Journal of Applied Microbiology 2000, **88**, 52–57

The growth of *Listeria monocytogenes* in cheese packed under a modified atmosphere

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6936/10/98: received 21 October 1998, revised 7 June 1999 and accepted 14 June 1999

E. WHITLEY, D. MUIR AND W.M. WAITES. 2000. The effect of Modified Atmosphere Packaging (MAP) on the growth of *Listeria monocytogenes* in mould ripened cheeses was studied at refrigeration temperatures (2–8·3 °C) over a storage period of 6 weeks. Control experiments in cling film with no atmospheric modification produced a lag time before growth of up to 1 week and rapid subsequent growth. MAP with a CO_2 concentration of less than 20% allowed growth to occur but when O_2 was incorporated; the lag time was reduced from 3 to 2 weeks and subsequent growth was also faster, producing an increase in cell numbers of 1·4 log cycles over the incubation period. N_2–MAP in the absence of O_2 increased the lag time to 3 weeks and slowed growth, while the inclusion of CO_2 extended the lag to 3 weeks and slowed subsequent growth even more. In MAP with 80:10:10 (v/v/v) N_2:CO_2:O_2, there was a lag period of 2–3 weeks before growth of *L. monocytogenes* occurred, while the total viable aerobic count (TVAC) decreased by 2–3 log cycles and the total *Lactobacillus* count showed little change. It was concluded that MAP was not suitable for preventing the growth of *L. monocytogenes* in such cheeses.

INTRODUCTION

During the period 1987–1989, there was a significant increase in the numbers of cases of food-borne illness attributed to the micro-organism *Listeria monocytogenes* (Newton *et al.* 1993), with a variety of foods identified as sources of infection and with soft cheeses, e.g. Mexican soft cheese (Linnan *et al.* 1988) and Vacherin Mont D'Or (Bille 1990), being implicated in several outbreaks. *Listeria monocytogenes* has also been implicated in outbreaks of human listeriosis from coleslaw (Schlech *et al.* 1983), prepared salads (Gellin and Broome 1989) and paté (Morris and Ribeiro 1989). Of foods tested by the Public Health Laboratory Service (PHLS), some samples of soft cheeses and paté yielded in excess of 10 000 cells g^{-1} (Committee on the Microbiological Safety of Food 1990). Subsequently, the number of reported cases in the UK has declined from about 300 to about 100 cases per year. However, the organism continues to be a significant problem and in 1992, for example, there was a major outbreak in France associated with pork tongue *en gelée* in which there were 279 cases reported and 63 deaths (Goulet *et al.* 1993).

Changes to the permitted storage temperatures for cheese as a result of the UK Food Safety (Temperature Control) Regulations 1995 (HMSO) mean that storage temperatures which do not inhibit growth of *L. monocytogenes* may be used. These regulations state that appropriate foods must be stored at a temperature of less than 8 °C or greater than 63 °C. However, certain groups of food are exempt from the regulations; ''food which must be ripened or matured at room temperature'' is one of these groups. Soft or mould ripened cheeses fall into this category, although once fully ripened or matured, industry must abide by the temperature controls stated. *Listeria* is a mesophilic genus with a growth range of −1 to 45 °C. This is a significant factor when considering its presence in foodstuffs, as most chilled produce will be stored at 1–5 °C and the Food Safety (General Food Hygiene) Regulations 1995 (HMSO) permit storage at temperatures up to 8 °C. However, the organism is not generally considered to be thermoduric so most pasteurization processes should render foods free from *Listeria* species (Donnelly and Briggs 1986).

The use of modified atmosphere packaging (MAP) has

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increased with a wide range of foods. It has potential as a packaging system for mould ripened cheese, enabling continued growth of the mould during the shelf-life period. Furthermore, with the rapid increase in the sales of prepared sandwiches in recent years, coupled with the increasing use of modified atmospheres in packaging sandwiches for supermarkets, a combination of soft cheese and MAP is likely to be met more frequently in the future. However, the ability of *L. monocytogenes* to grow as a facultative anaerobe raises the question of its ability to grow under MAP.

The potential for growth of *L. monocytogenes* in mould ripened soft cheeses is well documented and Ennahar *et al.* (1994) demonstrated that soft and red smear cheeses (for example, Port Salut) were frequently contaminated with the organism at levels in excess of 10^5 cfu g^{-1}. Red smear cheeses are characterized by a typical red flora growing on the rind and are commonly produced in France from both pasteurized and unpasteurized milks. In soft and mould ripened cheese, the final pH of the product (ranging from pH 5·3–7·5 at the surface and from 5·0 to 7·3 in the core) may not be inhibitory to *L. monocytogenes.* As *L. monocytogenes* has a pH range for growth of 4·1 to 9·6 with an optimum in the region of pH 6, it can be seen that there is a potential for growth in such cheeses.

This work examines whether MAP storage is appropriate for mould ripened cheeses with respect to *L. monocytogenes*.

MATERIALS AND METHODS

A mature (7 weeks) whole Stilton cheese was cut horizontally into eight rings of approximately 5 cm depth. These rings were then cut into eight equal segments, with each portion including the same areas and proportion of areas to ensure reproducible results. The cheese was assessed for the presence of *L. monocytogenes* prior to commencing the trials, both on the coat and in the core of the cheese, in duplicate, using the standard method described. No *L. monocytogenes* was detected in 25 g of sample. The trials were conducted over a period of several months so portions of cheese were frozen in a domestic freezer (−20 °C) until needed.

Inoculum

In order to model post-processing contamination, an inoculum of *L. monocytogenes* serotype 4a (NCTC 5214 01 (ATCC 19114)) was applied evenly to the surface of the cheese by pipetting, drop-wise, the required amount of culture using a *Volac* micropipetter (John Poulten Ltd, Barking, UK). The inoculum level was changed during the initial experiments (Table 1). The samples were refrigerated at 5 °C for 2 d to allow the culture to diffuse into the cheese before packaging. The level of inoculum was estimated by making serial dilutions in Oxoid maximum recovery diluent (CM733), subsequently plating on Listeria Oxford agar and incubation for 24 h at 30 °C. The count was checked after a further 24 h, but in no cases were there differences between 24 and 48 h.

Packaging and storage

The inoculated samples were placed in nylon:polyethylene laminate bags of known gas transmission rates and packaged under selected modified atmospheres (Table 1) using a Multivac A 300 packaging machine (Wolfertschwenden, Germany). Following MAP, the samples were incubated at refrigeration temperatures (2·1–7·2 °C) for the duration of the trials.

Listeria detection

Samples were tested in duplicate using the standard *Listeria* testing method described above and assessed for growth on *Listeria* selective agar (Oxford formulation) after incubation at 30 °C for 2 d. Plates showing presumptive positive colonies were then examined further for catalase reaction, Gram reaction and motility. If positive reactions were recorded, the presence of *Listeria* species was assumed and the CAMP test (Oxoid Manual) was carried out to identify the species present.

pH and other measurements

The pH of the cheeses under test was measured by selecting random samples (10 g) of cheese, which were homogenized in a blender with 25 ml distilled water for 2 min, and the pH measured with a portable pH meter (pHep 3; Hanna Instruments, Mauritius), calibrated with pH 4 and 7 buffers.

In addition to *L. monocytogenes*, the total *Lactobacillus* and total viable count was also determined using de Man Rogosa Sharpe agar, CM 361 (Bridson 1995) and milk agar, respectively, after incubation at 30 °C for 3 d. *Lactobacillus* colonies were confirmed by the catalase test.

Experimental design and trials

The experimental design was varied in experiments 1–3 to assess the difference between the atmospheres in order to select an appropriate incubation level. Two experiments used the same atmospheric conditions thought to represent potential commercial practice for mould ripened cheeses. As a

Table 1 Increase in cfu of *Listeria monocytogenes* after 6 weeks of storage in modified atmosphere packaging

Trial number	Atmosphere $N:CO_2:O_2$	Inoculum size (ml g^{-1})	Inoculation rate (log cfu g^{-1})	Log count at week 6 cfu g^{-1}	Increase (%)
1	80 : 10 : 10	0·01	2·5	3·9	2300
2a	100 : 0 : 0	0·5	3·3	3·9	350
2b	80 : 20 : 0	0·5	3·3	2·6	−17
2c	No modification	0·001	1·4	1·7	180
3–9	80 : 10 : 10	0·001	1·6	3·27 ± 0·11	5000 ± 878

result of these trials, an inoculum of 0·001 ml g^{-1} was selected for the main trials. The conditions used throughout this investigation are summarized in Table 1.

Trials 4–6 and 7–9 were run as two concurrent batches of trials and therefore, incubation temperatures for these two sets of three trials were the same.

RESULTS

Preliminary experiments 1–3 indicated that an atmosphere of 80:10:10 v/v/v nitrogen:carbon dioxide:oxygen, and an inoculation rate of log 1·5 cells g^{-1} and 0·001 ml g^{-1}, were appropriate, giving reproducible results.

An increase in the count of *L. monocytogenes* over the inoculation rate was recorded during all trials with 80:10:10 v/v/v N_2:CO_2:O_2 atmosphere (trials 4–9) (Fig. 1). In these trials, an initial period of delay, during which the number of viable cells initially declined from the number inoculated, was followed by a period of growth, although some of this apparent growth may have represented recovery of the cells in the initial inoculum. Nevertheless, in all trials with Stilton cheese and an atmosphere of 80:10:10 v/v/v N_2:CO_2:O_2, there was a significant increase in count of *L. monocytogenes* over the period of storage. The increase for all trials is summarized in Table 1 and ranged between 1 and 2 log cycles for the main trials 4–9.

When compared with the other gas mixtures used, i.e. 80:20 v/v N_2:CO_2 and 100% N_2, and with the control with no modification of atmosphere, there was a greater increase in the number of viable cells when no modification of the atmosphere occurred. In those atmospheres where O_2 was included, the increase in viable counts was significantly higher than in the atmospheres deficient in O_2.

The general trend over the trials was for the total viable (aerobic) count to decline over the incubation period (Fig. 2). The *Lactobacillus* count also showed an overall decrease in numbers (Fig. 3), but it is interesting that it increased in all trials up to week 4 and then gradually declined. Given the ability of members of the genus to grow under conditions of reduced oxygen tension, initially there would be sufficient O_2

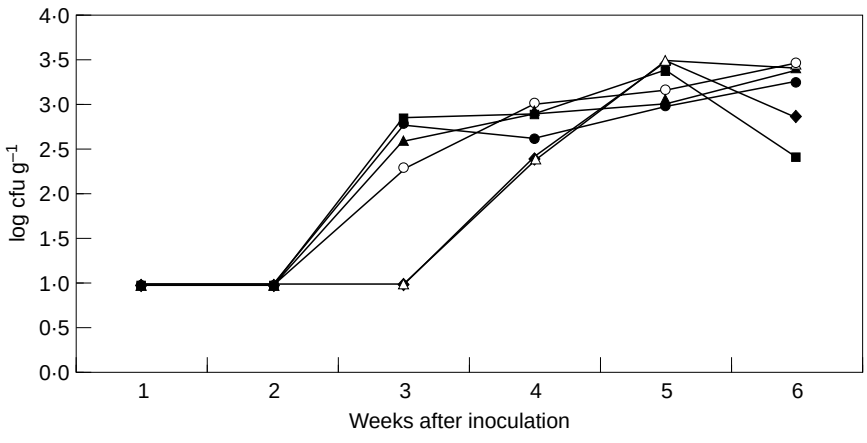

Fig. 1 Viable counts from six trials of *Listeria monocytogenes* in Stilton cheese packaged in an atmosphere of 80:10:10 v/v/v N_2:CO_2:O_2 during refrigerated storage

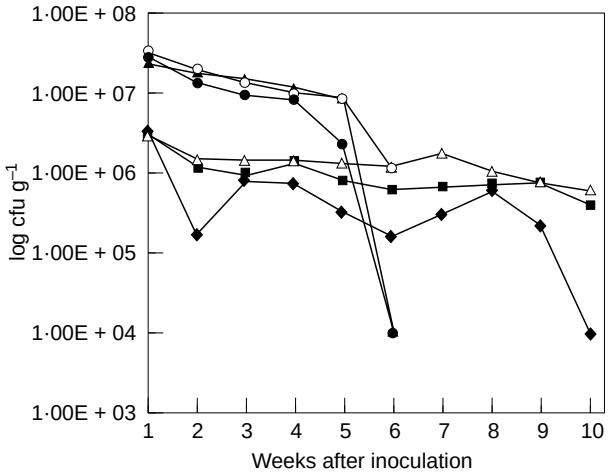

Fig. 2 Total viable aerobic counts from six trials of Stilton cheese during refrigerated storage under an atmosphere of 80:10:10 v/v/v N_2:CO_2:O_2

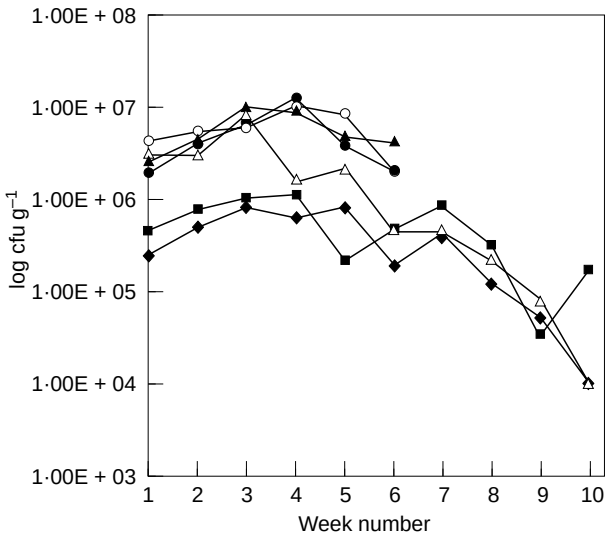

Fig. 3 Changes in the total *Lactobacillus* count from six trials of Stilton cheese during refrigerated storage in an atmosphere of 80:10:10 v/v/v N_2:CO_2:O_2

for growth. However, other aerobic micro-organisms, such as *Penicillium roquefortii*, would act as competitors for the O_2 and result in the decline in count observed later in the storage period. Anti-microbial agents may also have been present and may have acted as growth inhibitors, or may have produced the cell death observed. All trials showed a very similar pattern, despite the fact that lactobacilli are not included in the starter bacteria of Stilton cheese.

During the trials, the pH values (Fig. 4) varied in a similar, though inverse, pattern to the *Lactobacillus* count. The increase in the *Lactobacillus* count may be linked to this reduction in pH, with the growth of members of the genus resulting in decreased pH due to the end products of the lactose fermentation. The pH values observed during the first set of trials (4–6) were higher than those of the second set, which may be due to the effect of freezing the cheese used in trials 7–9.

The pH levels observed within the cheese were well within the growth range of *L. monocytogenes* and it was expected that there would be no effect on growth. This was reflected by an analysis of variance, which demonstrated no significant relationship between the pHs observed and the growth of *L. monocytogenes* ($P = 0{\cdot}23$). With respect to the total viable count, the effect of pH was significant ($F = 26{\cdot}1$ and $P = <0{\cdot}001$) and an even stronger effect between the change in pH and the total *Lactobacillus* count ($F = 27{\cdot}74$ and $P = <0{\cdot}0001$) was noted.

DISCUSSION

During the preliminary trials, *L. monocytogenes* was sufficiently versatile in its metabolism to grow under conditions of modified atmosphere. An increase in the concentration of CO_2 to 20% inhibited growth to a greater extent than a simple reduction in the O_2 content of the package. However, the aim of this work was not to establish an atmosphere that would totally prevent growth, but to determine the ability of the species to grow under MAP. Studies by Hudson *et al.* (1994), Bell *et al.* (1995) and Avery *et al.* (1995) on *L. monocytogenes* in sliced roast beef, smoked blue cod and raw beef, respectively, centred on the inhibitory effects of CO_2. However, these studies produced differing results; Hudson *et al.* (1994) reported that an atmosphere of saturated CO_2 was not inhibitory to growth, while both the other groups found that a 100% CO_2 atmosphere was inhibitory.

In atmospheres containing CO_2, the numbers of *L. monocytogenes* after 3 weeks of incubation reached a level at which they remained throughout the rest of the trial. In the atmosphere containing 100% N_2, the numbers appeared to be increasing at the end of the trial. This implied that growth was affected to some extent by the addition of CO_2, and was consistent with the observations reported by Church and Parsons (1995) that the inhibitory effect of CO_2 on bacteria increases linearly up to 50–60% v/v CO_2.

Farber (1991) showed that the inhibitory action of CO_2 in susceptible species resulted in an increase in the duration of the lag phase and a reduction in growth rate during the logarithmic phase. In the current study, the lag phase was unaffected by the presence of the gas when comparing the 80:20 v/v and 100 (N_2:CO_2:O_2) atmospheres. Thus, the main influence of CO_2 is in reducing the growth rate during the logarithmic phase. However, when the atmosphere was not modified, the lag phase was much reduced. This implies that

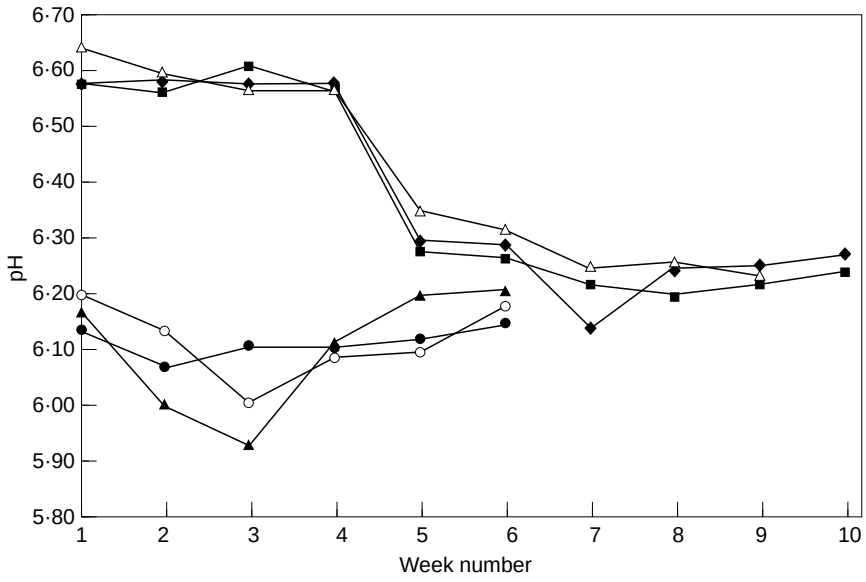

Fig. 4 pH values obtained from Stilton cheese during refrigerated storage under an atmosphere of 80% N_2 : 10% CO_2 : 10% O_2

when the concentration of CO_2 is as low as 10% (or less), the presence of O_2 has more effect on the length of the lag phase than does the CO_2. Comparing these results with those of the control samples, it is significant that the lag phase was between 2 and 3 weeks longer in the MAP cheeses.

Listeria monocytogenes occurring in cheese as a result of environmental contamination may be at greater risk of shock when subjected to storage at cold temperatures, low pH and/or MAP. Stress responses appear to be universal and have been detected in all organisms examined. In addition to the well studied heat stress response, cold and acid shock, salt stress, starvation and O_2 limitation responses have been demonstrated in bacteria. Such responses make cells more resistant to the stress and there is some overlap between the different responses. For example, in *Bacillus subtilis* (Völker *et al*. 1994), some stress response proteins are produced as a result of heat shock, salt stress, oxidative stress and O_2 limitation, while others are produced only in response to heat stress. The production of such response proteins may be important in the ability of *L. monocytogenes* to survive and grow in Blue Stilton cheese.

This study demonstrates (i) that film wrapping is insufficient to control growth of *L. monocytogenes* in Blue Stilton cheese and (ii) that MAP, whether using atmospheres containing O_2 or not, does not control growth in such cheese. It also demonstrates that an atmosphere of 80:10:10 v/v/v N_2:CO_2:O_2 is not suitable where *L. monocytogenes* may be present.

The decline in TVC during storage was expected as the count is of aerobic micro-organisms and the atmosphere used had a reduced O_2 content. With aerobic metabolism of bacteria and the blue mould, *P. roquefortii*, the ratio of CO_2 to O_2 could be expected to increase over time, resulting in greater inhibition of the aerobic micro-organisms. The presence of CO_2 in the packaging atmosphere and the presence of antimicrobial substances produced by the starter organisms could also be expected to inhibit the growth of aerobic species.

Non-starter lactic acid bacteria (NSLAB), to which the lactobacilli belong, make up a large proportion of most cheeses, reaching levels of 10^7–10^9 g^{-1} (Fox 1993). The decline in the *Lactobacillus* count was not anticipated because of their ability to respire anaerobically or aerobically, but a relationship between their growth and the pH values was demonstrated.

Modified atmospheres have not, as yet, been used commercially for the packaging of mould ripened cheeses. Such atmospheres are currently used for packaging of goods, including sandwiches, containing Brie, Camembert and other similar cheeses and chilled recipe dishes. However, the storage time of these sandwiches is relatively short (of the order of day of manufacture + 2 d), although temperature abuse may be significant. In fact, in a study evaluating the microbiological quality of various MAP sandwiches, *L. monocytogenes* was present in five of 58 samples (Farber *et al*. 1990). In the current study, the potential for growth has been demonstrated and it is clear that the utmost care must be taken to avoid contamination with the organism and to control other growth parameters carefully. This must be considered during risk analysis of new products. However, the results of this study indicate that even in conditions of controlled temperature and modified atmosphere, *L. monocytogenes* may eventually grow to high levels.

It is apparent from this study that once CO_2 levels are reduced to 10%, and with the incorporation of O_2 into the

pack, MAP does not inhibit the growth of *L. monocytogenes* at refrigeration temperatures. In conclusion, it is probable that the use of MAP and controlled atmosphere packaging is likely to increase in the future, and that the range of foods packaged using such a system will expand. Further studies on the type of atmospheres suitable for the inhibition of *L. monocytogenes* should be carried out in order to assess the suitability of this packaging system for high risk foods.

ACKNOWLEDGEMENTS

The authors acknowledge J. Slack for practical assistance during the trials. Long Clawson Dairy is thanked for the Stilton cheese used in the trials.

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