EFFECT OF MODIFIED ATMOSPHERE PACKAGING ON GROWTH OF *LISTERIA MONOCYTOGENES* AND NONPROTEOLYTIC *CLOSTRIDIUM BOTULINUM* IN COOKED TURKEY

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(ABSTRACT)

The growth of *Listeria monocytogenes* and nonproteolytic *Clostridium botulinum* type B spores in cooked, uncured turkey was investigated separately under varying conditions of modified atmosphere packaging (MAP), refrigerated and temperature-abuse storage, and lactic acid bacteria (LAB) competition. *L. monocytogenes* (LM) growth was suppressed when initially outnumbered 3.5 logs:1 or 2.1 logs:1 by naturally-occurring LAB, but not when the initial LAB:LM population ratio was equivalent. Lowering storage temperature from 10° to 4^oC enhanced the inhibitory effect of CO_2 in the packaging atmosphere, and extended the period of product olfactory acceptability. When the LAB:LM population ratio was equivalent, objectionable odors were not detected in most of the samples, despite LAB counts in excess of 10^8/g. This raises concerns with respect to public health, since high levels of *L. monocytogenes* can be present in MAP meat and poultry products without accompanying signs of overt spoilage.

Cellular fatty acid (CFA) analysis was a valuable tool for distinguishing between phenotypically distinct isolates of LM inoculated into MAP turkey. Fatty acid composition of foodborne outbreak-associated (serotype 4) and non-outbreak-associated (serotype 1) strains of LM correlated with antigenic type (4 or 1) and agglutination reaction (granular or flocculent). Strain ATCC 43256 (serotype 4b) produced a consistently unique CFA profile, making it the easiest of the four test strains to be differentiated. Analysis of additional LM serotypes, as well as examination of existing clinical and environmental CFA databases for correlations between fatty acid profiles and diagnostic characteristics of LM, is necessary before CFA analysis can be

effectively applied as an epidemiological tool for tracking the distribution of LM strains in food products and throughout the farm-to-table food chain.

Reduced storage temperature significantly delayed botulinal toxin production and extended the period of olfactory acceptability of cooked turkey, but even strict refrigeration did not prevent growth and toxigenesis by nonproteolytic *C. botulinum* type B. Toxin was detected on day 7 for product stored at $15^{\circ}C$ and on day 14 for product stored at $10^{\circ}C$, irrespective of packaging atmosphere. At $4^{\circ}C$, toxin was detected on day 14 for product packaged without CO_2, and on day 28 for product packaged with 30% CO_2. At all three storage temperatures, toxin detection preceded or coincided with olfactory unacceptability, demonstrating the potential for consumption of toxic product when spoilage-signalling sensory cues are absent.

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I. INTRODUCTION

Changing lifestyles and growing consumer demand for freshness, nutrition, and convenience have prompted the marketing of an ever-increasing number and variety of cooked, ready-to-eat and microwave-ready food products. Many of these items receive only minimal thermal processing, contain no added preservatives, and depend upon refrigeration ($4^{o}C$ or less) as the primary, if not sole, barrier to growth of pathogenic and spoilage microorganisms during product distribution and storage. Since "refrigeration" temperatures employed by retailers and consumers can often be as high as $15.5^{o}C$ (Van Garde and Woodburn, 1987; Hutton et al., 1991), simply chilling these foods cannot guarantee their safety or stability. Additional antimicrobial barriers are needed to ensure wholesomeness and quality throughout their expected storage life.

Modified atmosphere packaging (MAP) has become a popular means of extending the shelf-life of refrigerated pre-cooked foods, especially meat and poultry products. MAP replaces the atmospheric air surrounding these foods with carbon dioxide (CO_2), nitrogen (N_2), or a mixture of gases, thereby delaying onset of the foods' inherent biochemical and microbiological spoilage mechanisms, and extending product shelf-life without the use of traditional chemical preservatives (viewed by many consumers as undesirable). Although MAP foods have met with considerable market success in recent years, many questions have been raised concerning their safety and potential for involvement in the transmission of foodborne disease (Hintlian and Hotchkiss, 1986; Marth, 1998). Chief among these concerns is that MAP environments -- by excluding oxygen (O_2) and augmenting CO_2 -- suppress the growth of normally-occurring spoilage organisms, thus facilitating population shifts which can favor rapid and unrestricted proliferation by anaerobic or facultatively anaerobic pathogens, particularly under conditions of

temperature abuse. Without the development of off-odors and other sensory changes indicative

of spoilage, consumers might unwittingly ingest foods containing potentially harmful levels of

these dangerous microbes and/or their toxic metabolites.

Of particular concern to processed meat and poultry manufacturers is the potential for

product contamination by psychrotrophic pathogens, such as *Listeria monocytogenes* and

nonproteolytic *Clostridium botulinum*. These organisms are ubiquitous in nature, and capable of

anaerobic growth at refrigeration temperatures without the accompanying signs of spoilage used

by many consumers to judge product safety. *L. monocytogenes* has a high prevalence and

environmental persistence in meat and poultry processing facilities (Pearson and Marth, 1990;

Wenger et al., 1990), and has been recovered from both raw and cooked retail chicken and turkey

products (Pini and Gilbert, 1988; Gilbert et al., 1989; Kerr et al., 1990; Rijpens et al., 1997). *L.

monocytogenes* has been linked to outbreaks of listeriosis resulting from consumption of chilled

ready-to-eat chicken, inadequately heated turkey franks, and undercooked chicken (Schwartz et

al., 1988; Barnes et al., 1989; Kaczmarski and Jones, 1989; Kerr et al., 1990). Some variability

exists in apparent virulence among *L. monocytogenes* strains, with only three of the thirteen

recognized serovars being responsible for the majority of foodborne listeriosis outbreaks

(NACMCF, 1991). The incidence of botulinal spores in processed meat and poultry products is

generally less than 1 spore/kg (Hauschild, 1989), but the organism's widespread occurrence in

nature suggests that environmental contamination is, at some point, inevitable. *C. botulinum*

growth and toxin production have been well documented in smoked turkey (Abrahamsson and

Riemann, 1971), chicken pot pies (Tompkin, 1980), chicken emulsions (Sofos et al., 1980),

turkey frankfurters (Barbut et al., 1986a,b), vacuum-packaged cook-in-bag turkey products

(Anders et al., 1989; Maas et al., 1989), and cooked turkey breast rolls (Genigeorgis et al., 1991). It is thus evident that, by lacking traditional curing agents or back-up preservative systems to prevent or delay growth of these pathogens during prolonged storage or under conditions of temperature abuse, MAP meat and poultry products have the potential to become vehicles for the transmission of both listeriosis and botulism.

Data describing the effects of MAP (and more specifically, CO_2) on pathogens in meat and poultry products are limited. CO_2 has been shown to kill, inhibit, have no effect on, or stimulate the growth of, microorganisms (Day, 1992; Parry, 1993). High levels of CO_2 (50-100%) have been shown to inhibit *Staphylococcus aureus*, *Salmonella* spp., *Escherichia coli*, *Yersinia enterocolitica*, and *L. monocytogenes* (Hintlian and Hotchkiss, 1986; Gill and Reichel, 1989; Hart et al., 1991; Krämer and Baumgart, 1993), with decreasing temperatures yielding increasing degrees of inhibition. *C. botulinum* and *C. perfringens* are not adversely affected by CO_2. In fact, their growth is stimulated by the anaerobic conditions present in MAP (Goodburn and Halligan, 1988). Refrigeration will control *C. perfringens* but not nonproteolytic *C. botulinum*, which can grow and produce toxin (without overt signs of spoilage) at temperatures as low as $3.3°C$ (Kautter et al., 1981; Genigeorgis, 1985).

For optimal control of spoilage bacteria, manufacturers typically employ headspace CO_2 levels of up to 25 or 30% in the modified atmosphere packaging of ready-to-eat meat and poultry products. Little is known about the effects of such packaging environments on *L. monocytogenes* and nonproteolytic *C. botulinum*, or about the influence of indigenous microflora on the overall ecology of these pathogens in MAP meat and poultry products. The present study was undertaken to assess the potential growth of *L. monocytogenes* and nonproteolytic *C. botulinum*

in ready-to-eat MAP cooked turkey stored for extended periods of time under refrigeration and mild to moderate temperature abuse. The effect of varying levels of naturally-occurring and inoculated lactic acid bacteria on growth of the organisms in MAP turkey was investigated. The applicability of using whole-cell fatty acid composition to distinguish between foodborne outbreak-associated and non-outbreak-associated strains of *L. monocytogenes* in cooked turkey was also evaluated.

II. REVIEW OF LITERATURE

A. *Listeria monocytogenes*

1. Organism and disease characteristics

Listeria monocytogenes is a motile, Gram-positive, rod-shaped, facultatively anaerobic, nonsporeforming bacterium that is widely distributed in nature and is responsible for causing human infections (listeriosis) ranging in severity from localized skin lesions and mild "flu-like" illness to septicemia, meningitis, and fetal abortion. Most cases of listeriosis occur among pregnant women, neonates, the elderly, and immunocompromised individuals, and are thought to result from foodborne infection (McLauchlin, 1987; NACMCF, 1991). Of the 13 recognized serovars of *L. monocytogenes*, only three (4b, 1/2a, 1/2b) are attributable to most cases of human listeriosis. Infection by this opportunistic pathogen normally occurs via the intestinal route, following ingestion of contaminated food. The organism penetrates the intestinal epithelial barrier, colonizes hepatic and splenic macrophages, and subsequently destroys them by producing a hemolytic protein (listeriolysin O) that binds to host cell membrane lipids (Swaminathan et al., 1995). As a result, macrophage-mediated presentation of bacterial antigen to T-lymphocytes does not occur, and host cell-mediated immunity is inhibited (Cluff and Ziegler, 1987). The incubation period for listeriosis varies from approximately 2 days to 6 weeks (ICMSF, 1996). The minimal infectious dose is unknown, due to variability in such factors as organism virulence, host susceptibility, and gastric

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6

acidity (Farber and Peterkin, 1991; Hof and Rocourt, 1992; Datta, 1994; Swaminathan et al., 1995). While the illness can be treated with antibiotic therapy, the overall mortality rate from listeriosis is estimated at approximately 25% (NACMCF, 1991).

2.   Significance in cooked, uncured poultry

L. monocytogenes is of particular concern to processed meat and poultry manufacturers because of its high incidence in raw poultry and other raw meats (Farber et al., 1988; Pini and Gilbert, 1988; Farber et al., 1989a; Genigeorgis et al., 1989), and its ability to survive mild heat treatments and grow under environmental conditions considered unfavorable to most nonsporeforming bacteria of foodborne disease significance (Ryser and Marth, 1991). *L. monocytogenes* can survive microwave heating of chicken to an endpoint temperature of 70°C and cooking of beef to a "medium" degree of doneness (71°C endpoint) (Coote et al., 1991). The organism's heat resistance can be enhanced in the presence of fat, as Fain et al. (1991) observed in determining heat lethalities for strain Scott A in lean (2.0% fat) and fatty (30.5% fat) ground beef. They reported a $D_{62.8C}$-value of 1.2 min. in fatty ground beef, compared to a $D_{62.8C}$-value of 0.6 min. in lean ground beef. *L. monocytogenes* is capable of growth over a wide temperature range (1° to 45°C) (Seeliger and Jones, 1986). The organism has been shown to survive and multiply in cooked ham, wieners, bologna, and bratwurst stored at 4.4^{o}C (Glass and Doyle, 1989), and to grow prolifically on sliced chicken and turkey stored at 4.4^{o}C (Glass and Doyle, 1989). Survival and growth of *L. monocytogenes* have been reported in

turkey sandwiches (Farber et al., 1990), chicken and/or turkey frankfurters (Wenger et al., 1990; Wang and Muriana, 1994), and poultry wieners (McKellar et al., 1994). *L. monocytogenes* is able to grow in cooked chicken loaf under aerobic, micro-aerophilic (5-10% CO_2 preferred), and anaerobic conditions (Ingham et al., 1990), and tolerates relatively high levels of salt (up to 10% (w/v) sodium chloride (NaCl) in nutrient broth). Some strains can even survive 16% (w/v) NaCl at pH 6.0 for over a year (Seeliger and Jones, 1986). The incidence of *L. monocytogenes* in raw meats is estimated at up to 92% (Farber et al., 1988; Farber et al., 1989a); the incidence in cooked, ready-to-eat meat products is estimated at 3-13% (WHO, 1988), with some poultry products having an incidence as high as 12-27% (Gilbert et al., 1989; Kerr et al., 1990; Rijpens et al., 1997). Gilbert et al. (1989) found low levels (<100/g) of *L. monocytogenes* in 12% of pre-cooked ready-to-eat chicken obtained from retail establishments and in 1.6% of cook-chill catering meals (mainly from hospitals). This latter finding is of particular concern, since the contaminated products were intended for use without further cooking.

Sporadic cases of listeriosis have been linked to consumption of a variety of meat products, including cooked-and-chilled chicken (serotype 4b; Kerr et al., 1988), ready-cooked chicken nuggets (serotype 1/2a; Kaczmarski and Jones, 1989), microwave-heated turkey frankfurters (serotype 1/2a; Barnes et al., 1989), and paté (serotypes 4b and 1/2a; McLauchlin et al., 1991). To date, turkey franks have been the only meat product microbiologically implicated as the source of infection in a case of clinical listeriosis (Barnes et al., 1989). The infection source in the current

multi-state outbreak of listeriosis involving hot dogs and other deli-style meat products (serotype 4b; CDC, 1998) has yet to be determined.

Case-control studies of the dietary risk factors for sporadic listeriosis have associated consumption of uncooked hot dogs and undercooked chicken with increased risk of listeriosis (Schwartz et al., 1988; Schuchat et al., 1992), particularly among those having suppressed immune systems or at least one underlying medical condition. Schwartz and co-workers (1988) reported that individuals who had eaten hot dogs without first cooking them had 6.1 times the risk of listeriosis compared to controls, and those who had consumed undercooked (still pink) chicken had 3.2 times the risk of listeriosis compared to controls. Schuchat et al. (1992) found a 1.6 times greater risk of listeriosis for people who ate foods purchased from store delicatessen counters. In addition to dietary exposures, these researchers found that the use of laxatives and antacids increased the risk of listeriosis, likely due to alterations in the intraluminal environment of the gastrointestinal tract and decreased gastric acidity, respectively (Schuchat et al., 1992). In epidemiological studies, Pinner et al. (1992) observed that refrigerated foods that were ready-to-eat, contained high levels (by direct plating) of *L. monocytogenes*, and contained serotype 4b were, respectively, 5 times, >3 times, and 4 times, more likely to cause listeriosis than other *Listeria*-contaminated foods.

Given the persistence of *L. monocytogenes* in poultry processing facilities (Lawrence and Gilmour, 1994), and its incidence in both raw (Genigeorgis et al., 1989) and cooked (Gilbert et al., 1989; Rijpens et al., 1997) poultry products, there is

justifiable concern that this psychrotrophic pathogen may pose a hazard in cooked, uncured turkey products, particularly if present as a post-processing contaminant. These products typically contain less than 2% NaCl, are packaged under modified atmospheres, and are distributed and stored at refrigeration temperatures -- conditions which are all conducive to the survival and growth of *L. monocytogenes* (Seeliger and Jones, 1986; Ingham et al., 1990).

3. **Factors affecting growth in cooked, uncured poultry**

   a. **Available nutrients**

   The nutritional requirements of *L. monocytogenes* include several amino acids (cysteine, glutamine, isoleucine, leucine, valine), vitamins (biotin, riboflavin, thiamin, alpha-lipoic acid), and simple carbohydrates (typically glucose) (Seeliger and Jones, 1986). Arginine, histidine, methionine, and tryptophan have been shown to stimulate growth (Gray and Killinger, 1966). The organism is able to readily multiply in synthetic culture media, such as brain heart infusion broth, blood agar (Seeliger and Jones, 1986), and trypticase soy agar/broth containing 0.6% (w/v) yeast extract (Hitchins, 1992).

   b. **pH**

   L. monocytogenes is capable of growth over a broad range of pH. Under otherwise optimal conditions in tryptose broth or tryptic soy broth, the lower limit for growth was found to be 4.5 (George et al., 1988; Ahamad and Marth, 1989; Parish and Higgins, 1989; Sorrells et al., 1989; Buchanan and Klawitter, 1990). Under similarly ideal conditions in tryptic soy broth, Petran and Zottola

(1989) found the optimum to be pH 7.0 and the upper limit for growth to be pH 9.2. Numerous reports indicate that the acidulant used to adjust the pH of culture media strongly influences the observed lower limiting pH. At the same pH, organic acids (acetic, lactic, citric, malic) prove to be considerably more inhibitory to listerial growth than inorganic acids (hydrochloric) (Ahamad and Marth, 1989; Farber et al., 1989b; Sorrells et al., 1989). In the studies of Sorrells et al. (1989), Ahamad and Marth (1989), and Buchanan and Klawitter (1990), incubation at refrigeration temperatures appeared to confer protection on *L. monocytogenes* against the stressful effect of low pH. This observation was also made by Conner and co-workers (1986), who investigated the growth of this pathogen in cabbage juice. These reports add to the growing body of evidence that many bacterial species respond to environmental stresses (such as low pH or cold temperatures) by inducing stress tolerance, thus enabling them to better adapt to, and survive under, harsh conditions (Berry and Foegeding, 1997). Data describing the effects of pH on survival and growth of *L. monocytogenes* in meat products are limited, and refer principally to fermented products (where active lactic starter cultures play an important role in controlling this organism during refrigerated storage). Johnson et al. (1988) documented the survival of *L. monocytogenes* at a pH as low as 4.3-4.5 in hard salami stored under refrigeration. Schillinger et al. (1991) reported rapid growth of *L. monocytogenes* at 15°C in spreadable fresh pork sausage made from high pH (6.3) meat; but in sausage made from normal pH (5.6-5.8) meat,

the organism survived without multiplication (even in the absence of added lactobacilli). These researchers indicated that the pH value of the meat was the most important factor for listerial proliferation.

c.   **Water activity (a_W)**

The water activity (a_W) limits for survival and growth of *L. monocytogenes* are related to both the type of a_W-lowering solute used and the osmotic conditions created by the solute. Farber et al. (1992) found the minimum a_W limit for growth of *L. monocytogenes* in broth containing glycerol as the a_W-lowering agent to be approximately 0.90 at $30^{\circ}C$. Petran and Zottola (1989) reported growth of strain Scott A at $30^{\circ}C$ in tryptic soy broth (TSB) containing from 9.1 to 39.4% sucrose (corresponding to a_W values from 0.97 to 0.92), but no growth in TSB containing >39.4% up to 65% sucrose (a_W values of <0.92 to 0.82). Tapia de Daza et al. (1991) observed a_W minima for strains Scott A and Brie 1 (TSB, $30^{\circ}C$) of 0.90 using glycerol, 0.92 using NaCl, and 0.92-0.96 using sucrose. They found that the solute effects were accentuated at $4^{\circ}C$, yielding a_W minima of 0.92 with glycerol, 0.94 with NaCl, and 0.93-0.96 with sucrose. Miller (1992) obtained similar a_W minima for Scott A using brain heart infusion (BHI) broth containing glycerol and NaCl; in addition, he obtained an a_W minimum of 0.97 using propylene glycol. He reported that, at equal a_W levels, survival and growth of *L. monocytogenes* were longest in glycerol, shortest in propylene glycol, and intermediate in NaCl. Chen and Shelef (1992) found that growth of Scott A was inhibited in cooked strained

beef having an a_w of 0.93 (at 20°C), a moisture content of 25%, and no added

solutes.

d.   Oxidation-reduction potential (E_h)

> *L. monocytogenes* is characterized as a micro-aerophile (prefers 5-10%
>
> CO_2) (Seeliger and Jones, 1986), but is capable of growth in food and broth
>
> systems under oxidizing (aerobic), reducing (anaerobic), and slightly reducing
>
> (micro-aerophilic) conditions – corresponding, respectively, to E_h values of
>
> approximately +200 millivolts (mV), -200 mV, and -50 mV (Jay, 1992; Joklik
>
> et al., 1992). This versatility allows the organism to survive and proliferate at
>
> many locations (from surface to interior) within a food product, and in the
>
> presence of competing microflora which lower substrate E_h during logarithmic
>
> growth. Ingham et al. (1990) found that generation times for strain Scott A in
>
> cooked chicken were similar whether product was stored under aerobic,
>
> anaerobic, or micro-aerophilic environments.

e.   Chemical preservatives

(1)   Sodium chloride (NaCl)

> *L. monocytogenes* is known to be quite resistant to sodium chloride
>
> (NaCl). Seeliger and Jones (1986) reported that the organism can grow
>
> in nutrient broth containing up to 10% (w/v) NaCl. Borovian (1989)
>
> observed growth of *L. monocytogenes* at 10°C in broth adjusted to pH 4.5
>
> and supplemented with up to 4% NaCl, and in broth adjusted to pH 6.0
>
> and supplemented with up to 7% NaCl. Extended survival of the

organism has also been found to occur over a wide range of salt

concentrations. Stenberg and Hammainen (1955) reported survival of

ten *L. monocytogenes* strains for more than a year at 20°-24°C in nutrient

broth containing 10% NaCl and 1% dextrose. At 12% NaCl, the

organism survived for 34-68 days, and at 24% NaCl, it survived for 24

days. Kukharkova et al. (1960) reported survival of *L. monocytogenes*

for more than 60 days in meat held at 4°C in a 30% NaCl brine.

(2)   Phosphates

Phosphates are the salt form of phosphoric acid, and are used in

meat products principally for their functional properties (e.g., moisture

retention, improved tenderness, pH adjustment, ion sequestration,

emulsification, and reduced shrinkage during cooking) (Wagner, 1986).

At levels of 0.5% or less, phosphates appear to exhibit little, if any, effect

on the growth of *L. monocytogenes.* Many listerial cultivation media

contain approximately 0.25% phosphate (Difco Laboratories, 1984), and

0.4% phosphate acts as a thermal protectant to reduce the degree of

destruction of *L. monocytogenes* during heat processing of ground pork

(Yen et al., 1991). Flores et al. (1996) reported that a 0.5% phosphate

blend (Bekaplus MSP) had minimal or no effect on the growth of *L.*

monocytogenes in cured smoked ham and linked smoked sausage at 4°,

12°, or 20°C.

Phosphate levels of 1% or more exhibit marked effects on *L.*

monocytogenes in both culture media and meat systems. Knabel et al.

(1991) found that 1% tetrasodium pyrophosphate completely inhibited

growth of the organism in solid media. They speculated that this effect

was due to the removal of essential metals from cell wall cation-binding

sites, since the effect was reversed by supplementing the medium with

iron. Hwang and Beuchat (1995) observed that a 1% trisodium

phosphate (TSP) wash solution was more effective at reducing *L.*

monocytogenes populations on chicken skin than was a 10% wash

solution of sodium tripolyphosphate (STPP), monosodium phosphate

(MSP), sodium acid pyrophosphate (SAPP), or sodium

hexametaphosphate (SHMP). Rodriguez de Ledesma et al. (1996)

reported a significant reduction in *L. monocytogenes* (LM) populations

on chicken wings following a 10% TSP dip. Lowering the storage

temperature of the treated wings from 10° to 4^oC more than doubled the

reduction in LM counts. Dickson and co-workers (1994) also noted a

temperature-related response of LM to TSP treatment. They found that

increasing the temperature of a TSP immersion solution (8, 10, or 12%)

for sliced beef tissue from 25° to 55^oC greatly reduced the populations of

viable *L. monocytogenes* on both lean and adipose tissue. In a similar

vein, Somers et al. (1994) observed that exposure of LM cells in a

biofilm to 8% TSP required twice as much time at 10^oC to effect a 1-log

reduction in cell numbers as it did at room temperature.

f.   Storage temperature

L. monocytogenes is capable of growth over a wide temperature range. Lower and upper limits for growth are approximately 1°C and 45°C, respectively (Seeliger and Jones, 1986), and the optimum is between 30° and 37°C (Seeliger and Jones, 1986). Walker et al. (1990) have observed growth in chicken broth at temperatures as low as -0.1° to -0.4°C . They reported generation times ranging from 13-24 h at 5°C and 77-131 h at 0°C. Papageorgiou et al. (1996) reported similar generation times (16-20 h) in fresh whey cheeses at 5°C. In addition, these latter researchers reported generation times of 5.1-5.8 h at 12°C and 1.7-2.7 h at 22°C. Andrews and Grodner (1997) reported a generation time of 1.2 h in tryptic soy broth at 20°C. In turkey slurries prepared without any food additives, Schlyter et al. (1993) found the generation time of *L. monocytogenes* to be 1.7 h at 25°C.

Although *L. monocytogenes* is generally unable to grow below 0.5°C, the organism can easily survive extended periods of storage at much lower temperatures (Ryser and Marth, 1991). Palumbo and Williams (1989) found that populations of *L. monocytogenes* inoculated into ground turkey and frankfurters decreased by only 1 to 3 orders of magnitude during 8 weeks of frozen storage at -18°C. In addition to the effect of temperature on growth rate, there have been reports suggesting that virulence of *L. monocytogenes* may increase when propagated at low, as opposed to high, temperatures (Durst, 1975; Wood and Woodbine, 1979).

g.   Gaseous atmosphere

Although *L. monocytogenes* is able to grow under aerobic, micro-aerophilic, and anaerobic conditions (Ingham et al., 1990), the organism prefers environments containing approximately 5% O_2 and 5-10% CO_2 (Bahk and Marth, 1990). Higher levels of CO_2 have not been shown to be inhibitory, except at low temperatures (Ingham et al., 1990). Buchanan et al. (1989) and Buchanan and Phillips (1990) observed noticeable differences between growth rates obtained in shaken versus anaerobic cultures, and the latter authors noted that limiting oxygen enhanced the organism's ability to grow under adverse conditions. Buchanan and Klawitter (1990) reported that, for strain Scott A in tryptose phosphate broth at $37^{\circ}C$, aerobic incubation resulted in relatively rapid inactivation, but oxygen restriction (i.e., a 10-min. N_2 flush) resulted in recovery and survival for extended periods. They found that the growth rate at $19^{\circ}C$ was also enhanced when O_2 was restricted.

h.   Microbial competition

Cooked meat and poultry products subjected to post-processing contamination with *L. monocytogenes* are likely to also contain psychrotrophic spoilage organisms which compete for growth and thus influence product safety and stability through storage (Roberts et al., 1981; Gill, 1986; Ray et al., 1992). These aerobic psychrotrophs usually out-compete *L. monocytogenes*, preventing growth of the pathogen to levels that would be dangerous to susceptible individuals (Day, 1992; Parry, 1993). However, under modified

atmosphere conditions, growth of aerobic organisms such as *Pseudomonas fragi* and *Ps. fluorescens* may be suppressed or slowed to such an extent that even low numbers of *L. monocytogenes* may grow to high levels before evidence of organoleptic spoilage occurs. Ingham et al. (1990) and Marshall et al. (1991) observed this effect in cooked chicken.

Lactic acid-producing bacteria (such as lactobacilli, pediococci, leuconostocs, *Carnobacterium* spp., and *Brochothrix* spp.) also compete with *L. monocytogenes* in refrigerated, pre-cooked meat products (Döring et al., 1988; Yang and Ray, 1994), often inhibiting listerial growth via the production of both lactic acid and antagonistic metabolites known as bacteriocins. Juven et al. (1998) reported inhibition of *L. monocytogenes* in ground beef using a lactic acid-producing strain (FloraCarn L-2) of *Lactobacillus alimentarius*. Schillinger et al. (1991) demonstrated inhibition of *L. monocytogenes* in MRS (deMan, Rogosa and Sharpe) broth and comminuted raw pork sausage by a bacteriocin-producing strain of *Lactobacillus sake*. Degnan et al. (1992) used a bacteriocin-producing strain of *Pediococcus acidilactici* to control three strains of *L. monocytogenes* in temperature-abused, vacuum-packaged beef wieners.

B.   Nonproteolytic *Clostridium botulinum*

1.   Organism and disease characteristics

Clostridium botulinum is a ubiquitous, Gram-positive, rod-shaped, anaerobic sporeforming bacterium whose potent neurotoxins cause the syndrome of progressive

muscular paralysis known as botulism. The toxins may be acquired by ingestion (foodborne botulism) or by *in vivo* formation in infected tissues (wound botulism) or the colonized intestinal tract (infant botulism). The foodborne form of botulism is of greatest interest, since it is a severe type of food poisoning with a mortality rate of 10-25% if not immediately and properly treated (Hauschild, 1989; Sugiyama, 1990; CDC, 1992). The intoxication results from consumption of contaminated foods containing preformed botulinal toxin. The toxin binds to skeletal neuromuscular junctions, blocking release of the neurotransmitter acetylcholine, resulting in muscle weakness, blurred vision, dizziness, impaired breathing, paralysis, and death from respiratory failure (Sugiyama, 1990). The incubation period for botulism is approximately 12-36 hours (shorter if more toxin is consumed), and the reported 50% human lethal dose (LD_{50}) is about one nanogram of toxin per kilogram of body weight (Jay, 1992).

The foodborne strains of *C. botulinum* belong to two groups: proteolytic group I strains (types A, B, and F), and nonproteolytic group II strains (types B, E, and F). Nonproteolytic strains are highly cold-tolerant and capable of producing lethal toxin without the accompanying signs of putrefactive spoilage used by many consumers to judge product safety. *C. botulinum* growth and toxin production have been well documented in smoked turkey (Abrahamsson and Riemann, 1971), chicken pot pies and cured chicken livers (Tompkin, 1980), chicken emulsions (Sofos et al., 1980), turkey frankfurters (Barbut et al., 1986a,b), vacuum-packaged cook-in-bag turkey products (Anders et al., 1989; Maas et al., 1989), and cooked turkey breast rolls

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(Genigeorgis et al., 1991). Consequently, these pathogens are of particular concern

to manufacturers who produce and distribute refrigerated, uncured meat and poultry

products.

2.   Significance in cooked, uncured poultry

Although the natural occurrence of *C. botulinum* spores in North American

meat products is quite low (0.00031/g in raw meat; 0.00057/g to 0.00167/g in

processed meat (Roberts and Smart, 1976; Hauschild, 1989)), occasional outbreaks

of botulism resulting from ingestion of these products are reported. From 1988-

1992, there was a total of 60 botulism outbreaks in the United States, involving 133

cases and 11 deaths (Bean et al., 1997). Meat products or stews were confirmed as

the transmission vehicle in 10 of those outbreaks, and inadequate cooking and

improper holding temperatures were identified as the principal factors contributing to

the outbreak (Bean et al., 1997). Temperature abuse was also implicated in two

outbreaks of type B botulism involving commercially processed chopped garlic in oil

(St. Louis et al., 1988; Morse et al., 1990). Although the outbreak strains were

proteolytic, St. Louis et al. (1988) demonstrated that nonproteolytic type B strains

could also produce toxin in garlic-in-oil preparations when stored at $25^{\circ}C$ for 2

weeks. As a consequence of these outbreaks, FDA recommended adding

antimicrobial inhibitors or acidifying agents to all such products distributed in oil

(FDA, 1989).

Nonproteolytic strains of *C. botulinum* produce spores of relatively low heat

resistance ($D_{82.2C}$(type B)=1.49-32.3 min.); $D_{82.2C}$ (type E)=0.15-4.90 min.; $D_{82.2C}$(type

F)=0.25-0.84 min.) (Lynt et al., 1982; Scott and Bernard, 1982; Simunovic et al., 1985). Consequently, inadequate pasteurization treatments or post-processing contamination (such as in retail or consumer slicing operations) could create conditions favorable for botulinal proliferation, particularly in the absence of a terminal cook step or in the event of temperature abuse during product distribution or storage (Conner et al., 1989; Hutton et al., 1991). Because of their ability to grow at temperatures as low as 3.3°C (Eklund et al., 1967; Lynt et al., 1982; Sperber, 1982; Simunovic et al., 1985) and produce lethal toxin without evidence of organoleptic spoilage, the nonproteolytic strains of *C. botulinum* are of critical concern with respect to the safety of increasingly popular pre-cooked, uncured, refrigerated poultry products (Hutton et al., 1991). The mild thermal process (70°-71°C endpoint temperatures) employed in the manufacture of these products, together with elimination of competitive microflora, absence of nitrite and reduced levels of other preservatives (e.g., NaCl), modified atmosphere packaging, and extended storage at refrigeration (4°C) or mild/moderate abuse (8°-16°C) temperatures all contribute to a favorable environment for growth and toxigenesis by this organism (Conner et al., 1989; Hutton et al., 1991).

3.   Factors affecting growth in cooked, uncured poultry

a.   Available nutrients

The nutritional requirements of nonproteolytic *C. botulinum* are twofold: those necessary for spore germination and outgrowth, and those necessary for vegetative cell growth. Germination-stimulating agents include amino acids

(L-alanine, L-cysteine, L-proline), ribosides (adenosine, inosine), sugars

(glucose), metal ions (Mn^{2+}, Na^+), enzymes (lysozyme, protease), and

bicarbonate or CO_2 (Rowley and Feeherry, 1970; Ando, 1971; Ando, 1974a;

Foegeding and Busta, 1983a,b). Metabolic intermediates, such as L-lactic and

glycolic acids, appear to act as co-germinants with L-alanine (Ando and Iida,

1970). Nutrients such as sulfur, phosphorus, nitrogen, carbon, amino acids,

and metal ions (especially Mn^{2+}) stimulate outgrowth of germinated spores into

vegetative cells (Strange and Hunter, 1969). The nutritional requirements for

vegetative cell growth are complex and include amino acids (particularly

arginine, phenylalanine, and tyrosine), B vitamins (thiamin, niacin,

pyridoxamine), and trace minerals (calcium, magnesium, phosphorus,

potassium, sodium) (Smith, 1977; Sneath, 1986; Jay, 1992). Growth is also

readily supported by synthetic culture media, such as trypticase-peptone-

glucose-yeast extract broth/agar and cooked meat medium (Sneath, 1986; Jay,

1992).

b.   pH

The pH range within which botulinal germination occurs varies widely,

apparently reflecting the pH optima exhibited by the various enzymes involved

in germination (Gould, 1969). Ando and Iida (1970) observed that

germination of type E spores in a complex medium occurred over a relatively

broad pH range, 6.0-8.1, but not as low as pH 5.3. They found the optimum to

be pH 6.6, which was similar to that reported by Strasdine (1967) for strain

Minnesota E. Gould (1969) reported the lower limiting pH value for

germination of type E spores in complex media to be 4.8, and Smith (1977)

reported the upper limit at pH 9.0, with an optimum from pH 6.5 to 7.0. It is

important to note, however, that under practical conditions the limiting pH

value is influenced by other factors (such as temperature, water activity,

preservatives, etc.), so consideration of pH value by itself can be misleading

(Gould, 1969). In general, botulinal germination can occur at pH values

slightly above and below the limits permitting vegetative growth (Gould,

1969). Extensive studies in broth and food systems have shown the upper limit

for botulinal growth and toxigenesis to be pH 8.5-8.8 (Banwart, 1989), the

optimum to be pH 6.0-8.0 (Banwart, 1989), and the lower limit to be

approximately pH 4.6-4.8 (Hauschild, 1989; Jay, 1992). Segner et al. (1966)

documented the failure of *C. botulinum* to grow and produce toxin in broth at

pH values below 4.8. Lerke (1973) reported similar results in crabmeat

cocktails. Although a pH of ≤4.6 is considered inhibitory to *C. botulinum*

under most circumstances, toxigenesis by type E at pH values as low as 4.0-4.5

has been observed in broth (Tsang et al., 1985) and in pickled herring (Dolman

et al., 1950). Growth and toxin production in broth at pH 4.0-4.4 has only been

reported to occur in the presence of large amounts of precipitated protein

which may create micro-environments of higher pH within the larger protein

aggregate (Tanaka, 1982), thus permitting localized growth of type B *C.*

botulinum even though the overall pH may be ≤4.6. In acidified foods,

botulinal toxigenesis may be due to delayed pH equilibration, as Dolman et al.

(1950) observed in a type E botulism outbreak in which the pH of home-

pickled herring ranged from 4.5 to 4.8.

c.   **Water activity (a_w)**

The water activity limits for germination of botulinal spores are generally

lower than those for vegetative growth (Gould, 1969). Baird-Parker and

Freame (1967) found that, under otherwise optimal conditions, botulinal spores

can germinate at a_w levels as low as 0.89 (obtained with glycerol), and that

under suboptimal conditions the effect of low a_w on germination can become

more pronounced. Germination of type E spores at low pH values is more

sensitive to low a_w than at optimal pH values, and suboptimal temperatures

enhance the inhibitory effect of low a_w on botulinal germination (Gould,

1969). Some a_w depressants (e.g., NaCl, $CaCl_2$, sucrose) also exhibit

inhibitory properties, thereby enhancing their usefulness in food processing.

Jakobsen and Murrell (1977) found that, at an a_w of 0.95, germination of

clostridial spores was completely inhibited by NaCl (when used as the a_w-

lowering agent), but not by urea, glycerol, or glucose under the same

conditions. The minimal a_w required for growth and toxigenesis by

nonproteolytic type B *C. botulinum* is approximately 0.97 (determined using

NaCl as the a_w-lowering solute) (Hauschild, 1989). Under optimum pH and

temperature conditions, Ohye and Christian (1967) found that the growth-

limiting a_w level for type E was 0.97, which is consistent with the reported

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water activity depression ratio of approximately 0.006 units per 1% NaCl (Hauschild, 1989), and corresponds to an NaCl concentration of 5% (Sperber, 1982). Baird-Parker and Freame (1967) observed that when NaCl was replaced by glycerol as the a_w-lowering agent, the a_w minimum for type E *C. botulinum* dropped from 0.97 to 0.94. Emodi and Lechowich (1969) reported a limiting a_w value for type E of 0.98 with NaCl and 0.95 with glycerol. While differences exist among individual strains of nonproteolytic *C. botulinum*, the solute-related water activity minima described above apply generally across the entire group of nonproteolytics.

d.   Oxidation-reduction potential (E_h)

Redox potential is not a critical factor for germination of botulinal spores. Ando and Iida (1970) showed that the presence of oxygen does not interfere with the ability of type E spores to germinate in complex media at $37^{o}C$. In their studies, type E spores germinated at E_h values as high as +414 mV, but post-germinative development (outgrowth) was not possible until E_h dropped to below +198 mV. Rapid cell division began when E_h was about -300 mV. Commonly-used E_h-lowering (reducing) agents such as L-cysteine and sodium thioglycollate have been shown to exhibit variable effects (either stimulatory or inhibitory) on germination and outgrowth of a variety of clostridial spores, depending upon culture medium, species, and phase of development (Holland et al., 1969; Rowley and Feeherry, 1970; Barker and Wolf, 1971).

Vegetative growth and toxigenesis by *C. botulinum* occurs over a wide range of redox potentials (E_h). Lund and Wyatt (1984) observed that type E was just as likely to grow at an E_h of +60 mV as it was at an E_h of -400 mV. Foods which have low or negative E_h values (such as commercially canned or vacuum-packaged items) tend to favor growth of *C. botulinum*, particularly if they lack dissolved oxygen which can be inhibitory to the organism even at low E_h levels (Lund and Wyatt, 1984). The E_h of pre-reduced media commonly used for the cultivation of *C. botulinum* is below -150 mV (Holdeman et al., 1977). Foods which are exposed to atmospheric oxygen (such as comminuted products or items packed in air or O_2-permeable films) can also permit growth, provided sufficiently anaerobic micro-environments exist within them to support botulinal growth (Sperber, 1982; Hauschild, 1989). This may partially explain the observation of Christiansen and Foster (1965) that *C. botulinum* can grow equally well in cured meats that are air-packed, vacuum-packaged, and gas-flushed. Actively-respiring foods and competing aerobic microflora also encourage growth since they consume free oxygen, thereby reducing E_h and allowing botulinal proliferation and toxigenesis to occur (Sugiyama and Yang, 1975).

The respective lower and upper E_h levels for *C. botulinum* growth are approximately -450 mV and +250 mV. Montville and Conway (1982) demonstrated growth of type B *C. botulinum* in canned beef stew at an E_h as low as -446 mV. Hauschild (1989) found the upper E_h level for outgrowth of

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nonproteolytic type B spores to be approximately +200 mV. Smoot and

Pierson (1979) observed that the critical E_h level for botulinal outgrowth was

lowered if the spores were subjected to stressful conditions (e.g., salt, acidity).

Huss et al. (1979) determined that the maximum E_h for growth of type E in

smoked fish was +250 mV.

e.   **Chemical preservatives**

(1)   **Sodium chloride (NaCl)**

Nonproteolytic strains (types B, E, and F) of *C. botulinum* are

inhibited by NaCl concentrations on the order of 5-6% (w/v) (Lynt et l.,

1982). Ando (1974b) showed that germination of type E botulinal spores

in a chemically defined medium at optimum pH could be inhibited by

5% NaCl. Whiting and Oriente (1997) found that NaCl levels below 3%

had little effect on the growth of nonproteolytic type B spores in broth.

While studying radiation resistance of types B and E spores in media,

also at optimal conditions, Kiss and co-workers (1978) found that 4%

and 2.5% NaCl, respectively, inhibited growth from nonproteolytic type

B and E spores. Emodi and Lechowich (1969) demonstrated that

lowering temperature reduced the concentration of NaCl required to

inhibit growth from type E spores in broth; 4.9% NaCl was required at

15.6^{o}C, while 3% NaCl was required at 5°C. Riemann et al. (1972)

observed that, in semi-preserved meat products, lowering pH also

lowered the level of salt necessary in the brine to inhibit growth from

types B and E spores. At pH 7, 9.5% NaCl was required to inhibit type

B spores, while only 6% NaCl was required at pH 5.5. At pH 7, 5.5%

NaCl was required for inhibition of type E spores, while only 4% NaCl

was required at pH 5.5. Barbut et al. (1986b) found that increasing the

NaCl content of turkey frankfurter emulsions from 1.0 to 3.0% delayed

type B toxigenesis by 4 days at 27°C. Juneja and Eblen (1995) reported

that heat resistance of nonproteolytic type B spores decreased with

increasing levels of NaCl (2 and 3%) in turkey slurry and in reinforced

clostridial medium.

It is recognized that NaCl alone is ineffective as a spore inhibitor,

and must be used in combination with other preservatives or inhibitory

factors. In fact, Ohye and Christian (1967) noted that, under otherwise

optimal temperature and pH conditions, as much as 10% NaCl may not

be sufficient to inhibit botulinal germination and outgrowth. Such a

concentration would clearly be organoleptically unacceptable in food

products.

(2)   Phosphates

Phosphate and phosphate compounds have been shown to exhibit

antibotulinal activity in culture media and certain meat products. In a

study of long-chain polyphosphates, Schoeni et al. (1980) found that

2.0% sodium polyphosphate (having an average chain length of 22

phosphate units) prevented growth of *C. botulinum* for 16 weeks at 27°C

in reinforced clostridial medium adjusted to pH 5.4 to 6.0. Wagner and

Busta (1984, 1985) demonstrated the effectiveness of sodium acid

pyrophosphate in delaying type B botulinal growth from spores and

vegetative cells in peptone-yeast extract-glucose broth, when present at

levels of 0.2 or 0.4% and in combination with potassium sorbate (0.13 or

0.26%). Nelson and co-workers (1983) observed that type B botulinal

toxigenesis in chicken frankfurter emulsions was delayed for a longer

period of time when sodium acid pyrophosphate was used in

combination with nitrite-sorbate or sorbate than when sodium

tripolyphosphate or sodium hexametaphosphate was used alone. Wagner

and Busta (1983) reported similar results for type B toxigenesis in

beef/pork frankfurter emulsions. Barbut et al. (1986b) found that type B

toxin production in turkey frankfurters was delayed by 1.5 or 2.0%

sodium acid pyrophosphate (SAPP). Toxigenesis was unaffected by

similar levels of hexametaphosphate (HMP), but accelerated by similar

levels of tripolyphosphate (TPP). Data are not available on the effects of

phosphates on botulinal growth and toxigenesis in uncured meat and

poultry products.

f.   Storage temperature

Germination of botulinal spores can occur over a wide range of

temperatures (often outside the limits for vegetative growth). Ando and Iida

(1970) found that, in a complex medium, type E spores were able to germinate

at 10° to 40°C, with the optimum at 37°C. At 20°C germination was slow with a fairly long lag period, and at 45°C germination was inhibited. Grecz and Arvay (1982) reported that type E strain VH spores were able to germinate in trypticase-peptone-glucose-thioglycollate (TPGT) broth at 2° to 50°C. Germination was exceptionally rapid at 50°C, and the optimum for both partial and full germination was 9°C. Post-germinative development, on the other hand, was restricted to a narrower temperature range. Growth occurred at 6°C to 41°C with a distinct optimum at 32.5°C. No growth occurred at 50°C, and only marginal growth was observed at 6° to 14°C. Schmidt et al. (1961) observed outgrowth by spores of four type E strains (VH, Beluga, Iwanai, 8E) in beef stew at 3.3°C but not at 2.2° or 1°C. Eklund and co-workers (1967) reported outgrowth of nonproteolytic type B spores in cooked meat medium at 3.3° to 5.6°C, but not at 2.2°C. Stringer et al. (1997) found that refrigerated storage (10° or 5°C) prevented outgrowth of nonproteolytic types B, E, and F spores for up to 23 weeks following mild heat treatment in broth. Whiting and Oriente (1997) observed an increase in time to outgrowth of nonproteolytic B spores in broth as temperature was lowered from 28° to 4°C. The optimal temperature for outgrowth of most strains is between 31° and 37°C (Smith, 1977).

All strains of *C. botulinum* are mesophilic (Smith, 1977) and, in general, produce toxin under the same conditions that permit vegetative growth (Sperber, 1982). The established minimum/maximum temperature limits for

vegetative growth depend on otherwise optimal growth conditions (e.g., pH, water activity, etc.) in the food or culture system (Hauschild, 1989; Jay, 1992). Temperatures at the lower end of the growth range require longer time periods for initiation of toxigenesis than do temperatures at the upper end of the range (Solomon et al. 1977; Simunovic et al., 1985). Maximum growth temperatures are usually no more than a few degrees above the optimum (Banwart, 1989).

The optimum temperature for growth of nonproteolytic strains is 25°-37°C (Sneath, 1986), with a maximum of 45°C (Jay, 1992). The minimum temperature for growth and toxin production is 3.3°C, as observed by Schmidt et al. (1961) for type E in beef stew, and Eklund et al. (1967) for type B in cooked meat medium. As the minimum temperature is approached, growth and toxigenesis become influenced by substrate composition, with some foods apparently exhibiting an inhibitory effect. Solomon et al. (1977) reported that type E was able to grow and produce toxin in broth at temperatures as low as 4°C, but in crabmeat the minimum required temperature to obtain the same result was 12°C. In 1982, Solomon and co-workers found that nonproteolytic types B and F grew and produced toxin in TPGY broth at temperatures as low as 4°C, but in crabmeat, growth and toxigenesis by these strains were inhibited below 26°C. Meng and Genigeorgis (1994) observed faster onset of toxicity from nonproteolytic type B and E spores in 'sous-vide' salmon than in 'sous-vide' beef or chicken stored at 4°C.

g.   Gaseous atmosphere

Although *C. botulinum* is a strict anaerobe, it is able to grow in products exposed to atmospheric oxygen, provided there are either micro-environments of sufficiently low redox potential to support botulinal growth (Sperber, 1982) or actively-respiring tissues/competitive microflora which consume free oxygen and lower E_h (Sugiyama and Yang, 1975). In vacuum-packaged, 'sous-vide' products, Meng and Genigeorgis (1994) observed outgrowth of nonproteolytic type B and E spores at temperatures ranging from 30° to 4^oC. Low levels of CO_2 or sodium bicarbonate (e.g., 0.1%) have been shown to markedly stimulate botulinal spore germination (Wynne and Foster, 1948; Ando and Iida, 1970), and even to eliminate the lag period (Ando and Iida, 1970). Ando (1971) determined the optimal bicarbonate concentrations for rapid and complete germination of type E spores in simple and complex media to be 60 to 120 mM. Postgerminative development was observed to occur only under anaerobic conditions (E_h below -200 mV) (Ando and Iida, 1970).

h.   Microbial competition

Pre-cooked meat and poultry products contaminated post-process with *C. botulinum* are likely to contain the same types of competitive microflora as described in the section of this review dealing with *L. monocytogenes* (Roberts et al., 1981; Gill, 1986; Hutton et al., 1991; Ray et al., 1992). During growth, aerobic pseudomonads metabolize oxygen and reduce product E_h to levels low enough to support botulinal proliferation (Day, 1992), thus permitting

toxigenesis to occur if there are no other inhibitory agents or organisms present (Hutton et al., 1991). Lactic acid bacteria can inhibit botulinal growth and toxigenesis, through production of lactic acid (Tanaka et al., 1985) and other antagonistic (bacteriocinogenic) compounds (Okereke and Montville, 1991a,b). The "Wisconsin process" (lactic acid starter culture plus sucrose) has been used for years to prevent botulinal toxin production in bacon formulated with reduced levels of nitrite (Tanaka et al., 1985). Okereke and Montville (1991a) observed (under otherwise optimal conditions) bacteriocin-like inhibition of nonproteolytic type B botulinal spores in culture media by strains of *Pediococcus pentosaceus*, *Lactococcus lactis* subsp. *lactis*, and *Lactobacillus acidophilus*. The *Pediococcus* strain effected the greatest degree of inhibition. Hutton and co-workers (1991) employed *P. acidilactici* to prevent nonproteolytic botulinal toxigenesis in chicken salad subjected to temperature abuse. In their study, toxin was detected only in samples held at or above 15°C.

C. Properties of cooked, uncured poultry that can promote proliferation of *L. monocytogenes* and nonproteolytic *C. botulinum*

1. Nutrient supply

Poultry products are rich in the vitamins, amino acids, and minerals known to stimulate growth of *L. monocytogenes*, including thiamin (Seeliger and Jones, 1986), tryptophan (Gray and Killinger, 1966), and iron (Sword, 1966). Poultry products are

also replete with the native proteins, amino acids, and minerals (Schweigert, 1987;

Stadelman et al., 1988; Godber, 1994) required for germination, outgrowth, and

multiplication of nonproteolytic *C. botulinum*, including alanine, cysteine (Ando and

Iida, 1970), manganese, sulfur (Strange and Hunter, 1969), thiamin, niacin, and

phosphorus (Smith, 1977; Sneath, 1986; Jay, 1992). Simple carbohydrates (such as

dextrose) added during formulation, together with CO_2 augmentation during

modified atmosphere packaging, serve as additional stimulants to promote rapid

proliferation by these pathogens.

2.   Near-neutral pH

While the pH of fresh (post-rigor) red meats is about 5.5, and that of poultry is

about 5.6 (Faustman, 1994), further processing often results in increased pH, due to

the addition of ingredients such as alkaline phosphates which aid in moisture

retention and prevention of pink color development (particularly in uncured poultry

products). The ideal pH for functional performance and quality of these latter

products is 6.9-7.0 (Stadelman et al., 1988) -- a range which is also ideal for

proliferation of both *L. monocytogenes* and nonproteolytic *C. botulinum*.

3.   High water activity (a_W)

Barring surface dehydration, the water activity of fresh meat and poultry is

generally 0.99 or higher -- near the optimum for growth of many bacterial species

(Niven, 1987; Banwart, 1989). Salting during further processing reduces the a_W to

levels largely related to the salt (brine) concentration used in processing. Canned

hams, for example, typically have a_W values between 0.95 and 0.97 (corresponding to

about 8% and 5% NaCl, respectively) (Niven, 1987). Cooked, uncured turkey roasts

and rolls contain up to 1.8% NaCl (finished weight basis) (Stadelman et al., 1988),

corresponding to an a_W value of 0.989; their chicken counterparts contain up to 1%

NaCl (finished weight basis) (Stadelman et al., 1988), corresponding to an a_W of

0.996. Sucrose and/or dextrose may contribute additional a_W-lowering effects to the

final formulation of cooked, uncured poultry, but finished products still possess

water activities which are sufficient, under otherwise optimal conditions, to support

the growth of both *L. monocytogenes* and nonproteolytic *C. botulinum*.

4.   Low oxidation-reduction potential (E_h)

The E_h of meat reportedly ranges from approximately -200 mV to +250 mV,

with solid meats having an E_h of about -200 mV and comminuted meats having an E_h

around +200 mV (Banwart, 1989; Jay, 1992). Although information on the redox

potentials of processed poultry products is not readily available, it is reasonable to

assume that their E_h values are sufficiently low (either locally or in aggregate) to

support growth of both *L. monocytogenes* and *C. botulinum*, since there is ample

evidence of this in modified atmosphere-packaged turkey sandwiches (Farber et al.,

1990), vacuum-packaged retail franks (Wang and Muriana, 1994), and vacuum-

packaged turkey breasts (Anders et al., 1989; Maas et al., 1989).

5.   Minimal levels of preservatives

Cooked, uncured turkey roasts and rolls typically contain up to 1.8% sodium

chloride (NaCl) on a finished weight basis, and their chicken counterparts contain up

to 1% NaCl on a finished weight basis (Stadelman et al., 1988). The salt levels in

these and other further-processed poultry products are far below those considered

necessary for inhibiting *L. monocytogenes* (>10% NaCl; Ryser and Marth, 1991;

Marth, 1993) and inhibiting *C. botulinum* (2.5-6.0% NaCl; Banwart, 1989).

Cooked, uncured poultry products typically contain phosphate levels up to

0.5% (finished weight basis). The type and amount of phosphate employed are

governed -- and federally regulated -- by chemical functionality, including water

binding (improved yield), metal ion sequestration (retardation of oxidative rancidity),

emulsification, and color stabilization (prevention of pink color development)

properties. The ideal pH for functional performance and quality of these products is

6.9-7.0 (Stadelman et al., 1988) -- well within the optimal range for proliferation by

L. monocytogenes and *C. botulinum*. In the absence of other inhibitory substances

(such as nitrite or high salt), the phosphate levels found in cooked, uncured poultry

products may not be sufficient to provide protection against growth of *L.*

monocytogenes and nonproteolytic *C. botulinum*.

6.   Favorable storage temperatures

A wide range of temperatures may be encountered during the distribution and

storage of processed meat and poultry products at the retail and consumer levels. In

a study evaluating the degree of temperature abuse in retail chill cabinets, Hutton et

al. (1991) found product temperatures ranging from -1.1° to 15.5°C. Fifty percent

(50%) of the products they purchased from convenience store refrigerated display

cases had temperatures $\geq 7.2^{\circ}$C, and 15% had temperatures above 10°C. In a survey

of household food discard practices, Van Garde and Woodburn (1987) reported

finding refrigerator temperatures ranging from 1.7° to 20°C. Twenty-one percent

(21%) of the household refrigerators evaluated had temperatures of $\geq 10^{\circ}$C. The

ability of *L. monocytogenes* (LM) and nonproteolytic *C. botulinum* (CB) to grow not

only under improper refrigeration or temperature abuse conditions, but also under

what may be considered "good" refrigeration conditions (4°C or less), poses a serious

threat to refrigerated food manufacturers, particularly given the possibilities of

enhanced LM virulence and CB toxigenesis without accompanying signs of spoilage

during extended periods of cold storage.

7.   Favorable package atmospheres

Many commercially-available, cooked, uncured poultry products are packaged

and distributed under vacuum or modified atmospheres, which extend refrigerated

shelf-life by inhibiting the growth of aerobic spoilage microorganisms. For example,

inclusion of >20% CO_2 in modified atmosphere packages (MAP) effectively inhibits

poultry spoilage aerobes such as pseudomonads, and significantly extends product

shelf-life (Day, 1992). However, by excluding oxygen and/or increasing carbon

dioxide levels, MAP environments may actually encourage the growth of

psychrotrophic pathogens such as *L. monocytogenes* and nonproteolytic *C. botulinum*

(Notermans et al., 1990; Day, 1992; Lund and Notermans, 1992). These organisms

may grow to dangerously high levels without accompanying signs of organoleptic

spoilage, thereby placing consumers at risk for potentially life-threatening infections

or intoxications (Farber, 1991). Ultimate success in maintaining optimal product

quality, delaying spoilage, and minimizing food poisoning risks relies upon good

manufacturing and handling practices, contamination control, the combination of modified atmospheres used, strict refrigeration, and the use of additional antimicrobial barriers where appropriate (NFPA, 1988; NACMCF, 1990; Hutton et al., 1991).

D. Use of modified atmosphere packaging (MAP) to delay microbial growth and extend product shelf-life

1. Definition of MAP

Modified atmosphere packaging (MAP) is a form of packaging in which atmospheric air in a package is removed and replaced with some other gas or gas mixture having a proportion different from that of air (21% oxygen, 78% nitrogen, <0.1% carbon dioxide), depending upon the type of product being packaged. Throughout storage, the gaseous atmosphere in the package continually changes, as a result of product respiration, biochemical deterioration, microbial growth, and container gas permeability (Parry, 1993).

2. Methods and gases used

The most common methods used to modify the gaseous atmosphere within a package are vacuum packaging and gas packaging. In vacuum packaging, the product is placed in an oxygen-barrier film, air is evacuated, and the film is sealed, effectively reducing the level of residual oxygen to less than 1% (Parry, 1993). In gas packaging, headspace atmosphere is modified by mechanical replacement of air or by atmosphere generation within the package. Mechanical air replacement can be

achieved by continuous gas flushing (which yields residual oxygen levels of 2-5%) or by a two-stage process of vacuum removal of air followed by gas filling (which yields residual oxygen levels of 0.1% or less). Atmosphere generation can be achieved either passively through post-harvest respiration, or actively through the incorporation of additives such as oxygen absorbents (reducing O_2 levels to $\leq 0.01\%$), carbon dioxide absorbents/emitters, ethanol emitters, or ethylene absorbents into the package or the packaging film (Parry, 1993).

The principal atmospheric gases used in MAP are carbon dioxide (CO_2), oxygen (O_2), and nitrogen (N_2). From a microbiological standpoint, CO_2 is the most important of these gases, for it may kill, inhibit, have no effect on, or stimulate the growth of microorganisms (Day, 1992). The efficacy of CO_2 varies with concentration, temperature, pH, a_W, and organism type, age, and numbers present (Wodzinski and Frazier, 1961; Daniels et al., 1985). While the specific mechanism of action is not clearly understood, the inhibitory effect of CO_2 results in an increase in the lag phase and generation time of susceptible microorganisms (Daniels et al., 1985; Day, 1992). CO_2 has both fungistatic and bacteriostatic properties, and retards the growth of most molds and aerobic bacteria. CO_2 is particularly effective against Gram-negative spoilage aerobes (such as pseudomonads, which produce off-flavors/-colors in meat and poultry), but has little effect on yeasts and lactic acid bacteria. In fact, the growth of lactic acid bacteria is enhanced in the presence of CO_2 and a low O_2 content (Day, 1992; Parry, 1993). The inhibitory effect of CO_2 increases with decreasing temperature, due to increased solubility (179.7 mL per 100 mL water at

0^oC) (Parry, 1993). Absorption of CO_2 is dependent upon product moisture and fat content, with excess absorption causing such conditions as package collapse, unsightly purge/drip, and discoloration and sharp acid taste in meat and poultry products (Parry, 1993).

Due to the stimulatory effect of oxygen on biochemical deterioration and the growth of aerobic spoilage organisms, O_2 levels in MAP foods are generally kept as low as possible (or even excluded). The only exceptions are in cases where O_2 is needed to retain fresh meat color, avoid anaerobic conditions in packaged white fish, or allow for fruit/vegetable respiration (Day, 1992).

Nitrogen is used chiefly to displace oxygen in MAP, and thereby retard oxidative deterioration and aerobic growth through the creation of an anaerobic or micro-aerophilic environment. Consequently, facultative, micro-aerophilic, or anaerobic microorganisms may replace aerobes as the predominant microflora. N_2 is an inert gas with low solubility in water and fat, and also serves as a filler gas to prevent package collapse in CO_2-absorbing foods (Day, 1992).

3.   Effect on foodborne pathogens

Data on the effects of MAP (particularly CO_2) on foodborne pathogens are limited. It has generally been found that high levels of CO_2 (50-100%) have an inhibitory effect on *Staphylococcus aureus*, *Salmonella* species, *Escherichia coli*, *Yersinia enterocolitica*, and *Listeria monocytogenes* (Hintlian and Hotchkiss, 1986; Gill and Reichel, 1989; Hart et al., 1991; Krämer and Baumgart, 1993), with decreasing temperatures yielding increasing degrees of inhibition. *Clostridium*

botulinum and *Clostridium perfringens* are not markedly affected by CO_2, and their growth is stimulated by anaerobic conditions present in MAP (Goodburn and Halligan, 1988). Temperature reduction will control *C. perfringens* but not nonproteolytic *C. botulinum*, which can grow and produce toxin (without overt signs of spoilage) at temperatures as low as $3.3^{\circ}C$ (Kautter et al., 1981; Genigeorgis, 1985).

4.   **Effect on spoilage microflora**

CO_2 concentrations greater than 5% inhibit the growth of most food spoilage bacteria and fungi. Depending upon the particular food and microflora involved, inhibition increases almost linearly with increasing CO_2 concentration from 5% to approximately 25-50% (Day, 1992); higher concentrations show little or no improvement. The degree of inhibition varies considerably, but certain generalizations can be made (Day, 1992; Parry, 1993). Most food spoilage molds have an absolute requirement for oxygen, and are sensitive to high levels of CO_2. Yeasts are comparatively resistant, except for some nonfermenters. Bacteria are highly variable, but Gram-negatives are typically more sensitive than Gram-positives. The common spoilage organisms of fresh meat and poultry -- *Pseudomonas* species and *Acinetobacter/Moraxella* species -- are strongly inhibited by CO_2, as are the *Micrococcus* species and *Bacillus* species. *Lactobacillus* species are among the most resistant spoilage microflora to CO_2. They are generally slow-growing (causing spoilage only at very high levels) and commonly outgrow aerobic spoilage organisms in MAP products (Day, 1992).

5.   Advantages/disadvantages of MAP

Parry (1993) has summarized the benefits and drawbacks of MAP to the manufacturer, retailer, and consumer. Advantages include: increased shelf-life, allowing less frequent loading of retail display shelves; reduction in retail waste; improved product visibility/presentation; drip- and odor-free sealed, stackable packages; easy separation of sliced products; little or no need for chemical preservatives; centralized packaging and portion control; reduced production and storage costs, due to better labor, space, and equipment utilization; and increased distribution area and reduced transportation costs, due to less frequent deliveries. Disadvantages include: capital cost of gas packaging machinery; cost of gases and packaging materials; cost of analytical equipment to ensure utilization of correct gas mixtures and maintenance of seal integrity; cost of quality assurance systems to prevent distribution of leakers, etc.; increased package volume, which will adversely affect transportation costs and retail display space; potential growth of foodborne pathogens, due to temperature abuse by retailers and consumers; and, the benefits of MAP are lost once the package is opened or leaks. Parry (1993) has concluded however that, given rapid market growth for MAP products, the benefits of the system clearly outweigh the drawbacks.

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MANUSCRIPTS FORMATTED FOR PUBLICATION

IN

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IV. EFFECT OF MODIFIED ATMOSPHERE PACKAGING (MAP) ON GROWTH OF *LISTERIA MONOCYTOGENES* IN COOKED TURKEY STORED UNDER REFRIGERATION AND MILD TEMPERATURE ABUSE

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ABSTRACT

The growth of *Listeria monocytogenes* (LM) in modified atmosphere packaged (MAP) cooked, uncured turkey was investigated at refrigeration (4°C) and mild abuse (10°C) temperatures. Turkey breast chunks containing low ($<10^2$/g) and high (10^4-10^5/g) levels of naturally-occurring lactic acid bacteria (LAB) were inoculated (10^1-10^2/g) with a five-strain mixture of LM, and stored for up to 4 weeks in high-barrier bags containing 100% N_2 or 30% CO_2 : 70% N_2. When the initial contamination ratio between indigenous LAB and inoculated LM populations (LAB:LM) was 3.5 logs:1 or 2.1 logs:1, LM growth was suppressed, particularly at the lower storage temperature. When the initial LAB:LM ratio was equivalent, LM growth was not suppressed. Instead, LM populations increased at the same rate, and reached the same ultimate levels, as LAB populations. Lowering storage temperature enhanced the inhibitory effect of CO_2 in the packaging atmosphere. When LM was outnumbered by indigenous LAB, olfactory characteristics of spoilage were detected in all samples. However, when the two populations were equivalent, olfactory spoilage characteristics were not detected in most of the samples, despite the fact that they contained final LAB levels of 10^9/g or higher. This latter finding raises concerns with respect to public health, since very high levels of *L. monocytogenes* can be present in ready-to-eat food products in the absence of overt signs of spoilage.

INTRODUCTION

Modified atmosphere packaging (MAP) has become increasingly popular as a means of extending the shelf-life of pre-cooked, ready-to-eat (RTE) and microwave-ready convenience foods, which ordinarily rely upon refrigeration as the primary, if not sole, barrier to microbial growth during product distribution and storage. MAP replaces the atmospheric air surrounding these foods with carbon dioxide (CO_2), nitrogen (N_2), or a mixture of gases, thereby delaying onset of the foods' inherent biochemical and microbiological spoilage mechanisms, and extending product shelf-life without the use of traditional chemical preservatives (viewed by many consumers as undesirable). MAP foods have met with considerable market success in recent years, owing to their high quality and nutritional value, excellent product visibility, controlled-portion sizing, and ease of use. However, many questions have been raised concerning their safety and potential for involvement in the transmission of foodborne disease (*10,18*). Chief among these concerns is that MAP environments -- by excluding oxygen (O_2) and augmenting CO_2 -- suppress the growth of normally-occurring aerobic spoilage organisms, thus facilitating population shifts which can favor rapid and unrestricted proliferation by anaerobic or facultatively anaerobic pathogens, particularly under conditions of temperature abuse (*5,12*). Without the development of off-odors and other sensory changes indicative of spoilage, consumers might unwittingly ingest foods containing potentially harmful levels of these dangerous microbes and/or their toxic metabolites.

Of particular concern to processed meat and poultry manufacturers is the potential for product contamination by the facultative anaerobe, *Listeria monocytogenes*. This organism has

been linked to outbreaks of listeriosis resulting from consumption of RTE and inadequately reheated refrigerated meat and poultry products (*1,14,15,16,24*). Because of its high prevalence and environmental persistence in meat and poultry processing facilities (*20,27*), incidence in both raw and cooked retail products (*6,16,21,22*), and ability to grow at refrigeration temperatures (*26*) and under anaerobic conditions (*13*), *L. monocytogenes* poses a threat to the safety of RTE muscle food products in general, and to those packaged and stored under modified atmospheres in particular.

Data describing the effects of MAP (and more specifically, CO_2) on pathogens in meat and poultry products are limited. High levels of CO_2 (50-100%) have been shown to inhibit *Staphylococcus aureus*, *Salmonella* spp., *Escherichia coli*, *Yersinia enterocolitica*, and *L. monocytogenes* (*7,9,10,17*), with decreasing temperatures yielding increasing degrees of inhibition. For optimal control of spoilage bacteria, manufacturers typically employ headspace CO_2 levels of up to 25 or 30% in the modified atmosphere packaging of RTE meat and poultry products. Little is known, however, about the efficacy of such levels in inhibiting or delaying proliferation of *L. monocytogenes* in RTE products, or about the influence of indigenous microflora on the overall ecology of *L. monocytogenes* in these products. The present study was undertaken to address some of these issues. The growth of *L. monocytogenes* in MAP cooked turkey was investigated at refrigeration and mild abuse temperatures and in the presence of varying levels of naturally-occurring lactic acid bacteria.

MATERIALS AND METHODS

Culture preparation and maintenance. *L. monocytogenes* strains Scott A, 101M, LCDC 81-861, V7, and Brie 1 were obtained from the culture collection of the Department of Food Science and Technology (FST) at Virginia Polytechnic Institute and State University (VPI&SU), Blacksburg, VA. Stock cultures were maintained on slants of trypticase soy agar (TSA; Difco Laboratories, Detroit, MI) supplemented with 0.6% yeast extract (YE; Difco) (TSAYE) and stored at 2°C. Before use, stock cultures were grown (through three successive transfers) to mid-log phase (based on prior growth curve studies) at 30°C in trypticase soy broth (TSB; Difco) supplemented with 0.6% yeast extract (YE; Difco) (TSBYE). Culture counts were determined in duplicate using TSAYE pour plates.

Product preparation. Cooked, uncured turkey breast was obtained from a commercial turkey processor. Samples were collected on separate occasions for three experiments that represented the following contamination ratios (indigenous lactic acid bacteria: inoculated *L. monocytogenes*): 1) 3.5 logs:1, 2) 2.1 logs:1, and 3) ca. 1 log:1. For experiments evaluating contamination ratios of 3.5 logs:1 and 2.1 logs:1, product was collected in barrier bags (4.5-kg increments) as it exited the carving machine (approximate carved size range: 1.9 cm x 1.3 cm x 1.3 cm to 3.8 cm x 1.9 cm x 1.3 cm). Bags were vacuum-sealed and shipped on ice (via overnight express carrier) directly from the manufacturing facility to the FST Department at VPI&SU. For the experiment evaluating a contamination ratio of ca. 1 log:1 (equivalent numbers of LAB and LM), vacuum-sealed, boneless cook-in-bag turkey breasts (ca. 2 kg ea.)

were obtained immediately after the final process chill step (core temperature: 32°F), packed in ice-filled coolers, and immediately transported to the FST Department at VPI&SU.

Product surface temperature was measured upon arrival (Omega HH-99A-T1 digital thermometer with surface probe, Omega Engineering, Inc., Stamford, CT), and the bags immediately transferred to a cold room (0.5^o ± 1.5^{o}C) for subdivision and packing. Where carving was required, whole breasts were aseptically removed from their cooking bags and hand-carved into chunks of the aforementioned size range. For each experiment, product chunks were commingled and distributed in 25-g aliquots into 22.5 cm x 15.5 cm high-barrier (coextruded ethylene-vinyl alcohol (EVOH)) bags (O$_2$ transmission rate: 3-6 cc/m^2/24 h @ 40°F and 0% R.H.; CO$_2$ transmission rate: 9-16 cc/m^2/24 h @ 40°F and 0% R.H.; H$_2$O vapor transmission rate: 0.5-0.6 g/100 in.^{2}/24 h @ 100°F and 100% R.H. (Cryovac Division, Sealed Air, Inc., Duncan, SC)), color-coded by treatment variable. To accommodate work flow, product bags (pouches) were held on ice in the cold room until inoculation (ca. 2 h).

Eight (8) product samples were randomly collected throughout the packing operation for subsequent zero-time microbiological and physical analyses. Proximate analysis results (composite of twelve samples) were provided by the manufacturer.

Product inoculation and MAP. A portion of the product samples was reserved prior to inoculation, to serve as a negative, uninoculated control for all treatments evaluated in this study. Each individual product pouch was treated, sealed, inspected, and placed on ice before advancing to the next pouch. The inoculum volume was previously determined, so as not to alter product water activity (a$_W$). The inoculating pipettor was calibrated before use, and separate, sterile

disposable pipet tips were used for each inoculation (as well as for each volume of sterile peptone water dispensed).

A stock cell suspension containing approximately equal numbers (mid-log phase) of each of the five *L. monocytogenes* test strains was prepared in sterile 0.1% peptone (Difco) water immediately prior to inoculation. After serial dilution, a working suspension was produced, which contained the necessary cell concentration to achieve the target inoculation level (Experiments 1 and 3: 30 cells/g of product; Experiment 2: 100 cells/g of product). This working suspension (inoculum) was held in an ice water bath under constant agitation during inoculation and MAP operations. Aliquots were withdrawn at the start and finish of all inoculation procedures and pour-plated in duplicate onto TSAYE to determine actual inoculum cell counts.

Ice-filled product coolers were transferred as needed from the cold room to the laboratory for inoculation. Individual product pouches were removed from the cooler and 200 μl of the inoculum was aseptically dispensed over the top surface of all product chunks in the pouch (Eppendorf Model 4710 adjustable pipettor, Brinkmann Instruments, Inc., Westbury, NY). To ensure uniformity in sample treatment, negative product controls received 200 μl sterile 0.1% peptone water in place of the inoculum.

After inoculation, the pouch was gently shaken to evenly distribute its contents. The designated modified atmosphere (100% N_2 or 30% CO_2:70% N_2) was applied via a two-stage process employing vacuum removal of air followed by gas filling (Multivac A300 vacuum sealer, Multivac, Kansas City, MO; Smith 180 SCFH proportional tri-gas blender, Smith Equipment, Watertown, SD). The pouch was impulse-sealed (Multivac A300 vacuum sealer),

visually inspected for seal integrity and headspace volume, and briefly held in an ice-filled cooler until all replicate pouches of the same treatment variable were modified atmosphere-packaged (MAP) (ca. 20 min). The MAP pouches were then removed from the cooler and placed in random, labeled, pre-assigned locations in a pre-assigned incubator.

Product incubation and sampling. MAP product was incubated for 4 weeks at 4° and 10^oC in separate Precision Scientific Model 815 low temperature incubators (Fisher Scientific Company, Pittsburgh, PA). Incubator temperature was monitored daily, using a mercury thermometer (Fisher Scientific) partially submerged in anhydrous glycerol (J. T. Baker Chemical Co., Philadelphia, PA), and some variation ($+ 1^o$ to 1.5^oC) was observed. At predetermined intervals (0, 7, 14, and 28 days), triplicate samples of each inoculated and uninoculated treatment variable were evaluated for gas production (swelling), headspace composition, aroma/appearance, *Listeria monocytogenes* (LM) count, aerobic plate count (APC), modified psychrotrophic (mPSY) plate count, and lactic acid bacteria (LAB) count. Product pH and water activity (a_w) were determined on day zero only.

Analyses of pH, a_w, and headspace gases. Product pH and a_w were determined on day zero using eight (8) random turkey breast samples collected during the pouch filling operation. Intact chunks (1.9 cm x 1.3 cm x 1.3 cm) were used for a_w measurement (Decagon CX-1 water activity system, Decagon Devices, Inc., Pullman, WA), while homogenized samples (25 g turkey + 25 g freshly-distilled water, blended for 2 min; Stomacher 400 lab blender, Tekmar Co., Cincinnati, OH) were used for pH measurement (Accumet Model 610A pH meter with

combination electrode, Fisher Scientific).

Headspace gas composition (percent CO_2, O_2, N_2) was determined at every sampling time on triplicate intact packages from each treatment variable (inoculated and uninoculated), using a Fisher Hamilton Model 29 gas partitioner (Fisher Scientific), equipped with DEHS/Chromosorb P and molecular sieve 13X columns, four-filament thermal conductivity detector, and Hewlett-Packard Model 3396A reporting integrator (Hewlett-Packard Co., Avondale, PA). Helium flow rate was 40 ml/min, cell temperature was 70°C, and column temperature was 25°C. An Airco BOC® blood gas mixture (5.13% CO_2 : 14.92% O_2 : 79.95% H_2; The BOC Group, Inc., Murray Hill, NJ) was used to standardize the partitioner. Headspace samples (1 cc) were withdrawn through a 1.3 cm-thick foam septum and collected in a 1-cc Gastight® #1001 glass syringe (Hamilton Co., Reno, NV).

Sensory evaluation. Following headspace analysis, random samples from each treatment condition were assigned random 3-digit codes and presented to a four-member untrained sensory panel for observations related to product acceptability, based collectively on aroma and visual appearance. Sample packages were opened immediately before evaluation, and panelists were asked to judge olfactory acceptability using the following five-point category scale: "definitely would use," "probably would use," "might use/might not use," "probably would not use," "definitely would not use." Olfactory unacceptability was determined as that point at which panelists reported they "probably would not use" or "definitely would not use" the product.

Microbiological analyses. After sensory evaluation, the product pouch was briefly kneaded with a cold cylindrical roller to produce a product "paste" suitable for microbiological sampling. At all sampling times, triplicate inoculated and uninoculated samples of each treatment variable were analyzed for LM, APC, mPSY, and LAB. Eleven-gram (11-g) aliquots of the sample "pastes" were blended with 99 ml of 0.1% peptone (Difco) diluent (2 min, Stomacher 400 lab blender), serially diluted as necessary, and pour-plated in duplicate using: Modified Oxford (MOX) Agar (Oxford Medium Base plus Modified Antimicrobic Supplement; Difco) for LM; Standard Methods Agar (Difco) containing 0.005% (w/v) 2,3,5-triphenyltetrazolium chloride (Fisher Scientific) for APC and mPSY; and All-Purpose Tween (APT) Agar (Difco) containing 0.0032% (w/v) bromcresol purple (BCP) dye (Fisher Scientific) (APT+BCP) for LAB. APT+BCP plates were overlaid with approximately 10 ml of APT+BCP prior to incubation. LM and APC plates were incubated at 35°C/48 h; mPSY and APT+BCP plates were incubated at 20°C/72 h. Esculin-positive (black) colonies on MOX were streaked for purity on TSAYE and then biochemically identified using the procedures described by Hitchins (*11*). Short, Gram-positive rods exhibiting the following characteristics were identified as LM: Iridescent blue colonies on TSAYE; microscopic end-over-end tumbling motility; umbrella-like growth in semisolid motility medium; catalase-positive; oxidase-negative; discrete β-hemolysis on 5% sheep blood agar; rhamnose-positive; and xylose-negative. Lenticular, sub-surface yellow (acid-producing) colonies on APT+BCP (purple background) were Gram-stained and tested for catalase reaction. Acid-producing, catalase-negative, Gram-positive nonsporeforming rods/cocci were presumptively identified as LAB (*25*).

Statistical analysis. Treatments were arranged as a 4 x 2 factorial in a randomized complete block design. All treatments were performed in triplicate, and the entire study was repeated twice. Bacterial counts were transformed into base-10 logarithms, and sensory ratings were converted from a word scale into numerical values ranging from 5 ("definitely would use") to 1 ("definitely would not use"). Analysis of variance was used to determine if significant ($P<0.05$) differences existed between treatments, using the general linear models procedure of SAS Institute, Cary, NC (*23*). Means were separated using the least significant difference method (SAS), employing a significance level of 0.05. Within individual experiments, there was no significant variation ($P>0.05$) between replicate trials, so results were combined for analysis.

RESULTS

Within each experiment, there was no difference (P>0.001) in mean indigenous microbial populations, mean headspace CO_2 concentration, or mean sensory evaluation scores between uninoculated and *L. monocytogenes*-inoculated product at each sampling time, regardless of packaging atmosphere or storage temperature. Therefore, only results from inoculated turkey are presented and discussed in this paper. No indigenous strains of *L. monocytogenes* were recovered from uninoculated turkey.

Experiment 1: Growth of *L. monocytogenes* when outnumbered 3.5 logs:1 by naturally-occurring lactics.

Product composition. The cooked turkey used in this experiment contained 24.2% protein, 71.2% moisture, 0.4% fat, 1.5% sucrose, 1.3% salt, and 0.3% phosphate. Initial product pH was 6.40 and water activity (a_W) was 0.951.

Changes in headspace CO_2 levels. Figure 1 (Part A) shows the changes in headspace CO_2 levels over time in inoculated turkey packaged under 100% N_2 (MA1) and 30% CO_2 : 70% N_2 (MA2). Samples packed under MA2 absorbed some CO_2 after packaging, so the actual zero-time headspace CO_2 concentration in these samples was approximately 3% below the target of 30% CO_2. Mean CO_2 levels increased by 18-20% (P<0.05) in MA1 samples and by 10-12% (P<0.05) in MA2 samples during storage. Increasing storage temperature dramatically increased (P<0.05) CO_2 accumulation, particularly during the first week of storage. Headspace CO_2 levels in samples held at $10^{o}C$ peaked after just 1 week, whereas CO_2 levels in samples held at $4^{o}C$

continued to increase (P<0.05) through 4 weeks of storage.

Changes in microbial populations. Initial populations of indigenous microflora in cooked turkey inoculated with *L. monocytogenes* are shown in Table 1. Total APC, mPSY, and LAB counts each approximated 10^5/gm at time zero. Morphological and biochemical characterization (*25*) confirmed that lactic acid bacteria constituted the predominant microflora on all APC and mPSY plates, thereby outnumbering *L. monocytogenes* 3.5 logs to 1. LAB levels increased (P<0.05) to 10^9/gm by week 2 and were sustained at 10^9-10^{10}/gm through week 4 in all products, irrespective of packaging atmosphere or storage temperature (Figure 2). Under both modified atmospheres, *L. monocytogenes* survived, but grew only slightly (P>0.05), increasing by 1 log at 10^oC and by only 0.5 log at 4^oC.

Sensory evaluation. Olfactory acceptability of inoculated turkey was affected by storage temperature (P<0.05) but not by packaging atmosphere (P>0.05). Lowering storage temperature extended the period of product acceptability by 2 weeks; 10^oC product was considered unacceptable at 2 weeks, but 4^oC product was not considered unacceptable until 4 weeks.

Experiment 2: Growth of *L. monocytogenes* when outnumbered 2.1 logs:1 by naturally-occurring lactics.

Product composition. The cooked turkey used in this experiment had the same proximate composition as reported in Experiment 1. Initial pH was 6.36 and a_W was 0.951.

Changes in headspace CO_2 levels. Figure 1 (Part B) shows the changes in headspace CO_2 levels over time in inoculated turkey packed under MA1 and MA2. As in Experiment 1, MA2 samples absorbed some CO_2 after packaging, effectively reducing the target zero-time

headspace CO_2 concentration by about 3%. Patterns and rates of CO_2 accumulation were similar to those observed in Experiment 1, except that the final levels of CO_2 reached were lower. Mean headspace CO_2 concentrations increased ($P<0.05$) by only 10-12% in MA1 samples and by about 6-9% in MA2 samples during storage.

Changes in microbial populations. Initial populations of background microflora in *L. monocytogenes*-inoculated turkey are shown in Table 1. Total APC, mPSY, and LAB counts were again high ($10^{4.5}$/gm), and lactic acid bacteria similarly constituted the predominant flora on APC and mPSY plates throughout the study. To counter this initial competitive challenge, the *L. monocytogenes* (LM) inoculum size was increased to 10^2/gm. This produced a contamination ratio in which LAB outnumbered LM 2.1 logs:1. As in Experiment 1, LAB levels increased ($P<0.05$) to 10^9/gm in all products by week 4, irrespective of packaging atmosphere or storage temperature (Figure 3). In comparison to 100% N_2-packaged product, LM populations were approximately 1 log ($P<0.05$) lower when product was packed under 30% CO_2 : 70% N_2 (MA2), and approximately 1.5 logs ($P<0.05$) lower when storage temperature was dropped from 10°C to 4°C. Other researchers have observed a similar increase in the inhibitory effect of CO_2 on *L. monocytogenes* with decreasing temperature (*7,9,17*), which is probably due to increased CO_2 solubility at lower temperatures (*19*).

Sensory evaluation. Olfactory acceptability of inoculated turkey was unaffected ($P>0.05$) by packaging atmosphere and storage temperature. All products were considered unacceptable at 1 week.

Experiment 3: Growth of *L. monocytogenes* when numerically equivalent to naturally-occurring lactics.

Product composition. The cooked turkey used in this experiment contained 15.2% protein, 79.4% moisture, 0.8% fat, 1.5% sucrose, 1.8% salt, and 0.4% phosphate. Initial product pH was 6.20 and a_w was 0.951.

Changes in headspace CO_2 levels. Figure 1 (Part C) shows the changes in headspace CO_2 levels over time in inoculated turkey packed under MA1 and MA2. MA2 samples absorbed some CO_2 after packaging (as was observed in Experiments 1 and 2), reducing the target zero-time headspace CO_2 level by about 4%. Overall CO_2 accumulation was considerably lower (P<0.05) for all treatment variables, compared to Experiments 1 and 2. Only slight increases in CO_2 levels were observed throughout storage for MA1 and MA2 samples (irrespective of incubation temperature), but none of these were found to be significant (P>0.05).

Changes in microbial populations. Initial populations of native microflora in *L. monocytogenes*-inoculated turkey are shown in Table 1. Background microflora in this product (hand-carved) differed considerably from that encountered in the previous two experiments (machine-carved product). APC, mPSY, and LAB counts at time zero were all under 10^2/gm, such that inoculated LM populations were essentially equivalent to, rather than outnumbered by, competing microbes. Lactic acid bacteria constituted the predominant flora isolated from APC and mPSY plates, but gas-producing strains were less prevalent than in earlier experiments. Greater variety in colony morphology and size/shape of rods was observed in this experiment (compared to the two previous ones), and for each treatment, LM and LAB populations increased (5 to 7 logs; P<0.05) in a parallel manner during the 4-week storage period. As incubation

temperature was lowered, packaging atmosphere exerted a noticeable effect (P<0.05) on final

LM and LAB population levels. MA2 suppressed ultimate populations approximately 1.5 logs

(P<0.05) in product held at 4°C, but had no effect (P>0.05) on ultimate population levels in 10°C

product.

Sensory evaluation. Product packaged under MA1 was considered acceptable

throughout the storage period, regardless of incubation temperature. Product packaged under

MA2 was considered unacceptable after 4 weeks at 10°C, but acceptable throughout the entire

storage period at 4°C.

DISCUSSION

In all three experiments, lactic acid bacteria (LAB) constituted the predominant background microflora of cooked turkey breast. Consequently, APC and mPSY counts were equivalent to LAB counts instead of declining over time, as would have been expected for a mixed flora.

When initial populations of indigenous lactics were 3.5 logs (Experiment 1) or 2.1 logs (Experiment 2) higher than LM populations, LM growth was suppressed (Figures 2 and 3). The degree of suppression increased as the LAB:LM contamination ratio increased, producing noticeably different patterns of growth and olfactory acceptability. Higher initial levels of LM in Experiment 2 conferred a slightly greater competitive advantage on the organism, allowing it to reach higher ultimate population levels than in Experiment 1. The lack of a distinct lag period in the growth of LAB populations (compared to Experiment 1) would account for the faster onset of olfactory unacceptability that was seen in Experiment 2. In both Experiments 1 and 2, sensory panelists commented that products stored at 10°C had a primarily "acrid" aroma, whereas those stored at 4°C had a primarily "fruity" aroma. This may be a manifestation of temperature-induced shifts in either metabolic pathways or relative proportions of lactic species present in the contaminating microflora.

When the initial LAB:LM population ratio was equivalent (Experiment 3), LM growth was not suppressed (Figure 4). Instead, for any given treatment, LM populations increased at the same rate and reached the same ultimate levels as LAB populations. Most of the lactics were morphologically different from those recovered in the two previous experiments (likely the result

of thermal processing variations, not product composition). They grew more slowly (Figure 4),

produced less gas (Figure 1), were more sensitive to environmental CO_2 (Figure 4), and impacted

olfactory acceptability less adversely. These properties would account for the substantial

differences observed in organism recovery on APC, mPSY, and LAB media at time zero,

compared to Experiments 1 and 2 (Table 1). In the presence of 30% CO_2, LM and LAB

populations were both negatively affected, with a stronger inhibitory effect occurring at lower

temperature (P<0.05). As mentioned earlier, this temperature-dependent inhibitory effect has

been widely observed (*7,9,17,18*). Sensory panelists found most of the samples to be acceptable

through 4 weeks of storage. Sample aroma was generally described as "sweet/metallic," but not

deemed objectionable. As a consequence, all of the 4°C samples and half of the 10°C samples

were never considered unacceptable, even though their LAB populations were comparable to

those found in unacceptable samples from Experiments 1 and 2.

The results of this study demonstrate that lactic competition can serve as a barrier to

listerial growth, but that indigenous microflora cannot be relied upon to inhibit *L. monocytogenes*

in ready-to-eat MAP poultry products. As observed at time zero, numbers and types of naturally-

occurring bacteria can vary considerably from one production lot to another. These can further

be radically altered as a consequence of inadequate sanitation, improper handling practices, and

temperature abuse. High levels of *L. monocytogenes* can be present without overt signs of

spoilage, thereby rendering evaluation of aroma and appearance ineffectual as a means of

indicating product safety. To ensure the safety of ready-to-eat MAP meat and poultry products,

strict temperature control throughout distribution and storage is necessary, together with

additional antilisterial barriers. Intervention strategies which employ bacteriocinogenic

compounds or standardized antagonistic cultures (*2,3,4,8*) hold great promise for improving the

safety of these products, as well as for providing viable commercial production alternatives that

are consistent with consumers' demands for "natural" ingredients and processes.

ACKNOWLEDGMENT

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TABLE 1. *Initial microbial populations in cooked turkey inoculated with* L. monocytogenes

	Microbial populations at time zero (mean log CFU/g ± SD; *n*=24)			
Experiment number	Aerobic plate count (APC)	Modified psychrotrophic (mPSY) plate count	Lactic acid bacteria (LAB) count	*Listeria monocytogenes* (LM) count
1	5.10 ± 0.09A[a]	4.97 ± 0.06A	4.99 ± 0.11A	1.43 ± 0.05B
2	4.64 ± 0.15A	4.67 ± 0.14A	4.67 ± 0.18A	2.20 ± 0.03B
3	0.72 ± 0.06C	1.81 ± 0.08A	1.28 ± 0.04B	1.34 ± 0.07B

[a] Within the same experiment number, means followed by different letters are significantly different (P<0.05).
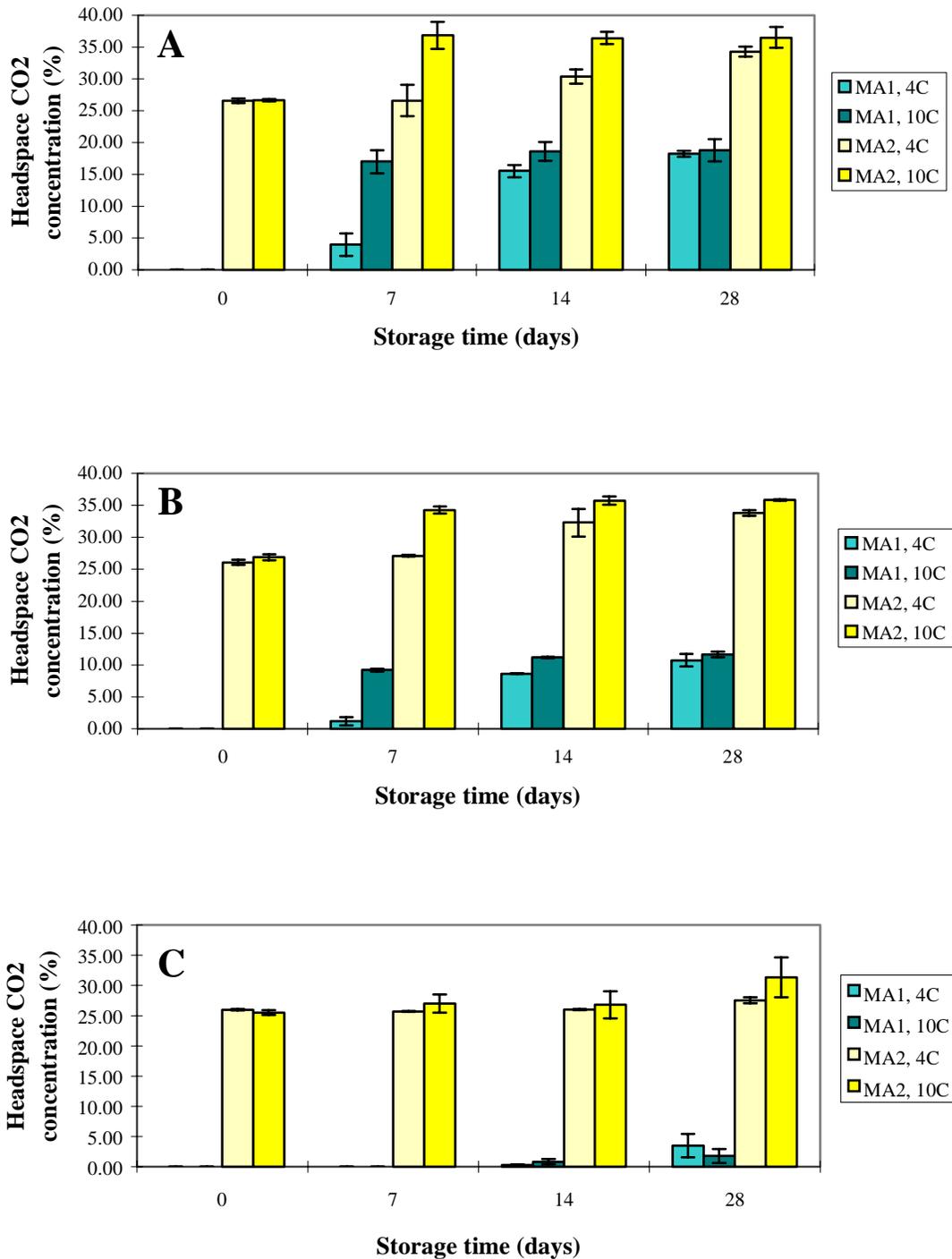
FIGURE 1. *Mean headspace CO_2 concentrations of* L. monocytogenes-*inoculated cooked turkey from: (A) **Experiment 1** (10^1 cells/g inoculum); (B) **Experiment 2** (10^2 cells/g inoculum); and (C) **Experiment 3** (10^1 cells/g inoculum). MA1 = 100% N_2 ; MA2 = 30% CO_2 : 70% N_2 . Error bars indicate standard deviations.*
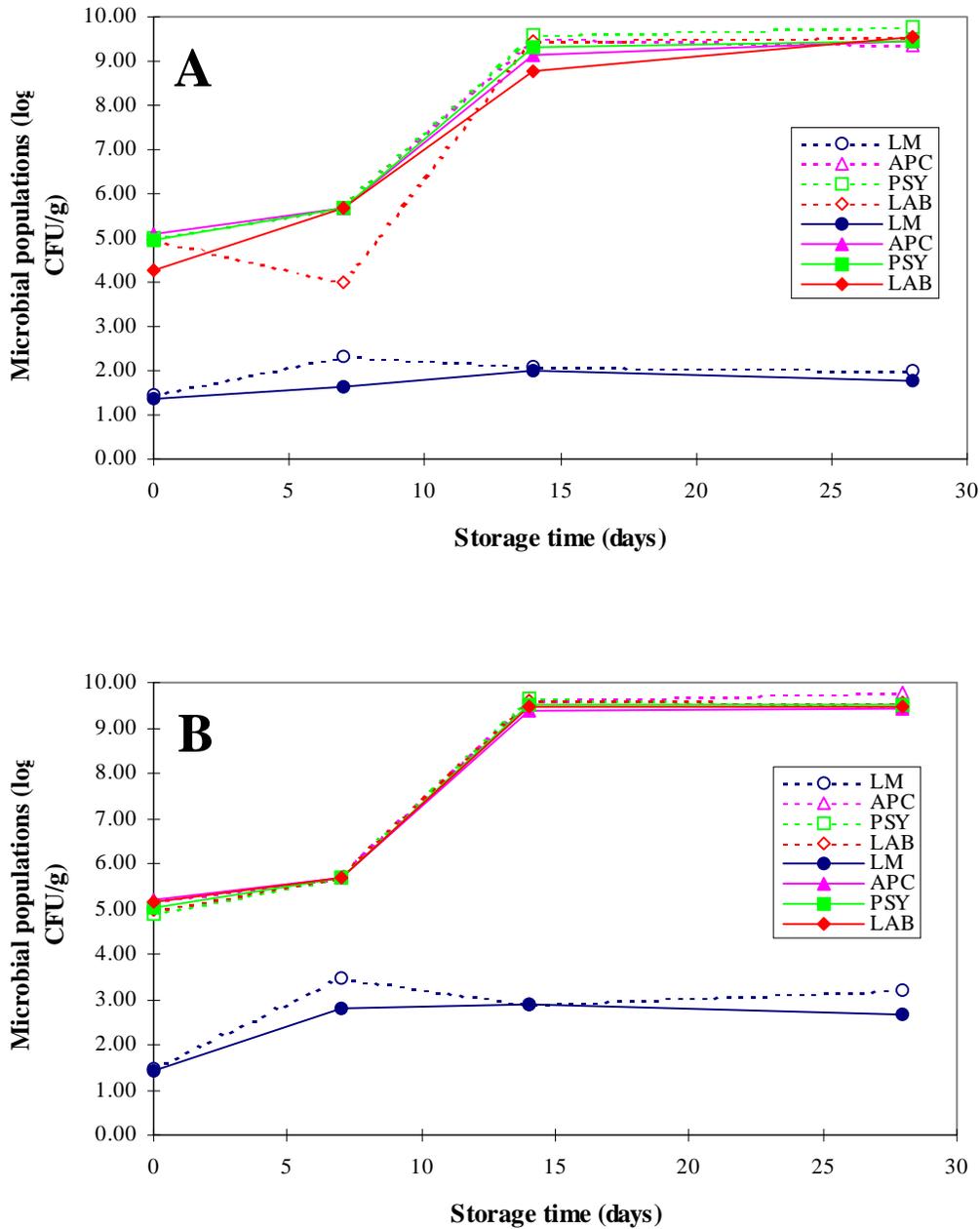
FIGURE 2. *Experiment 1. Mean microbial populations of cooked turkey inoculated with a five-strain mixture of* L. monocytogenes *and stored at (A) 4°C and (B) 10°C. Dashed lines represent product packaged under 100% N_2 (MA1); solid lines represent product packaged under 30% $CO_2 : 70\% N_2$ (MA2). LM = L. monocytogenes count; APC = aerobic plate count; PSY = (modified) psychrotrophic plate count; LAB = lactic acid bacteria count.*
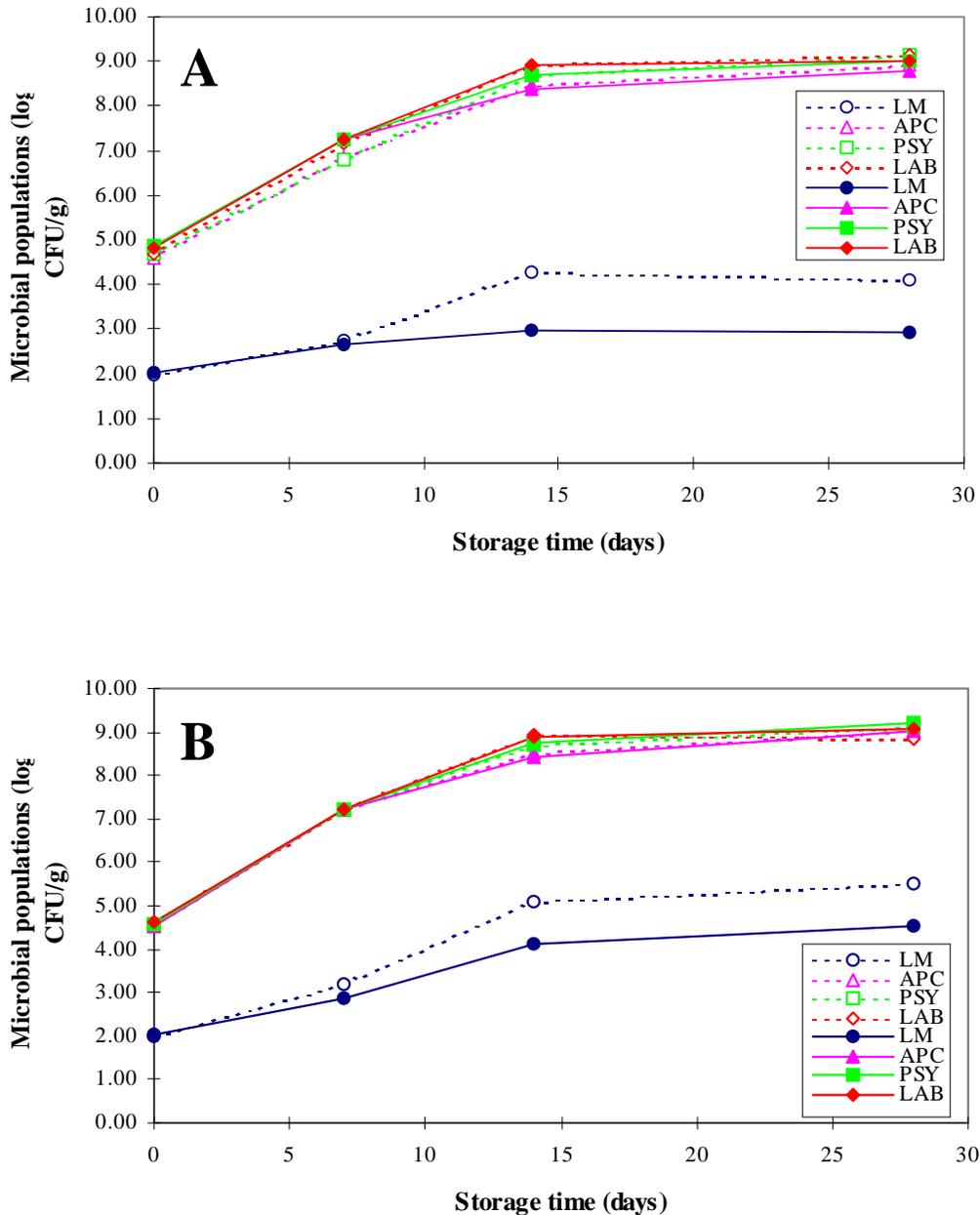
FIGURE 3. *Experiment 2. Mean microbial populations of cooked turkey inoculated with a five-strain mixture of* L. monocytogenes *and stored at (A) 4ºC and (B) 10ºC. Dashed lines represent product packaged under 100% N_2 (MA1); solid lines represent product packaged under 30% CO_2 : 70% N_2 (MA2). LM = L. monocytogenes count; APC = aerobic plate count; PSY = (modified) psychrotrophic plate count; LAB = lactic acid bacteria count.*
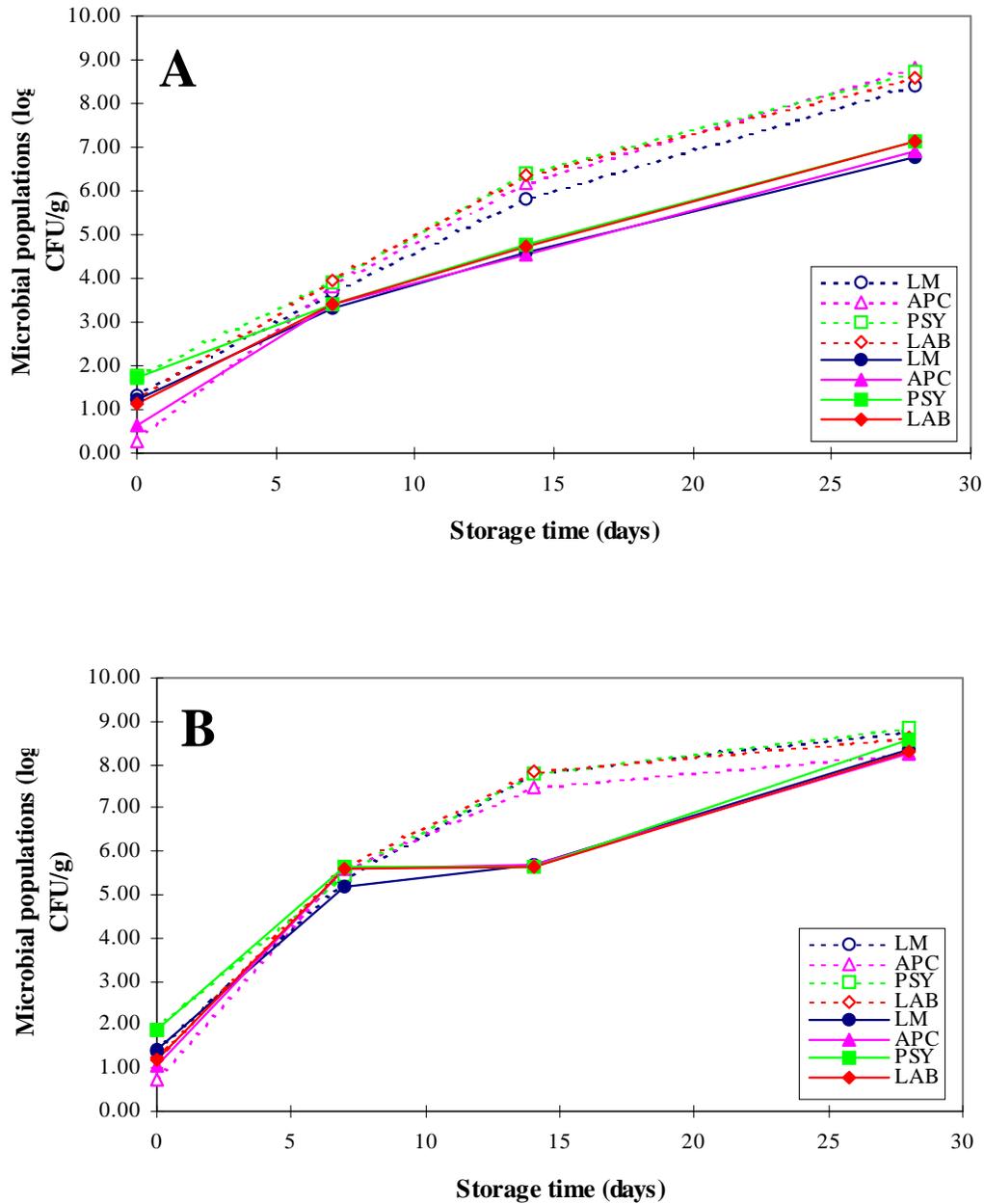
FIGURE 4. *Experiment 3.* *Mean microbial populations of cooked turkey inoculated with a five-strain mixture of* L. monocytogenes *and stored at (A) 4°C and (B) 10°C. Dashed lines represent product packaged under 100% N_2 (MA1); solid lines represent product packaged under 30% CO_2 : 70% N_2 (MA2). LM* = L. monocytogenes *count; APC = aerobic plate count; PSY = (modified) psychrotrophic plate count; LAB = lactic acid bacteria count.*

V. USE OF CELLULAR FATTY ACID ANALYSIS TO DISTINGUISH BETWEEN FOODBORNE OUTBREAK-ASSOCIATED AND NON-OUTBREAK-ASSOCIATED STRAINS OF *LISTERIA MONOCYTOGENES* IN COOKED TURKEY

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ABSTRACT

A feasibility study was conducted to determine if cellular fatty acid (CFA) composition could be used to distinguish between outbreak-associated (serotype 4) and non-outbreak-associated (serotype 1) strains of *Listeria monocytogenes* (LM) inoculated into cooked turkey. Replicate cultures (79 total) of four test strains (antigenic type 4: ATCC 43256 and 49594; antigenic type 1: CDC F7535 and F7631) were analyzed for CFA composition before inoculation into, and after recovery from, product. CFA profiles of pre-inoculation cultures clustered into 14 subgroups (Euclidian distance (ED)=1.65), corresponding exactly to antigenic type for 95.4% of the isolates. CFA profiles of post-recovery cultures clustered into 12 subgroups (ED=2.52), corresponding exactly to antigenic type for 61.1% of the isolates, and to a mixture of types 4 and 1 for the remaining 38.9% of isolates. Strain ATCC 43256 produced a consistently unique CFA profile, making it the easiest of the four test strains to differentiate. CFA composition of all 79 isolates correlated strongly with agglutination reaction, indicating that perhaps other phenotypic characteristics might be useful in differentiating between LM strains isolated from foods. There is a great and immediate need to examine existing clinical and environmental CFA databases for correlations between fatty acid composition and morphological and diagnostic characteristics of LM that could facilitate strain differentiation. CFA profiling could then become a valuable adjunct to more complex procedures for epidemiologically tracking the distribution of LM strains in food products and throughout the farm-to-table food chain.

INTRODUCTION

Since the emergence of *Listeria monocytogenes* (LM) as a foodborne pathogen, both FDA and USDA have adopted a zero-tolerance policy with regard to this organism in ready-to-eat food products. Although there are thirteen recognized LM serovars, only three are responsible for the majority (>97%) of human listeriosis cases, prompting some to question whether all foodborne strains of the organism should be viewed as posing the same risk potential (*14*). The reason for the variability in apparent pathogenicity across LM strains is unknown, but may be related to strain-specific virulence factors, stress response mechanisms, or host susceptibility (*9,14,30,37*). Whatever the cause, there is a need for developing laboratory methodologies that can rapidly, accurately, and inexpensively differentiate between outbreak- and non-outbreak-associated strains of LM. Such methods would be extremely valuable in epidemiological investigations, as well as in the study of the ecology and control of *L. monocytogenes* at all stages in the farm-to-table food chain.

Many techniques have been employed to identify bacterial pathogens in food products and processing environments. Simple procedures such as biotyping, serotyping, and phage typing (*12,19,20,35*) are not sufficiently discriminating by themselves to type strains, but are useful as initial screening tools. More sophisticated methods such as pulsed-field gel electrophoresis (PFGE), multilocus enzyme electrophoresis (MEE), ribotyping, and restriction enzyme analysis (REA) (*1,3,5,6,33,35*) are highly discriminating, but are also costly, time-consuming, and usually require specialized skills not readily available in the routine testing laboratory. An intermediate technique, in terms of speed, cost, and labor/skill requirements, is

gas chromatographic (GC) analysis of whole-cell fatty acids (CFAs) (*22*). Since the total fatty acid composition of a particular microorganism is recognized as a reliable taxonomic characteristic (*25*) and correlates with DNA homology (*26*), quantitative CFA analysis can be used to identify the organism to the species level (*10,11,15,26,35,36,41*). Recent work by Mukwaya and Welch (*23*) and Birnbaum and co-workers (*4*) suggests that CFA analysis might even be used to characterize some bacteria to the subspecies level. If CFA profiles of specific pathogens could additionally be linked to virulence factors, this technique might serve as a valuable adjunct to more complex procedures for epidemiologically tracking strains implicated in foodborne disease outbreaks. The purpose of the present study was to assess whether CFA profiles could be used to distinguish between phenotypically distinct outbreak- and non-outbreak-associated strains of *L. monocytogenes*. The test organisms were inoculated into cooked, ready-to-eat turkey in the presence of indigenous and inoculated lactic acid bacteria, and stored under modified atmospheres at refrigeration and mild abuse temperatures.

MATERIALS AND METHODS

Culture preparation and maintenance. Four strains of *Listeria monocytogenes* (two outbreak-associated, two non-outbreak-associated) and three strains of lactic acid-producing bacteria (*Carnobacterium gallinarum*, *Lactobacillus plantarum*, *Enterococcus faecalis*) were used in this study. *L. monocytogenes* strains CDC F2380 (ATCC 43256, serotype 4b, Mexican-style cheese outbreak) and Scott A (ATCC 49594, serotype 4b, milk outbreak), *C. gallinarum* (ATCC 49517, ice slush from chicken carcasses), and *L. plantarum* (ATCC 49445, ground pork) were obtained from the American Type Culture Collection (Rockville, MD). *L. monocytogenes* strains F7535 (serotype 1/2a, frozen green beans) and F7631 (serotype 1/2a, hamburger) were obtained from the laboratory surveillance culture collection of the Centers for Disease Control and Prevention (Atlanta, GA). Serotypes 1 and 4 were chosen because of the commercial availability of homologous antisera and the ease and speed of differentiation (no antigenic cross-reactivity; two distinct agglutination reactions: flocculent (ATCC 43256; F7535) and granular (ATCC 49594; F7631)). *E. faecalis* (ATCC 29212, human) was obtained as a Bactrol™ Disk culture from Difco Laboratories (Detroit, MI). *C. gallinarum* and *L. plantarum* were chosen to represent typical post-processing contaminants, while *E. faecalis* was chosen to represent potential thermoduric nonsporeformers which might survive a cook-in-bag thermal process. The distinguishing characteristics which enabled easy differentiation between the test organisms are shown in Table 1.

L. monocytogenes stock cultures were maintained on slants of trypticase soy agar (TSA; Difco Laboratories, Detroit, MI) supplemented with 0.6% yeast extract (YE; Difco) (TSAYE).

C. gallinarum and *L. plantarum* stock cultures were maintained on slants of deMan, Rogosa, and Sharpe agar (MRS; Difco). *E. faecalis* stock cultures were maintained on slants of All-Purpose Tween agar (APT; Difco). All culture slants were stored at 2°C. Before use, stock cultures were grown (through three successive transfers) to mid-log phase (based on prior growth curve studies) at 30°C in broth (tryptic soy broth (TSB; Difco) for *L. monocytogenes*; MRS broth (Difco) for *C. gallinarum* and *L. plantarum*; and APT broth (Difco) for *E. faecalis*). Culture counts were determined in duplicate using TSAYE pour plates for the *L. monocytogenes* strains, and APT agar (containing 0.0032% (w/v) bromcresol purple (BCP) dye (APT + BCP)) pour plates for the lactic strains.

Product preparation. Cook-in-bag turkey was prepared in a manner similar to commercial practice. Fresh skinned and deboned whole turkey breasts were collected immediately after slaughter from a commercial Virginia supplier and transported on ice to the Department of Food Science and Technology at Virginia Polytechnic Institute and State University (Blacksburg, VA) (ca. 2.5 h). Further-processing operations were performed in a sanitary manner in a controlled temperature cold room. Breasts were ground (0.5-cm particle size) using a Hobart Model 4532 grinder (Hobart Manufacturing Company, Troy, OH), and subdivided into 11-kg batches for blending with the remaining ingredients (added on a meat-weight basis): sodium chloride (1.8%), sodium tripolyphosphate (0.4%), sucrose (1.5%), and distilled water (16%). To aid in dispersion, the dry ingredients were solubilized in the process water and then blended into the ground turkey using a Hobart Model A-200 blender (2 min. on low speed, followed by 2 min. on medium speed). The formulated product was vacuum-

packaged (Multivac Model A300 vacuum sealer, Multivac, Kansas City, MO) in 500-g

increments in P-460-2.6 mil linear low-density polyethylene (LLDPE) barrier bags (O_2

transmission rate: 4.0 cc/100 in.^{2}/day @ 73°F and 0% R.H. (Cryovac Division, Sealed Air, Inc.,

Duncan, SC)). Bags (14 cm x 12 cm x 2.5 cm) were placed vertically in a wire mesh supporting

basket (4 bags to a basket, oriented parallel to water flow) and immersion-cooked (6 baskets at a

time) in an 82°C circulating water bath (Omega Model HH23 thermostatic controller; Omega

Engineering, Inc., Stamford, CT) to an internal temperature of 72°C. Three centrally-placed bags

in the water bath were equipped with calibrated Type K thermocouples to measure product

temperature at the cold spot during cooking. A Type K thermocouple was also positioned in the

bath to measure water temperature farthest from the heater throughout the cooking process.

Thermocouples were attached to an electronic datalogger (Model 5100, Campbell Scientific, Inc.,

Logan, UT) programmed to record temperature every 60 seconds during the cooking process

(approximately 1 h). When product endpoint temperature was reached, baskets were removed

from the water bath and submerged in a chlorinated ice water bath until product temperature

reached $\leq 2^o$C. Chilled intact bags (excluding those with thermocouples) were then transferred to

a cold room (0.5^o $\pm$ 1.5^{o}C), aseptically opened, and the product carved into chunks ranging in

size from 1.9 cm x 1.3 cm x 1.3 cm to 3.8 cm x 1.9 cm x 1.3 cm. Chunks were commingled and

distributed in 25-g aliquots into 22.5 cm x 15.5 cm high-barrier (coextruded ethylene-vinyl

alcohol (EVOH)) bags (O_2 transmission rate: 3-6 cc/m^2/24 h @ 40°F and 0% R.H.; CO_2

transmission rate: 9-16 cc/m^2/24 h @ 40°F and 0% R.H.; H_2O vapor transmission rate: 0.5-0.6

g/100 in.^{2}/24 h @ 100°F and 100% R.H. (Cryovac Division, Sealed Air, Inc., Duncan, SC)),

color-coded by treatment variable. To accommodate work flow, product bags (pouches) were

held on ice in the cold room until inoculation (ca. 2 h). Eight (8) random product samples were

collected during packing operations for subsequent zero-time microbiological and physical

analyses.

Product inoculation and MAP. A portion of the product samples was reserved prior to

inoculation, to serve as a negative, uninoculated control for all treatments evaluated in this study.

Each individual product pouch was treated, sealed, inspected, and placed on ice before advancing

to the next pouch. The inoculum volume was previously determined, so as not to alter product

water activity (a_W). The inoculating pipettor was calibrated before use, and separate, sterile

disposable pipet tips were used for each inoculation (as well as for each volume of sterile

peptone water dispensed).

Immediately prior to inoculation, like organisms were pooled, and separate stock cell

suspensions containing approximately equal numbers (mid-log phase) of the component test

strains of either *L. monocytogenes* (LM) or lactic acid bacteria (LAB) were prepared in sterile

0.1% peptone (Difco) water. Two suspensions (high- and low-count) were prepared for each

inoculation pool, and serially diluted to contain the necessary cell concentration to achieve the

target inoculation level (LM: 10^2/g of product; LAB: 10^3/g of product).   These working

suspensions (inocula) were held in separate ice water baths under constant agitation during

inoculation and MAP operations. Aliquots were withdrawn at the start and finish of all

inoculation procedures and pour-plated in duplicate onto TSAYE (for LM) and APT + BCP (for

LAB) to verify inoculum counts.

Ice-filled product coolers were transferred as needed from the cold room to the laboratory

for inoculation. Individual product pouches were removed from the cooler and 200 μl of the

inoculum was aseptically dispensed over the top surface of all product chunks in the pouch

(Eppendorf Model 4710 adjustable pipettor, Brinkmann Instruments, Inc., Westbury, NY). For

products receiving only LM, 200 μl of the low-count LM inoculum was used; for products

receiving only LAB, 200 μl of the low-count LAB inoculum was used; for products receiving

LM and LAB, 100 μl each of the high-count LM and high-count LAB inocula was used. To

ensure uniformity in sample treatment, negative product controls received 200 μl sterile 0.1%

peptone water in place of the inoculum.

      After inoculation, the pouch was gently shaken to evenly distribute its contents. The

designated modified atmosphere (100% N_2 or 30% CO_2:70% N_2) was applied via a two-stage

process employing vacuum removal of air followed by gas filling (Multivac A300 vacuum

sealer, Multivac, Kansas City, MO; Smith 180 SCFH proportional tri-gas blender, Smith

Equipment, Watertown, SD). The pouch was impulse-sealed (Multivac A300 vacuum sealer),

visually inspected for seal integrity and headspace volume, and briefly held in an ice-filled cooler

until all replicate pouches of the same treatment variable were modified atmosphere-packaged

(MAP) (ca. 20 min). The MAP pouches were then removed from the cooler and placed in

random, labeled, pre-assigned locations in a pre-assigned incubator.

      Product incubation and sampling. MAP product was incubated for 4 weeks at 5° and

10°C in separate Precision Scientific Model 815 low temperature incubators (Fisher Scientific

Company, Pittsburgh, PA). Incubator temperature was monitored daily, using a mercury

thermometer (Fisher Scientific) partially submerged in anhydrous glycerol (J. T. Baker Chemical

Co., Philadelphia, PA), and some variation ($+ 1^{o}$ to 1.5^{o}C) was observed. At predetermined intervals (0, 7, 14, 21, and 28 days), triplicate samples of each inoculated and uninoculated treatment variable were evaluated for gas production (swelling), headspace composition, *Listeria monocytogenes* (LM) count, modified psychrotrophic (mPSY) plate count, and lactic acid bacteria (LAB) count. Aerobic plate count (APC), pH, and water activity (a_W) were determined on day zero only. Whole-cell fatty acid analysis was performed on selected LM isolates before inoculation and after recovery from product that was stored at 5°C for 28 days.

Analyses of pH, a_W, and headspace gases. Zero-time product pH and a_W were determined using eight (8) random turkey breast samples collected during the pouch filling operation. Intact chunks (1.9 cm x 1.3 cm x 1.3 cm) were used for a_W measurement (Decagon CX-1 water activity system; Decagon Devices, Inc., Pullman, WA), while homogenized samples (25 g turkey + 25 g freshly-distilled water, blended for 2 min; Stomacher 400 lab blender; Tekmar Co., Cincinnati, OH) were used for pH measurement (Accumet Model 610A pH meter with combination electrode; Fisher Scientific). pH measurements of zero-time MAP samples were made on the initial product dilution (11 g turkey blended with 99 ml 0.1% peptone diluent), using both the pH meter and Hydrion paper (Fisher Scientific). Since there were no differences ($P>0.05$) in zero-time pH values between water-homogenized and peptone-homogenized samples, all subsequent MAP sample pH measurements were made using the peptone-diluted product. There were no significant differences ($P>0.05$) in pH values obtained by the meter and paper methods at 0 and 7 days so, for reasons of efficiency, all subsequent measurements were made using pH paper.

Headspace gas composition (percent CO_2, O_2, N_2) was determined at every sampling time on triplicate intact packages from each treatment variable (inoculated and uninoculated), using a Fisher Hamilton Model 29 gas partitioner (Fisher Scientific), equipped with DEHS/Chromosorb P and molecular sieve 13X columns, four-filament thermal conductivity detector, and Hewlett-Packard Model 3396A reporting integrator (Hewlett-Packard Co., Avondale, PA). Helium flow rate was 40 ml/min, cell temperature was $70^{o}C$, and column temperature was $25^{o}C$. An Airco BOC® blood gas mixture (5.13% CO_2 : 14.92% O_2 : 79.95% H_2; The BOC Group, Inc., Murray Hill, NJ) was used to standardize the partitioner. Headspace samples (1 cc) were withdrawn through a 1.3 cm-thick foam septum and collected in a 1-cc Gastight® #1001 glass syringe (Hamilton Co., Reno, NV).

Microbiological analyses. At all sampling times, triplicate inoculated and uninoculated samples of each treatment variable were analyzed for LM, mPSY, and LAB. Aerobic plate counts (APC) and KF streptococcal (KF) counts (to confirm the presence of *E. faecalis*) were performed at time zero only. Sample pouches were briefly kneaded with a cold cylindrical roller to produce a "paste"-like product consistency suitable for microbiological sampling. Eleven-gram (11-g) aliquots of each sample "paste" were blended with 99 ml of 0.1% peptone (Difco) diluent (2 min, Stomacher 400 lab blender), serially diluted as necessary, and pour-plated in duplicate using: Modified Oxford (MOX) Agar (Oxford Medium Base plus Modified Antimicrobic Supplement; Difco) for LM; Standard Methods Agar (Difco) containing 0.005% (w/v) 2,3,5-triphenyltetrazolium chloride (TTC; Fisher Scientific) for APC and mPSY; All-Purpose Tween (APT) Agar (Difco) containing 0.0032% (w/v) bromcresol purple (BCP) dye

(Fisher Scientific) (APT+BCP) for LAB; and KF Streptococcus Agar (KF; Difco) containing

0.01% (w/v) TTC for *E. faecalis*. APT+BCP plates were overlaid with approximately 10 ml of

APT+BCP prior to incubation. LM, KF, and APC plates were incubated at 35°C/48 h; mPSY

and APT+BCP plates were incubated at 20°C/72 h. Esculin-positive (black) colonies on MOX

were streaked for purity on TSAYE and then biochemically identified using the procedures

described by Hitchins (*13*). Short, Gram-positive rods exhibiting the following characteristics

were identified as LM: Iridescent blue colonies on TSAYE; microscopic end-over-end tumbling

motility; umbrella-like growth in semisolid motility medium; catalase-positive; oxidase-negative;

discrete β-hemolysis on 5% sheep blood agar; rhamnose-positive and xylose-negative in purple

carbohydrate broth; DIM-negative (API Listeria test; bioMérieux Vitek, Inc., Hazelwood, MO);

and type 1 or 4 Listeria O agglutination (rapid slide test; Difco). Deep red, acid-producing

(yellow halo) colonies on KF agar (purple background) were Gram-stained and tested for

catalase production. Acid-producing, catalase-negative, Gram-positive cocci were presumptively

identified as fecal streptococci (*E. faecalis*). Lenticular, sub-surface yellow (acid-producing)

colonies on APT+BCP (purple background) were Gram-stained and tested for catalase

production. Acid-producing, catalase-negative, Gram-positive nonsporeforming rods/cocci were

presumptively identified as LAB (*40*).

Cellular fatty acid (CFA) analysis. Four morphologically distinct confirmed LM

isolates from each inoculum pool at zero-time (prior to inoculation of turkey) and from each

treatment condition (following recovery from stored turkey) were subjected to CFA analysis,

using the Microbial Identification System (MIS) (Microbial ID, Inc. (MIDI), Newark, DE). A

total of 79 cultures was analyzed (8 reference cultures, 71 isolates). Bacteria were cultivated

aerobically under standardized growth conditions (Trypticase Soy Broth Agar, TSBA (Difco),

$30^{o}C/48h$), scraped from the agar using a sterile 4-mm inoculating loop, and collected in 13 x

100 mm borosilicate tubes with Teflon®-lined screw-cap closures. The harvested cells were

saponified for 30 min at $100^{o}C$ in a solution of 15% (w/v) NaOH in 50% aqueous methanol,

cooled, methylated for 10 min at $80^{o}C$ in a solution of 6N HCl in aqueous methanol, cooled, and

the resulting fatty acid methyl esters (FAMEs) extracted with hexane/methyl-tert butyl ether

(MBTE) and washed in dilute (1.2%, w/v) NaOH. The FAMEs were analyzed by high-

performance capillary gas-liquid chromatography (GLC), using a system comprised of a

Hewlett-Packard (Avondale, PA) HP5890 gas chromatograph, HP 7673A controller and

automatic sampler, Ultra 2 fused-silica capillary column, flame ionization detector, HP 3396A

recording integrator, and Zenith Z-Note 325L portable computer. System operating conditions

were as specified by Microbial ID, Inc. (*26*). Fatty acids were identified by comparing their

retention times to those of reference calibration standards, and peak area values were calculated

as percentages of the total peak area. The MIS computer software (*22*) compared the fatty acid

composition of each isolate with the mean fatty acid composition of the strains used to create the

LM library entry listed as the isolate's match. This comparison generated a numerical value

known as the Similarity Index (SI), which measured the relative distance (in multi-dimensional

space) of the sample CFA profile from the library entry population mean (*26*).

Statistical analysis. Treatments were arranged as a 5 x 2 x 2 factorial in a randomized

complete block design. All treatments were performed in triplicate, and the entire study was

repeated twice. Bacterial counts were transformed into base-10 logarithms. Analysis of variance

was used to determine if significant (P<0.05) differences in microbial populations, pH,

headspace composition, or CFA profiles existed between treatments, using the general linear

models procedure of SAS (*31*). Means were separated using the least significant difference

method (SAS), employing a significance level of 0.05. Cluster analysis (unweighted pair

matchings based on isolate CFA composition) (*26*) was used to generate a tree diagram

(dendrogram), graphically showing the relatedness of LM isolates in units of Euclidian Distance

(the distance between two samples in n-dimensional space). The dendrogram was used to

separate isolates into CFA subgroups (*26,29*). Significance of potential associations between

subgroups and antigenic type or agglutination reaction of each isolate was determined using a

chi-square test (*28*).

RESULTS

Microbial growth patterns. There was no significant difference (P>0.05) in mean microbial populations, mean pH values, or mean headspace CO_2 concentrations between uninoculated and *L. monocytogenes*-inoculated treatment variables throughout the study; therefore, only results from inoculated product are reported. No indigenous strains of *L. monocytogenes* (LM) were recovered from uninoculated turkey. Lactic acid bacteria (LAB) constituted the predominant background microflora of cooked turkey, and were present at levels equivalent (P>0.05) to the levels of inoculated LAB (Table 2).

LM grew well in the presence of both indigenous and inoculated LAB (Table 2), and over a pH range of 6.3 to 4.7 (Table 3). LM levels increased more slowly at 5°C than at 10°C, but final levels were equivalent to, and not significantly different (P>0.05) from, final levels of LAB. Packaging atmosphere did not significantly affect (P>0.05) ultimate levels of LM or LAB. Water activity values and headspace CO_2 accumulation rates and patterns (data not shown) were similar to those reported previously (*17*). Four morphologically and antigenically distinct culture types, matching those of the four inoculated LM test strains, were recovered from cooked turkey at each treatment condition.

Cellular fatty acid (CFA) composition. Seventy-six (76) presumptive LM isolates (71 typical, 5 atypical) were selected from inoculum pools at time zero and recovered from inoculated turkey after 28 days of storage at 5°C. The 5 atypical isolates (recovered from product) were weakly esculin-positive, but only after 48 h of incubation. Upon further

examination, they were found to be non-motile, non-hemolytic, non-agglutinating (types 1 and 4 Listeria O antisera), catalase-negative cocci, and were therefore discontinued from consideration for CFA analysis. The 71 typical isolates were strongly esculin-positive within 18-24 h, and typed conclusively (morphologically, biochemically, and serologically) as LM, without any ambiguities. Antigenic type 4 isolates outnumbered type 1 isolates by approximately 2:1 both before inoculation into, and after recovery from, cooked turkey (Table 4). Confirmed LM isolates were subjected to CFA analysis, together with duplicate cultures of the four reference strains.

No unsaturated fatty acids were detected in any samples, including the reference cultures. All LM isolates had the following saturated fatty acids in common: tetradecanoic ($C_{14:0}$), 13-methyltetradecanoic ($C_{i-15:0}$), 12-methyltetradecanoic ($C_{a-15:0}$), 14-methylpentadecanoic ($C_{i-16:0}$), hexadecanoic ($C_{16:0}$), 15-methylhexadecanoic ($C_{i-17:0}$), and 14-methylhexadecanoic ($C_{a-17:0}$) (Figure 1). The $C_{15:0}$ and $C_{17:0}$ chain-length acids predominated, with anteiso-branched chains being more prevalent than iso-branched chains. These results are in agreement with the findings of other researchers (*8,10,16,27,38*). Strain ATCC 43256 was the only reference culture to produce 10-methyldodecanoic acid ($C_{a-13:0}$), and strain F7535 was the only reference culture to produce 16-methyloctadecanoic acid ($C_{a-19:0}$). The only significant difference observed in CFA profiles between pre-inoculation and post-recovery isolates was the relative proportion of $C_{a-15:0}$ and $C_{a-17:0}$ (each of which accounted for $\geq 30\%$ of the total fatty acid composition). This could be the result of age variations among cells harvested from different regions of TSBA plates (*32*). Minor variations in trace quantities ($<1\%$) of $C_{12:0}$ and other branched-chain acids between the two groups of isolates could also have the same cause.

Reproducibility of reference strain CFA profiles. Two CFA analyses were performed on each reference strain prior to the start of this study, and two were performed during the study, yielding a total of four repetitions per strain. When cultivated at optimal temperatures and analyzed repeatedly under standardized conditions, each reference strain produced CFA profiles with good reproducibility (Table 6). Within each strain, the coefficient of variation (CV) for each fatty acid produced was generally under 20% (and in some cases, under 10%). This contrasts with the results of Steele et al. (*34*), who observed high variability in fatty acid profiles from each of 5 LM strains tested, leading them to conclude that CFA analysis was of limited use for studying the epidemiology of LM isolates. Strains F7631 and ATCC 49594 had the least amount of variation, while strains F7535 and ATCC 43256 had the greatest amount of variation (especially among $C_{16:0}$, $C_{i-17:0}$, and $C_{a-17:0}$ acids). F7535 additionally showed substantial variation in $C_{i-14:0}$ production. The signature fatty acids, $C_{a-13:0}$ and $C_{a-19:0}$, also showed higher levels of variability for their respective strains (ATCC 43256 and F7535).

Cluster analysis and distribution of subgroups. Cluster analysis (*26*) of CFA profiles for 39 LM isolates before inoculation into cooked turkey (together with 4 reference cultures) showed that the isolates were all very closely related (Figure 2). At a Euclidian distance of 1.65, the isolates could be distributed into 14 subgroups, based principally on the relative amounts of $C_{12:0}$, $C_{a-13:0}$, $C_{i-14:0}$, $C_{14:0}$, $C_{i-15:0}$, $C_{a-15:0}$, $C_{i-16:0}$, $C_{16:0}$, $C_{i-17:0}$, and $C_{a-17:0}$. The 14 subgroups corresponded exactly to antigenic type (1 or 4) for 41 of the 43 isolates (95.4%), an observation which has been made by others (*32*). At a Euclidian distance of 0.66 (data not shown), these isolates could be further separated according to agglutination reaction (granular or flocculent).

Cluster analysis (*26*) of CFA profiles for 32 LM isolates recovered from cooked turkey (together with 4 reference cultures) showed that the isolates were still related, but somewhat less closely than before inoculation (Figure 3). At a Euclidian distance (ED) of 2.52, 12 subgroups were apparent, based primarily on the relative quantities of $C_{12:0}$, $C_{i-14:0}$, $C_{i-15:0}$, $C_{a-15:0}$, $C_{i-17:0}$, $C_{a-17:0}$, and $C_{a-19:0}$. Nine (9) of the 12 subgroups (comprising 61.1% of the isolates) corresponded exactly to antigenic type (1 or 4), with overlap between types 1 and 4 in the remaining 3 subgroups (4, 6, 8). All isolates were further separated by agglutination reaction (granular or flocculent) at an ED of 0.69 (data not shown).

DISCUSSION

Although the four antigenically distinct *L. monocytogenes* (LM) test strains were pooled in equal numbers at time zero, they were not recovered from inoculum pools or inoculated turkey in equal proportions. Approximately twice as many antigenic type 4 isolates as type 1 isolates were recovered. Since the strain pool was held in an ice water bath during product inoculation procedures, the type 1 strains (F7535, F7631) may have been less able to tolerate the shift to low temperature, thus resulting in lower populations than their type 4 counterparts (ATCC 43256, ATCC 49594). Microbial adaptation to low temperatures ("cold shock" response) has been widely observed in many species (*2*). No data are available comparing the thermal adaptability characteristics of the 13 recognized serotypes of *L. monocytogenes*, but this property may be a possible contributing factor to the higher observed frequency of type 4 strains (versus types 1 and 2) associated with foodborne listeriosis outbreaks (*24*).

Strain-specific growth rates may also play a role in determining the relative proportions of LM serotypes after storage in cooked turkey. Growth curves generated in conjunction with this study showed that, under optimal growth conditions, the lag phase for strains F7535 and F7631 was 1 h longer than for strains ATCC 43256 and ATCC 49594 (data not shown). In addition, type 1 strain lag phases were 2-3 times as long as those of lactic acid-producing bacteria inoculated into the turkey. Buncic and Avery (*7*) have observed that increased lag phases accompany declines in pathogenicity for LM strains held at $4^{o}C/4$ wks and then cultivated at $37^{o}C$. Perhaps an extended lag phase, together with a competitive disadvantage (with respect to initiation of logarithmic growth) in a mixed-microflora food product, also contribute to a

reduction in the frequency of type 1 strain occurrence in, and recovery from, foods.

A surprising observation was that all type 4 isolates (including type 4 reference cultures) exhibited both end-over-end tumbling motility and rapid conical spinning (about a fixed point) in wet mount preparations. Type 1 isolates (including type 1 reference cultures), on the other hand, exhibited only end-over-end tumbling motility. Wherever spinning cells were observed, they appeared to be attached to the microscope slide at one end of the cell. *L. monocytogenes* has been shown to produce proteins which aid in adhesion to surfaces (preparatory to biofilm formation) (*18*), which might account for this behavior. Another possibility is the formation of actin tails, which aid in locomotion (*30*). These tails are known to extend only from one end of the bacterium and are thought to be involved in virulence, since they allow LM to move between and within intestinal mucosa cells, where the surrounding temperature (37°C) significantly diminishes production of flagella (*39*). Perhaps the additional motility characteristics of the type 4 strains evaluated here would afford them improved colonization ability over type 1 strains, and thus provide another possible reason for the greater incidence of type 4 strains associated with foodborne disease outbreaks.

Strain ATCC 43256 consistently occupied its own fatty acid subgroup (due to exclusive production of $C_{a-13:0}$), making it the easiest of the four LM test strains to differentiate. There was some variability in clustering patterns among the 3 other test strains, which complicated differentiation. This might be due to variations in cell age or growth phase at the time of harvesting from TSBA plates (*32*). Strain ATCC 43256 had the most pliable colonial consistency of the four cultures tested, which facilitated cell harvesting operations. The other strains were either very wet or tacky on TSBA, leading to difficulty in harvesting. This might

have affected the relative amounts of new and old growth collected from plates and, thus, the resultant CFA profiles. In addition, differences in harvesting techniques among researchers could also have contributed to within-strain variability in CFA patterns (*26,32*). Agglutination reaction (granular or flocculent) for all strains was distinguishable at the same ED (0.7), regardless of inoculation or harvesting condition. This could be coincidental or possibly hint of an environmentally-stable genetic determinant (marker) which might be useful as an ancillary property for strain discrimination employing more complex procedures. Analysis of additional LM serotypes is warranted, in order to define which diagnostic properties best afford discrimination between strains.

The results of this study indicate that CFA analysis can distinguish between phenotypically different, foodborne outbreak-associated and non-outbreak-associated strains of *L. monocytogenes* recovered from refrigerated, ready-to-eat food products. Usefulness of the technique at the present time is limited, however, due to a lack of published data correlating CFA profiles with morphological and other diagnostic characteristics of LM that serve as valuable tools for differentiation. There is a great and immediate need to examine existing clinical and environmental CFA databases to identify diagnostic correlations and to quantify CFA pattern similarity and discrimination ability across strains. CFA profiling could then be used as a valuable adjunct to more complex procedures for epidemiologically tracking the distribution of LM strains in food products and throughout the farm-to-table food chain.

ACKNOWLEDGMENTS

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TABLE 1. *Distinguishing characteristics of* L. monocytogenes *(Lm) and lactic acid bacteria (*C. gallinarum *(Cg),* L. plantarum *(Lp),* E. faecalis *(Ef)) strains inoculated into cooked turkey*

Characteristic	Strain						
	Lm1[a]	Lm2[b]	Lm3[c]	Lm4[d]	Cg[e]	Lp[f]	Ef[g]
Cell morphology	Long thin rods, singly and pairs, many 1-end spinners	Long thin rods, singly and pairs, many 1-end spinners	Medium rods with bulbous ends, singly and short chains	Medium to long thin rods with beaded appearance, most singly, some pairs	Short stubby rods, pairs and short chains	Short to medium grainy rods, singly and pairs	Elongated diplococci and short chains
Motility (25°C)	+	+	+	+	−	−	−
Colony Morphology							
- MOX	Opaque, sl. dull, lt. grey w/ dk. grey depr. ctr.	Translucent, shiny, med. grey to black, flat, irreg. edge, (umbonate over time)	Opaque, dull brown w/ sl. depr. ctr.	Opaque, shiny, grey w/ lg. brownish depr. ctr. somewhat metallic cast	No growth	No growth	No growth
- TSAYE	Shiny bluish white, cvx., soft, liquidy	Shiny off-wh., bluish, cvx., sl. translucent, soft, liquidy	Opaque, bluish white, cvx., smooth, moist but tacky	Shiny, bluish translucent, cvx., sl. dry and tacky	Opaque, wh., smooth, cvx., soft and moist	Opaque, sl. dull, ivory, cvx., soft and very liquidy	Opaque, sl. dull, ivory, cvx., soft and very liquidy
Catalase	+	+	+	+	−	−	−
Esculin hydrolysis	+	+	+	+	−	−	−
β-hemolysis (sheep)	+	+	+	+	−	−	−
Agglut. - Type 1	−	−	+	+	−	−	−
- Type 4	+	+	−	−	−	−	−

[a] ATCC 43256   [b] ATCC 49594   [c] CDC F7535   [d] CDC F7631   [e] ATCC 49517   [f] ATCC 49445   [g] ATCC 29212

TABLE 2. *Growth of inoculated* L. monocytogenes *(Lm) and indigenous and inoculated lactic acid bacteria (LAB) in modified atmosphere packaged cooked turkey*

Temp.[a]	MA[b]	LAB type[c]	*Lm* populations (mean log CFU/g ± SD; *n*=6) at day:				
			0	7	14	21	28
5°C	1	N	2.30±0.21[B][d]	5.75±0.00[A,B]	7.26±0.27[A,B]	7.34±0.09[A,B]	7.20±0.10[A,B]
		I	2.21±0.04[B]	4.26±0.51[A,B]	4.65±0.18[A,B]	5.20±0.46[A,B]	4.39±0.05[A,B]
	2	N	2.23±0.11[B]	4.79±0.29[A,B]	5.51±0.04[A,B]	6.14±0.58[A,B]	6.59±0.01[A,B]
		I	2.31±0.10[B]	2.87±0.15[B]	3.74±0.13[A,B]	4.49±0.35[A,B]	4.36±0.25[A,B]
10°C	1	N	2.23±0.14[B]	6.66±1.00[A,B]	8.24±0.10[A]	8.12±0.23[A]	7.95±0.21[A]
		I	2.17±0.07[B]	6.33±0.64[A,B]	7.11±0.66[A,B]	7.10±0.47[A,B]	7.12±0.60[A,B]
	2	N	2.21±0.09[B]	6.21±0.05[A,B]	7.73±0.58[A,B]	7.83±0.37[A,B]	7.65±0.47[A,B]
		I	2.20±0.07[B]	6.35±0.66[A,B]	6.81±0.71[A,B]	6.68±0.91[A,B]	7.41±0.07[A,B]

Temp.	MA	LAB type	LAB populations (mean log CFU/g ± SD; *n*=6) at day:				
			0	7	14	21	28
5°C	1	N	2.86±0.17[B]	7.10±0.49[A,B]	8.99±0.10[A]	8.70±0.40[A]	8.25±0.72[A]
		I	3.16±0.18[B]	8.08±0.46[A,B]	8.39±0.58[A]	8.82±0.06[A]	8.76±0.10[A]
	2	N	2.70±0.32[B]	4.44±0.37[A,B]	8.83±0.01[A]	8.54±0.28[A]	8.70±0.09[A]
		I	2.96±0.29[B]	4.87±0.96[A,B]	8.77±0.11[A]	8.48±0.41[A]	9.28±0.12[A]
10°C	1	N	2.91±0.14[B]	8.61±0.04[A]	8.99±0.15[A]	8.79±0.20[A]	8.59±0.05[A]
		I	3.01±0.30[B]	8.14±1.53[A]	8.69±0.54[A]	8.77±0.09[A]	8.60±0.11[A]
	2	N	2.70±0.35[B]	7.35±0.75[A,B]	8.77±0.10[A]	8.70±0.13[A]	8.50±0.24[A]
		I	3.07±0.30[B]	8.14±0.52[A]	8.68±0.42[A]	8.83±0.09[A]	8.68±0.10[A]

[a] Temp., storage temperature
[b] MA, modified atmosphere (MA1=100% N_2; MA2=30% CO_2:70% N_2)
[c] LAB type, lactic acid bacteria type (N=indigenous; I=inoculated)
[d] Within each storage temperature group, means followed by different letters are significantly different (P<0.05).

TABLE 3. *pH profiles of* L. monocytogenes-*inoculated modified atmosphere packaged cooked turkey in the presence of indigenous and inoculated lactic acid bacteria (LAB)*

Temp.[a]	MA[b]	LAB type[c]	Mean sample pH ($\pm$ SD; $n=6$) at day:				
			0	7	14	21	28
5°C	1	N	6.3±0.0A [d]	5.6±0.0A,B	5.2±0.0B	5.1±0.1B	5.2±0.2B
		I	6.3±0.0A	5.7±0.0A,B	5.4±0.0A,B	5.2±0.1B	5.0±0.2B
	2	N	6.3±0.0A	5.7±0.1A,B	5.5±0.0A,B	5.4±0.1A,B	5.3±0.1B
		I	6.3±0.0A	5.8±0.0A,B	5.3±0.0A,B	5.2±0.1B	5.2±0.1B
10°C	1	N	6.3±0.0A	5.1±0.1A,B	5.0±0.1A,B	4.8±0.1B	4.7±0.0B
		I	6.3±0.0A	5.2±0.1A,B	5.2±0.0A.B	4.9±0.1B	4.9±0.1B
	2	N	6.3±0.0A	5.4±0.0A,B	5.3±0.1A,B	4.8±0.1B	4.8±0.0B
		I	6.3±0.0A	5.5±0.1A,B	5.2±0.1A,B	4.9±0.1B	4.8±0.1B

[a] Temp., storage temperature
[b] MA, modified atmosphere (MA1=100% N_2; MA2=30% CO_2:70% N_2)
[c] LAB type, lactic acid bacteria type (N=indigenous; I=inoculated)
[d] Within each storage temperature group, means followed by different letters are significantly different (P<0.05).

TABLE 4. *Agglutination reactions and fatty acid subgroups of* L. monocytogenes *isolates before inoculation into, and after recovery from, cooked turkey*

Antigenic type/ agglutination reaction	Before inoculation			After recovery		
	No. of isolates	Isolate designation	Fatty acid subgroup[a]	No. of isolates	Isolate designation	Fatty acid subgroup[b]
4/granular	1	Sample	2	1	Sample	3
	3	Sample	3	1	ATCC 49594	4
	2	Sample	4	1	Sample	6
	1	Sample	9	2	Sample	7
	2	Sample	10	2	Sample	8
	7	Sample	12	1	Sample	9
	1	ATCC 49594	14	2	Sample	10
				3	Sample	12
4/flocculent	1	Sample	1	1	ATCC 43256	1
	1	Sample	3	1	Sample	3
	4	Sample	4	1	Sample	4
	1	Sample	8	1	Sample	5
	1	Sample	9	1	Sample	6
	1	Sample	10	1	Sample	8
	1	Sample	11	1	Sample	10
	1	Sample	12	3	Sample	12
	1	ATCC 43256	13			
1/granular	1	Sample	3	2	Sample	4
	4	Sample	5	1	F7631	6
	1	Sample	6	1	Sample	6
	3	Sample	8	2	Sample	8
	1	F7631	8	1	Sample	11
1/flocculent	1	F7535	5	1	F7535	2
	2	Sample	7	2	Sample	8
	1	Sample	8	3	Sample	11

[a] Subgroup designations as in Figure 2
[b] Subgroup designations as in Figure 3

TABLE 5. *Cellular fatty acids produced by* L. monocytogenes *isolates prior to inoculation into, and following recovery from, cooked turkey, together with corresponding reference cultures*

Fatty acid detected	Fatty acids[a] of reference (R)[b] cultures and isolates (I)										
	R1	R2	R3	R4	I[c]		R1	R2	R3	R4	I[d]
$C_{9:0}$	−	−	−	−	+		−	−	−	−	−
$C_{10:0}$	−	−	−	−	+		−	−	−	−	−
$C_{i-12:0}$	−	−	−	−	+		−	−	−	−	−
$C_{12:0}$	+	+	+	+	+		+	−	−	−	+
$C_{a-13:0}$	+	−	−	−	+		+	−	−	−	−
$C_{i-14:0}$	+	+	+	+	+		+	−	+	+	+
$C_{14:0}$	+	+	+	+	+		+	+	+	+	+
$C_{i-15:0}$	+	+	+	+	+		+	+	+	+	+
$C_{a-15:0}$	+	+	+	+	+		+	+	+	+	+
$C_{15:0}$	−	−	−	−	+		−	−	−	−	−
$C_{i-16:0}$	+	+	+	+	+		+	+	+	+	+
$C_{16:0}$	+	+	+	+	+		+	+	+	+	+
$C_{i-17:0}$	+	+	+	+	+		+	+	+	+	+
$C_{a-17:0}$	+	+	+	+	+		+	+	+	+	+
$C_{i-18:0}$	−	−	−	−	−		−	−	−	−	+
$C_{18:0}$	−	−	−	−	+		−	−	−	−	−
$C_{a-19:0}$	−	−	+	−	+		−	−	−	−	+
Subgroup(s)	13	14	5	8	1-12		1	4	2	6	3-12

[a] Fatty acid detected= +; fatty acid not detected= −
[b] R1=ATCC 43256; R2=ATCC 49594; R3=CDC F7535; R4=CDC F7631
[c] 39 isolates before inoculation into cooked turkey
[d] 32 isolates after recovery from cooked turkey

TABLE 6. *Cellular fatty acid compositions of reference strains of* L. monocytogenes

Fatty acid	% Mean ± SD (range) [coefficient of variation][a] by strain[b]:			
	ATCC 43256	ATCC 49594	F7535	F7631
$C_{12:0}$	0.43 ± 0.08 (0.38-0.52) [18.16]	0.67 ± 0.08 (0.61-0.76) [11.85]	0.30 ± 0.04 (0.27-0.33) [14.14]	0.43 ± 0.09 (0.34-0.51) [19.87]
$C_{a-13:0}$	0.44 ± 0.22 (0.28-0.59) [50.39]	– [c]	–	–
$C_{i-14:0}$	0.52 ± 0.07 (0.44-0.61) [13.60]	0.45 ± 0.11 (0.35-0.57) [25.16]	0.41 ± 0.17 (0.28-0.61) [42.07]	0.50 ± 0.08 (0.40-0.57) [15.08]
$C_{14:0}$	1.16 ± 0.11 (1.07-1.31) [9.13]	1.64 ± 0.12 (1.50-1.78) [7.50]	1.35 ± 0.35 (1.01-1.70) [26.06]	1.26 ± 0.20 (1.04-1.31) [15.86]
$C_{i-15:0}$	8.98 ± 1.45 (7.66-10.60) [16.16]	8.81 ± 0.83 (7.64-9.60) [9.42]	6.71 ± 1.71 (4.83-8.71) [25.44]	5.79 ± 0.82 (4.68-6.59) [14.17]
$C_{a-15:0}$	54.21 ± 8.80 (44.77-65.23) [16.22]	49.48 ± 3.77 (45.22-54.39) [7.62]	49.85 ± 6.56 (44.47-59.30) [13.15]	49.26 ± 4.54 (45.00-53.77) [9.22]
$C_{i-16:0}$	1.51 ± 0.28 (1.27-1.88) [18.49]	1.50 ± 0.17 (1.32-1.72) [11.47]	1.68 ± 0.17 (1.50-1.89) [10.39]	1.70 ± 0.31 (1.24-1.31) [18.15]
$C_{16:0}$	2.69 ± 0.75 (2.05-3.59) [27.92]	3.60 ± 0.88 (2.56-4.70) [24.29]	3.19 ± 0.81 (2.43-4.27) [25.36]	3.37 ± 0.27 (3.04-3.68) [7.95]
$C_{i-17:0}$	1.55 ± 0.96 (0.57-2.67) [61.71]	2.29 ± 0.39 (2.00-2.85) [16.87]	1.98 ± 0.53 (1.39-2.63) [26.70]	1.75 ± 0.47 (1.14-2.15) [26.80]
$C_{a-17:0}$	28.72 ± 8.47 (17.63-36.69) [29.50]	31.50 ± 2.47 (29.05-34.86) [7.84]	34.17 ± 7.26 (24.08-40.40) [21.25]	35.64 ± 3.70 (32.49-39.65) [10.38]
$C_{a-19:0}$	–	–	0.45 ± 0.16 (0.33-0.56) [36.55]	–

[a] Coefficient of variation=(SD/mean) x 100
[b] Percentage of total cellular fatty acids. Each strain was analyzed on four occasions.
[c] –, not detected

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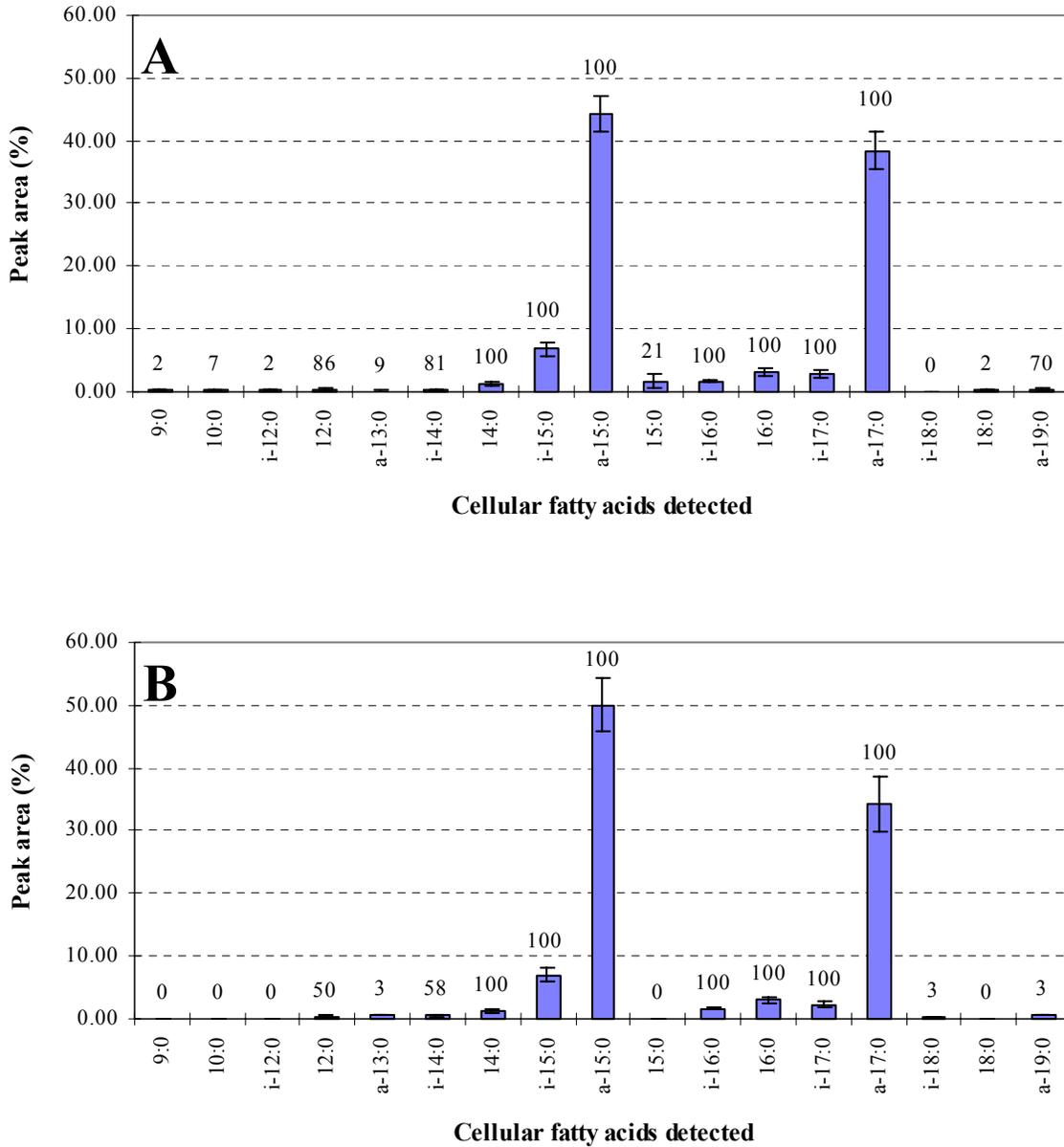
FIGURE 1. *Cellular fatty acid (CFA) composition of* L. monocytogenes *(A) before inoculation into cooked turkey (39 isolates plus 4 reference cultures) and (B) after recovery from cooked turkey (32 isolates plus 4 reference cultures). The number above each bar indicates the percentage of isolates containing that fatty acid.*
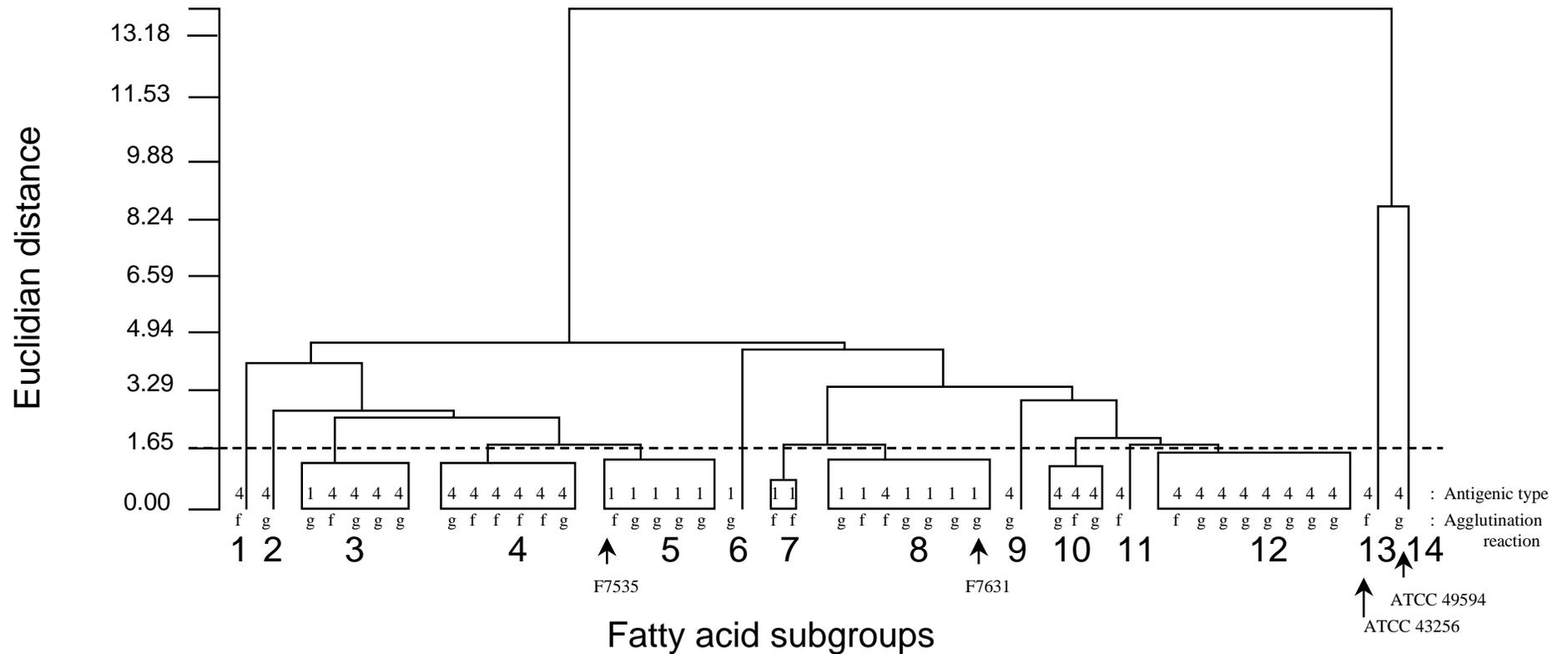
FIGURE 2. *Dendrogram showing the relatedness of 39* L. monocytogenes *isolates before inoculation into cooked turkey (together with 4 reference cultures), as generated by cluster analysis of whole-cell fatty acid profiles. Dashed line indicates cut-off value for subgroup determination. Fatty acid subgroup designations are cross-referenced in Table 4. Agglutination reactions are shown as "f" (flocculent) or "g" (granular).*
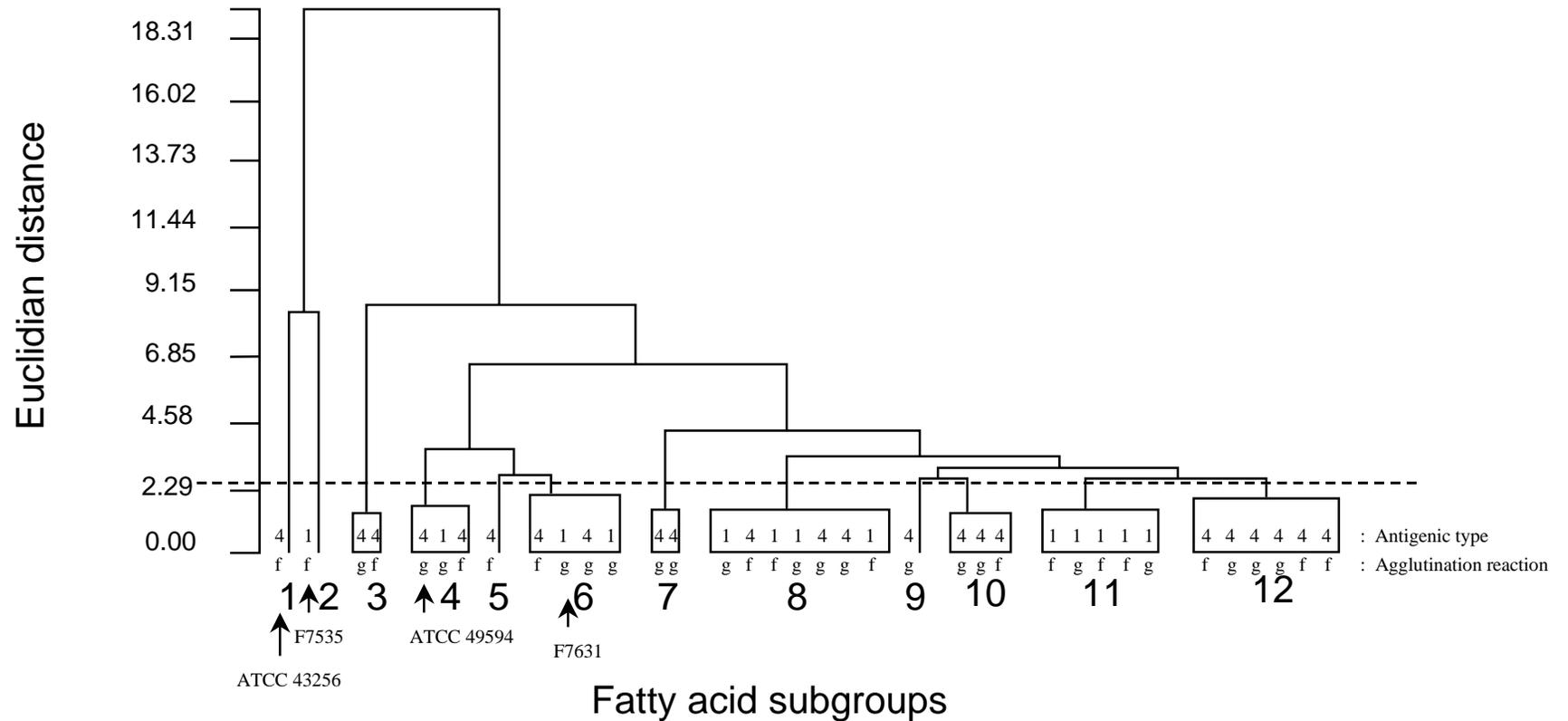
FIGURE 3. *Dendrogram showing the relatedness of 32* L. monocytogenes *isolates after recovery from cooked turkey (together with 4 reference cultures), as generated by cluster analysis of whole-cell fatty acid profiles. Dashed line indicates cut-off value for subgroup determination. Fatty acid subgroup designations are cross-referenced in Table 4. Agglutination reactions are shown as "f" (flocculent) or "g" (granular).*

VI. NONPROTEOLYTIC *CLOSTRIDIUM BOTULINUM* TOXIGENESIS IN COOKED TURKEY STORED UNDER MODIFIED ATMOSPHERES

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Key words: Nonproteolytic *Clostridium botulinum*, botulinal toxin, modified atmosphere packaging, turkey

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ABSTRACT

The ability of nonproteolytic *Clostridium botulinum* type B spores to grow and produce toxin in cooked, uncured turkey packaged under modified atmospheres was investigated at refrigeration and mild to moderate abuse temperatures. Cook-in-bag turkey breast was carved into bite-size chunks, surface-inoculated with a mixture of nonproteolytic *C. botulinum* type B spores, packaged in O_2-impermeable bags under two modified atmospheres (100% N_2 and 30% CO_2:70% N_2), and stored at 4°, 10°, and 15°C. Samples were analyzed for botulinal toxin and indigenous microflora, and subjected to olfactory evaluation on days 0, 7, 14, 28, 42, and 60. Given sufficient incubation time, nonproteolytic *C. botulinum* type B eventually grew and produced toxin in all temperature/modified atmosphere treatment combinations. At moderate temperature abuse (15°C), toxin was detected on day 7, independent of packaging atmosphere. At mild temperature abuse (10°C), toxin was detected on day 14, also independent of packaging atmosphere. At refrigeration temperature (4°C), toxin was detected on day 14 in product packaged under 100% N_2, and on day 28 in product packaged under 30% CO_2:70% N_2. Reduced storage temperature significantly delayed toxin production and extended the period of olfactory acceptability of cooked turkey, but even strict refrigeration did not prevent growth and toxigenesis by nonproteolytic *C. botulinum*. At all three storage temperatures, toxin detection preceded or coincided with development of olfactory characteristics of spoilage, demonstrating the potential for consumption of toxic product when spoilage-signalling sensory cues are absent.

INTRODUCTION

Changing lifestyles and growing consumer demand for freshness, nutrition, and convenience have prompted the marketing of an ever-increasing number and variety of cooked, ready-to-eat and microwave-ready food products. Many of these items receive only minimal thermal processing, contain no added preservatives, and depend upon refrigeration (4°C or less) as the primary, if not sole, barrier to growth of pathogenic and spoilage microorganisms during product distribution and storage.   Since "refrigeration" temperatures employed by retailers and consumers can often be as high as 15.5°C (*10,26*), simply chilling these foods cannot guarantee their safety or stability. Additional antimicrobial barriers are needed to ensure wholesomeness and quality throughout their expected storage life.

Modified atmosphere packaging (MAP) has become a popular means of extending the shelf-life of refrigerated pre-cooked foods, especially meat and poultry products. By replacing the air in the package headspace with CO_2, N_2, or a mixture of gases, MAP can delay the food's inherent biochemical and microbiological spoilage mechanisms, thereby imparting a preservative effect without the use of chemical additives (considered by many consumers as undesirable). However, by suppressing the growth of normally-occurring aerobic spoilage organisms, the combination of O_2 exclusion and CO_2 augmentation used in MAP can encourage proliferation of anaerobic pathogens before evidence of spoilage occurs, thus raising questions about the safety of products packaged and stored under modified atmospheres (*8*). Of particular concern to processed poultry manufacturers is the potential for growth of psychrotrophic (nonproteolytic) strains of *Clostridium botulinum* in uncured, ready-to-eat products. These strains are capable of

producing toxin at temperatures as low as 3.3°C (*6,12,23,24,25*) without the accompanying signs

of putrefactive spoilage used by many consumers to judge whether or not a product is fit for

human consumption. Although the incidence of botulinal spores in processed meat and poultry

products is quite low (less than 1 spore/kg) (*7*), the ubiquitous nature of the organism suggests

that environmental contamination is, at some point, inevitable. Thus, in the absence of

traditional curing agents or back-up preservative systems to prevent toxigenesis during prolonged

storage or under conditions of temperature abuse, these products have the potential to become

vehicles for the transmission of foodborne botulism.

While many investigators have studied the ability of proteolytic (putrefactive) strains of

C. botulinum to grow and produce toxin in processed poultry products, few have reported on the

behavior of nonproteolytic strains of the organism in such products (*14,15,19,20*). Where data

are available, they have generally been obtained under conditions of moderate to severe

temperature abuse (15°-30°C), using products formulated with multiple combinations of

antibotulinal agents (e.g., salt, nitrite, sorbate, phosphate, organic acids), exposed to substantial

spore challenges (10^3-10^6/g), and stored under vacuum. Very few studies have evaluated the

behavior of nonproteolytic *C. botulinum* in products formulated without high levels of traditional

preservatives, and under conditions more commonly encountered in commercial practice (i.e.,

low numbers of contaminating spores, and storage under refrigeration ($\leq$4°C) or mild

temperature abuse (8°-12°C)) (*14,15*). We are not aware of reports addressing the behavior of

nonproteolytic *C. botulinum* in uncured poultry products packaged and stored under modified

atmospheres, where the presence of CO_2 (a known botulinal spore germinant (*1,28*)) might

actually promote the growth of this anaerobic pathogen. The purpose of the present study was to

assess the potential for toxin production in cooked, uncured, low-salt turkey products inoculated with low levels of nonproteolytic *C. botulinum* spores, packaged under modified atmospheres and stored for extended periods of time under refrigeration and mild to moderate temperature abuse.

MATERIALS AND METHODS

Spore preparation and maintenance. Nonproteolytic *C. botulinum* strains 2B, 17B, and 25765B were obtained from the culture collection of the Department of Food Science and Technology at Virginia Polytechnic Institute and State University (VPI&SU), Blacksburg, VA. Spore suspensions of individual strains were prepared at 30°C in Fernbach flasks using a biphasic culture system (*2,3*). The liquid phase contained sterile distilled water; the solid phase contained (w/v): 5% trypticase (Difco Laboratories, Detroit, MI), 0.5% peptone (Difco), 0.4% glucose (BBL, Cockeysville, MD), 0.2% sodium thioglycollate (BBL), and 3% agar (BBL). Spores of each strain were harvested by centrifugation at 2,520 x g (4°C/20 min; Sorvall® RC-5B refrigerated superspeed centrifuge, DuPont Instruments, Wilmington, DE), washed in sterile distilled water, centrifuged, and the resultant pellet enzymatically cleaned at 45°C for 2 h in a pH 8.1 K_2HPO_4 (Fisher Scientific Company, Pittsburgh, PA) buffer containing 100 μg/ml lysozyme (Sigma Chemical Company, St. Louis, MO) and 50 μg/ml trypsin 1:250 (Difco). The enzyme-treated suspension was centrifuged, washed an additional six times in sterile distilled water, and the final pellet suspended in sterile distilled water. The cleaned spore suspension (ca. 10^6 spores/ml) was subdivided into 2-ml aliquots and stored at -20°C until needed. Viable spore counts for each strain were determined in duplicate at the time of use by the trypticase-peptone-glucose-yeast extract (TPGY) roll tube method (*9*).

Product preparation. Boneless cook-in-bag turkey breasts (uncured, ca. 3.2 kg each) were obtained from a commercial turkey processor immediately after the final process chill step

(core temperature: 32°F). Vacuum-sealed breasts were packed in ice-filled coolers and immediately transported to the Department of Food Science and Technology at VPI&SU in Blacksburg, VA (ca. 2.5 h). Product surface temperature was measured upon arrival (Omega HH-99A-T1 digital thermometer with surface probe, Omega Engineering, Inc., Stamford, CT). Product was then transferred to a cold room ($0.5° \pm 1.5°C$) for cutting and packing. Breasts were aseptically removed from their vacuum cooking bags and carved into chunks ranging in size from 1.9 cm x 1.3 cm x 1.3 cm to 3.8 cm x 1.9 cm x 1.3 cm. Chunks were commingled and distributed in 25-g aliquots into 22.5 cm x 15.5 cm high-barrier (coextruded ethylene-vinyl alcohol (EVOH)) bags (O_2 transmission rate: 3-6 cc/m^2/24 h @ 40°F and 0% R.H.; CO_2 transmission rate: 9-16 cc/m^2/24 h @ 40°F and 0% R.H.; H_2O vapor transmission rate: 0.5-0.6 g/100 in.^{2}/24 h @ 100°F and 100% R.H. (Cryovac Division, Sealed Air, Inc., Duncan, SC)), color-coded by treatment variable. To accommodate work flow, product bags (pouches) were held on ice in the cold room until inoculation (ca. 2 h).

Six (6) random product samples were collected throughout the packing operation for subsequent zero-time microbiological and physical analyses. Proximate analysis results (composite of twelve samples) were provided by the supplier.

Product inoculation and modified atmosphere-packaging. A portion of the product samples was reserved prior to inoculation, to serve as a negative, uninoculated control for all treatments evaluated in this study. Each individual product pouch was treated, sealed, inspected, and placed on ice before advancing to the next pouch. The inoculum volume was previously determined, so as not to alter product water activity (a_W). The inoculating pipettor was calibrated

before use, and separate, sterile disposable pipet tips were used for each inoculation (as well as for each volume of sterile distilled water dispensed).

A stock spore suspension containing approximately equal numbers of each of the three nonproteolytic *C. botulinum* test strains was prepared in sterile distilled water immediately prior to inoculation, and serially diluted in sterile distilled water to obtain the spore concentration necessary to achieve a target inoculum level of 100-500 spores/g of product. This working suspension (inoculum) was heat-shocked at $60^{\circ}C/10$ min (TE45 thermostatic circulating water bath, Neslab Instruments, Inc., Portsmouth, NH), cooled and held in an ice water bath under constant agitation during inoculation and packaging operations. Actual inoculum spore counts were determined by plating in duplicate onto TPGY roll tubes (*9*) at the start and finish of all inoculation procedures.

Ice-filled product coolers were transferred as needed from the cold room to the laboratory for inoculation. Individual product pouches were removed from the cooler and 200 μl of the inoculum was aseptically dispensed over the top surface of all product chunks in the pouch (Eppendorf Model 4710 adjustable pipettor, Brinkmann Instruments, Inc., Westbury, NY). To ensure uniformity in sample treatment, negative product controls received 200 μl sterile distilled water in place of the inoculum.

After inoculation, the pouch was gently shaken to evenly distribute its contents. The designated modified atmosphere (100% N_2 or 30% CO_2:70% N_2) was applied via a two-stage process employing vacuum removal of air followed by gas filling (Multivac A300 vacuum sealer, Multivac, Kansas City, MO; Smith 180 SCFH proportional tri-gas blender, Smith Equipment, Watertown, SD). The pouch was impulse-sealed (Multivac A300 vacuum sealer),

visually inspected for seal integrity and headspace volume, and briefly held in an ice-filled cooler until all replicate pouches of the same treatment variable were modified atmosphere-packaged (MAP) (ca. 20 min). The MAP pouches were then removed from the cooler and placed in random, pre-assigned locations in a pre-assigned incubator.

Product incubation and sampling. MAP product was incubated for up to 60 days at 4°, 10°, and 15^oC in separate Precision Scientific Model 815 low temperature incubators (Fisher Scientific). Incubator temperature was monitored daily, using a mercury thermometer (Fisher Scientific) partially submerged in anhydrous glycerol (J. T. Baker Chemical Co., Philadelphia, PA), and some variation ($+ 1^o$ to 1.5^oC) was observed. At predetermined intervals (0, 7, 14, 28, 42, and 60 days), triplicate samples of each inoculated and uninoculated treatment variable were evaluated for gas production (swelling), headspace composition, aroma/appearance, aerobic plate count (APC), modified psychrotrophic plate count (mPSY), lactic acid bacteria count (LAB), and botulinal toxin production. Any treatment variable testing positive for botulinal toxin on two consecutive sampling days was discontinued from the study. Product pH, water activity (a_W), and botulinal spore counts were determined on day zero only.

Analyses of pH, a_W, and headspace gases. Product pH and a_W were determined on day zero using six random turkey breast samples collected during the pouch-filling operation. Intact chunks (approximately 1.9 cm x 1.3 cm x 1.3 cm) were used for a_W measurement (Decagon CX-1 water activity system, Decagon Devices, Inc., Pullman, WA), while homogenized samples (25 g turkey + 25 g distilled water, blended for 2 min; Stomacher 400 lab blender, Tekmar Co.,

Cincinnati, OH) were used for pH measurement (Accumet Model 610A pH meter with combination electrode, Fisher Scientific).

Headspace gas composition (percent CO_2, O_2, N_2) was determined at every sampling time on triplicate intact packages from each treatment variable (inoculated and uninoculated), using a Fisher Hamilton Model 29 gas partitioner (Fisher Scientific), equipped with DEHS/Chromosorb P and molecular sieve 13X columns, thermal conductivity detector, and Hewlett-Packard Model 3396A reporting integrator (Hewlett-Packard Co., Avondale, PA). Helium flow rate was 40 ml/min and cell temperature was $70^{\circ}C$. An Airco blood gas mixture (5.13% CO_2 : 14.92% O_2 : 79.95% N_2) was used to standardize the partitioner. Headspace samples (1 cc) were withdrawn through a 1.3 cm-thick foam septum and collected in a 1-cc Gastight® #1001 glass syringe (Hamilton Co., Reno, NV).

Sensory evaluation. Following headspace analysis, random samples from each treatment condition were assigned random 3-digit codes and presented to a three-member untrained sensory panel (immunized with pentavalent botulinal toxoid) for observations related to product acceptability, based collectively on aroma and visual appearance. Sample packages were opened immediately before evaluation, and panelists were asked to judge olfactory acceptability using the following five-point category scale: "definitely would use," "probably would use," "might use/might not use," "probably would not use," "definitely would not use." Olfactory unacceptability was determined as that point at which panelists reported they "probably would not use" or "definitely would not use" the product.

Microbiological analyses. Nonproteolytic *C. botulinum* spore counts in triplicate inoculated and uninoculated samples of each treatment variable were determined in duplicate on day zero only (to verify inoculum levels), using the TPGY roll tube method (*9*).

Following sensory evaluation, each product pouch was briefly kneaded with a cold cylindrical roller to produce a "paste" suitable for microbiological sampling. At all sampling times, triplicate inoculated and uninoculated samples of each treatment variable were analyzed for APC, PSY, and LAB. Eleven-gram (11-g) aliquots of the sample "pastes" were blended with 99 ml 0.1% peptone diluent (Difco) (2 min, Stomacher 400 lab blender), serially diluted as necessary, and pour-plated in duplicate using Standard Methods Agar (Difco) containing 0.005% (w/v) 2,3,5-triphenyltetrazolium chloride (Fisher Scientific) for APC and mPSY, and All-Purpose Tween (APT) Agar (Difco) containing 0.0032% (w/v) bromcresol purple (BCP) dye (Fisher Scientific) (APT+BCP) for LAB. APT+BCP plates were overlaid with approximately 10 ml of APT+BCP prior to incubation. APC plates were incubated at $35^{o}C/48$ h; mPSY and APT+BCP plates were incubated at $20^{o}C/72$ h. Lenticular, sub-surface yellow (acid-producing) colonies on APT+BCP (purple background) were Gram-stained and tested for catalase reaction. Acid-producing, catalase-negative, Gram-positive nonsporeforming rods/cocci were presumptively identified as LAB (*27*).

Mouse bioassay for botulinal toxin. At each scheduled sampling time, triplicate inoculated and uninoculated samples of each treatment variable were analyzed for the presence of botulinal toxin using the mouse bioassay, according to FDA Bacteriological Analytical Manual (BAM) procedures (*11*). Three extracts of each test sample were prepared in gelatin

phosphate buffer (pH 6.2): 1) untreated, 2) trypsin-treated, and 3) heat-treated. For each sample extract preparation, duplicate male ICR mice (18-20 g; Harlan Sprague-Dawley, Inc., Indianapolis, IN) were each injected intraperitoneally (i.p.) with 0.5 ml of the extract, and observed for 72 h for clinical signs/symptoms of, and death resulting from, botulism.

Statistical analysis. Treatments were arranged as a 6 x 2 factorial in a randomized complete block design. All treatments were performed in triplicate, and the entire study was repeated twice. Bacterial counts were transformed into base-10 logarithms, and sensory ratings were converted from a word scale into numerical values ranging from 5 ("definitely would use") to 1 ("definitely would not use"). Analysis of variance was used to determine if significant ($P<0.05$) differences existed between treatments, using the general linear models procedure of SAS (*22*). Means were separated using the least significant difference method (SAS), employing a significance level of 0.05. Analysis of botulinal toxin production was performed using a chi-square (2 x *k*, contingency) test (*21,22*), wherein toxic samples were scored as 1 and nontoxic samples were scored as 0.

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RESULTS AND DISCUSSION

Product composition. The product used in this study contained 15.2% protein, 79.4% moisture, 0.8% fat, 1.5% sucrose, 1.8% salt, and 0.4% phosphate. Initial product pH was 6.20 and water activity (a_w) was 0.951.

Changes in headspace CO_2 levels. Mean headspace CO_2 levels increased by up to 2% (P<0.05) in inoculated and uninoculated product during the first week of storage at 15°C (regardless of packaging atmosphere), and there was approximately a 3-log increase in lactic acid bacteria counts (data not shown). There was no further CO_2 accumulation by week 2 (the point at which the 15°C treatment variable was terminated), consistent with a leveling-off of lactic populations. Package headspace composition remained unchanged (P>0.05) over time in the inoculated and uninoculated product stored at 10° and 4°C (regardless of packaging atmosphere), consistent with the failure of low levels of background lactic acid bacteria to proliferate at these temperatures.

Changes in indigenous microflora. No indigenous *C. botulinum* spores or toxin were detected in uninoculated turkey at any time during the study. Aerobic plate counts (APC) of all product samples were ≤10/g at time zero. Storage temperature significantly affected (P<0.05) mean APC of uninoculated turkey, producing overall population increases of 4 to 5 logs at 15°C, but only 1.5 logs or less at 10° or 4°C (Figure 1). In *C. botulinum*-inoculated turkey, no significant differences (P>0.05) in ultimate aerobe population levels were observed between

storage temperatures. Packaging atmosphere had no significant effect (P>0.05) on APC of

uninoculated product, but did significantly affect (P<0.05) aerobic counts of *C. botulinum*-

inoculated product. Total aerobic populations of inoculated turkey packaged under 30%

CO_2:70% N_2 were 1 to 2.5 logs lower than in inoculated turkey packaged under 100% N_2, an

effect which became more pronounced as storage temperature decreased. Although competition

by *C. botulinum* and other anaerobes may account for a portion of this APC decline, the

increased solubility of CO_2 at lower temperatures (*18*) and well-documented inhibitory effect of

CO_2 against aerobic spoilage organisms (*5*) is the more likely cause.

Modified psychrotrophic plate counts (mPSY) of all product samples were <100/g at time

zero, and increased (P<0.05) by 4 to 6 logs throughout storage (Figure 2). For each treatment

condition, growth patterns between uninoculated and *C. botulinum*-inoculated samples were

similar. Storage temperature affected the rate of growth of psychrotrophs in both uninoculated

and inoculated turkey, but generally not the ultimate population levels reached. Packaging

atmosphere significantly affected (P<0.05) only those samples stored at 4°C. At this

temperature, the presence of 30% CO_2 in the package headspace delayed onset of logarithmic

growth in uninoculated and inoculated product -- an effect observed by others (*4,5*) -- and

reduced final PSY levels in inoculated product by more than 2 logs, compared to product

packaged under N_2 alone. It would seem likely that increased CO_2 solubility at 4°C is

responsible for this effect.

Lactic acid bacteria (LAB) counts of all product samples were <10/g at time zero.

Overall levels of LAB in uninoculated turkey packaged under 100% N_2 or 30% CO_2:70% N_2

increased approximately 3 logs within the first week of storage at 15°C, but this was the only

significant effect (P<0.05) observed (data not shown). There were no overall changes (P>0.05) in LAB populations in uninoculated product at 10° or 4^oC (regardless of packaging atmosphere) or in inoculated product under any treatment condition. This explains the lack of measurable changes (P>0.05) in headspace CO_2 concentration in these samples.

Toxin detection and sensory evaluation. Although the target *C. botulinum* spore inoculum level was 2.0-2.7 logs/g of product, the actual level achieved was 1.6-1.7 logs/g. There were no significant differences (P>0.05) in inoculum spore counts between the start and finish of all inoculation procedures. Variations observed in toxin detection time between trials were likely due to the difficulties ordinarily inherent in uniformly distributing spores throughout solid products.

Production of botulinal toxin was significantly affected by storage temperature and incubation time, but not by packaging atmosphere. Lowering storage temperature delayed toxigenesis in product packed under both 100% N_2 and 30% CO_2:70% N_2, and reduced the number of samples that tested positive for toxin (Table 1). Toxigenesis in 100% N_2-packed product was delayed by 1 week when storage temperature was dropped from 15^oC to either 10° or 4^oC. Toxigenesis in product packed under 30% CO_2:70% N_2 was delayed by 1 week when storage temperature was dropped from 15^oC to 10^oC, and by 3 weeks when the temperature was dropped from 15^oC to 4^oC. These results are not surprising, given the anaerobic growth environment provided by both packaging atmospheres, and the fact that, as incubation temperatures approach the low end of the botulinal growth range (3.3^oC), longer periods of time are required for elaboration of toxin (*24,25*).

Time to olfactory unacceptability did not always coincide with product toxicity, resulting in many instances where product that was actually toxic was rated as acceptable, and therefore considered suitable for consumption. In some cases, sensory panelists did not uniformly regard deteriorative changes in aroma/appearance as necessarily constituting unacceptability; so product that was given a collective sensory panel rating of unacceptable was rated as acceptable by at least one panel member. At 15°C (regardless of packaging atmosphere), detection of botulinal toxin (day 7) either coincided with, or preceded, olfactory unacceptability by 1 week. At 10°C (regardless of packaging atmosphere), detection of botulinal toxin (day 14) coincided with olfactory unacceptability (day 14). At 4°C, detection of botulinal toxin preceded olfactory unacceptability by a month or more. Product packaged under 100% N_2 was not considered unacceptable until day 42, even though a third of the samples were already toxic by day 14; and product packaged under 30% CO_2:70% N_2 was not considered unacceptable until day 60, even though a third of the samples were toxic by day 28.

These results demonstrate that modified atmosphere packaging (MAP) and refrigerated storage are not sufficient barriers to prevent nonproteolytic *C. botulinum* growth and toxigenesis in ready-to-eat poultry products formulated without intrinsic antibotulinal safety factors. Aroma and visual appearance cannot be relied upon to provide consumer protection, since olfactory unacceptability does not always coincide with toxigenesis. Background contaminants (such as lactic acid bacteria or psychrotrophs) cannot be relied upon to out-compete or inhibit *C. botulinum*, since both types and numbers of indigenous flora can vary considerably from one product lot to another. In order to ensure the safety of refrigerated, pre-cooked poultry products throughout their expected storage life, additional antibotulinal barriers are necessary.

Intervention strategies employing lactate and other organic acid salts (*13,14,15*), microbially-derived bactericidal/bacteriostatic proteins or glycoproteins (bacteriocins) (*16*), or competitive inhibition/antagonism by lactic starter cultures (*17*), could provide viable alternatives to the use of traditional preservatives, and would be consistent with consumers' demands for "natural" ingredients and processes. Lactic acid and its lactate salts are already FDA-approved and commercially available for use in muscle food products. Nisin, the bacteriocin produced by *Lactobacillus lactis* subsp. *lactis*, is FDA-approved for use in processed cheese spreads, but may be applicable to other food products. More research is needed to evaluate the efficacy of other bacteriocinogenic compounds or organisms in food systems and, ultimately, their safety and stability in commercial practice. Stricter control of refrigeration ($<3^{o}$C) throughout the retail, food service, and consumer handling chain would also serve to enhance the inhibitory effects of these and other antibotulinal agents (*17,18,20*), and thus reduce the risk of pre-cooked poultry products becoming vehicles for the transmission of foodborne botulism.

ACKNOWLEDGMENT

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TABLE 1. *Time to toxin detection and olfactory unacceptability in cooked turkey inoculated with a mixture of nonproteolytic type B* C. botulinum *spores,[a] packaged under modified atmospheres, and stored at refrigeration and abuse temperatures*

Storage temperature (°C)	Modified atmosphere	Trial 1		Trial 2	
		Days to toxin detection	Days to olfactory unaccept-ability[b]	Days to toxin detection	Days to olfactory unaccept-ability
15	100% N_2	7 (3/3)[c]	7	7 (3/3)	14
	30% CO_2:70% N_2	7 (3/3)	14	7 (3/3)	7[d]
10	100% N_2	14 (1/3)	14[d]	14 (2/3)	14[d]
	30% CO_2:70% N_2	14 (2/3)	14	28 (2/3)	28
4	100% N_2	28 (1/3)	60	14 (1/3)	42
	30% CO_2:70% N_2	28 (1/3)	60	42 (2/3)	28

[a] Mean inoculum size: 54 spores/g in trial 1; 43 spores/g in trial 2
[b] Time of olfactory unacceptability, as determined by evaluation of aroma and appearance
[c] Number of toxic samples/number of samples tested
[d] Although mean sensory rating was unacceptable, at least one of the three panelists would consume this product.
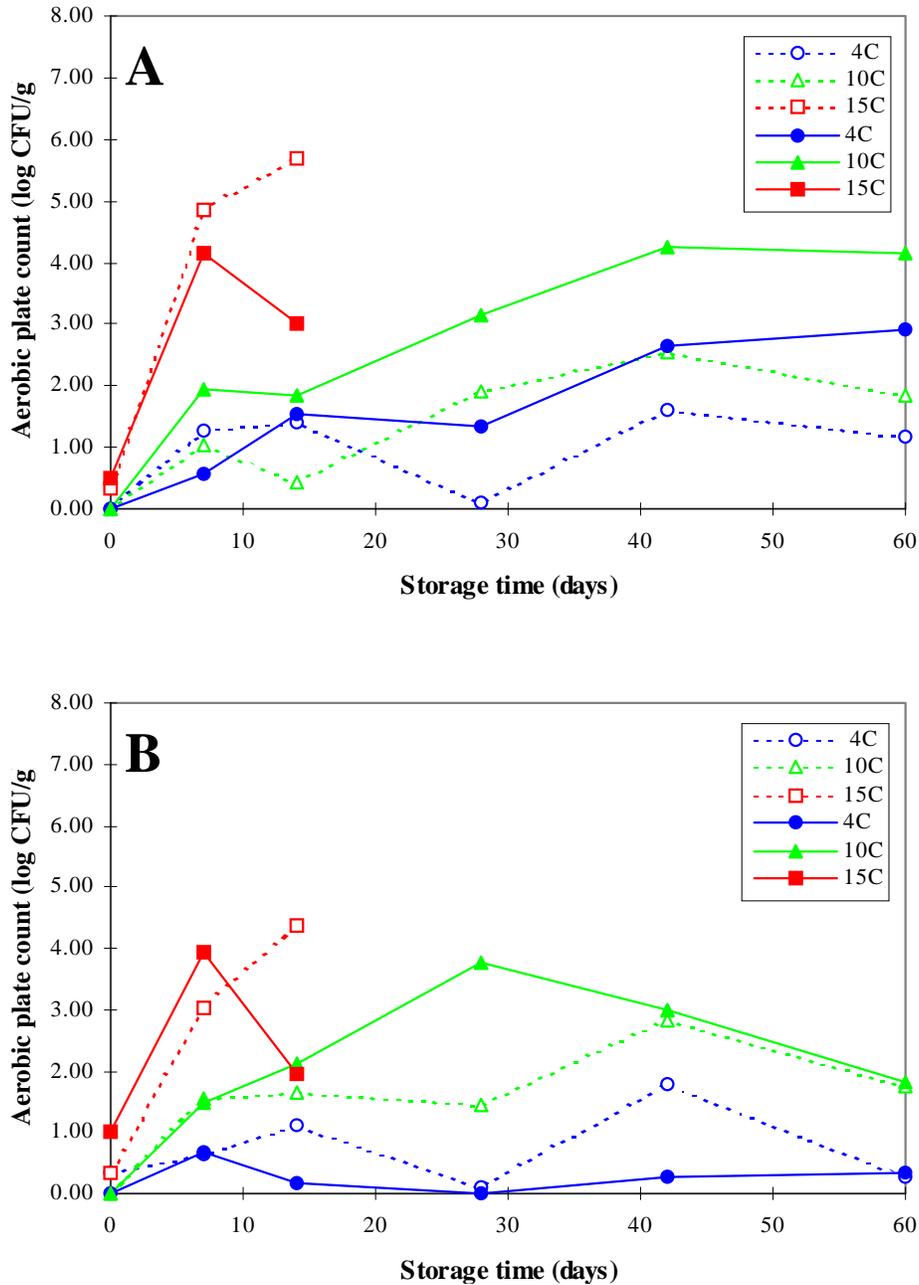
FIGURE 1. *Mean aerobic plate counts (APC) of cooked turkey packaged under (A) 100% N_2 and (B) 30% CO_2 : 70% N_2 , and stored at refrigeration and abuse temperatures. Dashed lines represent uninoculated (control) product; solid lines represent product inoculated with a mixture of nonproteolytic type B* C. botulinum *spores. 15°C storage was terminated after 14 days, due to detection of botulinal toxin in inoculated product from two consecutive sampling periods.*
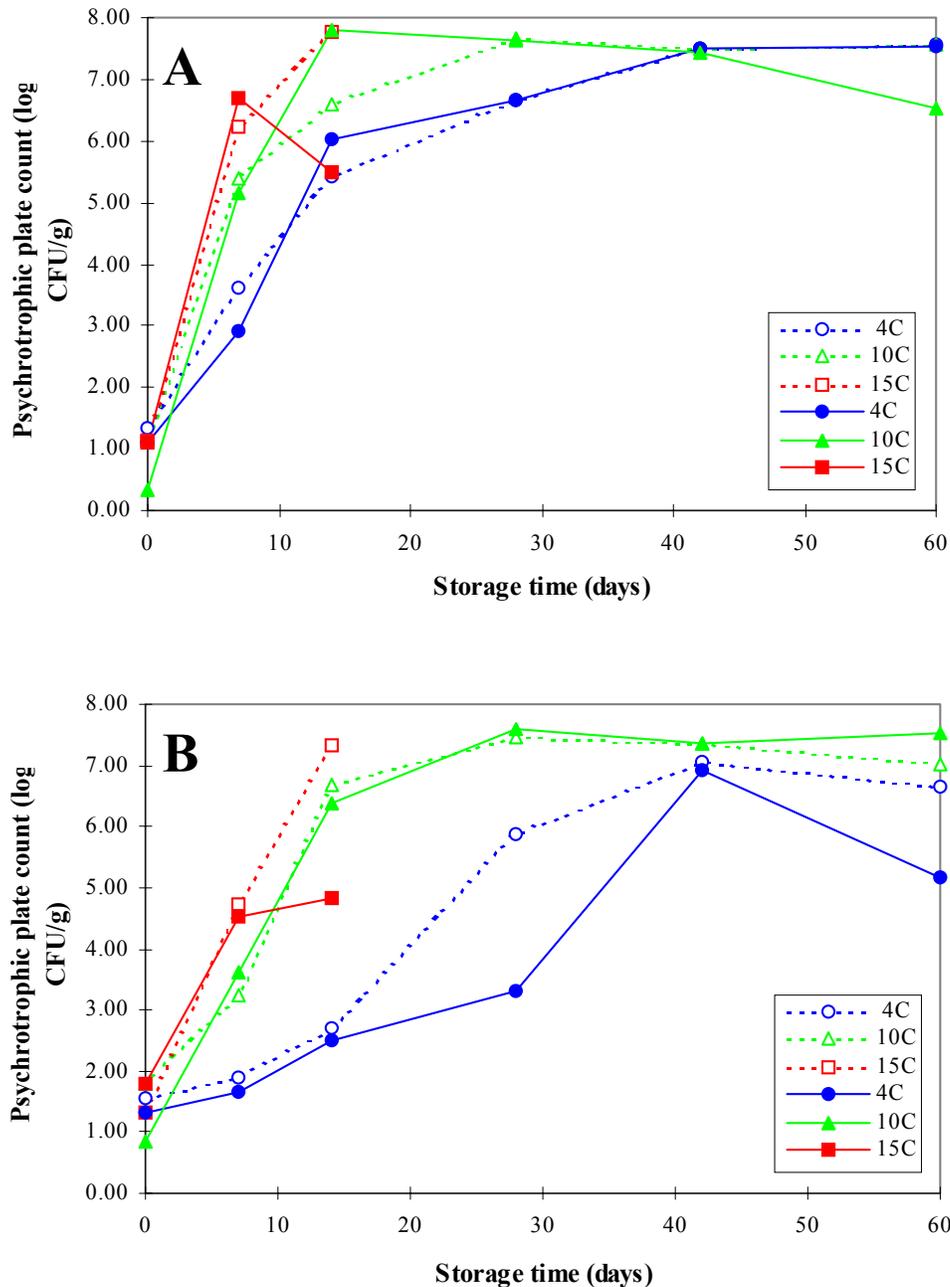
FIGURE 2. *Mean modified psychrotrophic plate counts (mPSY) of cooked turkey packaged under (A) 100% N_2 and (B) 30% CO_2 : 70% N_2 , and stored at refrigeration and abuse temperatures. Dashed lines represent uninoculated (control) product; solid lines represent product inoculated with a mixture of nonproteolytic type B C.* botulinum *spores. 15ºC storage was terminated after 14 days, due to detection of botulinal toxin in inoculated product from two consecutive sampling periods.*

VII. VITA

Kathleen Lawlor was born and raised in New Jersey, where she received her primary and secondary education. She received her undergraduate training in microbiology at the University of Illinois (Urbana, IL), and her graduate training in food science at Rutgers, The State University of New Jersey (New Brunswick, NJ) and Virginia Polytechnic Institute and State University (Blacksburg, VA). Following completion of the baccalaureate degree, she was employed by General Foods Corporation (Tarrytown, NY and Cranbury, NJ), where she worked as a microbiologist until beginning her doctoral studies. She is a member of the American Society for Microbiology (ASM), the International Association of Milk, Food and Environmental Sanitarians (IAMFES), and the Institute of Food Technologists (IFT).

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