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ORIGINAL ARTICLE

The growth of *Listeria monocytogenes* in fresh goat cheese (Cameros cheese) packaged under modified atmospheres

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The effect of modified atmosphere packaging (MAP) on the growth of Listeria monocytogenes *in inoculated and non-inoculated Cameros cheese was evaluated. Three different modified atmosphere conditions were studied (20%CO_2/80%N_2, 40%CO_2/60%N_2 and 100%CO_2). Control cheeses were packaged in air. The product was stored at 4°C and evaluated periodically to investigate its microbiological quality.*

MAP presented an extended shelf-life. Those containing CO_2 reduced the growth rate of mesophiles, psychrotrophs and anaerobes, which was lower when the CO_2 concentration increased. A concentration of 100% CO_2 showed the lowest microbial counts. L. monocytogenes *growth was lower when the CO_2 concentration increased. However, after 28 days the* L. monocytogenes *population was 1·3 log units lower in inoculated cheeses packaged at 100% CO_2 than in those packaged in air.* Listeria monocytogenes *can grow in atmospheres containing 20, 40 and 100% CO_2.* L. monocytogenes *were not found in any of the non-inoculated samples. It was concluded that MAP was not a suitable means to prevent the growth of* L. monocytogenes *in Cameros cheese.*

Introduction

Cameros cheese is a fresh cheese made from pasteurized goat milk. It takes its name from the Cameros geographical area in the province of La Rioja (Spain). Cameros cheese is a fat cheese (54·2±6·5% of total scuds TS) with a high pH close to neutral (6·35±0·14), a high moisture (TS value of 42·5±4·7%) and a low salt content (0·78±0·30% of TS) (Olarte et al. 1999). Given the presence of oxygen, high water activity and the high pH of fresh cheese, the micro-organisms responsible for spoilage can grow easily. Thus, the shelf-life of fresh cheese under refrigeration is only 7 days.

The increased consumer demand for fresh, preservative-free foods has led to the use of modified atmosphere packaging (MAP) as a technique to improve product image and extend the shelf-life of various foods, including cheeses (Pergiovanni et al. 1993, Maniar et al. 1994, González-Fandos et al. 2000). The optimal composition of modified atmospheres for Cameros cheese has been established at 50%CO_2/50%N_2 or 40%CO_2/60N_2 (González-Fandos et al. 2000). However, there is a great deal of concern about the microbiological safety of foods as regards packaging because of the ability of this pathogen to grow as a facultative anaerobe (Hotchkiss and Banco 1992). Using packaging

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with modified atmospheres, pathogen growth may occur or even be stimulated before spoilage becomes evident.

Listeria monocytogenes has been known to be a foodborne pathogen since the 1970s. In addition to vegetables and meat, certain types of cheeses were shown to be ideal media for *Listeria* growth (Genigeorgis et al. 1991, Mossel et al. 1995). Moreover, certain types of fresh cheeses (Mexican soft cheese) have been implicated in several outbreaks (Linnan et al. 1988).

The potential for growth of *L. monocytogenes* in Cameros cheese is well documented (Olarte et al. 1999). In this fresh cheese, the final pH ($6{\cdot}35 \pm 0{\cdot}14$) may not be inhibitory to *L. monocytogenes*. As this pathogen has a pH range for growth of 5·1–9·6, the optimum being in the region of 6 (ICMSF 1996), it can be seen that there is a potential for growth in this cheese. Moreover, *L. monocytogenes* is a psychrotrophic pathogen with a growth range from −1°C to 45°C (ICMSF 1996). Bahk and Marth (1990) reported that this micro-organism can grow at temperatures as low as 4°C in milk. This is a significant factor since Cameros cheese is stored under refrigeration at 4°C.

The aim of this work was to evaluate the potential growth of *L. monocytogenes* in Cameros cheese packaged under modified atmospheres.

Materials and Methods

Preparation of L. monocytogenes *inoculum*

The *L. monocytogenes* serovar 4b strain NCTC 11994 was grown in brain–heart infusion broth (Oxoid) for 18 h. The culture was centrifuged at 10 000 *g* for 10 min at 4°C. The supernatant fluid was decanted and the pellet resuspended in pasteurized goat milk. The suspension of cells was diluted in pasteurized milk to obtain the appropiate cell concentration for the inoculation of the product after pasteurization to simulate post-pasteurization contamination (frequently from equipment or handling) (ICMSF 1980).

Preparation of cheeses

The sample cheeses were manufactured in our pilot plant from pasteurized (72°C/15 s) goat milk, following the same procedure as the producers. After pasteurization of the milk some batches were inoculated to provide final levels of 3 log cfu ml^{-1} (Table 1). A quantity of $0{\cdot}2\ g\ l^{-1}$ milk of NaCl, $0{\cdot}2\ g\ l^{-1}$ milk of $CaCl_2$ and $0{\cdot}25\ ml\ l^{-1}$ milk of commercial calf rennet (strength 1:10000) (Lab. Arroyo, Santander, Spain) was also added. Curd formation was achieved at 32–33°C after 45 min. Next, the curd was cut, whey was removed and the molds were filled (Olarte et al. 1995). After 12 h of refrigeration, the cheeses were removed from the molds and the control samples were packaged in air. The other cheeses were packaged under different modified atmosphere conditions: $20\%CO_2/80\%N_2$, $40\%CO_2/60\%N_2$ and $100\%CO_2$. The plastic films used were provided by Dixie (Dixie, Bern, Switzerland) with a CO_2 permeability of less than $13\ cm^3\ m^{-2}\ 24\ h^{-1}$ at 1 atm and O_2 permeability of $5\ cm^3\ m^{-2}\ 24\ h^{-1}$ at 1 atm. The packages were evacuated, flushed and sealed in a Vaessen-Schoemake machine with gas injection. The gases used were industrial

Table 1. Atmosphere composition and inoculation of different cheese batches

Batch	Atmosphere	*Listeria monocytogenes* inoculation
C	Air	No
C20	$20\%CO_2/80\%N_2$	No
C40	$40\%CO_2/60\%N_2$	No
C100	$100\%CO_2$	No
L	Air	Yes
L20	$20\%CO_2/80\%N_2$	Yes
L40	$40\%CO_2/60\%N_2$	Yes
L100	$100\%CO_2$	Yes

mixtures provided by Carburos Metálicos (Spain).

All the cheeses were stored at 4°C for 28 days. Samples were analysed on day 0 and after 7, 14, 21 and 28 days of storage.

Two experiments were carried out. In each experiment eight batches were studied. The atmosphere composition and batches inoculated with *L. monocytogenes* are described in Table 1.

Physicochemical analyses

pH was measured with a Crison model 2002 pHmeter with a penetration electrode (Crison Instruments, Barcelona, Spain). TS were determined according to FIL-IDF 4A:1982 (Anon 1982). All analyses were carried out in duplicate.

Gas determination

Carbon dioxide and oxygen were determined using an O_2 and CO_2 head space gas analyser, Checkmate model 9900 (PBI-Dansensor, Denmark). Determinations were performed in duplicate.

Microbiological analyses

Cheese samples (25 g) were homogenized for 1 min in 225 ml of a sterile solution of 2% (w/v) sodium citrate using the Stomacher (IUL, Barcelona, Spain). Further decimal dilutions were prepared with the same diluent. Analyses were carried out using the following procedures.

Mesophilic micro-organisms were enumerated on plate count agar (Difco, Detroit, Michigan, US) following the pour plate method and incubated at 30°C ± 1°C for 72 h (ICMSF 1978).

Psychrotrophs were determined on plate count agar at an incubation temperature of 7°C ± 1°C for 10 days following the pour plate method (ICMSF 1978).

Anaerobes were determined on plate count agar following the pour plate method and incubated under anaerobic conditions at 30°C ± 1°C for 2 days (ICMSF 1978).

The presence of *Listeria* spp. was investigated by the following procedure: a 25 g sample was homogenized with 225 ml of Listeria enrichment broth (LEB, Merck, Darmstadt, Germany) in a Stomacher. The enrichment broth was incubated at 30°C for 48 h. LEB cultures were streaked onto Palcam agar (Merck) and the plates were then incubated at 37°C for 48 h and analysed for the presence of *Listeria* colonies. Suspected colonies grown on Palcam agar were subcultured for purity on tryptone soya agar (TSA) and confirmed as *L. monocytogenes* by the aminopeptidase, catalase and oxidase test, growth at 37°C, tumbling motility at 20–25°C, umbrella motility in the SIM medium (Oxoid, Unipath, UK), aesculin and urea hydrolysis and CAMP test (Seeliger and Jones 1986). Suspected isolates were also identified by using API *Listeria* strips (BioMérieux, Marey Lètoile, France).

All analyses were performed in duplicate.

Statistical analysis

Analysis of variance was performed using the SYSTAT program for Windows; Statistics version 5.0 (Evanston, Illinois, US, 1992). Tukey's test was performed for comparison of means using the same program. Significance was defined as $P \leq 0{\cdot}05$.

Plate count data were written as logarithms before statistical treatment.

The data reported in this paper represent means of values from two replicated experiments in which all analyses were performed in duplicate.

Results and Discussion

Slight changes in gas concentrations in the packages were detected. The initial CO_2 concentrations were approximately 20, 40 and 100% (v/v) depending on the batch. CO_2 concentrations declined during the first 7 days of storage in all the batches packaged in MAP (2–5%). Moir et al. (1993) also observed this decrease in carbon dioxide concentrations; this may be because CO_2 dissolves into the cheeses and spreads out of the package.

The oxygen levels detected in cheeses packaged in MAP at day 0 were below 2%. These levels are expected since the structure of the cheese tends to trap air (Moir et al. 1993). The oxygen levels decreased during the first 7 days

(<0·6%) because of intake of micro-organisms. After 28 days, oxygen levels were around 0·01%.

The physicochemical results are shown in Figs 1 and 2. The characteristics of the cheeses prepared were similar to those of previously studied commercial cheeses (Olarte et al. 1999), except as regards their humidity percentages, which were higher in the cheeses prepared in our pilot plant (65·71% ± 2·61).

The average moisture decreased during storage for all cheese samples. Significant differences were not found between inoculated and non-inoculated cheeses packaged under the same conditions. The mean moisture losses in cheeses packaged under 20%CO_2/80%N_2 and 40%CO_2/60%N_2 were very similar (3·78% and 4·15%, respectively) after 28 days. These moisture losses were lower than in cheeses packaged in the other conditions studied. The moisture losses in cheeses packaged in air were around 6·75%. The highest loss (10·67%) was found in cheeses packaged under 100%CO_2.

The pH decreased slightly during the storage period. After 28 days, it had only decreased by 0·6–0·8 units. This decrease is characteristic of fresh cheese produced without a starter culture (Martín-Hernández et al. 1990). The decrease in pH became evident after 7 days in cheeses packaged under 100%CO_2 and after 14 days in cheeses packaged under 40%CO_2/60%N_2. The lowest final pH values were observed in cheeses packaged under 100%CO_2 (5·9). The depression of pH caused by CO_2 atmospheres has been reported by other authors (Koskeli 1988, Farber 1991) and proposed as one of the mechanisms by which CO_2 inhibits microbial growth (King and Nagel 1967, Daniels et al. 1985). The decrease in pH could be the cause of the higher moisture losses found in cheeses packaged under 100%CO_2, and it is related to the inhibitory effect of CO_2 on microbial growth.

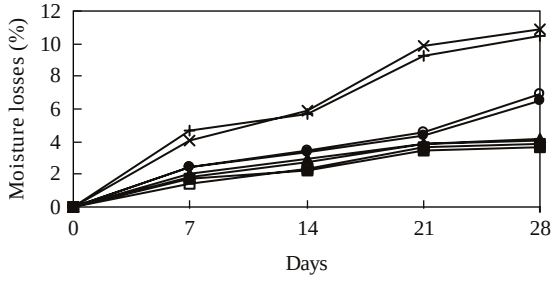

Figure 1. Evolution of humidity losses in cheeses under different packaging conditions. Batch C (○), Batch C20 (□), Batch C40 (△), Batch C100 (×), Batch L (●), Batch L20 (■), Batch L40 (▲) and Batch L100 (+) (see Table 1). The data are the mean values of two experiments.

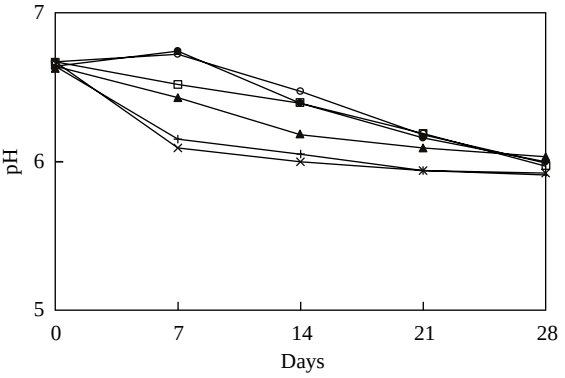

Figure 2. Evolution of pH values in cheeses under different packaging conditions. Batch C (○), Batch C20 (□), Batch C40 (△), Batch C100 (×), Batch L (●), Batch L20 (■), Batch L40 (▲) and Batch L100 (+) (see Table 1). The data are the mean values of two experiments.

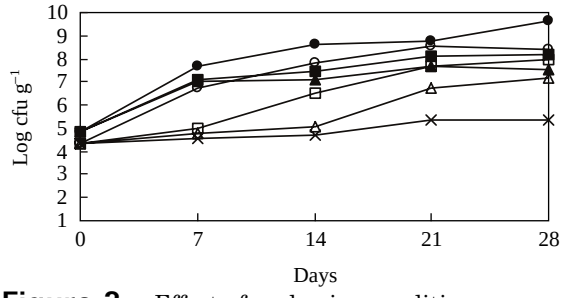

Figure 3. Effect of packaging conditions on mesophile counts in cheese. Batch C (○), Batch C20 (□), Batch C40 (△), Batch C100 (×), Batch L (●), Batch L20 (■), Batch L40 (▲) and Batch L100 (+) (see Table 1). The data are the mean values of two experiments.

Figures 3, 4 and 5 show the results obtained in the microbiological analyses. The prime effect of MAP is an extended shelf-life. The delay of the microbial population in reaching spoilage levels is due in part to an increase in the lag phase. In fact, the effect of 40% and 100%CO_2 atmospheres was particularly impor-

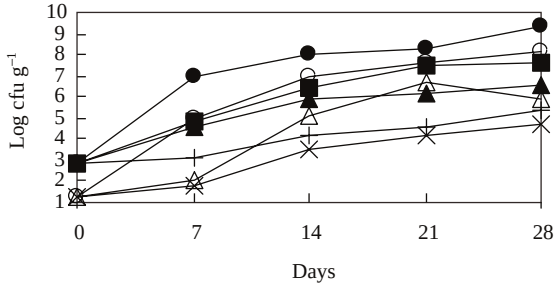

Figure 4. Effect of packaging conditions on psychrotroph counts in cheese. Batch C (○), Batch C20 (□), Batch C40 (△), Batch C100 (×), Batch L (●), Batch L20 (■), Batch L40 (▲) and Batch L100 (+) (see Table 1). The data are the mean values of two experiments.

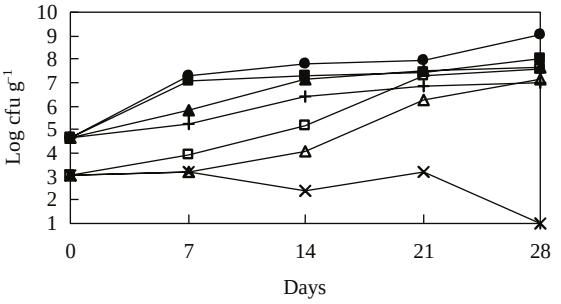

Figure 5. Effect of packaging conditions on anaerobe counts in cheese. Batch C (○), Batch C20 (□), Batch C40 (△), Batch C100 (×), Batch L (●), Batch L20 (■), Batch L40 (▲) and Batch L100 (+) (see Table 1). The data are the mean values of two experiments.

tant between days 0 and 7, when non-growth in populations of mesophiles, psychrotrophs and anaerobes was detected in non-inoculated batches. This fact could be explained because the micro-organisms had to adapt to the new atmospheric conditions. This increase in the lag phase was only detected in the inoculated cheeses packaged under 100%CO_2 for psychrotrophs, anaerobes and *L. monocytogenes*.

The mean mesophile counts of the inoculated and non-inoculated samples packaged in air increased quickly, and were higher than 7 log cfu g^{-1} after 7 or 14 days of storage at 4°C, respectively. However, non-inoculated cheeses packaged under modified atmospheres only reached populations above 7 log cfu g^{-1} on day 21 or 28 of storage in cheeses packaged in 20%CO_2/80%N_2 and 40%CO_2/60%N_2, respectively. These populations were not reached in cheeses packaged under 100%CO_2, which gave a value of $5{\cdot}35 \pm 0{\cdot}59$ log cfu g^{-1} after 28 days of storage. By contrast, inoculated cheeses packaged under 20%CO_2/80%N_2 and 40%CO_2/60%N_2 reached populations of 7 log cfu g^{-1} on day 7. However, those packaged in 100%CO_2 reached 7 log cfu g^{-1} on day 28. The inhibitory effect of CO_2 was particularly important since the populations reached after 28 days in inoculated batches were 1·5–2·5 log units lower than those obtained in samples packaged in air. Moreover, in non-inoculated cheeses packaged in MAP, mesophile counts after 28 days were 0·5–3 log units lower than in those packaged in air. The atmosphere composition had a significant effect ($P<0{\cdot}05$) on mesophile counts. This effect had been identified by other authors in cheese (Scott and Smith 1971, Eliot et al. 1998). These results agree with the finding of Farber (1991) who reported that the inhibitory action of CO_2 in susceptible organisms results in an increase in the duration of the lag phase and a reduction in the growth rate during the logarithmic phase.

In all the conditions tested, psychrotroph and anaerobe counts were higher in batches inoculated than in those non-inoculated until day 7, since *L. monocytogenes* is a psychrotroph (Fig. 4) and a facultative anaerobe (Fig. 5). The atmospheric composition and the inoculation have a significant effect ($P<0{\cdot}05$) on psychrotroph and anaerobe counts. The lower counts obtained when the carbon dioxide increased could be due to the inhibitory effect of CO_2. The psychrotroph and anaerobe evolution was similar to the mesophile evolution, although the population was lower.

Since *Listeria* is able to grow in soft cheeses, even at low temperatures (Farber et al. 1987, Lovett 1989) and was detected in 5·6% of commercial Cameros cheese (Olarte et al. 1999), its possible presence in the samples was investigated. *Listeria* spp. were not found in non-inoculated samples.

At day 0, *L. monocytogenes* counts were 4·4–4·53 log cfu g^{-1}. These levels were 1 log unit higher than the initial levels in inoculated milk. This fact could be explained by the retention of micro-organisms in the curd during whey draining (Medina et al. 1992).

Cameros cheese supported the growth of *L. monocytogenes* in all the conditions studied (Fig. 6). After 28 days, there was a great increase in *L. monocytogenes* counts when inoculated cheeses were packaged in air (3·86 log cycles). When gas mixtures of 20%CO_2/80%N_2 and 40%CO_2/60%N_2 were used, the *L. monocytogenes* counts increased 3·40 and 3·00 log cycles, respectively, after 28 days of storage. Only when an atmosphere of 100%CO_2 was used did the lag phase increase, but after 28 days the pathogen counts increased 2·5 log cycles.

The results of the present study showed that *L. monocytogenes* could grow in the cheese samples stored at 4°C for up to 28 days. The absence of any antimicrobial barriers in the cheese composition (no starter culture, near neutral pH, low salt content and high moisture content) might enable quick growth of the micro-organisms in this type of cheese. The pH levels (6–6·7) of these cheeses were within the growth range of *L. monocytogenes* and did not affect its growth.

Farber et al. (1990) detected *L. monocytogenes* in cheese sandwiches stored in 50%CO_2/50%N_2, which indicates that *L. monocytogenes* can survive in atmospheres containing high CO_2 levels, although these authors suggested that this does not necessarily imply a capability to multiply. The present results demonstrate that *L. monocytogenes* can grow in atmospheres containing high CO_2 levels, although the populations reached after 28 days were 0·5–1·3 log units lower than in those found in cheeses packaged in air. Also, Whitley et al. (2000) found that MAP in 10% and 20% CO_2 was not effective in controlling growth of *L. monocytogenes* counts in a mould-ripened cheese.

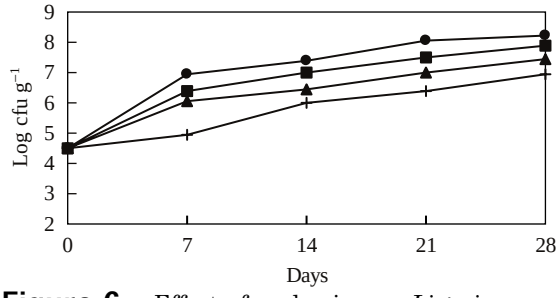

Figure 6. Effect of packaging on *Listeria monocytogenes* growth. Batch C (○), Batch C20 (□), Batch C40 (△), Batch C100 (×), Batch L (●), Batch L20 (■), Batch L40 (▲) and Batch L100 (+) (see Table 1). The data are the mean values of two experiments.

However, in contrast to these results, Moir et al. (1993) did not observe growth of *L. monocytogenes* inoculated into cottage cheese packaged in air or 40%CO_2/60%N_2. This lack of growth in cottage cheese probably resulted from the low pH (Bahk and Marth 1990).

Hudson et al. (1994) reported that an atmosphere of saturated CO_2 was not inhibitory to *L. monocytogenes* growth on sliced roast beef. Moreover, Bell et al. (1995) and Avery et al. (1995) found that a concentration of 100%CO_2 was not inhibitory to the pathogen on smoked blue cod and raw beef, respectively.

According to some authors, the oxygen MAP content seems to be an important factor in the control of *L. monocytogenes* growth. Whitley et al. (2000) observed that MAP in the absence of O_2 increases the lag phase. Wimpfheimer and Hotchkiss (1989) found that *L. monocytogenes* was able to grow equally well at 4°C on raw chicken stored in an aerobic MAP (75·5%CO_2, 22·5%N_2, 5%O_2) or in air. However, these authors reported that the same atmosphere containing no oxygen inhibited its growth. In the present study, the low oxygen levels (0·01–0·60%) could explain the lower growth of the pathogen.

Taking the results of the microbial evaluations as a starting point, it can be concluded that the use of packaging systems under a 100%CO_2 atmosphere for Cameros cheese inhibits the development of micro-organisms involved in its spoilage and extends the shelf-life of this product, but *L. monocytogenes* can survive and grow in atmospheres containing high CO_2 levels, thus being a health risk.

In previous work we observed that cheeses packaged in 40%CO_2/60%N_2 had a better sensory quality (González-Fandos et al. 2000). However, there is a high risk under these conditions since *L. monocytogenes* grew faster than under 100%CO_2.

In conclusion, the present study has shown that fresh goat cheese having a pH value of 6·6, moisture of 60–70% and manufactured without the use of a starter culture is an excellent substrate for the growth of *L. monocyto-*

genes. Thus, special care must be taken to avoid contamination with *L. monocytogenes*, and other growth parameters should be controlled.

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