Effect of High Oxygen and Carbon Dioxide Conditions on the Microbial Quality of Fresh-cut Butter Lettuce

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Abstract

The effects of controlled atmospheres containing 5 kPa O₂ and 0 kPa CO₂, 5 kPa O₂ and 15 kPa CO₂, 75 kPa O₂ and 0 kPa CO₂, 75 kPa O₂ and 15 kPa CO₂, and 21 kPa O₂ and 0 kPa CO₂ (as control) on the growth of Listeria innocua, aerobic mesophilic bacteria, lactic acid bacteria and yeasts on fresh-cut butter lettuce at 7°C were studied. The gas composition did not show a clear influence on the growth of lactic acid bacteria and yeasts. High CO₂ conditions increased the growth of Listeria innocua. No O₂ effect was found on the growth of Listeria innocua. However, when high O₂ and CO₂ conditions were combined, a reduction in the aerobic mesophilic count was observed. Growth of those bacteria was also slightly reduced in 75 kPa O₂ and 0 kPa CO₂ and 5 kPa O₂ and 15 kPa CO₂. Therefore, a high O₂ condition alone could reduce the mesophilic count to the same extent as low O₂ combined with high CO₂ levels while avoiding anaerobic fermentation reactions.

Keywords: Listeria innocua, superatmopheric O₂, minimally processed, modified atmosphere packaging, vegetables

INTRODUCTION

Modified atmosphere packaging (MAP) is a normal technique used to commercialise fresh-cut produces. Often the plastic film chosen to produce a favourable atmosphere may cause excessive accumulation of CO₂ and/or depletion of O₂ when the packages designed for a cold temperature range are stored at higher temperatures increasing the respiration rate of the produce higher than the permeability of the film (Kader et al., 1989). When the CO₂ concentrations are above a critical level some psychrotrophic pathogens as Listeria monocytogenes can survive at low temperatures favoured by the elimination of natural competitors and the prolonged shelf life period (Francis et al., 1999).

According to Day (1996) a novel type of MAP using superatmopheric O₂ conditions are effective for inhibiting enzymatic discoloration, preventing anaerobic fermentation reactions, undesirable moisture and odour losses, and influencing aerobic and anaerobic microbial growth reducing decay of the fresh produce. High oxygen concentrations are effective for fresh-cut vegetables that are sensitive to enzymatic browning and spoilage by yeasts (Jacxsens et al., 2001; Pérez and Sanz, 2001). Exposure to high O₂ levels did not inhibit microbiol growth strongly, while CO₂ reduced growth to some extent in most cases. The combined treatment of high O₂ concentration and 10 to 20 kPa CO₂ may provide adequate suppression of microbial growth and prolong the shelf-life (Amanatidou et al., 1999; Allende et al., 2004; Geysen et al., 2005). Applying a high O₂ atmosphere in a high-barrier film had a beneficial effect on the microbial and sensory shelf life of raspberries and strawberries, by inhibiting the development of moulds and by maintaining fresher sensory properties, as long as atmospheric conditions did not induce
fermentation (Van der Steen et al., 2002). No growth of \textit{L. monocytogenes} was observed on fresh-cut salads packaged in a low or a high barrier film with an initial oxygen concentration of 95\% or conventional MAP conditions at 4\°C (Allende et al., 2002).

The objective of the present research was to study the effect of an elevated CO\(_2\) levels and low and high O\(_2\) levels on the microbial quality with special attention to growth of \textit{Listeria innocua}, in order to analyse the effect of both gases and the advantages of increasing the oxygen concentration from low (normal retail condition) to high levels Storage temperature was 7\°C, because this is the maximum allowed for refrigerated products in Belgium (Anonymous, 1982). \textit{Listeria innocua} was used as a model organism for the pathogenic \textit{L. monocytogenes}.

**MATERIALS AND METHODS**

**Sample Preparation**

Butterhead lettuces (\textit{Lactuca sativa} L.) ‘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, after soiled and decayed external leaves were eliminated the butterheads were processed. 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 using a domestic centrifuge, which removed excess water without causing any visible damage to the lettuce.

**Listeria Inoculation**

\textit{Listeria 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 growth ability of the selected \textit{Listeria innocua} strain on nutrient agar at 7\°C under ambient atmosphere was confirmed in preliminary tests (data not shown).

**Experimental Set-up**

Samples of 161.3 \pm 0.4 g cut lettuce were put into 1.7 litre glass jars in a cool room set at 7\°C. Lettuce samples were inoculated with 5 mL of an appropriate dilution of \textit{L. innocua} to obtain a concentration of 3 to 4 log cfu g\(^{-1}\). For each gas mixture in the study, four jars were connected in series and flushed continuously with a flow rate of 10 L h\(^{-1}\). 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. 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 \textmu m pore size filter (Nylon Filter Media with Polypropylene Housing, Whatman, Clifton NJ, USA) was used to avoid the external contamination on the lettuce samples. Controlled atmosphere (CA) of 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 O₂ + 0 kPa CO₂ (as control) were applied. Temperature was monitored every 10 minutes with temperature loggers (Escort Junior, Tech-nnovators, New Zealand). Fresh-cut lettuce was stored in the room during 10 days at 7ºC (6.8 ± 0.5ºC according to temperature loggers).

Microbiological Analysis

In order to determine microbial growth on days 0, 3, 5, 7, and 10, three random samples were taken. A jar per treatment was analysed in each evaluation period being divided in three sub-samples. A 40 g 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 bacteria microflora and 0.1 mL for yeast and mould. The following media and incubation conditions were used: *Listeria innocua* by spread plating on *Listeria* selective agar base (CM0856, Oxoid, Hampshire, England) incubated for 2 days at 35ºC; plate count agar (PCA, CM0325, Oxoid, Hampshire, England) for mesophilic microflora, 48 h at 30ºC; MRS agar (CM0361, Oxoid, Hampshire, England) for lactic acid bacteria (LAB) counted by pour plating with top layer, 25ºC for 4 days; and Rose Bengal Chloramphenicol agar base (CM0549, Oxoid, Hampshire, England) for yeast and mould by spread, 5 days at 22ºC. All microbial counts were reported as log colony forming units per g sample (log cfu g⁻¹). Microbial quality was evaluated following different European recommend limits (CNERNA-CNRS, 1996; Debevere, 1996).

Experimental Design and Statistical Analysis

A completely randomised design with three replicates per treatment from each jar was performed. A three-way analysis of variance (ANOVA) with a 95% confidence level was carried out. Statistical analysis permits evaluation of CA effect on the microbial growth on fresh-cut lettuce. The factors O₂ and CO₂ levels and storage time were studied. By comparing mean values by the Duncan multiple range test significant differences among treatments were analysed and significant interactions between factors were determined. SAS/STAT® version 8.2 (SAS Institute Inc., Cary, North Carolina, United State) was used for statistical analysis.

RESULTS

A low or high O₂ condition (5 or 75 kPa) without CO₂ did not have a significant effect on the general microbiology quality of fresh-cut butter lettuce. However, the high CO₂ level (15 kPa) showed a significant decrease on most microbial spices.

*Listeria innocua* numbers reached 6 log units in 10 days on lettuce stored under atmospheres with CO₂. The growth of *Listeria* in 15 kPa CO₂ was stimulated reaching a significantly higher counts higher than without CO₂ (Table 2, Fig. 1A). From the 3rd day of storage *Listeria*’s count was significantly higher in 15 kPa CO₂. Therefore at the end of storage, treatments in CO₂ enriched atmospheres obtained 6.1 log cfu g⁻¹ in comparison with fresh-cut lettuce stored in 0 kPa CO₂ where the counts were 4.8 log cfu g⁻¹. The different O₂ levels (5 to 75 kPa) were not significantly effecting on the growth of *Listeria* (*P < 0.05).

Aerobic mesophilic bacteria were 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 O₂ plus 0 kPa CO₂, the counts were significantly higher than in an atmosphere with 75 kPa O₂ alone during
the complete storage time. High O2 levels had a significant decreasing effect on the aerobic mesophilic count until day 7 about 1 log cfu g⁻¹ (*P < 0.05). However, when 5 or 75 kPa O2 were combined with 15 kPa CO2 the aerobic mesophilic counts on fresh-cut lettuce were significantly diminished (Table 2, Fig. 1B). Therefore, superatmosphere O2 condition (75 kPa) and high CO2 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 (Fig. 1B).

The LAB growth was significantly increased until day 5, after which the differences between the treatments could be attributed to high CO2 level (Table 2, Fig. 1C). Under 15 kPa CO2 the counts were higher than those without CO2. Most of the cases, the growth of these micro-organism was not dependent from the O2 concentrations. After 10 days of storage, the counts were about 6.87 log cfu g⁻¹ for CO2 treatments and 5.53 log cfu g⁻¹ for those with 0 kPa CO2.

CA with 15 kPa CO2 could significant delay the growth of yeast around 1 log unit until 7 days of storage. Atmosphere with 0 kPa CO2 had a shelf life according to the European standard (5 log cfu g⁻¹) of 3 or 5 days at 7°C while CA of 15 kPa CO2 extended this period to 7 days. O2 concentration between 21 to 75 kPa did not have any effect on the growth of yeast (Table 1). Treatments with 5 kPa O2 alone showed counts lower than those with higher O2 concentrations during day 7 and 10 of the storage (Fig. 1D).

The growth of mould was always below 4 log cfu g⁻¹ (European standard limit) and did not show a constant growing during the storage. Neither O2 nor CO2 levels had any effect on the counts (data not shown).

DISCUSSION

Kader and Ben-Yehoshua (2000), suggested that among the many factors that may help explain the toxicity of hyperbaric O2 are the unfavorable effects on the oxidation-reduction potential of the system, the oxidation of certain enzymes especially those having sulphydryl groups or disulfide bridges and the accumulation of injurious reactive O2 species (ROS). The formation of superoxide radicals (O2⁻) would be the explanation for O2 toxicity, which is destructive to some components of cell metabolism.

In the current study, the growth of L. innocua, mesophilic aerobic counts, Lactic acid bacteria, and yeasts was evaluated under high O2 and CO2 conditions.

Several studies have demonstrated that the behaviour of Listeria innocua and L. monocytogenes is comparable as affected by temperature, acidification and modified atmosphere (Hugas et al., 1998; Thomas et al., 1999). During a storage at 4°C of mushroom slices, grated celeriac and shredded chicory endive, the reducing effect of the growth of L. monocytogenes is the most detectable under 95% O2 than in others high O2 conditions as 70 and 80% (Jacxsens et al., 2001). Amanatidou et al. (1999), showed that for L. monocytogenes growth rates were slightly decreased under 90% O2 alone or in combination with 10% CO2 compared to air conditions at 8°C. However, in our experiment L. innocua growth on fresh-cut butter lettuce was not affected by 75 kPa O2 alone or in combination with 15 kPa CO2. Probably, the O2 concentration should be higher than 75 kPa O2 to reduce the growing of Listeria. Results obtained in mixed salad did not show any effect on L. monocytogenes growth after 10 days at 4°C by using conventional and superatmospheric O2 MAP (Allende et al., 2002). Geysen et al. (2005), studied the effect of superatmospheric oxygen and carbon dioxide concentrations on the growth of L. innocua. These authors reported than the bacterial growth under in vitro conditions was not significantly influenced by high oxygen concentrations. Carbon
dioxide had a prolonging effect on lag time and reduced the maximum specific growth rate.

Aerobic mesophilic counts increased during storage in all treatments. The inhibition of microbial populations in those conditions with CO₂ has been reported in others fresh-cut produces. Allende et al. (2004), did not find an aerobic microbial growth with superatmospheric O₂ applied in MAP. However, those authors found a significant reduction of those bacteria growth when fresh-cut baby spinach was packed in MAP by using a barrier film. The accumulation of high CO₂ levels in those treatments was probably responsible for this inhibition. The antimicrobial activity of CO₂ at high concentration has been well known (Kader et al., 1989). Reports on the effect of conventional and super atmospheric O₂ MAP on microbial growth vary considerably in literature.

Allende et al. (2002), reported that LAB counts analysed in fresh processed salad under active conventional MAP (3-5 kPa O₂ and 6-8 kPa CO₂) were always higher than those stored in superatmospheric O₂ during 6 days at 4°C. Nevertheless, the LAB limiting recommended level (7 log cfu g⁻¹) was surpassed after 8 days. Following this recommendation, 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 O₂ showed different results. Jacxsens et al. (2001), found a yeast growth reduced and Amanatidou et al. (2000) stimulated around 80 kPa O₂. Allende et al. (2002), reported yeast count higher than the limit after 3 days at 4°C on mixed salad stored using superatmospheric O₂ MAP. However, the same salad under 3-4 kPa O₂ and 7-8 kPa CO₂ could be stored 3 days more below 5 log cfu g⁻¹.

To conclude: superatmospheric O₂ levels in combination with 15 kPa CO₂ retarded aerobic mesophilic bacteria growth allowing and extending shelf-life from 5 to 7 days at 7°C according to recommended limit criteria (total aerobic counts lower than 7.7 log cfu g⁻¹). However, under these conditions the growth of L. innocua was promoted.

Acknowledgements
The authors are grateful to Fundación Séneca, Centro de Coordinación de la Investigación (Murcia-Spain) for the postdoctoral grant to V.H. Escalona. The Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT) is gratefully acknowledged for financial support (CO – project 020803).

Literature Cited


Table 1. Statistical analysis of the microbial growth on fresh-cut butter lettuce during storage at 7°C. Values expressed as log cfu g⁻¹ and corresponding to average of CO₂ treatments.

<table>
<thead>
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<th>Micro-organism</th>
<th>CO₂ (kPa)</th>
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<th>5</th>
<th>7</th>
<th>10</th>
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<tr>
<td>Listeria innocua</td>
<td>0</td>
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<td>4.72</td>
<td>4.68</td>
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<td></td>
<td>15</td>
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<td>4.92</td>
<td>5.24</td>
<td>5.58</td>
<td>6.16</td>
</tr>
<tr>
<td>Aerobic mesophilic</td>
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<td>6.94</td>
<td>7.77</td>
<td>8.42</td>
<td>8.83</td>
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<tr>
<td>bacteria</td>
<td>15</td>
<td>5.60</td>
<td>6.03</td>
<td>6.97</td>
<td>7.66</td>
<td>8.43</td>
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<tr>
<td>Lactic acid bacteria</td>
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<td>5.29</td>
<td>5.72</td>
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<td>5.53</td>
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<tr>
<td></td>
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<td>5.54</td>
<td>6.57</td>
<td>5.93</td>
<td>6.87</td>
</tr>
<tr>
<td>Yeast</td>
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<td>4.02</td>
<td>6.23</td>
<td>6.64</td>
<td>6.53</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>3.17</td>
<td>3.41</td>
<td>5.59</td>
<td>5.67</td>
<td>6.71</td>
</tr>
</tbody>
</table>

Values within a row followed by the same capital letter and within a column by micro-organism followed by the same small letter are not significantly different (*P < 0.05) according to Duncan multiple range test.

Fig. 1. Changes in the population of Listeria innocua (A), aerobic mesophilic bacteria (B), lactic acid bacteria (C), and yeast (D) on fresh-cut butter lettuce during 10 days at 7°C. Bars represent ± SE (◇: 75 kPa O₂ + 15 kPa CO₂; ◇: 75 kPa O₂ + 0 kPa CO₂; △: 5 kPa O₂ + 15 kPa CO₂; ▲: 5 kPa O₂ + 0 kPa CO₂; ■: 21 kPa O₂ + 0 kPa CO₂).
Effet de haute concentrations en oxygène et en dioxyde de carbone sur la qualité microbienne de laitue fraîche.

Mots clés : Listeria innocua, oxygène superatmosphérique, transformation minimale, emballage à atmosphère contrôlée, légumes

Résumé:
Cet article étudie les effets d’atmosphères contrôlées contenant respectivement 5 kPa d’O₂ et 0 kPa de CO₂, 5 kPa d’O₂ et 15 kPa de CO₂, 75 kPa d’O₂ et 0 kPa de CO₂, 75 kPa d’O₂ et 15 kPa de CO₂, et enfin 21 kPa d’O₂ et 0 kPa de CO₂ (témoin) sur la croissance de Listeria innocua, de bactéries mésophiles aérobiennes, de bactéries et de levures lactiques, sur des feuilles de laitue maintenues à 7°C. La composition du gaz ne semble pas avoir d’influence claire sur la croissance des bactéries lactiques et des levures. De hautes concentrations en CO₂ favorisent le développement de Listeria innocua. Aucun effet de la concentration en O₂ n’a été montré sur le développement de Listeria innocua. Cependant une réduction des souches aérobiennes mésophiles a été observée pour de fortes concentrations en O₂ et en CO₂ combinées, la croissance de ces bactéries a été fortement réduite sous des atmosphère à 75 kPa d’O₂ et 0 kPa de CO₂ et à 5 kPa d’O₂ et 15 kPa de CO₂. De plus, une haute concentration en O₂ suffit à elle-seule à réduire le nombre de mésophiles dans les mêmes proportions qu’une faible concentration d’O₂ combinée à de fortes quantités de CO₂, tout en évitant l'apparition de fermentations anaérobies.