Food Microbiology 21 (2004) 131–141

FOOD MICROBIOLOGY

www.elsevier.nl/locate/jnlabr/yfmic

Challenge studies with *Listeria monocytogenes* and proteolytic *Clostridium botulinum* in hard-boiled eggs packaged under modified atmospheres

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Received 25 February 2003; received in revised form 30 June 2003; accepted 30 June 2003

Abstract

Studies were conducted to determine the sensory shelf-life and microbiological safety of hard-boiled eggs packaged under various gas atmospheres, challenged with *Listeria monocytogenes* and proteolytic strains of *Clostridium botulinum* and stored at various temperatures. Growth of *L. monocytogenes* occurred in all inoculated egg samples stored at 4°C, 8°C, and 12°C with counts increasing from $\sim 10^2$ to $> 10^6$ cfu g^{-1} after 3–20 days, depending on the packaging atmosphere and storage temperature. Growth of *L. monocytogenes* to these levels either preceeded, or occurred simultaneously with, spoilage. These results showed that packaging eggs, even under elevated CO_2 concentrations (80%), had a limited inhibitory effect on the growth of this psychrotrophic pathogen. In challenge studies with proteolytic strains of *C. botulinum*, botulinum neurotoxin was not detected in any samples after 21 days at 12°C. However, neurotoxin was detected in all inoculated eggs that had been initially stored at 12°C, then transferred to 25°C for a further 7 days, regardless of the packaging atmosphere. Spoilage preceded toxigenesis in all cases and toxin was not detected in any uninoculated samples. This study has shown that modified atmosphere packaging cannot be regarded as an adequate barrier to control the growth of *L. monocytogenes* in hard-boiled eggs, even at refrigerated storage temperatures. However, refrigerated temperatures were essential to extend the sensory shelf-life and prevent the growth of proteolytic strains of *C. botulinum* in these packaged products.

Keywords: *Listeria monocytogenes*; *Clostridium botulinum*; Challenge studies; Modified atmosphere packaging; Cooked eggs

1. Introduction

Eggs are one of the most important and nutritious food products in North America. In Canada, 5.4 billion eggs were produced in 2002 i.e., a 75% increase in production since 1987 (Statistics Canada, 2002) while in the USA more than 70 billion eggs are produced annually. While the majority of eggs (~70%) are distributed to the food industry either in a frozen or dried form for use as basic ingredients in product formulations, the remaining 30% are marketed as fresh eggs. Recently, hard-boiled eggs, bulk packaged under modified atmospheres (MAP), have appeared on the market for use by catering services, fast food outlets, etc., in value-added egg products, such as salads and sandwiches, or individually packaged as a ready-to-eat breakfast item at convenience stores. These MAP eggs have a pH of ~7.7 and an a_w >0.99 i.e., conditions conducive to the growth of micro-organisms of public health concern in minimally processed MAP products, specifically *Listeria monocytogenes* and *Clostridium botulinum*. However, little is known about the safety of MAP hard-boiled eggs with respect to the growth of these two pathogens.

While *L. monocytogenes* should be readily destroyed during the boiling of eggs due to its low thermal resistance (*D*) value, hard-boiled eggs may be subjected

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0740-0020/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0740-0020(03)00062-5

to post-processing contamination by food handlers or work surfaces during preparation and packaging. Sionkowski and Shelef (1990) reported that when heat-treated eggs (121°C, 15 min) were contaminated with *L. monoctyogenes* post-processing, growth of this pathogen occurred in cooked whole eggs, yolk and albumen stored at 5°C and 20°C.

Although *C. botulinum* is not among the normal microflora on egg shells, Lubin et al. (1985) demonstrated that if spores of *C. botulinum* are present in the egg wash water, they can readily pass through the porous egg shell. Furthermore, if such contamination occurred, botulinum neurotoxin could be formed in hard-boiled eggs within 2–7 days depending on the storage temperature (Lubin et al., 1985). More recently, home-pickled eggs were implicated in an outbreak of botulism in the USA with *C. botulinum* type B neurotoxin being found in egg yolks (Rifkin et al., 2000).

These studies and report clearly show that eggs are a suitable substrate for the growth of both *L. monocytogenes* and *C. botulinum*. However, no studies to date have examined the growth of these pathogens in hard-boiled eggs packaged under modified atmospheres. Therefore, the objective of this study was to monitor the microbiological, and sensorial changes in hard-boiled eggs challenged with *L. monocytogenes* and *C. botulinum*, packaged under various gas atmospheres and stored at various temperatures.

2. Materials and methods

2.1. Raw materials and product preparation

Fresh eggs (eighty dozen) were obtained from the Poultry Unit, Macdonald Campus of McGill University. Only large Grade A eggs (~65 g) were used throughout this study. The first batch of hard-boiled eggs (30 dozen) were prepared by placing eggs in 5 l stainless-steel pots (one dozen/pot) each containing 2 l of cold tap water. The pots were then heated on a domestic stove at high setting for the following time/treatment combinations (i) 15 min come-up time until the water was boiling, (ii) 10 min boiling, and (iii) 10 min cooling in cold running tap water. After cooling, the shells were removed aseptically and the hard-boiled eggs were separated into egg white and egg yolk. Egg whites and egg yolks were placed in separate Ziploc® bags (1 kg bag^{-1}) and stored at 4°C until use.

A second batch of 30 dozen eggs was prepared in a similar manner. After the shells were removed, the hard-boiled eggs were mashed to a uniform consistency using a pre-sterilized domestic potato masher and then stored as described previously.

2.2. Thermal profile of eggs and lethality values

The thermal profile of eggs was measured during the heating and cooling process. A small hole (0.5 cm in diameter) was made at the apex of 24 large Grade A eggs using a sterilized needle. Thin pre-calibrated thermocouples (Type T, Omega, Stanford, CT, USA) were then inserted inside 12 egg whites (~1 cm inside the shell) while another set of thermocouples was inserted directly inside the yolk of another batch of 12 eggs (~2.5 cm below the surface of the shell). A silicon seal was attached to the hole of the apex of each egg to hold the thermocouples in place and to prevent the escape of any contents during boiling of the eggs. The eggs were then placed in the refrigerator overnight to ensure hardening of the silicon sealant. One dozen eggs, in duplicate, (six thermocouples in egg white, six thermocouples in egg yolk) were placed in a stainless steel pot (5 l capacity) and 2 l of cold tap water were added. The eggs were then subjected to same time and temperature processing conditions used previously to make hard-boiled eggs. The thermal profile of water during the heating/cooling process, was measured by placing two unattached pre-calibrated thermocouples directly into the water. The temperature profile of the water, egg white and egg yolk were recorded every 15 s using a Hewlett-Packard Data Recorder (Model 34970 A, Hewlett-Packard Company, Loveland, CO, USA). The F_{100} and the lethality value (F_0 value) were calculated by the method of Bigelow and Esty (1920) as modified by Townsend et al. (1938) using the following equation:

$$F_T = u^* \sum 10^{(t-T)/z},$$

where u is the time interval (min), T the reference temperature (°C), and $z(=10°C)$ the temperature sensitivity indicator.

2.3. Inoculum preparation

2.3.1. L. monocytogenes challenge study

Five strains of *L. monocytogenes* were obtained from Dr. J. Farber, Bureau of Microbial Hazards, Health Products and Food Branch (HPFB), Health Canada, Ottawa, Ontario, Canada. The strains included Scott A (human isolate), and HPFB strains 10 (chicken isolate), 11 (chicken isolate), 482 (egg wash isolate) and 2319 (egg wash isolate).

All cultures were stored at −80°C in tryptic soy broth (TSB) (Difco, Becton Dickinson, Sparks, MA, USA) supplemented with 20% glycerol to ensure viability. The inoculum was prepared by transferring isolated colonies of each strain from TSB into separate tubes containing tryptic soy broth supplemented with 0.6% yeast extract (TSBYE) (Difco) and incubating overnight at 30°C to give cultures of ~10^8 cfu ml^{-1}. These cultures were then

serially diluted with sterile peptone water (0.1% v/v) to $\sim 10^4$ cfu ml^{-1}. Equal aliquots (1×10^4 cfu ml^{-1}) of each suspension were then mixed in a sterile bottle to give a five-strain mixture with a final concentration of 5×10^4 cfu ml^{-1}. This was verified by plate counts on TSA.

2.3.2. *C. botulinum* challenge study

A composite inoculum of *C. botulinum* was prepared from four proteolytic type A strains (A6, 17A, 62A, and CK2A) and five proteolytic type B strains (MRB, IB1-B, 13983IIB, 368B, and 426B). Spore crops of each strain were prepared separately, enumerated as described by Hauschild and Hilsheimer (1977) and stored frozen at −80°C. Equal numbers of spores of each strain were mixed to form a single suspension of 2×10^5 spores ml^{-1}. The spore mixture was heat shocked at 75°C for 20 min prior to sample inoculation. Inoculum levels were verified prior to inoculation of samples by plating appropriate dilutions in duplicate on McClung Toabe Agar (Difco) containing 0.5% yeast extract (Difco) and egg yolk (McClung and Toabe, 1947). All plates were incubated anaerobically in an atmosphere of 10% H_2, 10% CO_2 and 80% N_2 at 35°C for 3 days.

2.4. Product inoculation and packaging

Fifty gram samples (in duplicate) of egg white, egg yolk, and mashed eggs were placed in 210×210 mm^2 Cryovac bags (O_2 transmission rate = 3–6 cm^3 m^{-2}, 24 h, atmosphere at 4.4°C, 0% relative humidity). In the *L. monocytogenes* challenge study, samples were inoculated with 0.5 ml of inoculum, to give a final inoculum level of $\sim 10^2$ cfu g^{-1}. In the *C. botulinum* challenge study, eggs were inoculated with 90 μl of spore suspension to give a final inoculum of 10^2 cfu g^{-1}. In both studies, control samples were inoculated with a similar volume of sterile 0.1% peptone water. Following inoculation, all samples were massaged manually to facilitate distribution of the inoculum.

Both control and inoculated egg white, egg yolk, and mashed egg samples were packaged under the following atmospheres, (i) air, (ii) CO_2:N_2 (40:60), and (iii) CO_2:N_2 (80:20). For air packaged samples, bags were sealed using an impulse heat sealer (Tew Electric Heating Equipment Co. LTD., Taipeh, Taiwan). Gas packaging was achieved using a chamber-type heat-seal packaging machine (model KM100-3 M, Multivac Inc., Kansas City, MO, USA). A Smith's proportional gas mixer (Model 299-028, Tescom Corp., Minneapolis, MN, USA) was used to give the desired proportions of O_2, CO_2, and N_2 in the package headspace.

In the *L. monocytogenes* challenge study, all packaged control and inoculated samples were stored at 4°C, 8°C, and 12°C and monitored for physical, chemical, microbiological, and sensorial changes at day 1, 3, 7, 14 and 21, or until shelf-life was terminated.

In the *C. botulinum* challenge study, samples were stored only at 12°C for 21 days and then transferred to 25°C for 7 days. Duplicate samples of each packaging treatment were analysed at day 7, 14, 21, and 28 for toxin production and sensory shelf-life.

2.5. Headspace gas analysis

At day 0, and after each storage interval, samples were analysed for changes in headspace gas composition by withdrawing samples through silicon seals attached to the outside of each package. Ten milliliters were withdrawn for O_2 measurements, and 5 ml for CO_2, using a 10 ml gas-tight pressure-Lok syringe (Model 790-002, Mocon, Minneapolis, MN, USA). Headspace gas was analysed with a previously calibrated Mocon O_2 and CO_2 analyser (Model HS-750 and PG-100, respectively).

2.6. Changes in pH

In the *L. monocytogenes* challenge study, the pH of inoculated samples was measured with a previously calibrated pH meter (Model 220, Corning Glass Works, Corning New York, USA) with a gel-filled polymer body combination electrode with Ag/AgCl reference (model 13-520-104, Fisher Scientific, Montreal, Quebec), by placing the electrode into a 1:3 dilution of sample:distilled water that had been stomached for 1 min. Analysis (in duplicate) was carried out for inoculated samples at day 0, 3, 7, 14, and 21, and only after day 0 and 21 for control samples. In the *C. botulinum* challenge study, pH was determined as previously reported (Daifas et al., 2000; Dufresne et al., 2000a, b).

2.7. Sensory analysis

All packaged egg samples were evaluated subjectively for texture, odor, and color at each sampling time by a six-member untrained panel using the method of Larmond (1979). Egg samples were scored on a five-point hedonic scale (1 = unacceptable, 5 = very acceptable). For each attribute, a score of 3 was considered to be the lower limit of acceptability, implying that shelf-life was terminated when this score was reached (Poole et al., 1990). Fresh boiled eggs were used as control samples for sensory evaluation.

2.8. Microbiological analysis

On each sampling day, packaged inoculated samples were aseptically opened and emptied into a stomacher bag on a tared balance. Sterile peptone water was added

to give an initial 1:3 dilution and the bag was stomached in a Colworth Stomacher (Model 400, A. J. Seward, London, UK) for 1 min. All subsequent decimal dilutions were made from this dilution.

L. monocytogenes was enumerated by placing 0.1 ml of the appropriate dilution on *Listeria* selective agar base (Oxoid, Code CM856) supplemented with *Listeria* selective supplement (Oxoid, code SR140E) according to manufacturer's specifications. Plates, in duplicate, were incubated aerobically at 35°C for 48 h as outlined by Lyver (1997).

Total aerobic and anaerobic counts were determined by spread-plating appropriate dilutions on Bacto Tryptic Soy Agar (Difco) as described by Lyver (1997). For aerobic counts, duplicate plates were incubated aerobically at 35°C for 48 h. For anaerobic counts, duplicate plates were incubated anaerobically at 35°C in a 3.5 l anaerobic jar (BBL Microbiology Systems, Becton Dickinson, MA, USA) containing an AnaeroGen sachet (Oxoid, England, UK).

Control samples were enumerated in a similar manner but only at day 0 and 21.

2.9. Botulinum neurotoxin assay

At each sampling test interval, egg samples were weighed into stomacher bags and twice the weight of 0.1% sterile peptone-water was added. The mixture was then stomached for 2 min (400 LabBlender A.J. Seward, London) and the homogenate was centrifuged at 17 500 g for 20 min at 4°C. An aliquot of the sample supernatant was filter sterilized through a 0.45 μm cut-off filter (Acrodisc, Gelman Sciences, Ann Arbor MI, USA) and 0.55 ml were injected intraperitoneally into each of two mice (20–25 g) and observed for up to 72 h for symptoms of botulism. Mice showing severe distress (pinched waist, labored breathing, limb paralysis) were euthanized with CO_2 (Daifas et al., 2000; Dufresne et al., 2000a, b). Two additional mice were injected if only one mouse showed typical signs of botulism. Samples were considered positive for botulinum neurotoxin if 2/2 or $\geqslant$ 2/4 mice showed severe distress. Control samples were prepared and injected in a similar manner. Neutralization of toxin was performed on positive egg extract samples using antisera (Aventis Pasteur, Toronto, ON, Canada) to botulinum neurotoxins as described by Daifas et al. (2000).

2.10. Statistical analysis

Two factorial designs were used in this study. In the *L. monocytogenes* challenge study, a $3 \times 2 \times 3 \times 3 \times 5$ factorial design was used while in the *C. botulinum* study, a $3 \times 2 \times 3 \times 2 \times 4$ split factorial design was employed. In both designs three types of egg samples (egg white, egg yolk, and mashed eggs, in duplicate) were subjected to three packaging treatments. In the *L. monocytogenes* study, packaged eggs were stored at three temperatures and analyzed on five test intervals while in the *C. botulinum* study, packaged eggs were stored at two temperatures and analysed on four test intervals. Analysis of variance (ANOVA) and comparison of means was done using Statistical Analysis System (SAS, 1998). A P-value of <0.05 was considered to be significantly different.

2.11. Predicted time models

The predicted time, in days, for growth of *L. monocytogenes* to reach $\sim 10^6$ cfu ml^{-1} was generated using the US Department of Agriculture (USDA) Pathogen Modeling program (version 5.1) (Buchanan and Klawitter, 1991; Buchanan et al., 1989). The predicted time (days) to toxicity was generated using the above model for proteolytic strains of C. *botulinum* (Whiting and Call, 1993; Whiting and Oriente, 1997). Actual values of a_w, pH, and inoculum levels used in this study were incorporated into the predictive models.

3. Results and discussion

3.1. Thermal profile of eggs and lethality values

The thermal profile of fresh eggs during boiling is shown in Fig. 1. Although water boiled after 15 min, its maximum recorded temperature was ~98°C due either to the latent heat of vaporization or variation in atmospheric pressure. The internal temperature of both egg white and egg yolk after this time was ~80°C (Fig. 1). However, when eggs were boiled for a further 10 min, their internal temperature reached the same temperature as water i.e., ~98°C (Fig. 1). During the cooling down period, the internal temperature of both egg white and egg yolk decreased more slowly than water. However, after 35 min, the temperatures of the water, egg white, and egg yolk were similar (~20°C, Fig. 1).

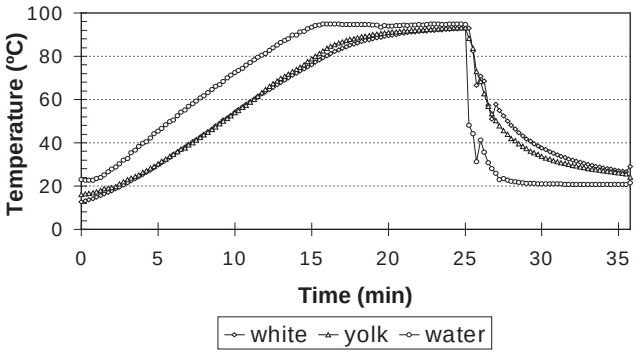

Fig. 1. Thermal profile of hard-boiled eggs.

The calculated F_{100} and F_0 values for both egg yolk and egg white based on these thermal profiles are shown in Table 1. Both values were higher in egg yolk compared to egg white and this difference can be attributed to the higher solids content of egg yolk compared to egg white. Based on an estimated D_{100} value of ~0.0002 min for *L. monocytogenes*, such a heat process treatment would readily destroy any *L. monocytogenes* present in fresh eggs (ACMSF, 1992). However, spores of proteolytic *C. botulinum*, which have a reported D_{100} value of 25 min, would easily survive the boiling process if initially present in raw eggs (ACMSF, 1992). To ensure destruction of spores of proteolytic *C. botulinum*, a heat process equivalent to an F_0 of 3 min is required (ACMSF, 1992). Since the maximum internal temperature was ~98°C (Fig. 1), the total heat process (area under the curve of Fig. 1) was equivalent to an F_0 of ~0.01 min at 121°C (Table 1). This means that the heat treatment was <1% of the target F_0 of 3 min and almost complete survival of any proteolytic spores of *C. botulinum,* if present in raw eggs, would be expected. Based on this data, hard-boiled eggs would be classified as minimally processed since they do not receive a heat treatment equivalent to a 12-D botulinum kill.

Table 1
F_{100} and F_0 values for hard-boiled eggs

	F_{100} (min)		$F_{121,1}$ (min)	
	White	Yolk	White	Yolk
Mean value[a]	1.201	1.620	0.009	0.013
Standard deviation	0.598	0.699	0.005	0.005

[a] Mean values from the thermal profile of 12 egg whites and 12 egg yolks.

3.2. Challenge study with L. monocytogenes

Summaries of changes in headspace gas composition, sensory and microbiological shelf-life of inoculated egg white, egg yolk, and mashed eggs packaged under various gas atmospheres at all storage temperatures are shown in Tables 2–4.

Only the initial levels (day 1) and levels of headspace gas at the termination of shelf-life, based on time in days for *L. monocytogenes* to reach a count of $>10^6$ cfu g^{-1} are shown. At the termination of shelf-life, higher levels of headspace O_2 (18% vs. 2%) and lower levels of CO_2 (2% vs. 31%) were consistently observed in all air-packaged eggs stored at 4°C and 8°C than at 12°C, respectively (Tables 2 and 4). The differences in the headspace gas composition of eggs stored at 12°C can be attributed solely to the more rapid growth of *L. monocytogenes* in air packaged eggs stored at moderate temperature abuse conditions since all eggs were sterile at the time of packaging (results not shown).

The headspace gas composition of all gas packaged inoculated eggs stored at 4°C and 12°C remained fairly constant throughout storage (Tables 2–4). This can be attributed to a lack of any competing microflora in the hard-boiled eggs and a lower growth rate of *L. monocytogenes* packaged under elevated levels of CO_2. Headspace CO_2 decreased initially in most samples throughout storage due probably to the dissolution of CO_2 in the aqueous phase of the product (Tables 2–4). This trend has been observed in previous gas packaging studies of foods resulting in slight changes in product pH and, in some instances, package collapse (Dufresne et al., 2000a, b; Lyver, 1997). Headspace gas composition remained constant in all uninoculated packaged control samples throughout storage (results not shown),

Table 2
Summary of headspace gas, sensory and microbial shelf-life of egg white, egg yolk and mashed whole eggs inoculated with a five-strain mixture of *L. monocytogenes*, packaged under various gas atmospheres and stored at 4°C

Egg samples	Storage temp. (°C)	Packaging treatment	Headspace gas (%v/v)				Sensory shelf-life (days)[b]			Microbial shelf-life[a] (days)
			Initial		Final		Texture	Color	Odor	
			O_2	CO_2	O_2	CO_2				
White	4	Air	20	<1	19.5	2.5	~14	~14	~14	~9
		40%CO_2/60%N_2	<1	40	<1	34.5	~21	~21	~14	~9
		80%CO_2/20%N_2	<1	80	<1	67.0	~21	~21	~21	~16
Yolk	4	Air	20	<1	19.5	1.5	~14	~14	~14	~7
		40%CO_2/60%N_2	<1	40	<1	34.0	~21	~21	~14	~8
		80%CO_2/20%N_2	<1	80	3.1	70.2	~21	~21	~21	~20
Mashed egg	4	Air	20	<1	19.0	1.5	~14	~14	~14	~7
		40%CO_2/60%N_2	<1	40	<1	31.8	~21	~21	~14	~9
		80%CO_2/20%N_2	<1	80	<1	75.2	~21	~21	~21	~15

[a] Based on time (days) to reach a *L. monocytogenes* count of ~10^6 cfu g^{-1}.
[b] Time (days) to reach a score of 3 or less on a hedonic scale of 1–5 (where 1 = unacceptable and 5 = very acceptable).

Table 3
Summary of headspace gas, sensory and microbial shelf-life of egg white, egg yolk and mashed whole eggs inoculated with a five-strain mixture of *L. monocytogenes*, packaged under various gas atmospheres and stored at 8°C

Egg samples	Storage temp. (°C)	Packaging treatment	Headspace gas (%v/v)				Sensory shelf-life (days)[b]			Microbial shelf-life[a] (days)
			Initial		Final		Texture	Color	Odor	
			O_2	CO_2	O_2	CO_2				
White	8	Air	20	<1	18.5	2.0	~14	~14	~14	~6
		40%CO_2/60%N_2	<1	40	<1	35.5	~21	~21	~14	~6
		80%CO_2/20%N_2	<1	80	<1	76.0	~21	~21	~21	~8
Yolk	8	Air	20	<1	17.5	3.0	~14	~14	~14	~6
		40%CO_2/60%N_2	<1	40	<1	34.5	~21	~21	~14	~6
		80%CO_2/20%N_2	<1	80	3.1	65.7	~21	~21	~21	~7
Mashed egg	8	Air	20	<1	17.5	3.2	~14	~14	~14	~5
		40%CO_2/60%N_2	<1	40	<1	35.8	~21	~21	~14	~6
		80%CO_2/20%N_2	<1	80	<1	76.2	~21	~21	~21	~6

[a] Based on time (days) to reach a *L. monocytogenes* count of $\sim 10^6$ cfu g^{-1}.
[b] Time (days) to reach a score of 3 or less on a hedonic scale of 1–5 (where 1 = unacceptable and 5 = very acceptable).

Table 4
Summary of headspace gas, sensory and microbial shelf-life of egg white, egg yolk and mashed whole eggs inoculated with a five-strain mixture of *L. monocytogenes*, packaged under various gas atmospheres and stored at 12°C

Egg samples	Storage temp. (°C)	Packaging treatment	Headspace gas (%v/v)				Sensory shelf-life (days)[b]			Microbial shelf-life[a] (days)
			Initial		Final[a]		Texture	Color	Odor	
			O_2	CO_2	O_2	CO_2				
White	12	Air	20	<1	1.8	31.0	~7	~7	~3	~3
		40%CO_2/60%N_2	<1	40	<1	36.0	~7	~7	~3	~3
		80%CO_2/20%N_2	<1	80	<1	76.0	~7	~7	~3	~3
Yolk	12	Air	20	<1	<1	37.0	~7	~7	~3	~3
		40%CO_2/60%N_2	<1	40	<1	34.5	~7	~7	~3	~3
		80%CO_2/20%N_2	<1	80	3.1	65.7	~7	~7	~7	~3
Mashed egg	12	Air	20	<1	<1	31.2	~7	~7	~3	~3
		40%CO_2/60%N_2	<1	40	<1	35.8	~7	~7	~3	~3
		80%CO_2/20%N_2	<1	80	<1	76.2	~7	~7	~7	~3

[a] Based on time (days) to reach a *L. monocytogenes* count of $\sim 10^6$ cfu g^{-1}.
[b] Time (days) to reach a score of 3 or less on a hedonic scale of 1–5 (where 1 = unacceptable and 5 = very acceptable).

confirming the sterile nature of the eggs and lack of competing microflora.

Sensory scores (texture, color and odor) decreased as the storage temperature and time increased (Tables 2 and 4). Furthermore, storage temperature had a significantly greater effect ($P<0.05$) on sensory scores and shelf-life than the packaging atmosphere. Odor scores were consistently the best indicators of consumer acceptability (Tables 2–4) and the shelf-life of eggs was terminated when an odor score of <3 was reached. These results are in agreement with Post et al. (1985) and Dufresne et al. (2000a, b) who reported that odor scores were the most reliable indicators of sensory rejection of fresh fish. Based on this criterion, the shelf-life of all inoculated packaged eggs could be extended to approximately 20 days at 4°C depending on the package atmosphere (Table 2) while most eggs would be rejected by consumers after 3–7 days at 12°C. Similar trends were observed for control packaged eggs although odor scores were marginally acceptable after 21 days.

The pH of inoculated egg whites decreased from ~8.2 to ~7.1 in most samples, while the pH of egg yolk (~6.6) and mashed eggs (~7.2) remained fairly constant throughout storage. The pH of all control packaged eggs remained fairly constant throughout storage and can be attributed to the lack of microbial metabolism due to the inactivation of indigenous

microorganisms during the heat processing of eggs (results not shown).

Since the growth trend of *L. monocytogenes* was similar in all packaged eggs, only the results for mashed eggs stored at 4°C, 8°C and 12°C are shown in Fig. 2. In air packaged mashed eggs, *L. monocytogenes* increased from approximately 10^2 to 10^6 cfu g^{-1} after 7 days (Fig. 2). However, growth of *L. monocytogenes* to this level was delayed for a further 2–8 days at 4°C in mashed eggs packaged in 40% and 80% CO_2 (v/v), respectively, balance N_2 (Fig. 2). At 8°C and 12°C, *L. monocytogenes* increased from its initial inoculum level to 10^6 cfu g^{-1} after approximately 6 and 3 days, regardless of the packaging atmosphere (Fig. 2, Tables 2–4).

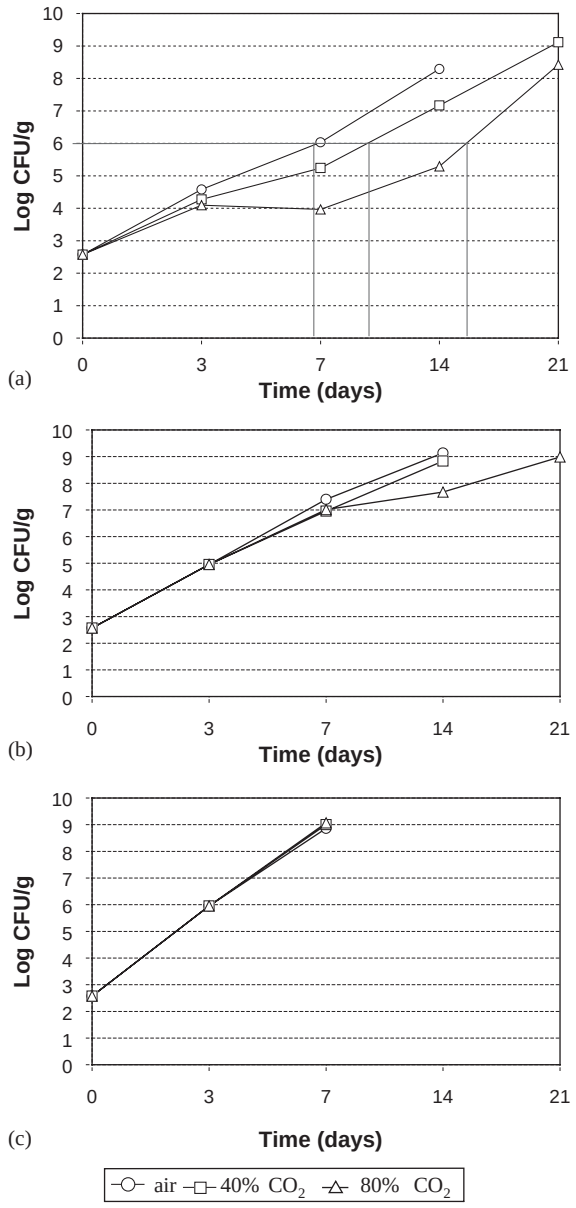

Fig. 2. Growth of *L. monocytogenes* in inoculated mashed egg stored under various modified atmospheres and at (A) 4°C, (B) 8°C, and (C) 12°C.

Growth *L. monocytogenes* in all egg white and yolk samples also increased from the initial inoculum level ($\sim 10^2$) to $>10^6$ cfu g^{-1} after approximately 3–20 days, depending on the storage temperature (Tables 2–4). Growth of *L. monocytogenes*—in both egg white and yolk was again more rapid at 8°C and 12°C than at 4°C and counts were $>10^6$ cfu g^{-1} in these egg samples by days 8 and 3 depending on the packaging atmosphere (Tables 3 and 4). These results confirm the importance of strict temperature control to delay the growth of this pathogen in conjunction with gas packaging. With the exception of products stored at 4°C, storage temperature had a more significant effect ($P > 0.005$) on growth of *L. monocytogenes* than the gas atmosphere in all packaged samples.

The observed times (3, 6 and 9 days) for air-packaged eggs to reach a count of $\sim 10^6$ cfu g^{-1} at 12°C, 8°C and 4°C respectively, (Tables 2–4) compared favourably with the predicted times of 2.5, 6 and 9 days for *L. monocytogenes* to reach these levels in broth stored under similar conditions (US Department of Agriculture Pathogen Modeling Program, Version 5.1, Buchanan et al., 1989, 1991). However, no modelling data was available on the predicted growth of this pathogen under various gas atmospheres. Although model broth studies can be used, as a guide, to predict the growth/no growth of pathogens under selected storage conditions, they should always be validated in challenge tests with the selected pathogen of concern in food under actual packaging and storage conditions.

No *Listeria* spp. were found in any of the control packaged egg samples. Furthermore, no aerobic or anaerobic bacteria were detected in any of the inoculated or control egg samples (results not shown), confirming the sterility of the eggs prior to packaging and at the end of shelf-life.

This study has shown that if MAP hard-boiled eggs products are contaminated with *L. monocytogenes*, they are an ideal substrate for the growth of this pathogen. Furthermore, MAP had little effect in controlling the growth of *L. monocytogenes* in egg white, egg yolk and mashed eggs particularly at mild (8°C) and moderate abuse (12°C) conditions of storage. More importantly, growth of *L. monocytogenes* preceded spoilage in nearly all instances, which is a cause for concern, since products may appear safe to the consumer from a sensory viewpoint (Tables 2–4). Marshall et al. (1991, 1992) reported that modified atmospheres, consisting of 80% CO_2, were effective in increasing the lag phase or reducing the growth rate of *L. monocytogenes* in chicken nuggets. However, several authors (Farber and Daley, 1994; Sheridan et al., 1995) reported that atmospheres containing 70–100% CO_2 completely inhibited the growth of *L. monocytogenes* in turkey roll slices and in lamb. While a complete inhibition of *L. monocytogenes* by 80% CO_2 was not

observed in our study, a delay in growth was observed in CO_2 enriched atmospheres, particularly at 4°C. Statistical analysis of our data also confirmed that the gas atmosphere did not play a significant role in delaying the growth of *L. monocytogenes* at 8°C and 12°C. However, at 4°C, the gas atmosphere had a highly significant effect ($P > 0.005$) on the growth of *L. monocytogenes* in egg white, egg yolk or mashed eggs. The enhanced antimicrobial effect of CO_2 at 4°C has been observed in previous studies and can be attributed to the greater solubility of CO_2 in the aqueous phase of products at lower storage temperatures (Dufresne et al., 2000a, b; Lyver, 1997).

Based on this study, a sensory and microbiological shelf-life of 14 days is possible if hard-boiled eggs are packaged in elevated levels of CO_2 (80%) and stored at 4°C (Fig. 2). However, even under these packaging and storage conditions, growth of *L. monocytogenes* would still increase to $>10^5$ cfu g^{-1} after 14 days in mashed eggs (Fig. 2). While these levels appear high, Nørrung (2000) observed that the average person consumes doses of 5×10^5 of *L. monocytogenes* per serving approximately four times per year, but only a small fraction of individuals actually contract the illness. A recent FDA/USDA risk assessment of contacting listeriosis from different types of ready-to-eat foods showed "that although US consumers are exposed to low levels of *L. monocytogenes* on a regular basis, the likelihood of acquiring listeriosis is very small" (Anonymous, 2001). Furthermore, ready-to-eat foods with known moderate to high contamination rates were not necessarily the highest risk products if consumed less frequently and in smaller amounts by fewer people (Anonymous, 2001). However, MAP hard-boiled eggs could pose a public health risk to susceptible consumers (pregnant women, immunocompromised individuals) if contaminated with this pathogen post-processing, regardless of the packaging atmosphere or storage temperature.

3.3. Challenge study with C. botulinum

A summary of the changes in headspace gas composition, sensory scores (texture, odor, and color), and pH of egg white, egg yolk, and mashed whole eggs inoculated with proteolytic *C. botulinum* ($\sim 10^2$ spores g^{-1}) packaged under various gas atmospheres and stored at 12°C for 21 days are shown in Table 5. Headspace gas changes were again similar to those observed in previous challenge studies with *L. monocytogenes*. One noticeable difference, however, was a slightly higher level of headspace O_2 throughout storage in several gas packaged samples indicating leakage problems (Table 5). This may have occurred at the sealing area due to the presence of moisture or fat in this area or traces of egg-shell that could have caused small pin-holes in the bags. However, several studies have shown that C. *botulinum* can grow under elevated levels of O_2 in the package headspace (Whiting and Naftulin, 1992; Clavero et al., 2000). Dufresne et al. (2000a) showed that *C. botulinum* type E can grow in trout fillets initially packaged in up to 100% O_2.

Odor scores were again the best indicators of shelf-life acceptability. Odor scores were approximately 4 and 3 after days 7 and 14, respectively, at 12°C. However, all inoculated eggs had unacceptable odor scores (~ 2) after 21 days at 12°C and would be rejected by consumers on this basis (Table 5). All texture and color scores, with the exception of egg white, were still acceptable for egg yolk and mashed eggs after 21 days at 12°C. All control eggs were also sensorially acceptable at the termination of shelf-life.

Table 5
Summary of headspace gas, sensory analysis scores, and pH of egg white, egg yolk, and mashed whole eggs inoculated with *C. botulinum* spores (10^2 g^{-1}), packaged in different gas atmospheres and stored at 12°C

Egg samples	Packaging treatment	Headspace gas (% v/v)				Sensory analysis scores[a]						pH	
		Initial		Day 21		Texture		Color		Odor		Day 0	Day 21
		O_2	CO_2	O_2	CO_2	Day 0	Day 21	Day 0	Day 21	Day 0	Day 21		
White	Air	20	<1	19	<1	5	2	5	3	5	2	8.2	7.1
	40% CO_2 / 60% N_2	<1	40	5	35	5	3	5	3	5	2	8.2	7.0
	80% CO_2 / 20% N_2	<1	80	7	76	5	3	5	3	5	2	8.2	7.1
Yolk	Air	20	<1	8	<1	5	3	5	4	5	2	6.5	6.0
	40% CO_2 / 60% N_2	<1	40	2	34	5	4	5	4	5	2	6.6	6.3
	80% CO_2 / 20% N_2	<1	80	2	65	5	4	5	4	5	2	6.6	6.8
Mashed	Air	20	<1	1.6	3	5	4	5	4	5	2	7.7	7.0
	40% CO_2 / 60% N_2	<1	40	2	35	5	4	5	4	5	2	7.6	7.3
	80% CO_2 / 20% N_2	<1	80	1.9	75	5	4	5	4	5	2	7.7	6.7

[a] Time (days) to reach a score of 3 or less on the hedonic scale of 1 to 5 (where 1 = unacceptable and 5 = very acceptable).

While the pH of egg white decreased by approximately 1 unit from pH 8.2–7.1, the pH of egg yolk remained relatively constant (approximately pH 6.0–6.8), while the pH of mashed eggs decreased from pH 7.6 to 7.3–6.7 depending on the packaging atmosphere (Table 5).

Botulinum neurotoxin was not detected in any uninoculated or inoculated samples after 21 days at 12°C (results not shown). Predictions from model broth studies with proteolytic strains of *C. botulinum* (10^2 spores ml^{-1}) indicated toxin production after 15 days at 15°C (US Department of Agriculture Pathogen Modeling Program, Version 5.1 (Whiting and Call, 1993; Whiting and Oriente, 1997). The lowest growth temperature in this model is 15°C, making direct comparison with our study impossible. However, our results show that when eggs were inoculated with proteolytic strains of *C. botulinum* they did not become toxic during 21 days at moderate temperature abuse (12°C) storage conditions. However, if any eggs had become toxic by this time, spoilage, as shown by odor scores, would have preceded toxigenesis. Overt spoilage, however, cannot always be regarded as a reliable barrier of food safety. This was demonstrated in the case of a botulism outbreak in Italy involving tiramisu that was described as "malodorous" at the time of consumption (Aureli et al., 1996).

To demonstrate the effect of severe temperature abuse on the safety of eggs challenged with proteolytic *C. botulinum*, eggs were transferred from 12°C after day 21 to 25°C for 7 days. Such temperature abuse conditions are not unrealistic in the retail or domestic environment. In a recent survey of the domestic storage temperatures of fresh whole shell eggs, Grijspeerdt et al. (1999) observed that some eggs were stored as high as 28°C for 2 weeks.

Major differences were observed in both the headspace gas composition and sensory scores of eggs stored at 25°C compared to eggs stored at 12°C. At the end of 7 days at 25°C, headspace O_2 decreased to undetectable levels in all packaged eggs with a concomitant increase in CO_2 resulting in most packages having a slightly blown appearance (results not shown). Furthermore, all eggs were sensorially unacceptable having scores of <1 for texture, odor and color. All egg whites were liquefied while egg yolks and mashed eggs were black in color and partially liquefied. Statistical analysis of the sensory scores showed that temperature (25°C vs. 12°C) had a more significant effect ($P<0.05$) on shelf-life of eggs than the packaging atmosphere.

While substantial changes in headspace gas composition and sensory evaluation scores were observed in inoculated egg samples, the changes for uninoculated eggs were less dramatic. Indeed, texture and color scores remained acceptable in most samples after 7 days even at severe temperature abuse storage conditions indicating that the changes observed in inoculated eggs were due to the growth of proteolytic *C. botulinum*. All uninoculated eggs, however, were rejected on the basis of odor scores (results not shown).

A summary of the time-to-toxin detection in inoculated packaged eggs stored at severe temperature abuse conditions is shown in Table 6. Botulinum neurotoxin was detected in all inoculated samples, regardless of the packaging atmosphere, at the end of storage at 25°C for 7 days. Toxin was again not detected in any of the uninoculated control samples. It is highly probable that botulinum neurotoxin was formed prior to day 7 since model broth studies with proteolytic strains of *C. botulinum* predicted neurotoxin production after ~2 days at 25°C (US Department of Agriculture Pathogen Modeling Program Version 5.1, Whiting and Call, 1993; Whiting and Oriente, 1997). Lubin et al. (1985) reported botulinum neurotoxin in hard cooked eggs within 2–7 days at 30°C. However, contrary to our observations, these authors found that although eggs had detectable levels of botulinum neurotoxin, many eggs were not overtly spoiled. In our study, all eggs were sensorially unacceptable at the time of toxigenesis.

While the use of MAP for shelf-life extension of food products is expanding worldwide, there are still safety concerns about this technology, particularly with respect to *C. botulinum*. Despite these concerns, the safety

Table 6
Summary of botulinum neurotoxin detection in egg white, egg yolk, and mashed whole eggs packaged in different gas atmospheres and stored at 25°C for 7 days

Egg samples	Packaging treatment	Inoculated[a]	Botulinum neurotoxin[b] detected at day 28
White	Air	+	4/4
	Air	–	0/4
	40% CO_2 / 60% N_2	+	4/4
	40% CO_2 / 60% N_2	–	0/4
	80% CO_2 / 20% N_2	+	4/4
	80% CO_2 / 20% N_2	–	0/4
Yolk	Air	+	4/4
	Air	–	0/4
	40% CO_2 / 60% N_2	+	4/4
	40% CO_2 / 60% N_2	–	0/4
	80% CO_2 / 20% N_2	+	4/4
	80% CO_2 / 20% N_2	–	0/4
Mashed	Air	+	4/4
	Air	–	0/4
	40% CO_2 / 60% N_2	+	4/4
	40% CO_2 / 60% N_2	–	0/4
	80% CO_2 / 20% N_2	+	4/4
	80% CO_2 / 20% N_2	–	0/4

[a] In duplicate.
[b] Number of positive samples/number of samples tested.

record of MAP foods with respect to this pathogen, has been excellent. Between 1960 and 1991, only seven outbreaks of botulism from MAP foods (five confirmed and two possible/unconfirmed) have been reported worldwide. While two of these outbreaks were attributed to *C. botulinum* type A in vegetable products, the majority of outbreaks were caused by *C. botulinum* type E in MAP minimally processed ready-to-eat smoked fish products (ACMSF, 1992). This present challenge study confirms that MAP hard-boiled eggs have the potential to support the growth of *C. botulinum* and may pose a safety risk but only if contaminated with this pathogen and subjected to severe temperature abuse at any stage during the processing and distribution chain. However, since no endogenous spores were found in any control samples, it can be assumed that the probability of contamination of raw shell eggs with proteolytic *C. botulinum* to be extremely low. According to Lioutas (1988) "an extremely low probability of contamination does not negate the hazard of botulism, it only reduces the risk". Nevertheless, MAP hard-boiled eggs appear, based on epidemiological statistics, to be extremely low botulism risk products. However, a good safety history does not always guarantee that this new generation of MAP products with extended shelf life will never be involved in botulism outbreaks in the future.

To date, no outbreaks of listeriosis or botulism have been reported from MAP minimally processed hard-boiled eggs. Elliot and Kveneberk (2000) reported that any contamination of ready-to-eat foods by *L. monocytogenes*, which includes a thermal kill step in their process for this pathogen, occurs post-processing from environmental sources. According to Elliot and Kveneberk (2000) control of *L. monocytogenes* in ready-to-eat foods, such as hard-boiled eggs, cannot be achieved through a Hazard Analysis Critical Control Point approach but only through pre-requisite programs, such as Good Manufacturing Practices (GMPs) and Sanitation Standard Operation Procedures (SSOPs). Both these programs are critical to minimize cross-contamination of products by *L. monocytogenes* at the source, during processing and during handling and packaging and hence assure the safety of MAP hard-boiled eggs (Elliot and Kveneberk, 2000). Maintenance of good hygenic conditions and compliance with GMPs are also essential to ensure that the levels of *L. monocytogenes* are within the regulatory standards for ready-to-eat foods. While the US has a zero tolerance policy for *L. monocytogenes* in a 25 g sample of a ready-to-eat food, Canada has a three tier policy based on the shelf-life of the food and whether the product has been linked to any cases of listeriosis. MAP hard-boiled eggs would be classified as a category 2 product i.e., products that have a shelf-life of >10 days and are capable of supporting the growth of *L. monocytogenes*. Category 2 products have a lower priority for inspection and if *L. monocytogenes* is found in these products, a class II recall, but nor necessarily a public alert, is required (Farber, 2000).

While refrigeration is not an adequate barrier to completely control the growth of *L. monocytogenes*, it is an essential barrier to prevent the growth of proteolytic strains of *C. botulinum*. Monitoring and control of this important critical control point in a HACCP program at all stages of the processing and distribution chain is essential to prevent the growth of proteolytic strains of *C. botulinum* should they be present in the final product. Other strategies that could be used to ensure the safety of MAP hard-boiled eggs would be the use of a time–temperature monitor (TTM) or a Fresh Tag™ in packages to warn consumers of temperature abuse or impending spoilage of products.

In conclusion, food safety is a complex issue and involves many inter-related variables including inoculum level of the hazard of concern, a_w, pH and preservatives, microbial ecology of the food matrix, redox potential, gaseous atmosphere surrounding the product and storage temperature. MAP hard-boiled eggs would appear, based on epidemiological statistics to be extremely low-risk products with respect to listeriosis and botulism. However, constant vigilance is required by regulatory authorities and the food industry to ensure, as far as reasonably possible, that ready-to-eat, MAP hard-boiled eggs are safe and fit for consumption.

Acknowledgements

The authors would like to acknowledge the Natural Sciences and Engineering Research Council for funds to support this study.

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