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Comparative Growth of Spoilage and Pathogenic Organisms on Modified Atmosphere-Packaged Cooked Beef

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ABSTRACT

The influence of CO_2 atmospheres on the growth of spoilage and pathogenic microorganisms on cooked, sliced roast beef was investigated. An atmosphere containing 75% CO_2, 15% N_2 and 10% O_2 was identified as the most effective in the simultaneous inhibition of *Pseudomonas fragi, Salmonella typhimurium, Staphylococcus aureus,* and *Clostridium perfringens*. Inoculated roast beef was exposed to two temperature abuse regimes to observe the resultant microbiological changes in air and three modified atmospheres containing 75% CO_2 and O, 5 or 10% O_2 (balance N_2). The modified atmospheres inhibited the growth of *S. aureus* during abusive storage, and atmospheres containing 5 or 10% O_2 inhibited the outgrowth of *C. perfringens*. The modified atmospheres were less effective in inhibiting the growth of *S. typhimurium*. However, high *Salmonella* counts were accompanied by high *Pseudomonas* counts. The concept of a Safety Index, which compares numbers of spoilage and pathogenic organisms, was developed to aid in the evaluation of modified atmospheres.

There is currently a strong commercial interest in extending the shelf life of foods. The extension of shelf life can be applied to both new and existing products to allow more centralized packaging and distribution of certain perishable products (*1*), to simplify distribution, or to decrease the requirement for frequent deliveries with a net result of lower manufacturing and retailing costs. To meet demands for longer product shelf life, food manufacturers are beginning to use modified atmosphere packaging (MAP) technology (*9*).

Inhibitory effects of CO_2 on common spoilage organisms, such as *Pseudomonas*, have been demonstrated (*4,5,16,17*). Although it is well-known that the use of modified atmospheres (MA's) containing elevated concentrations of CO_2 can significantly extend the microbiological shelf life of refrigerated products (*1,2,21,23*), there has been less research on the effects of MA's on pathogens and overall product safety.

Concern over the safety of foods packaged in MA's is a major reason for the failure to take greater advantage of the benefits of MAP. Although some researchers have advocated the inclusion of O_2 in MA's to help maintain the bright red color of fresh meats (*5*), most MA's contain high concentrations of CO_2 and N_2. While spoilage organisms are inhibited by CO_2 concentrations as low as 20% (*13*), CO_2 does not appear to have a significant inhibitory effect on the growth of anaerobic pathogens such as *Clostridium botulinum (22)*. Because *Clostridium perfringens* and *C. botulinum,* with the exception of certain aquatic type E strains (*15*), do not grow at refrigeration temperatures, their growth is more likely in instances of temperature abuse.

A major concern about the use of MAP is whether anaerobic pathogens, such as *C. botulinum,* can grow before nonpathogenic spoilage organisms, such as *Pseudomonas,* whose growth is evidenced by an offensive odor and a slimy surface on the product (*20,26*). A second concern is that CO_2 enhances *C. botulinum* spore germination (*10,14,18*). However, requirements for growth are more exacting than those for germination; the important factor is the ability to grow and produce toxin (*25*).

Facultative anaerobic food pathogens, such as *Staphylococcus aureus* and *Salmonella* spp., grow very slowly, if at all, at refrigeration temperature. Previous work has shown that MA's at refrigeration temperatures do not encourage the growth of these organisms (*19,22*) and that MA's may inhibit growth (*3,12*). However, product safety depends on the potential growth of these organisms at abusive temperatures.

The objectives of this research were to determine the effects of MA's on co-inocula of the spoilage organism *Pseudomonas fragi* and three pathogens, *S. aureus, Salmonella typhimurium* and *C. perfringens,* and to compare the outgrowth of these organisms in MA's at abusive temperatures.

MATERIALS AND METHODS

Samples and packaging

Prepared samples were packaged in sterilized glass 1-pint jars. Two holes were drilled in the jar lid and fitted with rubber septa, and the edges were caulked with silicone sealant.

Top round beef roasts were obtained from a local grocery on the day they were to be cooked. The roasts were cooked in a conventional oven at 162.8°C (325°F) to an internal temperature of 54.4°C (130°F). Cooked roasts were cooled overnight at 4.4°C (40°F) and sliced (4 mm thick) on a sanitized meat slicer. Excess fat was aseptically trimmed and 50.0 ± 0.3 g was put into the sterile jars.

Desired gas mixtures were attained by blending CO_2 and N_2 using a Scott Flowmeter (2-31B Series Gas Blender, Scott Environmental Technology). Standard curves were prepared by flushing empty jars with combinations of gases and analyzing the mixture by gas chromatography against a standard mixture of gases (Scott Environmental Technology).

After sealing, each jar was continuously flushed for 0.5 to 2.0 min with the appropriate gas mixture by inserting inlet and outlet needles through the septa. Jars containing samples to be stored were analyzed by gas chromatography to insure proper gas mixtures. In cases where O_2 was added to the jars, a volume of the N_2/CO_2 mixture was removed with a gastight syringe and replaced with O_2.

Cultures and inoculation of samples

Dehydrated media were manufactured by Difco except where indicated.

C. perfringens (ATCC #3624) was maintained on brain heart infusion agar (BHIA) and incubated anaerobically (BBL Anaerobic Jar, BBL Microbiology Systems, Cockeysville, MD) at 37°C. Broth cultures were prepared in brain heart infusion broth (BHIB) and sealed with vaspar. *P. fragi* (ATCC #27363) was grown at 23°C on plate count agar (PCA) and broth cultures were prepared in tryptic soy broth. *S. typhimurium* and *S. aureus* were maintained on BHIA and broth cultures were prepared in BHIB incubated at 37°C.

A standard curve for each organism was prepared by spectrophotometrically (600 nm) determining cell density after growth in broth culture for 24 h. Cultures were diluted and plated on the appropriate selective medium to determine cell density. A standard curve was prepared and the regression line determined for each organism. *C. perfringens* was diluted with peptone water; phosphate buffer was used for the other organisms.

For sample inoculation, the density of a 24-h culture was determined and the culture was diluted to produce a suspension containing 5.0×10^3 cells of each organism per volume such that the sum of the volumes was 0.5 ml (i.e., depending on the number of organisms being inoculated) and the 0.5-ml inoculum would contaminate each sample with ca. 100 cells/g. The inoculum was distributed onto the surface of the meat of each sample. Recovery of all organisms was better than 90% at time zero.

Enumeration of organisms

C. perfringens was enumerated on pour plates of tryptose sulfite cycloserine agar which consisted of SFP agar base plus 0.04 g of cycloserine per ml of media (*24*). Following anaerobic incubation at 37°C for 24 h, black colonies were counted. *P. fragi* was enumerated by surface plating on *Pseudomonas* agar base plus a supplement containing centrimide, fucidin and cephaloridine (Oxoid), and incubating. Plates at 23°C for 48 h. Following incubation, random isolates were inoculated into Oxi-Ferm Tubes (Roche Diagnostic Systems) to confirm their identity. Desoxycholate citrate agar was used to enumerate *S. typhimurium*. After spread plating and incubation at 37°C for 48 h, light pink colonies were counted. At each sampling period, random isolates were inoculated onto API20E System Strips (Analytab Products) to confirm identification. *S. aureus* was selectively enumerated on Baird-Parker agar plus Bacto egg yolk-tellurite enrichment. After spread plating, plates were incubated at 37°C for 48 h. Black colonies surrounded by a clear or white zone were counted.

At each sampling period, random isolates of each organism were Gram stained to check for the correct Gram reaction and cell morphology. Periodically, uninoculated samples were plated on all media to insure low numbers of background organisms.

Experiment A

Roast beef samples were inoculated with a mixture of *P. fragi* and *C. perfringens*, and flushed with atmospheres containing 75% CO_2 with 0, 2, 5, 10 or 25% O_2 (balance N_2) and stored at 12.8 or 26.7°C. Control samples were stored in air. One sample from each atmosphere and storage temperature was analyzed on Days 0, 5, 11, 18, 26 and 33.

Experiment B

Roast beef was inoculated with a mixture of *P. fragi*, *S. typhimurium* and *S. aureus* and stored at 12.8 or 26.7°C in atmospheres containing 75% CO_2 with 0, 2, 5, 10 or 25% O_2 (balance N_2) on air. One sample from each atmosphere and storage temperature was analyzed on Days 0, 5, 8, 14, 21, 28 and 35.

Experiment C

Samples were inoculated with a mixture of *C. perfringens*, *S. typhimurium*, *S. aureus* and *P. fragi*. Jars were flushed with 75% CO_2, 10% O_2 (balance N_2) or air and stored at 4.4°C. One set of samples remained at 4.4°C throughout the experiment and were analyzed on Days 0, 7, 14, 21, 28, 35 and 42. A second set of samples was stored at 4.4°C, but individual jars were stored at 12.8°C during the final 7 d of storage. A third set of samples, flushed only with 75% CO_2 with 10% O_2 (balance N_2) or containing air was moved from storage at 4.4°C on Day 21, held for 6 h at 26.7°C, and returned to 4.4°C until Day 28, when they were plated.

RESULTS AND DISCUSSION

Experiment A

Although the bacteriostatic and bactericidal effects of CO_2 are more pronounced at colder temperatures (*27*), an effect on *P. fragi* was seen at 12.8°C (Fig. 1). *P. fragi* counts were lower in jars with lower O_2 content. The MA containing 25% O_2 inhibited at least 1 $\log_{10}$ of *P. fragi* throughout the entire storage period, indicating the effect is not solely attributable to the reduced O_2 content of the MA's. Enfors and Molin (*8*) have previously demonstrated the inhibitory effect of CO_2 on *P. fragi*. At 26.7°C, *P. fragi* numbers declined in all atmospheres except air. The effect of limited O_2 was more pronounced at the higher temperature, which is greater than the optimum growth temperature of this psychrotrophic organism.

C. perfringens grew to 10^7 CFU/g in air at 12.8°C (data not shown). It is likely that oxygen consumption by *P. fragi* created a more favorable anaerobic atmosphere. Headspace analysis by gas chromatography con-

firmed that O_2 levels in 12.8 and 26.7°C air samples on Day 13 were less than 1%. At 12.8°C, the combination of lower temperature and MA's containing moderate O_2 concentrations prevented outgrowth of *C. perfringens* on samples co-inoculated with *C. perfringens* and *P. fragi*.

At 26.7°C, the atmosphere composition had no effect on *C. perfringens*, which flourished in all atmospheres (data now shown). These results reiterate the fact that MAP cannot be used effectively at room temperature, and cannot be used as a substitute for refrigeration (*11*).

The relationship between the ratios of the $\log_{10}$ counts of *P. fragi* and *C. perfringens* and the percentage O_2 in the MA at 12.8°C is shown in Figure 2. The dashed horizontal line represents the ratio of the $\log_{10}$ counts of the two organisms at the time of inoculation. Values above this line indicate that the *P. fragi* numbers increased relative to the numbers of *C. perfringens*. Some lines on the graph are incomplete because of the failure to recover one or the other of the organisms from some MA's after a period of time.

Oxygen levels greater than 2% represent a safety factor in that the numbers of *P. fragi* increased with time relative to the growth of *C. perfringens* (Fig. 2). The concept may be thought of as a Safety Index, where products with an increasing ratio of spoilage organisms to pathogenic organisms can be considered less hazardous than products whose ratios decrease from the original inoculum ratio.

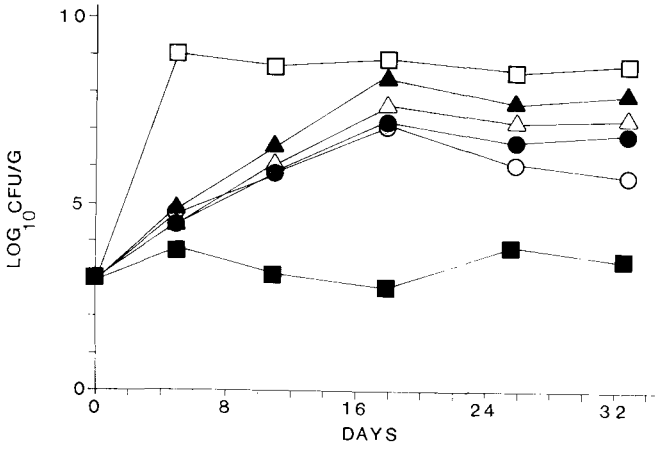

Figure 1. *Growth of* P. fragi *at 12.8°C on roast beef inoculated with* P. fragi *and* C. perfringens *and stored in air (□) or modified atmospheres containing 75% carbon dioxide with 0% (■), 2% (○), 5% (●), 10% (Δ) or 25% (▲) O_2 (balance N_2).*

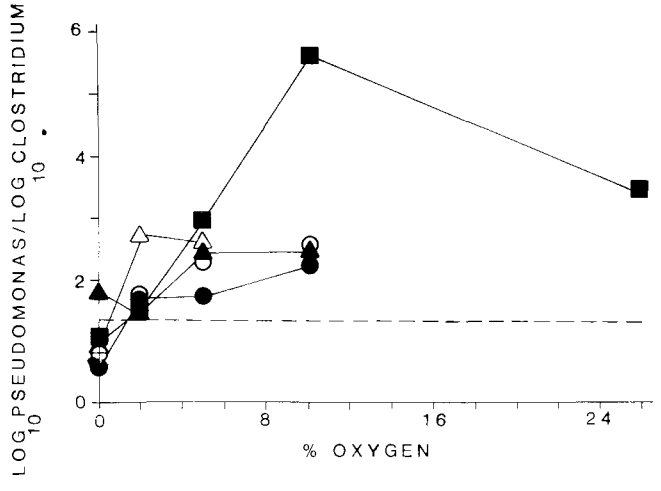

Figure 2. *Safety Index of cooked roast beef inoculated with* P. fragi *and* C. perfringens *and stored in 75% CO_2 with 0 to 25% O_2 (balance N_2) for 5 (■), 11 (○), 18 (●), 26 (Δ) or 33 (▲) d at 12.8°C. Dashed line represents ratio at time zero.*

Experiment B

The growth of *P. fragi* on samples co-inoculated with *P. fragi*, *S. typhimurium* and *S. aureus* and held at 12.8°C in air or MA's containing 75% CO_2 and 0 to 25% O_2 (balance N_2) is shown in Figure 3. At 12.8°C, 75% CO_2 almost completely inhibited *P. fragi* regardless of the O_2 content of the MA. Compared with results in Figure 1, there was an even greater inhibition of *P. fragi* in this case; this may be because *P. fragi* was competing for O_2 with the other two organisms. There was a loss in viability of *P. fragi* in the MA at 26.7°C to the point that the organism was not recovered after 20 d.

At 12.8°C, 75% CO_2 had a strongly inhibitory effect on *S. aureus* (Fig. 4). It is clear that the effect was due to the high CO_2 concentration rather than low O_2 levels because the different amounts of O_2 had little influence on counts. At 26.7°C (data not shown) the effect was due more to anoxia than elevated CO_2 levels. This is suggested by the observation that changes in the number of *S. aureus* were more dependent upon O_2 content; the growth of *S. aureus* in 25% O_2 was similar to that in air. Inhibition of *S. aureus* by CO_2 has been observed by other researchers (*3,6*). At 20°C, limited growth was observed in an MA containing 60% CO_2 (*22*).

Although the numbers of *S. typhimurium* increased by several log cycles in all atmospheres (Fig. 5), the MA had an inhibitory effect on the organism other than that

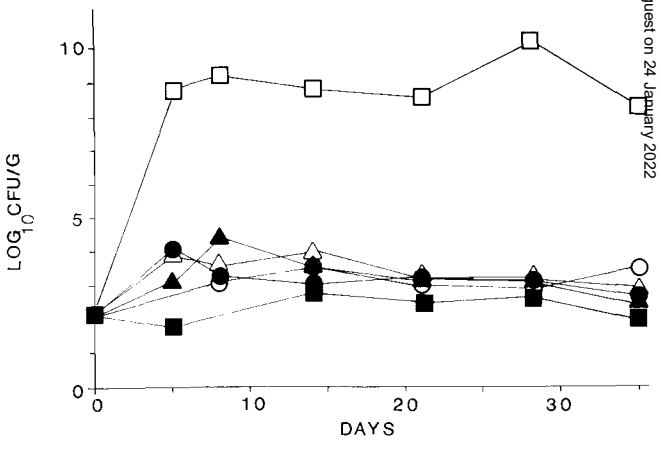

Figure 3. *Development of* P. fragi *on cooked roast beef inoculated with* P. fragi, S. typhimurium *and* S. aureus *and stored at 12.8°C in air (□) or modified atmospheres containing 75% CO_2 with 0% (■), 2% (○), 5% (●), 10% (Δ) or 25% (▲) O_2 (balance N_2).*

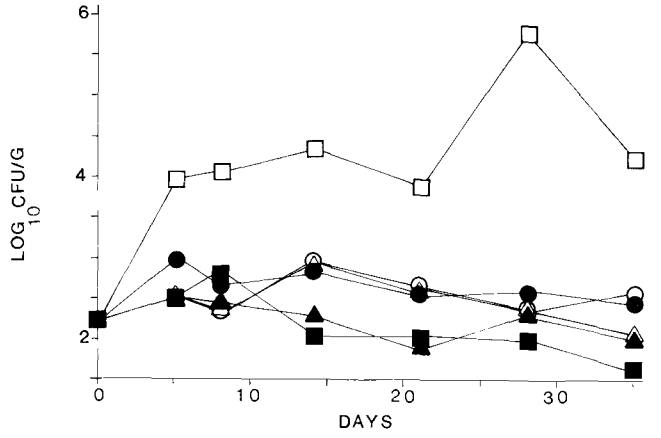

Figure 4. *Development of* S. aureus *on cooked roast beef inoculated with* P. fragi, S. aureus *and* S. typhimurium *and stored at 12.8°C in air (□) or modified atmospheres containing 75% CO_2 with 0% (□), 2% (○), 5% (●), 10% (Δ) or 25% (▲) O_2 (balance N_2).*

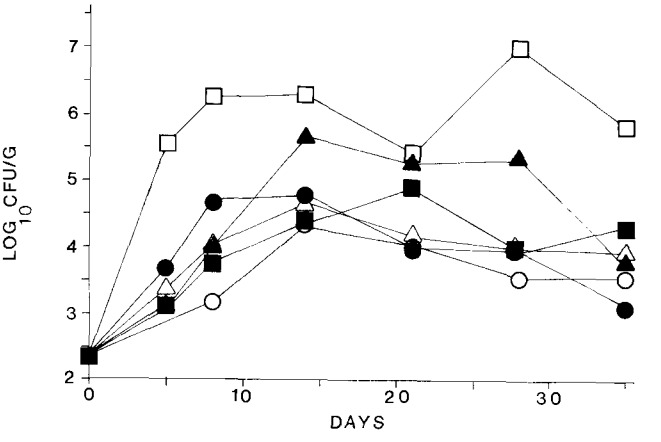

Figure 5. *Development of* S. typhimurium *on cooked roast beef inoculated with* P. fragi, S. aureus *and* S. typhimurium *and stored at 12.8°C in air (□) or modified atmospheres containing 75% CO_2 with 0% (■), 2% (○), 5% (●), 10% (Δ) or 25% (▲) O_2 (balance N_2).*

due to decreased levels of O_2. Like *S. aureus*, this organism has a preference for oxidative metabolism. Partial inhibition of *Salmonella* spp. by CO_2 on other substrates has been observed by others (*3,7,12,19*).

Plots of the $\log_{10}$ CFU of the ratio of *P. fragi* to *S. aureus* and *S. typhimurium* vs. the percentage of O_2 of the MA's are shown in Figures 6 and 7, respectively. Values above the dotted line (representing Day 0) in Figure 6 represent indices where *P. fragi* numbers increased relative to *S. aureus*. Values below the line, which are for MA's with less than 2% O_2, represent more hazardous atmospheres. In general, the ratio increases as the percentage of O_2 increases. A positive ratio was more difficult to attain for *Salmonella* (Fig. 7), a reflection of the moderate inhibition by those MA's which are strongly inhibitory to *P. fragi*.

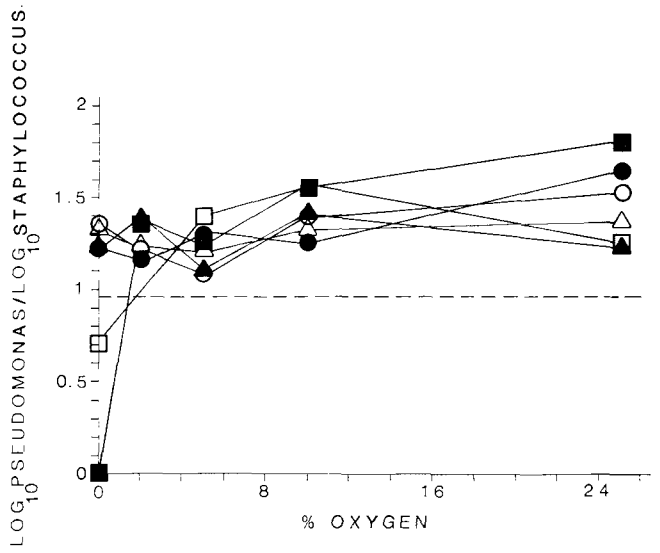

Figure 6. *Safety Index with respect to* S. aureus *of cooked roast beef inoculated with* P. fragi, S. aureus *and* S. typhimurium *and stored at 12.8°C in 75% CO_2 with 0 to 25% O_2 (balance N_2) for 5 (□), 8 (■), 14 (○), 21 (●), 28 (Δ) or 35 (▲) d. Dashed line represents ratio at time zero.*

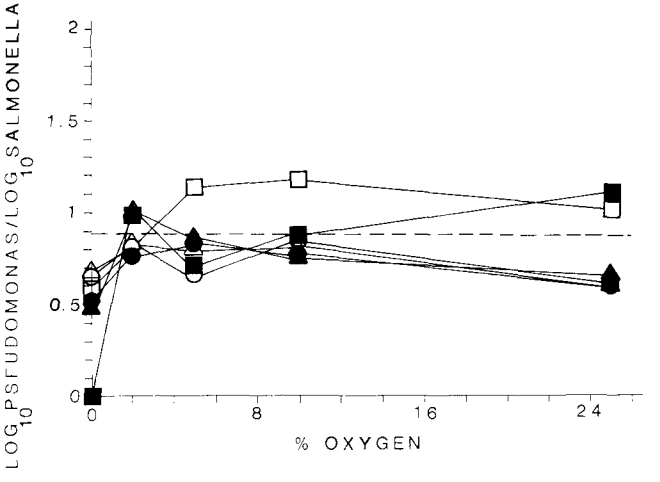

Figure 7. *Safety Index with respect to* S. typhimurium *of cooked roast beef inoculated with* P. fragi, S. aureus *and* S. typhimurium *and stored at 12.8°C* in 75% CO_2 with 0 to 25% O_2 (balance N_2) for 5 (□), 8 (■), 14 (○), 21 (●), 28 (Δ) or 35 (▲) d. *Dashed line represents ratio at time zero.*

Experiment C

The effects of 1 wk of storage at a temperature higher than refrigeration (12.8°C, broken lines) following optimum refrigerated storage (4.4°C, solid lines) on the growth of *S. typhimurium, S. aureus, C. perfringens* and *P. fragi* in air and 75% CO_2 and 10% O_2 (balance N_2) are shown in Figures 8 through 11, respectively. During storage at 4.4°C, *S. typhimurium* decreased to undetectable levels in all atmospheres (Fig. 8). Some cells remained viable to this low temperature and were able to multiply during holding at 12.8°C. MA's did not signific-

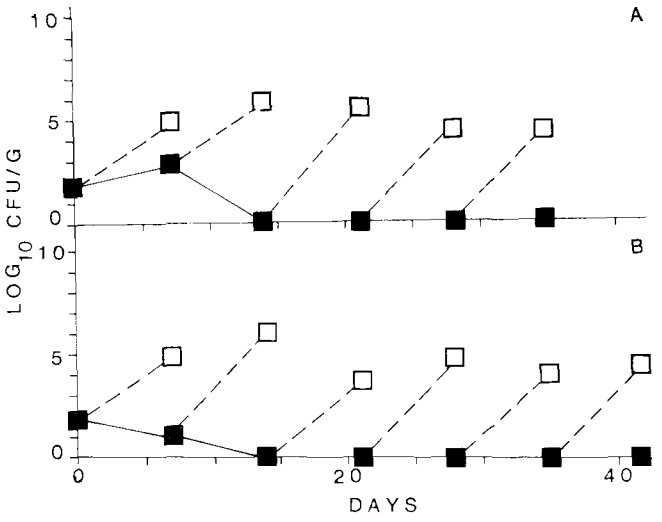

Figure 8. *Development of* S. typhimurium *on roast beef inoculated with* P. fragi, S. typhimurium, S. aureus *and* C. perfringens *and stored in air (A) or 75% CO_2 with 10% O_2 (B) (balance N_2) at 4.4°C (————) for 0 to 42 d or moved to storage at 12.8°C (--------) for the final 7 d.*

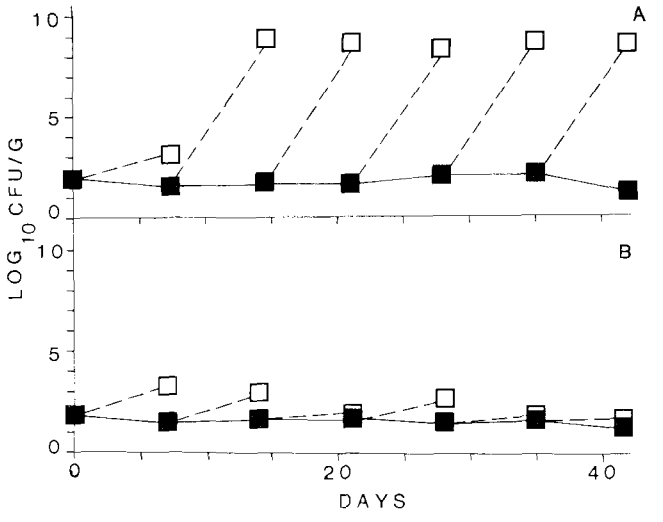

Figure 9. *Development of* S. aureus *on roast beef inoculated with P. fragi, S. typhimurium, S. aureus and* C. perfringens *and stored in air (A) or 75% CO_2 with 10% O_2 (B) (balance N_2) at 4.4°C (————) for 0 to 42 d or moved to storage at 12.8°C (--------) for the final 7 d.*

antly affect the ability of *S. typhimurium* to recover during storage at 12.8°C.

Growth of *S. aureus* was inhibited at 4.4°C (Fig. 9) and CO_2 had an inhibitory effect on the organism's growth during the period of abusive storage. In air, the growth of controls was as great as 7 $\log_{10}$ during the final 7 d of storage at 12.8°C.

The numbers of *C. perfringens* decreased during storage at 4.4°C (Fig. 10). The organism was not recovered following the transfer of samples to 12.8°C after Day 21. Recovery of the organism in samples stored less than 21 d was greatest in samples containing air. Including 10% O_2 in the MAP inhibited *C. perfringens* compared to the samples that contained only CO_2 and N_2.

In contrast to the other organisms, *P. fragi* is a psychrotroph, hence there was less of an increase in the counts of *P. fragi* than for the other organisms when samples were moved to 12.8°C (Fig. 11). There was essentially no change in *P. fragi* counts in samples held in air, but in the MA's, the higher temperature corresponded with a decrease in the effectiveness of the CO_2.

Storage at 26.7°C for 6 h on Day 21 followed by storage at 4.4°C for seven more days had little effect on the numbers recovered of any organism studied. *S. typhimurium* and *C. perfringens* remained undetectable and the numbers of *S. aureus* did not change. The numbers of *P. fragi* actually decreased by about 1.5 $\log_{10}$ (data not shown).

The most effective and safest gas combination was 75% CO_2, 15% N_2 and 10% O_2. *P. fragi* was inhibited by the high CO_2 concentration, *S. aureus* and *S. typhimurium* by the elevated CO_2 and reduced O_2 levels, and *C. perfringens* by the presence of O_2. For this reason, the inclusion of O_2 in MAP is recommended as a safety precaution.

Stier et al. (*26*) and Post et al. (*20*) conducted studies on fish fillets packaged in MA's and inoculated with *C.*

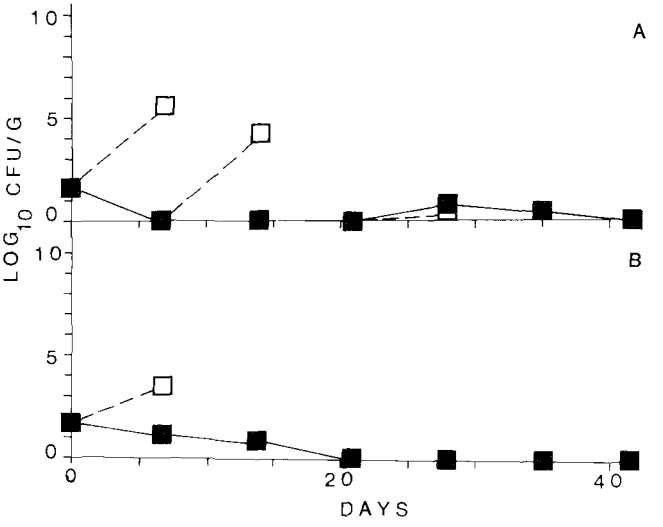

Figure 10. *Development of* C. perfringens *on roast beef inoculated with* P. fragi, S. typhimurium, S. aureus *and* C. perfringens *and stored in air (A) or 75% CO_2 with 10% O_2 (B) (balance N_2) at 4.4°C (————) for 0 to 42 d or moved to storage at 12.8°C (--------) for the final 7 d.*

botulinum type E to determine whether evidence of product spoilage would cause the product to be rejected before the fillets became toxic. Stier et al. (*26*) found that spoilage usually was evident before toxigenesis, but Post et al. (*20*) found that toxigenesis often preceded spoilage. Because fish and seafood are often contaminated with psychrotrophic strains of *C. botulinum* type E (*15*), vacuum or modified atmosphere packaging of these products is not recommended.

The concept of the relationship of spoilage to toxigenesis or pathogenicity must also be applied to non-

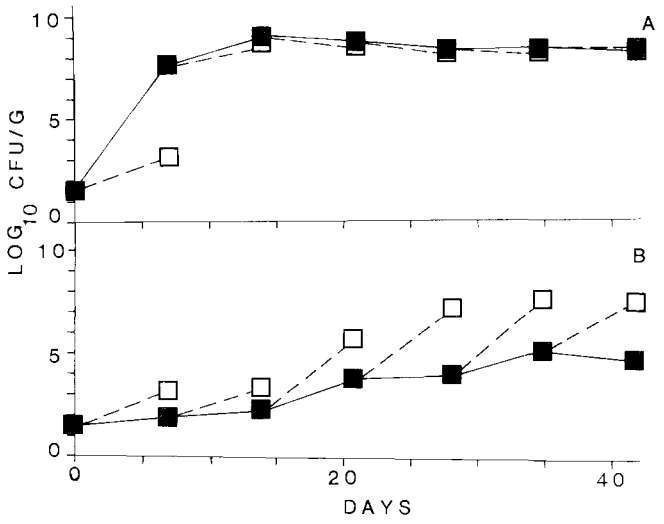

Figure 11. *Development of* P. fragi *on roast beef inoculated with* P. fragi, S. typhimurium, S. aureus *and* C. perfringens *and stored in air (A) or 75% CO_2 with 10% O_2 (B) (balance N_2) at 4.4°C (————) for 0 to 42 d or moved to storage at 12.8°C (--------) for the final 7 d.*

seafood products in the evaluation of MA's. The Safety Index can be used for this purpose.

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