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Effect of Superatmospheric Oxygen Packaging on Sensorial Quality, Spoilage, and *Listeria monocytogenes* and *Aeromonas caviae* Growth in Fresh Processed Mixed Salads

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ABSTRACT

Atmospheres with O_2 levels higher than 70 kPa have recently been suggested as an innovation to modified atmosphere packaging (MAP) for fresh processed vegetables to maintain sensory quality and safety. In the present work, mixed vegetable salad collected from a commercial processing plant and stored with the MAP technique was studied. Two gas mixtures were actively generated by using an initial O_2 concentration of 95 kPa and combined with two plastic films. The low-barrier film permeability for O_2 was 1,629 $mlO_2/m^2 \times 24$ h $\times$ atm with 30 μm of thickness (Hyplast, Hoogstraten, Belgium) and the O_2 permeability of the high-barrier film was 2 $mlO_2/m^2 \times 24h \times$ atm with 150 μm of thickness (Euralpack, Wommelgen, Belgium) at 23°C. As control, active conventional MAP with application of 3 to 5 kPa of O_2 and 6 to 8 kPa of CO_2 was used. Packaged salads were stored up to 8 days at 4°C and at temperatures simulating chilled distribution chain conditions. Microbial safety and sensory quality, as well as the survival of inoculated *Listeria monocytogenes* and *Aeromonas caviae,* were monitored. The effect of superatmospheric O_2 on the growth of aerobic microflora was variable. Under superatmospheric conditions, lactic acid bacteria and members of *Enterobacteriaceae* were inhibited. Nevertheless, growth of yeast and *A. caviae* seem to be stimulated by superatmospheric O_2, whereas growth of psychrotrophic bacteria and *L. monocytogenes* was not affected. The overall visual appearance (mainly color) of the mixed vegetable salads was better maintained and the shelf life prolonged when packaged under O_2 concentrations greater than 50 kPa.

Several assessments performed on selected fresh processed produce items showed that O_2 concentrations higher than 70 kPa under modified atmosphere packaging (MAP) inhibited microbial growth and decay *(2–5, 25, 28, 34)*. Therefore, it could be considered as an alternative to conventional MAP with moderate-to-low O_2 and elevated CO_2 concentrations *(14)*. Many articles have demonstrated the advantages of conventional MAP using 3 to 5 kPa of O_2 and 5 to 10 kPa of CO_2 (balanced with N_2) to reduce deterioration of fresh processed vegetables and proliferation of aerobic spoilage microorganisms *(1, 6, 21, 27, 31)*. Nevertheless, it is known that the effect of conventional MAP on aerobic mesophilic microflora is variable. In some fresh processed vegetables packaged under conventional MAP, O_2 levels could decrease rapidly, depending on several factors (respiration activity of the produce, film permeability, surface area, storage temperature) that progressively create anaerobic conditions (e.g., <2 kPa of O_2 and >20 kPa of CO_2) favorable for growth of anaerobic bacteria and undesirable fermentation reactions *(25, 31)*.

Fresh processed mixed vegetable salad is a perishable product. Current techniques used by the fresh processing vegetables industry have improved the overall quality and extended shelf life of these products, but safety is still an issue of concern. Although conventional MAP helps maintain the quality of fresh processed vegetables, the benefits were not systematically related to a reduction in growth of mesophilic flora *(31)*. In addition, psychrotrophic pathogens, such as *Listeria monocytogenes*, were not inhibited under conventional MAP *(9–11, 19, 26)*.

Data already exist on the sensory quality changes and the growth of different microorganisms associated with the spoilage and safety of fresh processed fruits and vegetables stored under elevated O_2 concentrations *(4, 5, 25, 28, 34)*. Exposure to superatmospheric O_2 may inhibit, have no effect, or even stimulate growth of different microorganisms from the same genus; however, the toxic effect of O_2 on microbial growth due to the formation of superoxide radicals (O_2^-) and their effects on cell metabolism have already been explained *(5, 20, 22)*. The fact that some microorganism growth was not affected or even stimulated under superatmospheric O_2 indicated the presence of a defense mechanism, such as O_2-decomposing enzymes or radical scavengers, to avoid lethal damage by O_2 *(5)*. Zhulin et al. *(35)* explained that in the presence of superatmospheric O_2, bacteria mainly used three strategies against reactive O_2 species generated by the partial reduction of O_2: regulation of the expression of genes involved in aerobic metabolism and in the enzymatic defense, use of noncoupled respiration and mild uncoupling mechanisms to accelerate the respiratory consumption of O_2, and escape from microenviron-

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ments where the O_2 level is too elevated. Because of the different behavior that microorganisms have under this atmosphere, it is necessary to study the effect of superatmospheric O_2 on the microflora of each product before it is used.

The aims of this study were (i) to evaluate the changes in sensory and microbial quality of mixed lettuce salad packaged under superatmospheric O_2 and under conventional MAP throughout cold storage, (ii) to study the effect of fluctuating temperatures during the chill distribution chain on the microbial and sensory quality of the product, and (iii) to determine the growth of inoculated *L. monocytogenes* and *Aeromonas caviae* under superatmospheric O_2 and conventional MAP throughout cold storage compared with that under the same atmosphere conditions of the chill distribution chain.

MATERIALS AND METHODS

Mixed vegetable salads packaged under superatmospheric O_2 at 4°C. Fresh processed mixed vegetable salad, including 20% endive (*Cichorium endiva*), 20% curly endive (*Cichorium endiva*), 20% radicchio (*Cichorium intybus*), 20% lollo rosso (*Lactuca sativa*), and 20% lollo bionda (*Lactuca sativa*) lettuces, was collected from a vegetable processing plant in Sint-Lievens Houtem (Belgium). The processing included reception, shredding, washing, rinsing, drying, and packaging in bulk at atmospheric conditions. Vegetable salads were transported to the laboratory (30 min) under refrigeration and immediately packaged into individual bags. Each bag (package dimensions, 21 × 17 cm) contained 150 g of mixed vegetable salad. To generate three different MAP treatments, two types of films and two gas concentrations (superatmospheric O_2 and conventional MAP) were applied as follows.

MAP 1. The MAP 1 technique consisted of salad packaged initially at approximately 3 to 5 kPa of O_2 and 6 to 8 kPa *(8)* of CO_2 balanced by N_2, using a low-barrier film. Based on previous studies *(24)*, the respiration rate of the mixed lettuce was calculated at 4°C (RO_2 = 4.52 $mlO_2/kg \times h$). The calculated film permeability, which is necessary to obtain an equilibrium at 3 kPa of O_2, was 1,266 $mlO_2/m^2 \times 24$ h × atm at 4°C. We worked with a low-barrier film with a O_2 permeability of 1,629 $mlO_2/m^2 \times 24$ h × atm and 30 μm of thickness (Hyplast, Hoogstraten, Belgium). The selected atmosphere (3 to 5 kPa of O_2 and 6 to 8 kPa of CO_2 balanced by N_2) was introduced into packages before thermal sealing by a gas-packaging device (gas mixer, WITT M618–3MS0, Gasetechnik, Germany; gas packaging Multivac A300/42 HagenmüllerKG, Wolferschwenden, Germany). Air Products (Vilvoorde, Belgium) supplied the gases (Fresh Line).

MAP 2. The MAP 2 technique consisted of salad packaged initially at 95 kPa of O_2, balanced by N_2, using the same low-barrier film. This atmosphere was introduced within bags directly from a cylinder with pure O_2 by using a flowmeter (Bronkhurst, Ruurlo, The Netherlands). The bags were flushed for 5 min with the desired atmosphere inside a zip bag and then heat sealed. When bags were sealed, the external zip bags were eliminated.

MAP 3. The MAP 3 technique consisted of salad packaged under an initial concentration of approximately 95 kPa O_2, using a high-barrier film with a O_2 permeability of 2 $mlO_2/m^2 \times 24$ h × atm at 23°C and 150 μm of thickness (Euralpack, Wommelgen, Belgium). The film was selected to maintain the highest possible initial O_2 concentrations within the packages. These bags were filled as described for MAP 2.

The selected MAP techniques were chosen based on results formerly obtained in previous experiments. This assay was performed twice.

Gas atmosphere. Changes in O_2 and CO_2 levels within packages throughout the shelf life were monitored. A 40-ml gas sample was taken from the headspace of the packages with a syringe and injected into a gas analyzer (Servomex, Series 1400, Crowborough, United Kingdom) to measure the composition of the gas before packages were opened.

Microbial quality. To determine the microbial quality of the mixed vegetable salads, standard enumeration methods were used. The following media and incubation conditions were used: plate count agar (CM325, Oxoid, Unipath, Basingstoke, United Kingdom) for total psychrotrophic counts by pour plating, incubated at 22°C for 72 h; deMan-Rogosa-Sharpe medium (pH 6.2) (CM361, Oxoid) for lactic acid bacteria (LAB) counts on pour plate and overlaid with the same medium, incubated at 30°C for 72 h; yeast glucose chloramphenicol (64894, Diagnostic Pasteur, Marnes-La-Coquette, France) to enumerate yeast by spread plating and incubating for 72 h at 30°C; violet red bile glucose agar (CM485, Oxoid) for *Enterobacteriaceae* bacteria counts on pour plate and overlaid with the same medium, incubated at 37°C for 24 h; and modified bile-salts-irgasan-brillant green agar *(30)* (pH 8.7) and ampicillin dextrin agar by spread plating for *Aeromonas* growth, incubated at 30°C for 24 h.

Microbial analyses were performed on days 0 (production day), 3, 5, 6, 7, and 8. Each microbial count was the mean of four packages. All the analyses were performed in duplicate. Thirty grams of lettuce from each bag was mixed with 270 ml of peptone saline solution (8.5 g/liter of NaCl [Vel 8605, Merck Eurolab, Leuven, Belgium] plus 1 g/liter of peptone [L34, Oxoid]) in a sterile stomacher bag and homogenized for approximately 1 min with a Colworth Stomacher 400 (Steward Laboratory, London, England). Tenfold dilution series were made in peptone saline solution as needed for plating. The microbial quality was evaluated following the French legislation *(13)* and the microbial criterion proposed by Debevere *(17)*. Microbial counts were expressed as $\log_{10}$ CFU/g.

Sensory quality. The sensory quality was assessed by six members of an expert sensory panel on the same days of microbial analysis, using a hedonic scale. All sensory tests were performed in a special tasting room with separated boxes and a red light. Organoleptical properties, such as taste, odor, and texture, were evaluated under the red light to exclude the influence of visual characteristics *(29)*. The visual properties (color and overall visual quality) were judged under normal light.. The panelists used a descriptive graduated scale to record their perceptions of overall visual quality (excellent or fresh appearance, very good, good, or poor appearance or not fresh), aroma (excellent or fresh; very good; good; fair or slight; or poor, none, or not typical), texture (excellent, crisp, fresh, or succulent; very good; good; fair; or poor or limp), flavor, and color (same scale as for aroma). The scales were transformed to a 10-point scoring system, where 1 and 10 were the best and worst scores, respectively, and 5 was the limit of marketability from the consumer point of view. Results were obtained comparing the evaluations of the three MAP treatments. Appraisal of the quality was performed by applying a Tukey and Duncan multiple range test as implemented in SPSS 9.0 for Windows 95 to determine significant differences ($P < 0.05$) between the applied atmospheres.

Shelf life. Shelf life was determined according to sensory properties and the mentioned microbial criterion. Product was not

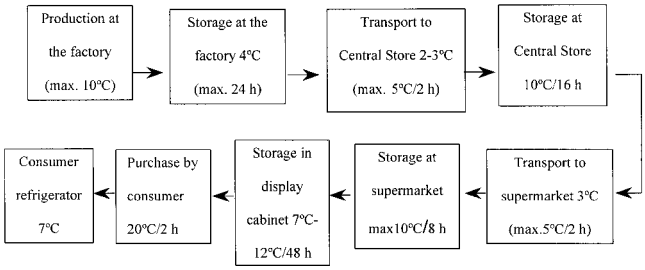

FIGURE 1. *Simulated distribution chain of fresh processed vegetables.*

any more accepted for each one of the evaluated variables when the mean score was above 5.

Mixed vegetable salads packaged under superatmospheric O_2 throughout the chill chain. A simulation of time and temperature conditions, from production to consumption by the consumer, of fresh processed mixed vegetable salads was performed. The whole chill distribution chain was divided into different consecutive handling and storing steps, including loading and unloading of the trucks and storage periods where the product was subjected to different time-temperature combinations. Taking into consideration previous studies concerning fresh processed vegetables throughout chill distribution chain *(13, 33)*, the steps shown in Figure 1 were followed. After production, fresh processed vegetables were conventionally stored at 4°C at the factory. The whole lot was transported to a central store and afterward to the distribution center, where pallets were loaded onto temperature-controlled trucks and transported to supermarkets and placed in cold rooms until moved to the shopping area. When packets were unloaded in the display cabinet, it was found that packaged fresh processed vegetables commonly stand for more than 1 h at room temperature *(33)*.

A temperature data logger (Escort, Tech Innovators, New Zealand) measured the temperature every 20 min. The data logger sensor was introduced into the package and the bag was mixed with the other prepared packages following the same steps. In this trial, salads were packaged under MAP 3 at two temperatures that simulated two temperatures at the display cabinet in the supermarket (7/12°C) and under MAP 1 stored at 7°C. A display cabinet temperature of 7°C was considered the maximum acceptable temperature, whereas a temperature of 12°C was considered temperature abuse. Comparisons between MAP 3 (7°C) and MAP 3 (12°C) and between MAP 3 (7°C) and MAP 1 (7°C) were made. To evaluate the influence of the temperature throughout the chill distribution chain on the salad quality, changes in gas composition, microbial growth (total aerobic psychrotrophic count, LAB, *Enterobacteriaceae, Aeromonas* spp., yeasts, and molds), and sensory quality of the product were monitored. Microbial and gas analyses were performed after 24, 42, 52, 100, 102, 118, 142, and 166 h of storage. Sensory quality was evaluated after approximately 4, 5, 6, and 7 days of storage.

Effect of superatmospheric O_2 on pathogenic microorganisms: inoculation. The inoculated cultures were one stock culture of *A. caviae* (HG4), previously isolated at the Laboratory of Food Microbiology and Food Preservation (University of Ghent) from fresh commercial spinach, and two strains of *L. monocytogenes,* one from stock culture (Scott A) and another isolated from fresh commercial green and red bell peppers at the same laboratory (LM LJ1) *(26)*. Each strain was consecutively subcultured twice in brain heart infusion broth (CM225, Oxoid) for 24 h at 37°C for *L. monocytogenes* and 30°C for *A. caviae*. After the

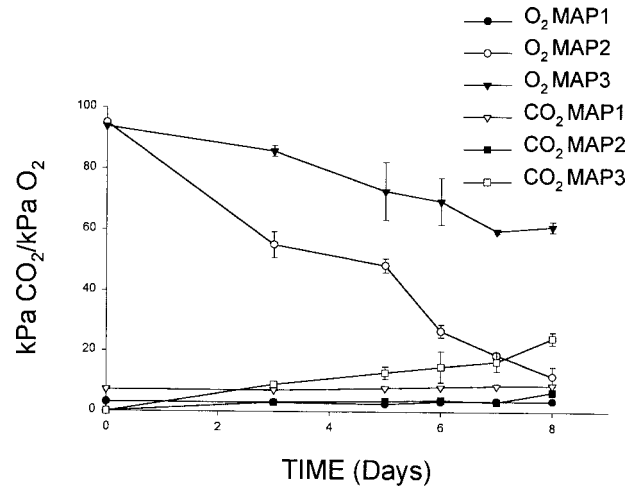

FIGURE 2. *Changes in oxygen and carbon dioxide concentrations during storage time within mixed vegetable salads bags under three MAP: conventional (MAP 1) and superatmospheric O_2 (MAP 2 and MAP 3) at 4°C. Bars indicate a 95% confidence interval.* n = 4.

cultures were transferred the second time, they were allowed to adapt to the final temperature of 4°C for 6 h. After this period of incubation, concentrations of approximately 5×10^8 CFU/ml of *L. monocytogenes* and approximately 1×10^9 CFU/ml of *A. caviae* grown in the broth (confirmed via plate counts) were found. A final concentration of 10^3 to 10^4 CUF/g of each microorganism was desired in the product. This final concentration was reached by adding 0.5 ml from the adequate dilution of each microorganism inside the bags containing 150 g of mixed lettuces. Product was carefully mixed to ensure a homogenized distribution of the inoculate.

To evaluate the effect of the selected MAP (MAP 1, MAP 2, and MAP 3) on pathogen microorganisms, 30 bags were stored up to 10 days under each MAP at 4°C and analyzed on days 3, 5, 6, 7, and 10. Another 32 bags were packaged with the MAP 3 method and subjected to time and temperature changes, to simulate a chill distribution chain, and stored at 7 or 12°C (16 bags at each temperature) in the display cabinets. As control, 16 bags were packaged with the MAP 1 method and subjected to time and temperature changes and stored at 7°C in the display cabinet.

Microbial analysis. Samples were analyzed after 24, 42, 52, 100, 102, 118, 142, and 166 h. To determine the survival or growth of *L. monocytogenes,* the following media and incubation conditions were used: *Listeria* selective agar base (Oxoid formulation: CM856 [Oxoid] + *Listeria* selective supplement and Oxford formulation: SRI40E [Oxoid]) was used and plates were incubated at 37°C for 48 h; the strains of *A. caviae* were surface plated on modified bile-salts-irgasan-brillant green agar (pH 8.7) and on ampicillin dextrin agar *(30)*, and the plates were incubated at 30°C for 24 h.

RESULTS

Gas composition changes. When MAP 3 was applied, O_2 concentration within the bags always remained higher than 60 kPa at any storage temperature (Fig. 2). But using MAP 2, the O_2 level decreased to approximately 12 kPa. Under an initial active high O_2 level, no optimal equilibrium atmosphere was attained. Final concentrations were similar to those obtained by Heimdal et al. *(23)* by applying

TABLE 1. *Growth of psychotrophic bacteria, lactic acid bacteria, and* Enterobacteriaceae *(log_{10} CFU/g) on mixed vegetable salad packaged under three MAP techniques*[a]

	Psychotrophic bacteria			Lactic acid bacteria			*Enterobacteriaceae*		
Day	MAP 1	MAP 2	MAP 3	MAP 1	MAP 2	MAP 3	MAP 1	MAP 2	MAP 3
0	5.98 (5.77–6.18)	5.78 (5.58–5.99)	5.78 (5.51–6.05)	3.48 (3.35–3.60)	3.34 (3.09–3.59)	3.34 (3.09–3.59)	4.08 (3.90–4.25)	4.08 (3.72–4.44)	4.08 (3.72–4.44)
3	6.58 (6.40–6.75)	7.10 (7.03–7.18)	6.61 (6.35–6.87)	4.63 (4.43–4.86)	4.38 (4.15–4.60)	3.91 (3.66–4.16)	4.28 (3.87–4.69)	4.19 (4.07–4.32)	3.93 (3.86–4.03)
5	7.08 (7.01–7.15)	7.52 (7.25–7.67)	7.16 (6.47–7.84)	5.25 (4.97–5.53)	4.50 (4.11–4.88)	4.76 (4.65–4.87)	5.49 (5.42–5.56)	4.91 (4.64–5.19)	4.43 (3.83–5.03)
6	7.27 (7.08–7.47)	8.02 (7.84–8.20)	7.80 (7.60–8.0)	5.34 (5.19–5.50)	4.87 (4.51–5.21)	5.03 (4.92–5.12)	5.54 (5.45–5.63)	5.51 (5.25–5.77)	5.26 (4.83–5.69)
7	7.46 (7.17–7.77)	8.11 (7.70–8.52)	7.80 (7.12–8.48)	5.43 (5.40–5.46)	4.93 (4.44–5.41)	5.50 (5.49–5.51)	5.57 (5.46–5.67)	4.98 (4.47–5.48)	5.41 (5.20–5.59)
8	7.76 (7.46–8.06)	8.18 (8.04–8.32)	7.70 (7.47–7.92)	5.92 (5.90–5.94)	5.10 (4.90–5.20)	6.06 (5.57–6.55)	5.34 (5.11–5.56)	5.28 (5.18–5.38)	5.37 (5.19–5.55)

[a] MAP techniques included conventional (MAP 1) and superatmospheric O_2 (MAP 2 and MAP 3) at 4°C. Values between parentheses indicate a 95% confidence interval. n = 4.

a 59-μm multilayer coextruded film and initial concentrations of 80 kPa of O_2 and 20 kPa of CO_2. When MAP 1 was applied, equilibrium concentrations were kept at approximately 3 to 4 kPa of O_2 and 7 to 8 kPa of CO_2. The storage temperature in the display cabinet (7 or 12°C) provoked slight differences in the atmospheric composition within the bags. As expected, the O_2 level decreased faster at 12 than 7°C, and the final CO_2 level was higher in bags stored at 12°C due to higher respiration rates.

Effect of superatmospheric O_2 on microbial quality. Comparison growth of psychrotrophic bacteria under superatmospheric O_2 (MAP 2 and MAP 3) and conventional MAP (MAP 1) after 7 days at 4°C showed no significant difference (Table 1). French legislation allows a maximum of 4.7 log CFU/g (5×10^4 CFU/g) at production and 7.7 log CFU/g (5×10^7 CFU/g) at consumption *(13)*. Using these criteria, the microbiological limit was exceeded on day 5 when salads were stored by MAP 2; however, salads stored by MAP 3 reached this limit on day 6 and salads stored by MAP 1 on day 8. The final psychrotrophic bacteria counts for all assayed MAPs were similar and close to the limiting level; however, the limiting level for the product at the production point was exceeded for all the samples. Because of repair mechanisms that some bacteria are able to develop for avoiding cell damage due to elevated O_2 concentrations *(5, 35)* and because some bacteria growth can be stimulated under superatmospheric O_2, the total psychrotrophic bacteria counts throughout storage did not change. A shift from mesophilic *Enterobacteriaceae* toward psychrotrophic *Enterobacteriaceae* can be expected during storage under chilled conditions. Our results agreed with those reported by Jacxsens et al. *(25)* for psychrotrophic growth on chicory endive (*Cichorium intybus foliosum* L.) stored at 4°C under conventional MAP and superatmospheric O_2. In the present assay, when salads were subjected to temperature changes throughout the chill chain, there was no difference in psychrotrophic growth under different MAPs (Table 2). Comparing psychrotrophic growth when the product was stored continuously at 4°C and at temperature fluctuations, the limiting criterion was exceeded 2 days before for MAP 1 and MAP 2 and 1 day before for MAP 3 when chill distribution chain was followed (Tables 1 and 2). As expected, the obtained bacteria counts were higher on product stored at 12°C in the display cabinet for most of the analyzed days.

Because of their influence on sensorial quality of the product, yeast counts seemed to be the limiting microorganisms for most of the fresh processed vegetables stored by MAP. The recommended limit was fixed at 5 log CFU/g (10^5 CFU/g) to guarantee the sensory quality *(17, 26)*. Several in vitro studies were performed on yeast growth under superatmospheric O_2 atmospheres. Jacxsens et al. *(25)* reported that growth of *Candida lambica* was reduced by superatmospheric O_2; however, Amanatidou et al. *(4)* observed that the growth rate of *Candida guilliermondii* and *Candida sake* was stimulated by approximately 80 kPa of O_2. On the other hand, the combined application of 80 kPa of O_2 and 20 kPa of CO_2 almost completely prevented growth of *C. guilliermondii* and *C. sake*. The effect of high O_2 on yeast growth was studied on fresh processed vegetables as well. Jacxsens et al. *(25)* reported that superatmospheric O_2 prolonged shelf life 3 more days when yeast growth was evaluated in chicory endive and grated celeriac (*Apium graveolens* var. *rapaceum* L.). In the present work, high initial yeast counts (close to 5 log CFU/g) were found in all experiments. Bags stored at 4°C exceeded this limit level after 3 days of storage by all applied MAPs (Fig. 3A). No difference was observed among the different MAPs before day 6. At that point, a difference of approximately 2-log CFU/g was observed between MAP 2 and MAP 3 and conventional MAP (MAP 1), which showed the lowest count. Yeast counts for MAP 1 and MAP 3 were similar on day 7; however, the same difference of 2 log CFU/g was kept to MAP 2. Equal tendency was observed on day 8. In storing the product at variable temperatures, the limiting level for yeast count was reached more quickly when prod-

TABLE 2. *Growth of psychotrophic bacteria, lactic acid bacteria, and* Enterobacteriaceae *(log_{10} CFU/g) on mixed vegetable salad packaged under conventional MAP (MAP 1) and superatmospheric O_2 (MAP 3) at variable temperatures, simulating the chill chain*[a]

	Psychotrophic bacteria			Lactic acid bacteria			*Enterobacteriaceae*		
Hour	7°C, MAP 1	7°C, MAP 3	12°C, MAP 3	7°C, MAP 1	7°C, MAP 3	12°C, MAP 3	7°C, MAP 1	7°C, MAP 3	12°C, MAP 3
0	6.33 (6.16–6.51)	6.67 (6.63–6.71)	6.67 (6.63–6.71)	4.01 (3.88–4.12)	3.45 (3.09–3.82)	3.45 (3.09–3.82)	3.47 (3.20–3.75)	3.47 (3.42–3.52)	3.47 (3.42–3.52)
24	6.82 (6.77–6.88)	6.54 (6.42–6.63)	6.54 (6.42–6.63)	4.80 (4.60–5.0)	4.53 (4.06–4.66)	4.53 (4.40–4.66)	3.63 (3.43–3.80)	4.06 (3.94–4.18)	4.06 (3.94–4.18)
42	7.06 (6.82–7.31)	6.61 (6.17–7.06)	6.61 (6.17–7.06)	5.80 (5.71–5.88)	4.55 (4.45–4.65)	4.55 (3.85–4.04)	4.30 (4.12–4.50)	4.63 (4.40–4.87)	4.63 (4.40–4.87)
52	7.07 (6.79–7.35)	7.22 (7.14–7.27)	7.22 (7.14–7.27)	5.77 (5.57–5.98)	4.45 (3.96–4.95)	4.45 (3.96–4.95)	4.52 (4.40–4.63)	4.69 (4.65–4.74)	4.69 (4.65–4.74)
100	7.49 (7.37–7.62)	7.33 (7.15–7.52)	7.70 (7.65–7.75)	5.78 (5.47–6.07)	5.32 (4.88–5.75)	5.15 (4.74–5.54)	5.14 (4.95–5.34)	5.77 (5.43–6.11)	5.50 (5.43–5.57)
102	7.82 (7.78–7.87)	7.57 (7.46–7.68)	8.03 (8.0–8.06)	5.73 (5.50–5.97)	5.18 (5.19–5.20)	5.47 (5.05–5.89)	5.20 (4.97–5.45)	5.55 (5.40–5.70)	5.94 (5.71–6.17)
118	7.82 (7.60–8.07)	7.74 (7.68–7.83)	8.24 (8.21–8.27)	5.81 (5.74–5.87)	5.83 (5.82–5.84)	5.26 (4.73–5.79)	5.38 (5.24–5.52)	6.18 (5.95–6.40)	6.39 (6.35–6.43)
142	8.04 (7.87–8.21)	8.13 (8.07–8.19)	7.99 (7.83–8.12)	5.88 (5.83–5.94)	6.03 (5.87–6.17)	5.37 (4.65–6.10)	5.45 (5.30–5.61)	6.17 (6.09–6.25)	6.32 (6.04–6.6)
166	8.06 (7.92–8.20)	8.11 (7.92–8.29)	8.04 (7.68–8.38)	6.34 (6.17–6.50)	5.91 (5.76–6.06)	5.94 (5.62–6.27)	5.59 (5.36–5.82)	6.09 (5.98–6.19)	6.13 (5.92–6.33)

[a] Bags stored under MAP 3 were subjected to 12 and 7°C at the display cabinet. Values between parentheses indicate a 95% confidence interval. n = 4.

uct was stored under MAP 3 (Fig. 3B). Product stored at 7°C under MAP 3 showed the lowest count after 7 days.

LAB growth under superatmospheric O_2 was tested in several in vitro studies. Amatinadou et al. *(5)* found an increase in the specific growth rate of *Leuconostoc mesenteroides* and *Lactobacillus lactis* from 0.036 to 0.044 μ (h^{-1}) and from 0.021 to 0.057 μ (h^{-1}), respectively, at 8°C in the presence of 90 kPa of O_2, whereas *Lactobacillus plantarum* was significantly reduced (63%). Day *(16)* reported that high O_2 levels (35 to 100 kPa) did not inhibit or stimulate growth of *Lactobacillus sake* at 8°C. Nevertheless, in the same report, other researchers found that superatmospheric O_2 levels inhibited the growth rate of several LAB (included *L. sake*). Jacxsens et al. *(25)* found that LAB were not able to growth under superatmospheric O_2 at 4°C because of the low temperature; however, Amatinadou et al. *(4)* reported that LAB were dominant in the presence of high O_2 and CO_2 concentrations (50 to 90 kPa of O_2 and 10 to 30 kPa of CO_2) in minimally processed carrots. When LAB growth was analyzed in fresh processed salads packaged under conventional MAP (MAP 1) at 4°C, LAB counts were always higher than those obtained under superatmospheric O_2 (MAP 2 and MAP 3) before day 6 of storage (Table 1). This represented an inhibition of LAB growth during the first 5 days of storage. On days 6, 7, and 8, there was no significant difference in LAB growth among the entire number of tested MAP techniques. Nevertheless, the LAB-limiting recommended level, 7 log CFU/g (10^7 CFU/g) *(17)*, was not surpassed in any case after 8 days at 4°C. Jacxsens et al. *(25)* reported that LAB were not important in the spoilage of mushrooms slices, grated celeriac, and shredded chicory endive stored at 4°C, because their numbers remained low. When temperature fluctuations were applied, simulating the chill chain, an initial LAB growth inhibition was found from the production day to day 4 of storage (Table 2). After that, LAB counts were similar among all the tested MAP techniques. Only on day 7 did LAB growth show a slight difference between superatmospheric O_2 and conventional MAP.

In vitro studies performed on *Enterobacteriaceae* reported an inhibition on their growth under superatmospheric O_2 conditions. *Salmonella* Typhimurium, *Salmonella* Enteritidis, and *Escherichia coli* showed a longer atmospheric lag phase in the presence of 90 kPa of O_2. Elevated O_2 concentrations alone reduced the final yield of *Salmonella* and slightly reduced the final yield of *E. coli (5)*; however, the *Enterobacteriaceae* growth cannot be the same in the real product. Amatinadou et al. *(4)* reported that *Enterobacteriaceae* growth based on plating in violet red bile glucose agar (Oxoid) was inhibited under 50 kPa of O_2 and 30 kPa of CO_2 but stimulated under 80 kPa or 90 kPa of O_2 in minimally processed carrots. In this study, *Enterobacteriaceae* showed greater growth in the product stored under MAP 1 without significant differences, except on day 5, where differences were significant (Table 1). On day 8, the obtained counts for *Enterobacteriaceae* were similar for all tested MAPs. Throughout the chill distribution chain, *Enterobacteriaceae* did not show any significant difference in its growth under any of the tested MAPs (Table 2). MAP 1 presented the lowest growth increase at variable temperatures after 7 days of storage.

Effect of superatmospheric O_2 on pathogenic microorganisms. An experimental study was performed to evaluate the survival or growth of inoculated *L. monocytogenes* on fresh-processed mixed vegetable salads. Product

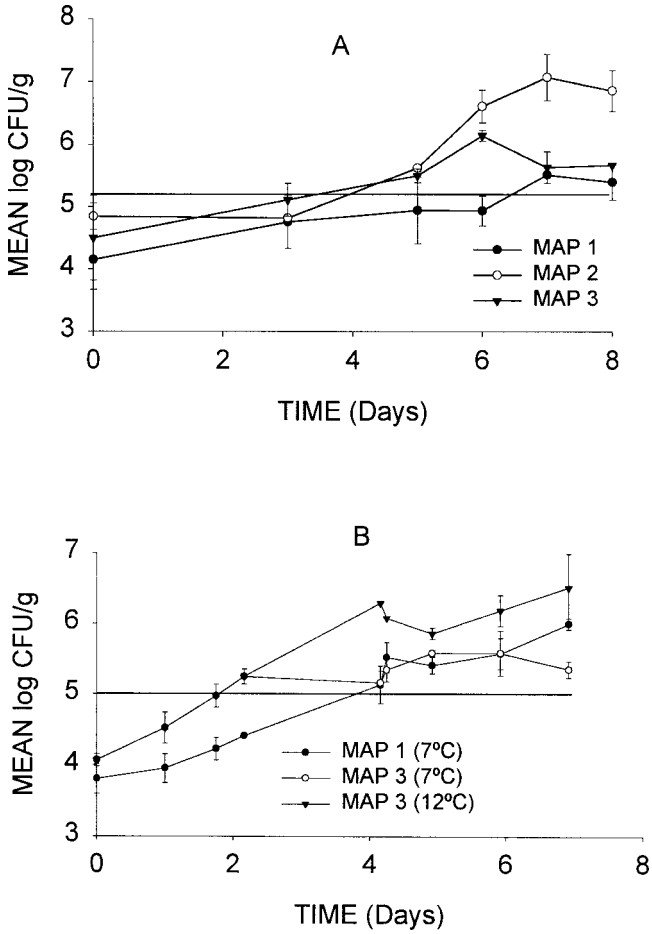

FIGURE 3. *(A) Yeast growth (log_{10} CFU/g) on mixed vegetable salads storage under three different MAP treatments: conventional (MAP 1) and superatmospheric O_2 (MAP 2 and MAP 3) at 4°C. (B) Yeast growth on mixed vegetable salad storage under conventional MAP (MAP 1) and superatmospheric O_2 MAP (MAP 3) at variable temperatures, simulating the chill distribution chain. Bags stored under MAP 3 were subjected to different temperatures at the display cabinet (12 and 7°C). Bars indicate the 95% confidence interval.* n = 4. *Straight lines indicate recommended microbial limit for yeast growth (10^5 CFU/g) proposed by Debevere (17).*

was packaged under superatmospheric O_2 (MAP 2 and MAP 3) and conventional MAP (MAP 1) at 4°C and analyzed to evaluate supporting growth of *L. monocytogenes.* Carlin et al. *(12)* reported that in several types of fresh processed green salads stored at 10°C for 7 days, *L. monocytogenes* showed a small growth increase. Various types of salad, stored at 4°C for 4 days, supported growth of *L. monocytogenes,* indicating that it can survive and multiply during storage of chilled precut salads, and, therefore, it is of concern as a potential contaminant of this type of product *(32).* Additionally, Farber et al. *(18)* revealed that the growth of this organism was less on cabbage packaged in high-permeable film, implying that in some cases the aerobic microflora, like the LAB, can compete successfully with *Listeria* spp. Supporting this statement, Aytac and Gorris *(7)* reported that although *L. monocytogenes* was unable to grow on air-stored chicory endive at 6.5°C, it did grow on the vacuum-packaged product stored at the same temperature. Results obtained in the present work on *L. monocytogenes* growth after 10 days of storage, when conventional MAP or superatmospheric O_2 was used, did not showed any significant change, except on day 5. On that day, *L. monocytogenes* counts were 0.85 ± 0.62 log CFU/g higher by using MAP 1 than MAP 2. Nevertheless, the rest of the changes were not significant (Tables 3 and 4), and consequently, *L. monocytogenes* only survived on mixed salads without any influence of the gas atmosphere within the bags. It was reported *(18)* that *L. monocytogenes* levels slightly increased or remained constant on various vegetable products (Caesar salad, coleslaw, onion, rutabaga, and stir-fry) stored at 4°C for 9 days. Our present results confirm these data. It is obvious that temperature has a strong effect on *L. monocytogenes* growth. Therefore, the influence of temperature changes along the chill distribution chain on mixed vegetable salads packaged under superatmospheric O_2 (MAP 3) was evaluated and compared with *L. monocytogenes* growth on conventional MAP (MAP 1) stored at the same temperature (Table 4). As expected, *L. monocytogenes* grew better when an abusive temperature (12°C) was applied; but again, the detected increase or decrease in *L. monocytogenes* growth under different MAP was not significant (Tables 3 and 4). Only survival of *L. monocytogenes* on lettuce was observed throughout the chill distribution chain.

There are not many studies on *A. caviae* on fresh processed vegetables. It was reported that *A. caviae* (HG4) was inhibited under 95 kPa of O_2 at in vitro conditions *(25).* In this case, *A. caviae* (HG4) growth was studied when inoculated in mixed vegetable salad stored following the chill distribution chain. Table 3 shows that *A. caviae* grew better under superatmospheric O_2 conditions (MAP 2 and MAP 3) when the product was stored continuously at 4°C. Similar results for product stored at 4°C were found at 7°C (Table 4). In this case, between days 2 and 5, stimulation of *A. caviae* growth was found. Finally, *A. caviae* counts were similar for all MAP after day 5.

Sensory quality. When comparing the evaluated sensory variables, color was the most determinative attribute for the overall visual quality of the product. Tissue browning due to oxidation of phenolic compounds, mainly by polyphenol oxidase enzyme *(27),* was also included in the color variable. No significant differences were obtained when the other sensory attributes were evaluated. Table 5 illustrates changes of the mean score for color and overall visual quality of mixed vegetable salads. Taking into account those sensory attributes, MAP 1 and MAP 2 exceeded the limit of marketability sooner than MAP 3 for both variables. This means that products kept until day 10 under superatmospheric O_2 (>60 kPa) conditions have a prolonged shelf life. On day 8 the product was not accepted any longer for all the studied MAPs. Off-odors were observed after 7 days of storage at 4°C, without any difference among treatments. Correlation of off-odors and aroma, measured sensorially with ethanol and acetaldehyde concentrations, was shown by López-Gálvez et al. *(29).* Heimdal et al. *(23)* reported that fresh processed iceberg lettuce

TABLE 3. *Growth of* Listeria monocytogenes *and* Aeromonas caviae *(log_{10} CFU/g) on mixed vegetable salad packaged under three MAP techniques*[a]

	Listeria monocytogenes			*Aeromonas caviae*		
Day	MAP 1	MAP 2	MAP 3	MAP 1	MAP 2	MAP 3
0	2.64 (2.55–2.75)	2.64 (2.55–2.74)	2.64 (2.55–2.74)	4.40 (4.22–4.58)	4.40 (3.79–5.01)	4.40 (3.79–5.01)
3	2.53 (2.13–2.90)	2.71 (2.48–2.85)	2.66 (1.93–3.39)	4.41 (4.20–4.62)	6.05 (5.75–6.96)	5.90 (5.75–6.05)
5	2.85 (2.56–3.14)	2.52 (2.46–2.58)	2.39 (2.21–2.55)	4.88 (4.47–5.29)	5.89 (4.98–5.94)	6.48 (6.34–6.61)
6	2.84 (2.54–3.13)	2.41 (2.31–2.51)	2.54 (2.53–2.55)	4.78 (4.65–4.91)	7.29 (7.04–7.53)	7.66 (7.57–7.75)
7	2.83 (2.47–3.23)	2.83 (2.72–2.95)	2.68 (2.59–2.77)	4.68 (4.48–4.88)	7.59 (7.53–7.64)	7.72 (7.58–7.86)
10	2.94 (2.68–3.20)	2.84 (2.76–2.92)	2.24 (1.76–2.70)	3.95 (3.70–4.20)	8.08 (7.71–8.44)	7.75 (7.36–8.15)

[a] MAP techniques included conventional (MAP 1) and superatmospheric O_2 (MAP 2 and MAP 3) at 4°C. Values between parentheses indicate a 95% confidence interval. $n = 4$.

packaged under 80 kPa of O_2 and 20 kPa of CO_2 showed less browning that those packaged in passive MAP. Instead of browning, a yellowing of leaves occurred in those bags, which was interpreted as a result of chlorophyll loss. No browning or yellowing of leaves occurred in MAP 2 and MAP 3 because of higher CO_2 levels. Jacxsens et al. *(25)* reported that superatmospheric O_2 was particularly effective in inhibiting enzymatic browning of different fresh processed vegetables, including shredded chicory endive. Beneficial effects of superatmospheric O_2 in sensory quality of different vegetable products sensitive to enzymatic discoloration (e.g., radicchio lettuce, lollo rossa lettuce) have already been reported *(15, 16)*.

CONCLUSIONS

It has been confirmed that superatmospheric O_2 does not affect all microorganisms in the same way. From our work, it can be concluded that psychrotrophic bacteria growing in mixed vegetable salad were not affected by use of different MAP techniques. Yeast growth was stimulated when superatmospheric O_2 was applied; however, LAB and *Enterobacteriaceae* showed growth inhibition when elevated O_2 and CO_2 concentrations were applied by MAP 2 and MAP 3. As expected, all bacteria counts were higher when the product followed the chill distribution chain, and the same happened when an abusive temperature (12°C) was

TABLE 4. *Growth of* Listeria monocytogenes *and* Aeromonas caviae *(log_{10} CFU/g) on mixed vegetable salad packaged under conventional MAP (MAP 1) and superatmospheric O_2 (MAP 3) at variable temperatures, simulating the chill distribution chain*[a]

	Listeria monocytogenes			*Aeromonas caviae*		
Hour	7°C, MAP 1	7°C, MAP 3	12°C, MAP 3	7°C, MAP 1	7°C, MAP 3	12°C, MAP 3
0	2.14 (1.95–2.31)	2.14 (1.85–2.44)	2.14 (1.85–2.44)	5.81 (5.67–5.92)	5.84 (5.83–5.85)	5.84 (5.83–5.85)
24	2.30 (2.02–2.58)	2.66 (2.47–2.88)	2.66 (2.47–2.88)	5.88 (5.77–5.99)	6.16 (5.66–6.65)	6.16 (5.66–6.65)
42	2.69 (2.67–2.71)	2.29 (2.14–2.44)	2.29 (2.15–2.44)	6.13 (6.02–6.22)	6.59 (6.30–6.89)	6.59 (6.30–6.89)
52	2.70 (2.50–2.88)	2.54 (2.42–2.65)	2.54 (2.42–2.65)	6.17 (5.99–6.34)	6.57 (6.50–6.65)	6.57 (6.50-6.65)
100	2.46 (2.35–2.56)	2.59 (2.46–2.70)	2.39 (2.26–2.50)	6.54 (6.46–6.63)	7.05 (6.73–7.34)	7.4 (7.11–7.67)
102	2.49 (2.30–2.70)	2.52 (2.43–2.61)	2.34 (2.25–2.43)	6.66 (6.41–6.93)	7.35 (7.08–7.61)	8.05 (8.04–8.06)
118	2.43 (2.36–2.49)	2.64 (2.55–2.74)	2.29 (2.20–2.39)	6.86 (6.71–7.00)	7.17 (6.82–7.51)	7.75 (7.60–7.92)
142	2.16 (2.03–2.29)	2.48 (2.47–2.49)	2.59 (2.57–2.61)	7.12 (7.02–7.22)	7.19 (6.95–7.44)	7.89 (7.65–8.14)
166	2.31 (2.18–2.44)	2.00 (1.80–2.20)	2.83 (2.72–2.95)	6.84 (6.65–7.03)	7.17 (6.99–7.34)	7.57 (7.39–7.75)

[a] Bags stored under MAP 3 were subjected to 12 and 7°C at the display cabinet. Values between parentheses indicate a 95% confidence interval. $n = 4$.

TABLE 5. *Score means for color and visual appearance of mixed vegetable salad under three MAP techniques*[a]

	Color			Visual appearance		
Day	MAP 1	MAP 2	MAP 3	MAP 1	MAP 2	MAP 3
0	1.00	1.00	1.00	1.00	1.00	1.00
3	1.50	1.50	1.50	1.50	1.50	1.00
	(0.98–2.00)	(1.21–2.04)	(1.39–1.61)	(1.05–1.94)	(0.98–2.00)	(0.93–1.06)
5	2.60	4.00	2.50	2.80	4.00	2.70
	(2.02–3.16)	(3.49–4.48)	(2.10–2.89)	(2.32–3.27)	(3.55–4.43)	(2.46–2.93)
6	3.30	5.00	3.00	4.00	5.40	3.30
	(2.62–4.02)	(4.60–5.39)	(2.91–3.29)	(3.50–4.49)	(4.50–6.29)	(3.27–3.33)
7	6.00	5.50	5.30	7.00	5.70	4.90
	(5.42–6.56)	(5.12–5.75)	(5.26–5.31)	(6.33–7.66)	(5.27–6.11)	(4.75–5.04)
8	6.25	6.00	5.40	6.50	7.00	5.40
	(5.49–6.70)	(5.46–6.53)	(5.33–5.44)	(5.95–7.04)	(6.38–7.60)	(5.36–5.43)

[a] MAP techniques include conventional (MAP 1) and superatmospheric O_2 (MAP 2 and MAP 3) at 4°C. Values between parentheses indicate a 95% confidence interval. $n = 4$.

used in the display cabinets. The challenge test showed that *L. monocytogenes* survived when it was inoculated in fresh processed mixed vegetable salads and there was no effect of gas atmosphere. However, superatmospheric O_2 stimulated *A. caviae* growth. The general appearance was maintained longer, and the shelf life of the product was prolonged by using O_2 concentrations higher than 60 kPa throughout the storage period. Color was the most important parameter that affected general appearance, and mixed vegetable salad stored under elevated O_2 and CO_2 concentrations obtained the best score for this parameter.

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