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Effect of high oxygen modified atmosphere packaging on microbial growth and sensorial qualities of fresh-cut produce

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Abstract

The application of High Oxygen Atmospheres (HOA) (i.e. $>70\%$ O_2) for packaging ready-to-eat vegetables was evaluated as an alternative technique for low O_2 Equilibrium Modified Atmosphere (EMA) packaging (3% O_2–5% CO_2–balance N_2) for respiring products. Comparative experiments between both techniques were performed in-vitro and in-vivo. Typical spoilage causing microorganisms (*Pseudomonas fluorescens*, *Candida lambica*), the moulds *Botrytis cinerea*, *Aspergillus flavus* and the opportunistic psychrotrophic human pathogenic microorganism associated with refrigerated minimally processed vegetables, *Aeromonas caviae* (HG4), showed a retarded growth during the conducted in-vitro studies at 4 °C in 70%, 80% and 95% O_2 as examples of HOA compared to the in-vitro experiments in 5% O_2 (as example of EMA packaging) and the effect was more pronounced in 95% O_2. The effect of the high O_2-concentrations on the human pathogen *Listeria monocytogenes* resulted in an extended lag phase (95% O_2). The plant pathogen *Erwinia carotovora* was increasingly stimulated by increasing high O_2-concentrations. During a storage experiment of three types of ready-to-eat vegetables (mushroom slices, grated celeriac and shredded chicory endive), which are sensitive to enzymatic browning and microbial spoilage, the effect of EMA and HOA (95% O_2–5% N_2) on their quality and shelf life was compared. High O_2 atmospheres were found to be particularly effective in inhibiting enzymatic browning of the tested vegetables. Also, the microbial quality was better as a reduction in yeast growth was observed. The HOA can be applied as an alternative for low O_2 modified atmospheres for some specific types of ready-to-eat vegetables, sensitive to enzymatic browning and spoilage by yeasts.

Keywords: High oxygen; Microbial quality; Sensorial quality; Vegetables; Safety

1. Introduction

An alternative for Equilibrium Modified Atmosphere packaging (EMA), used for prolonging the shelf life of respiring ready-to-eat vegetables, can be packaged under High Oxygen Atmospheres (HOA) (Day, 1996). At the present time, the key to successful EMA packaging of fresh-cut produce is to establish equilibrium modified atmospheres, optimally 3–5% O_2 and 3–10% CO_2 (balance N_2), by choosing the packaging film with the correct permeability for O_2 and CO_2. Due to the respiratory nature of fresh produce, the development of hazardous anaerobic conditions (e.g. $<2\%$ O_2 and $>20\%$ CO_2) and

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undesirable fermentation reactions within EMA packages may occur when stored at higher temperatures. During retail of EMA packages, temperature fluctuations are unavoidable. Jacxsens et al. (2000a,b, in press) demonstrated the use of EMA packages in a simulated distribution chain. In the temperature range of 2 to 10 °C, satisfactory O_2 and CO_2 levels were obtained with a new type of high permeable packaging material. But above these temperatures, anaerobic fermentation reactions occurred resulting in a negative evaluation of sensorial properties. Moreover, as the respiration rate differs strongly from vegetable to vegetable, packaging films with a different permeability for O_2 are necessary to package a range of vegetables (Jacxsens et al., 1999a). This is, from a logistic point of view, not very convenient for the producer. The application of high O_2-concentrations (i.e. $>70\%$ O_2) could overcome the disadvantages of low O_2 modified atmosphere (EMA) packaging for some ready-to-eat vegetables. High O_2 was found to be particularly effective in inhibiting enzymatic discoloration, preventing anaerobic fermentation reactions and inhibiting microbial growth (Day, 1996, 2000, 2001). It has been hypothesised that high O_2-levels may cause substrate inhibition of the enzyme polyphenoloxidase (PPO), or alternatively, that high levels of colourless quinones subsequently formed may cause feedback inhibition of PPO (Day, 1996). Amanatidou et al. (1999) screened microorganisms, associated with the spoilage and safety of minimally processed vegetables. In general, exposure to high oxygen alone (80 to 90% O_2, balance N_2) did not inhibit microbial growth but caused a variable reduction in the growth rate of some of the tested microorganisms at 8 °C. A prolongation of the lag phase was more pronounced at higher O_2-concentrations. Amanatidou et al. (1999) as well as Kader and Ben-Yehoshua (2000) suggested that these high O_2-levels could lead to intracellular generation of reactive oxygen species (ROS, O_2^-, H_2O_2, OH^*), damaging vital cell components and thereby reducing cell viability when oxidative stresses overwhelm cellular protection systems. Combined with increased CO_2-concentration (10 to 20%), a more effective inhibitory effect on the growth of all microorganisms was noticed rather than the individual gases alone (Gonzalez Roncero and Day, 1998; Amanatidou et al., 1999, 2000). Wszelaki and Mitcham (1999) found that 80–100% O_2 inhibited the in-vivo growth of *Botrytis cinerea* on strawberries.

Exposure to high O_2-levels may stimulate, have no effect or reduce rates of respiration of produce depending on the commodity, maturity and ripeness stage, O_2-concentration, time and temperature of storage, and the CO_2- and C_2H_4-concentrations (Kader and Ben-Yehoshua, 2000). Respiration intensity is directly correlated to the shelf life of produce (Kader et al., 1989). Therefore, the quantification of the effect of high O_2-levels on the respiratory activity is necessary.

High O_2 MAP of vegetables is not yet fully commercialised probably because of the lack of understanding of the basic biological mechanisms involved in inhibiting/reducing microbial growth, inhibiting enzymatic browning, effect on respiratory activity, its unknown effect on the nutritional quality of the packaged fruits and vegetables and concerns about possible safety implications during packaging. Concentrations higher than 25% O_2 are considered to be explosive and special precautions have to be taken on the work floor (BCGA, 1998).

The objective of this work was threefold: (1) to determine the effect of high oxygen concentrations, without the combination with CO_2, in-vitro on different types of pathogenic and spoilage causing microorganisms, associated with minimally processed vegetables, (2) to quantify the effect of high O_2-levels on the respiration rate of some minimally processed vegetables sensitive for enzymatic browning and microbial spoilage (shredded chicory endives, grated celeriac and sliced mushrooms), (3) to perform a comparative storage experiment between HOA and EMA packages of the investigated vegetables.

2. Materials and methods

2.1. In-vitro study

2.1.1. Isolation and cultivation of microorganisms

Spoilage microorganisms were isolated from industrially prepared minimally processed lettuce (20% endive, 20% curled endive, 20% radicchio lettuce, 20% lollo rossa and 20% lollo bionda lettuces), stored for 14 days at 4 °C in an industrial standard low O_2 package (PVdC-coated OPP film; O_2 perme-

ability = 15 ml O_2/m^2 24 h atm at 7 °C and 90% RH, 30 μm, Van der Windt Packaging, Hoogstraten, Belgium). The mixed lettuce was, after preparing a dilution seri (1/10) in Peptone Saline Solution (PPS, 8.5 g/l NaCl (Vel; 8605, Merck Eurolab, Leuven, Belgium) + 1 g/l peptone (Oxoid; L34, Unipath, Basingstoke, Hampshire, UK)), plated on Plate Count Agar (PCA (Oxoid; CM325); incubation for 5 days at 22 °C) as a spread plate to isolate G^- psychrotrophic microorganisms, on de Man, Rogosa, Sharpe agar (MRS (Oxoid; CM361); anaerobic incubation (addition of AeroGen™ sachet (Oxoid; AN025A) to a closed jar) for 3 days at 30 °C) as a spread plate to isolate lactic acid bacteria, on Yeast Glucose Chloramphenicol agar (YGC (Sanofi Diagnostics Pasteur; 64104, Marnes-La-Coquette, France); 3 days at 30 °C) as a spread plate to isolate yeasts. After incubation visually different colonies were purified by a 4 × 4 plating on Tryptone Soya Agar (TSA (Oxoid; CM131)). After incubation at 30 °C for 24 h, they were identified by classical identification tests (Gram-straining, oxidase (identification sticks oxidase (Oxoid; BR64A)), catalase (H_2O_2 30% (Vel; 90320, Merck Eurolab, Leuven, Belgium) diluted until 3%), microscopy) and by biochemical tests: API NE (for G^- microorganisms), API ID32 C (for yeasts) and API 50 CH (for lactic acid bacteria) (bioMérieux, Marcy l'Etoile, France). The identification was confirmed by LMG Culture Collection (Ghent University, Ghent, Belgium) applying protein gel electrophoresis (SDS-PAGE) for lactic acid bacteria and Biolog GN 2 (biochemical test based on d the ability to oxidize 95 different carbon sources) (Biolog, California, USA) for G^- microorganisms. Yeasts were confirmed by physiological tests (sugar fermentation) and applying the auxanographic method (IHEM Culture Collection, Brussels, Belgium) (Vanbreuseghem et al., 1978).

Listeria monocytogenes (LM LJ1) was isolated out of green and red bell peppers using a spread plate on Oxford selective agar (Oxoid; CM856 and SR140E) after incubation at 37 °C for 48 h. A further purification was conducted by a 4 × 4 plating on TSA and identified by using API Lis (bioMérieux) (Jacxsens et al., 1999a).

Aeromonas caviae (HG 4) was isolated from fresh spinach using a spread plate on modified bile-salt–irgasan–brilliant-green agar (mBIBG; Neyts et al., 2000). After 24 h incubation at 30 °C, suspicious colonies were purified by a 4 × 4 plating on TSA and further identified by BBL Crystal™ ID enteric/non-fermenter kit (Becton Dickinson, Cockeysville, MD, USA). The identification was confirmed (LMG, Ghent University) using FAME analyse techniques (Jacxsens et al., 1999a; Neyts et al., 2000).

Moulds (*B. cinerea* 5146 and *Aspergillus flavus* 3790) were obtained from culture collections (IHEM, Brussels, Belgium).

A stock culture was preserved on glass pearls at −75 °C. A working culture was stored at 4 °C and the following media were utilised for the slants/plates: MRS for lactic acid bacteria, YGC for yeasts and moulds, Nutrient Agar (Nutrient Broth (Oxoid; CM1) + 10 g/l agar (Oxoid; L11)) for *Aeromonas* spp., TSA for *L. monocytogenes* and Brain Heart Infusion agar (BHI broth (Oxoid; CM225) + 10 g/l agar (Oxoid; L11)) for the G^- microorganisms (*Pseudomonas fluorescens*, *Erwinia carotovora*).

An inoculum was prepared to inoculate agar plates: each strain was separately two times consecutively subcultured in BHI for 24 h at 30 °C (or 37 °C for *L. monocytogenes*). The second culture was allowed to adapt to the final temperature of 4 °C for 6 h. After these 6 h, the second culture was diluted (1/10) in PPS to such an extent that a contamination level of approximately 10^3–10^4 viable cells/cm^2 agar plate was obtained. The moulds were surface plated on YGC agar and incubated at 30 °C. Spores were collected by swabbing with PPS. To define the number of spores in the spore suspension, the suspension was diluted and surface plated on YGC. An inoculum of 10^3–10^4 spores/cm^2 agar plate was made for the in-vitro experiment.

BHI was chosen as cultivation medium for the in-vitro experiment because of its non-selective, low nutritive value. For yeasts and moulds, the richer and more selective YGC agar and for lactic acid bacteria MRS agar enriched with 3 g/l yeast extract (Yeast Extract (Oxoid; L21)) was applied because preliminary tests showed an inhibition in their growth on BHI agar in combination with low storage temperature (4 °C). Storage temperature during the in-vitro experiment was 4 °C, recognised as optimal temperature for the storage of minimally processed vegetables (Anonymous, 1988).

2.1.2. Packaging of agar plates

The purpose of the in-vitro study was to quantify the effect (stimulation/reduction/inhibition) of high oxygen concentrations (not in combination with CO_2) on the growth of microorganisms. Therefore, four gas combinations were applied (balanced by N_2): 70% O_2, 80% O_2, 95% O_2 and 5% O_2 as reference for low O_2 atmospheres (EMA) traditionally applied for minimally processed vegetables.

A flushing system was used for gas packaging of the inoculated agar plates. The inoculated agar plates were individually packaged in a package of 15×19 cm^2 barrier film of 150 μm thickness and an oxygen transmission rate of 0.98 ml O_2/m^2 24 h atm at 23 °C and 0% RH (WA6193-1, Hyplast, Hoogstraten, Belgium). The packages were flushed for 5 min with the selected gas combination which was mixed with a Witt unit (gas mixer, WITT M618-3MSO, Gasetechnik, Germany). Air Products (Vilvoorde, Belgium) supplied the gases (Fresh Line). After flushing, the packages were heat sealed (Sealboy 321, Audion Elektri, Weesp, the Netherlands) and stored at 4 °C.

2.1.3. Enumeration of in-vitro growth curve

The gas concentration inside the packages was analysed before opening with a CO_2/O_2 gas analyser (Servomex Food Package Analyser, Series 1400, Crowborough, Sussex, UK). The complete content of the agar plate was aseptically transferred in a stomacher bag and diluted with PPS. Dilutions (1/10) were made, surface plated on BHI and incubated at 30 °C for 24 h (*A. caviae*) and 3–4 days (lactic acid bacteria, G^- strains, yeasts), at 37 °C for 48 h (*L. monocytogenes*).

Regularly, the purity of the inoculated agar plates was checked by plating, next on to BHI, on a selective media for the specific microorganism. For *L. monocytogenes* Oxford selective agar was used and the plates were incubated at 37 °C for 48 h. *A. caviae* was surface plated on mBIBG (30 °C for 24 h). The spoilage microorganisms (Gram negative and Gram positive) and yeasts were regularly controlled on their identity by API (bioMérieux) or BBL Crystal™ ID enteric/non-fermenter kit (Becton Dickinson). The development of the moulds was visually followed daily and quantified as follows: duration (days) before visual appearance of mycelium, duration (days) before visual appearance of spores and the amount of spores after a certain period. The purity of the moulds was visually checked under the microscope.

2.2. Storage experiment

2.2.1. Minimally processing of the vegetables

All the vegetables were bought on a local wholesale company in Ghent at the day of their arrival of the public auction. They were transported to the laboratory (15 min) under refrigeration conditions and immediately processed. Celeriac (*Apium graveolens* var. *rapaceum* L.) was peeled and grated in thin sticks ($3 \times 3 \times 40$ mm) by Compacto Kitchen Cutter (Philips, the Netherlands). Chicory endives (*Cichorium intybus foliosum* L.) and mushrooms (*Agaricus bisporus* L.) were manually shredded in small slices (respectively 1 and 0.3 cm) with a sharp stainless steel knife. The vegetables were washed for 1 minute in potable water of 4 °C and manually spin dried (Zyliss, Bern, Switzerland).

2.2.2. Packaging of the vegetables

The vegetables were packaged under HOA and EMA. The same barrier film was applied for the HOA packages as for the in-vitro studies in order to maintain the high O_2-concentrations inside the packages. Packaging configurations were 150 g of vegetables packaged in 15×19 cm^2 packages. The same flushing technique was applied as during the in-vitro experiments.

A selective permeable packaging film was used for the EMA packages (Jacxsens et al., 2000a): oxygen permeability of the film for shredded chicory endives was 1446 ml O_2/m^2 24 h atm at 7 °C, 90% RH and for grated celeriac 2812 ml O_2/m^2 24 h atm at 7 °C, 90% RH (Hyplast, Hoogstraten, Belgium). The same packaging configurations as for the HOA packages were used. Mushroom slices are respiring very fast and no selective permeable packaging film until now is available. Therefore, a BOPP packaging film (oxygen permeability of 914 ml O_2/m^2 24 h atm at 12.5 °C and 90% RH, 30 μm, Klerks Group, Noordwijkerhout, the Netherlands), nowadays widely applied in the vegetable processing industry, was used to package the mushroom slices. These packages were heat sealed without gas packaging, so the

modified atmosphere was established in a passive way. For grated celeriac and shredded chicory endives, the modification of the atmosphere (3% O_2–5% CO_2–balance N_2) in the packages was performed in an active way by using a gas packaging unit (gas mixer, WITT M618-3MSO, Gasetechnik; vacuum compensation chamber, Multivac A300/42 Hagenmüller, Wolfertschwenden, Germany). Air Products supplied the gases (Fresh Line).

2.2.3. Microbial quality

The microbial quality of the packaged vegetables was followed with classical enumeration techniques. Lactic acid bacteria were counted by pour plating with top layer on MRS agar (30 °C for 3 days), aerobic psychrotrophic count was enumerated by pour plating on PCA (22 °C for 5 days), yeasts and moulds were quantified by spread plating on YGC after respectively 3 and 5 days of incubation at 30 °C. On mushrooms slices, the anaerobic mesophilic count was also monitored by pour plating with top layer on Reinforced Clostridial Agar (RCA (Oxoid; CM 151)) after 48 h of anaerobic incubation (addition of AeroGen™ sachet (Oxoid; AN025A) to a closed jar) at 30 °C.

2.2.4. Sensorial quality

The sensorial quality of the packaged vegetables was regularly evaluated by a trained taste panel consisting out of minimum six persons. Before the experiment started, the typical characteristics of the three types of applied minimally processed vegetables and the possibilities of deterioration were explained. All sensorial tests were performed in a special taste room with separated boxes and IR light. Organoleptical properties such as taste, flavour, odour and texture (perception of the mouthfeel) were evaluated under IR light to exclude influence of the visual characteristics. The visual properties (colour, dryness, general appearance) were judged under normal light. A numerical score between 1 (= excellent) and 10 (= extreme bad) was given for each property to describe the sensorial quality of the vegetables. The cut off score was defined at score 5. Below this score, the sample was still acceptable.

2.2.5. Statistical analysis

The storage experiment was conducted in duplicate. Results of gas concentration and microbial quality are given by the mean and standard deviation. Results of sensorial analysis were analysed by a Tukey and Duncan test by applying SPSS 9.0 for Windows 95 in order to determine significant differences ($P < 0.05$) between the applied modified atmospheres.

2.3. Respiration measurement

Vegetables were prepared in the same way as for the storage experiments. A sample of 100 g was introduced in a glass jar, specially designed for measuring respiration rates of produce by the closed system method (Jacxsens et al., 1999b). The jars were flushed with 95% O_2–balance N_2 with the same system as for the in-vitro study and the storage experiments, in order to quantify the respiration rate at high O_2-levels. Next to this, jars were closed with air inside in order to measure the respiration intensity at 3% O_2. The jars were placed at 4 °C and regularly, the headspace gas composition was analysed for O_2, CO_2 and N_2 by gas chromatography (Catharometer IGC11M-TCD, 2 faze column (Alltech, Laarne, Belgium), column temperature of 35 °C and helium as carrier gas (Air Liquide, Aalter, Belgium) with a flow of 0.33 ml/s). Out of the measured depletion of O_2 and production of CO_2, the respiration rate, expressed as ml O_2 consumed/kg h or ml CO_2 produced/kg h, could be calculated (Jacxsens et al., 1999b). The respiration measurements were conducted in triplicate.

3. Results and discussion

3.1. Effect of high O_2 on the growth characteristics of vegetable-associated microorganisms

3.1.1. Spoilage microorganisms (P. fluorescens, E. carotovora, Candida lambica)

The potential of high O_2 atmospheres (70%, 80% and 95% O_2) to reduce or inhibit the growth of a number of spoilage microorganisms was evaluated and compared with storage under a low O_2 atmosphere (5%). The gas composition inside the barrier bags, containing an inoculated plate, was controlled at every point of analysis. The O_2-levels did not change significantly until the stationary phase was reached at the end of the in-vitro test. In this way,

stable high or low surrounding O_2-levels could be assured for the performed in-vitro studies.

All isolated microorganisms were able to grow at 4 °C except *Lactobacillus brevis* and *Leuconostoc gasicomitatum*, whose minimal temperatures for growth are 2–4 °C (Björkroth et al., 2000; Teixeira, 2000). Table 1 gives an overview of the obtained results as the time necessary to reach $\geq 10^7$ CFU/cm^2 for G^- microorganisms and 10^5 CFU/cm^2 for the yeast *C. lambica*.

A difference in growth was found for *P. fluorescens* under 5% and 70%, 80% and 95% O_2. The time needed to obtain $\geq 10^7$ CFU/cm^2 was respectively 127.5 h at 5% and 173 h at 70%, 80% and

Table 1
Growth of spoilage microorganisms in the in-vitro experiment at 4 °C and under different O_2 concentrations (5%, 70%, 80% and 95% O_2, balance N_2)
Time necessary to reach $\geq 10^7$ CFU/cm^2 for *P. fluorescens* and *E. carotovora* and $\geq 10^5$ CFU/cm^2 for *C. lambica* is indicated in bold.

Time (h)	5% O_2 ($\log_{10}$ CFU/cm^2)	70% O_2 ($\log_{10}$ CFU/cm^2)	80% O_2 ($\log_{10}$ CFU/cm^2)	95% O_2 ($\log_{10}$ CFU/cm^2)
P. fluorescens				
0	3.181	3.273	3.181	3.217
34.5	4.283	3.905	3.880	3.427
43	4.475	3.763	3.854	3.632
57.5	4.795	3.854	3.905	4.357
76.5	5.339	4.972	5.029	4.552
101	6.488	6.218	6.181	5.206
127.5	**7.312**	6.645	6.252	5.928
149	7.649	6.553	6.542	mv
173	mv[a]	**7.482**	**7.795**	**7.154**
221	7.632	7.826	7.728	7.126
C. lambica				
0	1.796	1.427	1.552	1.853
118	**6.312**	**5.365**	4.273	3.011
144.5	6.303	mv	mv	3.455
166	6.455	6.240	**5.442**	4.599
242	6.181	6.229	6.589	mv
261.5	6.011	5.728	mv	**5.488**
289.5	6.168	5.795	6.312	mv
310	6.475	6.330	6.342	6.604
E. carotovora				
0	2.763	2.763	2.853	2.746
21.5	2.992	2.928	3.047	3.126
44.5	3.029	2.552	3.181	3.217
66	3.168	2.691	3.229	2.971
112.5	3.229	2.971	2.853	2.795
161.5	3.011	2.825	2.632	mv
258.5	3.029	2.365	2.029	3.064
330	3.649	2.574	mv	mv
354.5	2.928	2.552	mv	6.536
406.5	3.568	mv	mv	mv
426	3.168	5.917	**7.763**	**7.763**
454.5	6.181	**7.283**	7.154	7.640
471.5	mv	6.853	7.691	7.825
521.5	6.482	7.293	7.905	7.795
569.5	**7.374**	6.971	7.825	7.853
641.5	7.330	7.795	7.563	7.691

[a] mv = Missing value.

95% O_2 (Table 1). These results indicate a sensitivity of *P. fluorescens* for high O_2-levels. *Pseudomonas* spp. counted on high O_2 packaged fresh prepared potatoes showed to be inhibited by 70% O_2 (Day, 2001). An in-vitro test of *P. fluorescens* at 8 °C gave as well a reduced growth rate but these inhibitions were more pronounced when a combination of high O_2 (80%) and 20% CO_2 was used (Amanatidou et al., 1999). Gonzalez Roncero and Day (1998) reported that 90% O_2 alone did not prevent growth of *P. fragi* but reduced its growth rate by 14% at 8 °C.

The phytopathogenic *E. carotovora* seemed to be stimulated under increasing O_2-concentrations. Under 80% and 95% O_2, 426 h were necessary to reach $\geq 10^7$ CFU/cm^2, while it took under 70% 454.5 h and under 5% 569.5 h. This stimulated growth was also found for some microorganisms investigated by Amanatidou et al. (1999) during their conducted solid surface in-vitro studies (i.e. *Enterobacter agglomerans*) when high O_2 was applied alone.

The yeast *C. lambica* was clearly reduced in its growth by the application of high O_2-levels (Table 1). The effect of high O_2 was progressive: increasing O_2-concentrations resulted in an increasing reduction in growth: respectively under 5% and 70% 118 h, under 80% 166 h and under 95% 261.5 h. An O_2-level of 80% alone resulted in a stimulation of both *C. guilliermondii* and *C. sake* at 8 °C (Amanatidou et al., 1999). The combined application of 80% O_2 and 20% CO_2 almost completely blocked growth of *C. guilliermondii*. Similar growth characteristics were observed for *C. sake*, although to a lesser extent (Amanatidou et al., 1999).

The maximum cell amount of the three considered microorganisms was not affected by the different O_2-concentrations (Table 1). The effect of high O_2-levels is a reduction in their growth and development rather than a complete inhibition.

The experimental finding that high O_2 MAP is capable of inhibiting aerobic and anaerobic microbial growth can be explained by the growth profiles of aerobes and anaerobes (Day, 2000, 2001). It is hypothesised that active oxygen radical species damage vital cellular macromolecules and thereby inhibit microbial growth when oxidative stresses overwhelm cellular protection systems (Gonzalez Roncero and Day, 1998; Amanatidou et al., 1999). The belief that O_2 exerts its toxic effect through the formation of superoxide radicals (O_2^-), which destroy some aspects of cell metabolism, was already studied and reported before (Gregory and Fridovich, 1973). Haugaard (1968) attributed O_2 toxicity to such damaging events as the oxidation of sulfhydryl groups in various compounds, such as gluthion, or enzymes. He also suggested the possibility of peroxide formation, which can cause extensive destruction (particularly of lipids). In some cases, exposure to high O_2-concentrations does not affect or even stimulate the growth of the microorganisms. This indicates the presence of a protective strategy developed by the microorganisms such as induction of O_2-decomposing enzymes (catalase, peroxidase, superoxide dismutase) or radical scavengers (e.g. glutathion) in order to avoid lethal damage by oxygen (Amanatidou et al., 1999). At 30–37 °C, repair proteins were identified for *Salmonella typhimurium*, *Escherichia coli* and *L. lactis* (Farr and Kogoma, 1991; Amanatidou et al., 1999). However, few information is available about these systems in specific microorganisms at low temperatures.

3.1.2. Moulds

The development of two moulds, important in the spoilage of fresh plant products (*A. flavus* and *B. cinerea*), was monitored as the time (days) before visual appearance of the mycelium on the agar plate, duration (days) until visual appearance of spores and amount of spores after 120 days of storage (spores/ml). Table 2 summarises the obtained results. A clear reducing effect on the development of the mycelium of *A. flavus* and *B. cinerea* was found starting from 80% but more pronounced at 95% O_2. The amount of spores decreased by one log through the application of O_2-concentrations above 80% O_2 for *A. flavus*. The in-vitro studies for moulds were as well performed at 10 °C and the same evolution in reduction of fungal growth was found (data not shown). High O_2 (80%) in combination with 20% CO_2 was found to be particularly effective in inhibiting the growth of three common genera of fungi: *Rhizopus stolonifer*, *B. cinerea* and *Penicillium echinulatum* showed a clear reduction in mycelium diameter (Hoogerwerf et al., 2000). The high O_2-levels alone (80% O_2–20% N_2) were less effective in slowing down their development. Practical trials with

Table 2
The effect of high O_2 concentrations on development and sporulation of *A. flavus* and *B. cinerea* at 4 °C

Type of mould	Gas combination (%)	Duration (days) before visual appearance of mycelium	Duration (days) before visual appearance of spores	Amount of spores (spores/ml)
A. flavus	21% O_2–79% N_2 (air conditions)	33	56	1.4×10^6
	5% O_2–95% N_2	20	84	2.2×10^6
	70% O_2–30% N_2	20	71	1.2×10^6
	80% O_2–20% N_2	26	68	4×10^5
	95% O_2–5% N_2	72	95	4×10^5
B. cinerea	21% O_2–79% N_2 (air conditions)	30	70	nd[a]
	5% O_2–95% N_2	15	60	nd
	70% O_2–30% N_2	30	86	nd
	80% O_2–20% N_2	86	112	nd
	95% O_2–5% N_2	112	165	nd

[a] nd = No data available.

white grapes and other soft fruit showed that high O_2 MAP can be considered as a very effective treatment for inhibition of fungal growth (Bakker et al., 1998). An inhibition of moulds was also noticed by Wszelaki and Mitcham (1999) for *B. cinerea* (inhibition by 80–100% O_2 during in-vivo experiments) on strawberry fruits stored at 5 °C. The elevated O_2-concentration in the atmosphere might induce an increased activity of enzymes involved in protection against excess oxygen. In the cell, O_2 is converted via the superoxide anion to hydrogen peroxide and molecular oxygen. This hydrogen peroxide is scavenged by catalase and peroxidase. The peroxidase activity in *B. cinerea* doubled at 80% O_2 compared with the level found at ambient atmosphere (Hoogerwerf et al., 2000).

3.1.3. Pathogenic microorganisms

Two vegetable-associated psychrotrophic human pathogenic microorganisms were considered as well during the in-vitro studies in order to evaluate the safety of high O_2 MAP. The reducing effect on the growth of *L. monocytogenes* was expressed as a prolongation of the lag phase. The maximum cell amount between the different O_2-levels remained constant (Fig. 1). The reducing effect of the growth of *L. monocytogenes* is, however, the most detectable under 95% O_2. Amanatidou et al. (1999) published similar results for *L. monocytogenes*: growth rates were slightly affected under 90% O_2 alone or in combination with 10% CO_2 compared to air conditions at 8 °C. But the pathogen showed a prolonged lag phase in the presence of 90% O_2 (82 h) compared to air conditions (62 h). Maximum population densities (N_{max}) were neither affected in their study (Amanatidou et al., 1999).

A. caviae (HG4) was, as far as known, not tested before under high O_2 modified atmospheres. *A. hydrophila* was screened by Amanatidou et al. (1999) and Gonzalez Roncero and Day (1998) and showed a reduction under high O_2 (80–90%) combined with 10–20% CO_2 at 8 °C. The performed analysis at 4 °C and high O_2-concentrations alone showed a clear

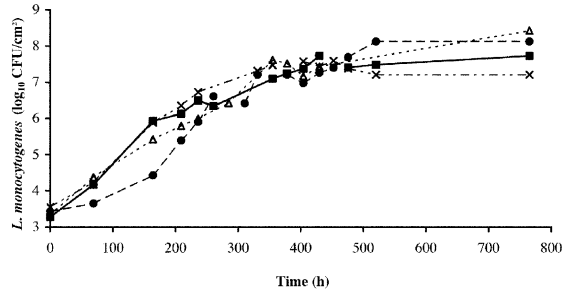

Fig. 1. Growth of *L. monocytogenes* on agar plates, stored at 4 °C under the following gas combinations: (■) 5% O_2–95% N_2, (△) 70% O_2–30% N_2, (×) 80% O_2–20% N_2, (●) 95% O_2–5% N_2.

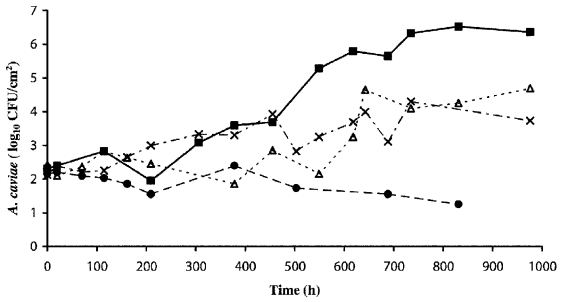

Fig. 2. Growth of *A. caviae* on agar plates, stored at 4 °C under the following gas combinations: (■) 5% O_2–95% N_2, (△) 70% O_2–30% N_2, (×) 80% O_2–20% N_2, (●) 95% O_2–5% N_2.

inhibition in the growth of *A. caviae* under 95% O_2 (Fig. 2). No significant growth (after 34 days) was found during the storage under 95% O_2. The growth was inhibited and the maximum cell yield decreased under increasing O_2-concentrations (70% and 80% O_2).

3.2. Effect of high O_2 on the respiration rate of minimally processed vegetables and residual O_2-concentration inside HOA packages

It could be concluded from the performed in-vitro experiments that if oxygen is considered as such (without inclusion of CO_2), concentrations of 95% O_2 are effective in reducing/inhibiting microbial growth. When fresh produce is packaged under a modified atmosphere, it is necessary to quantify the effect of the applied atmosphere on its respiration rate (Table 3). Grated celeriac and shredded chicory endive appeared to be less sensitive to the increased O_2-levels (no significant increase/decrease in respiration rate) compared to mushroom slices which are showing an increase in respiration intensity of respectively 59.5% and 64.8% at 80% and 95% O_2 compared to 3% O_2. Apparently, the tissue of mushrooms reacts completely different on the stress condition of HOA compared to the root tissue (grated celeriac) and leafy tissue (chicory endives). At 80% O_2, a CO_2-concentration around 5% was build up inside the closed respiration jars. These concentrations of CO_2 are expected not to have an inhibitory effect on the respiration rate (Kader et al., 1989; Jacxsens et al., 2000a).

Exposure to high O_2-levels may stimulate, have no effect or reduce rates of respiration, depending on the commodity, maturity and ripeness stage (Kader and Ben-Yehoshua, 2000). No results were found until now in literature concerning respiration rates of minimally processed vegetables subjected to high O_2-levels. Purvis (1997) suggested that during normal respiration (21% O_2), some reactive O_2 species are produced by the respiratory electron transport chain, but antioxidants (vitamins C and E) are present in sufficient concentration to prevent damage to the phospholipids and proteins of the membrane. In stressed plant tissue, when exposed to high O_2-levels, more reactive O_2 species are produced then there are antioxidants to destroy them which leads to damage of the membranes which can result in a wound respiration and consequently, in an increased respiration rate.

For the application of high O_2 modified atmospheres for minimally processed vegetables, an initial atmosphere of 95% O_2–5% N_2 was introduced inside the barrier packages. The CO_2-level accumulated inside these packages because of natural respiration and reached bacteriostatic or antimicrobial levels (i.e. > 20% CO_2) after 3 to 4 days of storage

Table 3
Respiration rate (RO_2 in ml O_2/kg h ± 95% confidence interval) of minimally processed vegetables stored at 4 °C and exposed to high and low O_2 concentrations

Minimally processed vegetable	RO_2 (ml O_2/kg h) at 3% O_2	RO_2 (ml O_2/kg h) at 80% O_2	RO_2 (ml O_2/kg h) at 95% O_2
Grated celeriac	7.43 ± 1.33	8.76 ± 4.07	10.86 ± 3.36
Mushroom slices	14.68 ± 4.51	36.29 ± 0.29	41.69 ± 2.95
Shredded chicory endive	9.84 ± 1.57	7.95 ± 1.07	10.18 ± 0.97

Table 4
Evolution of oxygen (% ± standard deviation) and carbon dioxide concentration (% ± standard deviation) of grated celeriac, mushroom slices and shredded chicory endive packaged under High Oxygen Atmosphere in barrier film and stored at 4 °C

Storage time days	Grated celeriac		Mushroom slices		Shredded chicory endive	
	% O_2 (± standard deviation)	% CO_2 (± standard deviation)	% O_2 (± standard deviation)	% CO_2 (± standard deviation)	% O_2 (± standard deviation)	% CO_2 (± standard deviation)
0	95 ± 0	0 ± 0	95 ± 0	0 ± 0	95 ± 0	0 ± 0
3	58.3 ± 1.3	21.5 ± 1.9	15.8 ± 2.4	46.9 ± 2.2	68.1 ± 2.8	16.6 ± 0.3
4	59.0 ± 6.2	27.2 ± 2.1	12.6 ± 3.4	57.5 ± 2.5	69.3 ± 6.5	22.3 ± 3.6
5	55.5 ± 3.2	32.1 ± 0.4	2.9 ± 3.8	75.85 ± 5.9	55.7 ± 15.1	21.9 ± 4.7
6	30.8 ± 1.6	38.7 ± 0.5	4.1 ± 5.6	63.0 ± 10.6	56.3 ± 3.3	31.7 ± 4.7
7	19.9 ± 8.8	45.5 ± 7.9	6.8 ± 0.9	47.5 ± 5.2	12.0[a]	12.3[a]

[a]Only one package was measured.

at 4 °C. The evolution of the gas concentration (% O_2, % CO_2) is illustrated in Table 4 for grated celeriac, mushroom slices and shredded chicory endive, packaged under high oxygen concentrations and stored at 4 °C. The oxygen concentration dropped fast the first 3 days of storage in the packages of grated celeriac, remained more or less constant to result in a sharp drop at days 6 and 7 of the storage period in agreement with a rise in microbial count, as microorganisms are consuming O_2 as well during their respiration (Tables 5 and 6). The gas composition inside the packages of shredded chicory endives changed in the same way, because their respiration rate is similar in high O_2-conditions (Table 3). The mushroom slices, on the other hand, respired faster and consequently the internal oxygen was consumed more quickly. Although no anaerobic conditions were created ($O_2 > 2\%$), the CO_2-levels reached very high values (Table 4).

3.3. Effect of high O_2 on the microbial quality of minimally processed vegetables

Lactic acid bacteria are not important in the spoilage of the three types of tested vegetables as their number remained low (average of 100 CFU/g) also under equilibrium modified atmosphere. The storage temperature of 4 °C was slowing down their growth compared to previously conducted experiments at 7 °C (Jacxsens et al., 1999a,b, 2001, submitted for publication).

Yeasts seemed to be the limiting microorganisms for the investigated EMA packaged minimally processed vegetables (Table 5). Their limiting level was fixed at 10^5 CFU/g because above this number spoilage of products becomes detectable for consumers (Debevere, 1996; Jacxsens et al., 1999a). On the celeriac, rich in sugar, yeasts were growing fast and exceeded the limit already after 3 days of stor-

Table 5
Growth of yeasts ($\log_{10}$ CFU/g ± standard deviation) on grated celeriac, shredded chicory endives and mushroom slices packaged under High O_2 Atmosphere (HOA) and Equilibrium Modified Atmosphere (EMA) stored at 4 °C
Values above the limiting level for ready-to-eat vegetables (not for mushrooms) of 10^5 CFU/g are indicated in bold.

Storage time (days)	Grated celeriac		Mushroom slices		Shredded chicory endive	
	HOA	EMA	HOA	EMA	HOA	EMA
0	2.44 ± 0.42	2.44 ± 0.42	5.65 ± 0.06	5.65 ± 0.06	3.21 ± 0.19	3.21 ± 0.19
3	3.39 ± 0.40	**5.56 ± 0.16**	5.25 ± 0.10	5.83 ± 0.61	4.33 ± 0.21	4.84 ± 0.19
4	4.72 ± 0.25	**5.00 ± 0.06**	6.16 ± 0.97	6.03 ± 0.47	4.30[a]	**5.40 ± 0.36**
5	4.71 ± 0.47	**5.99 ± 0.30**	4.37 ± 0.41	4.72 ± 0.12	4.49 ± 0.09	**5.90 ± 0.42**
6	**5.13 ± 0.07**	**6.52 ± 0.34**	4.92 ± 0.32	5.99 ± 1.00	4.85 ± 0	**6.28**[a]
7	**5.85 ± 0.61**	**6.82 ± 0.02**	5.46 ± 0.02	5.74 ± 0.06	**5.47 ± 0.34**	**6.08**[a]

[a]Result of only one package was usable.

age in the EMA packages. The high O_2 packages, on the other hand, were not acceptable anymore after 6 days of storage at 4 °C. A difference between the two atmospheres in the development of yeasts was detected as well for shredded chicory endives. The packages of shredded chicory endives under low O_2-concentrations exceeded the limit at day 4 while the high O_2 packages exceeded the limit at day 7. During previous conducted storage experiments with shredded chicory endives packaged under EMA, almost the same initial count was found (3.0 ± 0.0 log CFU/g) but after 6 days of storage at 7 °C, no limiting level was obtained for yeasts (Jacxsens et al., 1999a). At that temperature, lactic acid bacteria were also developing (until 5.66 ± 0.26 log CFU/g after 6 days of storage at 7 °C) and were probably playing a role in the competition of nutrients which was not the case during the storage experiment at 4 °C.

The limiting criteria for ready-to-eat vegetables, proposed by Debevere (1996) and CNERNA-CNRS (1996) cannot be applied for mushrooms slices. The tissue of mushroom differs in structure and composition from the one of vegetables. The initial contamination by yeasts of the mushroom slices was 5.65 ± 0.06 log CFU/g and did not significantly increase during storage under both EMA and HOA (Table 5).

The limiting amount of 10^8 CFU/g of aerobic psychrotrophic count was not obtained for both the grated celeriac and shredded chicory endives (increase of 1.04 $\log_{10}$ CFU/g on HOA packaged celeriac in 7 days, increase of 2.85 $\log_{10}$ CFU/g on EMA packaged celeriac in 7 days, increase of 1.49 $\log_{10}$ CFU/g on both EMA and HOA packaged chicory endives in 7 days). A difference in aerobic psychrotrophic count for grated celeriac packaged under the high O_2 and the low O_2 atmospheres was observed during 7 days (6.04 ± 0.80 $\log_{10}$ CFU/g on HOA packaged while 7.85 ± 0.32 $\log_{10}$ CFU/g on EMA packaged celeriac at day 7).

Also for the mushroom slices a difference in aerobic psychrotrophic count was detected (6.46 ± 0.06 $\log_{10}$ CFU/g on HOA packaged while 7.34 ± 0.87 $\log_{10}$ CFU/g on EMA packaged mushroom slices at day 7). Anaerobic mesophilic count did not change as much during the storage of the mushroom slices at 4 °C (initially 4.54 ± 0.04 $\log_{10}$ CFU/g, 5.35 ± 0.79 $\log_{10}$ CFU/g on HOA packaged and 5.00 ± 0.21 $\log_{10}$ CFU/g on EMA packaged mushrooms after 7 days of storage). Apparently, G^- microorganisms were responsible for the spoilage of the mushrooms slices (no outgrowth of yeasts and lactic acid bacteria either).

3.4. Effect of high O_2 on the sensorial quality of minimally processed vegetables

3.4.1. Visual qualities

Scores, given by the trained taste panel, at the individual properties of the minimally processed vegetables were analysed by a test of variance. The colour of the cut surfaces was the most determinative property of the visual quality. Table 6 illustrates the evolution of the mean score for colour of grated

Table 6
Evolution of score (average ± 95% confidence interval) for the colour of shredded chicory endives, grated celeriac and mushrooms slices during the storage at 4 °C under High Oxygen Atmosphere (HOA) or Equilibrium Modified Atmosphere (EMA)
Values increasing the cut off score of 5 are indicated in bold.

Storage time (days)	Grated celeriac		Mushroom slices		Shredded chicory endive	
	HOA	EMA	HOA	EMA	HOA	EMA
0	3.00 ± 1.56	3.00 ± 1.56	3.50 ± 1.31	3.50 ± 1.31	3.64 ± 2.02	3.64 ± 2.02
3	2.50 ± 0.71	4.50 ± 2.12	2.80 ± 1.48	**5.20 ± 1.30**	3.42 ± 1.81	**6.00 ± 2.00**
4	nd[a]	**7.80 ± 1.64**	4.00 ± 1.09	nd	3.00 ± 1.87	**5.60 ± 2.19**
5	3.00 ± 2.34	**8.17 ± 1.17**	5.00 ± 2.12	**5.40 ± 0.89**	2.00 ± 1.09	**7.50 ± 1.87**
6	3.40 ± 0.89	**9.60 ± 0.89**	**6.25 ± 0.96**	**5.40 ± 1.91**	1.60 ± 0.55	**6.60 ± 1.51**
7	4.40 ± 2.19	**9.60 ± 0.89**	nd	nd	nd	nd

[a] nd = No data available.

celeriac, shredded chicory endives and mushroom slices during storage time. Polyphenoloxidase (PPO) is the enzyme primarily responsible for initiating discoloration on the cut surfaces of prepared produce. PPO catalyses the oxidation of natural phenolic substances to colourless quinones which subsequently polymerise to coloured melanin-type compounds (McEvily et al., 1992). Low O_2 atmospheres cause a reduction in the enzymatic discoloration because O_2 is a necessary substrate for the enzymatic reaction (Kader et al., 1989), but high O_2 atmospheres were more effective in reducing or inhibiting the enzymatic discoloration of the three tested minimally processed vegetables (Table 6 and Fig. 3). The applied cut off score was fixed at 5. A score above 5 indicates an unacceptable sample. All EMA packages exceeded that score before the HOA packages (Table 6). The mushroom slices, packaged under high O_2-concentrations, were rejected after 6 days of storage, while after 3 days when packaged under low O_2 atmospheres. A more than 12-day shelf life based on sensorial qualities (appearance, odour) was described by Day (2001) for mushroom slices packaged under 80% O_2–20% N_2 and stored at 8 °C, while packaged in an OPP-film (low O_2 concentrations), only 2 days could be given as shelf life period.

The enzymatic discoloration of grated celeriac and shredded chicory endive was evolving fast as well under EMA: 4 and 3 days, respectively, while no unacceptable scores were obtained for the HOA packaged vegetables. For other types of fresh prepared produce which are sensitive for enzymatic discoloration (i.e. iceberg lettuce, radicchio lettuce, lollo rossa lettuce,...) high O_2 modified atmospheres were also found to be beneficial in sensorial quality (Heimdal et al., 1995; Day, 2000, 2001).

3.4.2. Organoleptical qualities

No significant difference between both methods of packaging could be found for the shredded chicory endives based on the organoleptical properties (taste, flavour, odour and texture) also no significant decrease in the organoleptical quality was found during storage of chicory endives (data not shown).

The loss of structure was the most determinative property for grated celeriac. The thin batons of grated celeriac were already rejected after 2 days of storage under EMA and 3 days of storage under HOA based on their loss of firmness. No significant difference could be made for the other organoleptical qualities except for the taste of the grated celeriac: day 7 average score of 7.6 for the EMA packages while an average score of 3.4 for the HOA packages. Oxygen is indeed known to be responsible for a fresh tasting and smelling product (Kader et al., 1989; BCGA, 1998). In texture and taste characteristics, no significant difference was found between the two atmospheres for the mushroom slices. Here the odour was determinative: during the 7 tested days, the mushrooms slices stored under the high O_2 atmospheres kept their fresh odour while on day 6 an unacceptable odour was detected for the EMA packaged mushroom slices. The effect of high O_2-concentrations on the organoleptical properties is not signifi-

Fig. 3. Grated celeriac packaged under Equilibrium Modified Atmosphere (EMA) packaging (left) and High O_2 Atmospheres (HOA) (right) after 7 days of storage at 4 °C.

Table 7
Overall achievable shelf life (days) obtained for fresh prepared mushroom slices, grated celeriac and shredded chicory endives, stored at 4 °C

Minimally processed vegetable	MAP treatment	Limit of sensorial quality (days)	Limit of microbial quality (days)	Overall achievable shelf life
Grated celeriac	HOA[a] in barrier film	> 7	6 (yeasts)	6
	EMA[b] in high permeable film	4 (colour)	3 (yeasts)	3
Mushroom slices	HOA in barrier film	6 (colour)	–[c]	6
	EMA in high permeable film	3 (colour)	–	3
Shredded chicory endives	HOA in barrier film	> 7	7 (yeasts)	7
	EMA in high permeable film	3 (colour)	4 (yeasts)	3

[a] High Oxygen Atmosphere.
[b] Equilibrium Modified Atmosphere packaging.
[c] No conclusions could be made because no limiting criteria are available for mushrooms.

cant except for the odour and taste: celeriac and mushroom slices are evaluated as fresher when stored under high O_2-levels.

3.5. Determination of overall achievable shelf life

Table 7 summarises the overall achievable shelf life obtained from the conducted comparative storage experiments at 4 °C. It can be concluded that the high oxygen atmosphere (95% O_2–5% N_2) had a beneficial effect on the colour retention of the three tested minimally processed vegetables which are sensitive for enzymatic browning. The other sensorial quality attributes were not significantly affected compared to the low O_2 stored vegetables. Although a trend towards a more fresh odour and taste was noticed by the application of high O_2-levels as initial atmosphere. Yeasts were reduced in their growth by HOA both in the in-vitro and the in-vivo studies. Again, yeasts appeared to be the limiting microorganisms in the microbiological quality of the minimally processed vegetables which was also found in previous work conducted at 7 °C (Jacxsens et al., 1999b). When high oxygen atmospheres are applied in combination with a barrier film, it is not necessary to introduce additional CO_2 initially inside the packages. Carbon dioxide is produced in a natural way by produce respiration and is reaching fast antimicrobial levels (> 20% CO_2). The overall achievable shelf life is at least doubled by using high oxygen atmospheres compared to the low EMA packages for the three tested minimally processed vegetables, when stored at 4 °C and this is based on the combination of suppressing enzymatic discoloration and yeast growth.

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