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Behavior of Psychrotropic Pathogens *Listeria monocytogenes, Yersinia enterocolitica,* and *Aeromonas hydrophila* in Commercially Pasteurized Eggs Held at 2, 6.7 and 12.8°C.

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ABSTRACT

Four commercially pasteurized liquid egg products were individually inoculated with *Listeria monocytogenes, Yersinia enterocolitica,* and *Aeromonas hydrophila.* They were unsalted whole egg blend, unsalted egg white, 5% NaCl whole egg blend, and 10% NaCl egg yolk. The inoculated samples and uninoculated controls were held at 2, 6.7, and 12.8°C (temperature abuse) for 14 d. Psychrotropic pathogen growth or survival risks in the unsalted and NaCl supplemented eggs were *Y. enterocolitica* > *A. hydrophila* > *L. monocytogenes,* and *L. monocytogenes* > *Y. enterocolitica* > *A. hydrophila,* respectively. *Y. enterocolitica* produced delayed ($\geq$4 d) growth responses in unsalted eggs held at $\leq$6.7°C but was inhibited by $\geq$5% NaCl at all three holding temperatures. *L. monocytogenes* growth was prevented at $\leq$6.7°C in the unsalted and NaCl supplemented eggs. The organism rapidly increased in the temperature abused 5% NaCl whole egg blend. *L. monocytogenes* and *A. hydrophila* were inactivated in the unsalted egg white and NaCl supplemented eggs, respectively. Psychrotropic pathogen behavior was unaffected by the competitive growth of indigenous spoilage microflora including pseudomonads, *Serratia* spp., and NaCl tolerant micrococci. Properly refrigerated and hygienically handled pasteurized liquid eggs are microbiologically safe against a broad range of psychrotropic pathogen strains.

Commercially pasteurized liquid eggs are used in a diverse array of processed foods including bakery goods, dried pasta, shelf-stable mayonnaise/salad dressings, frozen dairy desserts (ice cream), and various chilled, ready-to-eat products (custards, spoonable dressings, pasta, etc.). Many types of pasteurized liquid eggs are available ranging from customized whole egg blends to separated whites and yolks. Frequently, NaCl or sugar is added before pasteurization to extend the refrigerated keeping quality and modify sensory and emulsification properties. Egg ingredient suppliers must adhere to stringent good manufacturing practices and pasteurization regulations. These were federally mandated (*25*) in the early 1970's to ensure that consistent, wholesome quality was established and maintained by both the processor and end-user, and the eggs were free from *Salmonella* spp. contamination.

In recent years, three potential psychrotropic pathogens have been identified: *Listeria monocytogenes, Yersinia enterocolitica,* and *Aeromonas hydrophila. L. monocytogenes* is a particularly serious health concern for the food industry and regulatory agencies (*8,20*). Recalls have occurred in a wide variety of foods - cheese, ice cream, frozen seafoods, hot dogs, prepared salads and sandwiches, etc. All three psychrotropic pathogens are widely distributed in the general environment, and raw food supply including poultry products (*1,13-15,26*). Specifically, Leasor and Foegeding (*14*) detected sporadic, low level *L. monocytogenes* contamination in a raw liquid egg blend. Foegeding and Stanley (*6*) and Foegeding and Leasor (*7*) demonstrated that *L. monocytogenes* is significantly more heat resistant in whole liquid egg blend than *Salmonella* spp. *A. hydrophila* and *Y. enterocolitica* thermal sensitivity is similar to *Salmonella* spp. (*16*). Psychrotropic pathogen growth rates have been studied in several refrigerated foods including milk, shell, raw and experimentally pasteurized eggs, red meats, and seafoods (*2,3,5,7,9,10,19,21-23*). Liquid dairy products are particularly susceptible to psychrotropic pathogen growth under refrigeration.

The purpose of this study was to determine psychrotropic pathogen behavior in a cross-section of commercially pasteurized liquid egg products. Comparative survival or growth patterns were evaluated for *L. monocytogenes, Y. enterocolitica* and *A. hydrophila* in the presence of competitive microflora, NaCl, and under refrigeration and temperature abuse (12.8°C) storage conditions.

MATERIALS AND METHODS

Microbial strains

Twelve ATCC strains were used throughout the study: five *L. monocytogenes* (LM) - ATCC #19111 (poultry - serotype 1), ATCC 19115 (human - serotype 4b), ATCC #15313 (rabbit - type strain), ATCC 43256 and 42357 (Mexican cheese outbreak isolates); three *Y. enterocolitica* (YE) - ATCC #9610 (human facial infection-type strain), ATCC #23715 (blood), ATCC #27739 (stream water - serotype 8); and four *A. hydrophila* (AH) - ATCC #7966 (spoiled milk-type strain), ATCC #19570 (pike intestine), ATCC #23211 (water supply), and ATCC #23213 (river water).

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Microbial strain preparation

LM strains were individually streaked onto the surface of liver veal agar (Difco, Detroit, MI) plates and incubated at 35°C for 72 h. After incubation, each plate was surface washed with 3 to 5 ml of sterile physiological saline to remove growth and combined in a sterile container. The LM-pool inoculum was diluted to approximately 6 $\log_{10}$ CFU/ml. YE and AH strains were individually streaked onto the surface of tryptic soy agar (Difco) and incubated 30°C for 72 h. Separate YE and AH pool inocula were prepared and adjusted in the same manner as described for LM.

Product inoculation, storage, and sampling procedures

Four commercially pasteurized liquid egg varieties were obtained from a local supplier. They were: unsalted whole egg blend (LEB), unsalted egg white (LEW), 5% NaCl whole egg blend (SLEB), and 10% NaCl egg yolk (SLEY). The eggs were transported and stored at ≤4.4°C and inoculated within 24 h after pasteurization. For each egg lot tested, 500-ml portions were aseptically transferred to sterile 0.946 L (1 qt) plastic ball jars (Bel-Art, Pequannock, NJ) and separately inoculated with 5 ml of the LM, YE, or AH pool inoculum. Uninoculated controls were also evaluated. The target inoculum was 3 to 4 $\log_{10}$ CFU/ml. Immediately after inoculation, the jars were vigorously handmixed for 1 min. to ensure homogeneous distribution of indigenous and inoculated microorganisms, and incubated at 2, 6.7, and 12.8°C for 14 d in precalibrated Environette® incubators (Model No. 702ASHX, Labline, Melrose Park, IL). On a daily basis, each jar was hand mixed for 30 to 60 s to simulate the high aeration rates associated with manufacturing plant storage conditions. Inoculated and uninoculated egg samples were analyzed at zero time (within 1 h after inoculation), 2, 4, 7, 10, and 14 d. Samples were also visually and organoleptically (odor) examined for overt spoilage at each sampling interval. The type and severity of spoilage was described, as appropriate. The study was replicated three times for each pasteurized egg type and psychrotropic pathogen evaluated. Freshly pasteurized egg lots were used for each replicate set.

General analytical procedures

Each egg lot was examined for pH and water activity (a_w) immediately upon receipt. The pH analyses were performed on a digital laboratory-pH meter (Model No. EA-940, Orion Research Inc., Boston, MA); a_w analyses were conducted in the Decagon instrument (Model No. CX-2, Decagon Devices Inc., Pullman, WA).

Microbial enumeration was conducted using the Hydrophobic Grid Membrane Filtration® technique (HGMF-QA Labs Ltd, Toronto, Canada), and applicable AOAC (*4*) sample preparation and direct plating procedures. Enzyme treatments were required at the 10^{-1} dilution level to increase filtration efficiency. One ml of the 10^{-1} dilution was mixed with 0.5 ml of a 10% filter sterilized tryspin (Difco) suspension and incubated 10-15 min at 35°C.

Psychrotropic bacteria were analyzed on tryptic soy agar supplemented with 0.25% fast green dye (Sigma, St. Louis, MO), and incubated at 12.8°C for 72 h. Psychrotropic bacteria counts were performed at each storage testing interval. Samples were also randomly analyzed for coliforms, fecal streptococci, and yeast and mold contamination, using standard selective recovery and differential media. Indigenous counts were recorded as $\log_{10}$ CFU/ml.

Psychrotropic pathogen detection and enumeration procedures

YE population levels were enumerated on *Yersinia* selective agar (YSA-Difco) fortified with antimicrobial supplement CN (Difco). YSA plates were incubated at 30°C for 72 h. Typical YE colonies were picked and inoculated into triple sugar iron agar slants (Difco) and incubated at 30°C for 24-48 h. Final confirmation was performed using the API 20E (Analylab Products Inc., Plainview, NY) rapid identification test-strip system.

Modified selective recovery media were employed for LM and AH. LM was plated on selective liver veal agar (SLVA). Liver veal agar was prepared per manufacturers' directions except 0.25% fast green dye, 0.6% yeast extract (Difco), 0.05% aesculin (Difco), and 1% lithium chloride (Sigma) were added before autoclaving. The SLVA medium was cooled to 45-50°C, and supplemented with four antimicrobial compounds: colistin (Sigma), moxalactam-diammonium salt (Sigma), acriflavin (Sigma), and cyclohexamide (Sigma). The final concentrations were 10, 20, 7.5, and 25 μg/ml, respectively. SLVA plates were incubated at 35°C for 72 h, and suspect LM colonies were confirmed by phase contrast wet mount examination (Model No. BH-2, Olympus Corp, Lake Success, NY), catalase positive reaction, and umbrella shaped motility in Semisolid Indole Motility Agar Stabs (Difco).

AH was enumerated and presumptively identified using a specially developed semi-selective plating medium, and HGMF-adapted differential assay step. The basal medium consisted of tryptic soy agar (TSA-AH) supplemented with 0.15% bile salts No. 3 (Difco), and 0.1% glucose (Difco), which was incubated under anaerobic conditions (Gas-Pak®, BBL, Cockeysville, MD) at 35°C for 24-28 h. Only aeromonads, coliforms, and fecal streptococci grew on the anaerobic TSA-AH medium. The plates were screened for cytochrome oxidase positive colonies as follows: the top lid of each petri dish was removed; a Whatman® No. 4 (Whatman International Ltd, Maidstone, England) filter paper disc saturated with 5 ml of Kovac's oxidase reagent was placed inside the lid; and the HGMF membrane filter containing surface microbial growth was placed on top of the filter disc. Presumptive AH-positive colonies were quantified by counting individual colonies which elicited light to dark blue oxidase positive color reactions within 30 to 60 s. The method proved to be extremely sensitive and accurate. Cell viability was also maintained because direct contact with the toxic oxidase reagent was avoided. The TSA-AH medium was compared with phenol-red starch ampicillin agar (*17*), and demonstrated equivalent to superior recovery results. AH confirmation employed the same two-step procedure used for YE. Psychrotropic pathogen counts were recorded as $\log_{10}$ CFU/ml. For each egg type and storage temperature investigated, psychrotropic pathogen counts were averaged over the three replicate runs and plotted on semilog graph paper at zerotime, 4, 10, and 14 d analysis times.

RESULTS AND DISCUSSION

The two pasteurized, unsalted liquid eggs, LEB and LEW, were susceptible to spoilage at ≥2°C because of their extremely high a_w and near neutral to alkaline pH range (Table 1). Visible spoilage was observed in 4 to 14 d. The spoilage microflora varied significantly between the two products. LEB was exclusively spoiled by pseudomonads, whereas LEW was spoiled by *Serratia* spp. at ≤6.7°C. Under temperature abuse (12.8°C), LEW's spoilage population was composed of 75% coliform bacteria and 25% *Serratia* spp.

Psychrotropic pathogen growth responses in the commercially pasteurized unsalted liquid eggs were temperature dependent as follows: YE > AH > LM (Fig. 1 and 2). When psychrotropic pathogen growth occurred, maximum population levels exceeded 7 $\log_{10}$ CFU/ml before leveling off. YE behavior was identical in LEB and LEW, indicating that divergent pH (neutral to alkaline) and spoilage microflora conditions produced negligible impact on its

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TABLE 1. *Microbial physical attributes and spoilage profile of commercially pasteurized liquid eggs.*

Egg composition	Initial a_w*	Initial pH*	Storage temp.	Storage time (visible spoilage)	Key spoilage characteristics	Aerobic plate count** (CFU/ml)	Predominant spoilage microflora
Unsalted whole egg blend	0.99	6.56	2°C	14 d	putrid odor, discoloration	5.5 x 10^6	pseudomonads
			6.7°C	10 d	putrid odor, discoloration	3.1 x 10^7	pseudomonads
			12.8°C	4 d	putrid odor, discoloration	4.1 x 10^7	pseudomonads
Unsalted egg white	0.99	9.07	2°C	14 d	musty, sour odor	3.4 x 10^5	*Serratia* spp.
			6.7°C	10 d	musty, sour odor	5.4 x 10^6	*Serratia* spp.
			12.8°C	4 d	musty, sour odor	1.5 x 10^7	Coliforms and *Serratia* spp.
5% NaCl whole egg blend	0.94	7.07	2°C	14 d	none	2.7 x 10^3	---
			6.7°C	14 d	none	2.7 x 10^3	---
			12.8°C	14 d	musty, stale odor	1.3 x 10^6	Fecal streptococci and micrococci
10% NaCl egg yolk	0.85	6.17	2°C	14 d	none	1.4 x 10^3	---
			6.7°C	14 d	none	3.1 x 10^3	---
			12.8°C	14 d	none	2 x 10^3	---

* Average of three lots.
** Direct plating assay results from samples taken at time of visual spoilage or end (14 d) of study.

growth capabilities in unsalted eggs. At ≤6.7°C, YE growth was characterized by a ≥4 d lag phase, followed by 2 and 3 $\log_{10}$ CFU/ml increases in 10 and 14 d, respectively. Under temperature abuse, YE increased 2 $\log_{10}$ CFU/ml in ≤4 d, and reached 7 $\log_{10}$ CFU/ml 40% faster (≤10 d) than in refrigerated LEB and LEW samples. Overall, our results determined that YE psychrotropic growth risk in commercially pasteurized, unsalted liquid eggs was equivalent to liquid dairy and raw meat products. Amin and Draughon (*2,3*) studied YE behavior in freshly pasteurized milk and shell eggs. They reported 16.8 to 25.9 h generation times in skim milk held at 4°C, and the organism was unaffected by competitive microflora (*2*). YE penetrated and grew inside intact shell eggs stored at 10°C (3). Stern et al. (*23*) showed a 4-d lag phase, followed by linear YE population increases held at 3°C for 20 d. Gill and Rachel (*19*) demonstrated that YE growth capabilities exceeded AH and LM in high pH-beef packaged under ambient and controlled atmosphere conditions at ≥0°C.

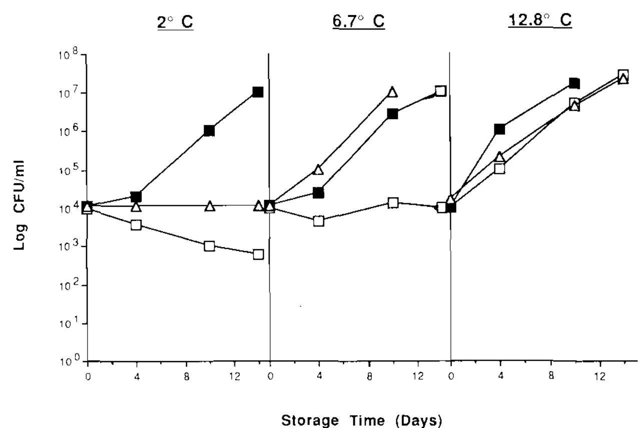

Figure 1. *Behavior of psychrotropic pathogens in commercially pasteurized liquid whole egg blend held at 2, 6.7, and 12.8°C for 14 d.* ❐, L. monocytogenes; ■, Y. enterocolitica; Δ, A. hydrophila. *Counts represent an average of three replicate runs, each using a freshly pasteurized egg lot.*

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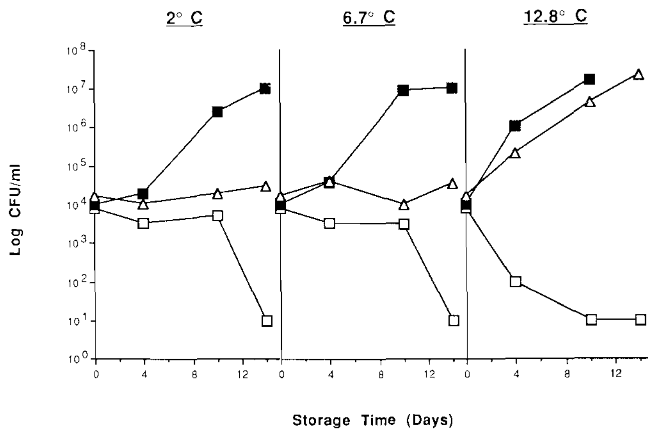

Figure 2. *Behavior of psychrotropic pathogens in commercially pasteurized liquid egg white held at 2, 6.7, and 12.8°C for 14 d.* ❐, L. monocytogenes; ■, Y. enterocolitica; Δ, A. hydrophila. *Counts represent an average of three replicate runs, each using a freshly pasteurized egg lot.*

AH survived but showed more temperature restrictive growth responses than YE in unsalted eggs. In LEB and LEW growth was prevented at 2 and 6.7°C, respectively, which indicated the organism was less versatile than YE regarding its ability to grow and proliferate in alkaline pH and low temperature (2°C) environments. This was consistent with Kirov et al. (*13*), and Palumbo et al. (*18*) observations that ≤5°C holding temperature suppressed AH growth in raw chickens and laboratory media.

LM demonstrated the lowest growth risk in refrigerated unsalted eggs. It was inhibited in LEB at ≤6.7°C and inactivated in LEW. The most efficient inactivation rate occurred at 12.8°C. Initial LM contamination levels remained stable for 10 d under refrigeration and decreased ≥3 $\log_{10}$ CFU/ml to below the direct plating detection threshold by 14 d. The temperature abused LEW showed >1 and ≥3 $\log_{10}$ CFU/ml decreases in 4 and 10 d, respectively. LM lethality was probably caused by high concentrations of lysozyme. Hughey et al. (*11*) and Hughey and Johnson (*12*) reported that hen egg white lysozyme produced anti-listerial activity in laboratory media and vegetable-based foods, but diminished in proteinaceous foods such as cheese and cooked sausage meat. Our superior results in protein rich LEW (>10% albumin) may be due to the presence of higher lysozyme concentrations than those investigated by Hughey and Johnson, or synergistic interaction with other unidentified anti-listerial compounds present in pasteurized liquid egg white. Our findings also supported and broadened the observations of Sionowski and Shelef (*22*). They studied LM behavior in unpasteurized whole egg blend and separated egg white and yolk portions, and reported growth inhibition and inactivation in the blend and white, respectively, stored at 5 and 20°C for up to 30 d. We demonstrated that standard pasteurization does not detrimentally affect egg white anti-listerial activity. Foegeding and Leasor (*17*) detected LM growth in ultrapasteurized whole liquid egg blend held at 4°C. Perhaps the ultrapasteurization processing conditions denatured egg white lysozyme, which led to greater LM growth risks than we found in commercially pasteurized eggs. Critically, we documented that LM growth risks in pasteurized unsalted liquid eggs was lower than liquid dairy products under comparable refrigeration conditions. Donnelly and Briggs (*5*) observed LM growth in sterilized milk with varying fat content and held at 4-37°C. Rosenow and Marth (*21*) reported similar findings in sterilized milk and whipping cream. They detected 5- to 10-d lag phases at 4°C and exponential population increases as low as 8°C.

The microbial quality and safety benefits of NaCl supplementation are clearly illustrated (Fig 3). The addition of ≥5% NaCl decreased a_w and correspondingly improved microbial shelf stability (Table 1). Microbial spoilage was limited to temperature abused SLEB which supported incremental NaCl tolerant streptococci and micrococci increases. Because of its extremely low a_w (0.85), SLEY was impervious to bacterial spoilage. Compared to unsalted eggs, psychrotropic pathogen growth or survival risks shifted as follows: LM > YE > AH. SLEB prevented YE and AH growth, even under temperature abuse. Due to its inherent NaCl tolerance, LM grew in the temperature abused SLEB, increasing 1, 3, and 4 $\log_{10}$ CFU/ml in 4, 10, and 14 d, respectively. Both YE and LM survived prolonged 5% NaCl exposure under refrigeration without any appreciable population reductions. YE growth was also inhibited in SLEB held at 12.8°C, and AH was rapidly inactivated in SLEB at all three holding temperatures. Unlike LM, AH inactivation rates were independent of storage temperature. In SLEY (data not shown), YE and LM contamination levels were stable or showed 0.5 to >2 $\log_{10}$ CFU/ml declines over 14 d, and AH was inactivated at the same rate as in SLEB. The low a_w did not moderate AH inactivation rates (cells in quiescent state), which suggested that high NaCl levels were directly toxic to the organism. Possible inactivation mechanisms include intracellular osmotic disruption or metabolic enzyme poisoning. Our results confirmed and expanded previously published findings. Stern et al. (*24*) reported ≥5% NaCl levels inhibited YE growth in laboratory media held at 3-7°C. Ingram and Potter (*10*) and Palumbo et al. (*18*) demonstrated that ≥3% NaCl inhibited AH growth in refrigerated cooked seafoods and laboratory media, respectively. As with unsalted pasteurized liquid eggs, LM growth suppression was superior in NaCl supplemented eggs compared with NaCl fortified liquid dairy products. Papageorgiou and Marth (*19*) observed LM growth in 6% NaCl skim milk and whey held at 4°C. Twelve percent NaCl levels inhibited LM growth at refrigeration and ambient holding temperatures.

The storage temperatures and holding times used in this study were carefully selected to reflect standard industry practices. Additionally, the research study simulated worst case postprocess contamination situations with 3 to 4 $\log_{10}$ CFU/ml nonstressed microorganisms. Taking these factors into account, our findings conclusively demonstrated that properly refrigerated and hygienically handled pasteurized liquid eggs represent minimal microbial safety risks. The combination of refrigerated storage at ≤5°C, and 5 to 10 d lot turnover rates effectively controls significant psychrotropic growth risks, especially for the serious pathogen *L. monocytogenes*. Unsalted eggs also exhibit obvious odor and texture decomposition defects which deter the

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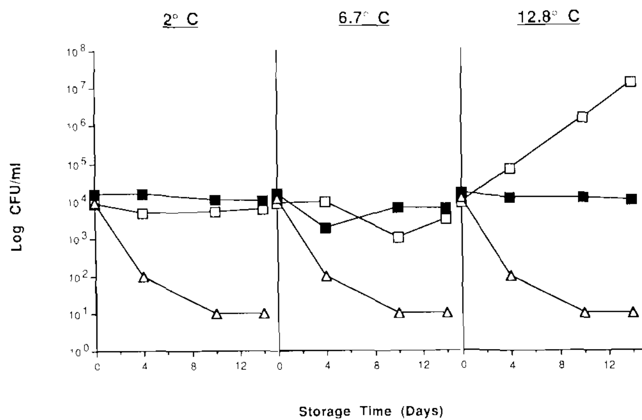

Figure 3. *Behavior of psychrotropic pathogens in commercially pasteurized 5% NaCl liquid egg blend held at 2, 6.7, and 12.8°C for 14 d.* ❐, L. monocytogenes; ■, Y. enterocolitica; Δ, A. hydrophila. *Counts represent an average of three replicate runs, each using a freshly pasteurized egg lot.*

accidental use of potentially dangerous temperature abused lots. Furthermore, we discovered that commercially pasteurized liquid eggs contain intrinsic and extrinsic protective factors such as lysozyme and NaCl supplements that inhibit or inactivate psychrotropic pathogens under refrigerated and abusive storage conditions.

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