogenes* and *B. cereus* during growth on agar at 8°C under 1·5% O_2 or 21% O_2.

Micro-organism	O_2 (%)	Parameter			
		b_0		b_1	
		Estimate	95% Conf. Int.[a]	Estimate	95% Conf. Int.
A. hydrophila	1·5	0·129	0·112 to 0·146	−0·00158	−0·00220 to −0·00095
	21	0·118	0·109 to 0·127	−0·00158	−0·00192 to −0·00125
Y. enterocolitica	1·5	0·133	0·097 to 0·168	−0·00136	−0·00265 to −6·3E-05
	21	0·115	0·103 to 0·127	−0·00144	−0·00188 to −0·00100
L. monocytogenes	1·5	0·082	0·067 to 0·097	−0·00089	−0·00146 to −0·00034
	21	0·089	0·062 to 0·116	−0·00108	−0·00208 to −8·5E-05
B. cereus	1·5	0·097	0·060 to 0·133	−0·00200	−0·00334 to −0·00065
	21	0·090	0·054 to 0·127	−0·00192	−0·00327 to −0·00057

[a]95% Confidence interval.

significant lag times were found for *A. hydrophila, Y. enterocolitica, L. monocytogenes* and *B. cereus* under the different gas phase compositions, except for *Y. enterocolitica* under 50% CO_2 and 21% O_2. To our knowledge, no reports are available on the influence of CO_2 on the lag times of *B. cereus*. Reported effects of CO_2 on the lag times of the other pathogens are variable, and always assessed at much higher CO_2 concentrations than applied in this study. Prolonged lag phases were observed for *A. hydrophila* grown on BHIA at 5°C in saturated CO_2 as compared to N_2 or air (Golden et al. 1989). Gill and Reichel (1989) observed an extended lag phase for *Y. enterocolitica* on high pH beef packaged under saturated CO_2 at 5°C and 10°C, while the growth of *A. hydrophila* and *L. monocytogenes* was completely inhibited at 5°C. Their study, however, demonstrated the absence of a significant lag phase and growth of *A. hydrophila* and *L. monocytogenes* at 10°C. Hudson et al. (1994) observed extended lag times for *A. hydrophila* and *L. monocytogenes*, but not for *Y. enterocolitica* on roast beef, packaged under saturated CO_2 at 3°C.

Although the behaviour of pathogens under the influence of CO_2 has been studied in several laboratory media and foodstuffs, the exact mechanism of the inhibitory effect of CO_2 is still obscure. A general effect of CO_2 is a decrease in pH. This effect increases at lower temperatures when solubility of the gas is enhanced (Butler 1982). Reduction of the medium pH by CO_2 is assumed not to be a major cause of inhibition of bacterial growth (King and Nagel 1975, Daniels et al. 1985). In our studies, a slight reduction of the maximum population densities was found for the pathogens under 50% CO_2. This reduction could not be attributed only to the acidification of the medium, since maximum population densities on a medium acidified to pH 6·7 (the equilibrium pH under 50% CO_2) were similar to those on medium of pH 7·2 in the absence of CO_2. However, the observed decrease of the maximum specific growth rates under elevated CO_2 concentrations for *A. hydrophila, Y. enterocolitica* and *L. monocytogenes* may be the result of a combined effect of acidification of the medium and direct inhibition by CO_2. In the case of *B. cereus*, the growth inhibition noted under 50% CO_2 might result from a direct inhibitory effect of CO_2, since growth on pH 6·7 and pH 7·2 medium was similar in the absence of CO_2.

Inhibition by CO_2 may result from diffusion of H_2CO_3 across the bacterial membrane, causing intracellular pH changes (Wolfe 1980). Such internal pH changes may effect enzymes that are involved in metabolic routes in the cell, and elevated CO_2 concentrations may inhibit decarboxylation reactions, in which CO_2 is released by feedback mechanisms (Dixon and Kell 1989). Direct

inhibition of enzymatic processes by CO_2 has been demonstrated in various studies (King and Nagel 1975, Gill and Tan 1979).

The growth patterns of *A. hydrophila, Y. enterocolitica, L. monocytogenes* and *B. cereus* were virtually the same under 1·5 and 21% O_2. Although 1·5% O_2 approaches the minimal requirement for MAP vegetables to maintain aerobic respiration (Kader et al. 1989, Gorris and Peppelenbos 1992, Peppelenbos 1995), this concentration would not be expected to be inhibitory to the aerobic respiratory growth of bacteria. Even growth rates of strictly aerobic bacteria such as *Pseudomonas fragi* are not reduced, unless O_2 concentrations are as low as 0·5 to 0·25% (Clark and Burki 1972, Enfors and Molin 1980).

Extrapolation of our results to MAP fruits and vegetables using typical O_2 concentrations of 1–5% and CO_2 concentrations of 5–10%, suggest that, with regard to O_2 concentrations, growth of all four pathogens may be possible at 8°C. Furthermore, the maximum specific growth rates and maximum population densities of *A. hydrophila, Y. enterocolitica, L. monocytogenes* and *B. cereus* could not effectively be inhibited by CO_2 concentrations generally prevailing in MAP vegetables.

Nutrient availability is a crucial factor affecting the growth of pathogens. Although a rich growth medium was used in this study, nutrient availability is not believed to be a limiting factor for bacterial growth on vegetable leaves (Nguyen-The, pers. comm.). This author demonstrated rapid growth of *L. monocytogenes* in 10-fold diluted leaf medium that was prepared by successively washing five batches of 20 g of intact vegetable leaves in 100 ml of distilled water. An increase of 3 log(cfu ml^{-1}) was observed within 3 days at 10°C.

Using the surface model system presented here and the data analyses performed, objective tools to determine the effects of CO_2 and other gases on growth of individual pathogenic bacteria were realized. This system may prove useful in studying the effects of a competitive or synergistic microflora on the growth of pathogens under different gas phase compositions. In terms of MAP vegetables, it must be remembered that the endogenous microflora consists mainly of pseudomonads (Nguyen-The and Carlin 1994), which are capable of excreting cell wall degrading enzymes to release nutrients (Liao et al. 1993). Since these organisms are sensitive to CO_2 (Enfors and Molin 1981), their suppression may influence nutrient availability for other organisms, e.g. for certain pathogens. To elucidate the impact of CO_2 on spoilage or other micro-organisms associated with MAP vegetables, it is necessary to study their growth in the absence of an interfering microflora using a systematic approach such as the model surface system.

Acknowledgements

The authors gratefully acknowledge Marcel Zwietering, Claire Wilkinson and Maarten Hertog for valuable advice on data handling. The capable technical assistance of Werner Vorstman and Marc Walpot is greatly appreciated. The work described was conducted with funds from the Commission of the European Union under contract AIR1-CT92-0125.

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Edited by S. Notermans