To the Graduate Council:

I am submitting herewith a thesis written by Robert Charles Williams entitled "Influence of Modified Atmosphere Packaging on Survival and Recovery of Sublethally Heat- and Acid-Injured *Listeria monocytogenes*." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

David A. Golden, Major Professor

We have read this thesis and
recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor and
Dean of The Graduate School

INFLUENCE OF MODIFIED ATMOSPHERE PACKAGING ON SURVIVAL AND RECOVERY OF SUBLETHALLY HEAT- AND ACID-INJURED *LISTERIA MONOCYTOGENES*

A Thesis
Presented for the
Master of Science
Degree
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Robert Charles Williams
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DEDICATION

This thesis is dedicated in loving memory of my grandfather

James Robert Warren
1919-1996

who provided me with many invaluable opportunities.

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ABSTRACT

This research was conducted as two separate studies. In the first study, the suitability of PALCAM and modified Oxford (MOX) agars for recovering sublethally heat- and lactic acid-injured *Listeria monocytogenes* was investigated. *L. monocytogenes* LM101M, LM103M (meat isolates), and Scott A were suspended in tryptose phosphate broth (TPB), heated for up to 40 min at 54°C, and surface plated onto tryptose phosphate agar (TPA), TPA + 4% NaCl (TPAS), PALCAM, and MOX. TPA and TPAS were used to determine total viable and sublethally injured populations, respectively. Heat-injured LM103M was recovered in the highest numbers on all media, followed by Scott A and LM101M ($P<0.01$). TPA allowed best recovery of all test strains, followed by PALCAM and MOX, which were not different, and TPAS ($P<0.01$). For acid-injury studies, uninjured and heat-injured (54°C for 20 min) test strains were suspended in phosphate-buffered TPB + 0.85% lactic acid (bTPBLA) at 25°C for up to 24 h and plated as described above. Uninjured and heat-injured *L. monocytogenes* were recovered better from bTPBLA on MOX than on PALCAM ($P<0.05$). Heat injured *L. monocytogenes* LM103M was recovered better than LM101M but similar to Scott A on MOX and PALCAM ($P<0.05$), whereas Scott A was recovered similarly to LM101M and LM103M on MOX and PALCAM ($P>0.05$). Acid-injury of *L. monocytogenes* LM103M was enhanced by prior heat stress.

In the second study, the effect of package atmosphere on survival of uninjured and sublethally heat-injured *L. monocytogenes*, inoculated onto tryptose phosphate agar containing 0.85% lactic acid and 2% NaCl (TPALAS) was investigated. Inoculated

TPALAS plates were packaged in air, 100% N_2 (N_2), 30% CO_2/70% N_2 (CO_2/N_2), and vacuum and stored at 4 and 20°C for up to 31 days. Recovery of *L. monocytogenes* from TPALAS was influenced by the injury status (i.e., injured and uninjured) of the inoculum, storage atmosphere (air, N_2, CO_2/N_2, and vacuum), storage temperature (4 and 20°C), and recovery media (tryptose phosphate agar [TPA] and modified Oxford agar [MOX]) ($P<0.05$). Storage in CO_2/N_2 atmosphere was more inhibitory to uninjured *L. monocytogenes* stored at 20°C than air or vacuum, but less than N_2 ($P<0.05$). When sublethally heat-injured *L. monocytogenes* was stored at 20°C, CO_2/N_2 atmosphere allowed better recovery than N_2 ($P<0.05$) but was not different than air ($P>0.05$). At 4°C, uninjured and sublethally heat-injured *L. monocytogenes* were recovered in highest numbers from samples packaged in N_2 followed by CO_2/N_2 ($P>0.05$). Overall, non-selective TPA allowed greater recovery of *L. monocytogenes* than the selective MOX ($P<0.05$). Uninjured cells stored at 4°C were recovered better than sublethally heat-injured cells on TPA ($P<0.05$). On TPA, *L. monocytogenes* stored under air was recovered better than *L. monocytogenes* stored under N_2 or CO_2/N_2 ($P<0.05$). However, recovery on MOX was best when *L. monocytogenes* was stored under N_2 ($P<0.05$).

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Part I

Literature Review

INTRODUCTION

The microorganism now known as *Listeria monocytogenes* was recognized as early as 1891 when a small, Gram-positive bacillus, isolated from human tissue, was first described in scientific literature (Donnelly, 1994). During the following years, several investigators isolated a similar bacillus and each assigned his newly discovered microorganism a different name. Hulphers, in 1919, isolated a Gram-positive bacillus from the livers of rabbits with necrotic lesions and named the organism *Bacillus hepatis* (Gellin and Broome, 1989). In 1924, Murray et al., noting monocytosis and hepatic lesions in rabbits, isolated a suspect microorganism and named it *Bacterium monocytogenes* (Gellin and Broome, 1989). In 1925, in the Tiger River region of South Africa, Pirie isolated a small, Gram-positive bacillus from rodents. This microorganism became popularly known as Tiger River Bacillus, but was later named *Listerella hepatolytica* in honor of Lord Joseph Lister (Gellin and Broome, 1989). Nyfeldt described a similar Gram-positive bacillus in 1929 (Jones et al., 1994). In possibly the first report of human listeriosis, Nyfeldt implicated this organism as the cause of infectious mononucleosis and named it *Bacterium monocytogenes hominis* (Gellin and Broome, 1989). It was found later that the monocytosis observed by Nyfeldt is not common to human listeriosis. The current name was proposed by Pirie in 1940 (Donnelly, 1994).

L. monocytogenes is a bacterial pathogen which causes illness in humans and animals. This organism has been recognized as the cause of circling-disease in sheep and keratoconjunctivitis in rabbits, although it is known to cause disease in other animals

(Lovett, 1989). Prior to the 1980's, *L. monocytogenes* infections were thought to occur especially in animal hosts and only rarely in human hosts, the latter thought to be primarily due to zoonosis from contact with infected animals (Donnelly, 1994). Several major outbreaks of listeriosis in humans during the 1980's, however, distinguished *L. monocytogenes* as an important human pathogen. In the early 1980's, epidemiological evidence implicated contaminated food as the vehicle of *L. monocytogenes* infection, and, ultimately, food was implicated as a vehicle of infection in every major listeriosis outbreak of that decade. (Gellin et al., 1991; Schuchat et al., 1991). Within the decade, it became readily apparent that listeriosis was, in fact, a disease of widespread human concern and that consumption of food contaminated with *L. monocytogenes* by susceptible hosts is associated with the development of characteristic clinical manifestations of listeriosis. Evidence of foodborne transmission of listeriosis became irrefutable as outbreaks of listeriosis continued to occur and continued to be associated with consumption of contaminated food. Outbreaks of listeriosis during the 1980's were linked to consumption of contaminated coleslaw, pasteurized milk, Vaccherin mont d'or cheese, Mexican-style cheese, ice cream, salami, vegetables, soft country cheese, and chicken (Jay, 1992).

GENERAL CHARACTERISTICS

L. monocytogenes is a small Gram-positive, nonsporeforming rod measuring 1.0-2.0 micrometers by 0.5 micrometers that may appear coccoid in older (> 48 hours) cultures (Donnelly et al., 1992; Jay 1992; Ryser and Marth, 1991). *L. monocytogenes* is a

psychrotroph and can grow over a temperature range of 1 - 45°C, with optimum growth at 30-37°C. The organism grows in substrates with a pH range of 4.1-9.6, with an optimum of neutral to slightly alkaline pH. (Jay, 1992; Ryser and Marth, 1991). It is facultatively anaerobic preferring an anaerobic to microaerophilic environment , and typically grows best in atmospheres containing elevated carbon dioxide (Jay, 1992). This microorganism is motile if cultured at or below 25°C, displaying characteristic tumbling and umbrella motility (Ryser and Marth, 1991). *L. monocytogenes* can grow in media containing up to 10% NaCl and survive in media containing up to 25.5% NaCl (Daza et al., 1991; Donnelly et al., 1992; Jay, 1992). *L. monocytogenes* is catalase positive and oxidase negative, hydrolyses esculin, and hydrolyses sodium hippurate (Donnelly et al., 1992). It produces acids, including lactic, acetic, isobutyric, isovaleric, and phenylacetic acid, but no gas from fermentation of glucose (Ryser and Marth, 1991). Additionally, *L. monocytogenes* produces acids from fermentation of maltose, rhamnose, salicin, levulose, and trehalose, but does not produce acid from mannitol, xylose, raffinose, or sucrose (Ryser and Marth, 1991). It displays delayed acid production during fermentation of lactose, galactose, sorbitol, and dextrin (Ryser and Marth, 1991).

DISTRIBUTION

L. monocytogenes is widespread in nature and can be found in silage, soil, decaying vegetation, sewage, damp earth, straw, and feces of humans and animals (Donnelly et al., 1992). In the agricultural ecosystem, *L. monocytogenes* has been recovered from fresh pig and cow feces as well as groundwater (Renterghem et al., 1991).

Listeriae, in general, occur in association with lactic acid bacteria, *Brochothrix*, and some coryneform bacteria (Jay, 1992).

L. monocytogenes has been recovered from a wide variety of foods, especially meat and dairy products. It has been recovered from fermented sausages, ground beef, ground pork, ground veal, chicken legs, ice cream, salami, ham, corned beef, vegetables, pate, and cheeses (Farber et al., 1989; Grau and Vanderline, 1992; De Simon et al., 1992; Trott et al., 1991; Varabioff, 1992; Wilson, 1995). *L. monocytogenes* has also been recovered from smoked salmon, shrimp, and minced fish (Rorvik and Yndestad, 1991).

L. monocytogenes has been detected within food processing facilities from drains, floors, processing equipment, and pooled water (Rocourt and Bille, 1997). In one survey of sources of *Listeria* spp. in dairy plants, *Listeria* were recovered from floors in storage, processing, and packaging rooms including cooler rooms (Klausner and Donnelly, 1991).

LISTERIOSIS

Although healthy adults are rather resistant to the most severe forms of *L. monocytogenes* illness, infection in immunocompromised hosts often results in severe disease with a high mortality rate (Jay, 1992). Conditions that predispose individuals to *L. monocytogenes* infections include acquired immunodeficiency syndrome (AIDS), cancer, old age, and pregnancy (Jay, 1992). Individuals with compromised immune function who contract clinical listeriosis typically suffer from meningitis and sepsis, although other manifestations include endocarditis, septic arthritis, and liver abscess (Schlech, 1997). Pregnant women with listeriosis often abort the fetus, deliver

prematurely, or the pregnancy results in stillbirth (Jay, 1992).

Historically, human listeriosis was thought to occur almost exclusively in immunocompromised hosts, and there was little evidence of listeriosis among individuals with normal immunity. However, recent reports link *L. monocytogenes* to outbreaks of gastroenteritis in previously healthy individuals (Dalton et al., 1997; Reido et al., 1994; Salamina et al., 1996). Gastroenteritis due to *L. monocytogenes* is recognized as an occasional prodrome in the immunocompromised host that is followed by severe invasive disease (Anonymous, 1997). Recent reports of gastroenteritis caused by *L. monocytogenes*, however, describe a self-limiting disease absent of further complications characteristically associated with listeriosis. Gastroenteritis caused by *L. monocytogenes* may not be new. Generally, clinical laboratories do not use media that are suitable for detecting *L. monocytogenes* from fecal specimens (Schlech, 1997). Therefore, it is difficult to determine how frequently gastroenteritis from listeriosis occurs.

Outbreaks of listeriosis have been associated with several different types of foods that include contaminated coleslaw, pasteurized milk, chocolate milk, Vaccherin mont d'or cheese, Mexican-style cheese, ice cream, salami, vegetables, soft country cheese, chicken, shrimp, rice salad, and rainbow trout (Bula et al. 1995; Dalton et al., 1997; Ericsson et al., 1997; Jay, 1992; Riedo et al., 1994; Salamina et al., 1996; Schwarz et al., 1989;). In most of these cases, improper processing, storage, or handling of implicated foods were implicated as contributing factors.

FERMENTED FOODS

Several investigations have been performed to determine the fate of *L. monocytogenes* in a wide variety of food products. However, survival of *L. monocytogenes* in fermented foods is of particular concern because of the hardiness of this organism and its ability to endure harsh environmental conditions. Fermented foods, including dairy, vegetable, and meat products, are at least partially preserved through the fermentation process. These foods typically are characterized by sugar fermentations that result in production of lactic acid.

Several investigators have reported on the ability of *L. monocytogenes* to survive during the production and storage of fermented milks (Ashenafi, 1994; Dalu and Feresu, 1996), yogurt (Griffith and Deibel, 1990; Shaack and Marth, 1988), kimchi (Lee et al., 1995) and tempeh (Ashenafi, 1991). However, most research on the fate of *L. monocytogenes* in fermented foods has targeted cheese and fermented sausages. Cheese is generally produced via the fermentation of milk by lactic acid bacteria (e.g., *Streptococcus thermophilus*, *Lactococcus lactis*) (Jay, 1992). During the production of cheese, milk is typically inoculated with lactic starter cultures and allowed to ferment. At the end of fermentation, rennin is added and curd formation results. The curd is cooked, shrunk, pressed, salted, and allowed to ripen (Jay, 1992). The characteristics of specific cheeses are derived from many variables such as milk source, starter culture constituents, cooking conditions, and ripening conditions.

Kinderlerer et al. (1996) reported that Bleu d'Auvergne cheese was more inhibitory to *L. monocytogenes* than Brie cheese, possibly due to the higher levels of

medium-chain fatty acids in the Bleu cheese. Nevertheless, *L. monocytogenes* was recovered from commercially prepared samples of both cheeses, showing the ability of this organism to survive the fermentation, ripening, and storage of these products (Kinderlerer et al., 1996). Buazzi et al. (1992a) reported that *L. monocytogenes,* inoculated in milk, was reduced to undetectable levels during the ripening of Swiss cheese. They also reported that *L. monocytogenes* was unable to survive during the production of Mozzarella cheese (Buazzi, 1992b).

Fermented sausages are meat products that are made from comminuted meat with the addition of fat, salt, curing agents, sugar, and spices and are fermented by the action of microorganisms (Lucke, 1985). In addition to salami, other fermented sausages such as pepperoni and Lebanon bologna, fall under this definition. The typical process of sausage fermentation begins with raw meat and includes mixing of ingredients, fermentation at an optimal incubation temperature, and a final drying step (Ward, 1989). Some sausages also are heated during processing or at some point prior to consumption (Lucke, 1985). The aim of fermentation of meats is to increase acidity (as lactic acid) while decreasing pH to yield a stable product with unique flavors (Ward, 1989). Commercial starter cultures generally are used to accomplish these objectives and typically are comprised of *Pediococcus* spp., *Micrococcus* spp. and/or *Staphylococcus carnosus* (Ward, 1989).

Sodium chloride (2.4 to 3%) and nitrite are used as curing agents in fermented meats (Lucke, 1985). The addition of NaCl decreases the initial water activity to approximately 0.97 to 0.96 and favors growth of desired lactic acid bacteria and

micrococci while also contributing flavor (Lucke, 1985). Nitrite serves to promote establishment of desired microorganisms, development of desired product color, and prevention of autoxidative rancidity (Lucke, 1985). Formulation of curing agents and processing of meats into fermented sausages are designed, in part, to prevent growth and survival of foodborne pathogens such as *Clostridium botulinum* and *Staphylococcus aureus*, (Lucke, 1985). However, other pathogens such as *Escherichia coli* O157:H7 may survive the process of meat fermentation. Glass et al. (1992) reported that *E. coli* O157:H7 was able to survive the fermentation, drying, and storage of fermented dry, sausage. Another investigation showed that *E.coli* O157:H7, initially present at 10^4 to 10^5 CFU/g of sausage batter would likely pose a health threat in salami stored at 5°C for 32 days (Clavero and Beuchat, 1996).

The incidence and ability of *L. monocytogenes* to grow and survive in fermented sausages also has been of interest. Farber et al. (1989) studied the incidence of *L. monocytogenes* in foods in Ottawa, Canada. They found that 6 of 30 (20%) fermented sausages sampled contained *L. monocytogenes,* therefore drawing into question post-processing contamination or the ability of this organism to survive sausage fermentation and subsequent processing. Several studies have been undertaken to investigate the ability of *L. monocytogenes* to grow and survive during the production of fermented sausages. Most of these studies focused on pre-fermentation contamination of raw meats used for sausage production. Johnson et al. (1988) found that *L. monocytogenes* did not grow during production or storage of hard salami, but survived in salami for up to twelve weeks during refrigerated storage.

Farber et al. (1993) found that *L. monocytogenes* numbers decreased during fermentation of uncooked German and American-style sausages, but low levels of *L. monocytogenes* were detected in the finished products. Results similar to these were reported by Buncic et al. (1991). Glass and Doyle (1989) found that typical processes used in the production of pepperoni and hard salami were inadequate to prevent *L. monocytogenes* survival during storage. They found that additional heating of the products to 51.7° C for 4 hours was needed to decrease *L. monocytogenes* to undetectable levels. D-values for *L. monocytogenes* in fermented sausages and ground beef roast were found to be similar, suggesting that this organism is much more tolerant to the combined treatments of heat and acid than other pathogens (Schoeni et al., 1991).

The effect of starter cultures and other additives on control of *L. monocytogenes* during fermented sausage manufacture has been examined. Campanini et al. (1993) found that naturally fermented sausages (i.e., sausages fermented by normal flora without the addition of starter culture) did not effectively inhibit *L. monocytogenes*. They found that addition of starter cultures enhanced the effectiveness of fermentation for controlling *L. monocytogenes*, but low levels of *L. monocytogenes* could still be recovered in the finished product. Sabel et al. (1991) also reported that addition of starter cultures to meat yielded a more effective fermentation than natural fermentation with regard to inhibition of *L. monocytogenes*. They also reported that TBHQ or BHA had minimal addition effect on *L. monocytogenes* survival. Bacteriocin-producing starter culture strains have shown inhibitory action against *L. monocytogenes* during sausage fermentation. When used as a starter culture for sausage fermentation, a bacteriocin-producing *Pediococcus acidilacti*

strain was more effective at inhibition of *L. monocytogenes* than bacteriocin-negative *P. acidilacti* (Foegeding et al., 1992).

SUBLETHAL INJURY

It is well known that bacterial cells may become damaged or injured as a result of various types of stress. When exposed to harsh conditions, a portion of the bacterial population will be killed, some will remain uninjured, and some will become sublethally injured. Unlike lethally injured cells, sublethally injured cells are reversibly injured, and upon placement in an appropriate environment, may repair and resume growth (Ray, 1993). Typically, the nutrient requirements of injured cells become more strictly defined (Ray, 1989). As a result, sublethally injured cells will grow on non-selective nutrient agar, but often will not form colonies on agar that contains selective agents.

Physical and chemical treatments applied to foods have been shown to induce sublethal injury. When foods are treated by heating, freezing, acidification, or addition of antibacterial compounds, microbial cells may become sublethally injured and remain undetected using common techniques which employ various selective media for isolation (Palumbo, 1989). After treatment, the food environment may return to conditions favorable for microbial growth allowing injured cells to repair and grow. Under normal testing for detection of foodborne pathogens, for example, a sample may appear negative for the target organism after processing, but may still pose a health threat.

L. monocytogenes has been shown to undergo sublethal injury upon exposure to various types of stress. Golden et al. (1988a) reported that injury of *L. monocytogenes* by

heating is a function of the temperature and the duration of heating. Injured cells comprised a substantial portion of the viable population when test samples were heated at 52, 54, and 56°C. Freezing *L. monocytogenes* at -18°C produced only 3 to 6% reductions in total viable population, but induced injury in up to 82% of the viable population (Golden et al., 1988a). Smith and Archer (1988) noted that heating *L. monocytogenes* cultures at 52°C for one hour resulted in injury as indicated by the difference between growth on non-selective media and media containing 5% NaCl.

Ahmad and Marth (1990) studied the effect of acetic, citric, and lactic acids on the development of injury in *L. monocytogenes,* and found that citric acid was the most effective for producing injury, followed by lactic and acetic acids. Temperature of incubation was found to be a contributing factor to the subsequent development of injury. Cultures that were incubated under acid treatment at 35°C experienced much shorter inactivation and injury times than cultures incubated at 13°C.

Knabel et al. (1990) found that incubation of heat-injured *L. monocytogenes* under anaerobic conditions significantly increased recovery as compared with aerobic controls. The addition of Oxyrase® and catalase to recovery media was also shown to significantly enhance recovery of injured *L. monocytogenes* (Patel et al., 1995).

MODIFIED ATMOSPHERE PACKAGING

Modified atmosphere packaging (MAP) has been used since the 1930's to increase the shelf-life of foods, in part, by inhibiting growth of spoilage microflora (Farber, 1991).

MAP may include controlled atmosphere packaging (CAP), packaging naturally respiring products in certain permeable films, vacuum packaging, and gas-flushing (Farber, 1991). Unlike CAP, where food products are subjected to a constant gas mixture, gas-flushing is applied once to food products during packaging (Farber, 1991). Gas-flushing may be applied to food packages with or without prior evacuation of the original atmosphere.

Spoilage microorganisms targeted by MAP generally are aerobic bacteria and cause spoilage at temperatures above freezing. The result of MAP is a longer product shelf-life. However, one concern is that during the lengthened storage time allowed by MAP, foodborne pathogens may grow uncontested by normal flora of the food product. The issue of MAP then becomes safety. Foods treated with MAP may appear to be of good quality (i.e., no off-odors), but may be unsafe to eat because of outgrowth of pathogenic bacteria.

L. monocytogenes is of particular concern in MAP food products because of wide distribution of *L. monocytogenes* in nature, its occurrence in foods, its ability to grow in low oxygen atmospheres, and its ability to grow at refrigeration temperatures (Farber, 1991). Several studies have investigated the fate of *L. monocytogenes* on MAP vegetables. Beuchat and Brackett (1990) found that *L. monocytogenes* growth on shredded lettuce was not appreciably inhibited in a MAP atmosphere of 3% O_2/97% N_2 during storage at 5 and 10°C. Similarly, Berrang et al. (1989) found that *L. monocytogenes* growth was unaffected on vegetables packaged in atmospheres of 15% O_2/6% CO_2/79% N_2, 11% O_2/10% CO_2/79% N_2, and 18% O_2/3% CO_2/79% N_2 during storage at 4 and 15°C. MAP atmosphere consisting of 30% CO_2/70% N_2 was ineffective

for reducing *L. monocytogenes* on shredded cabbage stored at 5°C (Kallander et al., 1991). Francis and O'Beirne (1997) found that storage of minimally processed lettuce under 100% N_2 at 8°C enhanced the growth of *L. monocytogenes*. Gill and Reichel (1989) found that *L. monocytogenes* was inhibited on high-pH beef (pH >6.0) when stored under 100% CO_2 at 5°C, but grew at 10°C. Additionally, they found that *L. monocytogenes* grew on high-pH beef stored under vacuum at 0, 2, 5, and 10°C. Wimpfheimer et al. (1990) found that *L. monocytogenes* was inhibited on minced raw chicken when stored under 25% O_2/ 75% N_2 at 4, 10 and 27°C; however, growth was not affected in air or 5% O_2/22.5% N_2/75% CO_2 atmospheres when stored at these temperatures. Pothuri et al. (1995) reported that MAP consisting of 10.4% O_2/14.8% N_2/ 74.8% CO_2 coupled with lactic acid (1%) inhibited *L. monocytogenes* growth better than air or vacuum and 1% lactic acid on crayfish tail meat stored at 4°C.

In a study of occurrence of foodborne pathogenic and related bacteria in refrigerated MAP food products, Farber et al. (1990) found *L. monocytogenes* in turkey, submarine, ham-beef-cheese sandwiches, and ham-cheese sandwiches. All sandwiches in which *L. monocytogenes* was detected were packaged under 5% CO_2/95% N_2 with the exception that the ham and cheese sandwich was stored under 50% CO_2/50% N_2.

PURPOSE OF CURRENT RESEARCH

L. monocytogenes is widely distributed in nature and is quite resistant to harsh environmental conditions, including high concentrations of NaCl and low pH (Rocourt and Bille, 1997). It has the ability to grow at refrigeration temperatures ($\leq$ 4°C), and can

survive in foods for long periods of time under harsh conditions (Rocourt and Bille, 1997). As a result, *L. monocytogenes* remains a problem for food processors throughout the world.

L. monocytogenes is a foodborne pathogen that poses a significant public health risk. Susceptible individuals who contract listeriosis often suffer severe illness with a high overall mortality rate (Jay, 1992). The likelihood of individuals consuming viable *L. monocytogenes* from fermented foods is significant. This organism has been found in a significant percentage of retail samples of fermented foods suggesting that this organism is capable of surviving in these products for common storage times (Farber et al., 1989; Ryser and Marth, 1991). Outbreaks of listeriosis have been associated with various cheeses (Jay, 1992) and one case of listeriosis has been epidemiologically linked to the consumption of salami (Ahmad and Marth, 1990).

Studies have shown the ability of *L. monocytogenes* to survive (and in some cases grow) during the production and storage of fermented foods (Ashenafi, 1991; Ashenafi, 1994; Buncic et al., 1991; Dalu and Feresu, 1996; Farber et al., 1993; Glass and Doyle, 1989; Griffith and Deibel, 1990; Johnson et al.,1988; Kinderlerer et al., 1996; Lee et al., 1995; Shaack and Marth, 1988). More importantly, these studies have shown the ability of *L. monocytogenes* to survive in certain fermented foods for long storage times. Schoeni et al. (1991) also suggest that *L. monocytogenes* is more tolerant to the combined effects of heat and acid than other pathogens.

Heating and drying steps that often occur after fermentation of certain products may produce injury in *L. monocytogenes*. Several studies have already established that *L.*

monocytogenes may become injured under heat stress (Golden et al. 1988a; Smith and Archer, 1988). It is likely that *L. monocytogenes* in fermented foods will become injured during the heating process. These injured organisms may be able to repair and become viable during storage. As an example, it is common practice today to vacuum package fermented sausages, such as pepperoni, and store them at room temperature. Studies have shown that this storage atmosphere and temperature combination may, in fact, promote the recovery of heat-injured *L. monocytogenes* in those products (Knabel et al., 1990; Patel et al., 1995).

Several studies have investigated the ability of certain media to select for and recover *L. monocytogenes* and injured *L. monocytogenes* (Amoaka et al., 1992; Curtis and Lee, 1995; Golden et al., 1988b; Gunasinghe et al., 1994; Patel and Beuchat, 1995). However, the ability of different media to recovery heat-injured *L. monocytogenes* from fermented foods has not been fully evaluated.

REFERENCES

Ahmad, N. and Marth., E.H. 1990. Acid-injury of *Listeria monocytogenes*. J. Food Prot. 53:26-29.

Amoaka, E.B., Toora, S., Ablett, R.F., and Smith, J. 1992. Evaluation of the ability of primary selective enrichment to resuscitate heat-injured and freeze-injured *Listeria monocytogenes* cells. Appl. Environ. Micro. 58:3177-3179.

Anonymous. 1997. Food poisoning, listeriosis, and febrile gastroenteritis. Nutrition Rev. 55(2):57-60

Ashenafi, M. 1991. Growth of *Listeria monocytogenes* in fermenting tempeh made from various beans and its inhibition by *Lactobacillus plantarum*. Food Microbiol. 8:303-310.

Ashenafi, M. 1994. Fate of *Listeria monocytogenes* during the souring of ergo, a traditional Ethiopian fermented milk. J. Dairy Sci. 77(3):696-702.

Berrang, M.E., Brackett, R.E., and Beuchat, L.R. 1989. Growth of *Listeria monocytogenes* on fresh vegetables stored under controlled atmosphere. J. Food Prot. 52:702-705.

Beuchat, L.R. and Brackett, R.E. 1990. Survival and growth of *Listeria monocytogenes* on lettuce as influenced by shredding, chlorine treatment, modified atmosphere packaging and temperature. J. Food Sci. 55: 755-758, 870.

Buazzi, M.M., Johnson, M.E., and Marth, E.H. 1992b. Fate of *Listeria monocytogenes* during the manufacture of mozzarella cheese. J. Food Prot. 55:80-83.

Buazzi, M.M., Johnson, M.E., and Marth, E.H. 1992a. Survival of *Listeria monocytogenes* during the manufacture and ripening of Swiss cheese. J. Dairy Sci. 75:380-386.

Bula, C.J., Bille, J., and Glauser, M.P. 1995. An epidemic of food-borne listeriosis in western Switzerland: description of 57 cases involving adults. 1995. Clin. Infect. Dis. 20:66-72.

Buncic, S., Paunovic, L., and Radisic, D. 1991. The fate of *Listeria monocytogenes* in fermented sausages and in vacuum-packaged frankfurters. J. Food Prot. 54:413-417.

Campanini, M., Pedrazzoni, I., Barbuti, S., and Baldini, P. 1993. Behaviour of *Listeria monocytogenes* during the maturation of naturally and artificially contaminated salami: effect of lactic-acid bacteria starter cultures. J. Food Microbiol. 20:169-175.

Clavero, M.R.S. and Beuchat, L.R. 1996. Survival of *Escherichia coli* O157:H7 in broth and processed salami as influenced by pH, water activity, and temperature and suitability of media for its recovery. Appl. Environ. Microbiol. 62:2735-2740.

Curtis, G.D.W. and Lee, W.H. 1995. Culture media and methods for the isolation of *Listeria monocytogenes*. Int. J. Food. Microbiol. 26:1-13.

Dalton, C.B., Austin, C.C., Sobel, J., Hayes, P.S., Bibb, W.F., Graves, L.M., Swaminathan, B., Proctor, M.E., and Griffin, P.M. 1997. An outbreak of gastroenteritis and fever due to *Listeria monocytogenes* in milk. New Eng. J. Med. 336:100-105.

Dalu, J.M. and Feresu, S.B. 1996. Survival of *Listeria monocytogenes* in three Zimbabwean fermented milk products. J. Food Prot. 59:379-383

Daza, M.S.T., Villegas, Y., and Martinez, A. 1991. Minimal water activity for growth of *Listeria monocytogenes* as affected by solute and temperature. Int. J. Food Microbiol. 14:333-337.

De Simon, M., Tarrago, C., and Ferrer, M.D. 1992. Incidence of *Listeria monocytogenes* in fresh foods in Barcelona (Spain). Int. J. Food. Microbiol. 16:153-156.

Donnelly, C.W. 1994. *Listeria monocytogenes*. In *Foodborne Disease Handbook: Diseases Caused by Bacteria*, Y.H Hui, J.R. Gorham, K.D. Murrel, and D.O. Cliver (Ed.), p. 215-252. Marcel Dekker, Inc., New York.

Donnelly, C.W., Brackett, R.E., Doores, S., Lee, W.H., and Lovett, J. 1992. Listeria. In *Compendium of Methods for the Microbiological Examination of Foods,* C. Vanderzant and D.F. Splittstoesser (Ed.), p. 637-663. American Public Health Association, Washington, D.C.

Ericsson, H., Eklow, A., Danielsson-Tham, M.L., Loncarevic, S., Mentzing, L.O., Persson, I., Unnerstad, H., and Tham, W. 1997. An outbreak of listeriosis suspected to have been caused by rainbow trout. J. Clinical Microbiol. 35:2904-2907.

Farber, J.M., Sanders, G.W., and Johnston, M.A. 1989. A survey of various foods for the presence of *Listeria* species. J. Food Prot. 52:456-458.

Farber, J. 1991. Microbiological aspects of modified-atmosphere packaging technology - a review. J. Food Prot 54(1):58-70.

Farber, J.M., Warburton, D.W., Gour, L., and Milling, M. 1990. Microbiological quality of foods packaged under modified atmospheres. Food Microbiol. 7:327-334.

Farber, J.M., Daley, E., Holley, R., and Usborne, W.R. 1993. Survival of *Listeria monocytogenes* during the production of uncooked German, American, and Italian-style fermented sausages. Food Microbiol. 10:123-132.

Foegeding, P.M., Thomas, A.B., Pilkington, D.H., and Klaenhammer, T.R. 1992. Enhanced control of *Listeria monocytogenes* by in situ-produced pediocin during dry fermented sausage production. Appl. Environ. Microbiol. 58:884-890.

Francis, G.A. and O'Beirne, D. 1997. Effects of gas atmosphere, antimicrobial dip and temperature on the fate of *Listeria innocua* and *Listeria monocytogenes* on minimally processed lettuce. Int. J. Food Sci. and Technol. 32:141-151.

Gellin, B.G. and Broome, C.V. 1989. Listeriosis. JAMA. 261:1313-1320.

Gellin, B.G., Broome, C.V., Bibb, W.F., Weaver, R.E., Gaventa, S., Mascola, L., and the Listeriosis Study Group. 1991. The epidemiology of listeriosis in the United States- 1986. Amer. J. Epidemiol. 133:392-401.

Gill, C.O. and Reichel, M.P. 1989. Growth of cold-tolerant pathogens *Yersinia enterocolitica*, *Aeromonas hydrophila*, and *Listeria monocytogenes* on high-pH beef packaged under vacuum or carbon dioxide. Food Microbiol. 6:223-230.

Glass, K.A., and Doyle, M.P. 1989. Fate and thermal inactivation of *Listeria monocytogenes* in beaker sausage and pepperoni. J. Food Prot. 52:226-231.

Glass, K.A., Loeffelholz, J.M., Ford, J.P., and Doyle, M.P. 1992. Fate of *Escherichia coli* O157:H7 as affected by pH or sodium chloride and in fermented, dry sausage. Appl. Environ. Microbiol. 58:2513-2516.

Golden, D.A., Beuchat, L.R., and Brackett, R.E. 1988a. Inactivation and injury of *Listeria monocytogenes* as affected by heating and freezing. Food Microbiol. 5:17-23.

Golden, D.A., Beuchat, L.R., and Brackett, R.E. 1988b. Evaluation of selective direct plating media for their suitability to recover uninjured, heat-injured, and freeze-injured *Listeria monocytogenes* from foods. Appl. Environ. Microbiol. 54:1451-1456.

Grau, F.H. and Vanderline, P.B. 1992. Occurrence, numbers, and growth of *Listeria monocytogenes* on some vacuum-packaged processed meats. J Food Prot. 55:4-7.

Griffith M. and Deibel, K.E. 1990. Survival of *Listeria monocytogenes* in yogurt with varying levels of fat and solids. J. Food Safety 10:219-230.

Gunasinghe, C.P.G.L., Henderson, C., and Rutter, M.A. 1994. Comparative study of two plating media (PALCAM and Oxford) for detection of Listeria species in a range of meat products following a variety of enrichment procedures. Lett. Appl. Micro. 18:156-158.

Jay, J.M. 1992. Modern food microbiology, 4th ed. Van Nostrand Reinhold, New York.

Johnson, J.L, Doyle, M.P., Cassens, R.G., and Schoeni, J.L. 1988. Fate of *Listeria monocytogenes* in tissues of experimentally infected cattle and in hard salami. Appl. Environ. Microbiol. 54:497-501.

Jones, E.M., McCulloch, S.Y., Reeves, D.S., and MacGowan, A.P. 1994. A 10 year survey of the epidemiology and clinical aspects of listeriosis in a provincial English city. Epidemiol. 91-104.

Kallander, K.A., Hitching, A.D., Linette, G.A., Schmieg, J.A., Garcia, G.R., Solomon, H.M., and Sofos, J.N. 1991. Fate of *Listeria monocytogenes* in shredded cabbage stored at 5 and 25°C under a modified atmosphere. J. Food Prot. 54:302-304.

Kinderlerer, J.L., Mathias, H.E., and Finner, P. 1996. Effect of medium-chain fatty acids in mould ripened cheese on the growth of *Listeria monocytogenes*. J. Dairy Res. 63:593-606.

Klausner, R.B. and Donnelly, C.W. 1991. Environmental sources of *Listeria* and *Yersinia* in Vermont dairy plants. J. Food Prot. 54:607-611.

Knabel, S.J., Walker, H.W., Hartman, P.A., and Mendonca, A.F. 1990. Effects of growth temperature and strictly anaerobic recovery on the survival of *Listeria monocytogenes* during pasteurization. Appl. Environ. Microbiol. 56:370-376.

Lee, S.H., Kim, M.K., and Frank, J.F. 1995. Growth of *Listeria monocytogenes* Scott A during kimchi fermentation and in the presence of kimchi ingredients. J. Food. Prot. 58:1215-1218.

Lovett, J. 1989. *Listeria monocytogenes*. In *Foodborne Bacterial Pathogens*, M.P. Doyle (Ed.), p. 283-310. Marcel Dekker, Inc., New York

Lucke, F.K. 1985. Fermented sausages. In *Microbiology of Fermented Foods, vol 2*, B.J.B. Wood (Ed.), p. 41-84. Elsevier Applied Science Publishers, New York.

Palumbo, S.A. 1989. Injury in emerging foodborne pathogens and their detection. Ch. 4 In *Injured index and pathogenic bacteria*, B. Ray (Ed.). CRC Press, Boca Raton, FL.

Patel, J.R., Hwang, C.A., Beuchat, L.R., Doyle, M.P., and Brackett, R.E. 1995. Comparison of oxygen scavengers for their ability to enhance resuscitation of heat-injured *Listeria monocytogenes*. J. Food Prot. 58:244-250.

Patel, J.R. and Beuchat, L.R. 1995. Evaluation of enrichment broths for their ability to recover heat-injured *Listeria monocytogenes*. J. Appl. Bacteriol. 78:366-372.

Pothuri, P., Marshal, D.L., and McMillin, K.W. 1995. Combined effects of packaging atmosphere and lactic acid on the growth and survival of *Listeria monocytogenes*

in crayfish tail meat at 4°C. J. Food Prot. 59:253-256.

Ray, B (Ed.). 1989. *Injured Index and Pathogenic Bacteria.* CRC Press, Inc., Boca Raton, Fl.

Ray, B. 1993. Sublethal injury, bacteriocins, and food microbiology. ASM News. 59:285-291.

Reido, F.X., Pinner, R.W., de Lourdes Tosca, M., Cartter, M.L., Graves, L.M., Reeves, M.W., Weaver, R.E., Plikaytis, D., and Broom, C.V. 1994. A point-source foodborne listeriosis outbreak: documented incubation period and possible mild illness. J. Infect. Dis. 170:693-696.

Renterghem, B.V., Huysman, F., Rygole, R., and Verstreate, W. 1991. Detection and prevalence of *Listeria monocytogenes* in the agricultural ecosystem. J. Appl. Bacteriol. 71:211-217.

Rocourt, J. and Bille, J. 1997. Foodborne listeriosis. Rapp. Trimest. Statist. Sanit. Mond. 50:67-73

Rorvik, L.M. and Yndestad, M. 1991. *Listeria monocytogenes* in foods in Norway. Int. J. Food. Microbiol. 13:97-104.

Ryser, E.T. and Marth, E.H.. 1991. *Listeria*, Listeriosis, and Food Safety. Marcel Dekker, Inc., New York.

Sabel, D., Yousef, A.E., and Marth, E.H. 1991. Behaviour of *Listeria monocytogenes* during fermentation of beaker sausage made with or without a starter culture and antioxidant food additives. Lebensm.-Wiss.-Technol.-Food-Sci.-Technol. 24:252-255.

Salamina, G., Dalle-Donne, E., Niccolini, A., Poda, G., Cesaroni, D., Bucci, M., Fini, R., Maldini, M., Schuchat, A., Swaminathan, B., Bibb, W., Rocourt, J., Binkin, N., and Salamaso, S. 1996. A foodborne outbreak of gastroenteritis involving *Listeria monocytogenes*. Epidemiol. Infect. 117:429-436.

Schlech, W.F. 1997. Listeria gastroenteritis- old syndrome, new pathogen. New Eng. J. Med. 336:130-132.

Schoeni, J.L., Brunner, K., and Doyle, M.P. 1991. Rates of thermal inactivation of *Listeria monocytogenes* in beef and fermented beaker sausage. J. Food Prot. 54:334-337.

Schuchat, A., Swaminathan, B., and Broome, C.V. 1991. Epidemiology of human listeriosis. Clin. Microbiol. Rev. 4:169-183.

Schwartz, B., Hexter, D., Broome, C.V., Hightower, A.W., Hirschorn, R.B., Porter, J.D., Hayes, P.S., Bibb, W.F., Lorber, B., and Faris, D.G. 1989. Investigation of an outbreak of listeriosis: new hypotheses for the etiology of epidemic *Listeria monocytogenes* infections. J. Infect. Dis. 159:680-685.

Shaack, M.M. and Marth, E.H. 1988. Survival of *Listeria monocytogenes* in refrigerated cultured milks and yogurt. J. Food Prot. 51:848-852.

Smith, J.L. and Archer, D.L. 1988. Heat-induced injury in *Listeria monocytogenes*. J. Indust. Microbiol. 3:105-110.

Trott, D., Seneviratna, P., and Robertson, J. 1991. *Listeria* in cooked chicken, pate, and mixed smallgoods. Austral. Vet. J. 68(7):249-250.

Varabioff, Y. 1992. Incidence of *Listeria* in smallgoods. Lett. Appl. Microbiol. 14:167-169.

Ward, O.P. 1989. Fermentation biotechnology. Prentice Hall, Englewood Cliffs, N.J.

Wilson, I.G. 1995. Occurrence of *Listeria* species in ready to eat foods. Epidemiol. Infect. 115:519-526.

Wimpfheimer, L., Altman, N.S., and Hotchkiss, J.H. 1990. Growth of *Listeria monocytogenes* Scott A, serotype 4 and competitive spoilage organisms in raw chicken packaged under modified atmospheres and in air. Int. J. Food Microbiol. 11:205-214.

Part II

Suitability of selective media for recovery and enumeration of sublethally heat- and acid-injured *Listeria monocytogenes*

ABSTRACT

The suitability of PALCAM and modified Oxford (MOX) agars for recovering sublethally heat- and lactic acid-injured *Listeria monocytogenes* was investigated. *L. monocytogenes* LM101M, LM103M (meat isolates), and Scott A were suspended in tryptose phosphate broth (TPB), heated for up to 40 min at 54°C, and surface plated onto tryptose phosphate agar (TPA), TPA + 4% NaCl (TPAS), PALCAM, and MOX. TPA and TPAS were used to determine total viable and sublethally injured populations, respectively. Heat-injured LM103M was recovered in the highest numbers on all media, followed by Scott A and LM101M ($P<0.01$). TPA allowed best recovery of all test strains, followed by PALCAM and MOX, which were not different, and TPAS ($P<0.01$). For acid-injury studies, uninjured and heat-injured (54°C for 20 min) test strains were suspended in phosphate-buffered TPB + 0.85% lactic acid (bTPBLA) at 25°C for up to 24 h and plated as described above. Uninjured and heat-injured *L. monocytogenes* were recovered better from bTPBLA on MOX than on PALCAM ($P<0.05$). Heat injured *L. monocytogenes* LM103M was recovered better than LM101M but similar to Scott A on MOX and PALCAM ($P<0.05$), whereas Scott A was recovered similarly to LM101M and LM103M on MOX and PALCAM ($P>0.05$). Acid-injury of *L. monocytogenes* LM103M was enhanced by prior heat stress.

INTRODUCTION

The ability to detect sublethally injured foodborne pathogens is crucial for ensuring the safety of ready-to-eat foods. Food processing treatments, such as heating and acidification, have been shown to induce sublethal injury in *Listeria monocytogenes* (Ahmad and Marth, 1990; Golden et al., 1988b; Smith and Archer, 1988). Sublethally injured organisms have strictly defined nutrient requirements which may cause increased sensitivity to various components of selective/differential media (Ray, 1989). As a result, sublethally injured *L. monocytogenes* may grow poorly or not at all on selective media that are used for the detection of pathogens. Upon placement into an appropriate environment, however, sublethally injured foodborne pathogens may repair, become viable, and present a health threat (Ray, 1993). This scenario is supported by research findings. McCarthy (1991) reported that heat-stressed *L. monocytogenes* may be less pathogenic than nonstressed *L. monocytogenes*. However, upon resuscitation, heat-stressed *L. monocytogenes* regains pathogenicity equivalent to that of nonstressed cells (McCarthy, 1991). Additionally, under routine testing, a food product may appear to be free of a particular pathogen, but upon storage, may become unsafe for consumption.

It is important to determine injury characteristics and factors that influence recovery of sublethally injured foodborne pathogens from processed food products in order to aid in their detection. In this pursuit, investigators have demonstrated the ability of heating (Golden et al., 1988b; Smith and Archer, 1988) and acidification (Ahmad and Marth, 1990) to produce injury in *L. monocytogenes.* These investigations indicate that sublethal injury of *L. monocytogenes* is quite variable and may be dependent on the type of

sublethal stress as well as strain variation. Other studies have investigated the ability of various media to select for and recover sublethally injured *L. monocytogenes* (Amoaka et al., 1992; Curtis and Lee, 1995; Golden et al., 1988a; Gunasinghe et al., 1994; Mackey et al., 1994; Patel and Beuchat, 1995). Generally, these investigations have concluded that selective media commonly used for the isolation and enumeration of *L. monocytogenes* do not effectively recover sublethally injured *L. monocytogenes* without time-consuming enrichment steps. Although the heat and acid-injury characteristics of *L. monocytogenes* have been described in some detail, research to date inadequately has addressed the question of recovery of *L. monocytogenes* (via direct plating) subjected to combined stresses of heating and acidification.

This study was undertaken to investigate the suitability of PALCAM and modified Oxford agars to recover heat-injured *L. monocytogenes* and heat-injured *L. monocytogenes* that are further subjected to exposure to lactic acid.

MATERIALS AND METHODS

Cultures and Culture Maintenance

L. monocytogenes Scott A (serotype 4b, human isolate), LM101M (serotype 4, meat isolate), and LM103M (serotype 1, meat isolate) were used. Test strains were grown in tryptose phosphate broth (TPB) at 37°C and were subjected to a minimum of two successive 24-h transfers before use.

Heat Injury

Cultures were heated and enumerated using protocols described by Golden et al.

(1988b). One milliliter of a 24-h culture of *L. monocytogenes* was inoculated into 99 ml sterile TPB, throughly mixed to ensure adequate dispersal of cells, and dispensed (3 ml) into 13 x 100 ml screw-cap test tubes. Tubes were placed into a test tube rack with sufficient space between tubes for water circulation in a shaker waterbath adjusted to 54°C. An electronic temperature recorder was placed into one tube to determine the time required for the temperature of the liquid in the test tubes to equilibrate with the temperature of the waterbath (come-up time). Test samples were heated at 54°C with samples being withdrawn at 10-min intervals for up to 40 min. Heated cell suspensions were serially diluted in 0.1% peptone water, surfaced plated (0.1 ml) onto tryptose phosphate agar (TPA; Difco Laboratories, Detroit, MI), TPA + 4% NaCl (TPAS), PALCAM agar (Oxoid, Inc., Ogdensburg, NY), and modified Oxford agar (MOX; *Listeria* selective agar base [Oxford formulation] plus selective supplements; Oxoid, Inc., Ogdensburg, NY) to compare the ability of these media to recover heat-injured *L. monocytogenes*. Furthermore, TPA and TPAS were used to determine total viable and sublethally injured populations. Media were prepared according to manufacturer instructions, including addition of selective supplements per instructions for preparation of PALCAM and MOX. Sublethal injury was determined by the inability of heat-stressed cells to form colonies on TPAS in comparison to their ability to form colonies on TPA and was calculated as the difference between TPA and TPAS counts, divided by counts from TPA. All plating media were incubated aerobically at 30°C for 72 h before *L. monocytogenes* was enumerated.

Acid-injury

Studies were conducted to investigate the recovery of heat-injured and uninjured *L. monocytogenes* from acidified TPB. Titratable acidity and pH of dry, fermented salami were determined, and TPB was adjusted to simulate the pH and acidity (as lactic acid) of the salami.

Uninjured and heat-injured (54°C for 20 min) *L. monocytogenes* Scott A, LM101M, and LM103M were inoculated into buffered TPB (bTPB; pH 6.9) and bTPB containing 0.85% lactic acid (pH 4.7), and samples were withdrawn for plating at intervals during 24-h incubation at 25°C. Portions of test samples were serially diluted in 0.1% peptone water and surface plated (0.1 ml) onto TPA, TPAS, PALCAM, and MOX and incubated as described above. Sublethal injury of strains exposed to lactic acid was determined as described for determining heat injury.

Statistical Analysis

This experiment was performed in triplicate. Statistical analysis was conducted using SAS version 6.1 (SAS Institute; Cary, NC) to determine differences in recovery of uninjured and injured test strains on TPA, TPAS, PALCAM, and MOX. Data were fit to a randomized block design, blocked on replication, and were analyzed using PROC MIXED.

RESULTS AND DISCUSSION

Heat-injury (Fig. 1)

Results of this investigation reveal that recovery of *L. monocytogenes* on selective media is influenced by type of sublethal injury (i.e. heat vs. heat + acid injury) and strain variation. Sorqvist (1994) reported that differences in heat resistance of *L. monocytogenes* strains were found between serovars and strains of the same serovar. Results of our study indicate that *L. monocytogenes* LM103M and Scott A were more resistant to heating than LM101M ($P<0.01$). *L. monocytogenes* LM103M, Scott A , and LM101M total viable populations decreased 0.82, 1.15, and 1.56 log CFU/ml, respectively, upon heating at 54°C for 40 min in TPB. Differences in development of heat-induced injury as indicated by the difference between counts on TPA (non-selective) and TPAS (selective) were also observed. After 40 min of heating, viable populations of *L. monocytogenes* LM103M, Scott A, and LM101M consisted of 71.6, 97.8, and 98.9% injured cells, respectively. Golden et al. (1988) also reported differences in development of heat- injury between strains of *L. monocytogenes*. Reasons for variation in resistance to heating and development of sublethal injury among strains are unclear. However, the environment from which isolates are recovered may play a role in enhancing or reducing heat-resistance and resistance to development of injury.

Generally, methods for recovery of *L. monocytogenes* from foods include enrichment procedures followed by plating onto selective media. The ability to recover sublethally heat-injured *L. monocytogenes* via direct plating onto selective media commonly used for isolation of *L. monocytogenes* from food samples was of interest in

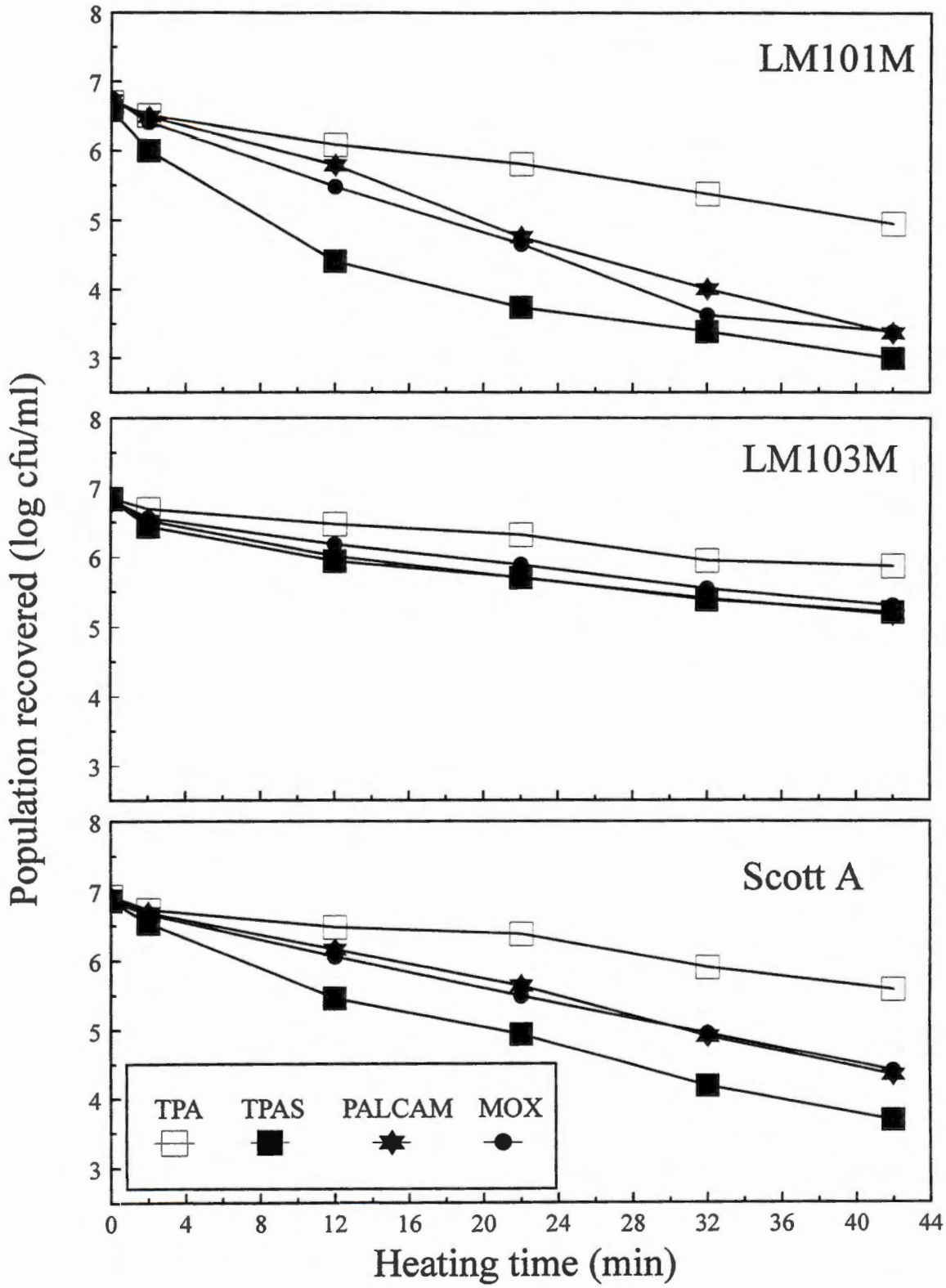

Figure 1. Recovery of *L. monocytogenes* Scott A, LM101M, and LM103M on various media after heating strains at 54°C in Tryptose phosphate broth.

this study. Overall, recovery of heat-stressed test strains followed the order TPA > MOX = PALCAM > TPAS ($P<0.01$). While this indicates that there are no differences overall between MOX and PALCAM for their ability to recover all strains of heat-stressed *L. monocytogenes*, there were differences in recovery of individual strains for each medium. Recovery of test strains on PALCAM followed the order LM103M = Scott A > LM101M ($P<0.01$), while recovery of test strains on MOX followed the order LM103M > Scott A > LM101M ($P<0.01$). It is important to note that, while neither PALCAM nor MOX displayed a clear advantage in recovering heat stressed *L. monocytogenes*, both media inadequately recovered injured *L. monocytogenes* cells.

Acid-injury (Fig. 2, 3, and 4)

Overall, uninjured test strains incubated in bTPBLA were recovered similarly on each medium, with the exception that recovery of LM103M was poorer on PALCAM than on other media ($P<0.05$). Heat-injured *L. monocytogenes* incubated in bTPBLA was recovered best by TPA followed by MOX, PALCAM, and TPAS ($P<0.05$). Heat-injured *L. monocytogenes* Scott A, LM101M, and LM103M populations decreased 0.99, 1.30, and 1.12 log CFU/ml, respectively, during 24-h incubation in bTPBLA, although these decreases were not statistical different ($P<0.05$).

Wang and Doyle (1996) reported that prior sublethal heat treatment (48°C for 10 min) of *Escherichia coli* O157:H7 substantially increased tolerance of the organism to subsequent exposure to acid. In the present study, we saw no evidence that prior heat treatment (54°C for 20 min) of *L. monocytogenes* enhanced acid resistance. Uninjured *L.*

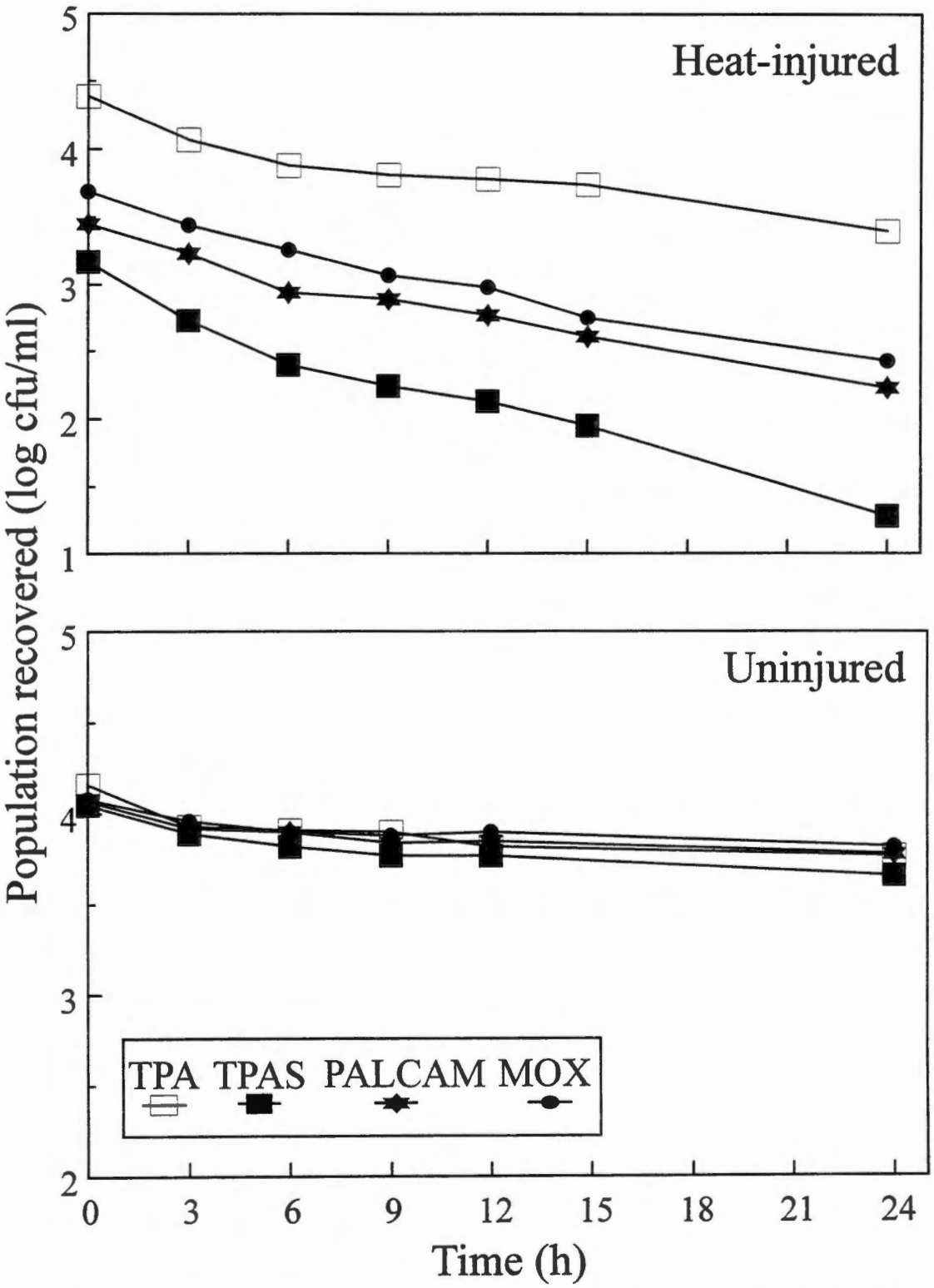

Figure 2. Recovery of heat-injured *L. monocytogenes* Scott A on various media after incubating strains at 25°C in tryptose phosphate broth containing 0.85% lactic acid.

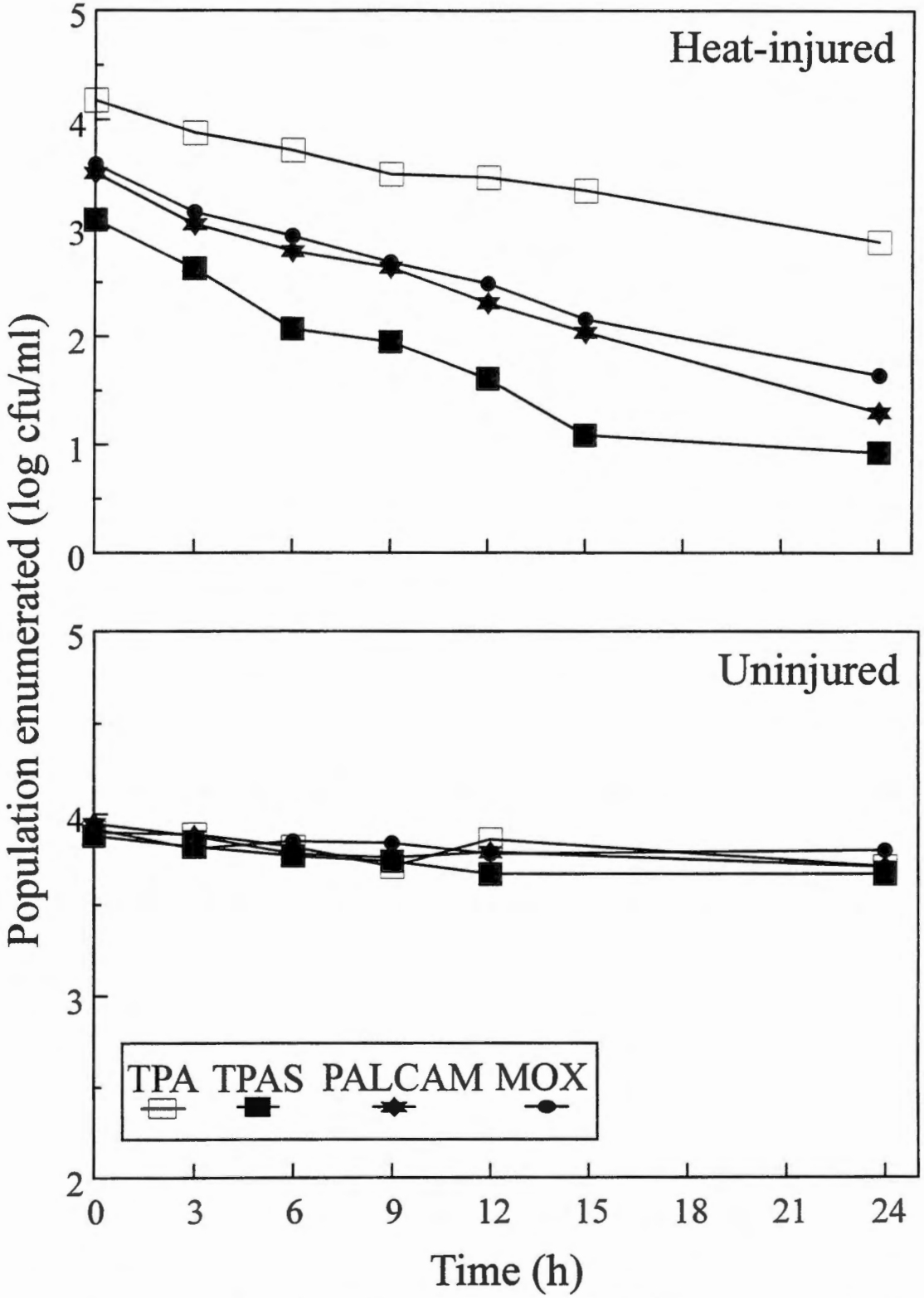

Figure 3. Recovery of heat-injured *L. monocytogenes* LM101M on various media after incubating strains at 25°C in tryptose phosphate broth containing 0.85% lactic acid.

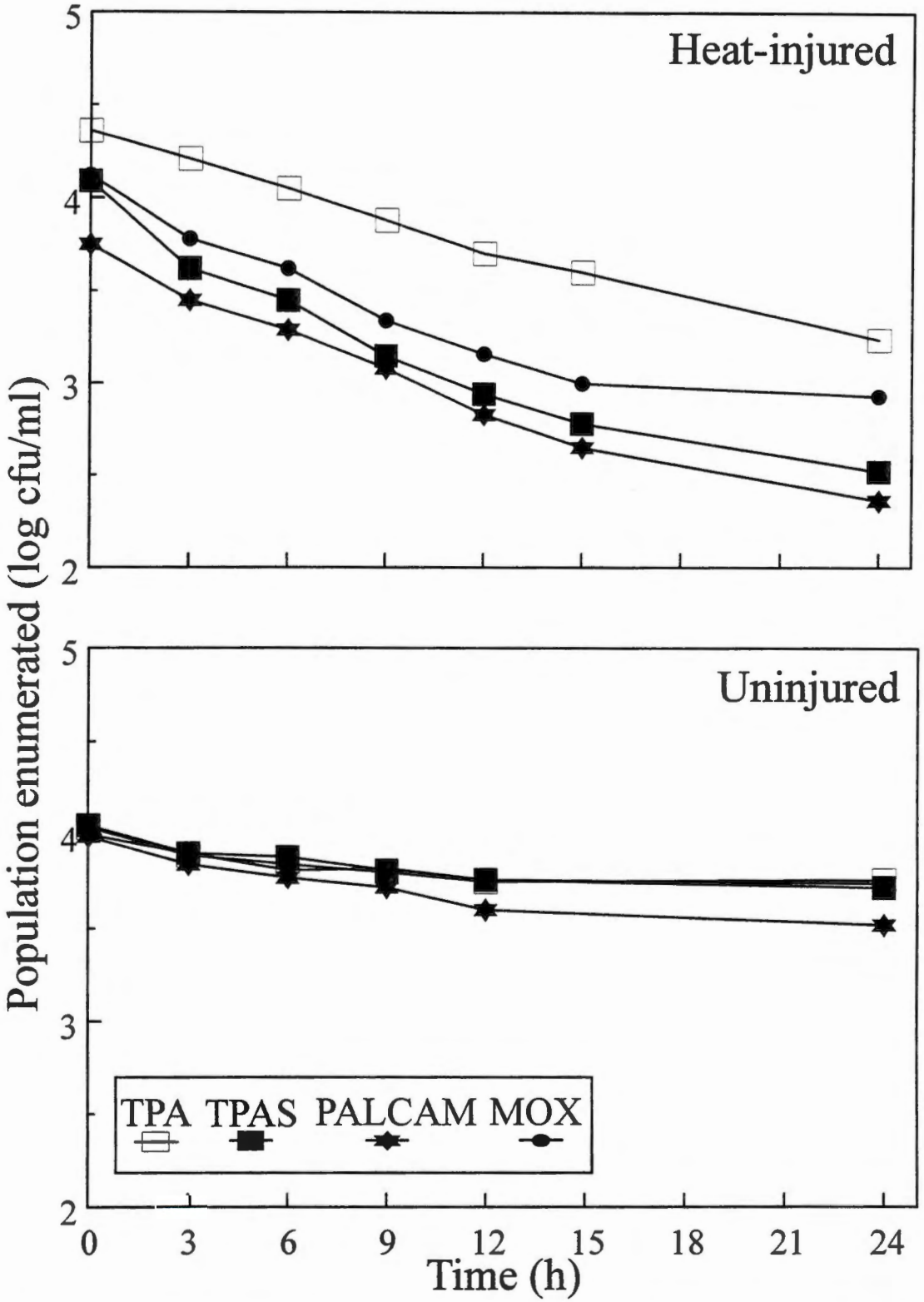

Figure 4. Recovery of heat-injured *L. monocytogenes* LM103M on various media after incubating strains at 25°C in tryptose phosphate broth containing 0.85% lactic acid.

monocytogenes Scott A, LM101M and LM103M sustained 19.5, 16, and 6.3% injury, respectively, after a 24-h exposure to lactic acid in bTPBLA. Heat-injured *L. monocytogenes* Scott A and LM101M sustained additional injury (i.e., in addition to injury resulting from heat) of 4.9 and 6.1% upon subsequent exposure to lactic acid. However, heat-injured *L. monocytogenes* LM103M sustained an additional 40.4% injury after a 24-h exposure to lactic acid, indicating that acid-tolerance of this strain was reduced by prior exposure to heat. This may have practical implications with respect to production of fermented food products such as dry fermented sausages, in which the combination of heat and acid can be used to minimize growth and/or survival of *L. monocytogenes*.

Overall (i.e., all strains combined), recovery of heat-injured *L. monocytogenes* strains was similar on MOX and PALCAM, although recovery was better on MOX than on PALCAM after heat-injured strains were incubated in bTPBLA ($P<0.05$). Additionally, recovery of acid-injured *L. monocytogenes* is better on MOX than PALCAM. These results indicate that the type of sublethal injury (i.e., heat vs. acid) plays a role in tolerance of *L. monocytogenes* to selective agents in recovery media. It should be noted, however, that, although statistically significant, the magnitude of differences in recovery on these two media was not appreciable. Furthermore, *L. monocytogenes* colonies develop more quickly on PALCAM and are more distinctive than *L. monocytogenes* colonies on MOX. The variability in recovery of *L. monocytogenes* on MOX and PALCAM indicates the need for careful selection of selective media for recovery of *L. monocytogenes*, and that consideration should be given to the type of sublethal injury to which the organism has been exposed.

REFERENCES

Ahmad, N. and Marth, E.H. 1990. Acid-injury of *Listeria monocytogenes*. J. Food Prot. 53:26-29.

Amoaka, E.B., Toora, S., Ablett, R.F., and Smith, J. 1992. Evaluation of the ability of primary selective enrichment to resuscitate heat-injured and freeze-injured *Listeria monocytogenes* cells. Appl. Environ. Microbiol. 58(9):3177-3179.

Curtis, G.D.W. and Lee, W.H. 1995. Culture media and methods for the isolation of *Listeria monocytogenes*. Int. J. Food Microbiol. 26:1-13.

Golden, D.A., Beuchat, L.R., and Brackett, R.E. 1988a. Evaluation of selective direct plating media for their suitability to recover uninjured, heat-injured, and freeze-injured *Listeria monocytogenes* from foods. Appl. Environ. Microbiol. 54(6):1451-1456.

Golden, D.A., Beuchat, L.R., and Brackett, R.E. 1988b. Inactivation and injury of *Listeria monocytogenes* as affected by heating and freezing. Food Microbiol. 5:17-23.

Gunasinghe, C.P.G.L., Henderson, C., and Rutter, M.A. 1994. Comparative study of two plating media (PALCAM and Oxford) for detection of *Listeria* species in a range of meat products following a variety of enrichment procedures. Lett. Appl. Microbiol. 18:156-158.

Mackey, B.M., Boogard, E., Hayes, C.M. and Baranyi, J. 1994. Recovery of heat-injured *Listeria monocytogenes*. Int. J. Food Microbiol. 22:227-237.

McCarthy, S.A. 1991. Pathogenicity of nonstressed, heat-stressed, and resuscitated *Listeria monocytogenes* 1A1 cells. Appl. Environ. Microbiol. 57:2389-2391.

Patel, J.R. and Beuchat, L.R. 1995. Evaluation of enrichment broths for their ability to recover heat-injured *Listeria monocytogenes*. J. Appl. Bacteriol. 78:366-372.

Ray, B. (Ed.). 1989. Injured index and pathogenic bacteria. CRC Press, Inc., Boca Raton, Fl.

Ray, B. 1993. Sublethal injury, bacteriocins, and food microbiology. ASM News 59(6):285-291.

Smith, J.L. and Archer, D.L. 1988. Heat-induced injury in *Listeria monocytogenes*. J. Ind.

Microbiol. 3:105-110.

Sorqvist, S. 1994. Heat resistance of different serovars of *Listeria monocytogenes.* J. Appl. Bacteriol. 76:383-388.

Wang, G., and Doyle, M.P. 1996. Heat shock response protects *Escherichia coli* O157:H7 against lethal acidity. Abst. Annu. Mtg. Intl. Assoc. Milk, Food, and Environ. Sanit., Seattle, WA, June 30 - July 3. p. 78.

Part III

Fate of sublethally heat-injured *Listeria monocytogenes* on tryptose phosphate agar containing 0.85% lactic acid and 2% NaCl during storage under modified atmosphere packaging

ABSTRACT

The effect of package atmosphere on survival of uninjured and sublethally heat-injured *L. monocytogenes*, inoculated onto tryptose phosphate agar containing 0.85% lactic acid and 2% NaCl (TPALAS) was investigated. Inoculated TPALAS plates were packaged in air, 100% N_2 (N_2), 30% CO_2/70% N_2 (CO_2/N_2), and vacuum and stored at 4 and 20°C for up to 31 days. Recovery of *L. monocytogenes* from TPALAS was influenced by the injury status (i.e., injured and uninjured) of the inoculum, storage atmosphere (air, N_2, CO_2/N_2, and vacuum), storage temperature (4 and 20°C), and recovery media (tryptose phosphate agar [TPA] and modified Oxford agar [MOX]) ($P<0.05$). Storage in CO_2/N_2 atmosphere was more inhibitory to uninjured *L. monocytogenes* stored at 20°C than air or vacuum, but less than N_2 ($P<0.05$). When sublethally heat-injured *L. monocytogenes* was stored at 20°C, CO_2/N_2 atmosphere allowed better recovery than N_2 ($P<0.05$) but was not different than air ($P>0.05$). At 4°C, uninjured and sublethally heat-injured *L. monocytogenes* were recovered in highest numbers from samples packaged in N_2 followed by CO_2/N_2 ($P>0.05$). Overall, non-selective TPA allowed greater recovery of *L. monocytogenes* than the selective MOX ($P<0.05$). Uninjured cells stored at 4°C were recovered better than sublethally heat-injured cells on TPA ($P<0.05$). On TPA, *L. monocytogenes* stored under air was recovered better than *L. monocytogenes* stored under N_2 or CO_2/N_2 ($P<0.05$). However, recovery on MOX was best when *L. monocytogenes* was stored under N_2 ($P<0.05$).

INTRODUCTION

Listeria monocytogenes has been recovered from many foods, including raw beef, raw pork, milk, and cabbage (Jay, 1992). These foods are commonly fermented for the purpose of preservation or to impart desired organoleptic characteristics. The aim of acid-type fermentations is to increase the level of acid (e.g., lactic acid) in the fermentation mixture while decreasing pH to yield a product with desirable characteristics and increased inhibition of spoilage microorganisms (Booth and Kroll, 1989).

Production of fermented foods includes acidification of raw or pasteurized products via the acid-producing action of starter cultures or naturally occurring microflora and may include further processing treatments such as heating (Jay, 1992). Food processing treatments, including heating and acidification, have been shown to induce sublethal injury in *L. monocytogenes* (Ahmad and Marth, 1990; Golden et al., 1988; Smith and Archer, 1988). Sublethally injured organisms may exhibit increased sensitivity to various components of selective/differential media because of increased nutrient requirements (Ray, 1989). As a result, sublethally injured *L. monocytogenes* may grow poorly or not at all on selective media used for its detection. Upon placement into an appropriate environment, sublethally injured *L. monocytogenes* may repair, grow, and present a health threat (Ray, 1993). Heat-stressed *L. monocytogenes* has been shown to be less pathogenic than nonstressed *L. monocytogenes*, but regains pathogenicity equal to nonstressed cells upon resuscitation in an appropriate environment (McCarthy, 1991). Consequently, under routine testing, a food product may appear to be free of *L. monocytogenes*, but upon storage, may become unsafe for consumption.

Modified atmosphere packaging (MAP) has been shown to affect survival of *L. monocytogenes* in a variety of food products (Chen and Hotchkiss, 1993; Francis and O'Beirne, 1997; Gill and Reichel, 1989; Grau and Vanderline, 1990; Pothuri et al., 1995). *L. monocytogenes* was inhibited by packaging atmospheres comprised of high carbon dioxide levels (35 to 100%) in packaged cottage cheese (Chen and Hotchkiss, 1993), beef (Gill and Reichel, 1989), and crayfish tail meat (Pothuri et al., 1995) when stored at about 5°C or below. However, one study reported that an atmosphere containing 75% CO_2 did not effect survival of *L. monocytogenes* in packaged cabbage stored at 5°C (Kallander et al., 1991). *L. monocytogenes* survival was enhanced by an atmosphere comprised of 96% N_2/4% O_2 in packaged lettuce stored at 3°C (Francis and O'Beirne, 1997), but not affected by 97% N_2/3% O_2 atmosphere in packaged lettuce stored at 5 or 10°C (Beuchat and Brackett, 1990). Thus, influence of MAP on survival and growth of *L. monocytogenes* may be affected by composition of atmosphere and storage conditions. The influence of MAP on sublethally injured *L. monocytogenes* in fermented foods has not been extensively investigated.

Several investigations have shown the ability of *L. monocytogenes* to survive during production and storage of fermented foods, including fermented meats (Buncic et al., 1991; Farber et al., 1993; Glass and Doyle, 1989; Johnson et al., 1988), fermented milks (Ashenafi, 1994; Dalu and Feresu, 1996), kimchi (Lee et al., 1995), and tempeh (Ashenafi, 1991). *L. monocytogenes* grew during the fermentation of milk, kimchi, and tempeh, but generally declined during storage, with low levels of *L. monocytogenes* persisting. Although growth of *L. monocytogenes* was not observed during either

production or storage of fermented sausages, low levels of *L. monocytogenes* persisted in the fermented meat products, even after lengthy storage.

L. monocytogenes may undergo injury during production of fermented foods due to exposure to stresses such as acidification and heating. Sublethal injury of *L. monocytogenes* may complicate detection of this microorganism in fermented foods and may result in underestimation or missed detection of *L. monocytogenes*. The influence of packaging atmosphere and storage temperature on resuscitation of sublethally injured *L. monocytogenes* from fermented foods is not known. Therefore, the objective of this study was to determine the fate of sublethally heat-injured *L. monocytogenes* at 4 and 20°C on media formulated to simulate fermented food products and packaged with four different modified atmospheres.

MATERIALS AND METHODS

Culture Maintenance

L. monocytogenes LM103M (serotype 1, meat isolate) was used in this study because of its demonstrated tolerance to heat and lactic acid (Williams and Golden, 1997). The culture was maintained in tryptose phosphate broth (TPB; Difco, Detroit, MI) through transfer at 24-h intervals and incubation at 37°C.

Heat Injury

L. monocytogenes LM103M was heated to produce greater than 90% injury using protocols described by Golden et al. (1988). A healthy 24-h, 37°C culture of *L. monocytogenes* LM103M, grown in TPB, was thoroughly mixed and dispensed in 3-ml

portions into ten 13 x 100 ml test tubes. These tubes were placed in a test tube rack with sufficient space between tubes to allow for water circulation and placed into a shaker waterbath (Dubnoff Metabolic Shaking Incubator; Precision Scientific, Chicago, IL) that had been previously set to 54°C and 90 rpm. An electronic thermometer was placed into one 13 x 100 ml test tube to determine the time at which the temperature of the contents reached equilibrium with the temperature of the waterbath. Upon reaching 54°C (~ 2 min equilibration time) cultures were heated at 54°C for 20 min. After 20 min, the test tubes were immediately submerged in an ice-water bath until test tube contents reached approximately room temperature (~ 25°C). After cooling, the contents of the test tubes were recombined and centrifuged twice at 10,000 rpm for 10 min, then washed with 0.1% peptone water (PW). After the final washing, cultures were resuspended in 0.1% PW. The purpose of dividing the test culture among ten 13 x 100 ml test tubes was to facilitate heat transfer during heating and cooling.

Preparation of Test Media

A nutrient medium was formulated to simulate the pH, acidity (as lactic acid), and NaCl content of a typical fermented food product. Buffered tryptose phosphate agar containing 0.85% lactic acid and 2.0% NaCl (TPALAS) was prepared by combining TPB (29.5 g), agar (15 g), NaCl (15 g), $K_2HPO_4 \cdot 3H_20$ (15.02 g), KH_2PO_4 (4.76 g), and deionized water (1000 ml). The medium was thoroughly mixed and brought to boiling on a hot plate/stirrer, sterilized in an autoclave at 121°C for 15 min, and cooled to about 55°C. Upon cooling, 85% lactic acid syrup (DL-Lactic Acid; Acros) was added and the medium was poured into 60x15 mm Petri dishes. The final medium (TPALAS, pH 4.8±

0.1) contained 0.85% lactic acid and 2% NaCl (Note: TPB inherently contains 0.5% NaCl).

Fate of Sublethally-Injured *L. monocytogenes* on TPALAS

An uninjured *L. monocytogenes* LM103M control was prepared as described for the heat-injured culture, with the omission of the heating step. The uninjured, washed cell suspension was diluted ten-fold in 0.1% PW prior to inoculation. Uninjured and injured cell suspensions were dispensed separately (0.1 ml) onto TPALAS. Prior to packaging, three holes (approx. 0.4 cm in diameter) were burned in the lid of each TPALAS plate with a Creative Woodburner, Jr. (Walnut Hollow; Dodgeville, WI) to facilitate gas exchange. Inoculated TPALAS plates were packaged in atmospheres of air, 100% nitrogen (N_2), 30% carbon dioxide/70% nitrogen (CO_2/N_2), and vacuum in Cryovac B-Series High Barrier Bags (Sealed Air-Cryovac; Duncan, S.C.) using a Multivac A300 Packaging Machine (Multivac; Wolfertschweden, Germany). Inoculated and packaged plates were incubated at 4 and 20°C. "Samples" were plated initially, after day 1, and then every other day throughout 31 days of storage. At each sampling interval, packaged samples were removed from storage and headspace gases were withdrawn from each package, through adhesive septum tape (Modern Controls; Minneapolis, MN) applied to each package, using a 5 cc syringe and 20 gauge needle. Gas samples were analyzed for O_2, CO_2, and N_2 using a Hewlett-Packard 5890 Series II gas chromatograph fitted with an Alltech CTR-1 column (Alltech; Deerfield, IL) and a thermal conductivity detector.

Microbiological Examination

Sample packages previously subjected to headspace gas analysis were opened and

plates were removed for microbiological examination. The entire block of agar from each sample plate was aseptically transferred to a stomacher bag, diluted in 50 ml PW, and pummeled for 2 min using a Stomacher 400 Lab Blender (Seward Medical, London). Pummeled samples were serially diluted (1/10) in PW and surface plated (0.1 ml) onto tryptose phosphate agar (TPA; Difco, Detroit, MI) and modified Oxford media (MOX; Oxoid, Ogdensberg, NY). Inoculated media were incubated at 30°C for 72 h before colony counts were obtained. Colony counts were reported as $\log_{10}$ CFU/cm^2 of TPALAS surface. The limit of detection by direct plating was calculated to be 0.95 $\log_{10}$ CFU/cm^2 using the plating scheme described. When *L. monocytogenes* was no longer recovered by direct plating, test samples were subjected to enrichment procedures. Test samples were pummeled, as described above, in 50 ml *Listeria* enrichment broth base (minus selective supplements) (UVM formulation; Oxoid, Ogdensburg, NY) and incubated at 30°C for 48 h. Enriched samples were pummeled for 1 min and inoculated onto MOX using a 10μl loop. Inoculated MOX plates were incubated at 30°C for 72 h.

Statistical Analysis

This experiment was performed in triplicate. The design of this experiment was a randomized block design, split-plot, with a whole plot factorial treatment arrangement, blocked on replication. Data were analyzed using Proc Mixed (SAS versions 6.12 and 7.00beta; Cary, N.C.).

RESULTS

It is possible that the storage conditions themselves used for this study could result in sublethal injury of *L. monocytogenes*. However, use of "uninjured" in the text that follows refers to the physiological state of *L. monocytogenes* prior to inoculation onto TPALAS, to differentiate this inoculum from that subjected to heat-injury.

Neither uninjured nor sublethally heat-injured *L. monocytogenes* grew under any of the storage conditions used in this study. Overall, populations of uninjured *L. monocytogenes* were recovered better than populations of sublethally heat-injured cells ($P<0.05$). Sublethally heat-injured *L. monocytogenes* stored at 4°C and recovered on TPA (Fig. 1), decreased from 4.33 $\log_{10}$ CFU/cm^2 by 2.99, 3.00, and 2.77 $\log_{10}$ CFU/cm^2 in air, N_2, and CO_2/N_2, respectively, over 31 days of storage, but was not detected by direct plating from vacuum packaged samples after 23 days. Sublethally heat-injured *L. monocytogenes* stored at 4°C was not detectable using MOX (Fig. 2) after 19, 29, and 19 days in air, N_2, and vacuum, respectively, and decreased from 3.51 $\log_{10}$ CFU/cm^2 by 2.39 $\log_{10}$ CFU/cm^2 in CO_2/N_2 over 31 days of storage. Uninjured *L. monocytogenes* stored at 4°C and recovered on TPA (Fig. 3) decreased from 5.42 $\log_{10}$ CFU/cm^2 by 2.11, 1.87, 2.08, and 2.88 $\log_{10}$ CFU/cm^2, in air, N_2, CO_2/N_2, and vacuum respectively, over 31 days storage. Uninjured *L. monocytogenes* stored at 4°C (Fig. 4), recovered on MOX decreased from 5.43 $\log_{10}$ CFU/cm^2 by 3.44, 2.69, 3.44, and 4.42 $\log_{10}$ CFU/cm^2, in air, N_2, CO_2/N_2, and vacuum, respectively, over 31 days storage.

Sublethally heat-injured *L. monocytogenes* stored at 20°C and recovered on TPA (Fig. 5) decreased from 4.33 $\log_{10}$ CFU/cm^2 to the limit of detection (0.95 $\log_{10}$ CFU/cm^2)

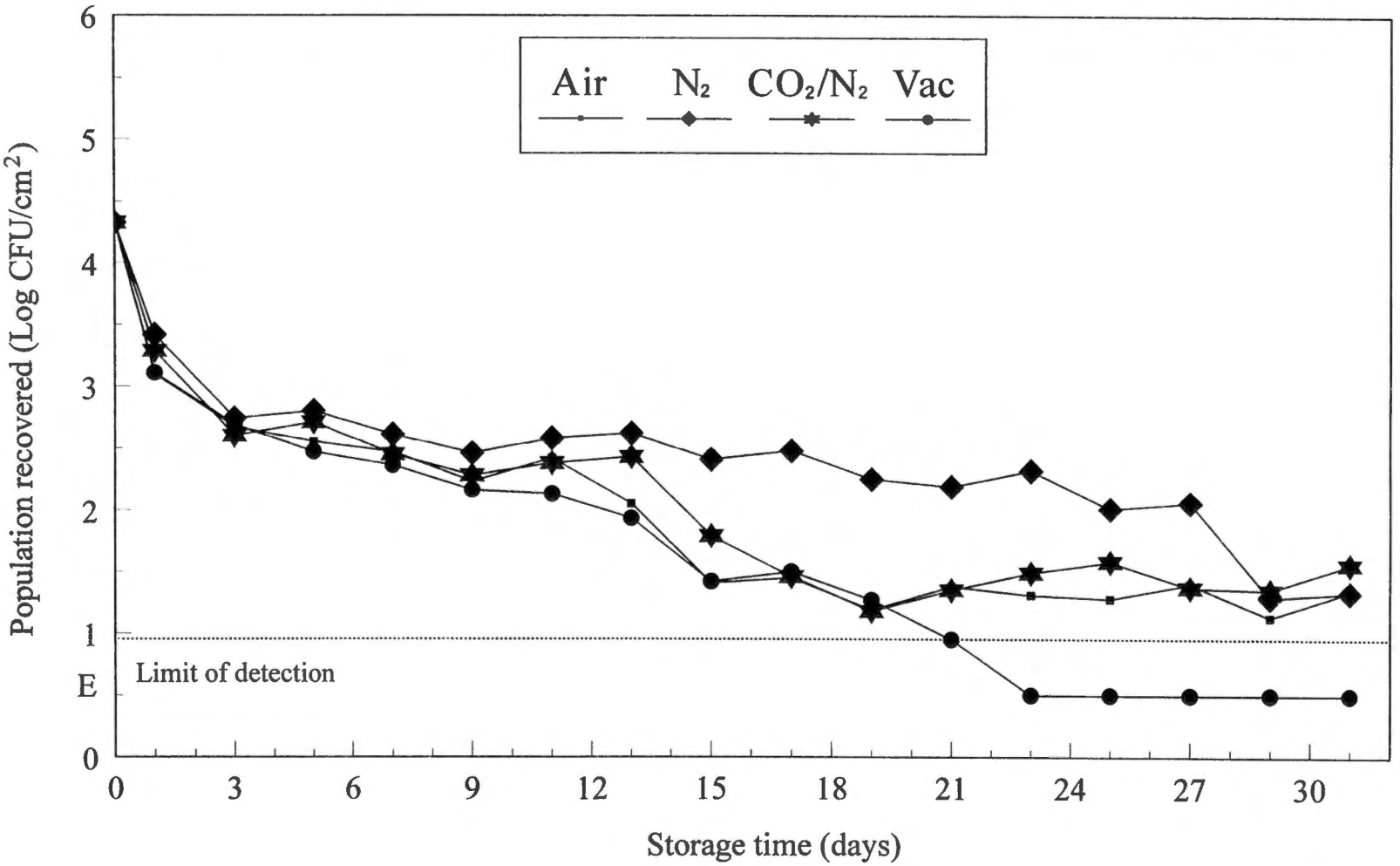

Figure 1. Survival of sublethally heat-injured *L. monocytogenes* on TPALAS media stored under air, 100% N_2, 30% CO_2/70% N_2, and vacuum at 4°C for up to 31 days and recovered on TPA. E = recovered after enrichment.

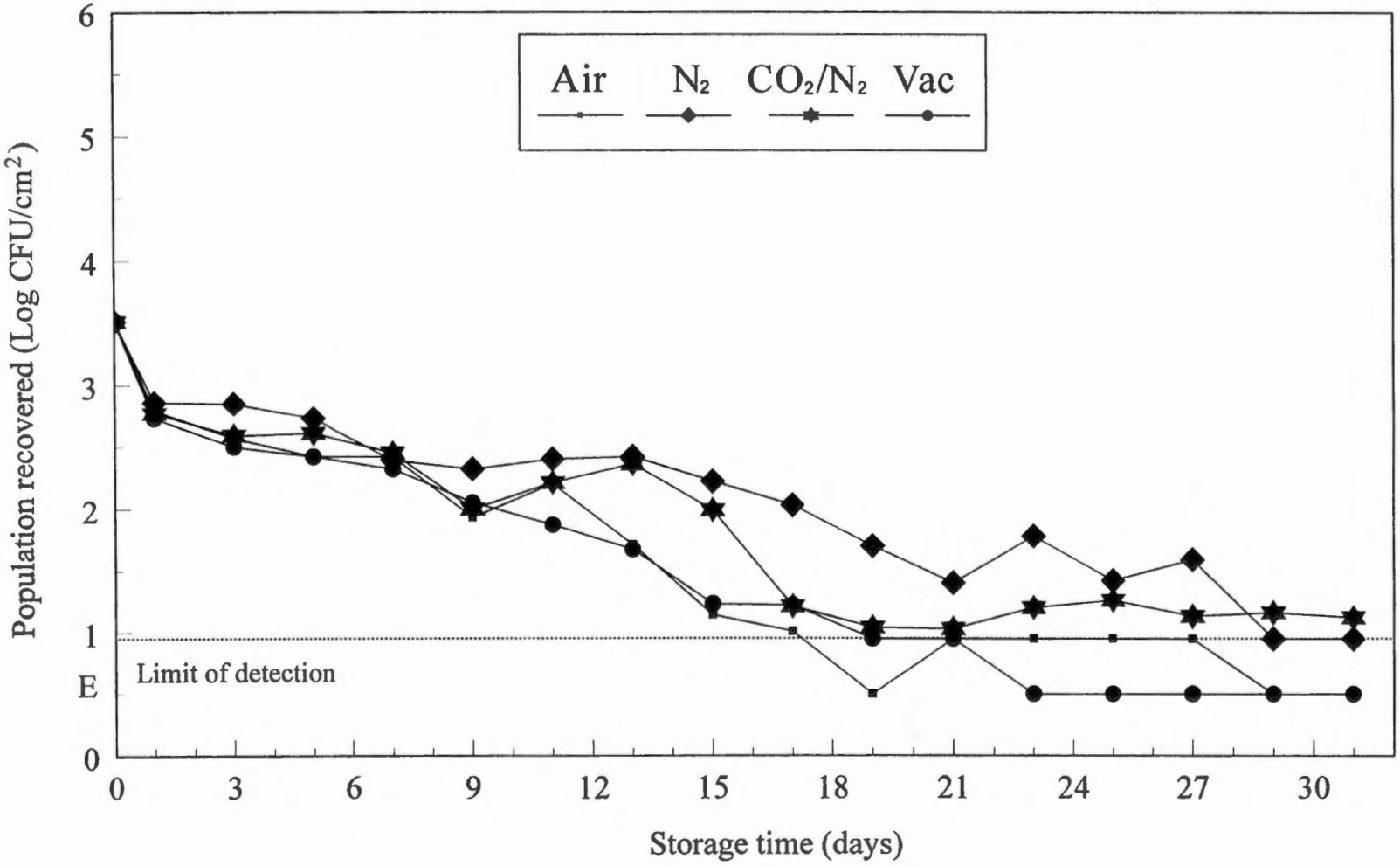

Figure 2. Recovery of sublethally heat-injured *L. monocytogenes* on MOX from TPALAS media stored under air, 100% N_2, 30% CO_2/70% N_2, and vacuum at 4°C for up to 31 days. E = recovered after enrichment.

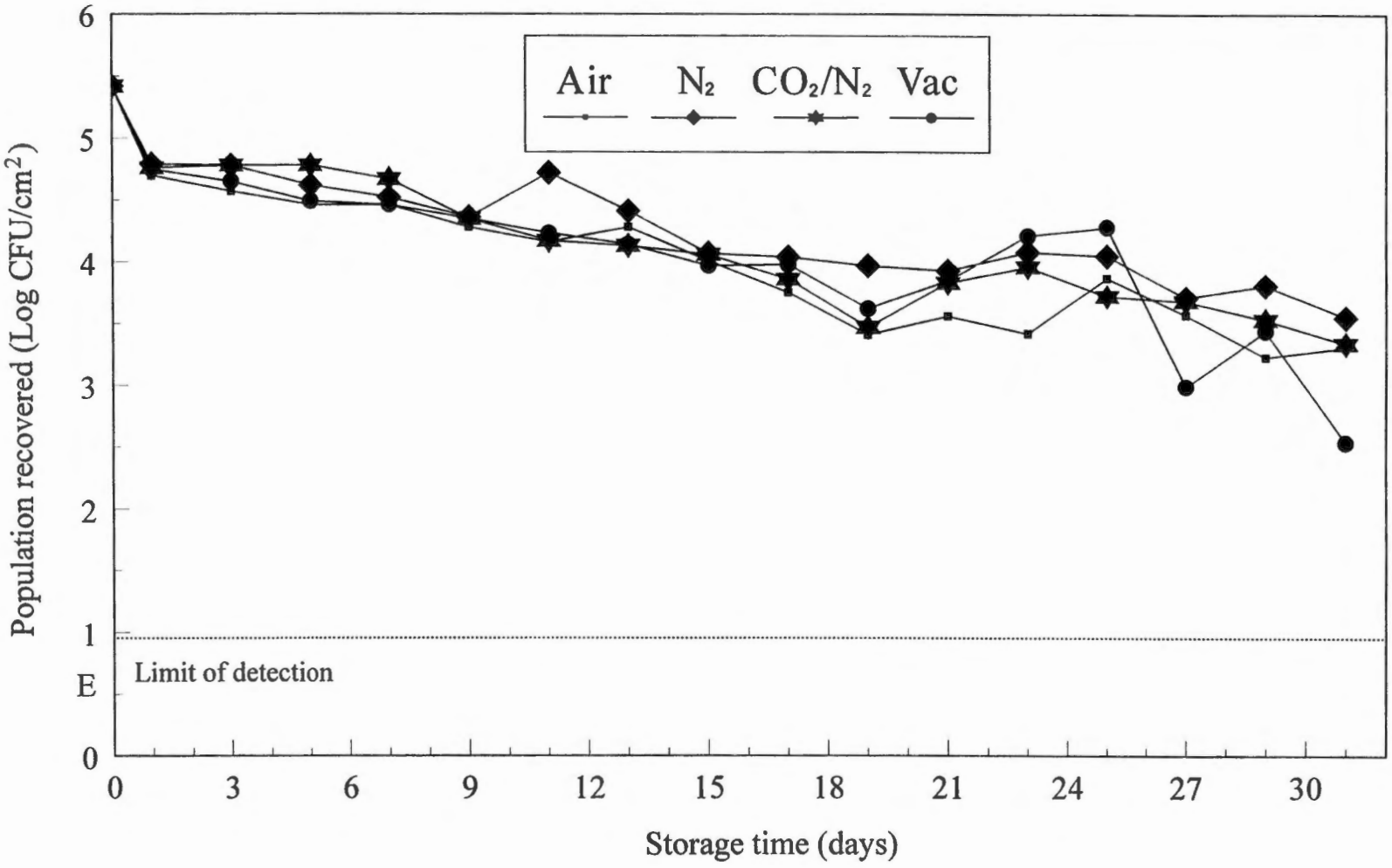

Figure 3. Survival of uninjured *L. monocytogenes* on TPALAS media stored under air, 100% N_2, 30% CO_2/70% N_2, and vacuum at 4°C for up to 31 days and recovered on TPA. E = recovered after enrichment.

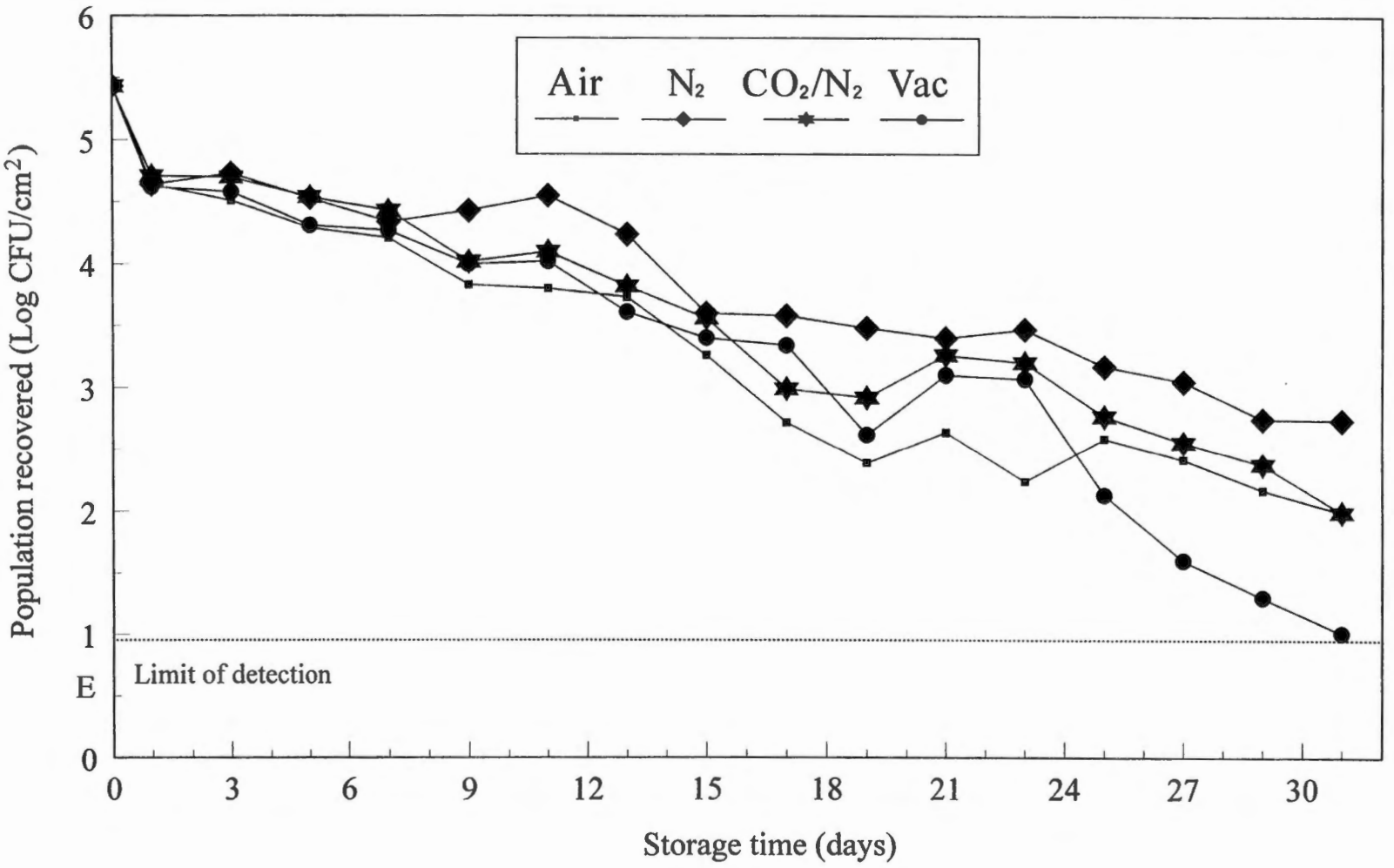

Figure 4. Recovery of uninjured *L. monocytogenes* on MOX from TPALAS media stored under air, 100% N_2, 30% CO_2/70% N_2, and vacuum at 4°C for up to 31 days. E = recovered after enrichment.

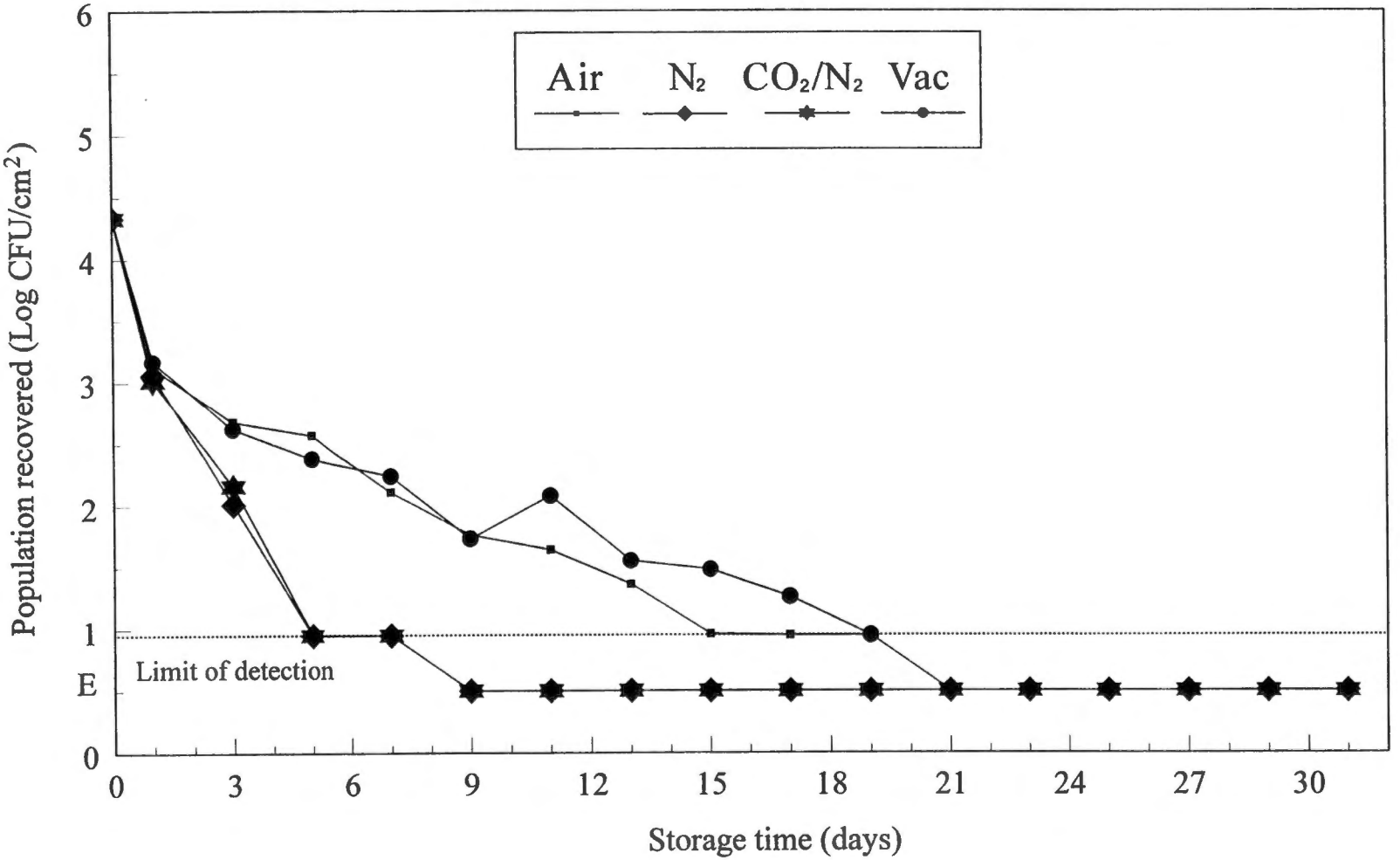

Figure 5. Survival of sublethally heat-injured *L. monocytogenes* on TPALAS media stored under air, 100% N_2, 30% CO_2/70% N_2, and vacuum at 20°C for up to 31 days and recovered on TPA. E = recovered after enrichment.

after 17, 5, 5, and 19 days, in air, N_2, CO_2/N_2, and vacuum, respectively. Sublethally heat-injured *L. monocytogenes* stored at 20°C decreased from 3.51 $\log_{10}$ CFU/cm^2 to undetectable levels in 17, 5, 5, and 19 days, in air, N_2, CO_2/N_2, and vacuum, respectively, as indicated by recovery on MOX (Fig. 6) Uninjured *L. monocytogenes* stored at 20°C and recovered on TPA (Fig. 7), decreased from 5.42 $\log_{10}$ CFU/cm^2 to levels not detectable by direct plating after 25, 13, and 15 days in air, N_2, and CO_2/N_2, respectively, and decreased 3.91 $\log_{10}$ CFU/cm^2 under vacuum over 31 days. Using MOX, counts for uninjured *L. monocytogenes* stored at 20°C (Fig. 8) decreased from 5.43 $\log_{10}$ CFU/cm^2 to non-detectable levels after 27, 13, 15, and 31 days in air, N_2, CO_2/N_2, and vacuum, respectively. Regardless of storage temperature and package atmosphere, *L. monocytogenes* was detected after enrichment from all samples through 31 days of storage, with the exceptions that sublethally heat-injured *L. monocytogenes* stored at 20°C under CO_2/N_2 and N_2 were not recovered by enrichment after 23 and 21 days, respectively.

Overall, air and vacuum atmospheres allowed greater survival of uninjured and heat-injured *L. monocytogenes* than N_2 and CO_2/N_2 atmospheres ($P<0.05$), and storage at 4°C allowed greater survival than storage at 20°C ($P<0.05$). Survival of uninjured *L. monocytogenes* followed the order vacuum > air > $CO_2/N_2 = N_2$ ($P<0.05$), while survival of sublethally heat-injured *L. monocytogenes* followed the order air > CO_2/N_2 ($P<0.05$) (other combinations did not differ [$P>0.05$]). Uninjured *L. monocytogenes* stored at 4°C was recovered on TPA better than sublethally heat-injured *L. monocytogenes* stored at 4°C ($P<0.05$). Overall, survival of *L. monocytogenes,* as indicated by recovery on TPA followed the order air > $CO_2/N_2 = N_2$ (note: air = vacuum, vacuum = CO_2/N_2 and N_2),

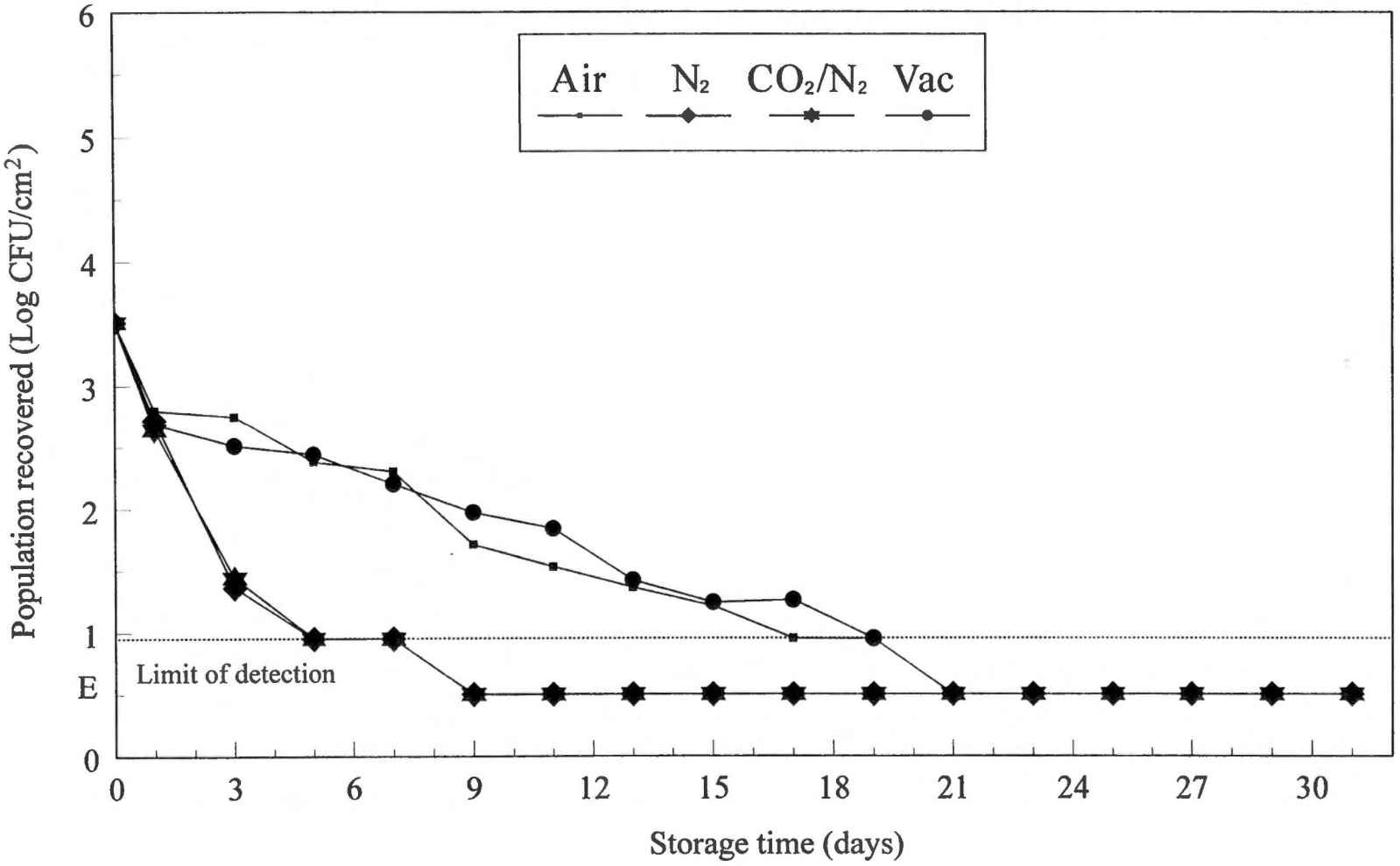

Figure 6. Recovery of sublethally heat-injured *L. monocytogenes* on MOX from TPALAS media stored under air, 100% N_2, 30% CO_2/70% N_2, and vacuum at 20°C for up to 31 days. E = recovered after enrichment.

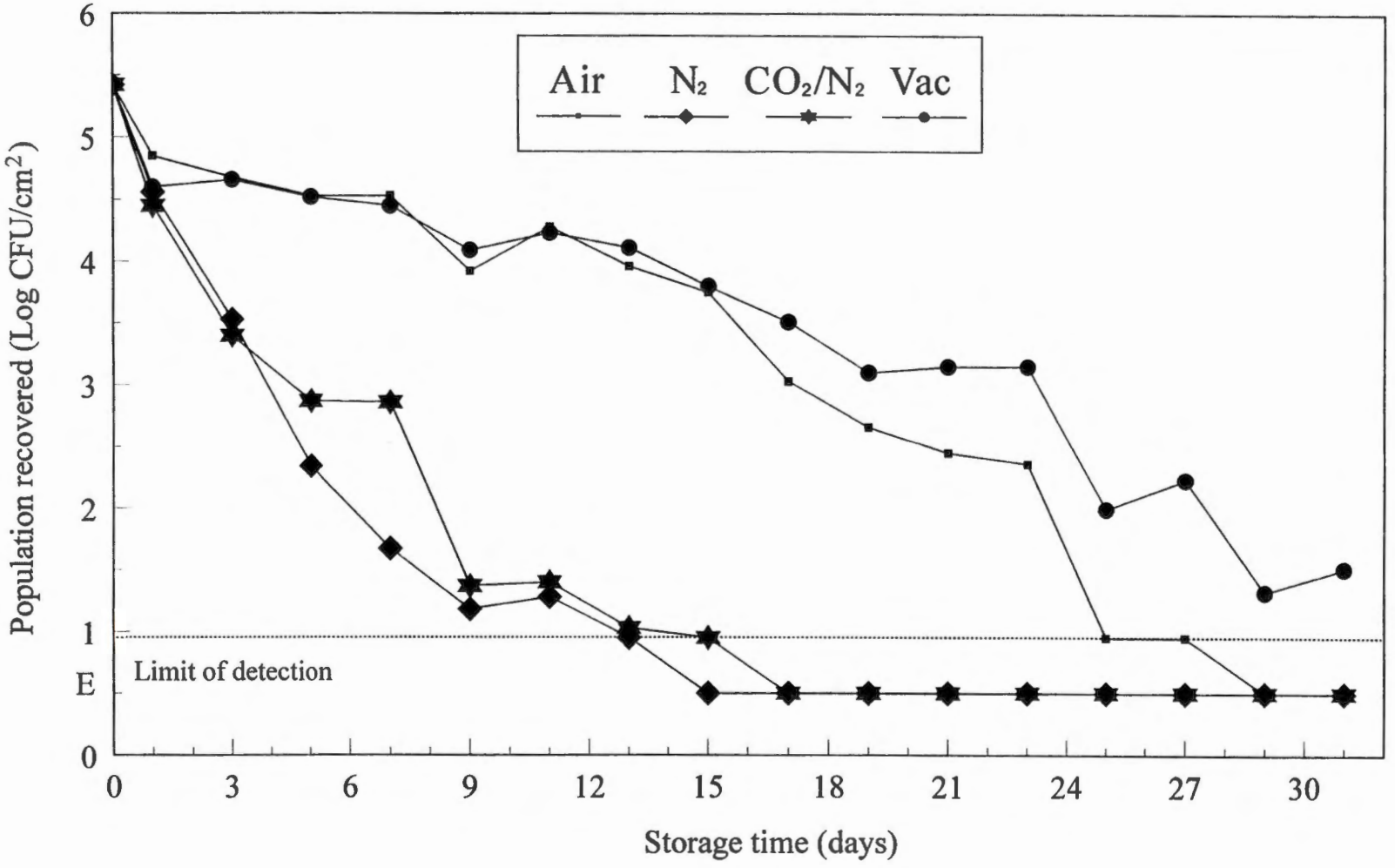

Figure 7. Survival of uninjured *L. monocytogenes* on TPALAS media stored under air, 100% N_2, 30% CO_2/70% N_2, and vacuum at 20°C for up to 31 days and recovered on TPA. E = recovered after enrichment.

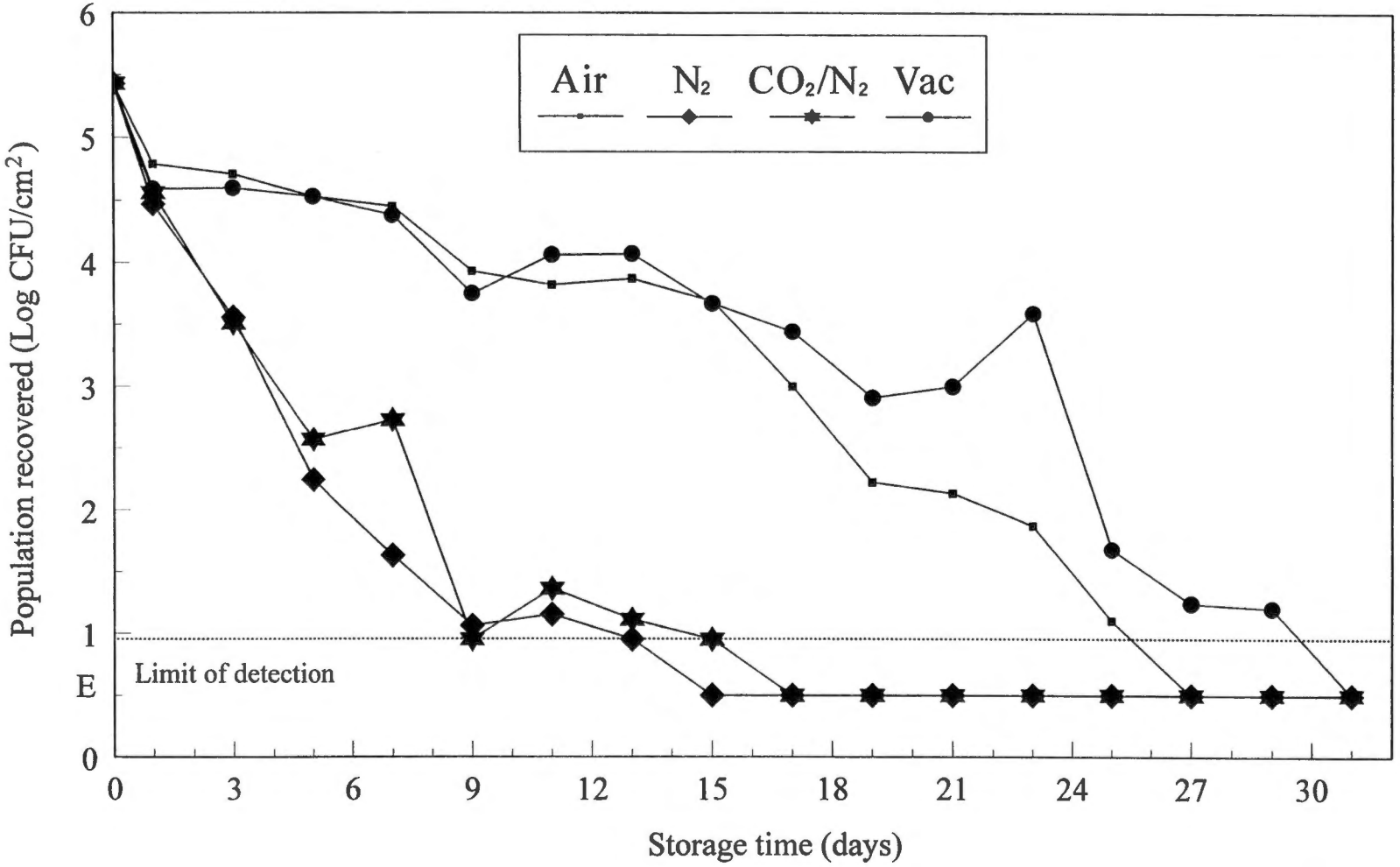

Figure 8. Recovery of previously uninjured *L. monocytogenes* on MOX from TPALAS media stored under air, 100% N_2, 30% CO_2/70% N_2, and vacuum at 20°C for up to 31 days. E = recovered after enrichment.

while recovery on MOX followed the order N_2 > vacuum and air, CO_2/N_2 > air (note: CO_2/N_2 = vacuum, vacuum = air) ($P<0.05$). Recovery of sublethally heat-injured *L. monocytogenes* stored at 4°C followed the order N_2 > CO_2/N_2 > air > vacuum ($P<0.05$), whereas recovery of uninjured *L. monocytogenes* stored at 4°C followed the order N_2 > CO_2/N_2 > vacuum > air ($P<0.05$). Recovery of sublethally heat-injured *L. monocytogenes* stored at 20°C followed the order vacuum > CO_2/N_2 > N_2 (note: air = CO_2/N_2 and vacuum) ($P<0.05$), whereas recovery of uninjured *L. monocytogenes* stored at 20°C followed the order vacuum > air > CO_2/N_2 > N_2 ($P<0.05$). Uninjured *L. monocytogenes* stored under N_2 at 4°C was recovered best, whereas sublethally heat-injured *L. monocytogenes* stored under N_2 at 20°C was recovered poorest ($P<0.05$).

DISCUSSION

Survival of *L. monocytogenes* on TPALAS is influenced by the injury status (i.e., injured and uninjured) of the inoculum, storage atmosphere (air, N_2, CO_2/N_2, and vacuum), storage temperature (4 and 20°C), and recovery media (TPA and MOX) ($P<0.05$). Research has shown that *E. coli* O157:H7 in acidic environments survives better at temperatures near refrigeration than at higher temperatures. Zhao et al. (1993) reported that *E. coli* O157:H7 in apple cider stored at 8°C survived for 31 days, but survived for only 3 days in apple cider stored at 25°C. Likewise, Fisher and Golden (1998) reported that *E. coli* O157:H7 survived better in apple cider 4°C than 12°C. In mayonnaise based sauces (pH <4.5), *E. coli* O157:H7 survived for 35 days when stored under refrigeration. Results of the present study show that *L. monocytogenes* in an acidic environment (i.e.,

TPALAS) survives better at 4°C than 20°C.

Previous studies have shown the inhibitory effect of high levels of CO_2 in package headspace gases coupled with refrigerated storage on survival of *L. monocytogenes*. Pothuri et al. (1995) found that an atmosphere comprised of 74.8% CO_2/ 10.4% O_2/ 14.8% N_2 inhibited *L. monocytogenes* on crayfish tail meat stored at 4°C better than air or vacuum. *L. monocytogenes* was unable to grow in cottage cheese at 4°C when stored under an atmosphere containing 35% CO_2 but grew under the same atmosphere at 7°C (Chen and Hotchkiss, 1993). *L .monocytogenes* was inhibited in beef at 5°C when packaged in a 100% CO_2 atmosphere (Gill and Reichel, 1989). Wimpfheimer et al. (1990) reported that *L. monocytogenes* growth was inhibited on raw chicken by an atmosphere of 75% CO_2/ 25% O_2 at 4, 10, and 27°C, but was not inhibited by an atmosphere of 72.5% CO_2/ 22.5% N_2/ 5% O_2. The latter modified atmosphere, while not inhibitory to *L. monocytogenes*, did inhibit spoilage microflora. In the present study, storage in CO_2/N_2 atmosphere was more inhibitory to uninjured *L. monocytogenes* stored at 20°C than air or vacuum, but less than N_2 ($P<0.05$). However, when sublethally heat-injured *L. monocytogenes* was stored at 20°C, CO_2/N_2 atmosphere allowed better recovery than N_2 ($P<0.05$), but was not different than air ($P>0.05$). At 4°C, uninjured and sublethally heat-injured *L. monocytogenes* were recovered in highest numbers from samples packaged in N_2 followed by CO_2/N_2 ($P>0.05$).

While this data demonstrates the influence of injury status, temperature of storage, and gas atmosphere on the recovery of *L. monocytogenes*, media used for recovery are also critical. Recovery media intended for the selection of *L. monocytogenes* also influence

recovery of *L. monocytogenes*. Overall, non-selective TPA allowed greater recovery of *L. monocytogenes* than the selective MOX ($P<0.05$). This is likely due to increased sensitivity of *L. monocytogenes* to selective agents found in MOX as a result of previous sublethal heat-injury or possible acid injury induced during storage on TPALAS. The temperature at which samples were stored and the status of the inocula prior to inoculation of TPALAS affected recovery on TPA and MOX. After storage at 4°C, uninjured cells were recovered better than sublethally heat-injured cells on TPA ($P<0.05$). It should be noted that sublethally heat-injured and uninjured inocula were likely acid-injured as a result of the acidic TPALAS environment. The atmosphere under which samples were stored also affected recovery of *L. monocytogenes*. On TPA, *L. monocytogenes* stored under air was recovered better than *L. monocytogenes* stored under N_2 or CO_2/N_2 ($P<0.05$). However, recovery on MOX was best when *L. monocytogenes* was stored under N_2 ($P<0.05$).

Results of this study demonstrate the variability in recovery of *L. monocytogenes* on selective vs. non selective media. Survival of *L. monocytogenes* on media formulated to simulate fermented food products is influenced by injury status, storage atmosphere, and storage temperature. It is concluded that factors involved in the production, storage, and distribution of fermented foods must be thoroughly evaluated when determining strategies for control and detection of *L. monocytogenes* in such products.

REFERENCES

Ahmad, N. and Marth, E.H. 1990. Acid-injury of *Listeria monocytogenes*. J. Food Prot. 53:26-29.

Ashenafi, M. 1991. Growth of *Listeria monocytogenes* in fermenting tempeh made from various beans and its inhibition by *Lactobacillus plantarum*. Food Microbiol. 8:303-310.

Ashenafi, M. 1994. Fate of *Listeria monocytogenes* during the souring of ergo, a traditional Ethiopian fermented milk. J. Dairy Sci. 77:696-702.

Beuchat, L.R. and Brackett, R.E. 1990. Survival and growth of *Listeria monocytogenes* on lettuce as influenced by shredding, chlorine treatment, modified atmosphere packaging and temperature. J. Food Sci. 55: 755-758, 870.

Booth, I.R. and Kroll, R.G. 1989. The preservation of foods by low pH. In *Mechanisms of Action of Food Preservation Procedures*, G.W. Gould (Ed.), p. 119-160. Elsevier Science Pub., New York.

Buncic, S., Paunovic, L., and Radisic, D. 1991. The fate of *Listeria monocytogenes* in fermented sausages and in vacuum-packaged frankfurters. J. Food Prot. 54:413-417.

Chen, J.H. and Hotchkiss, J.H. 1993. Growth of *Listeria monocytogenes* and *Clostridium sporogenes* in cottage cheese in modified atmosphere packaging. J. Dairy Sci. 76:972-977.

Dalu, J.M. and Feresu, S.B. 1996. Survival of *Listeria monocytogenes* in three Zimbabwean fermented milk products. J. Food Prot. 59:379-383

Farber, J.M., Daley, E., Holley, R., and Usborne, W.R. 1993. Survival of *Listeria monocytogenes* during the production of uncooked German, American, and Italian-style fermented sausages. Food Microbiol. 10:123-132.

Fisher, T.L. and Golden, D.A. 1998. Fate of *Escherichia coli* O157:H7 in ground apples used in cider production. J. Food Prot. (in press).

Francis, G.A. and O'Beirne, D. 1997. Effects of gas atmosphere, antimicrobial dip and temperature on the fate of *Listeria innocua* and *Listeria monocytogenes* on minimally processed lettuce. Int. J. Food Sci. and Technol. 32:141-151.

Gill, C.O. and Reichel, M.P. 1989. Growth of cold-tolerant pathogens *Yersinia*

enterocolitica, *Aeromonas hydrophila*, and *Listeria monocytogenes* on high-pH beef packaged under vacuum or carbon dioxide. Food Microbiol. 6:223-230.

Glass, K.A., and Doyle, M.P. 1989. Fate and thermal inactivation of *Listeria monocytogenes* in beaker sausage and pepperoni. J. Food Prot. 52:226-231.

Golden, D.A., Beuchat, L.R., and Brackett, R.E. 1988. Inactivation and injury of *Listeria monocytogenes* as affected by heating and freezing. Food Microbiol. 5:17-23.

Jay, J.M. 1992. Modern Food Microbiology, 4th ed. Van Nostrand Reinhold, New York.

Johnson, J.L, Doyle, M.P., Cassens, R.G., and Schoeni, J.L. 1988. Fate of *Listeria monocytogenes* in tissues of experimentally infected cattle and in hard salami. Appl. Environ. Microbiol. 54:497-501.

Kallander, K.A., Hitching, A.D., Linette, G.A., Schmieg, J.A., Garcia, G.R., Solomon, H.M., and Sofos, J.N. 1991. Fate of *Listeria monocytogenes* in shredded cabbage stored at 5 and 25°C under a modified atmosphere. J. Food Prot. 54:302-304.

Lee, S.H., Kim, M.K., and Frank, J.F. 1995. Growth of *Listeria monocytogenes* Scott A during kimchi fermentation and in the presence of kimchi ingredients. J. Food. Prot. 58:1215-1218.

McCarthy, S.A. 1991. Pathogenicity of nonstressed, heat-stressed, and resuscitated *Listeria monocytogenes* 1A1 cells. Appl. Environ. Microbiol. 57:2389-2391.

Pothuri, P., Marshal, D.L., and McMillin, K.W. 1995. Combined effects of packaging atmosphere and lactic acid on the growth and survival of *Listeria monocytogenes* in crayfish tail meat at 4°C. J. Food Prot. 59:253-256.

Ray, B. 1993. Sublethal injury, bacteriocins, and food microbiology. ASM News. 59:285-291.

Ray, B (ed.). 1989. Injured Index and Pathogenic Bacteria. CRC Press, Inc., Boca Raton, Fl.

Smith, J.L. and Archer, D.L. 1988. Heat-induced injury in *Listeria monocytogenes*. J. Industr. Microbiol. 3:105-110.

Weagent, S.D., Bryant, J.L., and Bark, D.H. 1994. Survival of Escherichia coli O157:H7 in mayonnaise and mayonnaise-based sauces at room and refrigerated temperatures. J. Food Prot. 57:629-631.

Williams, R.C. and Golden, D.A. 1997. Suitability of selective media for recovery and enumeration of sublethally heat- and acid-injured *L. monocytogenes*. J. Food Prot. 60(Suppl. A):33.

Wimpfheimer, L., Altman, N.S., and Hotchkiss, J.H. 1990. Growth of *Listeria monocytogenes* Scott A, serotype 4 and competitive spoilage organisms in raw chicken packaged under modified atmospheres and in air. Int. J. Food Microbiol. 11:205-214.

Zhao, T., Doyle, M.P., and Besser, R.E. 1993. Fate of enterohemorrhagic Escherichia coli O157:H7 in apple cider with and without preservatives. Appl. Environ. Microbiol. 59:2526-2530.

APPENDIX

Statistical Analyses

Part II — Suitability of Selective Media for Recovery and Enumeration of Sublethally Heat- and Acid-injured *Listeria monocytogenes*

Statistical analysis was conducted using SAS version 6.12 (SAS Institute; Cary, NC) to determine differences in recovery of uninjured and injured test strains on TPA, TPAS, PALCAM, and MOX. Data were fit to a randomized block design, blocked on replication, and were analyzed using PROC MIXED.

SAS program for Heat-Injury studies:

```
options ls=75 ps= 66;
data; input time rep media strain count;
cards;
;
proc mixed;
class time rep media strain;
model count= time | media | strain /predicted;
lsmeans time | media | strain /pdiff;
make 'predicted' out = rrr noprint;
make 'lsmeans' out = mmm noprint;
make 'diffs' out = ppp noprint;
run;
proc univariate plot normal data = rrr;
var _resid_;
run;
%include 'c:\sas\pdmix612.sas';
%pdmix612 (ppp,mmm);
run;
```

SAS program for Lactic Acid-Injury studies:

```
options ls=75 ps=66;
data one;
input stn $ inoc $ med $ tm $ rep $ cnt;
```

```
cards;
;
proc mixed;
 class inoc med stn tm rep;
 model cnt= stn | inoc | med
     tm(inoc) | stn | med/predicted;
 random rep rep*stn*inoc;
lsmeans stn | inoc | med
  tm(inoc)/pdiff;
 make 'predicted' out=rrr noprint;
 make 'lsmeans' out=mmm noprint;
 make 'diffs' out=ppp noprint;
run;
%include 'c:\sas\pdmix612.sas';
%pdmix612 (ppp,mmm);
proc univariate plot normal data=rrr;
 var _resid_;
run;
```

Part III -- Fate of Sublethally Heat-Injured *Listeria monocytogenes* on Tryptose Phosphate Agar Containing 0.85% Lactic Acid and 2% NaCl During Storage Under Modified Atmosphere Packaging.

Statistical analysis was conducted using SAS versions 6.12 and 7.00beta (SAS Institute; Cary, NC) to determine differences in recovery of uninjured and sublethally heat-injured test cultures on TPA and MOX as influenced by storage atmosphere and temperature. Data were fit to a randomized block design, blocked on replication, split-plot, with factorial treatment arrangement under the whole plot were analyzed using PROC MIXED and PROC GLM.

Note: There were numerous computer/software difficulties in analyzing the data from this study. Problems stemmed from a large data model and memory allocation difficulties in SAS.

SAS program for Modified Atmosphere Packaging studies:

Analysis by DAY:

```
data one; infile 'mapsas3.dat';
input day $ atms $ temp $ inoc $ med $ rep $ count;
run ;
proc sort; by rep inoc atms temp day med;
proc means noprint; by rep inoc atms temp day med;
var count; output out=xxx mean=mcount;
run;
proc sort; by day;
proc mixed covtest noprofile; by day;
class day atms temp inoc med rep;
 model mcount = inoc | atms | temp| med   ;
 random rep  rep*inoc*atms*temp;
 lsmeans inoc|atms|temp|med/pdiff;
 make 'lsmeans' out=mmm noprint;
 make 'diffs' out=ppp noprint;
run;
%pdmix612(ppp,mmm);
options fullstimer;
```

Full analysis including DAY:

```
data one; infile 'mapsas3.dat';
input day $ atms $ temp $ inoc $ med $ rep $ count;
run  ;
proc sort; by rep inoc atms temp day med;
proc means noprint; by rep inoc atms temp day med;
var count; output out=xxx mean=mcount;
run;
proc mixed covtest noprofile;
class day atms temp inoc med rep;
```

```
 model mcount = inoc | atms | temp | day | med@4  ;
 random rep  rep*inoc*atms*temp*day;
 parms (.1281) (.1662) (.07209)/noiter;
 lsmeans inoc|atms|temp|day|med@4/pdiff;
 ods listing exclude diffs;
 ods listing exclude lsmeans;
 ods output diffs=ppp;
 ods output lsmeans=mmm;
run;
%pdmix700(ppp,mmm);
```

Analysis to overcome memory difficulties:

```
data one; infile 'mapsas3.dat';
input day $ atms $ temp $ inoc $ med $ rep $ count;
run  ;
proc sort; by rep inoc atms temp day med;
proc means noprint; by rep inoc atms temp day med;
var count; output out=xxx mean=mcount;
run;
proc means noprint data=xxx; by rep inoc atms temp day;
 var mcount;
 output out=yyy mean=wpcount;
run;
proc means noprint data=xxx;
 var mcount;
 output out=zzz mean=overall;
run;

******part one*********;
proc glm data=yyy;
 class rep;
 model wpcount=rep;
 output out=rrr r=rcount;
run;
data rrr; merge rrr zzz;
 retain om;
 if _n_=1 then om=overall;
 mcount=rcount+om;
run;
proc glm data=rrr;
title2 'WP means adjusted for rep analysis';
```

```
class day atms temp inoc rep;
 model mcount = inoc | atms | temp | day ;
 lsmeans inoc|atms|temp|day/stderr  ;
run;

********part two*********;
proc glm data=xxx;
 title2 'Adjust out whole plot';
 class day atms temp inoc rep;
 model mcount = rep*inoc*atms*temp*day;
 output out=rrr r=rcount;
run;
data rrr; merge rrr zzz;
 retain om;
 if _n_=1 then om=overall;
 mcount=rcount+om;
run;
proc glm data=rrr;
 title2 'Analyze subplot after whole plot removed';
 class day atms temp inoc med;
 model mcount = med*day|med*atms|med*temp|med*inoc med;
 lsmeans med inoc*med|atms*med|temp*med|day*med@4/stderr ;
run;
/* */
```

Pdmix612.sas* was provided courtesy of Dr. Arnold Saxton.

*Saxton, A.M. 1988. A macro for converting mean separation output to letter groupings in PROC MIXED. In SAS 1998. Proceedings of the 23rd Annual SAS Users Group International Conference. pp. 1243-1246.

VITA

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