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Effect of modified atmosphere packaging on the growth and survival of listeria in raw minced beef

Efecto del envasado en atmósferas modificadas sobre el crecimiento y la supervivencia de listeria en carne picada de ternera

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A study on the behaviour of *Listeria monocytogenes* and *L. innocua* in raw minced meat packaged under modified atmospheres was carried out. Three gas atmospheres were tested with various CO_2 concentrations (100% CO_2; 65% CO_2, 25% O_2, 10% N_2; 20% CO_2, 80% O_2). Packages containing minced meat were inoculated or uninoculated with *L. monocytogenes* and *L. innocua* and were stored at 4 °C for 18 d. Samples were taken every 3 d, and the development of the bacterial species, pH and water activity (a_w) were monitored. The 100% CO_2 atmosphere was the most effective for the inhibition of growth of both species; pH influenced microbial inhibition but low pH values were not the most important factor in the inhibition of *Listeria*, the direct effect of the CO_2 was necessary for that inhibition. Water activity values did not change during storage. None of the gas mixtures were bactericidal. The numbers of *L. innocua* recovered from all the modified atmospheres tested were always lower than those of *L. monocytogenes*.

Keywords: *Listeria monocytogenes*, *Listeria innocua*, packaging, modified atmospheres, beef

Se ha realizado un estudio del comportamiento de *Listeria monocytogenes* y *Listeria innocua* en carne picada envasada en atmósferas modificadas. Se han probado tres atmósferas con distintas concentraciones de CO_2 (100% CO_2; 65% CO_2, 25% O_2, 10% N_2; 20% CO_2, 80% O_2). Los envases con la carne picada no inoculada o inoculada con *L. monocytogenes* y *L. innocua* se almacenaron a 4 °C durante 18 días. Se tomaron muestras cada tres días y se determinó el desarrollo de ambas especies así como los valores de pH y a_w. La atmósfera con un 100% de CO_2 fue la más efectiva en la inhibición del crecimiento de ambas especies. El pH pudo tener influencia en la inhibición microbiana en envases con 100% de CO_2, sin embargo fue necesario el efecto directo del CO_2 para conseguir dicha inhibición. Ninguna de las mezclas gaseosas tuvo efecto bactericida. Los recuentos de *L. innocua* fueron inferiores a los de *L. monocytogenes* en las tres mezclas.

Palabras clave: *Listeria monocytogenes*, *Listeria innocua*, envasado atmósfera modificada, carne de ternera

INTRODUCTION

Carbon dioxide (CO_2) is capable of inhibiting the growth of aerobic spoilage microorganisms and moulds, although lactic acid bacteria are still able grow (Brody, 1989). CO_2 shows an inhibitory effect

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on food microflora, which is dependent on several factors: CO_2 concentration, partial pressure of CO_2, temperature, pH, water activity (a_w), type of microorganism and microbial growth phase. The highest inhibition is obtained by the use of 40–60% CO_2 (Farber, 1991), although some studies have shown that 20–30% CO_2 concentrations could be sufficient (Seideman and Durland, 1984).

Modified atmosphere packaging is capable of extending the shelf-life of fresh beef from 50 to 400% compared with packaging in air (Hotchkiss, 1988). One of the risks associated with packaging meat under modified atmospheres is the differential survival and/or the stimulation of the growth of pathogens, such as *Yersinia enterocolitica, Aeromonas hydrophila, Campylobacter jejuni, Clostridium botulinum* and *Listeria monocytogenes* (Farber, 1991). The extra time which pathogens may have for growth during an extended shelf-life increases this risk. The behaviour of *L. monocytogenes* in beef packaged in modified atmospheres is of concern because of its ability to survive and grow under adverse conditions (Wimpfheimer *et al.*, 1990). However, not much work on the effect of modified atmospheres on *Listeria* spp. has been published (Wimpfheimer *et al.*, 1990; Farber, 1991).

Listeriosis outbreaks linked to consumption of meats contaminated with *L. monocytogenes* have been described: among them, an outbreak of listeriosis in the USA in 1989 and one in the UK in 1988 should be cited (Centers for Disease Control, 1989). The risk of contamination of food with *L. monocytogenes* increases according to the amount of processing carried out; it has been found that minced meats generally showed higher contamination levels than unminced meat (Johnson *et al.*, 1990).

This report studies the effect of three gas atmospheres on the behaviour of two strains of the genus *Listeria*, (one strain of *L. monocytogenes* and one strain of the apathogenic *L. innocua*) when inoculated into fresh minced beef packaged under modified atmospheres.

MATERIAL AND METHODS

Preparation of samples

Organisms

The *Listeria* strains were previously isolated in our laboratory from meat and identified as typical *Listeria* spp. colonies on LSAMM (*Listeria* Selective Agar Modified Medium) [i.e. black point in the centre (tellurite reduction), with a surrounding black halo (esculin hydrolysis), gram stain, catalase, motility at 25 °C, haemolytic profile using the overlay technique, and API *Listeria* kits (Biomerieux, France)].

Preparation of inocula

Tubes of BHI (brain heart infusion) broth were inoculated with *L. monocytogenes* or *L. innocua* from pure cultures on BHI agar slants. BHI broth tubes were incubated at 37 °C for 24 h to obtain a final concentration of 1×10^{10} cfu/ml for both species. Appropriate dilutions were then made in sterile distilled water to obtain the final inocula.

Preparation and inoculation of minced meat

Fresh beef (chuck) was purchased from a local market, deboned and minced for 10 s to homogeneity. The minced meat was then immersed for 5 min in diluted cultures [in BHI broth (Difco, USA)] of *L. monocytogenes* and *L. innocua* (1×10^8 cfu/ml) to obtain a final concentration of approximately 10^4–10^6 cfu/g of minced meat (Kim and Slavik, 1994). The absence of Listeria in the uninoculated minced meat was demonstrated.

Packaging under modified atmospheres

Inoculated minced beef was divided into 36 portions of 50 g providing 12 samples for each modified atmosphere. Each sample was placed in a 17×25 cm Cryovac barrier bag (O_2 transmission rate between 2 and 5 ml/m^2. 24 h.atm at 4.4 °C and 0% RH) and randomly assigned to one of three gas atmospheres: 100% CO_2; 65% CO_2, 25% O_2, 10% N_2; 20% CO_2, 80% O_2. These atmospheres were based on published research (Gill and Reichel, 1989; Farber, 1991; Madrid-Vicente *et al.*, 1991). Packaging was carried out with a chamber-type, heat seal packaging machine (Model Vac-210. Talleres Guasch. Barcelona, Spain). A proportional gas mixer (Model KM 100–3M. Witt-Gassetechnik, Germany) was used to give the desired proportions of CO_2, O_2 and N_2.

Bags were stored at 4 °C for up to 18 d. Two bags containing inoculated beef and an uninoculated control sample per gas mixture were opened aseptically every 3 d for analysis. All analyses were performed in duplicate.

Methods

Microbiological analyses

Uninoculated meat (10 g) was homogenized with 90 ml FDA (Food and Drugs Administration) enrich-

ment broth (Lovett, 1988) in a sterile bag using a Stomacher blender. Homogenates were incubated at 30 °C for 48–72 h. After incubation, a loopful of homogenate was streaked onto LSAMM to confirm the absence of *Listeria* spp. in uninoculated minced meat (Lovett, 1988).

Packaged meat (25 g) was homogenized for 1 minute in a sterile bag containing 225 ml of peptone water (Difco Labs, Detroit, USA). Serial dilutions were prepared with the same medium; 0.1 ml of suitable dilutions were surface plated in duplicate on LSAMM and incubated at 37 °C for 48 h. Colonies growing on this agar which had a black point in the centre (tellurite reduction) and a surrounding black halo (esculin hydrolysis) were assumed to be *Listeria* spp. (Blanco *et al.*, 1989; Domínguez *et al.*, 1990).

Differentiation between *L. monocytogenes* and *L. innocua* and enumeration were conducted using the overlay technique. LSAMM plates with colonies of *L. monocytogenes* and *L. innocua* were covered with a semisolid medium (8 ml per plate) containing suspended sheep blood red cells. Plates were preincubated at 4 °C for 2 h and then incubated at 30 °C for 14 h (Blanco *et al.*, 1989; Domínguez *et al.*, 1990). Two suspected colonies from each plate were tested for catalase activity and motility at 25 °C and identified using API *Listeria* kits (Biomerieux, France).

Physico-chemical analyses

pH was measured with a calibrated pH-meter (Model 2001. Crison Instruments, Spain) using a spear-tip electrode (Presidencia del Gobierno, 1979).

Water activity was determined from dew point measurements obtained with an AquaLab hygrometer (Model CX-2. Decagon Devices, Inc., USA), which uses the chilled mirror technique.

Statistical analyses

A mean value for each analysis was obtained from two replicates. Significant differences among means ($p < 0.05$) were determined by the paired t-test.

RESULTS

Modified atmospheres

Pure CO_2 atmosphere

A continuous decrease in numbers of *L. monocytogenes* (Figure 1) and *L. innocua* (Figure 2) was detected, although some fluctuations were observed. pH values fluctuated, with a maximum at 9 d and a minimum at 18 d (Table 1). Water activity values showed no important fluctuations, with maxima at 9 and 12 d and a minimum at 6 d (Table 2).

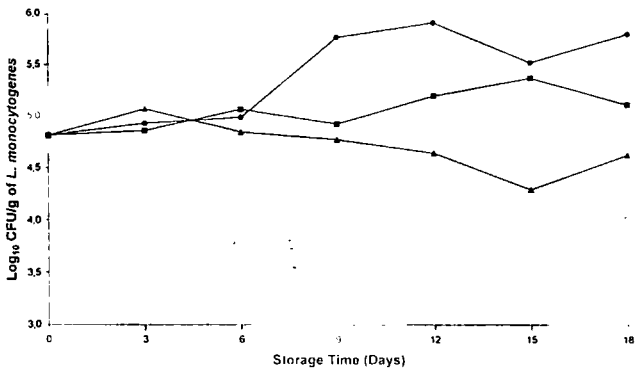

Figure 1. Growth of *L. monocytogenes* under the three different atmospheres tested. ▲ 100% CO_2, ■ 65% CO_2, ● 20% CO_2.

Figura 1. Evolución de *L. monocytogenes* para cada una de las tres atmósferas ensayadas. ▲ 100% CO_2, ■ 65% CO_2, ● 20% CO_2.

65% CO_2 plus O_2 and N_2 atmosphere

L. monocytogenes grew throughout the entire storage period and was highest at 15 d, although it declined slightly on days 9 and 18 (Figure 1). The growth of *L. innocua* was not as high as that of *L. monocytogenes*. A slight decrease was detected from 6 d but with a

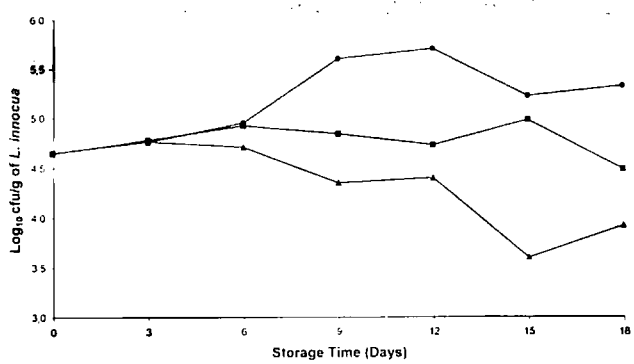

Figure 2. Growth of *L. innocua* under the three different atmospheres tested. ▲ 100% CO_2, ■ 65% CO_2, 20% ● CO_2.

Figura 2. Evolución de *L. innocua* para cada una de las tres atmósferas ensayadas. ▲ 100% CO_2, ■ 65% CO_2, ● 20% CO_2.

Table 1. Comparison of pH values between inoculated (I) and uninoculated (U) beef samples packaged under three different modified atmospheres.

Tabla 1. Comparación entre los valores de pH de muestras de carne inoculadas (I) y no inoculadas (U) envasadas con tres atmósferas diferentes.

	100% CO_2		65% CO_2		20% CO_2	
Days	U	I	U	I	U	I
0	5.60	5.79	5.60	5.79	5.60	5.79
3	5.72	5.84	5.81	5.82	5.83	5.83
6	5.68	5.74	5.69	5.84	5.71	5.85
9	5.71	5.87	5.70	5.73	5.80	5.90
12	5.67	5.39	5.59	5.25	5.67	5.92
15	5.65	5.42	5.33	5.21	5.87	5.88
18	5.25	5.22	5.27	5.13	5.88	5.83

Table 2. Comparison of water activity values between inoculated (I) and uninoculated (U) beef samples packaged under three different modified atmospheres.

Tabla 2. Comparación entre los valores de actividad de agua de muestras de carne inoculadas (I) y no inoculadas (U) envasadas con tres atmósferas diferentes.

	100% CO_2		65% CO_2		20% CO_2	
Days	U	I	U	I	U	I
0	0.994	0.996	0.994	0.996	0.994	0.996
3	nd	0.997	0.993	0.997	0.997	0.998
6	0.996	0.996	0.995	0.997	0.996	0.997
9	0.991	0.998	0.995	0.998	0.996	0.996
12	0.996	0.998	0.996	0.998	0.997	0.998
15	0.991	0.997	0.995	0.996	0.997	0.997
18	0.991	0.997	0.996	0.998	0.999	0.997

nd: not determined

maximum at 15 d (Figure 2). pH values decreased throughout storage time (Table 1). Water activity showed a fluctuation for all the storage conditions, but it was very slight (Table 2).

20% CO_2 and O_2 atmosphere

The increase in levels of *L. monocytogenes* was always very apparent, except on day 15 (Figure 1). Growth of *L. innocua* was also apparent (Figure 2), but the increase was lower than for *L. monocytogenes*. pH values fluctuated between a maximum at 12 d and a minimum at 3 and 12 d (Table 1). Water activity fluctuated only slightly and may thus be considered constant (Table 2).

DISCUSSION

Behaviour of *L. monocytogenes* and *L. innocua* did not differ at 3 and 6 d. Differences between the two *Listeria* species became apparent at 9 d at which time the different effects of the three modified atmospheres could be assessed. Paired contrasts did not indicate significant differences between numbers of *L. monocytogenes* in pure CO_2 and $CO_2/O_2/N_2$ (65:25:10) atmospheres ($p = 0.08$) or between $CO_2/O_2/N_2$ (65:25:10) and CO_2/O_2 (20:80) atmospheres ($p = 0.05$). Significant differences were found between pure CO_2 and CO_2/O_2 (20:80) atmospheres ($p = 0.02$). All of the comparisons among numbers of *L. innocua* from the three modified atmospheres showed significant differences (pure CO_2 and $CO_2/O_2/N_2$ (65:25:10) atmosphere, $p = 0.04$; $CO_2/O_2/N_2$ (65:25:10) and CO_2/O_2 (20:80) atmosphere, $p = 0.04$; pure CO_2 and CO_2/O_2 (20:80) atmosphere, $p = 0.02$).

The pure CO_2 atmosphere was the only one that showed a slight effect on the inhibition of *L. monocytogenes* and *L. innocua* growth; the CO_2/O_2 (20:80) atmosphere shows the lowest effect. The differences between *Listeria* populations in the three gas mixtures became important only after the ninth day, probably due to the higher generation time at low temperature (33 h at 4 °C and pH = 7) (Petran and Zottola, 1989).

A pH of 5.6 was found in uninoculated meat; this is a normal post-rigor mortis level (Moreno, 1991). A pH of less than 6.0 is not optimal for *L. monocytogenes* growth (Petran and Zottola, 1989) but this bacterium is capable of growing below pH 5.0 (George *et al.*, 1988; Farber *et al.*, 1989; Parish and Higgins, 1989; Sorrells *et al.*, 1989) although its growth begins after a prolonged incubation period. Other factors, such as temperature, could influence microbial behaviour, but in this study the highest temperature was 4 °C, at which *L. monocytogenes* is capable of growth. Uncontrolled factors could inhibit the growth of *L. monocytogenes*, such as antibiotic remains, but *L. monocytogenes* and *L. innocua* should be able to grow for the full 18 d according to the pH values obtained.

Samples in the 20% CO_2 atmosphere showed the highest pH values (Table 1) because this mixture contains the lowest proportion of CO_2. It is well known that CO_2 contributes to the decrease in pH; this is why the pH was lower in samples from 65% CO_2 and 100% CO_2 atmospheres. Significant differ-

ences were not found between pH values in samples from the pure CO_2 atmosphere and those from 65% CO_2 atmosphere ($p = 0.1$). However, *Listeria* did not grow in the pure CO_2 atmosphere. Therefore, low pH values are not the most important factor influencing on the inhibition of *Listeria* and the direct effect exerted by CO_2 is necessary for that inhibition.

There were no significant differences between the pH values of inoculated and uninoculated samples in any atmosphere (pure CO_2, $p = 0.67$; 65% CO_2, $p = 0.37$; 20% CO_2, $p = 0.15$). Changes in pH during storage were caused by the effect of the three atmospheres on beef and tested microorganisms did not influence those pH changes.

It was expected that the inoculation procedure would increase water activity (a_w) values in inoculated samples (Table 2): a_w values in the inoculated samples were significantly higher than those in the uninoculated samples. The minimum a_w in uninoculated samples was 0.991. The lowest a_w required for growth of pathogenic bacteria is approximately 0.920–0.930 (Skovgaard, 1987; Petran and Zottola, 1989), and the optimum for growth is 0.970 (Petran and Zottola, 1989). Practically all microorganisms can grow when the a_w is 0.980 (Mossel and Moreno-García, 1984). Measured levels of water activity were not important in the inhibition of the microorganisms tested.

The effects of the modified atmospheres seems to be responsible for the data obtained, together with a probable contribution from pH which in turn is likely to be influenced by the gas atmosphere. It is known that CO_2 has a bacteriostatic effect (Brody, 1989; Farber, 1991; Madrid-Vicente *et al.*, 1991) which verifies the results obtained, a 100% CO_2 concentration was reported to be necessary to reach that effect (Gill and Reichel, 1989; Avery *et al.*, 1994).

Numbers of the apathogenic species (*L. innocua*) were lower than numbers of the pathogenic one (*L. monocytogenes*) (Figures 1 and 2). When paired contrasts were used to determine significant differences between the counts of these two species, they were found for all three gas mixtures. The initial rate of *L. innocua* growth was lower than that of the pathogenic species, and although this fact could have had some influence on the results, it had very little, if any. This could indicate that *L. monocytogenes* is more resistant to these adverse environmental conditions.

The growth of *L. monocytogenes* and *L. innocua* in meat exudate in packages was investigated because this product is optimum for development of spoilage and pathogenic microorganisms. Counts were made after 12 d at which time the exudate had reached a significant volume, due to the inoculation procedure used (Table 3). Bacterial counts in the exudates were more elevated than those for the meat in each atmosphere; counts for the 65% CO_2 atmosphere were higher than those for 100% CO_2 in agreement with the differences observed between counts in the meat. Samples packaged in the 20% CO_2 atmosphere had counts greater than 1×10^6cfu/g.

Table 3. Counts ($\log_{10}$ cfu) of *L. monocytogenes* (L.m.) and *L. innocua* (L.i.) in meat exudates in the packages.

Tabla 3. Poblaciones ($\log_{10}$ cfu) de *L. monocytogenes* (L.m.) y *L. innocua* (L.i.) en el exudado de los envases de carne.

	100% CO_2		65% CO_2		20% CO_2	
Days	L.m.	L.i.	L.m.	L.i.	L.m.	L.i.
12	5.38	4.22	5.23	4.08	5.72	5.06
15	4.28	nd	5.02	3.84	> 6.00	> 6.00
18	5.47	4.56	6.02	5.03	> 6.00	> 6.00

nd: not determined

In conclusion, a CO_2 concentration of greater than 65% was necessary to inhibit the growth of *L. monocytogenes* and *L. innocua*. In addition, all three atmospheres failed to exert any bactericidal effect on either species. The greater sensitivity of *L. innocua* to modified atmospheres was detected for all CO_2 concentrations tested.

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