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Effect of elevated oxygen and carbon dioxide on the surface growth of vegetable-associated micro-organisms

A. Amanatidou, E.J. Smid and L.G.M. Gorris*
Agrotechnological Research Institute, Wageningen, The Netherlands

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A. AMANATIDOU, E.J. SMID AND L.G.M. GORRIS. 1999. The impact of a novel type of Modified Atmosphere (MA), referred to as high O_2-MA, on micro-organisms associated with the spoilage of minimally-processed vegetables was studied. Pure cultures of *Pseudomonas fluorescens, Enterobacter agglomerans*, *Aureobacterium* strain 27, *Candida guilliermondii*, *C. sake*, *Salmonella typhimurium*, *Salm. enteritidis, Escherichia coli*, *Listeria monocytogenes, Leuconostoc mesenteroides* var. *mesenteroides, Lactobacillus plantarum* and *Lactococcus lactis* were cultured on an agar-surface model system and incubated at 8 °C under an atmosphere composed of O_2 (80 or 90%, balanced with N_2), CO_2 (10 or 20%, balanced with N_2), or a combination of both gases. In general, exposure to high O_2 alone did not inhibit microbial growth strongly, while CO_2 alone reduced growth to some extent in most cases. Consistently strong inhibition was observed only when the two gases were used in combination. With minimally-processed vegetables, where CO_2 levels of around 20% or above cannot be used because of physiological damage to the produce, the combined treatment of high O_2 and 10–20% CO_2 may provide adequate suppression of microbial growth, allowing a safe, prolonged shelf-life.

INTRODUCTION

Modified atmosphere packaging (MAP) is used to extend shelf-life and maintain high quality of minimally-processed fruits and vegetables. The rapid increase in the market share of MAP of 'ready-to-eat vegetables' reflects the trends of today's consumers for fresh, additive-free foods.

Respiring products, like raw or processed vegetables and ready-made salads, generate equilibrium gas conditions inside the package that are typically very low in O_2 (2–3%) and moderately high in CO_2 (5–20%). These conditions reduce deterioration by limiting product respiration and maturation (Kader 1986; Day 1992; Gorris and Peppelenbos 1992) as well as by slowing down the proliferation of aerobic spoilage micro-organisms (Hotchkiss 1988; Kader *et al*. 1989; Moleyar and Narasimham 1994). The antimicrobial effect of CO_2 on micro-organisms has been intensively documented (Molin 1983; Eklund 1984; Daniels *et al*. 1985; Dixon and Kell 1989).

Correspondence to: Dr Athina Amanatidou, Agrotechnological Research Institute, PO Box 17, 6700 AA Wageningen, The Netherlands (e-mail: A.AMANATIDOU@ATO.DLO.NL).
**Present address: Unilever Research Laboratorium, Olivier van Noortlaan 120, 3133 AT Vlaardingen, The Netherlands.*

Although MA packaging of respiring produce has come into substantial use in practice, some potential problems with regard to product quality and safety remain to be solved. The O_2 and CO_2 levels in an MA package are achieved mostly by the active respiration of the produce and are often difficult to predict and control (Ahvenainen 1996). All too frequently, O_2 is completely depleted, resulting in the production of off-odours and rapid deterioration of the product (Zagory and Kader 1988). In addition, excessive levels of CO_2 (generally over 20%) cause specific disorders such as the development of brown stains (Lougheed 1987; Kader *et al*. 1995). Bennik *et al*. (1995) showed that CO_2 levels from 20–50%, in combination with low O_2 (1·5%), affect the growth rate of spoilage bacteria relevant to minimally-processed vegetables but have no effect on the maximum population densities or the lag phase duration.

Concerning product safety, psychrotrophic pathogens such as *Listeria monocytogenes* are not suppressed under MA conditions that are optimal for respiring produce (Berrang *et al*. 1989; Brackett 1994; Bennik *et al*. 1995; Carlin *et al*. 1996; Francis and O'Beirne 1997). On the contrary, growth may be enhanced in certain cases because of suppression of the natu-

ral flora (Hotchkiss 1988; Bennik *et al*. 1996). Evidently, alternatives to the current, low oxygen, MA packaging need to be investigated to better assure the safety of MA-packaged respiring produce.

Recent experimental trials on fresh commodities have indicated that high O_2 (70–90%) may be advantageous for product quality (Day 1996a, b). The use of high O_2-MAP packaging for respiring produce in practice is still in its infancy and needs to be supported by research. At present, little literature is available documenting a possible inhibitory effect of high O_2 on surface growth of micro-organisms (Ogihara *et al*. 1993). In the present study, therefore, the effect of high O_2, alone and in combination with moderately high CO_2 (10–20%), on the growth of pure cultures of a number of yeasts and bacteria relevant to MA-packaged vegetables was investigated. A surface model system was used in which micro-organisms are exposed to controlled gas conditions at 8 °C, a refrigeration temperature generally used for retail storage.

MATERIALS AND METHODS

Micro-organisms

Salmonella typhimurium DSM 4780, *Salm. enteritidis* DSM 13076, non-pathogenic *Escherichia coli* DSM 11755, *Leuconostoc mesenteroides* subsp. *mesenteroides* DSM 20343, *Lactobacillus plantarum* ATCC 8014 and *Lactococcus lactis* subsp. *lactis* NCDO 495 are type strains obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSM, Braunsweig, Germany). *Listeria monocytogenes* Scott A was from the culture collection of the Department of Food Science, University of Wageningen (The Netherlands). Strain 27 was isolated from ready-made salads incubated under 90% O_2 : 10% N_2 at 8 °C and identified by DSM as *Aureobacterium* spp. Two yeast strains also isolated from ready-made salads stored under modified atmospheres (10% O_2 : 5% CO_2) were identified by the Centraal Bureau voor Schimmelcultures (CBS, Baarn, The Netherlands) as *Candida sake* (Saito & Ota) van Uden & Burkley and *Candida guilliermondii* (Castelalani) Langeron & Guerra, respectively. *Pseudomonas fluorescens and Enterobacter agglomerans* were isolated from fresh produce and identified with the Biolog MicroPlate 2N system (Biolog®, Hayward, USA) in combination with classical identification methods.

Growth conditions

Listeria monocytogenes was grown on Palcam Listeria Selective Medium (PLSM, Oxoid), and *Leuc. mesenteroides, Lact. plantarum* and *L. lactis* on de Man Rogosa Sharp agar (MRS agar, Oxoid). All other strains were cultivated on Nutrient Agar (NA, Oxoid). All media were prepared according to the manufacturers' instructions.

Storage and preparation

Listeria monocytogenes was routinely stored at −80 °C in Brain Heart Infusion broth (BHI, Oxoid), lactic acid bacteria in MRS broth, yeasts in Yeast Nitrogen Base (YNB, Difco), and all other strains in Nutrient broth (NB, Oxoid) supplemented with 20% glycerol. A 0·1% inoculum (v/v) of a 5 ml full-grown culture was sub-cultured in MRS broth (lactic acid bacteria) or NB (all other strains) for 24 h at 25 °C, and subsequently transferred to agar plates of the same medium for further incubation. *Listeria monocytogenes* was transferred on PLSM. Stationary phase cells were harvested from plates, washed twice in 0·1 mol l^{-1} sodium phosphate buffer (pH 7·0) and then resuspended in the same buffer to an optical density of 0·5 at 660 nm. From this suspension, serial dilutions were made in saline and 50 ml samples of the diluted cultures were spread on 60 mm Petri dishes containing 9 ml medium, which gave an initial population on the agar surface of 10^3–10^4 cfu cm^{-2}.

Experimental set-up

The surface model system employed here was previously described by Bennik *et al*. (1995). In short, agar plates were prepared as described above and incubated in a series of 1 litre jars. The jars were placed in a temperature-controlled room at 8 °C and connected in sequence to a gas flow-through system. The following mixtures of O_2:N_2:CO_2 (% v/v) were used: (i) 90 : 10 : 0 (ii) 90 : 0:10 (iii) 80 : 20 : 0 (iv) 80 : 0:20 (v) 0 : 90 : 10 and (vi) 0 : 80 : 20. A mixture of 20% O_2 and 80% N_2 was used as the control. The compositions were prepared from pure gases using mass-flow controllers (5850 TR series, Brooks Instrument b.v., The Netherlands), humidified to a level close to saturation by passage through a water bottle, and finally introduced into the jars at a flow-rate of 200 ml min^{-1}. The composition was checked daily with a gas analyser (ADC 7000, ThIS Analytical B.V., Breda, Holland).

Enumeration of viable counts

Plate samples were taken in duplicate at each sampling time by removing the plates from two jars that were last in their sequence. The agar from one plate was removed aseptically and homogenized for 1 min in a stomacher bag containing 50 ml saline. The drop count method was used to estimate the viable counts in cfu ml^{-1}, which were converted to cfu cm^{-2} assuming that all counts originated from micro-organisms growing on the surface of the agar.

The pH of uninoculated medium was measured on each day of sampling in order to detect acidification of the medium.

In none of the incubations was the pH lower than 6·6 at the end of the incubation time. The pH of uninoculated MRS was 5·9 at the end of the incubation time.

Quantification of microbial growth parameters

Viable count data were fitted to the mathematical model described by Baranyi *et al.* (1993) and reparameterized with the DMFit program (1996, IFR, Reading Laboratory) kindly provided by Dr J. Baranyi. The model was used to estimate the lag phase (h, hours), the growth rate (h^{-1}) and the maximum population density y_{max} (log cfu cm^{-2}) as well as the associated standard error (S.E.) and correlation coefficient r^2.

RESULTS

Effect of high O_2 and CO_2 on the growth characteristics of selected micro-organisms

This study evaluated the potential of high O_2-MAP to reduce the growth of a number of spoilage micro-organisms and pathogenic bacteria typically associated with minimally-processed vegetables as compared with storage under an ambient atmosphere. The possible antimicrobial effect of traditional low O_2-MA packaging was assessed as well. Pure cultures of selected isolates were subjected to different controlled atmosphere conditions in an agar-surface model system incubated at 8 °C. The impact of gas atmosphere compositions on microbial growth was evaluated quantitatively on the basis of general growth characteristics and on three specific growth parameters (lag phase duration, growth rate and maximum yield) with the D-Model (Baranyi *et al.* 1993).

Listeria monocytogenes, *Salm. enteritidis*, *Salm. typhimurium* and *E. coli*

Growth of the pathogens was monitored in the presence of 90% O_2, or 10% CO_2, or the combination of the two gases (90% O_2 plus 10% CO_2), and compared with growth under normal air (Table 1). All pathogens could grow at 8 °C under air but growth of *Salm. typhimurium* was extremely slow. Growth rates of *E. coli* and *Salm. enteritidis* were found to be inhibited in the presence of 90% O_2 by 22% and 44%, respectively. On the other hand, growth rates of *L. monocytogenes* and *Salm. typhimurium* were hardly affected under these conditions. All four pathogens showed a lag phase in the presence of 90% oxygen which was longer than that under normal air. High oxygen alone reduced the final yield of *Salmonella* and slightly reduced the final yield of *E. coli.*

In the presence of 10% CO_2, growth of *E. coli* was hardly affected (Fig. 1a). Growth rate of *Salm. enteritidis* was inhibited by approximately 91%, while growth of *Salm. typhimurium* and *L. monocytogenes* was stimulated under these conditions (Table 1). An increase in the lag phase in the presence of 10% CO_2 was observed only for *L. monocytogenes* and *Salm. typhimurium.* On the other hand, no lag phase was observed for *E. coli* and *Salm. enteritidis*. For the latter strain, the effect on the final yield (4·5) was significant.

The combination of 90% O_2 and 10% CO_2 significantly reduced growth rates of *E. coli* and *Salm. enteritidis* (Table 1). Slight increases in the growth rate of the other two pathogens (*L. monocytogenes* and *Salm. typhimurium*) were observed. The lag phase of *E. coli* and *Salm. typhimurium* increased moderately to strongly, respectively, compared with the control. As all four pathogens grew at relatively slow rates at 8 °C, unequivocal conclusions about the effects of the gas conditions on the final yield cannot be drawn from the data presented.

Lactobacillus plantarum, *Leuc. mesenteroides* and *L. lactis*

All three lactic acid bacteria tested grew very well under ambient O_2 conditions at 8 °C. The growth rate of *L. lactis* and *Leuc. mesenteroides* was stimulated by the presence of 90% O_2 alone, while that of *Lact. plantarum* was significantly (63%) reduced (Table 2). *Lactococcus lactis* (Fig. 1b) showed a prominent lag phase (71 h) and a significant reduction in the final yield (6·6) with high O_2 alone (Table 2). A reduction in the final yield was also observed with *Lact. plantarum* but not with *Leuc. mesenteroides.*

In the presence of 10% CO_2, all three strains grew very well. No effect on growth rate was observed with *L. lactis* and *Leuc. mesenteroides* compared with the control. With *Lact. plantarum,* a 20% increase in growth rate was found (Table 2). A lag phase (17 h) was only apparent with *Lact. plantarum.* Final maximum population densities were not affected in any case.

All growth characteristics of the three strains were affected by the combination of 90% O_2 and 10% CO_2. Moderate reduction of growth rates was observed for *Leuc. mesenteroides* and *Lact. plantarum* but not for *L. lactis*. A lag phase of $\pm$ 30 h was observed with all three strains. Maximum population densities were reduced for all three strains.

Pseudomonas fluorescens, *Ent. agglomerans* and *Aureobacterium* strain 27

Growth of the three strains of spoilage bacteria tested was rapid under ambient conditions at 8 °C; 80% O_2 alone increased the growth rates of *Ps. fluorescens* (Fig. 2a) and *Ent. agglomerans* by 200% and 185%, respectively. Final yields were also increased. With strain 27, incubation under 90% O_2 resulted in a slight extension of the lag phase, while there

Table 1 Estimated parameters of lag time (λ), specific growth rate (μ) and maximum population densities (y_{max}), for the growth of *Listeria monocytogenes*, *Salmonella enteritidis*, *Salm. typhimurium* and *Escherichia coli* derived from fitting data to the D-model. The standard errors (S.E.) of lag are also presented

Micro-organism	O_2 (%)	CO_2 (%)	lag λ, (h)	(S.E.) (lag)	Specific growth rate, μ (h^{-1})	y_{max} (log cfu cm^{-2})
Listeria monocytogenes	20	0	62	20	0·031	(7·2)*
	90	0	82	21	0·039	(7·8)
	90	10	75	9	0·041	(8·1)
	0	10	123	16	0·052	(8·1)
Salmonella typhimurium	20	0	101	0	0·011	(6·5)
	90	0	353	32	0·015	(5·3)
	90	10	371	64	0·023	(5·6)
	0	10	123	17	0·022	(7·8)
Salm. enteritidis	20	0	0	0	0·091	(7·8)
	90	0	91	4	0·051	6·1
	90	10	0	0	0·021	4·5
	0	10	0	0	0·008	4·1
Escherichia coli	20	0	0	0	0·027	9·3
	90	0	31	18	0·021	8·9
	90	10	65	24	0·009	(7·2)
	0	10	0	0	0·024	9·1

* Numbers in brackets indicate population densities at the end of the sampling period for the cases in which y_{max} was not detected due to slow growth.

was no effect on either growth rate or final yield (Fig. 1c, Table 3).

Under 20% CO_2, growth rate and lag phase of *Ent. agglomerans* were not affected (Table 3). An increase in the lag phase (26 h) was only observed with *Ps. fluorescens*. With strain 27, 10% CO_2 resulted in a reduction in the growth rate (28%) and a significant increase in the lag phase (211 h compared with 97 h at ambient conditions). Carbon dioxide alone did not affect apparent final yields with *Ps. fluorescens* and *Ent. agglomerans*, while strain 27 did not reach stationary phase within the incubation period.

The combination of the gases significantly affected growth rates of all three strains; 80% O_2 and 20% CO_2 together caused a reduction in the growth rates of *Ps. fluorescens* and *Ent. agglomerans* (Table 3). The combination of 90% O_2 and 10% CO_2 almost halved the growth rate of strain 27. An increase in lag phase duration was apparent for all three strains, most prominently with strain 27 (170 h).

Candida sake and *C. guilliermondii*

High O_2 (80%) considerably stimulated the growth rate of both *C. guilliermondii* (Fig. 2b) and *C. sake*. High oxygen alone did not affect the lag phase of the two strains compared with the ambient control condition (Table 4). With *C. guilliermondii*, the growth yield under ambient O_2 was higher than under 80% O_2. The increase in viable counts of *C. sake* did not reach a maximum value under the test conditions.

With *C. guilliermondii* under 20% CO_2, a slightly reduced growth rate and a strongly increased lag phase were found. Also with *C. sake*, a decreased growth rate was evident.

The combined application of 80% O_2 and 20% CO_2 almost completely blocked growth of *C. guilliermondii* (Table 4). An apparent maximum yield was not reached due to the virtual absence of growth. For the same reason, a lag phase could not be deduced. Similar growth characteristics were observed with *C. sake*, although to a lesser extent.

DISCUSSION

The micro-organisms tested here have been reported in the literature as possible contaminants of minimally-processed vegetables (Lund 1992; Marchetti *et al.* 1992; Nguyen-the and Carlin 1994; Fain 1996). Under ambient O_2 conditions (control conditions), full growth in the model system was detected between 120 and 500 h at 8 °C in most cases. Growth of *L. monocytogenes* and *Salm. typhimurium* was rather slow. On the basis of the current evaluation, the following general

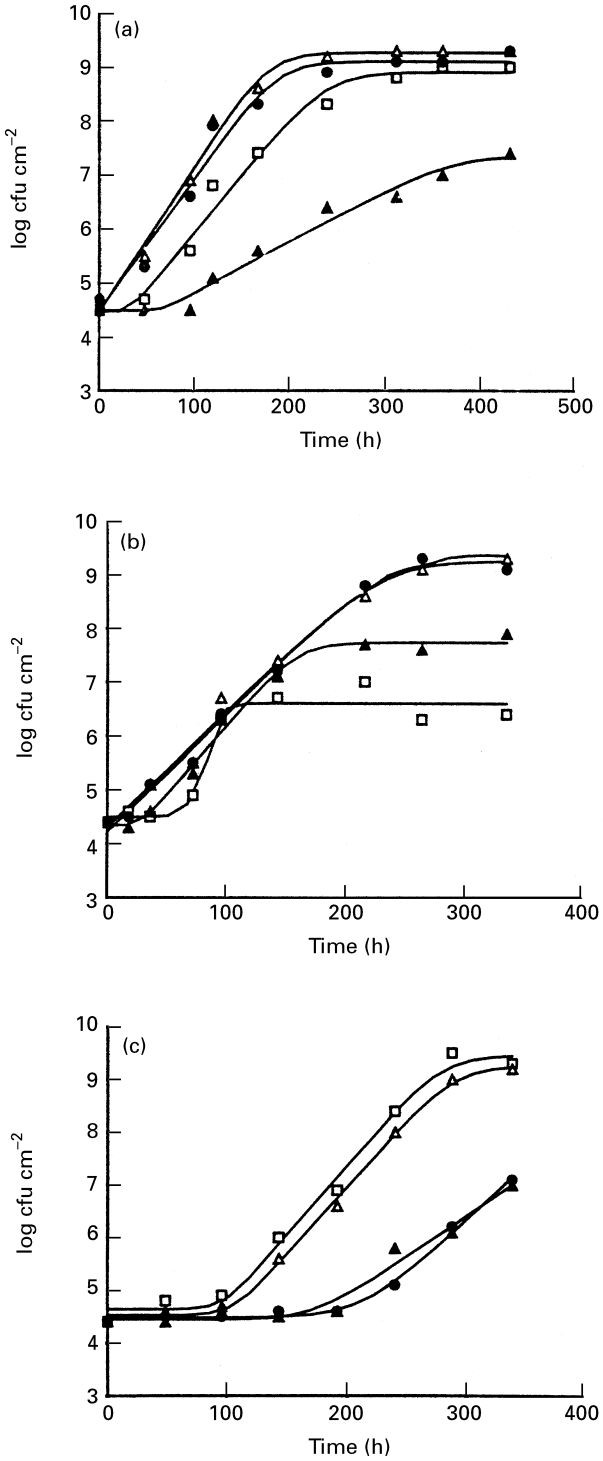

Fig. 1 Growth of *Escherichia coli* (a), *Lactococcus lactis* subsp. *lactis* (b) and strain 27 (c) on the surface of Nutrient Agar or MRS broth under the following combinations of gases: (△) 20% O_2 : 80% N_2; (□) 90% O_2 : 10% N_2; (●) 10% CO_2 : 90% N_2; (▲) 90% O_2 : 10% CO_2. Data are fitted with the D-model

observations can be made regarding the effects of high O_2 (80 or 90%) or CO_2 (20 or 10%), applied alone (balanced to 100% with N_2) and in combination:

(i) 80 or 90% O_2 alone did not inhibit growth of most test micro-organisms, but caused a significant reduction in the growth rate and/or maximum yield of *Salm. typhimurium*, *Salm. enteritidis* and *C. guilliermondii*. Growth of all other strains tested here was stimulated;

(ii) 10 or 20% CO_2 alone was found to reduce growth rates of *Ps. fluorescens*, *C. guilliermondii* and *Salm. enteritidis* significantly. A prolonged lag phase was noticed with strain 27 and *C. guilliermondii*. Growth rates of the lactic acid bacteria, *Salm. typhimurium, L. monocytogenes* and the non-pathogenic *E. coli* were either not affected or stimulated.

(iii) The combined application of high O_2 and CO_2 had an inhibitory effect on the growth rate of all micro-organisms, except for two of the strains that grew only poorly in the experimental set-up, i.e. *L. monocytogenes* and *Salm. typhimurium*. In most cases, a notable prolongation of the lag phase and a reduction in the final population density was observed. The most prominent effect of the combined application of high O_2 and CO_2 was found with the yeast strains that were almost completely inhibited in their growth.

Although only a limited number of prokaryotic and eukaryotic micro-organisms was evaluated in this study, it may be concluded that exposure of micro-organisms to high O_2 alone has an inhibitory effect on growth in a few cases only, and may occasionally have a stimulatory effect. It would be expected that the high O_2 levels applied would lead to intracellular generation of reactive oxygen species (ROS, O_2^-, H_2O_2, OH*) that would affect vital cell components and reduce cell viability (Halliwell and Guteridge 1984; Fridovich 1986). Evidently, micro-organisms have developed strategies such as induction of O_2-decomposing enzymes (catalase, peroxidase, superoxide dismutase) or radical scavengers (e.g. glutathione) in order to avoid lethal damage by oxygen. Studies on the effects of oxygen stress on *Salm. typhimurium, E. coli* and *L. lactis* have identified the presence of inducible multi-gene systems which destroy ROS. Other proteins such as the SOS system serve to repair oxidative damage at 30–37 °C (Demple and Halbrook 1983; Farr and Kogoma 1991; Sanders 1997). However, to our knowledge, no information is available in the literature about the responses of pure cultures of micro-organisms to oxidative stress at lower temperatures. The ability of *Salm. typhimurium* to respond to oxidative stress is reduced as metabolic processes are slowed down at 8 °C. This observation is, to a lesser degree, valid for *Salm. enteritidis* that can adapt easily at low temperature. Considering the sensitivity of the salmonellas to high oxygen, as found in the present study, incubation temperature will

Table 2 Estimated parameters of lag time (λ), specific growth rate (μ) and maximum population densities (y_{max}), for the growth of *Lactococcus lactis*, *Leuconostoc mesenteroides* var. *mesenteroides* and *Lactobacillus plantarum* derived from fitting data to the D-model. The standard errors (S.E.) of lag are also presented

Micro-organism	O_2 (%)	CO_2 (%)	lag λ, (h)	(S.E.) (lag)	Specific growth rate, μ (h^{-1})	y_{max} (log cfu cm^{-2})
Lactococcus lactis	20	0	0	0	0·021	9·2
	90	0	71	15	0·057	6·6
	90	10	27	8	0·025	7·7
	0	10	0	0	0·022	9·3
Leuconostoc mesenteroides	20	0	0	0	0·036	9·2
	90	0	10	5	0·044	9·2
	90	10	30	13	0·023	7·5
	0	10	0	0	0·038	9·2
Lactobacillus plantarum	20	0	0	0	0·047	9·3
	90	0	0	0	0·030	7·4
	90	10	30	6	0·032	(8·1)*
	0	10	17	6	0·056	9·5

* As in Table 1.

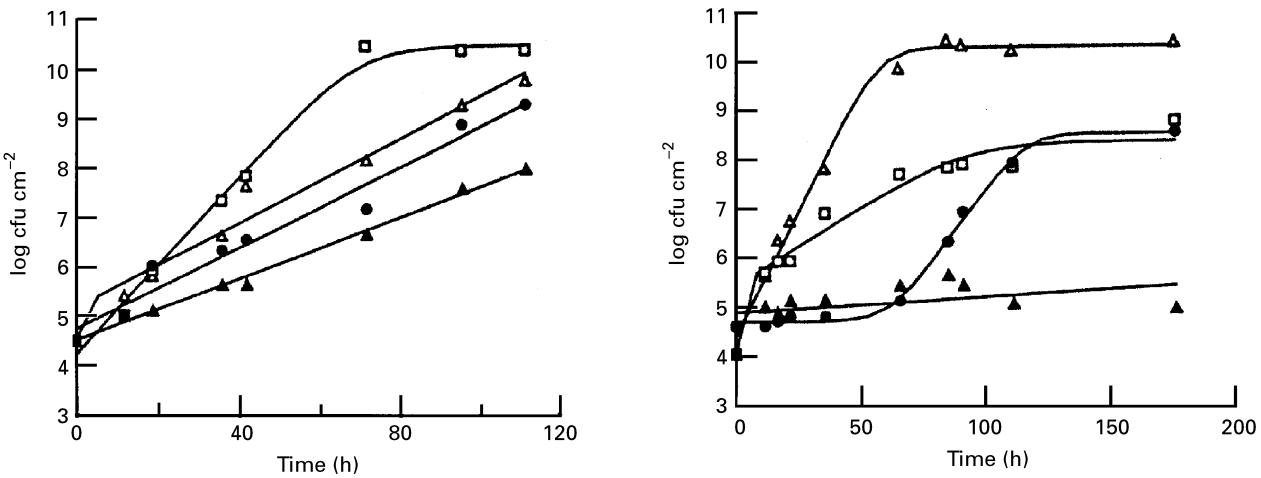

Fig. 2 Growth of *Pseudomonas fluorescens* (a) and *Candida guilliermondii* (b) on the surface of Nutrient Agar under the following combinations of gases: (△) 20% O_2 : 80% N_2; (□) 80% O_2 : 20% N_2; (●) 20% CO_2 : 80% N_2; (▲) 80% O_2 : 20% CO_2. Data are fitted with the D-model

play a role in the ability of a micro-organism to encounter oxidative stress.

Lactic acid bacteria can tolerate oxygen, or even use oxygen, in the presence of a carbon source by a closely coupled NADH oxidase/peroxidase system and, in some cases, superoxide dismutase (Archibald and Fridovich 1981; Condon 1987; Piard and Desmazeaud 1991). *Lactobacillus plantarum* ATCC 8014 growing in flasks with APT broth could tolerate

Table 3 Estimated parameters of lag time (λ), specific growth rate (μ) and maximum population densities (y_{max}), for the growth of strain 27, *Pseudomonas fluoresecens* and *Enterobacter agglomerans* derived from fitting data to the D-model. The standard errors (S.E.) of lag are also presented

Micro-organism	O_2 (%)	CO_2 (%)	lag λ, (h)	(S.E.) (lag)	Specific growth rate, μ (h^{-1})	y_{max} (log cfu cm^{-2})
Aureobacterium strain 27	20	0	97	12	0·061	9·5
	90	0	106	16	0·059	9·3
	90	10	170	18	0·035	(7·4)*
	0	10	211	6	0·048	(7·3)
Pseudomonas fluorescens	20	0	16	3	0·097	9·9
	80	0	24	5	0·200	10·4
	80	20	37	10	0·069	(7·9)
	0	20	26	9	0·092	(9·6)
Enterobacter agglomerans	20	0	0	0	0·141	9·9
	80	0	17	11	0·261	10·4
	80	20	5	3	0·110	9·3
	0	20	6	1	0·130	9·6

* As in Table 1.

Table 4 Estimated parameters of lag time (λ), specific growth rate (μ) and maximum population densities (y_{max}), for the growth of *Candida sake* and *C. guilliermondii* derived from fitting data to the D-model. The standard errors (S.E.) of lag are also presented

Micro-organism	O_2 (%)	CO_2 (%)	lag λ, (h)	(S.E.) (lag)	Specific growth rate, μ (h^{-1})	y_{max} (log cfu cm^{-2})
Candida guilliermondii	20	0	0	0	0·100	10·3
	80	0	0	0	0·190	8·3
	80	20	–	–	0·002	4·9
	0	20	61	12	0·070	8·4
Candida sake	20	0	10	5	0·029	8·8
	80	0	10	9	0·040	(9·3)*
	80	20	31	10	0·010	(7·4)
	0	20	–	–	0·018	(7·3)

* As in Table 1.

oxygen but its growth rate was reduced when incubation was under a stream of 100% O_2 (Gregory and Fridovich 1974). Pure oxygen reportedly had a positive effect on growth and biomass production of *Leuc. mesenteroides* in batch cultures (Plihon *et al.* 1995). Similar results were obtained for *Ps. fluorescens* (Onken 1990).

Our results concerning the effect of high oxygen on yeasts are in agreement with previous reports. Whereas an 'excess oxygen state' (60% or higher) has been found to initiate high cell yields and low levels of fermentation products of *Saccharomyces cerevisiae*, the cellular response of eukaryotic micro-organisms to oxidative stress remains to be resolved (Brown and Johnson 1971; Moradas-Ferreira *et al.* 1996; Pinheiro *et al.* 1997).

High levels of CO_2 have frequently been reported to reduce microbial growth significantly, although results were quite variable when comparing different spoilage and pathogenic bacteria (Dixon and Kell 1989; Eyles *et al.* 1993; Ogihara *et al.* 1993). Extension of the lag phase and reduction of the growth rate is often considered to be a prominent effect of CO_2

(Farber 1991). Bennik *et al.* (1995) have shown that this effect is consistent only at very high CO_2 concentrations but CO_2-enriched modified atmospheres are not a reliable way to control the fate of *L. monocytogenes* in vegetable products (Carlin *et al.* 1996). *Listeria monocytogenes* growing on fresh produce was not inhibited by CO_2 up to 70% (Kallander *et al.* 1991). Several studies showed that *Pseudomonas* spp. expressed a rather high sensitivity towards CO_2 (also at 20%), although total inhibition may not be achieved if O_2 is reduced to 0·2–0·6% but not fully depleted (Gill and Tan 1979; Enfors and Molin 1980; Molin 1983). Enterobacteriaceae were reported to be unaffected by 20% CO_2 at chill temperatures (Gill and Tan 1980; Bennik *et al.* 1998). In general, growth of Enterobacteriaceae, including *Salmonella* spp., is only retarded at very high levels of CO_2, but the inhibitory effect is temperature-dependent (Sawaya *et al.* 1995; Woolfe *et al.* 1995). Most reports for growth of *Salmonella* at elevated CO_2 levels concern highly proteinaceous foods. Prolongation of the lag phase by CO_2 of *Salm. typhimurium* at low temperature (10 °C) has been reported by Silliker and Wolfe (1980). The high sensitivity of *Salm. enteritidis* to only 10% CO_2 is probably a strain-specific phenomenon. Lactic acid bacteria were generally favoured by elevated CO_2 or reduced O_2 concentrations (Roth and Clark 1975), while the effect on yeasts was variable (Jones and Greenfield 1982; Eklund and Jarmund 1983; Ison and Gutteridge 1987; Barriga *et al.* 1991).

In this study, growth characteristics of all pathogenic micro-organisms were significantly reduced at combinations of high O_2 and CO_2 compared with air. In the case of *Listeria*, the growth rate was stimulated compared with air but was retarded compared with 10% CO_2. Ogihara *et al.* (1993) found that pure cultures of facultative anaerobic bacteria such as *Enterobacter* spp., *L. monocytogenes* and *Salm. typhimurium* are not completely inhibited by high O_2 or CO_2 or a mixture of both, but growth rate was reduced in proportion to the increase in the ratio of CO_2 in the gas mixtures.

From the results of the current study, as well as from the available literature, it is concluded that when high O_2 and CO_2 are applied alone, the inhibitory effect on microbial growth is highly variable. Stronger and much more consistent inhibition of microbial growth can be obtained when the two gases are used in combination. This result is very relevant because it enables a further improvement of the low O_2-MA packaging systems currently used for minimally-processed vegetables; CO_2 levels in the combined treatment need not be higher than 20% to give consistent suppression of microbial growth, although this finding remains to be validated in product studies.

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