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Microbial Quality and Browning of Fresh-cut Butter Lettuce under Superatmospheric Oxygen Condition

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Summary

Fresh-cut butter lettuce inoculated with *Listeria innocua* was stored under controlled atmospheres (CA) containing 5 kPa O_2 and 0 kPa CO_2, 5 kPa O_2 and 15 kPa CO_2, 75 kPa O_2 and 0 kPa CO_2, 75 kPa O_2 and 15 kPa CO_2, and 21 kPa O_2 and 0 kPa CO_2 (as control) for 10 days at 7 °C. The growth of *L. innocua,* aerobic mesophilic bacteria, lactic acid bacteria and yeasts was studied. Additionally, the respiration rates and visual appearance of the butter lettuce were monitored in all treatments. The browning was measured on the lettuce stems using L*, chroma and hue values. High CO_2 conditions promoted the growth of *L. innocua.* No O_2 effect was found on the growth of *L. innocua*. However, when high O_2 and CO_2 conditions were combined, a reduction in the aerobic mesophilic bacterial count was observed. A high O_2 condition alone could reduce the mesophilic count to the same extent as low O_2 levels combined with high CO_2 levels while avoiding anaerobic fermentation reactions. The studied CA conditions slightly affected the respiration rates of fresh-cut butter lettuce. CO_2 treatments decreased the browning of the lettuce stems. Colour changes were also slightly delayed on the stems under high O_2 levels. At the end of the storage period, fresh-cut butter lettuce stored under 75 kPa O_2 with 15 kPa CO_2 had the best visual appearance.

Key words. high oxygen – high carbon dioxide – *Listeria innocua* – *Listeria monocytogenes* – minimally processed – modified atmosphere packaging

Introduction

Controlled atmosphere (CA) or modified atmosphere packaging (MAP) with low O_2 and moderate or high CO_2 levels are generally used to reduce the respiration rate of fruits and vegetables. However, the effects of reduced O_2 and elevated CO_2 concentrations vary greatly among the species (Gorny 1997; Kader et al. 1989). According to the recommendations, concentrations of 1–8 kPa O_2 combined with 10–20 kPa CO_2 maintain a good quality of fresh-cut lettuce at 0–2 °C. Under this condition, the phenolic compounds production in fresh-cut lettuce is delayed (López-Gálvez et al. 1996; Gorny 1997; Hertog et al. 1998).

As for the respiration rate, Kader and Ben-Yehoshua (2000) reported that exposure to superatmospheric O_2 concentration may stimulate, have no effect, or reduce rates of respiration and ethylene production, depending on the commodity, maturity and ripeness stage, O_2 concentration, storage time and temperature, and concentrations of CO_2 and C_2H_4 present in the atmosphere. Respiration intensity is directly correlated to the shelf life of produce, and the quantification of the effect of high O_2 levels on the respiratory activity is necessary to determine the gas compositions of MAP (Jacxsens et al. 2001b).

According to Day (1995, 2001) a novel type of MAP using superatmopheric O_2 conditions is effective in inhibiting enzymatic discoloration, preventing anaerobic fermentation reactions and undesirable moisture and odour losses and reducing aerobic and anaerobic microbial growth.

High oxygen concentrations could be particularly effective for fresh-cut vegetables that are sensitive to enzymatic browning and spoilage by yeasts (Jacxsens et al. 2001b; Pérez and Sanz 2001; Hoogerwerf et al. 2002). Tissue browning due to oxidation of phenolic compounds by polyphenol oxidase results from loss of compartmentalisation within the cells when exposed to physical and /or physiological stresses. There are few published reports on the effects of elevated O_2 on enzymatic browning (Kader and Ben-Yehoshua 2000). An atmosphere of 80 kPa O_2 plus 20 kPa CO_2 delayed browning of shredded lettuce under MAP for 10 days at 5 °C compared to air (Heimdal et al. 1995).

Several studies on the effect of high O_2 and CO_2 concentrations have been performed (Day 1995, 2001; Allende et al. 2004; Geysen et al. 2005a, b). Exposure to high O_2 levels did not inhibit microbial growth strongly, while a high CO_2 level reduced the growth to some extent in most cases. The combined treatment of a high O_2 level and 10–20 kPa CO_2 may provide adequate suppression of microbial growth and prolonged shelf-life (Amanatidou et al. 1999; Allende et al. 2004; Geysen et al. 2005a, b). Applying a high O_2 atmosphere in a high-barrier film had a

beneficial effect on the microbial and sensory shelf life of raspberries and strawberries inhibiting the development of moulds and maintaining fresher sensory properties (Van Der Steen et al. 2002). Fresh-cut salads packaged in a low or a high barrier film with an initial oxygen level of 95 kPa or conventional MAP conditions did not show growth of *Listeria monocytogenes* at 4 °C (Allende et al. 2002). Nevertheless Jacxsens et al. (2001a) found that *L. monocytogenes* was able to grow on the green top of strawberry fruit packed in superatmospheric O_2 levels at 7 °C.

Based on the results of controlled atmosphere storage experiments, the most effective high O_2 gas mixtures were found to be 80–85 kPa O_2 / 15–20 kPa CO_2; these had the most noticeable sensory quality and microbial benefits on a range of fresh prepared produce items (Day 2001).

Butter lettuce is mechanically and physiologically fragile and according to some reports it has a short shelf life period when minimally processed (Varoquaux et al. 1996). An increased demand for butter lettuce from European Nordic countries has induced an expansion of the butter lettuce cultivated area under greenhouse (1.750 ha in 2003) in Belgium (Vbt 2003). There is no information available in the literature about the effect of superatmospheric oxygen conditions on the quality of fresh cut butter lettuce.

The objective of the present research was to study the effect of elevated CO_2 and low and high O_2 levels on the microbial quality with special attention on growth of *Listeria innocua*. Storage temperature was selected at 7 °C, as fresh-cut vegetables are often stored or distributed between 5–8 °C (maximum allowed temperature) according to the several European countries regulations (Francis et al. 1999). *L. innocua* was used as a model organism for the pathogenic *L. monocytogenes*. The virulence of different strains of *L. monocytogenes* and *L. innocua* were studied inoculating pregnant mice. In this work, *L. innocua* had an invasiveness and colonization of mice livers and spleens comparable to *L. monocytogenes* (Lammerding et al. 1992). According to Hugas et al. (1998) and Thomas et al. (1999), *L. innocua* and *L. monocytogenes* showed a similar response to changes of temperature, acidification and gas atmospheres. Therefore, *L. innocua* can be utilized to study the growing of *L. monocytogenes* avoiding the health risks associated with the use of a virulent strain. In a previous report, our group developed a predictive growth model of *L. innocua* in vitro under superatmospheric oxygen and carbon dioxide conditions (Geysen et al. 2005a). Now in a second step the in vitro findings need to be validated for an in vivo situation. The influence of the CA conditions on the respiration rate, the browning and the visual appearance of fresh-cut butter lettuce were evaluated considering the importance of this vegetable for the Belgian grower.

Materials and Methods

Sample preparation

Butter lettuces (*Lactuca sativa* L.) cv. 'Zendria' grown in a commercial greenhouse in Mechelen (Belgium) in August were used for the experiment. Lettuces were transported to the laboratory (40 km) and stored at 1 °C until used the next morning. In a clean room at 7 °C, the lettuces were processed after removing soiled and decayed external leaves.

The lettuce was hand cut in pieces of about 2 cm wide using a sharp knife discarding the central stem as would have been done on an industrial line. Immediately the cut lettuce was immersed for 1 min in tap water at 5 °C and centrifuged for 30 sec using a hand operated domestic centrifuge with a plastic container with a diameter of 30 cm. This operation removed excess water without causing any visible damage to the lettuce.

Experimental design and set-up

Samples of 160±2 g cut lettuce were put into 1.7 L glass jars and stored in a cool room set at 7 °C (6.8±0.5 °C). The temperature was monitored every 10 min with temperature loggers (Escort Junior, Tech Innovators, New Zealand). Five controlled atmospheres (CA), 75 kPa O_2+0 kPa CO_2, 75 kPa O_2+15 kPa CO_2, 5 kPa O_2+0 kPa CO_2, 5 kPa O_2+15 kPa CO_2 and 20 kPa O_2+0 kPa CO_2 (as control) were applied. Pure gases (oxygen, carbon dioxide and nitrogen) were mixed using mass-flow controllers (model 5850S, Brooks Instrument, The Netherlands). The obtained gas mixtures were humidified by passage through a distilled water bottle. For each gas mixture in the study, four jars were connected in series and flushed continuously with a flow rate of 10 L h^{-1}. Gas concentrations inside the jars were checked using a micro-GC (Varian-Chrompack, Bergen op Zoom, The Netherlands), and were found to be constant during the experiment and identical in all jars within a series. Before the input of each jar a 0.45 µm pore size filter (Nylon Filter Media with Polypropylene Housing, Whatman, Clifton NJ, USA) was used to avoid the external contamination on the lettuce samples.

The respiration rate of the butter lettuce was measured in each of the four jars of each of the five CA conditions on day 2. On day 3, 5, 7 and 10 of storage one jar was removed from each CA series for microbiological analysis. An additional set of five jars was prepared to determine the initial microbial load at day 0. The visual appearance was evaluated on day 0, 5 and 10 on the remaining jars.

Listeria innocua inoculation

L. innocua Seeliger 1983 strain CIP 80.12 (isolated from faeces), obtained from the Institute Pasteur (Paris, France), was used. Bacteria were stored at −80 °C in nutrient broth (NB, Oxoid, Hampshire, England) supplemented with 25 % glycerol. Two subsequent subcultures were grown in NB at 37 °C until stationary phase, respectively for 24 h and 21 h. From the second subculture a dilution series was made in PPS (Peptone Physiological Salt containing 8.5 g L^{-1} NaCl and 1 g L^{-1} bacteriological peptone, L37-Oxoid). The ability of the selected *L. innocua* strain to grow at 7 °C was confirmed in preliminary tests on selective nutrient agar under ambient atmosphere (data not shown). Lettuce samples were inoculated with 5 mL of *L. innocua* dilution (5 log cfu g^{-1}) to obtain a concentration of 3–4 log cfu g^{-1} in 160 g cut lettuce. Firstly, 2.5 mL of dilution (5 log cfu g^{-1}) was poured when the jar was filled with the half of the sample (80 g cut lettuce). The other 2.5 mL of dilution was poured when the

jar was completed with cut lettuce. The jar was closed and shaken for approximately five seconds to homogenise the inoculation.

Microbiological analysis

From a jar containing 160 g of lettuce, three random samples of 40 g were taken. The remainder of the jar was discarded and not further used in the experiment. Each sample of lettuce was blended with 360 mL of PPS for 1 min into a sterile stomacher bag (Model 400 Bags 6141, London, UK) by using a Masticator (Basic 0470, IUL Instrument, Barcelona, Spain). Serial dilutions were prepared in 9 mL PPS.

From each dilution, 1 mL aliquots were aseptically pipetted for accessing bacterial microflora and 0.1 mL for yeasts and moulds plating. The following media and incubation conditions were used: selective agar base (CM0856, Oxoid, Hampshire, England) for *L. innocua* counts by spread plating, incubated for 2 days at 35 °C; plate count agar (PCA, CM0325, Oxoid, Hampshire, England) for mesophilic microflora counts by pour plating, for 48 h at 30 °C; MRS agar (CM0361, Oxoid, Hampshire, England) for lactic acid bacteria (LAB) counts by pour plating with top layer, for 4 days at 25 °C; and Rose Bengal Chloramphenicol agar base (CM0549, Oxoid, Hampshire, England) for yeasts and moulds counts by spread, 5 days at 22 °C. All microbial counts were reported as log colony forming units per gram sample (log cfu g^{-1}). Microbial quality was evaluated following European recommended limits (Cnerna-Cnrs 1996; Debevere 1996).

Respiration rate measurements

The respiration rates of fresh-cut butter lettuce were measured on day 2 of storage. Per CA treatment, four jars were closed and the initial head space composition (O_2, CO_2 and N_2) was measured with a micro-GC (Chrompack CP 2002, Bergen op Zoom, The Netherlands). The headspaces were analysed again after 2 h. The difference in gas concentration was converted to the molar concentration according to the ideal gas law. The O_2 consumption and CO_2 production rates were expressed in mol per fresh weight of sample (kg) and per unit time (s).

Visual appearance evaluation

Immediately after cutting and inoculating the lettuce sample (day 0) and on day 5 and 10 of the storage period, five people from our laboratory with professional experience in fresh quality of vegetables evaluated the visual appearance of the fresh-cut lettuce. The panellists evaluated the lettuces samples placed and closed within its jar as a consumer in supermarket looking at a display cabinet. All jars were exposed simultaneously to the panellist. To judge the cut lettuce the panellist was asked to choose either acceptable or unacceptable. Samples were acceptable when the visual quality was higher than the limit of marketability and unacceptable when they were not. The samples were not eaten for safety reasons.

Colour measurements

Colour changes during storage were measured on lettuce stems of 3 mm thick. These discs were hand cut from the first 10 mm of the lettuce stem using a sharp knife. Immediately the discs were immersed for 1 min in tap water at 5 C and centrifuged using the same hand domestic centrifuge as was used for the cut lettuce.

The stems were stored at the same condition as the fresh-cut lettuce but in separate jars. In one jar, nine stems were placed and two jars were used per treatment. A colorimeter (Minolta CR-300, Ramsey, NJ, USA), with 8 mm viewing aperture diameter was used. A white plate (Y=94.3; x=0.3142; y=0.3211, standard CIE illuminate, 2 ° observer) was used as reference and the colour values were expressed as L*, hue angle ($H°=\tan^{-1}b^*/a^*$) and chroma $[(a^{*2}+b^{*2})']$ (McGuire 1992). The measurements were done on the middle of the stem and on both sides on day 0, 3, 5, 7 and 10 of storage. This method has been previously applied for lettuce. L* was found to be the best parameter related with enzymatic browning (Casta er et al. 1999; Escalona et al. 2004).

Statistical analysis

The effect of O_2 or CO_2 levels and storage time on the microbial growth of fresh-cut lettuce and the colour changes on the lettuce stems was evaluated using a two-way analysis of variance (ANOVA) with a 95 % confidence level. To asses the effect of CA treatments on the respiration rates a one-way ANOVA with a 95 % confidence level was carried out. By comparing mean values by the Duncan multiple range test significant differences among treatments were analysed. The SAS/STAT® version 8.2 (SAS Institute Inc., Cary, North Carolina, United State) software package was used for statistical analysis.

Results

Respiration rate

The respiration rate of fresh-cut butter lettuce was 119.9 nmol CO_2 kg^{-1} s^{-1} and 165.8 nmol O_2 kg^{-1} s^{-1} in air atmosphere at 7 °C (Table 1). A significant reduction of the respiration rate was observed when 5 kPa O_2 was combined with 15 kPa CO_2. Oxygen consumption significantly increased under 5 kPa O_2 and 0 kPa CO_2 and decreased significantly under 75 kPa O_2 and 0 kPa CO_2 compared to air atmosphere storage. The respiratory quotient was less than one in all treatments. At O_2 concentrations of 5 kPa RQ values similar to air atmosphere (about 0.7) were obtained. Under CA with 75 kPa O_2, the RQ was about 0.9.

Microbial growth

From the 3rd day of storage *L. innocua* counts were significantly higher on lettuce stored at 5 or 75 kPa O_2 plus 15 kPa CO_2. At the end of storage, treatments in CO_2 enriched atmospheres obtained around 6.1 log cfu g^{-1} whereas the final counts were around 4.8 log cfu g^{-1} in atmosphere without CO_2 (Fig. 1A). Nevertheless, the O_2 levels 5 and 75 kPa were not significantly affecting the growth of *L. innocua* ($P < 0.05$) compared to the air atmosphere.

Aerobic mesophilic counts increased during storage from 5.6 log cfu g^{-1} at day 0 to 8.3–9.2 log cfu g^{-1} at day 10. In air atmosphere and 5 kPa O_2 plus 0 kPa CO_2, the

Table 1. Respiration rate and respiratory quotient (RQ) of fresh-cut butter lettuce in CA at 7 °C. Mean (n=4) ± SE.

CA treatment		Respiration rate		RQ
O_2 (kPa)	CO_2 (kPa)	nmol CO_2 kg^{-1} s^{-1}	nmol O_2 kg^{-1} s^{-1}	(mol CO_2/mol O_2)
5	0	132.8±2.2 ab [z]	184.5±1.6 a	0.72±0.01 b
5	15	104.2±2.0 c	137.9±2.4 d	0.75±0.01 b
75	0	131.9±4.7 ab	144.3±5.1 dc	0.91±0.00 a
75	15	141.7±8.7 a	154.1±5.3 bc	0.92±0.06 a
21	0	119.9±2.0 b	165.8±3.7 b	0.72±0.01 b

[z] Values within a column followed by the same letter are not significantly different (P<0.05) according to Duncan multiple range test.

counts were significantly higher in comparison to 75 kPa O_2 alone during 7 days at 7 °C. High O_2 level had a significant delaying effect of 1 log cfu g^{-1} on the aerobic mesophilic growth until day 7 (P<0.05). Additionally, when 5 or 75 kPa O_2 were combined with 15 kPa CO_2 the aerobic mesophilic counts on fresh-cut lettuce were significantly diminished in comparison to 5 or 75 kPa O_2 without CO_2 (Fig. 1B). Therefore, superatmospheric O_2 condition (75 kPa) and high CO_2 level (15 kPa) maintained the microbial count of aerobic mesophilic bacteria below the maximum limit (7.7 log cfu g^{-1}) allowing a longer shelf life of lettuce to 7 days (Fig. 1B). In 5 kPa O_2 plus 15 kPa CO_2 and 75 kPa O_2 plus 0 kPa CO_2 the shelf life decreased to 5 days.

The LAB growth was significant until day 5 in all treatments. After 5 days of storage, the counts under 15 kPa CO_2 were higher than those without CO_2 (Fig. 1C). After 10 days of storage, the counts were about 6.9 log cfu g^{-1} for CO_2 treatments and 5.5 log cfu g^{-1} for those with 0 kPa CO_2. The growth of these microorganisms was not dependent on the O_2 concentration.

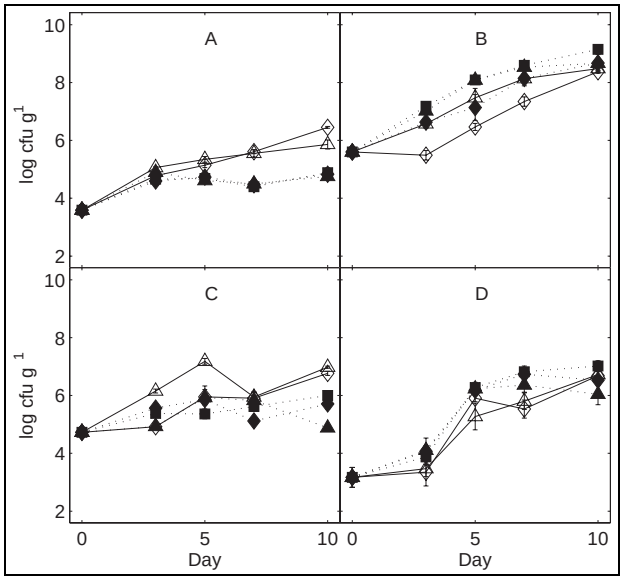


Fig. 1. Changes in the population of *Listeria innocua* (A), aerobic mesophilic bacteria (B), lactic acid bacteria (C), and yeasts (D) on fresh-cut butter lettuce during 10 days at 7 °C. The symbols represent the average value for three replicates and bars represent ± SE (◇: 75 kPa O_2+15 kPa CO_2; ◆: 75 kPa O_2+0 kPa CO_2; △: 5 kPa O_2+15 kPa CO_2; ▲: 5 kPa O_2 +0 kPa CO_2; ■: 21 kPa O_2+0 kPa CO_2).

Fresh–cut lettuce under CA had a shelf life according to the maximum limit for yeasts indicated by European standard (5 log cfu g^{-1}) of 3 days at 7 °C. CA with 15 kPa CO_2 significantly delayed the growth of yeasts with around 1 log unit until 7 days of storage. O_2 concentration between 21–75 kPa did not have any effect on the growth of yeasts. Treatments with 5 kPa O_2 alone showed counts lower than those with 20 or 75 kPa O_2 during day 7 and 10 of the storage (Fig. 1D). The growth of moulds was always below 4 log cfu g^{-1} (European standard limit) during the storage. Neither O_2 nor CO_2 levels had any significant effect on the growth of moulds (data not shown).

In general, a low or high O_2 condition (5 or 75 kPa) alone did not have a significant effect on the microbial quality of fresh-cut butter lettuce compared to control. However, a high CO_2 level (15 kPa) showed a significant decreased growth rate on most microbial species except the opposite was observed for *L. innocua*.

Visual appearance

All the panellists scored the samples independently. However, the effect of the different conditions was so obvious that all panellists agreed on every individual sample and attributed the same score (acceptable/unacceptable) (Table 2).

Compared to acceptability scores at harvest a drastic decrease was detected in visual appearance after 5 days of storage due to loss of freshness of the lettuce and browning on the cut surface. However, fresh-cut lettuce stored under CO_2 enriched atmospheres was still acceptable for commercial purposes. Superatmospheric O_2 condition combined with 15 kPa CO_2 caused the best visual appearance at the end of the storage period, by reducing the enzymatic browning and maintaining the visual quality. This treatment was the only one that maintained acceptable visual appearance of fresh-cut lettuce during 10 days at 7 °C.

Stem colour

Significant browning of the stems occurred during storage. This was reflected in a decrease of L* value and hue angle and an increase in chroma (Fig. 2). The lightness

Table 2. Visual appearance in fresh-cut butter lettuce at harvest and after 5 and 10 days of storage at 7 °C in CA.

CA treatment		Time (day)		
O_2 (kPa)	CO_2 (kPa)	0	5	10
5	0	A[y]	U	U
5	15	A	A	U
75	0	A	U	U
75	15	A	A	A
21	0	A	U	U

[y]A: Acceptable; U: Unacceptable.

(L*) values decreased from about 55 at the beginning of the storage to an average value of 46.5 after 10 days at 7 °C for treatments without CO_2 and to average value of 50 for treatments with CO_2. CO_2 treatments slowed down the decreasing trend of L* values of the lettuce stems (Fig. 2). The browning was also affected by the O_2 concentration. Under 75 kPa O_2 the average L* value of the stems was significantly higher (53.3) compared with 5 kPa O_2 (51.5) and 21 kPa O_2 (50.7).

Increased chroma values, particularly for the treatments with 0 kPa CO_2 and air atmosphere were observed. CO_2 treatments significantly delayed the unfavourable chroma increase for 10 days at 7 °C. However, the low O_2 level (5 kPa) maintained chroma values significantly lower than under CA of 21 and 75 kPa O_2 ($P<0.001$). Furthermore, the chroma increase was even smaller when a high CO_2 level (15 kPa) was combined with a low O_2 (5 kPa) or the superatmospheric O_2 condition (75 kPa) (Fig. 2).

A significant decrease of hue angle values for all treatments throughout storage was found ($P<0.001$). However the superatmospheric O_2 condition retarded the decreasing evolution of the hue angle the most. Furthermore, the combination with high CO_2 levels increased the retarding effect.

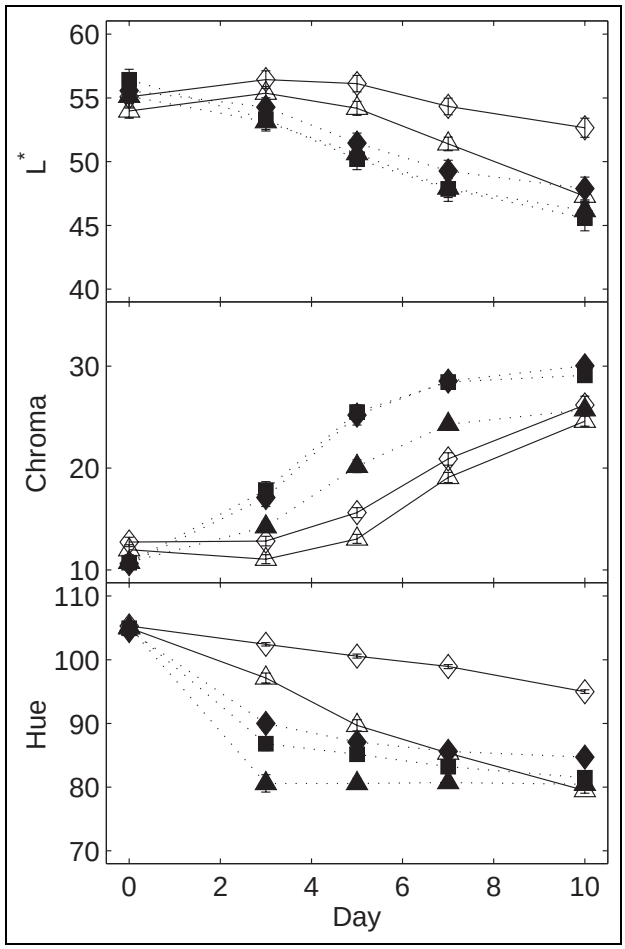


Fig. 2. Colour change of fresh-cut butter lettuce stems during 10 days at 7 °C. The symbols represent the average value for 36 measurements and bars represent ± SE (◇: 75 kPa O_2+15 kPa CO_2; r: 75 kPa O_2+0 kPa CO_2; △: 5 kPa O_2 +15 kPa CO_2; ▲: 5 kPa O_2+0 kPa CO_2; ■: 21 kPa O_2+0 kPa CO_2).

Discussion

Respiration rate

In this experiment, 15 kPa CO_2 combined with 5 kPa O_2 delayed the respiratory activity showing that this level of CO_2 is probably not yet damaging the lettuce. Some reports (Kader 1986; Kays 1991; Wills et al. 1998) have showed that CO_2 concentrations higher than 5 kPa would decrease intracellular pH influencing enzymatic activity. Changes in enzymatic activity might affect the cellular metabolism and decrease the respiration rate of fresh-cut butter lettuce. The respiration results were in the range found in fresh-cut butter lettuce under 20 or 75 kPa O_2 combined with 0 or 10 kPa CO_2 at 5 °C by Escalona et al. (2004). The influence of different O_2 (0–100 kPa) and CO_2 (0–20 kPa) levels were studied by the same authors who found that CO_2 levels affected the respiration rates more than O_2 levels. A CO_2 concentration of 10 kPa significantly reduced the respiration rate of fresh-cut lettuce and when the CO_2 level was raised to 20 kPathe respiration rate increased probably due to metabolic disorders at that concentration of CO_2 (Escalona et al. 2006).

In this study we found that in the absence of CO_2 the oxygen consumption rate was higher under 5 kPa O_2 compared to air conditions. This was a rather unexpected result. However in a previous report (Escalona et al. 2006), fresh-cut butter lettuce exposed to 5 kPa O_2 at 1, 5, and 9 °C showed the same oxygen consumption rate as in air conditions.

According to Pérez and Sanz (2001), high O_2 (80 kPa) and high CO_2 (20 kPa) levels induced flavour alterations of strawberry stored at 8 °C. Additionally, exposure of fresh-cut carrot to 50 kPa O_2 plus 30 kPa CO_2 showed an accelerated ethylene evolution possibly due to an injury response at 8 °C after 2–3 days (Amanatidou et al. 2000). This stress might also explain the higher respiration rate (as CO_2 produced) of fresh-cut lettuce under 75 kPa O_2 and 15 kPa CO_2 compared with 5 kPa O_2 and 15 kPa CO_2 at 7 °C. In comparison with others fresh-cut products, butter lettuce samples had a lower respiratory activity than baby

spinach, which reached 146–202 nmol CO_2 kg^{-1} s^{-1} in air atmosphere at 5 °C (ALLENDE et al. 2004).

Apparently the respiration rate of some fresh-cut vegetables could show a different sensitivity to high O_2 levels. JACXSENS et al. (2001b), found at 4 °C a lower sensitivity in grated celeriac and shredded chicory endive. In CA of 3, 80 and 95 kPa O_2, grated celeriac reached values of 120, 97 and 124 nmol O_2 kg^{-1} s^{-1} respectively and for shredded chicory endive the respiration rate was 90, 107 and 132 nmol O_2 kg^{-1} s^{-1} under the same conditions.

Microbial growth

Several studies have demonstrated that the behaviour of *L. innocua* and *L. monocytogenes* is comparable as affected by temperature, acidification and modified atmosphere (HUGAS et al. 1998; THOMAS et al. 1999). Therefore, we considered *L. innocua* as a valid model organism for the pathogenic *L. monocytogenes*. During storage at 4 °C, an atmosphere of 95 kPa O_2 significantly reduced the growth of *L. monocytogenes* in *in vitro* studies (JACXSENS et al. 2001b). AMANATIDOU et al. (1999), showed that *L. monocytogenes* growth rates were slightly decreased under 90 kPa O_2 alone or combined with 10 kPa CO_2 compared to air atmosphere at 8 °C. However, in our experiment a high O_2 concentration (75 kPa) alone did not reduce the growth of *L. innocua* on fresh-cut butter lettuce. GEYSEN et al. (2005b), who studied the effect of superatmospheric oxygen and carbon dioxide concentrations on the in vitro growth of *L. innocua* reported than the bacterial growth was not significantly influenced by high O_2 levels up to 100 kPa. This is in agreement with our results. However GEYSEN et al. (2005b), also found that high CO_2 concentrations had a prolonging effect on lag time and reduced the maximum specific growth rate in vitro, which was in contradiction with our results on produce. This can be due to interaction with other organisms present on the lettuce. In air conditions *L. innocua* might be much more suppressed by the abundant spoilage flora. However, when the growth of these microorganisms (e.g. aerobic mesophilic bacteria) is suppressed or delayed by high CO_2 other opportunistic organisms (e.g., *L. innocua)* might grow much better. Similar results were found by ALLENDE et al. (2002) in either conventional or high O_2 MA packages of mixed salads. These MA packages are both expected to have increased CO_2 concentration and showed no difference in the growth of *L. monocytogenes* after 10 days at 4 °C.

Aerobic mesophilic counts increased on fresh-cut butter lettuce during storage in all treatments. The inhibition of microbial populations in high CO_2 condition has been reported in other fresh-cut produces. ALLENDE et al. (2004) found a reduction of aerobic microbial growth in superatmospheric O_2 applied in MAP using a barrier film. The accumulation of high CO_2 levels in these treatments was probably responsible for this inhibition. The antimicrobial activity of CO_2 at high concentration has been well known (KADER et al. 1989). Reports on the effect of conventional and superatmospheric O_2 MAP on microbial growth vary considerably in the literature (GEYSEN et al. 2005a).

ALLENDE et al. (2002), reported that LAB counts analysed in fresh processed salad under active conventional MAP (3–5 kPa O_2 and 6–8 kPa CO_2) were always higher than those stored in superatmospheric O_2 during 6 days at 4 °C. The recommended limiting level of LAB (7 log cfu g^{-1}) was surpassed after 8 days. In our experiment, the O_2 condition did not affect the growth of LAB. However, the CO_2 levels promoted the growth of LAB after 5 days of storage. FRANCIS and O'BEIRNE (1998), found that LAB were more resistant to elevated CO_2 concentrations than others spoilage bacteria as *Enterobacteriaceae* and *Pseudomonas*. Following the recommendation of 7 log cfu g^{-1}, the fresh-cut butter lettuce could be marketed during 10 days at 7 °C under all CA conditions.

Several in vitro studies on yeast growth under superatmospheric O_2 showed different results. JACXSENS et al. (2001b), found a reduced growth of the yeast *Candida lambica,* in contrast to AMANATIDOU et al. (1999) who found a stimulated growth of *C. guilliermondii* and *C. sake* around 80 kPa O_2 at 8 °C. VAN DER STEEN et al. (2003), found that growth of yeast can be reduced or stimulated under high O_2 concentrations depending on the species or strain and their sensitivity to high O_2. A gas mixture of 80 kPa O_2 and 20 kPa CO_2 almost blocked growth of *C. guilliermondiia* although this effect was less clear for *C. sake* (AMANATIDOU et al. 1999). ALLENDE et al. (2002), reported yeast counts higher than the limit (5 log cfu g^{-1}) after 3 days at 4 °C on mixed salad stored using superatmospheric O_2 MAP. However, the same salad under 3–4 kPa O_2 and 7–8 kPa CO_2 could be stored 3 days more with less than 5 log cfu g^{-1}.

In our study the aerobic mesophilic bacteria showed less growth in 75 kPa O_2 combined with 15 kPa CO_2 than in the other conditions showing that the inhibiting effect of CO_2 was enhanced by the high oxygen concentration. KADER and BEN-YEHOSHUA (2000) suggested many factors that may help to explain the toxicity of hyperbaric O_2 amongst which,

(i) are the unfavourable effects on the oxidation-reduction potential of the system,
(ii) the oxidation of certain enzymes especially those having sulfhydryl groups or disulfide bridges, and
(iii) the accumulation of injurious reactive O_2 species (ROS) such as the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (HO^-), which are the most important ones.

The formation of superoxide radicals (O_2^-) would be the explanation for O_2 toxicity, which is destructive to some components of the cell metabolism. On the other hand, carbon dioxide causes a decrease in intra- and extracellular pH and interferes with the cellular metabolism (DIXON and KELL 1989). The inhibition effect is stronger at low temperature when the solubility of CO_2 is enhanced.

Also microbial assessments on iceberg lettuce, cos lettuce, potatoes, bean sprouts, honeydew melon, sliced mushrooms, broccoli florets and baton carrots showed that high O_2 (70, 80, and 100 kPa) MAP treatments were capable of inhibiting total aerobic counts, total anaerobic counts, yeasts, moulds, *Pseudomonas* species, *Enterobacteriaceae* and coliforms compared with low O_2 MAP (DAY 2001). These results showed that the combination of high O_2 and CO_2 levels generated by respiration of the produce were primarily responsible for the antimicrobial effects observed, rather than the high O_2 or CO_2 levels alone.

Visual appearance

The fresh-cut butter lettuce maintained a good visual appearance in 75 kPa O_2 and 15 kPa CO_2 after 10 days at

7 °C reducing the browning of cut surface and the loss of freshness. An acceptable quality scores were found in spinach leaves treated with 80–100 kPa O_2 plus 20 kPa CO_2 (Allende et al. 2004). However, the sensory quality of spinach leaves was significantly affected by fermentation under low O_2 and high CO_2 injury (Allende et al. 2004). Low O_2 and /or high CO_2 concentrations still tolerated by the commodity can reduce the incidence and severity of certain physiological disorders such as those induced by C_2H_4 and chilling injury (Kader et al. 1989). Beneficial effects of superatmospheric O_2 on the sensory quality of vegetables have been reported for shredded chicory endive and mixed vegetable salads (Day 1995; Jacxsens et al. 2001b; Allende et al. 2002; Escalona et al. 2004).

Browning

L* and hue values were good browning indicators on the lettuce stems. They reflected the increase of browning on stems during the storage and the benefitial effect of 75 kPa O_2 plus 15 kPa CO_2 compared to other treatments. For the browning estimation, the L* value has been considered as one of the best indices and is frequently used (Sapers et al. 1989). Storage at 5 kPa O_2 did not show any benefits on the colour changes compared to air most probably due to the fact that this O_2 level is still high enough to allow browning reactions. Storage at 5 kPa O_2 plus 15 kPa CO_2 only inhibited the browning expressed as hue angle for 5 days at 7 °C, subsequently the values were similar to air and CA with 0 kPa CO_2. The best gas composition to reduce the enzymatic browning was 75 kPa O_2 and 15 kPa CO_2.

The O_2 concentration in active MAP for fresh-cut lettuce is commonly less than 1 kPa to slow the browning reaction caused by the enzyme polyphenol oxidase (PPO), and more than 10 kPa CO_2 to inhibit biosynthesis of reaction substrates (Gorny 1997). Therefore in this fresh-cut butter lettuce experiment, an O_2 concentration of 5 kPa was not useful to delay the enzymatic browning on the stems. Varoquaux et al. (1996), also found a smaller effect than expected on enzymatic browning of the cut surface of 3 fresh-cut butter lettuce cultivars stored at O_2 concentration of 1 and 10 kPa for 13 days at 8 °C. The effect of CO_2 concentration on browning was also less than expected, although 1 kPa O_2 with 20 kPa CO_2 almost inhibited it.

Jacxsens et al. (2001b), found that the colour of the cut surfaces was the most important visual quality attribute in fresh-cut vegetables. Low O_2 atmospheres caused a reduction in the enzymatic browning because O_2 is a necessary substrate for the enzymatic reaction. However, high O_2 atmospheres were more effective in reducing or inhibiting the enzymatic browning of iceberg lettuce, radicchio lettuce, lollo rossa lettuce. High O_2 MAP was also found to be beneficial in the sensory quality of those products. Day (2001) reported evidence of the effectiveness of high O_2 MAP on inhibiting iceberg lettuce enzymatic browning. It is hypothesised that high O_2 levels may cause substrate inhibition of PPO or, high levels of colourless quinines formed cause feedback inhibition of PPO. However Heimdal et al. (1995), found that fresh-cut iceberg lettuce under MAP with 80 kPa O_2 and 20 kPa CO_2 had more browning that under moderate vacuum MAP.

Conclusions

Superatmospheric O_2 condition in combination with 15 kPa CO_2 retarded growth of aerobic mesophilic bacteria allowing an extension of shelf-life until 7 days at 7 °C according to recommended limit criteria (total aerobic counts lower than 7.7 log cfu g^{-1}). Under this superatmospheric O_2 condition the growth of *L. innocua* was promoted. However the same growth promotion was found in low O_2 condition combined with CO_2, which would be applied in conventional low oxygen MAP.

Storage under CA of 5 kPa O_2 plus 15 kPa CO_2 reduced the respiration rate of fresh-cut butter lettuce. CO_2 level of 15 kPa retarded enzymatic browning of the lettuce stems. Browning was delayed under high O_2 and CO_2 levels. CA of 5 kPa O_2 did not prevent browning as compared with air atmosphere. Fresh-cut lettuce stored under 75 kPa O_2 combined with 15 kPa CO_2 had a better visual appearance than the other conditions after 10 days of storage at 7 °C. In conclusion, superatmospheric O_2 condition in combination with high CO_2 level (75 kPa O_2 plus 15 kPa CO_2) was recommended to assure a good sensorial and microbial quality of fresh-cut butter lettuce compared to the conventional gas atmospheres poor in O_2 and rich in CO_2 (5 kPa O_2 plus 15 kPa CO_2).

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