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The combined affects of modified atmosphere, temperature, nisin and ALTA[TM] 2341 on the growth of *Listeria monocytogenes*

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Abstract

A cocktail of seven *Listeria monocytogenes* isolates of food, human and environmental origin was used to assess the antilisterial activity of the bacteriocins nisin and ALTA[TM] 2341 in combination with various atmospheres: air, 100% N_2, 40% CO_2:60% N_2, or 100% CO_2. Buffered tryptone soya broth (pH 6.0) was used as the growth medium and incubation was at 4°C (21 days) or 12°C (7 days), or when temperature fluctuated between these values for defined periods. It was observed that atmosphere alone influenced the growth rate of *L. monocytogenes*, with 100% CO_2 exerting the greatest inhibition. A 5 log population increase was observed in all atmospheres after 7 days at 12°C. At 4°C a 4–5 log population increase was observed in air, 100% N_2 and 40% CO_2:60% N_2 within 21 days. Growth was prevented by 100% CO_2. In the presence of nisin (400 IU/ml), an increase in the lag phase was observed before growth (5 log population increase after 7 days) in all atmospheres at 12°C. This effect was enhanced at 4°C where a maximum 2 log population increase was observed in all atmospheres except 100% CO_2, in which growth was prevented. Increasing the concentration of nisin to 1250 IU/ml prevented *L. monocytogenes* growth in all atmosphere combinations at 4 and 12°C. Two concentrations of ALTA[TM] 2341 were also tested. In the presence of 0.1% ALTA[TM] 2341 and at 12°C, a 3–5 log population increase was observed in all atmospheres with the exception of 100% CO_2, which prevented *L. monocytogenes* growth. At 4°C, growth was observed in the combination of 0.1% ALTA[TM] 2341 and 100% N_2 only (3 log population increase). Use of a higher concentration of ALTA[TM] 2341 (1.0%) resulted in a population decrease below the detection level within 24 h in all atmosphere/temperature combinations. Re-growth occurred in the presence of 1.0% ALTA[TM] 2341 in all atmospheres at 12°C, and in combination with air or 100% N_2 at 4°C. When the effectiveness of either nisin or ALTA[TM] 2341 and atmosphere was tested against *L. monocytogenes* as temperature fluctuated for periods between 4 and 12°C, only the combination of 100% CO_2 and 1.0% ALTA[TM] 2341 prevented growth. Cells surviving exposure to nisin or ALTA[TM] 2341 were recovered from 28 of the 32 combinations tested that contained bacteriocin. Nisin survivors remained sensitive to the bacteriocin. ALTA[TM] 2341 survivors had become resistant to the bacteriocin. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Listeria; Modified atmosphere; Bacteriocin; Temperature

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1. Introduction

Modified atmosphere packaging (MAP) technology dates back to the 1930s and the shipping of fresh beef from Australia and New Zealand to distant markets under carbon dioxide. The potential of MAP to extend the shelf-life of other commodities as diverse as fish (Dalgaard et al., 1993), sandwiches (Farber, 1991), salads and vegetables (Day, 1990; Kwang Soo Lee et al., 1996; Segall and Scanlon, 1996) is well documented. However, there remains concerns about the microbiological safety of MAP food, in particular the growth of psychrotrophic pathogens in refrigerated ready-to-eat MAP food (Farber, 1991).

The psychrotrophic pathogen *Listeria monocytogenes* has caused concern to both the food industry and regulatory agencies since the early 1980s (Sutherland and Porritt, 1997). Its disease manifestations in humans vary from a non-specific flu-like illness to infection involving sepsis or meningitis. It is rare for listeriosis to occur in a human host in the absence of a predisposing risk factor. Those most at risk include the elderly, pregnant women, neonates and the immunocompromised.

L. monocytogenes can grow and survive under diverse environmental conditions that range from soil to cool, moist areas in food processing factories. Relevant to the present work, is the organism's ability to survive and grow in atmospheres with low or no oxygen content such as vacuum packaging or packaging in an atmosphere containing CO_2. The growth capacity of *L. monocytogenes* in vacuum-packaged products is well documented for beef (Grau and Vanderlinde, 1990), lamb (Garcia de Fernando et al., 1995), pork (van Laack et al., 1993) and smoked salmon (Hudson and Mott, 1993). However, there is conflicting evidence regarding the effect of CO_2 on the organism's growth and survival.

Workers elsewhere have reported that *Listeria* was unable to grow in lamb and beef at 5°C in an atmosphere of 100% CO_2 (Garcia de Fernando et al., 1995; Gill and Reichel, 1989). It was reported that chicken packaged in an atmosphere of 30% CO_2/70% N_2 did not support the growth of the organism at 6°C (Hart et al., 1991). Similarly, *L. monocytogenes* did not grow in chicken packaged in an atmosphere of 75% CO_2/25% N_2 at 4°C (Wimpfhimer et al., 1990) or 6°C (Hudson et al., 1994). Yet, *Listeria* growth has been shown in lamb at 5°C packaged in an atmosphere of 50% CO_2/50% N_2 (Nychas, 1994), in pork packaged in 40% CO_2/60% N_2 (Manu-Tawiah et al., 1993), and roast beef packaged in 100% CO_2 at 3°C (Hudson et al., 1994). In broth, Farber et al. (1996) observed that *L. monocytogenes* was unable to grow at low pH (5.5) and temperature (4°C) in the presence of $\geq$50% CO_2 (balance N_2). However, an increase in storage temperature to 7°C allowed for growth at low pH. Taken together, these studies indicate that additional barriers may be required to control the growth of *L. monocytogenes* in some CO_2-enriched MAP food, especially if the food is subjected to temperature abuse.

A suggested approach to improving the safety of CO_2-enriched MAP food, with respect to *L. monocytogenes*, is the use of antimicrobial substances derived from bacteria. These substances are usually low-molecular weight proteins and have been referred to as bacteriocins (Tagg et al., 1976), lantibiotics (Jung, 1991) or BLIS (bacteriocin-like inhibitory substances; Tagg, 1992).

Commercial preparations of nisin and ALTA™ 2341 were used in this study. Both are inhibitory to *L. monocytogenes* (Harris et al., 1991; Glass et al., 1995) and are representatives of two bacteriocin classes (Abee et al., 1995). Nisin, a Class I bacteriocin, is produced by certain strains of the dairy starter microorganism *Lactococcus lactis* subsp. *lactis* (Mattick and Hirsch, 1944, 1947). It contains several atypical amino acids. Two of the atypical amino acids, lanthionine and β-methyl-lanthionine, close single sulfur rings that are a structural characteristic of nisin. Nisin is inhibitory to a range of Gram-positive bacteria but Gram-negative bacteria (*Neisseria* excepted), yeasts and moulds are resistant (Abee et al., 1995). Nisin has been used by the food industry for the past 25 years, mainly in the production of processed cheeses and canned foods. About 50 countries, including Australia, allow the use of nisin in selected foods.

ALTA™ 2341, as described by the manufacturer (Quest International), is a crude fermentation product of lactic acid bacteria. The antimicrobial component of ALTA™ 2341 is not specified by the manufacturer. Schlyter et al. (1993) determined that ALTA™ 2341 had a similar intrinsic activity against *L. monocytogenes* to pediocin AcH, a fermentation by-

product of *Pediococcus acidilactici*. Unlike nisin, pediocin AcH does not contain the amino acids lanthionine and β-methyl-lanthionine, it is a Class II bacteriocin. It is postulated that the antimicrobial component of ALTA™ 2341 is a Class II bacteriocin.

In this study we investigated the application of nisin or ALTA™ 2341 in combination with modified atmospheres for controlling *L. monocytogenes* growth at refrigeration and abuse temperatures, and when temperature fluctuated between these values. These conditions were evaluated in a controlled atmosphere broth system.

2. Materials and methods

2.1. Isolates and culture media

The 21 strains of *L. monocytogenes* from clinical, food and environmental sources used in this study are listed in Table 1. All isolates were grown in tryptone soya broth (TSB, Oxoid) or on tryptone soya agar (TSA, Oxoid). The identity of all isolates was confirmed using the Microbact™ 12L rapid *Listeria* identification kit (Medvet, France).

2.2. Bacteriocin critical dilution plate assay

The sensitivity of *L. monocytogenes* to nisin (Sigma) and ALTA™ 2341 (Quest International) was determined using the critical dilution method (Parente et al., 1995). The bacteriocins were prepared according to the manufacturer's instructions in a doubling dilution series. Five μl of each dilution was spotted onto de Man, Rogosa, Sharpe agar (MRS, Oxoid), allowed to dry at room temperature for 30 min, and overlayed with semi-solid agar (TSB with 0.5% No. 1 Agar, Oxoid) containing approximately 10^5 cfu of *L. monocytogenes* per ml. Plates were incubated aerobically at 30°C for 16 h. The minimal inhibitory concentration (MIC) was taken as the lowest concentration exerting an inhibitory effect (definite zone of lysis) on the resulting *L. monocytogenes* lawn culture. In the case of nisin, the MIC was expressed as international units (IU) per ml, and for ALTA™ 2341 as % (w/v). The MIC was determined from two different bacteriocin batches, that were tested on different days.

2.3. Effect of modified atmospheres, nisin and ALTA 2341 in a broth system

A controlled-atmosphere broth system was achieved by modifications to 250-ml Duran Schott bottles (Duran, USA). Schott bottle caps (Duran, GL 45) were made air tight by use of a fitted seal. Bulkhead reducers (Sydney Swagelok Services) were fitted with dual 13-mm blue septa (Alltech Pty. Ltd., Australia) and the reducer inserted through an 11-mm hole in the fitted cap seal.

TSB (Oxoid) was the selected growth medium. The medium was supplemented with a buffering agent, piperazine dihydrochloride hydrate (15.9 g/l; Sigma-Aldrich), and the pH adjusted to 6.0 using HCl or NaOH. Varying combinations of nisin, ALTA™ 2341, 100% N_2, 40% CO_2 (balance N_2) and 100% CO_2 were added to the buffered TSB after sterilisation. Nisin was dissolved in 0.02 M HCl, diluted appropriately and added to the buffered TSB after sterilisation to give a final concentration of 400 or 1250 IU/ml. ALTA 2341 was dissolved in sterile water, diluted and added to the buffered TSB after sterilisation to give a final concentration of 0.1 or 1.0% (w/v). Prior to inoculation, each bottle was flushed (10 min) with the appropriate filter-sterilized gas, and allowed to equilibrate overnight. This process was repeated on three separate days. The head space atmosphere was checked using a Gaspace 2 gas analyser (Systech Instruments Ltd.).

Seven isolates of *L. monocytogenes* (Table 1: 1372, 1369, 1281, 1260, 1192, 1067, and 1059) were used as a mixed inoculum. The isolates were grown separately in 50 ml of TSB at 15°C for approximately 72 h until the cells had reached late log phase. Equal volumes of the cultures were combined, and serially diluted in 0.1% (w/v) peptone. A 0.5-ml volume of an appropriate dilution of the cocktail was used to inoculate 50 ml of TSB to give a final concentration of approximately 10^3 cfu/ml. Broths were incubated in shaking waterbaths (120 strokes per min; SS40-D, Grant Instruments) at either 12 or 4°C. The temperature of the waterbaths were constantly monitored using a Grant 1200 Series Squirrel meter/logger (Grant Instruments Ltd.).

Samples were taken periodically from the test

bottles for up to 7 days at 12°C, and up to 21 days at 4°C using a 26-gauge needle and syringe. *L. monocytogenes* were enumerated by spread plating onto TSA followed by incubation at 37°C for 18 h. Forty combinations of atmosphere, temperature and bacteriocin were tested. Each combination was tested on at least two occasions.

2.4. Bacteriocin resistance

At the completion of each broth study, 100 μl of broth was subcultured into 10 ml of fresh TSB and incubated at 30°C for 24–48 h to recover surviving *L. monocytogenes*. This culture was used to determine whether *L. monocytogenes* became resistant following exposure to bacteriocins. Resistance was determined by a change in MIC of bacteriocin-surviving cells compared to the MIC of the initial population using the critical dilution assay. TSB cultures were also streaked onto TSA to check for purity and selected colonies tested for haemolysis on horse blood agar (Oxoid).

2.5. Curve fitting

Curve fitting was carried out using the Baranyi 'D-model' for bacterial growth (Baranyi et al., 1993).

3. Results

3.1. Minimal inhibitory concentration of bacteriocins

The MIC values of nisin and ALTA™ 2341 are recorded in Table 1. The MIC for nisin varied from 312 to 1250 IU/ml among the isolates of *L. monocytogenes* tested. The MIC for ALTA™ 2341 varied from 0.08 to 0.6% among the isolates. The MIC of

Table 1
L. monocytogenes isolates and the minimal inhibitory concentrations (MIC) of nisin and ALTA™ 2341

Isolate[a]	Source	Nisin (MIC) (IU/ml)	ALTA™ 2341 (MIC) (%)
Serovar 4a	Clinical	1250	0.15
1372	Clinical (blood)	625	0.60
1369	Clinical (blood)	625	0.15
1281	Soft cheese	1250	0.30
1260	Salami	625	0.60
1192	Smoked mussels	625	0.08
1067	Environment (floor)	625	0.08
1059	Environment (drain)	625	0.60
1	Sandwich filling (pizza)	625	0.60
2	Sandwich filling (pizza)	625	0.60
3	Sandwich filling (pizza)	625	0.60
4	Sandwich filling (pizza)	625	0.60
5	Sandwich filling (beef)	312	0.30
6	Sandwich filling (pizza)	625	0.60
7	Sandwich filling (pizza)	625	0.60
8	Sandwich filling (chicken)	625	0.30
9	Sandwich filling (pizza)	312	0.30
10	Sandwich filling (chicken)	1250	0.15
11	Sandwich filling (beef)	625	0.15
12	Sandwich filling (pizza)	1250	0.60
13	Sandwich filling (pizza)	312	0.60

L. monocytogenes isolate serovar 4a was obtained from Dr B. Davidson, The University of Melbourne. Isolates 1372, 1369, 1281, 1260, 1192, 1067, and 1059 were obtained from Mr P. Sutherland, Pacific Industry Service Corporation, Sydney, Australia. Isolates 1–13 were obtained from Mrs C. Moir, Food Science Australia, North Ryde Laboratory, Australia.

the cocktail of isolates selected for use in the growth studies was 0.3% ALTA™ 2341 and 1250 IU/ml nisin.

Based on these results, two concentrations of each bacteriocin were selected for study in the broth system; 0.1 and 1.0% ALTA™ 2341, and 400 and 1250 IU/ml nisin.

3.2. Effect of atmosphere on the growth of L. monocytogenes in the broth system

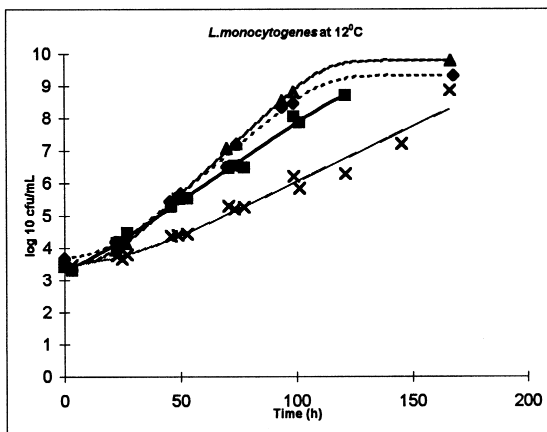
The effect of atmosphere alone on the growth of *L. monocytogenes* in broth at 4 and 12°C was evidenced by changes in growth rate and lag phase duration (Fig. 1). Of the four atmospheres tested, 100% CO_2 exerted the greatest effect on *L. monocytogenes*. The mean growth rates for *L. monocytogenes* at 12°C in air, 100% N_2, 40% CO_2:60% N_2 and 100% CO_2 were 0.073 h^{-1} (range 0.070–0.075), 0.0645 h^{-1} (range 0.062–0.065), 0.051 h^{-1} (range 0.049–0.053) and 0.035 h^{-1} (range 0.034–0.036), respectively.

At 4°C, the mean growth rates for 100% N_2, air, and 40% CO_2:60% N_2 were 0.014 h^{-1} (range 0.013–0.014), 0.015 h^{-1} (range 0.014–0.016) and 0.009 h^{-1}, respectively (Fig. 1). The growth of *L. monocytogenes* was not observed in 100% CO_2 for the 21 days of testing

3.3. Effect of atmosphere and ALTA™ 2341 in the broth system

The effectiveness of ALTA™ 2341 on the growth of *L. monocytogenes* appeared to depend upon the interaction of bacteriocin, atmosphere and temperature (Fig. 2). At 12°C the combination of 0.1% ALTA™ 2341 with air, 100% N_2, or 40% CO_2:60% N_2 increased the lag phase of *L. monocytogenes* when compared to controls. The combination of 100% CO_2 and 0.1% ALTA™ 2341 inhibited *L. monocytogenes* growth at 12°C, where the initial count was observed to decrease to ~50 cfu/ml (at 75 and 100 h) and then increase to ~10^4 cfu/ml (at 160 h). While these observations were reproducible, reproducibility of growth rate was poor. The development of bacteriocin resistance, which is discussed later, may have caused the poor reproducibility of growth rates observed under these conditions.

An initial decrease in *L. monocytogenes* numbers below the level of detection (<50 cfu/ml) was observed with all atmospheres in combination with 1.0% ALTA™ 2341 at 12°C (Fig. 2). Growth eventually occurred in all atmospheres. Again, the best control of growth was observed in combination with 100% CO_2. These observations were reproducible. As also observed with combinations using 0.1% ALTA™ 2341, reproducibility of growth rate was poor.



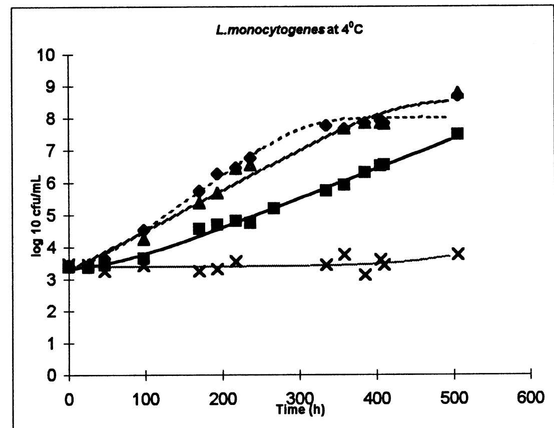


Fig. 1. Fitted growth curves showing the effect of of air (●), 100% N_2 (♦), 40% CO_2:60% N_2 (■) or 100% CO_2 (×) on the growth of *Listeria monocytogenes* at 4 or 12°C.

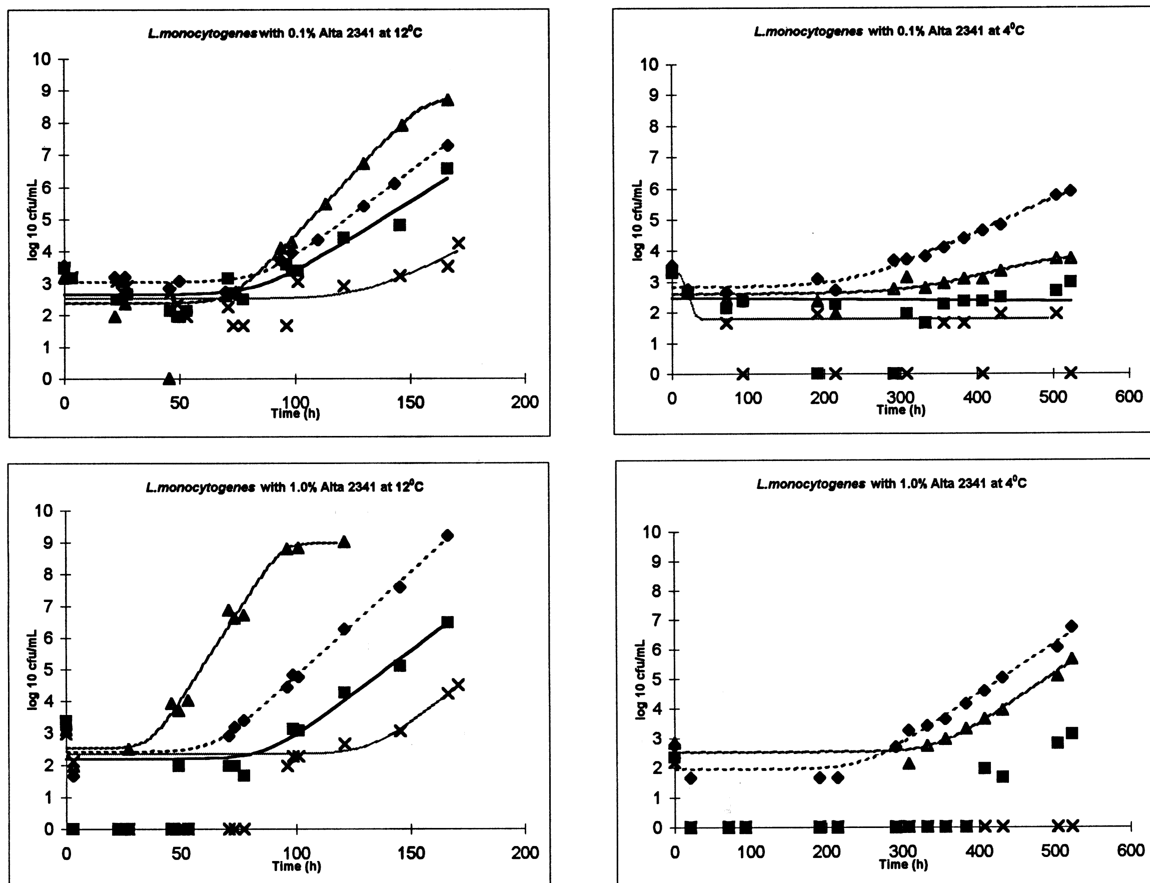


Fig. 2. Fitted growth curves showing the effect of 0.1 or 1.0% ATLA™ 2431 in combination with an atmosphere of air (●), 100% N_2 (♦), 40% CO_2:60% N_2 (■) or 100% CO_2 (×) on the growth of *Listeria monocytogenes* at 4 or 12°C.

ALTA™ 2341 was more effective against *L. monocytogenes* when the incubation temperature was lowered. At 4°C, the combination of 0.1% ALTA™ 2341 with air, 40% CO_2:60% N_2, or 100% CO_2 effectively controlled the growth of *L. monocytogenes* throughout the 21 days of testing (Fig. 2). Growth to elevated levels ($>10^6$ cfu/ml) was observed when 0.1% ALTA™ 2341 was combined with 100% N_2. These observations were reproduced in repeat experiments.

An initial decrease in *L. monocytogenes* numbers below the level of detection was observed with all atmospheres in combination with 1.0% ALTA™ 2341 at 4°C (Fig. 2). *L. monocytogenes* growth eventually occurred in air and 100% N_2, reaching detectable levels after 300 h in air or 200 h in 100% N_2. *L. monocytogenes* remained below the level of detection at 4°C for 400 h in an atmosphere containing 40% CO_2:60% N_2, and throughout the 21 days (504 h) of experimentation in 100% CO_2. These observations were reproducible.

3.4. Effect of atmosphere and nisin in the broth system

In all atmospheres, 400 IU/ml nisin extended the lag phase before growth at 12°C (Fig. 3). This observation was reproducible; however, the reproducibility of growth rate was poor. At 4°C, this level of nisin prevented growth when in combination with 100% CO_2 and permitted only a 2 log cfu/ml increase in all other atmospheres.

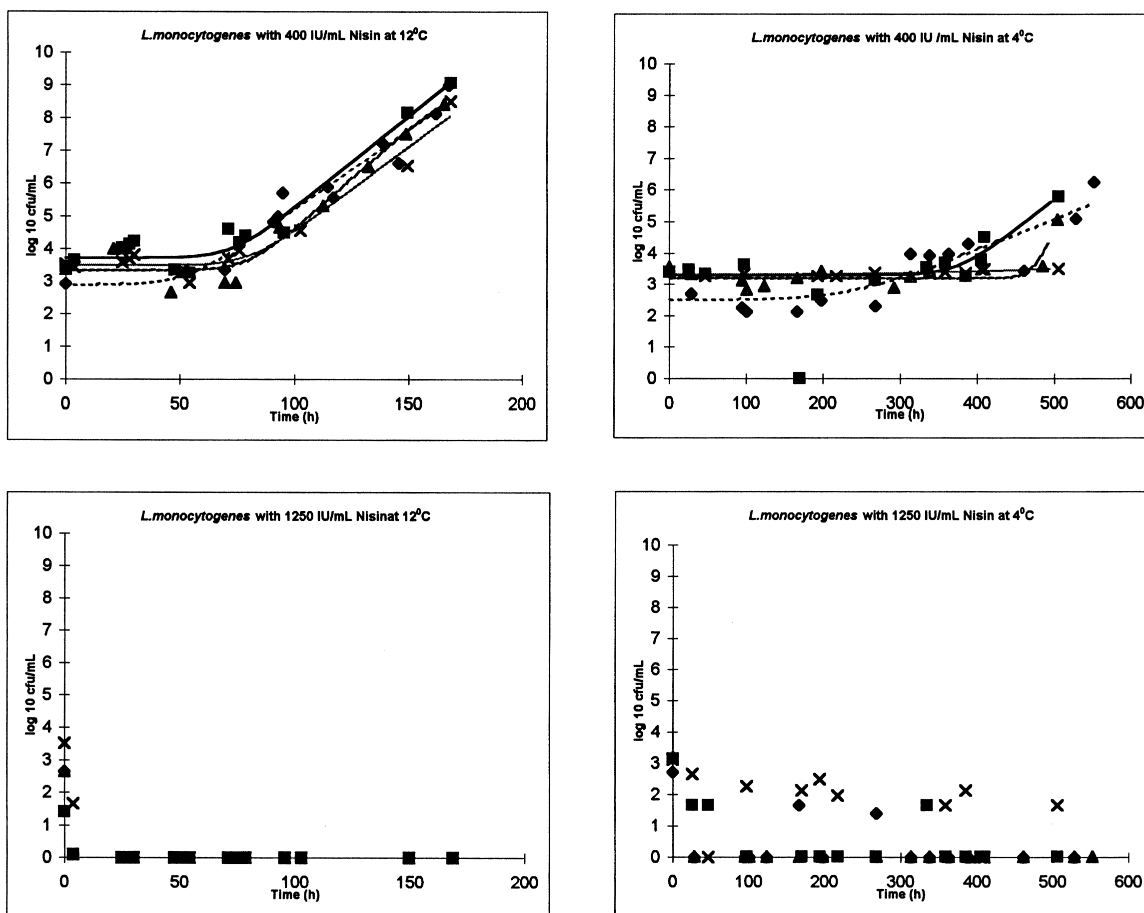


Fig. 3. Fitted growth curves showing the effect of 400 or 1250 IU/ml nisin in combination with an atmosphere of air (●), 100% N_2 (♦), 40% CO_2:60% N_2 (■) or 100% CO_2 (×) on the growth of *Listeria monocytogenes* at 4 or 12°C.

Raising the level of nisin to 1250 IU/ml increased the inhibitory effect on *L. monocytogenes* in all atmospheres. At both 4 and 12°C, the growth of *L. monocytogenes* was prevented (Fig. 3). These observations were reproducible.

3.5. Effect of shifting between temperatures

The combinations of 100% N_2 and 100% CO_2 with or without 400 IU/ml nisin or 1.0% ALTA™ 2341 were assessed for their inhibitory effect on *L. monocytogenes* growth when the temperature was shifted between 4 and 12°C (Table 2). Duplicate broths of each combination were held at 4°C for 5 days, shifted to 12°C for 3 days, and then either shifted to 4 or 8°C for 14 days.

Combinations with bacteriocin as well as 100% CO_2 acting alone prevented *L. monocytogenes* growth throughout the initial 5 days at 4°C. In 100% N_2, a 2 log population increase was observed. After incubation at 12°C for 3 days in the absence of bacteriocin, a 6 log population increase was observed in 100% N_2, and a 3 log population increase noted in 100% CO_2. A 2 log population increase was observed in the presence of bacteriocin, with the exception of the combination of 100% CO_2 and 1.0% ALTA™ 2341, which fell below the level of detection (<2 $\log_{10}$ cfu/ml). When shifted to 4 or 8°C for a further 14 days, growth continued in all combinations with the exception of 100% CO_2 and 1.0% ALTA™ 2341, where the population remained below the level of detection.

Table 2
Interaction of atmosphere, temperature and bacteriocin on the growth of *Listeria monocytogenes* as temperature fluctuated between 4 and 12°C

Day	100% N_2		100% N_2 + 400 IU/ml nisin		100% N_2 + 1.0% ALTA 2431		100% CO_2		100% CO_2 + 400 IU/ml nisin		100% CO_2 + 1.0% ALTA 2431	
Incubation at 4°C												
Initial	3.23[a]		2.53		2.00		3.04		2.92		2.15	
Day 1	4.15		2.56		nd		3.45		2.65		nd	
Day 2	4.81		2.40		nd		3.86		3.08		nd	
Day 3	5.70		2.32		nd		4.41		3.28		nd	
Day 4	5.88		2.32		nd		3.79		2.89		nd	
Incubation at 12°C												
Day 5	6.28		2.73		nd		4.28		2.56		nd	
Day 7	9.26		4.34		3.49		6.76		3.28		nd	
Incubation at (°C):	4	8	4	8	4	8	4	8	4	8	4	8
Day 8	8.7	9.28	4.00	3.98	4.86	5.98	8.23	8.95	4.45	5.77	nd	nd
Day 9	—	—	4.04	—	6.23	7.92	8.49	8.18	4.56	5.51	nd	nd
Day 11	—	—	6.93	6.49	8.73	8.38	6.95	8.08	4.56	6.41	nd	nd
Day 14	—	—	7.93	8.04	8.56	9.36	8.61	8.59	6.65	8.59	nd	nd
Day 17	—	—	7.82	—	9.15	—	9.11	—	7.08	—	nd	nd
Day 18	—	—	8.74	—	—	—	—	—	7.65	—	nd	nd

nd, not detected (<2 log_{10} cfu/ml); —, not tested.
[a]Counts of *L. monocytogenes* expressed as log cfu/ml, mean of two experiments.

3.6. Bacteriocin resistance

Twenty-eight of the 32 combinations that exposed *L. monocytogenes* either to nisin or ALTA™ 2341 were not 100% bactericidal as evidenced by the recovery of survivors following subculture. The exceptions were the combinations of: 1250 IU/ml nisin, 100% N_2, at 12°C and 4°C; 1250 IU/ml nisin, 40% CO_2:60% N_2, at 4°C; and 1% ALTA™ 2341, 100% CO_2, at 4°C.

In order to determine whether surviving cells had escaped the effects of the bacteriocin or become resistant to it, their susceptibility was tested by critical dilution assays. Resistance was determined by an observed change in MIC of surviving cells compared to that of the initial population. For *L. monocytogenes* that survived exposure to nisin, their susceptibility to the bacteriocin was unaltered. The susceptibility of nisin survivors to ALTA™ 2341 was also tested but this too had not changed.

A change in susceptibility was observed in *L. monocytogenes* that survived exposure to ALTA™ 2341. Regardless of temperature or atmosphere, *L. monocytogenes* recovered from broths containing 1.0% ALTA™ 2341 were resistant to a dilution series of 10–0.08% (w/v) ALTA™ 2341. *L. monocytogenes* recovered from broths containing 0.1% ALTA™ 2341 produced 'fuzzy' zones of inhibition against a dilution series from 10–0.08% (w/v) ALTA™ 2341. All ALTA™ 2341-resistant *L. monocytogenes* mutants remained sensitive to nisin.

4. Discussion

The emergence of *L. monocytogenes* as an important foodborne pathogen has led to a resurgence of interest in antimicrobials suitable for its control. At the same time, consumer demands for foods that contain fewer preservatives, are less processed, free from artificial additives and perceived as fresh and more natural have increased (Gould, 1992). To address both these issues, much research has focussed on the potential of bacteriocins derived from lactic acid bacteria for use in food preservation. In the present study, it was found that the growth of *L. monocytogenes* was influenced (to varying degrees) by a combination of temperature, bacteriocin and atmosphere.

In experiments assessing the effect of atmosphere in the absence of bacteriocin, 100% CO_2 was found to exert the greatest inhibitory effect on *L. monocyto-*

genes. While growth was observed in this atmosphere at 12°C, it was at a rate lower than any other atmosphere tested. At 4°C, growth was inhibited by 100% CO_2. These results in broth are consistent with studies using laboratory media (Farber et al., 1996; Fernandez et al., 1997), chicken breast meat (Hart et al., 1991), and lamb (Garcia de Fernando et al., 1995) where lower temperatures, which increase the solubility of CO_2 in the menstruum, were more inhibitory. There is inconsistency in the published data with respect to *L. monocytogenes* growth in atmospheres $< 80\%$ CO_2 in the absence of oxygen (Hart et al., 1991; Marshall et al., 1991). In this study, 40% CO_2:60% N_2 permitted *L. monocytogenes* growth to levels exceeding 10^6 cfu/ml at 12 and 4°C.

Bacteriocin, temperature and atmosphere were found to interact in their influence on *L. monocytogenes* growth, in some instances achieving a level of control not attained by atmosphere alone. In general, nisin and ALTA™ 2341 were more effective when the incubation temperature was lowered to 4°C. With the addition of nisin (400 and 1250 IU/ml), inhibition of *L. monocytogenes* growth was observed in all atmospheres compared to the controls. The combinations of: 1250 IU/ml nisin, 100% N_2 at 12 and 4°C; and 1250 IU/ml nisin, 40% CO_2:60% N_2 at 4°C were listericidal. ALTA™ 2341 (0.1 and 1.0%) enhanced the inhibitory capacity of 40% CO_2:60% N_2 (0.1 and 1.0% ALTA™ 2341), 100% N_2 (0.1% ALTA™ 2341), and was listericidal in combination with 100% CO_2 (1.0% ALTA™ 2341).

Viable *L. monocytogenes* were recovered from all culture conditions exposing cells to nisin except the combination of 1250 IU/ml nisin and 100% N_2 (4 and 12°C) or 40% CO_2:60% N_2 (4°C). Viable *L. monocytogenes* were recovered from broths exposing cells to ALTA™ 2341 under all experimental conditions except 1.0% ALTA™ 2341 and 100% CO_2 at 4°C. There is no clear explanation as to why these combinations were listericidal and others not. Differences in adsorption of nisin between strains of *L. monocytogenes* have been reported (Davies and Adams, 1994), and attributed to differences in cell wall architecture (Davies et al., 1996). Psychrotrophic microorganisms, such as *L. monocytogenes*, appear to adapt to low-temperature growth by altering the ratio of short and/or branched fatty acyl chains in the lipid content of the cell membrane (Gounot, 1991). It is possible that the conditions imposed on *L. monocytogenes* in 100% N_2 and 40% CO_2:60% N_2 at lower temperatures affected the cell wall of the strains used in this study, increasing their susceptibility to the effects of bacteriocin.

Because of the inhibitory effects observed with varying combinations of atmosphere and bacteriocin at 4 and 12°C, an experiment was designed to test the ability of 100% N_2 and 100% CO_2 in the presence or absence of 400 IU/ml nisin or 1.0% ALTA™ 2341 to control the growth of *L. monocytogenes* when temperature fluctuated. These conditions were chosen to simulate (in broth) vacuum packaging and CO_2-enriched MAP. The level of 400 IU/ml of nisin was chosen as this is reputed as the highest level of nisin that is still economically feasible for industrial use, and 1.0% ALTA™ 2341 as this is the level of use in food recommended by the manufacturer. When the temperature was shifted from 4°C for 4 days to 12°C for 3 days, then to 4 or 8°C for a maximum of 14 days, only the combination of 100% CO_2 and 1.0% ALTA™ 2341 effectively controlled *L. monocytogenes* growth. This combination of atmosphere and bacteriocin, at 4°C, was listericidal. The experiment demonstrated that the combination of 100% CO_2 and 1.0% ALTA™ 2341 offers inhibition of *L. monocytogenes* growth within a cold chain that fluctuates between good temperatures of refrigeration and abuse. This inhibition was not attained with atmosphere alone.

Despite the favorable inhibitory effects of combinations of atmosphere, bacteriocin and temperature, viable *L. monocytogenes* were detected from most combinations. In particular, mutants of *L. monocytogenes* resistant to ALTA™ 2341 were isolated from most atmosphere and temperature combinations containing the bacteriocin. This may have contributed the observed poor reproducibility of growth rate in conditions containing ALTA™ 2341. The occurrence of spontaneous bacteriocin-resistant mutants from an initial sensitive population of *L. monocytogenes* has been reported with other bacteriocins. Motlagh et al. (1992) examined the bactericidal activity of pediocin AcH against three strains of *L. monocytogenes* in ground beef and milk. These workers demonstrated that cells survived and grew in pediocin AcH-treated samples with prolonged storage at 4 and 10°C, even while residual levels of the bacteriocin were still detectable. Mathieu et al.

(1994) showed that extended exposure of *L. monocytogenes* to carnocin CP5 in broth or in skimmed milk lead to the development of resistance. They found that carnocin CP5 resistance persisted with multiple subculture in the absence of the bacteriocin. Larsen and Norrung (1993) screened 245 strains of *L. monocytogenes* from clinical, food and environmental sources for their sensitivity to bavaricin A and found three 'naturally' occurring resistant strains. There have been reports where *L. monocytogenes* developed resistance to pediocin AcH, and this conferred cross-resistance to other class II bacteriocins; mesenterocin 52, curvaticin 13 and plantaricin C19 (Ray and Daeschel, 1992; Rekhif et al., 1994). While a change in *L. monocytogenes* resistance to nisin was not noted in the present study, it has been reported by others (Harris et al., 1991; Davies and Adams, 1994; Mazzotta and Montville, 1997).

5. Conclusion

In this study, a combination of incubation at 4°C along with a CO_2-enriched atmosphere adequately controlled the growth of *L. monocytogenes* in a broth system (pH 6.0) for prolonged periods. With temperature abuse, a similar degree of control was achieved only with the addition of bacteriocin to the system. This control was dependent upon the bacteriocin/atmosphere combination. While our study shows that the use of bacteriocins assists with the control of *L. monocytogenes* in a range of atmospheres, we see that a major obstacle to the development of food applications for bacteriocins is the ability of pathogens such as *L. monocytogenes* to develop resistance. Our understanding of the mechanisms of bacteriocin resistance needs elucidation so strategies that minimise the occurrence of resistant populations can be developed.

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