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The effect of growth atmosphere on the ability of *Listeria monocytogenes* to survive exposure to acid, proteolytic enzymes and bile salts

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Abstract

Four isolates of *Listeria monocytogenes* from food, human and environmental sources were grown separately in broth (pH 6.0 at 8 °C) under atmospheres of air, 100% N_2, 40% CO_2:60% N_2 or 100% CO_2. Exponential and stationary phase cells were harvested to determine if growth atmosphere and growth phase influenced this pathogen's ability to survive exposure to an acid environment coupled with proteolytic enzymes, and the activity of bile salts. In general, isolates were more resistant to the acid environment than the bile salts environment and stationary phase cells were significantly more resistant to both environments than exponential phase cells. Irrespective of prior growth atmosphere, none of the isolates when in exponential phase remained detectable following full exposure to the acid environment (110 min at 37 °C) or the bile environment (3 h at 37 °C). With the exception of one isolate grown under the atmosphere of 40% CO_2:60% N_2, all isolates when in stationary phase were detectable following full exposure to the acid environment but death rates varied significantly. Stationary phase cells of all isolates grown under 40% CO_2:60% N_2 and 100% CO_2 were highly susceptible to the bile salts environment: cells were not detectable after a 2-min exposure whereas stationary phase cells grown under air or 100% N_2 were recovered following full exposure to the bile environment. Survival curves were characterised by a population decline of at least 3 $\log_{10}$/ml (from an initial level of 7 $\log_{10}$ CFU/ml) in the first 15 min; thereafter a constant population number of approximately 4 $\log_{10}$/ml was maintained over the remaining exposure period. No survival was observed when stationary phase cells of *L. monocytogenes* FRRB 2538 grown in air and 100% N_2 were subjected to the acid environment followed by immediate exposure to the bile salts environment. The results showed that growth atmosphere and growth phase could influence survival of this pathogen against conditions that imitate the extremes of the most important nonspecific defence mechanisms against microbial infection: the acid environment of the stomach coupled with the activity of proteolytic enzymes, and the activity of bile salts in the small intestine.

Keywords: *Listeria monocytogenes*; Modified atmosphere packaging; Acid; Proteolytic enzymes; Bile salts; Gastrointestinal tract

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1. Introduction

Listeria monocytogenes is a rare cause of foodborne disease with fewer than four to eight cases of listeriosis per million individuals per year reported in developed countries (Buchanan and Lindqvist, 2000). As the consequences of the disease may be serious, fatality rates are 20–30% among hospitalised patients, the food industry remains concerned about the presence of *L. monocytogenes* in food. Such concerns have been raised about its presence in refrigerated, minimally processed, ready-to-eat foods and where modified atmosphere packaging (MAP) is used to extend the shelf life. It is felt that these storage conditions may favour the survival of *L. monocytogenes* and, with sufficient time and condition, support its growth to levels in excess of 100 cells/g at the point of consumption (Alinorm 03/13, 2001; Hintlian and Hotchkiss, 1986; Berrang et al., 1989; Wimpfheimer et al., 1990; Kallander et al., 1991; Garcia-Gimeno et al., 1996).

The gases most commonly used in MAP, either alone or in combination, are CO_2, N_2 and O_2 (present in concentrations different to that occurring naturally in air). Of these gases, CO_2 is the gas mainly responsible for any direct antimicrobial activity in MAP foods. Temperature plays a critical role in its activity. As the temperature increases, the solubility of CO_2 decreases and consequently the antimicrobial effect is less pronounced. The precise mechanism for the antimicrobial activity of CO_2 is unknown. Mechanisms postulated to occur include: inhibition of growth energetics by alteration of the physicochemical properties of enzymes, changes to the rate of protein solution, feedback inhibition of decarboxylation reactions, and the lowering of substrate pH as dissolved CO_2 is hydrated to carbonic acid (Statham, 1984; Dixon and Kell, 1989). While such activity may, at the cellular level, cause protein denaturation or loss of functions of some bacterial cell structures, the effect of modified atmospheres at chill temperature, even those rich in CO_2, on *L. monocytogenes* (in low-acid foods) is not bactericidal (Gill and Reichel, 1989; Garcia de Fernando et al., 1995; Farber et al., 1996; Szabo and Cahill, 1998). Growth is usually restricted under these conditions, indicating that the action of CO_2 on this pathogen is sublethal.

Acknowledgement that a microorganism's prehistory bears influence on its response to the intrinsic and extrinsic properties of its environment has emerged as a key issue in studies of microbial food safety. In particular, many studies have focussed on the concept of cross-protection where prior exposure to one form of sublethal condition may cross-protect microorganisms against further homologous or seemingly heterologous sublethal conditions. For example, Davis et al. (1996) found that *L. monocytogenes* acquired acid tolerance during exponential growth after prior exposure to a sublethal acid environment. Okereke and Thompson (1996) found that acid resistance also conferred limited nisin resistance on *L. monocytogenes*. The concept of cross-protection then is of particular importance for minimally processed ready-to-eat MAP food in which contaminating foodborne pathogens are exposed to a sublethal environment (Lou and Yousef, 1997). In these food systems where exposure time is extended due to the desire to extend the shelf life of the product, a pathogen's response to the sublethal preservative hurdle may lead to cross-protective responses such as increased resistance to conditions found in the gastrointestinal tract of humans (Bolton and Frank, 1999).

The objective of this study was to conduct laboratory experiments with *L. monocytogenes* to determine if growth atmosphere (CO_2-rich atmospheres in particular) in combination with chill temperature influenced this pathogen's ability to survive conditions that imitate the extremes of some of the most important nonspecific defence mechanisms against microbial infection during gastric and small intestinal transit. An acid broth system was used to mimic the acid environment of the stomach coupled with the presence of proteolytic enzymes and a bile broth system was used to mimic the inhibitory activity of bile salts in the small intestine. As other studies have found that the ability of *L. monocytogenes* to survive sublethal conditions was a function of the bacterial growth phase (O'Driscoll et al., 1996), exponential phase cells and stationary phase cells were harvested and subjected to the acid and bile broth systems.

2. Materials and methods

2.1. Isolates and culture media

The four isolates of *L. monocytogenes* used in this study were obtained as lyophilised cultures from the

Food Science Australia Culture Collection (North Ryde NSW, Australia). The isolates were of clinical (FRRB 2472, blood, strain Scott A), food (FRRB 2619, packaged iceberg lettuce; FRRB 2696, pizza-based sandwich filling) and environmental (FRRB 2538, dairy factory floor) origin. All isolates were grown in tryptone soya broth (TSB; Oxoid, UK) or on tryptone soya agar (TSA, Oxoid). The identity of the isolates was confirmed by Gram-stain, the CAMP test and API *Listeria* biochemical test strips (bioMérieux, Marcy I'Etoile, France).

2.2. Preparation of the inoculum

Each of the isolates were streaked onto TSA plates and incubated for 24 h at 37 °C. Single colonies were restreaked onto fresh TSA plates and incubated for 24 h at 37 °C. An isolated colony was used to inoculate 20 ml of TSB at pH 6 and was incubated for 24 h at 37 °C. One hundred microliters of the resulting culture was then used to inoculate 20 ml of fresh TSB that was incubated at 15 °C for 72 h in a shaking water bath (30 strokes/min, SS40-D; Grant Instruments, England). After 72 h, the culture was serially diluted by conducting two successive 1:100 dilutions. A 0.5 ml volume of the final dilution was then added to the controlled atmosphere-broth system using a sterile 0.63 × 32 mm needle and 1 ml sterile syringe (Becton-Dickinson, Singapore). This consistently gave an initial cell density of approximately 10^3 cells/ml for each isolate that was enumerated by spread-plating onto TSA followed by incubation at 37 °C for 24 h. All serial dilutions were made using 0.1% (w/v) peptone (Amyl Media, Australia) as a diluent.

2.3. Growth under modified atmospheres

Four atmospheres were used: air and premixes of 100% N_2, 40% CO_2:60% N_2 and 100% CO_2 (BOC, Australia). A controlled-atmosphere broth system was achieved by modifications to 250 ml Duran Schott bottles (Duran, USA) as described elsewhere (Szabo and Cahill, 1998). TSB (50 ml) supplemented with the buffering agent piperazine dihydrochloride hydrate (15.9 g/l; Sigma-Aldrich, USA) was the selected growth medium. The pH was adjusted to 6.0 for all atmospheres except 100% CO_2 in which the pH of the media was adjusted to 6.4 to counter the acidification caused by CO_2 dissolving in the medium. Modified atmosphere bottles were held in an 8 °C water bath for 24 h prior to inoculation to allow equilibration of the media to the test temperature. This temperature was chosen as it represents a realistic value for a food storage temperature-abuse situation that could allow growth of *L. monocytogenes* to unacceptable levels. The temperature of the water bath was monitored using Tinytag temperature dataloggers (Gemini Dataloggers, UK). The headspace of each modified atmosphere bottle was analysed using the Gaspace 2 analyzer (Systech Instruments, England) before inoculation and at the end of experimentation. In order to ensure that atmospheres were maintained throughout the experiment, bottles were flushed with the appropriate gas once every 48 h for 5 min. The pH of the media from each modified atmosphere bottle was recorded after completion of each growth curve.

L. monocytogenes were enumerated as described above by taking a sample from the modified atmosphere bottle using a sterile 0.63 × 32 mm needle and a 1 ml sterile syringe (Becton-Dickinson). Samples were taken immediately after inoculation and then twice daily. Three growth curves per atmosphere were generated from three separate MAP bottles. Curve fitting and calculation of growth rates were carried out using the Baranyi "D-model" for bacterial growth (Baranyi et al., 1993). The replicates were compared to determine the appropriate time to harvest cells at exponential and stationary phases for inoculation of the acid and bile broth systems.

2.4. The acid and bile broth systems

The appropriate volume of stationary or exponential phase cells were harvested from the modified atmosphere bottles to yield an initial cell density of approximately 10^7 cells/ml upon inoculation into the acid and bile broth systems. Cells were centrifuged for 10 min at 5000 × *g* at 4 °C (Jouan, MR1822, France). The supernatant was removed and the remaining pellet was resuspended in 100 μl of 0.1% (w/v) phosphate-buffered saline (PBS; Oxoid). The suspension was then added to the appropriate broth system.

The acid broth system contained 12 ml of 0.1 mol/l HCl:KCl buffer pH 2 containing 500 U/ml pepsin A (Oxoid) and 1 g/l bacteriological peptone (Oxoid).

The broth was sterilised by filter sterilisation (0.22 μm, cellulose; Millipore, USA) and added to a sterile 30 ml screw-capped bottle. The *L. monocytogenes* isolates were added to the broth at an initial cell density of approximately 10^7 cells/ml. The sample was incubated for 110 min at 37 °C in a water bath (SS40 D; Grant Instruments). Samples (100 μl) were taken for enumeration immediately upon inoculation and then every 10 min. Samples, neat and serially diluted, were plated in duplicate onto TSA plates and TSA plates containing 1 g/l pyruvate (Sigma, St. Louis, MO).

The bile broth system contained 12 ml of 1 g/l phosphate buffer pH 6.5 containing 3 g/l oxbile (Oxoid). The broth was sterilised by filter sterilisation (0.22 μm, cellulose; Millipore) and added to a McCartney bottle. The *L. monocytogenes* isolates were added to the broth at an initial cell density of approximately 10^7 cells/ml. The sample was incubated for 3 h at 37 °C in a water bath (SS40 D; Grant Instruments). Samples (100 μl) were taken immediately upon inoculation and then every 15 min and were enumerated as described above.

A 500 μl sample was removed for enrichment at the completion of incubation in the acid broth system (110 min) and the bile broth system (3 h). The sample was added to a bottle containing 20 ml of TSB and incubated for 48 h at 37 °C. Where turbidity was observed, a loopful of the suspension was streaked onto a Columbia Horse Blood Agar plate (Oxoid, Australia). The detection of β haemolysis after 48 h at 37 °C was taken as a confirmation that the turbidity was due to the presence of *L. monocytogenes*.

Experiments investigating the survival of *L. monocytogenes* when exposed to sequential transit through both models involved exposing cells first to the acid broth system at an initial cell density of approximately 10^7 cells/ml. A sample was taken upon inoculation and plated onto TSA plates with 1 g/l pyruvate added. The broth was incubated at 37 °C. After 100 min, the samples were centrifuged for 10 min at 5000 × *g* at 4 °C (Jouan, MR1822). The supernatant was removed and the pellet was resuspended in 100 μl of 0.1% PBS. A sample was taken immediately after resuspension to constitute the count at 110 min. The suspension was then added to the bile broth system and a sample was taken. At the same time, 500 μl of the acid broth system was added to 20 ml of TSB and incubated at 37 °C for 48 h. Samples were taken at 15 and 180 min and plated onto TSA plates to which 1 g/l pyruvate was added; at the same time 0.5 ml of the bile broth system was added to 20 ml of TSB and incubated at 37 °C for 48 h.

2.5. Statistical analysis of death curves

Colony counts, expressed as $\log_{10}$ CFU/ml, were tabulated against time (min) and entered into MINITAB version 11 to generate death rates and standard errors. A program was created in FORTRAN (Dr. John Best, Food Science Australia) to compare the death rates using *t*-tests. As this involves 16–31 *t*-tests, there was a high probability that some *t*-tests would be significant by chance alone (the multiple comparisons problem). To adjust for this possibility, a 0.01% critical value of the *t*-statistic was used so that the overall significance level could still be claimed to be above the conventional 5%.

3. Results and discussion

The two environments tested simulated the extremes of the most important nonspecific defence mechanisms against microbial infection, i.e. the acid environment of the stomach, the excretion of proteolytic enzymes and the inhibitory activity of bile salts. The acid broth system did not simulate the gradual acidification that occurs in the stomach upon ingestion of a meal nor was the protective effect of food against the lethal action of acid or bile salts taken into account in the broth systems. Nevertheless, the broth systems used did serve to demonstrate that the survival of *L. monocytogenes* to these extreme conditions was possible and was influenced by growth atmosphere, growth phase and the isolate studied.

3.1. Growth under modified atmospheres

The pH of the media from each modified atmosphere bottle remained constant at a pH of 6.0 during experimentation. The growth rate, approximate final cell concentration and time taken, in TSB, for each growth atmosphere/phase of growth combination for the four isolates of *L. monocytogenes* tested are shown in Table 1. Each isolate grew under the atmospheres

Table 1
Approximate final cell concentrations (log_{10} CFU/ml), time and growth rate[a] (μ_{max} h^{-1}) in TSB (pH 6.0 at 8 °C) for each growth atmosphere/phase of growth combination for the *L. monocytogenes* isolates[b]

Isolate	Growth phase and rate	Atmosphere			
		Air	100% N_2	40% CO_2:60% N_2	100% CO_2
FRRB 2472	Exponential	7.07 ± 0.227	7.66 ± 0.155	7.89 ± 0.330	6.22 ± 0.362
		165 h	87 h	120 h	100 h
	Stationary	9.39 ± 0.083	9.20 ± 0.320	8.99 ± 0.214	7.82 ± 0.396
		366 h	218 h	261 h	250 h
	Growth rate	*0.031* ± 0.002	*0.037* ± 0.005	*0.039* ± 0.006	*0.038* ± 0.010
FRRB 2538	Exponential	6.62 ± 0.165	6.71 ± 0.306	6.19 ± 0.124	5.67 ± 0.497
		180 h	89 h	100 h	100 h
	Stationary	9.24 ± 0.019	9.09 ± 0.086	8.70 ± 0.185	7.53 ± 0.181
		363 h	239 h	261 h	261 h
	Growth rate	*0.031* ± 0.000	*0.040* ± 0.005	*0.040* ± 0.003	*0.039* ± 0.012
FRRB 2619	Exponential	7.12 ± 0.278	6.83 ± 0.302	5.91 ± 0.185	5.43 ± 0.230
		180 h	72 h	67 h	90 h
	Stationary	9.72 ± 0.014	9.13 ± 0.209	8.40 ± 0.309	7.67 ± 0.011
		350 h	216 h	212 h	281 h
	Growth rate	*0.029* ± 0.002	*0.048* ± 0.007	*0.035* ± 0.002	*0.031* ± 0.003
FRRB 2696	Exponential	8.70 ± 0.180	7.26 ± 0.151	6.77 ± 0.223	5.94 ± 0.597
		234 h	89 h	90 h	90 h
	Stationary	9.70 ± 0.038	8.67 ± 0.146	8.41 ± 0.002	7.47 ± 0.163
		370 h	255 h	230 h	257 h
	Growth rate	*0.028* ± 0.003	*0.049* ± 0.003	*0.054* ± 0.007	*0.036* ± 0.005

[a] Growth rate values of exponential phase cells are in italics.
[b] Values are the mean of three replicate growth curves.

tested but the rate of growth and final population size varied. Commonalities in the growth response of the isolates to the conditions, however, were observed: growth under air was not only slower for each isolate but the isolates had similar growth rates (such a uniform response with respect to growth rate was not observed when the isolates where grown under the anaerobic atmospheres tested); and of the four atmospheres tested, growth under 100% CO_2 lowered the final stationary population size of each isolate the most. Isolates of *L. monocytogenes* FRRB 2472 and FRRB 2538 responded similarly under each atmosphere, growing most slowly under air and more quickly under the other atmospheres. *L. monocytogenes* FRRB 2619 grew faster under 100% N_2 than 40% CO_2:60% N_2 followed by similar rates of growth in air and 100% CO_2. *L. monocytogenes* FRRB 2696 grew most quickly in 40% CO_2:60% N_2 then in 100% N_2, 100% CO_2 and air.

Analysis of the replicate growth curves enabled determination of the appropriate time to harvest exponential and stationary phase cells of each of the isolates under the test conditions that were subjected to the acid broth system and the bile broth system separately in addition to transit through both.

3.2. Survival in the acid and bile broth systems

Because exogenous pyruvate has been reported to aid in the recovery of injured cells (McDonald et al., 1983), it was added to the recovery or enumeration media, TSA. No differences in counts were observed between samples plated onto TSA and samples plated onto TSA plates with 1 g/l pyruvate added (data not shown). Therefore, results are reported for samples plated onto TSA plates containing pyruvate only. The initial concentration of inoculum added to each of the broth systems was approximately 10^7 CFU/ml. The use of this high inoculum level enabled calculation of and comparison of death rates in the acid and bile environments that served as a measure of the influence that modified atmosphere and chilled storage conditions had on the ability of this pathogen to survive the conditions encountered during gastric

transit. Although a defined infective dose cannot be calculated for *L. monocytogenes* due to variables such as the food matrix, amount of food consumed and host susceptibility, the inoculum level does bear relevance to the level of *L. monocytogenes* reported to cause listeriosis. This varies for "healthy" or "high-risk" populations. For healthy adults, reported levels vary from 10^5 to 10^7 CFU/ml and for high-risk groups, from 10 to 10^4 CFU/ml (Maijala et al., 2001).

3.2.1. Survival in the acid broth system

Previous studies by Davis et al. (1996) demonstrated that *L. monocytogenes* has both a pH-dependent acid tolerance response system as well as a pH-independent growth phase-dependent acid response system. These workers found the level of acid tolerance increased rapidly during the transition between exponential phase and stationary phase. These findings complement our observations in that the resistance of the *L. monocytogenes* isolates to the acid broth system was growth phase dependent. Fig. 1 shows the survival curves of each growth atmosphere/phase of growth combination for the *L. monocytogenes* isolates studied. The calculated death rates are presented in Table 2 and the corresponding statistical analysis in Table 3. With two exceptions, a significant difference ($P<0.05$) was observed between the death rates of all exponential phase and all stationary phase cells. No significant difference ($P>0.05$) was observed between exponential phase cells of *L. monocytogenes* FRRB 2538 grown in 40% CO_2:60% N_2 and stationary phase cells of both *L. monocytogenes* FRRB 2472 grown in 40% CO_2:60% N_2 and *L. monocytogenes* FRRB 2696 grown in 100% N_2. The results show that exponential phase cells were significantly ($P<0.05$) more susceptible to the acid broth system than stationary phase cells and the degree of resistance varied with growth atmosphere and the isolate studied.

For cells in the exponential phase, some general trends in the response of the isolates under the con-

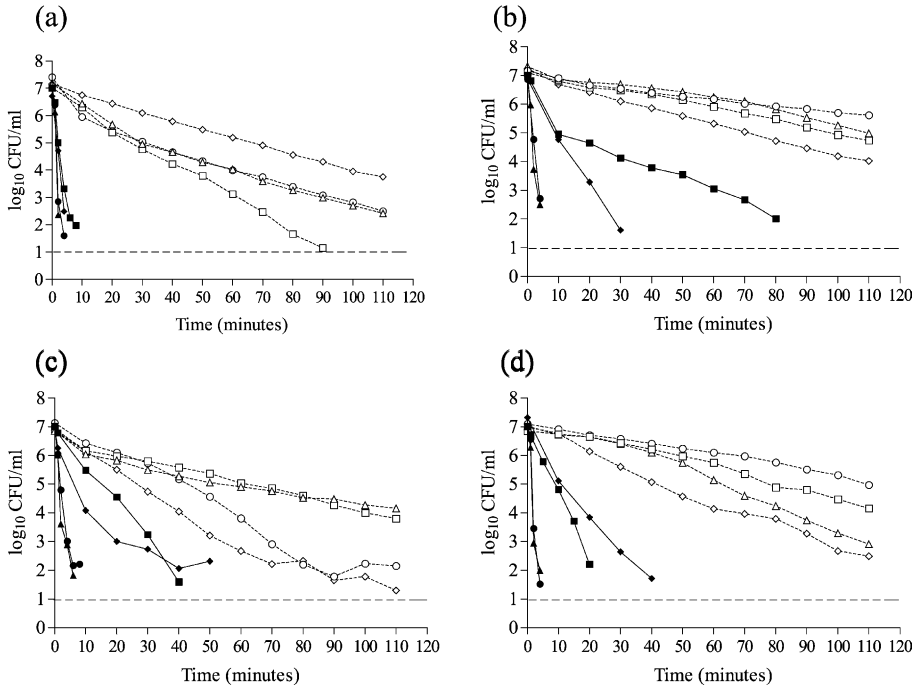

Fig. 1. Survival curves of exponential (—) and stationary (- - -) phase *L. monocytogenes* isolates: (a) FRRB 2472; (b) FRRB 2538; (c) FRRB 2696; and (d) FRRB 2619 after exposure to the acid broth system (37 °C) for 110 min. Exponential phase data points are represented by closed symbols and stationary phase points are represented by open symbols. Legend: (●) air, (♦) 100% N_2, (■) 40% CO_2:60% N_2 and (▲) 100% CO_2. Values are the average of three duplicates. The dashed line indicates the detection level of the enumeration method.

Table 2
The death rates (μ_{Max} h^{-1}) of each isolate after exposure to the acid broth system[a]

Isolate	Atmosphere	Death rate	
		Exponential	Stationary
FRRB 2472	Air	− 1.450 ± 0.124	− 0.045 ± 0.0016
	100% N_2	− 1.090 ± 0.065	− 0.030 ± 0.0005
	40% CO_2: 60% N_2	− 0.725 ± 0.031	− 0.066 ± 0.0008
	100% CO_2	− 2.030 ± 0.260	− 0.046 ± 0.0011
FRRB 2538	Air	− 1.040 ± 0.076	− 0.013 ± 0.0009
	100% N_2	− 0.184 ± 0.004	− 0.028 ± 0.0006
	40% CO_2: 60% N_2	− 0.068 ± 0.003	− 0.020 ± 0.0006
	100% CO_2	− 1.220 ± 0.073	− 0.016 ± 0.0013
FRRB 2696	Air	− 0.762 ± 0.052	− 0.054 ± 0.0014
	100% N_2	− 0.134 ± 0.010	− 0.063 ± 0.0021
	40% CO_2: 60% N_2	− 0.131 ± 0.003	− 0.031 ± 0.0012
	100% CO_2	− 0.970 ± 0.065	− 0.030 ± 0.0012
FRRB 2619	Air	− 1.400 ± 0.102	− 0.017 ± 0.0004
	100% N_2	− 0.141 ± 0.004	− 0.043 ± 0.0014
	40% CO_2: 60% N_2	− 0.231 ± 0.003	− 0.024 ± 0.0009
	100% CO_2	− 1.370 ± 0.120	− 0.034 ± 0.0014

[a] Values are the average of three replicates ± S.E.

ditions tested were observed. Exponential phase cells of *L. monocytogenes* FRRB 2472 were the least resistant to the acid broth system and those of *L. monocytogenes* FRRB 2696 were the most resistant, with the exception of prior growth in 40% CO_2:60% N_2. Exponential phase cells of all isolates grown under 100% CO_2 and air were consistently more susceptible to the acid broth system.

Prior exposure to growth under 100% N_2 or 40% CO_2:60% N_2 seemed to enable greater resistance to the conditions encountered in the acid broth system for exponential phase cells of *L. monocytogenes* FRRB 2538, 2696 and 2619 (Fig. 1 and Table 2). The rate of death differed significantly between each of these isolates under these conditions with the exception of *L. monocytogenes* FRRB 2619 under 100% N_2 and *L. monocytogenes* FRRB 2696 under 40% CO_2:60% N_2 where similar rates of death were observed (Tables 2 and 3). Although the rate of death was significantly slower with prior exposure to these atmospheres for *L. monocytogenes* FRRB 2538, 2696 and 2619 compared to that of air and 100% CO_2 (Tables 2 and 3), prior exposure to 100% N_2 or 40% CO_2:60% N_2 did not enable any of these isolates to remain detectable following full exposure to the acid broth system. At best, prior exposure to 40% CO_2:60% N_2 enabled exponential phase cells (of *L. monocytogenes* FRRB 2538) to remain detectable in the acid broth system for at least 80 min and prior exposure to 100% N_2 enabled cells (of *L. monocytogenes* FRRB 2696) to remain detectable for 50 min. At best, prior exposure to air and 100% CO_2 enabled one of the isolates (*L. monocytogenes* FRRB 2696) to remain detectable for 10 and 8 min, respectively. It must be noted that the acid broth system used in this study contained a static acid concentration and therefore may have exposed cells to harsher conditions than the gradual acidification that occurs in the in vivo situation and this aspect warrants further study, particularly for cells in exponential phase.

The resistance of stationary phase cells to the acid broth system was dependent upon the isolate and growth atmosphere (Fig. 1, Table 2). With one exception (*L. monocytogenes* FRRB 2472 grown in 40% CO_2:60% N_2), all isolates when in stationary phase and irrespective of the growth atmosphere were detectable following full (110 min) exposure to the acid broth system, although significant differences were found between the rate of death (Table 3). No specific atmosphere uniformly offered stationary phase-cells greater resistance to the acid broth system. Conditions that significantly enabled the most resistance to the acid broth system varied amongst the isolates: *L. monocytogenes* FRRB 2472 (100% N_2), FRRB 2538 (air, 100% CO_2), FRRB 2696 (40% CO_2:60%N_2, 100% CO_2), FRRB 2619 (air) (Tables 2 and 3). Stationary phase cells of *L. monocytogenes* FRRB 2538, 2696 and 2619 displayed the least resistance to the acid broth system with prior growth in 100% N_2 that contrasts to observations made when cells from these isolates were in exponential phase. Overall, *L. monocytogenes* FRRB 2538 was regarded as the most resistant isolate displaying the slowest death rates in the acid broth system with prior exposure to each atmosphere (Table 2).

Interestingly, cells of *L. monocytogenes* FRRB 2472 were found to be the most sensitive to the conditions encountered in the acid broth system after growth in most of the atmospheres/phase of growth combinations under study. *L. monocytogenes* FRRB 2472 (strain Scott A) is included in many studies. With extended laboratory storage, it is possible that

Table 3
Statistical comparison[a], by *t*-tests, of death rates between exponential phase cells[b] and between stationary phase cells[c] after exposure to the acid broth system

Isolate	Atmosphere	FRRB 2472				FRRB 2538				FRRB 2696				FRRB 2619			
		Air	100% N_2	40% CO_2:60% N_2	100% CO_2	Air	100% N_2	40% CO_2:60% N_2	100% CO_2	Air	100% N_2	40% CO_2:60% N_2	100% CO_2	Air	100% N_2	40% CO_2:60% N_2	100% CO_2
FRRB 2472	Air		-	+	-	-	+	+	-	+	+	+	-	-	+	+	-
	100% N_2	+		+	-	-	+	+	-	-	+	+	-	-	+	+	-
	40% CO_2:60% N_2	+	+		+	+	+	+	+	-	+	+	-	+	+	+	+
	100% CO_2	-	+	+		-	+	+	-	+	+	+	-	-	+	+	-
FRRB 2538	Air	+	+	+	+		+	+	-	-	+	+	-	-	+	+	-
	100% N_2	+	-	+	+	+		+	+	+	+	+	+	+	+	+	+
	40% CO_2:60% N_2	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+
	100% CO_2	+	+	+	+	-	+	-		-	+	+	-	+	+	+	-
FRRB 2696	Air	-	+	+	-	+	+	+	+		+	+	-	+	+	+	+
	100% N_2	+	+	-	+	+	+	+	+	-		+	+	+	+	+	+
	40% CO_2:60% N_2	+	-	+	+	+	-	+	+	+	+		+	+	-	+	+
	100% CO_2	+	-	+	+	+	-	+	+	+	+	-		-	+	+	-
FRRB 2619	Air	+	+	+	+	-	+	+	-	+	+	+	+		+	+	-
	100% N_2	+	+	+	-	+	+	+	+	+	+	+	+	+		-	+
	40% CO_2:60% N_2	+	+	+	+	+	-	-	+	+	+	-	-	+	+		+
	100% CO_2	+	-	+	+	+	-	+	-	+	+	-	-	+	-	+	

[a] "+" denotes there is a significant difference ($P<0.05$) and "−" denotes there is no significant difference ($P>0.05$).
[b] Values in bold type (+) represent *t*-test results between exponential phase cells.
[c] Values in regular type (+) represent *t*-test results between stationary phase cells.

attenuation of the isolate may have occurred. This highlights the need to use various *L. monocytogenes* isolates in any study from which conclusions regarding food safety are drawn.

3.2.2. Survival in the bile broth system

Bile appeared to have a greater deleterious effect on all isolates than the combined effects of the low pH and pepsin in the acid broth system. None of the exponential phase cells for any of the isolates grown under any of the atmospheres were detectable at the first time of sampling (2 min) after exposure to the bile broth system. Equally as susceptible to the bile broth system were stationary phase cells of all isolates grown under CO_2-rich atmospheres (40% CO_2:60% N_2, 100% CO_2). As CO_2 reputedly interacts with membrane lipids, cells may have become more permeable to the bile salts, which break down the cell wall, resulting in plasmolysis and cell death (Sung et al., 1993). Only stationary phase cells of *L. monocytogenes* FRRB 2538, FRRB 2696 and FRRB 2619 grown under air and *L. monocytogenes* FRRB 2472, FRRB 2538 and FRRB 2619 grown under 100% N_2 survived exposure to the bile broth system (Fig. 2).

L. monocytogenes FRRB 2538, FRRB 2696 and FRRB 2619 grown in air were fairly uniform in their

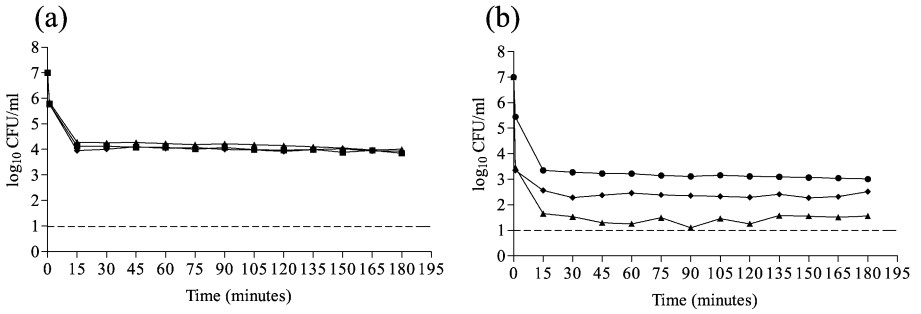

Fig. 2. The survival curves for stationary phase cells of each of the four *L. monocytogenes* isolates exposed to the bile broth system (37 °C for 180 min). Graphs: (a) isolates grown in the air and (b) isolates grown in 100% N_2. Symbols, *L. monocytogenes* isolates: (●) FRRB 2472; (◆) FRRB 2538; (■) FRRB 2696; and (▲) FRRB 2619. Values are averages of three replicates. The dashed line indicates the detection level of the enumeration method.

response to the bile broth system. After a 15-min exposure, an initial 3 log reduction was observed for each isolate and a constant population number of approximately 10^4 CFU/ml was then maintained over the remaining exposure period. A correlation can be seen between the survival of air-grown stationary phase cultures of *L. monocytogenes* FRRB 2538 and FRRB 2619 subjected to the bile broth system and acid broth system. As well as remaining viable for the entire time of exposure to the bile broth system, these isolates displayed the slowest death rates in the acid broth system compared to prior growth in all other atmospheres.

Significant ($P < 0.05$) variation in the initial response of cells harvested from 100% N_2 was observed (Fig. 2). After a 15-min exposure, an initial log reduction of 3.5, 4.5 and 5.5 was observed for *L. monocytogenes* isolates FRRB 2472, FRRB 2538 and FRRB 2619, respectively. Thereafter, the population number remained constant for the entire time of exposure but the number of surviving organisms varied for each isolate. This "tailing" effect may be due to some cells in the population being intrinsically more resistant than others (Xiong et al., 1999). It is possible that the population growing in the modified atmosphere bottles, and in turn subjected to the bile broth system, likely consisted of a cell mass containing an unknown complex mixture of diverse subpopulations (Bridson and Gould, 2000). Interestingly, as well as surviving the bile broth system, stationary phase cultures of *L. monocytogenes* FRRB 2472 grown in 100% N_2 also possessed the slowest death rate when exposed to the acid broth system.

The results obtained in this study are in accordance with the low bile tolerance of *L. monocytogenes* found by Taranto et al. (2000). However, it should be noted that the in vivo antibacterial activity in the presence of phospholipids might be much lower than that extrapolated from the broth system data reported here. The antimicrobial activity of bile salts is a function of the hydrophobicity of the molecules, with the hydrophobic bile salts being more cytotoxic than the hydrophilic bile salts (Sung et al., 1993). Most of these bile salts in human bile are complexed in micelles with phospholipids, thereby engaging the hydrophobic components of bile salts and attenuating the bacteriostatic property of these molecules (Sung et al., 1993).

3.2.3. Sequential transit through the acid and bile broth systems

Cells of *L. monocytogenes* FRRB 2538 were subjected to the acid broth system followed immediately by exposure to the bile broth system. This isolate was selected as it was the most resistant isolate to the acid broth system. Exponential phase cells were not used as no survival was observed for *L. monocytogenes* FRRB 2538 (nor any other of the isolates) after 110 min of exposure to the acid broth system. Only stationary phase cells of *L. monocytogenes* FRRB 2538 grown in air and 100% N_2 were tested as cells grown in 40% CO_2:60% N_2 and 100% CO_2 were not detectable at the first time of sampling after exposure to the bile broth system.

L. monocytogenes FRRB 2538 survived transit through the acid broth system but no viable cells were recovered from the bile broth system after 15 and 180

min exposure periods. Acid exposure can lead to damage to the lipopolysaccharide layer of the outer membrane and denaturation of cytoplasmic proteins (Brown and Booth, 1991). This may have resulted in greater permeability and susceptibility of the cells to the bile salts in the bile broth system.

While the results show that *L. monocytogenes* FRRB 2538 did not survive sequential transit through the acid and bile environments under the conditions studied, care must be taken in extrapolating these observations to in vivo conditions. First, the acid system represents the extreme of the in vivo condition. Under in vivo conditions, the pH in the stomach gradually decreases from approximately 6.0 to 2.0 over a 2-h period instead of maintaining a constant high-acid environment like that used in this study. Second, the protective effects of the food matrix were not taken into account. Gänzle et al. (1999) demonstrated the protective effect of meat against the lethal action of bile salts on lactobacilli *L. innocua* and *Escherichia coli*. Finally, it is rare for listeriosis to occur in a human host in the absence of a predisposing factor. Despite the limitations of the acid broth system in not providing a gradual acidification of the gastric fluid or the protective effects of a food matrix, it should be noted that all isolates in stationary phase under all atmospheres (apart from *L. monocytogenes* FRRB 2472 grown under 40% CO_2:60% N_2) were still detectable at 110 min. Given this, cells may have an increased ability to survive the intestine in vivo after transit through the stomach compared to the broth system results observed in this study.

4. Conclusions

Within food microbiology, an increasing emphasis is being placed on understanding what influence the ''state'' of a microorganism has on its ability to survive the myriad of environments it is exposed to within the food chain. In this study, we report the responses of four *L. monocytogenes* isolates to an acid broth system coupled with proteolytic enzymes and to a bile broth system in order to gain some insight into the effects prior exposure to growth under modified atmospheres at low temperature may have on the pathogen's survival. The results demonstrated that survival of *L. monocytogenes* to extreme acid and bile salt conditions was possible and was influenced by growth atmosphere, growth phase and the isolate studied. No particular atmosphere uniformly influenced the response of all the isolates in each phase of growth studied. It was noteworthy that prior exposure of *L. monocytogenes* to 40% CO_2:60% N_2 and 100% CO_2 led to rapid loss of viability upon exposure to the bile broth system irrespective of growth phase and the isolate studied.

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