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THE COMBINED EFFECT OF MODIFIED ATMOSPHERE PACKAGING (MAP) AND CHITOSAN ON THE GROWTH OF *LISTERIA MONOCYTOGENES* IN MODEL SYSTEMS AND IN FRESH PORK LOIN.

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ABSTRACT

M.Sc. Jennifer Morris Food Science

THE COMBINED EFFECT OF MODIFIED ATMOSPHERE PACKAGING (MAP) AND CHITOSAN ON THE GROWTH OF *LISTERIA MONOCYTOGENES* IN MODEL SYSTEMS AND IN FRESH PORK LOIN

Listeria monocytogenes is a pathogenic, psychrotrophic microorganism that is ubiquitous in nature. *L monocytogenes* has been isolated from numerous meat products, both fresh and processed, the incidence of contamination varying greatly. The ability of *Listeria* to grow in meats depends on temperature, pH, water activity (a_w), nutrients, species and numbers of competing microorganisms, gaseous conditions, and levels of additional barriers. Therefore, methods to control the growth of *L.monocytogenes* are of great importance to food processors since this organism can grow under a wide range of environmental and storage conditions. Two methods of control, in conjunction with temperature, were studied in this project: (i) modified atmosphere packaging (MAP) and (ii) chitosan, to determine the optimum levels of these "hurdles" needed to effectively control the outgrowth of *L.monocytogenes* in both model broth and agar systems and in fresh pork loin. Initial studies in broth and agar medium indicated that the optimum level of chitosan needed for complete inhibition of *Listeria* was 0.2%(w/v) at a pH of 6.0 over a wide temperature range. Modified atmosphere packaging studies in a model agar system showed that the optimal gaseous conditions for inhibition of *Listeria* were: a) 100%N_2 + an Ageless FX oxygen absorbent, b) 80%CO_2+20%N_2 and c) an Ageless SS Oxygen absorbent alone. These gaseous conditions were effective at temperatures of 10°C or less, indicating the importance of adequate storage temperature control, in conjunction with any other control methods used, i.e., "the hurdle approach" to food safety. On the basis of these preliminary studies, a combination of chitosan as a dipping solution and modified atmosphere packaging were investigated to control the growth of *L.monocytogenes* in fresh pork loin. Pork loin samples were dipped in a 0.2% chitosan solution for 60 seconds and packaged under various atmospheres in Cryovac bags and stored at 5, 10

and 15°C up to 28 days. Samples were monitored for physical, chemical and microbiological changes throughout the storage period. Optimum control over the growth of *L.monocytogenes* was achieved using a combination of 100%N_2 + an Ageless FX oxygen absorbent and dipping in a 0.2% chitosan solution. Based on these studies, a combination of 0.2% chitosan and MAP could be used to extend the shelf life of pork without adversely affecting color, odor and exudate loss while inhibiting the growth of the pathogenic microorganism, *L. monocytogenes*

RESUME

M.Sc. Jennifer Morris Sciences de l'alimentation

INFLUENCE DE L'EMBALLAGE SOUS ATMOSPHERES MODIFIEES COMBINEE AVEC CHITOSAN SUR LA CROISSANCE DE *LISTERIA MONOCYTOGENES* UTILISANT DES SYSTEMES MODELES ET DU PORC FRAIS

L. monocytogenes est un microorganisme psychrotrophique pathogène, fréquemment retrouvé dans la nature. *L.monocyotogenes* a été isolé à partir de plusieurs produits de la viande où la fréquence de contamination est sujette à de grande variation. L'habilitée de cette bactérie a se reproduire dépend principalement de la température, du pH, de l'activité de l'eau (a_W), des nutriments disponibles, des concentrations des gaz (O_2, N_2, CO_2), et finalement du nombre et des différentes espèces de microorganismes en compétition avec elle. En conséquences, les méthodes de contrôle de croissance de *L.monocytogenes* représentent un grand problème pour les producteurs de viande puisque cet organisme peut se reproduire sous plusieurs conditions environnementalles. Deux traitements: l'emballage sous atmosphères modifiées (ESM) d'un part et chitosan de l'autre, en conjonction avec la température, furent étudiés dans ce projet avec l'intention de déterminer les niveaux optimum des conditions qui pourraient effectivement contrôler la croissance de *L.monocytogenes*, en utilisant premièrement des systèmes modèles, puis du porc frais. Des études préliminaires utilisant des milieue liquides et solides (agar) ont montré qu'une concentration de 0.2% (p/v) de chitosan à un pH de 6.0 était requise pour l'inhibition complête de *L. monocytogenes* en utilisant une distribution variée de températures. De plus, les études utilisant l'emballage sous atmosphères modifiées de *L.monocytogenes* dans un système modèle solide (agar) ont démontrées que plusieurs conditions gazeuses pouvaient arrêter la croissance de cet organisme. Ces conditions comprennent: (a) 100% N_2 + un absorbant d'oxygène (Ageless FX), (b) 80% CO_2 + 20% N_2 et (c) un absorbant d'oxygène seul (Ageless SS). Ces conditions gazeuses sont valables seulement lorsque la température ne dépasse pas 10°C. Ceci démontre l'importance de l'utilisation de la réfrigération en conjonction avec d'autres méthodes de contrôle. En se basant sur ces études préliminaires, une méthode combinant l'utilisation du chitosan (comme solution trempage) et ESM fut etudiée pour contrôler la croissance de *L.monocytogenes* sur les filets du porc frais. Les échantillons de filet de porc frais furent trempés dans une solution de 0.2% de chitosan pour une période de 60 secondes furent emballés sous

differentes conditions atmosphériques. Les échantillons furent entreposés à 5, 10 ou 15°C pour une période maximale de 28 jours. Les échantillons furent examiner à differentes temps préetablis pour suivre les changements chimiques, physique et microbiologiques. L'utilisation d'une atmosphère de 100% d'azote, avec une absorbant d'oxygène (Ageless FX) contenu dans l'emballage, le tout combiné avec trempage des échantillons dans une solution de 0.2% chitosan furent les conditions optimales pour le contrôle de *L. monocytogenes* dans le porc frais. En conclusion de ces études, une combinaison, chitosan et ESM, pourrait être utilisée pour prolonger la période de fraîcheur du porc et arrêter la croissance des microorganismes pathogéniques tel que *L.monocytogenes* sans affecter les caractéristiques sensorielles du porc.

PREFACE

Claim of Original Research

(1) The use of Ageless oxygen absorbents, types FX, SS and SE to delay the growth of *Listeria monocytogenes* in model agar systems and in fresh pork loin.

(2) The use of combination treatments: chitosan hydrochloride in conjunction with modified atmosphere packaging (MAP) to control the growth of *Listeria monocytogenes* in broth, agar and pork systems.

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CHAPTER 1
LITERATURE REVIEW

1.1. INTRODUCTION

The incidence of food poisoning illnesses has been increasing steadily year by year (Knϕchel and Gould, 1995; Todd, 1992). The reason for the increase in reported illnesses is probably due to a combination of factors, including: improved reporting, changes in agricultural practices, changes in food marketing and eating habits, identification of new pathogens, development of superior microbiological methods and changing population sensitivities. There have been reports of the emergence of new pathogens (Schofield,1992) but in reality they are mostly the recognition of organisms that have probably caused food borne illness for thousands of years (Baird-Parker,1994). One such pathogen, relatively well known today, is *Listeria monocytogenes.* Two aspects of its control in foods are: (i) improvement in analytical methods and (ii) the development of new methods to inhibit or delay its growth in foods (Farber, 1993). At the same time, the demand for foods that are more convenient, safe, fresh and less heavily processed and preserved is ever increasing. Fresh pork represents a major class of food produced and consumed in Québec, that normally does not have a shelf-life of more than four days. Minimal processing methods are currently being applied to pork in order to extend its short shelf-life while *L.monocytogenes* has been implicated in several cases of food poisoning involving the consumption of pork (Cooksey, 1993; Farber and Peterkin, 1991). With such a reduction in the preservation of foods, how will it be possible to achieve a real reduction in the incidence of food poisoning from manufactured foods? Combination treatments, or the "hurdle approach" to food safety and shelf life extension could play a major role in the resolution of this important problem.

1.2. Pork: Shelf life and Spoilage

Fresh pork is a highly perishable product with a refrigerated shelf life of about four days, undergoing progressive deterioration from the time of slaughter to consumption. Pork is composed of water, proteins, lipids, carbohydrates (in the form of glycogen) and other minor components such as vitamins, minerals, enzymes and pigments, the relative proportions of which contribute to its structure, texture, flavor, color and nutritive value. The shelf life of pork is affected by temperature, atmospheric oxygen, enzymes, light,

moisture and most importantly, microorganisms. Meat spoilage is defined as "any single symptom or group of symptoms of overt microbial activity, manifested by changes in meat odor, flavor or appearance "(Gill, 1986).

1.2.1. Microbiological Concerns

Microbial growth is by far the most important factor influencing the shelf life of pork. Most microbial contamination occurs post-slaughter on the surface of the meat carcass when spoilage bacteria, pathogenic bacteria, molds and yeasts may be present. The deep muscle tissues remain relatively free from contamination if the slaughtering practices are hygienic. The microbial population of pork is affected by the species, health and handling of the live animal, slaughtering practices, proper chilling of the carcass, sanitation prior to fabrication, the type of packaging as well as the handling of the meat throughout distribution and storage. The spoilage microorganisms most likely to be found include species such as *Pseudomonas, Moraxella, Acinetobacter, Aeromonas, Alteromonas, Lactobacillus, Brocothrix.* Pathogenic bacteria commonly found include: *Salmonella, Staphylococcus aureus, Yersinia enterocolitica, Clostridium botulinum, Clostridium perfringens, Campylobacter, Aeromonas hydrophila* and *Listeria monocytogenes.* These microorganisms can originate from the skin of the animal, from fecal material, air, soil and water (Gill and Greer, 1993). Of particular concern to meat processors, consumers and regulatory authorities, both nationally and internationally,.is the growth of the psychrotrophic pathogen *L.monocytogenes.*

1.3. *Listeria monocytogenes*

L.monocytogenes is a pathogenic, psychrotrophic microorganism that is ubiquitous in nature and has been implicated in numerous food poisoning outbreaks. It is a gram positive, facultatively anaerobic, non-spore forming rod, capable of growth between 0.4 and 50°C (Farber and Peterkin, 1991). It is motile by peritrichous flagella, showing tumbling motility under a light microscope in a wet mount at 25°C. The colonies demonstrate a characteristic blue-green sheen by obliquely transmitted light. It is catalase positive, oxidase negative and expresses a β-hemolysin which produces zones of clearing on blood agar. The hemolysin is recognized as a major virulence factor whose secretion is essential for promoting intracellular growth and T-cell recognition of the organism. The hemolysin is called Listeriolysin-O.

L.monocytogenes was first recognized as a pathogen in animals 60 years ago, and was later implicated in human outbreaks of food poisoning. The disease that *L.monocytogenes* causes is termed Listeriosis, with most cases being sporadic. The source and route of infection is usually unclear in these cases, although a portion of them may be previously unrecognized common source clusters. The recent association of *L. monocytogenes* with several large food poisoning outbreaks suggests that contaminated food is the primary source of the organism. Meningitis (the inflammation of the brain or spinal cord) is the most common form of Listeriosis. The symptoms include respiratory illness, sore throat interrupted by vomiting, headache, confusion, lethargy and stiffness in the back and neck. There are other bacteria that cause meningitis such as *Streptococcus pneumoniae, Neisseria menigitidis, Staphylococcus aureus* and several others.

The majority of human cases of Listeriosis occur in individuals who have an underlying condition which leads to suppression of their T-cell mediated immunity. There are incidents in which apparently healthy individuals became ill. The highest incidence of infection appears to be in newborn babies, followed by those older than 60 years of age. Pregnant women can also be victims of Listeriosis- a mild flu-like illness is contracted, rarely leading to a full blown case of Listeriosis. Maternal Listeriosis is associated with abortion late in the third trimester of pregnancy. Listeriosis is also more common among cancer patients and tends to attack the central nervous system. The highest mortality rate from Listeriosis is associated with this group of individuals. Another widely affected group is patients who are taking drugs to suppress their immune system i.e., patients who are receiving organ transplants and taking immuno-suppressants so that the body will not reject the new organ (Farber and Peterkin, 1991).

1.4. Factors Influencing the Growth of *L.monocytogenes*

L monocytogenes is also highly adaptable and hardy and is capable of growth over a wide range of physical and chemical conditions. It can grow at pH values between 4.7 and 7.0 in tryptic soy broth supplemented with 0.6% yeast extract incubated at 30°C, with no growth occurring at pH 4.0 or lower (Parish and Higgins, 1989). Acetic acid was found to be the best growth inhibitor out of a range of acids used to lower pH. However, *L.monocytogenes* could grow at lower pH values when incubated at a higher storage temperature. Petran and Zottola (1989) found that *L.monocytogenes* could grow at

temperatures between 4 and 45°C and at pH values between 4.7 and 9.2. *L.monocytogenes* grew optimally at pH 7.0 and between 30 and 37°C (Petran and Zottola, 1989). Recently, a surface response mathematical model was developed describing the effects of temperature, pH and NaCl, $NaNO_2$, and gaseous conditions on the growth kinetics of *L.monocytogenes* Scott A in tryptone phosphate broth (Buchanan and Philips, 1990). In this experiment, a total of 709 growth curves were generated with individual curves fitted using non-linear regression analysis in conjunction with the Gompertz function. The data was adapted into an easy to use spreadsheet program commonly referred to as the USDA Pathogen Modeling Program (Smittle and Flowers, 1994). This study indicated that in model systems, *Listeria* is able to withstand reasonably acidic conditions and that its ability to grow at low pH is highly dependent on incubation temperature. These workers also concluded that *Listeria* was highly adaptable to anaerobic conditions when cultures were flushed with nitrogen and that in the absence of nitrate, the microorganism's growth kinetics were similar for both aerobic and anaerobic conditions. Buchanan and Klawitter(1990) also reported that the growth of *L.monocytogenes* under aerobic conditions was highly dependent on incubation temperature at pH 4.5, and, that when oxygen was restricted, the organism recovered and survived for extended periods. These results are not in agreement with those of George and Lund (1992) who determined that *L.monocytogenes* was capable of more rapid growth in air than under nitrogen at a pH of 4.5. McClure et al.(1989) used gradient plates to assess the effects of NaCl and pH in combination with different temperatures on the growth of *L.monocytogenes*. Their results suggested that growth did not occur below pH values of 4.95 at 20°C or pH 4.6 at 25, 30 and 35°C after 24 hours of incubation. These workers also determined that growth at high NaCl concentrations (10%) occurs only within a clearly defined pH range (6.6 to 7.0) at 30 and 35°C. An automated turbidimetric system was used to examine the effects of different combinations of NaCl, $NaNO_2$, pH and temperature on the growth of *L.monocytogenes* (McClure et al., 1991). The data presented illustrated the combinations that permitted the organism to grow to visible levels. In this study, the ability of *L.monocytogenes* to grow at low pH values was again strongly influenced by incubation temperature as well as $NaNO_2$ concentration. At 20°C and below, no visible growth was detected, even with 50ug/ml of $NaNO_2$ at pH 5.3 (or below) within 21 days. At pH 6 or above, $NaNO_2$ had little effect in delaying visible growth except at higher concentrations and also at lower incubation temperatures. Sorrels et al. (1989), studied the effect of different acids, pH, incubation time and incubation temperature on the growth and survival of *L.monocytogenes* in tryptic soy broth.

Hydrochloric, acetic, lactic, malic and citric acids were used to acidify the broth to pH values of 4.4, 4.6, 4.8, 5.0, and 5.2. The inhibition of *L.monocytogenes* again appeared to be a function of the type of acid used to modify the pH and incubation temperature. The antimicrobial activity of the acids was: acetic>lactic>citric>malic>hydrochloric at constant pH values and at all incubation temperature/time combinations. One significant observation was that *L.monocytogenes* was not only able to grow at pH levels of 4.4, but tolerate such an acidic pH at low storage temperatures. Kroll and Patchett (1992) studied the response of *L.monocytogenes* to increasing acidic pH in order to determine its ability to withstand a pH shock. *L.monocytogenes* incubated in broth at pH 5.0 did not increase growth of the organism at pH 7.0 after exposure to this low pH compared with cells initially icubated in broth at pH 7.0. However, growth at pH 5.0 significantly increased survival of cells at low pH as determined by plate counts compared with cells grown at neutral pH. These workers concluded that pH adaptation occurs in *L.monocytogenes* and that alterations in the cytoplasmic membrane could be responsible for this adaptation.

There have been few studies examining the effect of water activity (a_w) alone or the combined effect of a_w and temperature on the growth of *L.monocytogenes*. The ability of *L.monocytogenes* to initiate growth at five different temperatures in brain heart infusion broth adjusted to various a_w values using NaCl, sucrose or glycerol was investigated by Farber et al. (1992). Glycerol was found to be the least toxic of the three solutes tested and *L.monocytogenes* was capable of growth at an a_w value of 0.9 at 30°C compared to a minimum of 0.93 and 0.92 in the broths adjusted with sucrose and NaCl respectively. These workers also determined that the minimum a_w for growth was dependent on incubation temperature and, as temperature decreased the minimum a_w increased. Farber et al.,(1992) concluded that *L.monocytogenes* is one of the few food pathogens capable of growth at a_w values below 0.93. These results agree with those of Miller (1992) who determined that the minimum a_w for growth of *L.monocytogenes* was 0.9, 0.92 and 0.97 in broths adjusted with glycerol, NaCl and propylene glycol respectively at 28°C. Tapia deDaza et al. (1991) and Petran and Zottola (1989) also reported similar results. The relationship between water activity, lactate and the growth of *L.monocytogenes* was further studied in a meat model system consisting of cooked strained beef ranging in moisture content from 25 to 85% (w/w) by Chen and Shelef (1992). Lactate depressed meat water activity and differences between water activity values in control and lactate treated samples at each moisture level increased progressively with decrease in moisture, from 85% moisture to 25% moisture. The effect of NaCl on the survival of

L.monocytogenes at refrigeration and frozen temperatures was examined by Hudson (1992). All concentrations of salts tested (6, 16 and 26% (w/v)) were ineffective in reducing numbers over 6 hours of incubation at refrigeration temperatures. Over a longer time, (33 days) at refrigeration temperature, the organism grew in 6% NaCl, remained static in 16% NaCl and was destroyed in 26% NaCl. Storage at -18°C for 33 days caused no significant reduction in numbers at any of the combinations of salt and temperatures tested.

1.5. *Listeria monocytogenes* in Food Products

Table 1 outlines the wide range of food products that have been implicated in *Listeria* outbreaks and infections. The number of cases varies, as does the number of deaths. Foods implicated range from fresh produce to fish, milk and meats including pork. The tolerance levels for *L.monocytogenes* in foods have been set by Health and Welfare, Canada, as follows: (i) a zero tolerance level in foods that have been linked to Listeriosis outbreaks e.g., coleslaw, soft cheeses, pate, (ii) a zero tolerance for foods with a shelf life of more than 10 days that are capable of supporting its growth, e.g., vacuum packaged meats and (iii) in frozen foods, a tolerance level of less than 100 cfu/g of food is accepted as long as the food was processed and packaged under good manufacturing practices (GMP). There is limited evidence that indicates that foods containing 100 or fewer *L.monocytogenes* per gram do not pose a health risk for healthy individuals. However, foods in which *L.monocytogenes* has multiplied to high levels can pose a threat to healthy individuals. The risk of listeriosis in normal persons has been classified as "moderate, direct, potentially extensive spread type health hazard" and for immuno-compromised individuals as a "severe, direct health hazard" (ICMSF, 1994).

Table 1: Food borne outbreaks due to *Listeria monocytogenes*

Location & Year	Number of Cases	Number of Deaths	Foods Associated
Boston, 1979	20	5	Raw vegetables
New Zealand, 1980	29	9	Shellfish,raw fish
Maritimes, 1981	41	17	Coleslaw
Massachusetts, 1983	49	14	Pasteurized milk
California, 1985	142	48	Cheese
Philadelphia, 1987	36	16	Salami, pork
Connecticut, 1989	9	1	Shrimp
U.K., 1987-1989	300+	NA	Paté, pork

Adapted from Farber and Peterkin (1991)

1.5.1. Meat and Meat Products

A wide variety of meats are contaminated with *L.monocytogenes*, with the incidence of contamination varying greatly. The variation is due in part to differences in the detection methods including such factors as the method used, the sample size and the source from which the samples were purchased. Table 2 illustrates the incidence of *L.monocytogenes* in pork and pork products. Most observed contamination occurs on the meat surface. However, there have been reports of the presence of *Listeria* in the interior muscle tissue of beef, pork and lamb (Johnson et al., 1988). The growth of *Listeria* in meat is generally dependent on temperature, pH of the muscle tissue and the type and amount of background microflora present. However, there have been conflicting results with respect to *Listeria's* ability to grow in meats (Farber and Peterkin, 1991). Studies have shown that it is not capable of growth on meat at 4 and 25°C, while others have shown the reverse or refuted this observation. Buchanan and Klawitter (1991) observed that growth

of *L.monocytogenes* did not occur in either untreated or irradiation-sterilized raw ground beef, even though the culture had been incubated at 5°C prior to inoculation. However, *L.monocytogenes* was able to survive for extended periods of time in the sterile meat. At 7°C and lower, *Listeria* was able to grow in meat with a low initial background microflora present (10^5 cfu/g), whereas at 25°C, no growth of *Listeria* was observed in meat with a background microflora of 10^7 or higher (Farber and Peterkin, 1991). Growth of *L.monocytogenes* is highly dependent on product type and pH, and tends to grow well on meat products with a pH value of 6.0 or above and not well on meats with a pH of 5.0 or below (Glass and Doyle, 1989). McKellar et al. (1994) determined the factors influencing the survival and growth of *L.monocytogenes* on the surface of Canadian retail wieners, either all beef, poultry or beef/pork. The wieners were surface inoculated with *L.monocytogenes* and stored under vacuum at 5°C for up to 28 days. Of a total of 61 samples tested, 40 supported growth of this pathogen. The aerobic growth rate and the duration of the lag period was determined for *L.monocytogenes* on ground lean beef and on pieces of fatty tissue by Grau and Vanderlinde (1993). The organism grew at 0°C on lean tissue at pH 6 and on fatty tissue. It failed to grow at 0°C on lean tissue at pH 5.6 but did grow at 2.5°C at this pH. The growth rate was described by a modified Arhenius equation. The lag period increased with decreasing temperature and pH. In summary, most of the research to date confirms that *L.monocytogenes* is capable of growth in meats.

Table 2: Incidence of *Listeria monocytogenes* in pork

Product	Source	Number of Samples	%Positive	Reference
Minced Pork	Commercial	30	80	Schmidt et al., 1988
Pork	Retail	25	68	Lowry and Tiong, 1988
Paté	Retail	101	7	Farber and Daley, 1994
Frankfurters	Retail	46	5	Wang and Muriana, 1994
Pork	Various	71	1	Adesiyun, 1993
Ham	Commercial	71	29	Grau and Vanderlinde, 1992
Pork	Retail	25	60	Wang et al., 1992
Pork	Retail	153	35	Comi et al., 1992
Minced pork	Commercial	51	63	Skovgaard and Norrung, 1989

1.6 Methods to Control the Growth of *L.monocytogenes*

1.6.1. Thermal Processing

While high temperatures can be used to control many pathogenic bacteria in foods, there is concern over the thermotolerance of *L.monocytogenes* following the Massachusetts outbreak in 1983 in which pasteurized milk was implicated as the vehicle of infection (Farber and Peterkin, 1991). There is still some disagreement as to the question of the thermal resistance of *L.monocytogenes*. A phenomenon called heat shock response and the methods used to recover heat stressed organisms are the main causes of discrepancies found in the literature. If *L.monocytogenes* is exposed to sub-lethal temperatures of around 44 to 48°C before being subjected to the final test temperature, the cells acquire an enhanced thermotolerance (Farber and Peterkin, 1991). If the methods used in enumerating heat stressed cells are carried out under strict anaerobic conditions, significantly more cells are recovered. Also, the selectivity of the enumeration media appears to be a factor in the recovery of organisms. The oxygen sensitivity of the heat stressed cells is thought to be due to the inactivation of the enzymes catalase and superoxidase dimutase during heating. Limited studies on *Listeria*'s heat resistance have been carried out in model systems. The heat resistance of *L.monocytogenes* was determined in sucrose solutions with an aw range of 0.98 to 0.90 (Sumner, 1991). The $D_{65.5°C}$ value shifted from 0.36 to 3.8 minutes (a 10 fold increase) and the z value ranged from 7.6 to 12.9°C. In other words the D value increased as sucrose concentrations increased and as aw decreased. In tryptone soya broth containing 0.3% yeast extract, the D values at 60, 63 and 66°C were 7.3, 3.0 and 1.0 respectively, while the z value was 6.0°C (Quintvalla and Campanini, 1991). Table 3 illustrates the heat resistance of *L.monocytogenes* in various products. The survival of small populations of *L.monocytogenes* on poultry processed using a moist heating method was determined by Carpenter and Harrison (1989). In this study, various inoculum levels were applied to chicken breasts which were cooked to an internal endpoint of 73.9°C. After cooking, portions were either vacuum packaged or wrapped in an oxygen permeable film and stored for up to 4 weeks at 4°C or up to 10 days at

10°C. Some *L.monocytogenes* survived the cooking process regardless of the inoculum levels. Significant increases in the population occurred at both storage temperatures, and populations of *L.monocytogenes* were able to re-establish themselves to levels above the initial inoculum level, with no differences noted due to packaging. Line and Harrison (1992), inoculated cured and uncured turkey rolls with *L.monocytogenes* and vacuum packaged the samples prior to cooking to internal temperatures of 68 and 74°C in a steam injected chamber. These workers also inoculated battered chicken nuggets with *L.monocytogenes* and cooked them under moist heating conditions to an internal temperature of 71°C. No *L.monocytogenes* cells were recovered from the cooked products suggesting that similar commercial practices are adequate to reduce levels of *L.monocytogenes* to below detectable limits. Cooksey, (1993) inoculated pre-cooked vacuum packaged beef chunks with *L.monocytogenes*, and subjected them to pasteurization at 85°C for 16 minutes. All samples were stored at 4°C for up to 85 days and examined at regular intervals. Pasteurization reduced all background contamination and significantly reduced the population of *L.monocytogenes*. However, *L.monocytogenes* was recovered upon enrichment of the samples prior to plating. In a study by Yen et al.(1992), the effects of heating on *L.monocytogenes* in pork formulated with kappa-carrageenan, sodium lactate and the algin/calcium meat binder was investigated. Uncured or cured pork was inoculated with *L.monocytogenes*, mixed with different combinations of the compounds previously mentioned and subjected to heating to a final internal temperature of 62°C. Both kappa-carrageenan and sodium lactate had no effect on the extent of thermal destruction of *L.monocytogenes* in uncured ground pork. However, kappa-carrageenan reduced the protective effect of the curing mixture against thermal inactivation in cured pork. The algin/calcium meat binder had no effect on the thermal destruction of *L.monocytogenes*.

Table 3: Heat resistance of Listeria monocytogenes in some meat and fish products

Product	Temperature (°C)	D value (Minutes)	z value (°C)	Reference
Lean Ground Beef	52	81.3	5.4	Fain, (1991)
	57	2.6		
	63	0.6		
Fatty Ground Beef	52	71.1	6.3	Fain, (1991)
	57	5.8		
	63	1.1		
Pork Emulsion	60	12.95	6.8	Quintvalla and Campanini, (1991)
	63	5.4		
	66	2.3		
Fermented beaker Sausage	60	9.13	NA	Schoeni et al. (1991)
Ground beef roast	60	4.47	NA	Schoeni et al. (1991)
Crawfish tail meat	55	10.23	NA	Dorsa et al. (1993)
	60	1.98		
	65	0.19		
Cod fillets	60	1.98	NA	Embarek & Huss, (1993)
	65	0.28		
Salmon fillets	60	4.30	NA	Embarek & Huss, (1993)
	65	1.02		

1.6.2 Low Temperature Control of *Listeria monocytogenes*

Freezing and holding at or below -10°C commonly extends the shelf life of foods by 5 to 50 fold compared to chilling preservation (Ciobanu,1976 cited in El-Kest et al.,

1991). Liquid nitrogen (-198°C) is widely used to preserve bacterial cultures for later use in food processes and fermentations. Temperatures below optimum for growth reduce metabolic activity of microorganisms and so are bacteriostatic rather than bactericidal. However, death of microorganisms may occur as a consequence of extended frozen storage due to injury. Although found in frozen foods, there is little information about the behavior of *L.monocytogenes* during freezing and frozen storage. El-Kest et al., (1991) studied the effect of freezing and frozen storage of *L.monocytogenes* in phosphate buffer and tryptose broth by freezing cell suspensions for either 30 minutes at -18°C or 10 minutes in liquid nitrogen. Freezing and storage for one month in phosphate buffer at -18°C caused 87% death and 79% injury, while 54% death and 45% injury was observed in tryptose broth. Freezing and storage in liquid nitrogen for one month caused no death or injury of cells in phosphate buffer, whereas minimal death and injury was observed in tryptose broth. These authors also studied the effect of freezing and thawing and re-freezing, which caused significantly more death/injury than a single freeze thaw cycle. If liquid nitrogen is to be used to preserve food the fact that *L.monocytogenes* is resistant to death and injury during many months storage must be considered (El-Kest and Marth, 1992). Recently, the fate of *L.monocytogenes* on packaged, frozen seafood was determined by Harrison et al. (1991). No increase in numbers of the organism was noted, and populations decreased by less than 1 log cycle after 3 months storage.

1.7. Novel Methods for the Control of *L.monocytogenes*:

Many novel methods for controlling the growth of *L.monocytogenes* in foods have been proposed and applied with varying degrees of success.

Little is known about the influence of nutritional factors on the virulence of *L.monocytogenes*. The limited knowledge was reviewed by Benedict (1990) who stressed the importance of understanding the role of environmental parameters in the secretion of virulence factors. Listeriolysin-O (LLO) is probably the best studied virulence factor and the extent of its secretion may be influenced by temperature, NaCl and calcium. It has been established that the ferric ion has the most significant effect (McKellar, 1993). The influence of several preservatives and growth factors on LLO secretion by *L.monocytogenes* was examined and found to be maximal in tryptic soy broth (McKellar, 1993). Both growth and secretion was inhibited by nitrite while

secretion only was selectively inhibited by sorbate and NaCl. These results suggest that LLO secretion is more sensitive than growth to the inhibitory action of preservatives.

1.7.1. Bacteriocins

Of particular interest to the processed/fermented meat industry is the use of bacteriocin producing strains of *Lactobacillus, Pediococcus*, *Leuconostoc* and *Carnobacterium* species (Daba et al., 1991; Dallas and Hitchins, 1993; Degnan et al., 1992; Foegeding et al., 1992; Luchansky, 1992; Mattila-Sandholm et al., 1991; Motlagh et al., 1992; Schillinger and Mattila -Sandholm, 1990; Skytta, 1991; Sobrino, 1991; Winkowski and Montville, 1992). In recent years, antimicrobial metabolites from food-grade starter culture bacteria, especially certain bacteriocins and bactericidal peptides, have generated interest as potential biopreservatives in minimally processed foods. Traditionally, fermented foods have been considered as pathogen-free. However, *L.monocytogenes* has been shown to grow in these products, possibly due to the suspected emergence of bacteriocin resistant variants of *Listeria.*

Lactic acid bacteria produce a variety of antibacterial factors. The inhibitory spectrum varies between narrow and broad within different lactic acid bacteria. Lactic acid bacteria show a number of antibacterial mechanisms, i.e., production of acid, hydrogen peroxide, or carbon dioxide. More importantly, the antibacterial effects may be based on the competition for available nutrients and formation of antibacterial compounds, such as bacteriocins (Skytta and Mattila-Sandholm, 1991). Most of the experimental work has been carried out with *Pediococus damnosus* (Mattila-Sandholm et al., 1991), which has shown inhibitory activity towards the growth of Gram positive spoilage microorganisms such as lactobacilli and streptococci and gram positive pathogens such as *S.aureus* and *Bacillus cereus* and *L.monocytogenes. Pediococcus acidilacti* H produces pediocin AcH which has been shown to be inhibitory towards *L.monocytogenes* (Motlagh et al., 1992). *Leuconostoc mesenteroides* was found to produce a bateriocin called mesenterocin 5 active against *L.monocytogenes* strains (Daba et al., 1991). Studies with *Lactobacillus bavaricus* (Winkowski and Montville, 1992), showed that it also produces a bacteriocin that was able to inhibit *L.monocytogenes* at 10°C. Sensitivity of *L.monocytogenes* to nisin has also been

demonstrated (Harris et al., 1991). Nisin is an antibacterial peptide produced by *Lactobacillus lactis* subsp. *lactis* that exhibits a broad spectrum of inhibitory activity towards Gram positive bacteria and spores. Ming and Daeschel (1993) and Harris et al. (1991) were able to isolate a mutant of *L.monocytogenes* that was resistant to nisin due to fundamental changes that had occurred in bacterial membrane structure and function. Despite its bactericidal effect, nisin should not be relied on to control *L.monocytogenes* in the food supply.

The lactoperoxidase system is a natural antimicrobial system present in milk that has also been tested for its antilisterial abilities (Zapico, 1993; Gaya et al., 1991). The enzyme lactoperoxidase catalyses the oxidation of thiocyanate by hydrogen peroxide with the antimicrobial effect due to intermediate reaction products. In cow's milk, the lactoperoxidase system exhibited bactericidal activity against *L. monocytogenes* at 4 and 8°C, with activity being dependent on temperature, length of incubation and strain of *Listeria* used (Gaya et al., 1991). The lactoperoxidase system was also found to have antilisterial properties in raw goat's milk (Zapico, 1993).

1.7.2. Preservatives

In more recent studies, the effects of various traditional and novel preservatives have been studied (Table 4). Most of these compounds have been studied in model broth and model agar systems. Only a few preservatives have actually been applied in a food matrix to test their inhibitory effects on *L.monocytogenes*. The compounds listed in table 4 have also been studied in combination with one another (El-Shenawy and Marth, 1992; Johansen et al., 1994; McKellar et al., 1992; Moir and Eyles, 1992; Oh and Marshall, 1993a and b; Shelef and Yang, 1991; Stillmunkes, 1993; Wang and Johnson, 1992; Wang and Shelef, 1992; Zaika and Kim, 1993). In general, results of these combination studies show that most preservatives, when used together, illustrate a synergistic effect, enhancing the inhibitory properties of the individual compounds. Thus, lower levels of the individual compounds may be used. This effect is one of the underlying principles behind the hurdle approach to food safety.

Table 4: Preservatives and novel compounds studied to control growth of *Listeria monocytogenes* (various strains)

Preservative	Type of medium	MIC[a]	Temperature(°C)	Reference
Carrot juice	Tryptic phosphate broth	1% (v/v)	30	Beuchat & Brackett, 1990b & Beuchat, 1994
Chitosan	Nutrient broth	0.5% (w/v) at pH 5.5	30	Wang, 1992
Ethanol	Tryptic soy broth	50,000 ug/ml (5%)	35	Oh & Marshall, 1993a
Glycerol monolaurate	Tryptic soy broth	10 ug/ml (0.001%)	35	Oh & Marshall, 1993b
	Brain heart infusion broth	10-20 ug/ml at pH 5	4	Wang & Johnson, 1992
	Sterile whole milk	N.I.C.T.[b]	4	Wang & Johnson, 1992
	Sterile skim milk	200 ug/ml	4	Wang & Johnson, 1992
	Beef round	2.5% (v/w)	4	Stillmunkes, 1993
Hexanoic acid	Tryptose soya yeast glucose broth	6.89-8.61 mmol/l,pH 5	20	Kinderlerer & Lund, 1992
Lactic acid	Tryptic soy broth	5000 ug/ml (0.5%)	35	Oh & Marshall, 1993b
Lactic acid immobilized in alginate	Lean beef tissue	1.7%(v/v)	5	Siragusa & Dickson, 1992
Lactoferrocin	Peptone yeast glucose broth	1-3 ug/ml	37	Wakabayashi, 1992
Lysozyme	Tryptic soy broth	10,000 ug/ml, pH5.5	5	Johansen, et al. 1994
	Fresh cod fish	N.I.C.T.	5, 20	Wang & Shelef, 1992
Methyl- -hydroxybenzoate	Brain heart infusion broth	300-700 mg/l at pH 5	30	Moir & Eyles, 1992
	"	1300-1600 mg/ml at pH 6	30	"
	"	600-1500 mg/ml at pH 6	5	"
Myristoyl-phenylalanine	Tryptic soy agar	8.5 ug/ml	30	McKellar et al.,1992
Octanoic acid	Tryptose soya yeast glucose broth	1.41-3.39 mmol/l, pH 5	20	Kinderlerer & Lund, 1992
Potasium sorbate	Brain heart infusion broth	400-600 mg/ml, pH 5	30	Moir & Eyles, 1992
	"	>5000 mg/ml, pH 6	30	"
	"	1100-1500 mg/ml, pH 6	5	"
Sodium diacetate	Brain heart infusion broth	0.45% (w/v)	20	Shelef & Addala, 1994
Sodium gluconate	Beef round	>2.5% (v/w)	4	Stillmunkes, 1993
Sodium lactate	Beef round	2.0 % (v/w)	4	Stillmunkes, 1993
	Smoked salmon	2.0% (v/w)	5	Pelroy, 1994
	Tryptic soy broth	> 5% (v/v)	35	Shelef & Yang, 1991
	Sterile comminuted chicken	4% (v/w)	5, 20, 35[c]	Shelef & Yang, 1991
	Sterile comminuted beef	4% (v/w)	5, 20, 35[c]	Shelef & Yang, 1991
Sodium polyphosphates	Brain heart infusion broth	0.3% (v/v)	5, 10, 19, 28[c]	Zaika & Kim, 1993
Sodium propoionate	Tryptose broth	0.3% (v/v), pH 5.6	13, 35	El-Shenawy & Marth, 1992

a: minimum inhibitory concentration
b: not inhibitory at concentrations tested
c: growth suppression, not inhibition, was observed at these temperatures, suppression increased as temperature decreased

Other interesting "natural" antilisterial compounds include plant essential oils, such as cinnamon, clove, origanum, pimento and thyme which have all been shown to retard the growth of *L.monocytogenes* to a limited extent (Aureli et al., 1992). Pimento oil was able to completely inhibit growth within two hours, whereas clove, origanum and thyme oils showed a much milder inhibitory effect, taking four or more hours to completely inhibit growth.. The antilisteric effect of thyme oil was also tested in minced pork meat in which the *Listeria* population was reduced by approximately 100 fold over the first week of storage (Aureli et al., 1992). In another similar study by Pandit and Shelef (1994) a different effect was found, with rosemary and cloves the only spices to exhibit antilisterial activity. Addition of 0.5% finely ground rosemary or 1% rosemary oil to ready-to-eat pork liver sausage prior to cooking delayed growth of *L.monocytogenes* during refrigerated storage. Hefnawy et al. (1993) also studied the sensitivity of *L.monocytogenes* to selected spices in tryptose soya broth. Counts of *L.monocytogenes* decreased to less than 10/ml in one, four and seven days by 1% sage, 1% allspice and by 1% cumin, garlic powder, paprika and red pepper respectively. Black pepper and mace at 1% did not inactivate *L.monocytogenes* while white pepper appeared to enhance its growth.

1.7.3. Irradiation

Gamma or electron beam irradiation treatment of meat to eliminate *Listeria monocytogenes* has been proposed (Grant and Patterson, 1992; Thayer and Boyd, 1995) Radiation D-values were determined for inactivation of *L.monocytogenes* at 5°C intervals from -20 to 5°C in inoculated beef (Thayer and Boyd, 1995). This data was used to develop an equation that predicts the response to gamma irradiation within a selected temperature range. An increase in resistance occurred at sub-zero temperatures, i.e., -5°C and -20°C. The radiation D-values were 0.45 kGy at 0 and 5°C, 0.77 kGy at -5°C and 1.21 kGy at -20°C. This resistance has been attributed to the decreased OH mobility at subfreezing temperatures. The D-value results at 5°C are similar to those obtained by Grant and Patterson (1992) in roast beef meal stored at refrigeration temperatures. Lebepe (1990) studied the effects of irradiation (3.0 kGy) on the microbiological changes in fresh, vacuum packaged pork loins which were stored at 2 to 4°C for up to 98 days and at 25°C for approximately 48 hours. *L.*

monocytogenes was found in two irradiated samples. The microbiological shelf life of the pork loins stored at 2 to 4°C was more than 90 days compared with unirradiated loins Varabioff et al.(1992) determined the effects of irradiation on bacterial load and *L.monocytogenes* in raw chicken that was either vacuum packaged or packaged in air. After irradiation of chickens to a dose of 2.5 kGy, *L.monocytogenes* was only recovered from the vacuum packaged chickens after 7 days of cold storage. These authors concluded that irradiation may not be effective in destroying *L.monocytogenes*.

1.7.4. Sanitizers

Preventing contamination of processed foods requires emphasis on sanitation in the food plant environment. *L.monocytogenes* has been reported to attach itself to a wide variety of surfaces. In the case of stainless steel, *L.monocytogenes* demonstrated significant resistance to chemical sanitizers and heat (Krysinski et al., 1992), and it was often isolated from moist surfaces in the processing and packaging areas of food plants (Ren and Frank, 1993). These observations indicate inadequate cleaning and sanitation. The germicidal effect of three common sanitizers, iodophor, quartenary ammonium compounds and chlorine on *L.monocytogenes* were evaluated by Tuncan (1993) using the suspension test method at various exposure temperatures and times. All three sanitizers were effective against *Listeria* at 25°C regardless of their concentrations (200-800ppm), giving a 5.0 log reduction after 30 seconds of exposure. As temperature decreased, the effects of the sanitizers were diminished. However, this could be reversed by extending the exposure time. Sallam and Donnelly(1992) observed similar results with a variety of sanitizers, and, in addition, discovered that the sanitizers used induced injury, not death of the microorganism, and that the lethal effect of a sanitizer was found to increase by increasing its concentration or exposure time. These results illustrate that exposure to a sanitizer does not mean destruction of *L.monocytogenes* and that the organism becomes sublethally injured, and capable of repair and multiplication when conditions become more favorable, i.e., in food products. This poses a public health hazard. Biofilm prevention lies in adherence with GMP (good manufacturing principles) and HACCP (Hazard Analysis Critical Control Point) processing. Strict attention should be paid to sanitizing of food processing plants to use lethal concentrations of sanitizer for optimal exposure times.

1.7.5. Modified Atmospheres

Modified atmosphere packaging has been defined as "the enclosure of foods in high gas barrier materials in which the gaseous environment has been changed or modified to slow respiration rates, reduce microbial growth and retard enzymatic spoilage- with the intent of extending shelf life" (Young et al., 1988). When MAP is viewed as a total control quality packaging technology, rather than a simple packaging step combined with some special gases, it can then be realized to its fullest potential and can become the revolutionary technology that it has proven to be in the European market place (Lioutas, 1988). MAP is a rapidly expanding technology considering that the energy costs associated with other preservation methods are relatively high i.e., heat processing, freezing and frozen storage and distribution and drying. Consumers are also more and more reluctant to accept chemical additives and preservatives in their foods and are demanding high quality, fresh-like foods ever increasingly. There are several methods that can be employed for modifying the internal gas atmosphere of a product's package head space. These methods are: vacuum packaging or skin packaging and gas packaging. Gas packaging is the technique in which various combinations and mixtures of carbon dioxide, nitrogen and oxygen are used depending on the type of product. The air is moved physically out of the package head space and the desired gas mixture is flushed in.

1.7.5.1. Gases used in Gas Flush Packaging:

1.7.5.1.1. Carbon dioxide

Carbon dioxide is considered to be the most important gas used in most MAP applications. CO_2 is bacteriostatic, fungistatic, prevents insect growth, and is highly soluble in fat and water where it forms carbonic acid. This solubility may lower the pH of the product resulting in slight flavor changes. The mechanism of action of CO_2 against microbes is explained by several theories. It is known that CO_2 inhibits the growth of Gram negative bacteria such as pseudomonads and other related psychrotrophs. Carbon dioxide increases the lag phase of the growth curve of these bacteria. Lactic acid bacteria, such as streptococci and lactobacilli are less affected by

elevated CO_2 levels, and in fact, elevated levels of CO_2 enhance their growth. CO_2 interferes with enzyme systems attached to the bacterial cell, such as the dehydrogenating enzymes and enters into the mass-action equilibria for enzymatic decarboxylation (Finne, 1982). Changes in surface pH have also been suggested as a mechanism of inhibition. The change in pH is due to the absorption of CO_2 on the food surface and subsequent ionization of the carbonic acid. Other theories suggest that CO_2 may act on bacterial cell membrane permeability and fluidity and toxicity of carbonic acid in its undissociated form (Finne, 1982).

1.7.5.1.2. Nitrogen

Nitrogen is an inert gas which apparently has no antimicrobial properties. It is termed the "filler gas" because its main function is to prevent package collapse since it is less water and fat soluble than carbon dioxide. N_2 prevents oxidative rancidity by replacing the oxygen present, especially in low water activity foods.

1.7.5.1.3. Oxygen

Oxygen is generally not used in gas packaging applications unless it is used to prevent the growth of strict anaerobes such as *Clostridium botulinum* and to maintain the bright red color or "bloom" associated with fresh red meats. Oxygen is also used in low concentrations in the packaging of respiring products.

1.7.5.2. New Developments: Oxygen Absorbent Technology

Oxygen absorbents are defined as a range of chemical compounds introduced into the MAP package (and not the product) to alter the atmosphere within the package. Oxygen absorbent technology was developed in Japan and forms an important role in food preservation and distribution in Japanese supermarkets. Oxygen absorbents are composed of compounds chemically reactive to oxygen. When oxygen absorbers are placed in gas permeable materials (in the form of small pouches usually made out of co-polymers of propene and ethylene vinyl alcohol, EVA) and placed in packages, they

are capable of removing residual oxygen from the package head space. It is important to note that gas packaging, which is a physical process, usually does not remove all traces of oxygen present, whereas oxygen absorbents are capable of removing all residual oxygen when used properly. First developed by the Mitsubishi Gas Chemical Company, Inc., in 1977, an iron-powder based oxygen absorbent under the name of AGELESS was introduced. The principle behind these absorbents is simply the reaction that occurs when iron rusts. The reaction is explained in Figure 1.

Figure 1: Reaction of iron to rust that occurs in oxygen absorbents

$$Fe \longrightarrow Fe^{2+} + 2e^-$$

$$\tfrac{1}{2}O_2 + H_2O + 2e^- \longrightarrow 2OH^-$$

$$Fe^{2+} + 2OH^- \longrightarrow Fe(OH)_2$$

$$Fe(OH)_2 + \tfrac{1}{4}O_2 + \tfrac{1}{2}H_2O \longrightarrow Fe(OH)_3$$

There are several types of Ageless absorbents available including an absorbent known as Ageless SS which is a self reacting type of absorbent capable of absorbing oxygen at low temperatures and is of particular interest to the chilled and frozen food industry. Ageless type SE is another absorbent of interest, especially to the bakery industry, since it not only removes the oxygen present, thus lowering the chance for mold spoilage, but it also emits ethanol vapor into the package head space. Ethanol vapor has been shown to be an effective, broad spectrum antimicrobial agent.

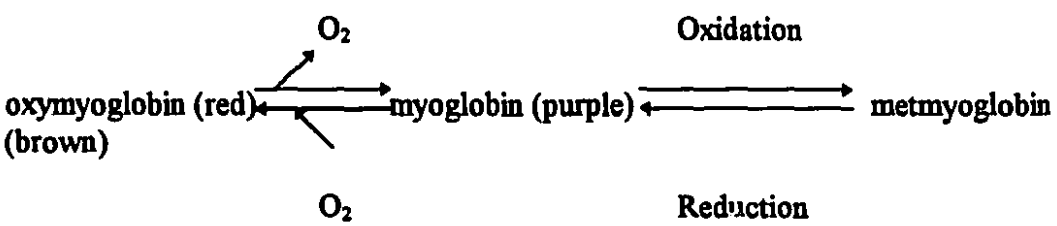
1.7.5.3. The Effect of MAP on Meat Sensory and Microbiological Characteristics

Several reviews and studies have been published on the topic of applying MAP to fresh meat products, the focus of which have been primarily extending the meat's shelf life from a microbiological and sensory perspective (Eyles et al., 1993; Finne, 1982; Gill and Tan, 1980; Greer et al., 1992; Holley et al., 1993; Venugopal et al., 1993; Wolfe, 1980; Young et al., 1988). In discussing the packaging of fresh meats, the two most important meat properties (aside from safety) are the color and microbial spoilage population.

1.7.5.3.1. Color

The color of fresh meat depends on the relative proportions of three forms of myoglobin; reduced myoglobin, oxymyoglobin and metmyoglobin (Holley et al., 1993; Young et al., 1988). Reduced myoglobin is the predominant muscle pigment in the absence of oxygen, producing the characteristic purple color meat exhibits when first cut as well as the expected color of vacuum packaged meats. Oxymyoglobin is the oxygenated form of the muscle pigment and is responsible for the bright red color consumers associate with fresh meat. Metmyoglobin is the form with an undesirable brown color that causes consumers to reject prepackaged meats, formed from the oxidation of oxymyoglobin and reduced myoglobin in low oxygen tensions or from the oxidation of iron heme in he myoglobin molecule (Young et al., 1988). Figure 2 illustrates this phenomenon.

Figure 2: The various forms of myoglobin



1.7.5.3.2. Microbial Population

High CO_2 levels (above 20%) cause a shift in the meat microflora, from fast growing aerobic spoilage bacteria to slow growing facultatively aerobic non-spoilage species. CO_2 inhibits pseudomonads and favors the outgrowth of their competitors that are less affected by CO_2 , mainly lactic acid bacteria. Since these organisms grow more slowly and generally yield metabolic products that are less offensive than those of

pseudomonads, such as lactic acid, spoilage of meat is delayed (Holley et al., 1993). The ability of CO_2 to inhibit respiration and growth of representative strains of seven species of meat spoilage bacteria was examined by Gill and Tan (1980). *Enterobacter* and *Brocothrix thermosphacta* were unaffected by CO_2 (25 and 75%). Both respiration and growth of the other species was inhibited (*Pseudomonas* (fluorescent and non-fluorescent), *Acinetobacter, Pseudomonas putrefaciens* and *Yersinia enterocolitica*). The degree of inhibition with a constant concentration of CO_2 in solution increased with decreasing temperature for all CO_2 susceptible species except non-fluorescent *Pseudomonas*. Anaerobic growth of CO_2 susceptible facultative anaerobes was unaffected by CO_2. Eyles et al.(1993) further studied the effects of MAP on the growth of psychrotrophic *Pseudomonads* on the surface of a model agar system. Atmospheres containing concentrations of CO_2 as low as 20% (balance nitrogen) inhibited the growth of *Pseudomonas fluorescens* and *Pseudomonas putida* on the surface of Brain heart infusion agar plates at pH 6.8 incubated at 5 and 15^{o}C in flexible packages. The modified atmospheres decreased the growth rates and reduced the populations attained at the end of the exponential phase of growth, but had no effect on the lag phase. Once again, the inhibitory effect of CO_2 increased as the temperature decreased. This effect is due to the increased solubility of CO_2 in the media, or muscle tissue at lower temperatures resulting in a lowering of the pH.

1.7.5.4. The Safety of MAP

MAP foods may pose a public health risk, especially if the MAP product is subjected to temperature abuse during storage and/or distribution. Consumers may also mishandle and/or subject the product to temperature abuse conditions, and they may also be prone to overextend the products' normal shelf life. Research on the safety of MAP foods, from a microbiological perspective, is lacking even though much is known about MAP in general, indicating that the wrong emphasis has been put into the development of MAP technology. Any research done should place emphasis on consumer safety first and freshness second. From a safety standpoint, in MAP foods, the normal aerobic spoilage microorganisms which would normally warn consumers of spoilage (by off odors and off colors) are inhibited, while the growth of pathogens may be allowed or even stimulated (Farber, 1991). In recent years, the focus of concern over the safety of MAP foods has shifted form the toxin producing Clostridia (Genigeorgis, 1985;

Hintlian and Hotchkiss, 1986; Silliker and Wolfe, 1980) to several "emerging pathogens". Due to the emergence of psychrotrophic pathogens, such as *L. monocytogenes, Aeromonas hydrophilia,* and *Yersinia enterocolytica,* new safety issues have been raised. Hotchkiss (1988) presents a detailed analysis on the experimental approaches to determine the safety of food packaged in modified atmospheres, however this review does not mention possible methods in evaluating whether or not the emerging pathogens pose health risks.

1.7.5.5 Effect of Modified Atmosphere Packaging (MAP) on the Growth of *L.monocytogenes*

Limited studies that have been done on the effect of MAP on *L.monocytogenes.* To date, few studies have been carried out in model systems. Razavilar and Genigeorgis (1992) studied the interactive effect of temperature, atmosphere and storage time on the probability of colony formation on blood agar by four *Listeria* species. The species used were *L.monocytogenes, L.seeligeri, L.ivanovii* and *L.innocua.* Sheep blood agar plates were inoculated with the test organisms and were stored at 4,8,20 and 30oC under air, vacuum, candle jar, 100% CO_2 or a MA consisting of $5\%O_2+10\%CO_2+85\%N_2$, for 7, 14, 21, 42 and 56 days. None of the interactions of temperature, time, or species with atmospheric conditions were found to be significant. An atmosphere of 100% CO_2 was found to be significantly more inhibitory to growth than any of the other atmospheres and its effect on delaying *Listeria*'s growth was enhanced at lower temperatures. *L.ianovii* was the most sensitive to CO_2. Packaging in 100% CO_2 extended the lag phase at less than 8°C and decreased the rate of growth at 4°C but not at higher temperatures.

1.7.5.5.1. Produce

The behavior of *Listeria* inoculated onto fresh vegetables and stored under CA (Controlled Atmosphere) conditions was examined by Berang et al. (1989). The CA used consisted of 11% O_2, 10% CO_2 and 79% N_2 . *Listeria* grew as well on CA vegetables as on control vegetables (air packaged) either at 4 or 15°C. Enumeration of asparagus at the end of storage showed that only slightly higher levels present in air

stored samples. In the same study, broccoli stored under 11%O_2, 10% CO_2 and 79% N_2 showed no decrease in the levels of *Listeria* present. The fate of *Listeria* in shredded cabbage at 5 and 25°C stored under MAP conditions (70% CO_2, 30%N_2) was determined by Kallander et al. (1991). In air packaged samples, *Listeria* populations increased by 2 log cycles within two days of storage at 25°C, but then decreased to undetectable levels within 6 days of storage. Packaging under a 70% CO_2 and 30%N_2 gas atmosphere and storage at 25°C also decreased levels of *Listeria* to undetectable levels within 6 days but with a less marked initial increase than the control samples. At 5°C, counts increased gradually but by only about 1 log in both the control and MAP stored samples. However, in control samples, a marked increase in *L.monocytogenes* was observed after 13 days of storage. The combined effects of shredding, chlorine treatment and MAP (97% N_2, 3% O_2) on the survival and growth of *L. monocytogenes* on lettuce stored at 5 and 10°C were determined by Beuchat and Brackett (1990a). None of the treatments, either alone or in combination, influenced the growth of this organism indicating that *Listeria* was able to grow on lettuce subjected to commonly used packaging and distribution procedures used in the food industry. An excellent review on the safety of produce packaged is under MAP conditions is provided by Hotchkiss and Banco (1992).

1.7.5.5.2. Poultry

The development of *L.monocytogenes* on minced raw chicken stored at 4, 10 and 27°C under various MAP conditions i.e., 75%CO_2, 25%N_2 (MA1) and 72.5%CO_2, 22.5%N_2, 5%O_2 (MA2) was studied by Wimpfheimer et al. (1990). *Listeria* grew under the aerobic control conditions at all temperatures. However, it failed to grow under any MAP conditions containing no oxygen, i.e., MA1. When oxygen was present (MA2) growth of *Listeria* was not inhibited and numbers increased by nearly 6 log cycles at 4°C after 21 days. However, growth of the aerobic spoilage microorganisms was inhibited. This study illustrates that under MAP conditions used commercially i.e., containing oxygen for bloom, *L.monocytogenes* is capable of growth while the spoilage microflora of chicken are inhibited. In another study, skinless chicken breast meat was inoculated with *L.monocytogenes* and stored at 1,6 or 15°C under aerobic conditions and atmospheres of 30%CO_2 + air (MA1), 30%CO_2/70%N_2 (MA2) and 100%CO_2 (MA3) (Hart et al., 1991). At 1°C the organism did not grow

under any of the test conditions. However, at 6°C *Listeria* counts increased 10 fold in aerobic conditions before spoilage of the meat was observed, but only when the culture was pre-incubated at 1°C rather than 35°C. In other words, subjecting the cells to refrigeration temperatures prior to inoculating the poultry appeared to enhance their ability to grow at lower temperatures since they were accustomed to these low temperatures. In CO_2 atmospheres, the growth of *Listeria* was inhibited on meat held at 6°C, especially under 100% CO_2. At 15°C, spoilage of the meat occurred within two days in all gaseous conditions and levels of *L.monocytogenes* increased 100 fold indicating the importance of strict temperature control to ensure the safety/quality of MAP muscle foods. The combined effect of MAP (90%CO_2/ 10%O_2) and lactic acid treatment on the growth of *L.monocytogenes* on chicken legs at 6°C was investigated by Zeitoun and Debevere, (1991). While MAP alone did not have an inhibitory effect on the growth of *Listeria*, combination treatments significantly reduced the numbers of *Listeria* present, illustrating a synergistic effect between lactic acid treatment and MAP on the growth of *L. monocytogenes* on chicken legs. The influence of various gaseous conditions, i.e., 76%CO_2, 13.3%N_2, 10.7%O_2 (MA1) and 80%CO_2, 20%N_2 (MA2) on the competitive growth *L.monocytogenes* and *Pseudomonas fluorescens* on pre-cooked chicken nuggets stored at 3,7 and 11°C was determined by Marshall et al.(1990). When the bacteria were co-inoculated in nuggets, growth of *Listeria* was stimulated at 3°C by the presence of *P.fluorescens* in air and under atmosphere MA1, but not in atmosphere MA2 where growth of *P.fluoresecens* was inhibited. This growth stimulation was also absent at higher temperatures. The stimulatory effect of *P.fluorescens* is thought to be due to a proteolysis production effect and certain amino acids which enhances the growth of *L.monocytogenes*.

1.7.5.5.3. Red Meats

Gill and Reichel (1989) studied the growth of *L.monocytogenes* on high pH beef (pH 6.0) stored under vacuum or 100% CO_2 at -2, 0, 2, 5 and 10°C. *L.monocytogenes* did not grow in vacuum packs at -2°C, and generally grew at rates less than the spoilage microflora at higher temperatures. In CO_2 packs, *Listeria* grew at 10°C, but not at the lower temperatures. The combined effect of MAP (25%CO_2/75%N_2) and irradiation (1.75 kGy) on the growth of *L.monocytogenes* in beef stored at 10 and 15°C was investigated by Grant and Patterson (1991). These authors showed that the safety of

MAP pork was less than that of MAP/irradiated pork indicating that combination treatments could provide an additional safety margin particularly when products are stored at temperature abuse conditions. Hudson et al. (1994) studied the growth of *L.monocytogenes* on sliced roast beef packaged under vacuum or saturated controlled CO_2 atmospheres at -1,.5 and 3°C. At -1.5°C, the pathogen declined in numbers in CO_2 packs but not under vacuum. However, at 3°C, growth occurred under all packaging conditions with maximum numbers being reached by the end of an extended storage period of 166 days.

1.7.6. Chitosan: A Novel Antimicrobial Agent

Chitin is the second most abundant natural polymer on earth (the first being cellulose) and is widely distributed throughout nature. Chitin is a polymer of N-acetylglucosamine units, while chitosan is the deacetylation product of chitin, i.e., a polymer of glucosamine. Figure 3 shows the structures of these compounds.

The most easily exploited sources of chitin are the protective shells of crustaceans, such as crabs and shrimps. It is estimated that fungi could provide approximately 32,000 tons of chitin annually. In the U.S., the main types of shellfish that are processed into chitin and chitosan are: Cancer magister (Dungeness crab), Paralithodes camshatica (King crab), and Pandalus borealis (Pacific shrimp) (Skjak-Braek, et al., 1989). Since crab and shrimp are harvested in different seasons, raw materials for chitin production are always readily available. Other sources of chitin include seafood waste, such as lobster, krill (an aggregate of small marine crustaceans), clams, oysters and squid. Chitin is also found in the cell walls of fungi as well as the cuticle of insects.

Chitosan is now produced in North America on a large scale. For some time it has been sold commercially as a solution, flaked and fine powder, and more recently in bead and fiber forms. Its availability in a variety of useful forms and its unique chemical and biological properties make it a very attractive biomaterial (Skjak-Braek et al., 1989). It is for the above mentioned reasons that chitosan already has a wide variety of applications, while more are currently the subject of extensive research. Recently, research on chitosan has been directed to its potential use as a food preservative and antimicrobial agent. It is estimated that the total sales of chitin and

chitosan will reach almost $2 billion U.S. in the next ten years, with sales of chitin and chitosan in the food and beverage industry comprising about 110 million dollars of this total.

1.7.6.1. Extraction of Chitin and Chitosan

The principle steps in the extraction of chitin from seafood waste are illustrated in Figure 4. Proteins are first removed from the ground up shells by treating the shells with sodium hydroxide. Using hydrochloric acid, minerals such as calcium carbonate and calcium phosphate are extracted. After rinsing, the chitin is dried as a flaked material. The general scheme to manufacture chitosan from chitin is shown in Figure 5. Chitin is first treated with concentrated sodium hydroxide to hydrolyze the N-acetyl linkages. It is then rinsed and the pH is adjusted. At this stage, chitosan can be dried to give what is called "flaked chitosan" which is a coarse mesh product whose particle size may be reduced by milling to give a finer mesh powder. The powder can then be dry blended with an organic acid, such as adipic acid, to give a chitosan acid blend that is self dissolving. One scheme to prepare chitosan of improved purity is to dissolve it in acetic acid and filter it to remove extraneous materials. The clarified product can then be reisolated to give an ultrapure product known as chitosonium acid salts, which are soluble in water. Another procedure used is to precipitate the chitosan out of solution using a base. This precipitate is then washed and dried to give chitosan in the free amine form.

Figure 3: Structures of the Repeating Units of:(A) Cellulose, (B) Chitin and (C) Chitosan

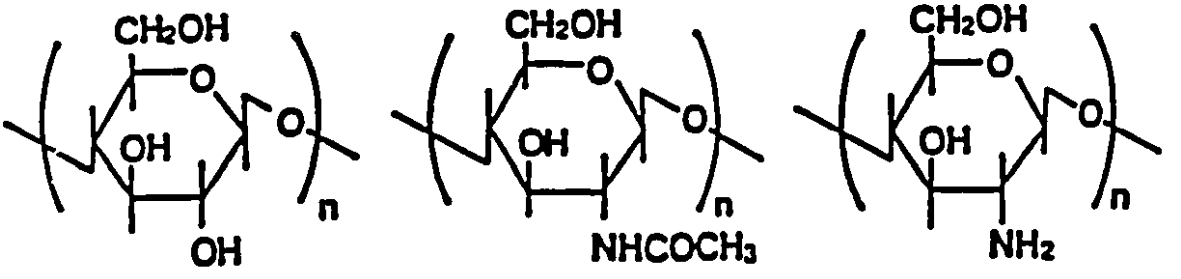


Figure 4. Flow Diagram of Chitin Extraction Process

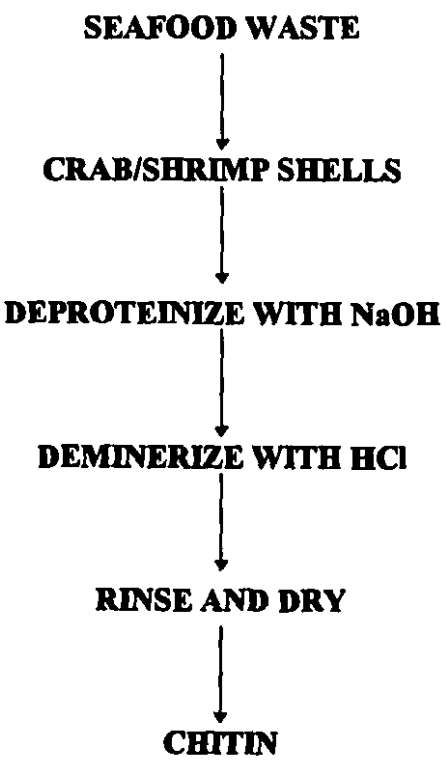
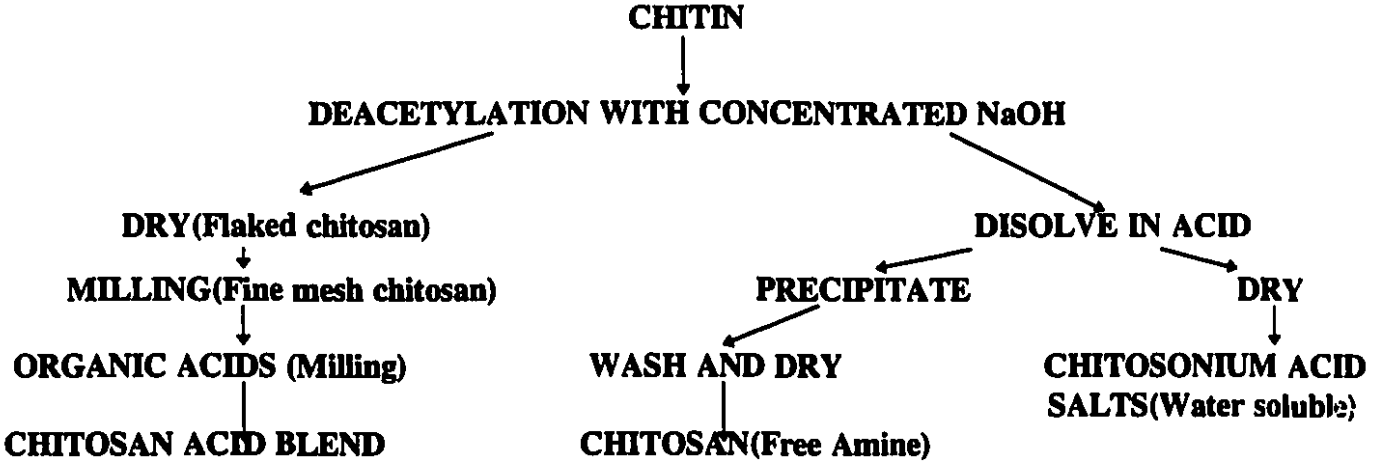


Figure 5: Flow Diagram of Chitosan extraction process



1.7.6.2. Applications of Chitosan

Chitosan has numerous applications, many of which depend on its cationic nature. It is a linear polyelectrolyte at acidic pH values and it has a high charge density, one per glucosamine unit. Since many materials carry negative charges (proteins, anionic polysaccharides, nucleic acids etc.) the positive charge of chitosan interacts strongly with negative surfaces to give electric neutrality. Due to the vast number of NH_3+ groups that can interact with negatively charged colloids, chitosan is also an excellent flocculating agent. It has been found that chitosan can also form complexes with many metal ions, thus it is useful in chelating iron, copper and toxic heavy metal ions, such as silver cadmium, mercury, lead, nickel and chromium (Muzzarelli, 1977).

Being a high molecular weight polymer, chitosan is a linear polyamine whose amino groups are readily available for chemical reactions and salt formation with acids. Since it can be viewed as a cellulose derivative, the primary and secondary hydroxyl groups can be used to make derivatives, many of which have commercial potential. In the free amine form, chitosan is not soluble in water at neutral pH. At acid pH values, the free amino groups become protonated to form cationic amine groups.

1.7.6.2.1. Agriculture

In agriculture, chitosan has been used as a seed coating and it has been shown to increase crop yields. The EPA (Environmental Protection Agency) in the U.S., permits the use of chitosan for this purpose and in 1989 it was being used in eleven states in the U.S. Chitosan triggers a response in the seed, signaling the plant to protect itself from natural predators, such as pathogenic fungi (a more detailed discussion on the effects of chitosan on fungi is presented later). Chitosan has also been approved for use as a flocculating agent to recover proteinaceous wastes for animal feed. Continuing studies investigate the use of chitosan coating as a controlled release agent for pesticides and herbicides. One other use in agriculture is the addition of chitosan to cropland to stimulate natural microbes that provide protection to certain crops. Hirano (1990) tested chitosan for use as an ingredient for domestic animal feeds. No abnormal symptoms were observed with hens and broilers by feeding <1.4 g of chitosan/kg of body weight per day for up to 239 days and with rabbits by feeding them with <0.8 g of chitosan/kg of body weight per day for up to 239 days. Both

chitin and chitosan were digested 35-83% by rabbits and 88-98% by hens and broilers. Chitosan also had an effect in decreasing serum cholesterol levels of the animals tested. However, for long term feeding, hen's appetites and egg laying rates decreased by feeding an excessive amount of chitosan due to its incomplete digestion.

1.7.6.2.2. Flocculating agent

The largest single use of chitosan is the clarification of waste water in Japan. Since chitosan is a natural polymer, it is preferred over synthetic polymer flocculants which may contain hazardous monomers. Several organizations in the U.S.A. (Division of Animal Feed, Department of Health and Human Service, FDA, Association of Animal Feed Control Officials (AAFCO)), have issued the following description of chitosan as a flocculent for recovering proteinaceous materials: "Chitosan is a cationic carbohydrate polymer intended for use as a precipitation agent of proteinaceous material from food processing plants. It is chemically derived by deacetylation of the naturally occurring chitin in crab and shrimp shells. It may be used in an amount not to exceed that necessary to accomplish its intended effect. Chitosan, when incorporated into the feed of livestock, should be present at no more than 0.1% of the feed. Proteinaceous material coagulated with chitosan must have safety and efficacy data approved before it can be registered or offered for sale" (Skjak-Braek et al., 1989). Imeri and Knorr (1988) and Soto Peralta et al. (1989), also found that acid soluble chitosan and water soluble chitosan proved equally effective as fining agents for apple and carrot juices. The one step application proved as effective as conventional treatments using silica/gelatin/bentonite. Knorr (1991) cited Bade and Wick (1988) who suggested the use of chitinous polymers for environmental protection and waste reduction. This is based on the fact that chitin and chitosan have proven film forming capabilities and that chitosan degrading microorganisms are abundant. Recently, it has been discovered that a chitosan based film had superior oxygen barrier properties, but less tensile strength, than synthetic packaging films, such as Mylar and polypropylene (Knorr, 1991).

1.7.6.2.3. Control of fungal diseases in plants

Fungal diseases are one of the major causes of post harvest losses of fruits and vegetables. Chemical control programs face may problems: (i) increasing amounts of

fungicide tolerant post harvest pathogens, (ii) increased public awareness and (iii) resistance to fungicide treated produce. As a result, a number of fungicides are now under review in many countries. Therefore, there is a growing need to develop alternative approaches for the efficient control of storage diseases. Chitosan has been shown to have an antimycotic effect against a wide range of fungi. Allan and Hadwinger (1979) studied the fungicidal action of chitosan on 46 fungi of varying cell wall composition. They observed that chitosan had more antifungal activity than chitin and, out of the 46 fungi tested, chitosan inhibited 32 of the fungi investigated. All fungi that were sensitive to chitin were more or equally sensitive to chitosan. At the concentrations used, chitosan seemed to have no effect on the fungi that contained chitosan in their cell walls. Stossel and Leuba (1984) confirmed the results of Allan and Hadwinger (1979). They reported that the antifungal mechanism of chitosan must resemble the mechanism described for plant cells, i.e., a polycationic effect that interferes with the membrane (Young et al., 1982).

Young et al. (1982), studied the effect of chitosan on the membrane permeability of plant cells. Treatment of plant cells with chitosan resulted in an increase in membrane permeability, as shown by leakage of electrolytes, protein, and UV absorbing materials. This effect, they concluded, was clearly due to the polycationic nature of chitosan. The effect of the chitosan was inhibited by divalent cations. An increase in membrane permeability would be expected in the presence of soluble chitosan, or when chitosan on the fungal cell wall surface makes contact with the host cell membrane. The cell wall may help to protect the plasma membrane from such effects by binding to chitosan, although its effectiveness as a natural barrier would be limited by its binding capacity. It seems possible that interactions of chitosan with membrane or cell wall components of plants might be involved in its ability to elicit phytoalexin synthesis, to induce lignification and to inhibit fungal growth. In contrast, increased permeability of the cell wall could also facilitate infection by enhancing the flow of nutrients to the pathogen and possibly enabling fungal products to enter host cells. This is an interesting dilemma and should be considered carefully when applying chitosan to plants.

Chitosan can activate specific genes in plants and, at similar concentrations, inhibit all RNA synthesis in some fungal organisms and thus suppress gene activity (Hadwinger, 1985). This phenomenon is explained by the interaction of chitosan's positively

charged amino groups and the negatively charged phosphate groups of nucleic acids. The chitosan must be 7 or more sugar units in length to optimally induce plant genes and to inhibit fungal growth (Kendra & Hadwinger, 1984). This length requirement suggests that a series of positive charges match up with phosphate negative charges in the grooves of the DNA helix in the form. Using radio-labeled chitosan, chitosan was applied to fungal cells in concentrations sufficient to inhibit their growth. DNA was found to precipitate into solution. Hirano and Nagao(1989) reported that strong growth inhibition was observed for low molecular weight chitosan but only weak inhibition was observed with high molecular weight chitosan. The effect of chitosan preparations with different levels of deactylation and other polyanions on the growth of post harvest pathogens was investigated by El Ghaouth et al. (1992 a and b). Chitosan markedly reduced the radial growth of all fungi tested, with a greater effect being observed at higher concentrations. Chitosan's inhibitory activity appeared to increase with increasing levels of deacetylation. In addition to inducing cellular leakage of amino acids and proteins in *Botrytus cinerea* and *Rhizopus stolinifer*, chitosan also induced morphological changes in *R.stolinifer*. An ultrastructural study, using electron microscopy, showed that chitosan caused deep erosion of the cell wall as well as increasing cell wall thickness. Chitosan was also found to stimulate the activity of chitin deacetylase, the enzyme involved in the biosynthesis of chitosan. This increase in activity of chitin deacetylase could upset the balance between biosynthesis turnover of chitin thereby rendering the cell wall more intact.

1.7.6.2.4. Control of Toxigenic Fungi

Toxigenic fungi are often found in pre-harvest crops, such as maize and peanuts, in association with other fungi, yeasts and bacteria. *Aspergillus parasiticus* and *Aspergillus flavus* are two such toxigenic fungi, both producing aflatoxin. Numerous attempts have been made to control pre-harvest aflatoxin production by limiting growth of toxigenic fungi through application of chemical or microbial agents with limited success. It has been reported that chitosan exhibits extensive inhibitory effects on *A.flavus* growth and/or aflatoxin production in liquid culture and in preharvest maize and groundnuts. In a study by Cuero et al. (1991) chitosan was used alone or in combination with microbial agents to inhibit growth of aflatoxin-producing fungal species and to control aflatoxin production in pre-harvest maize. They found that the growth of and aflatoxin production by *A.flavus* were significantly reduced in chitosan

treated pre-harvest maize. A single component treatment was found to be more effective than combination treatments. Fang et al. (1994) studied the inhibitory effects of chitosan on the growth of *Aspergillus niger* and the aflatoxin production of *A.parasiticus*. The inhibitory effect of chitosan on *A.niger* was increased as the chitosan concentration increased from 0.1 to 5.0 mg/ml at a pH of 5.4. The greatest inhibitory effect of chitosan of *A.parasiticus* was found at 3.0-5.0 mg/ml. In addition chitosan could completely prevent aflatoxin production at these concentrations.

1.7.6.2.5. Control of Senescence and Ripening

The effect of chitosan on respiration, ethylene production and quality attributes of tomatoes stored at 20°C was investigated by El Ghaouth et al. (1992a). Coating the tomatoes with chitosan solutions reduced their respiration rates and ethylene production, with the greatest effect being observed with 2%(w/v) chitosan solutions. Chitosan was also used as a dipping solution to extend the shelf life of fresh strawberries to approximately 30 days at 13°C (El-Ghaouth et al., 1991).

1.7.6.2.6. Control of Food Spoilage/Pathogenic Bacteria

In Japan, several patents have been issued on the use of chitosan as a food preservative to extend the shelf life of foods. Reports have shown that chitosan can inhibit the growth of *S.aureus, Escherichia coli, Pseudomonas aeruginosa and Bacillus subtilus*. Although no antibacterial mechanism has been elucidated, it has been shown that chitosan is only effective against certain bacteria and under certain conditions. Wang (1992) studied the inhibition of five species of food borne pathogens: *S.aureus, E. coli, Y.enterocolitica, L.monocytogenes,* and *Salmonella typhimurium*. Nutrient broths were supplemented with 0.5, 1.0, 1.5, 2.0 and 2.5% chitosan and adjusted to either pH 5.5 or 6.5. It was shown that the antibacterial action of chitosan was greatest on *S.aureus* followed by *S.typhimurium, E.coli,* and *Y.enterocolitica* at pH 6.5. At this pH, *L.monocytogenes* was not inhibited. However, at pH 5.5, the outgrowth of all bacteria was inhibited. Wang (1992) concluded that it was difficult to predict the chitosan concentrations actually needed to inhibit bacteria due to many factors, such as the character of the chitosan used, pH, water activity, storage temperature of the food

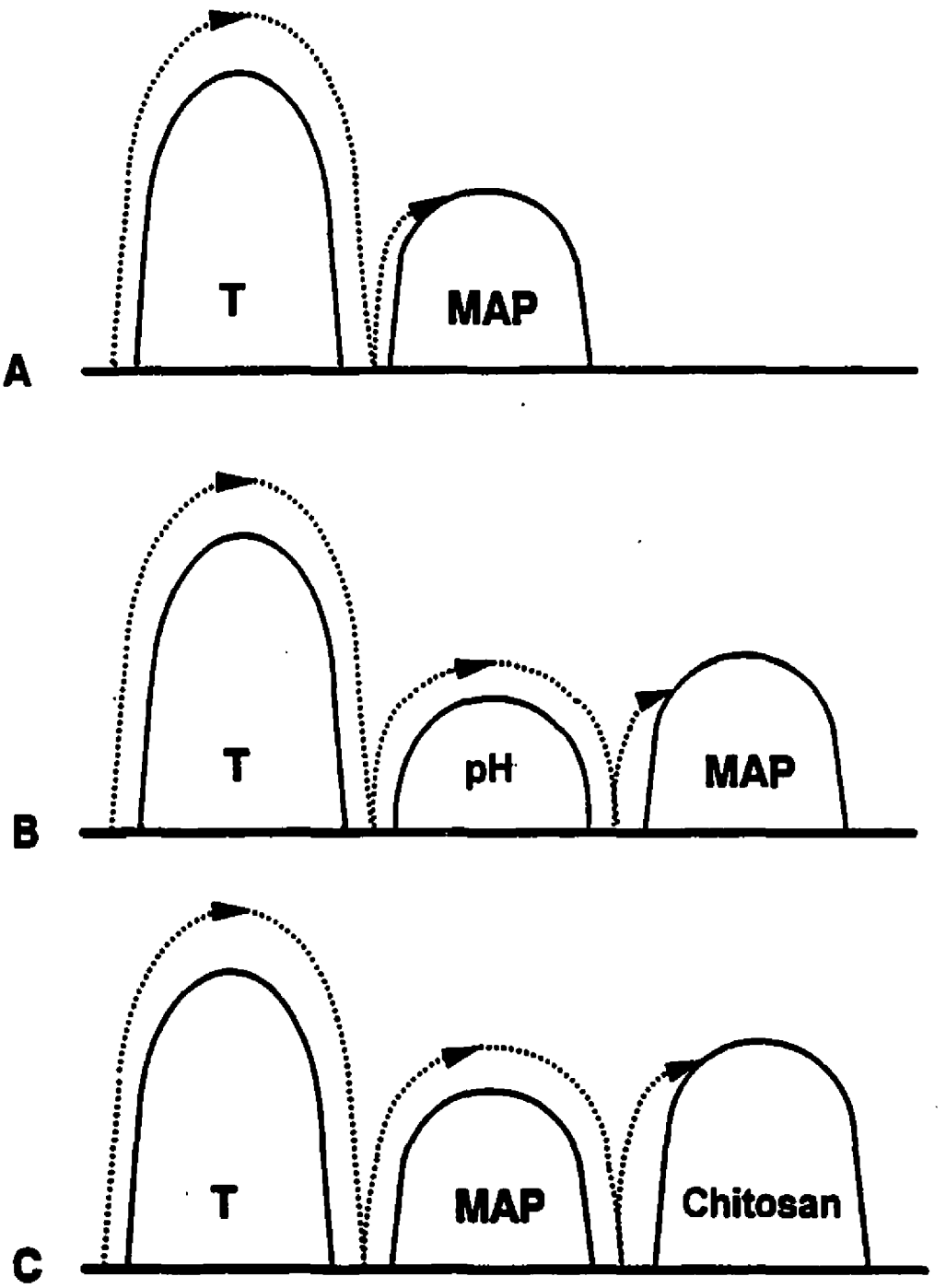
as well as the initial degree of contamination of the food. All of these factors can influence the effectiveness of chitosan as an inhibitor either alone or in conjunction with one another.

In another recent investigation, the effect of chitosan on meat preservation was studied (Darmadji and Izuminoto, 1994). In liquid medium, 0.01% chitosan inhibited the growth of spoilage bacteria e.g., *B.subtilis*, *E.coli*, *Pseudomonas fragi* and *S.aureus*. At higher concentrations (0.1% and 1.0%), it inhibited the growth of the meat starter cultures *Lactobacillus plantarium*, *Pediococcus pentosaceus* and *Micrococcus varians*. In beef, stored at 30°C for 48 hours or at 4°C for 10 days, 0.5 to 1.0% chitosan inhibited the growth of spoilage bacteria, reduced lipid oxidation and putrefaction and resulted in improved sensory qualities of beef. Chitosan also had a beneficial effect on the development of the red beef color during storage (Darmadji and Izuminoto, 1994).

1.8. Hurdle Approach to Food Safety

Also called combined methods, combined processes, combination preservation, combination techniques or barrier technology, the concept of the hurdle approach to food safety/preservation was developed several years ago as a comprehensive strategy for the production of safe, stable nutritious, tasty and economical foods. This technique deliberately combines existing and innovative methods in order to establish a series of preservative factors, hurdles, that any microorganisms present should not be able to overcome. These hurdles may be temperature, pH, water activity, redox potential, preservatives, MAP, irradiation, dipping solutions etc... The microorganisms must make a specific amount of effort to overcome each hurdle, the "larger" the hurdle, the greater the effort the microorganism must make. Because of their combined or synergistic effect, the individual hurdles may be set at lower levels than would be required if only a single hurdle was used as a preservation technique. Any hurdles that are included in a food should affect the microorganisms present in several different ways, e.g., by affecting the cell membranes' DNA, enzymes, internal pH etc. (Baker, 1989; Leistner and Gorris,1995). Examples of the hurdle approach to food safety are shown in Figure 6.

Figure 6: The hurdle approach to food safety and shelf-life extension (A) Combination of low temperature (T) and MAP, (B) combination of low temperature (T), pH and MAP, (C) combination of low temperature (T), MAP and Chitosan



CHAPTER 2
CHITOSAN STUDIES

2.1. INTRODUCTION

The effects of various antimicrobial agents have been studied for their inhibitory action against *L.monocytogenes* in model systems. However, the effect of chitosan and environmental factors such as pH, temperature and gaseous atmospheres on the growth of *L.monocytogenes* has not been studied extensively. In this preliminary study, the antimicrobial effect of various concentrations of chitosan on the growth of *L.monocytogenes* and *Listeria innocua* was investigated in a model broth and agar systems adjusted to various pH levels and stored at various temperatures prior to its application in pork.

Objectives:

The objectives of this study were:

(i) To construct a growth curve for *L.monocytogenes* in a model broth system at various storage temperatures;

(ii) To determine the effect of a non-water soluble chitosan on *L.monocytogenes* and *L.innocua*;

(iii) To determine the effect of pH and concentration of water soluble chitosan on the growth of *L.monocytogenes* in a model broth system;

(iv) To determine the level of chitosan needed to inhibit *L.monocytogenes* in a model agar system;

(v) To determine the optimum combination of temperature, pH and chitosan concentration for inhibition of *L.monocytogenes*.

Based on these studies, the level of chitosan as a potential dipping solution for fresh pork loin was determined.

2.2 MATERIALS AND METHODS

2.2.1. Microorganisms and Inoculum Preparation

L.monocytogenes Scott A (human isolate), and *L.innocua,* were obtained from the Microbial Hazards Bureau (Health Protection Branch, Health and Welfare Canada, Ottawa) from the culture collection of Dr.J.Farber. Cultures were maintained on tryptic soy agar (TSA) slants (Difco, Michigan, USA) at 4°C and were transferred monthly to ensure viability. The inoculum was prepared by transferring isolated colonies of *L.monocytogenes* Scott A from TSA into 5 ml of tryptic soy broth with 0.6% yeast extract (TSBYE, Difco) and incubating at 30°C for 24 hours to give a working suspension of $2x10^9$ cfu/ml. Appropriate dilutions were made in 0.1% peptone broth (Difco) to give an inoculum level of $2x10^6$cfu/ml for all the studies as described below.

2.2.2. Growth Curve

Experimental flasks were filled with 99 ml of tryptic soy broth and sterilized by autoclaving (STM-E, Sterilmatic, Market Forge Co., Everett Mass.) at 15 psi for 15 minutes (i.e., 121°C) and then cooled. Upon cooling, 1 ml of the working dilution of *L.monocytogenes* was asceptically transferred into each flask to give an initial inoculum level of $2x10^2$ cfu/ml. The flasks (three at each temperature) were stored at 5, 10 and 15°C and monitored for growth at regular intervals for up to 42 days. Counts were made at each sampling time in duplicate by spread plating 0.2 ml of the samples on tryptose soya agar (Difco). All plates were incubated at 30°C and the colonies were enumerated after 24 hours.

2.2.3. Antilisterial Activity of a Non-Water Soluble Chitosan

2.2.3.1. Preparation of Chitosan

A non-water soluble chitosan (Sigma, USA) was prepared according to the method of Allan and Hadwinger (1979) by dissolving it in 0.1N acetic acid and stirring for approximately 30 hours. This solution was then centrifuged at 10,000 rpm for 10 minutes at 5°C using a Beckman centrifuge (model #J2-21, Beckman). The supernatant was neutralized with 1N NaOH until a precipitate formed. The precipitate

was then washed with distilled deionized water 10 times and then freeze dried using a Labconco Freeze drier (Lyph-lok 18, Labconco, Kansas, Missouri) for 48 hours. The dried chitosan was then ground into a fine powder and dissolved in distilled deionized water to give a 1% working solution.

2.2.3.2. Preparation Growth Media

Experimental flasks were filled with appropriate amounts of tryptose soya broth (Difco) and the 1% stock solution of chitosan (prepared as described previously) giving a total volume of 100 ml in each flask and the following concentrations of chitosan: 0, 0.05, 0.10, 0.15 and 0.20%. The flasks were then autoclaved as described previously and cooled. The pH was monitored in control flasks using a previously calibrated pH meter (Model 220, Corning Glass Works, Corning, NY) with a gel filled polymer body combination electrode with Ag/AgCl reference (Fisher Scientific, model 13-620-104). The pH of all the broths was 7.0±.1. The flasks were then inoculated with either *L.monocytogenes* Scott A or *L.innocua* to give an initial inoculum level of $2x10^4$ cfu/ml. All flasks were incubated at 30°C. Counts were enumerated in duplicate on TSA plates using the spread plate technique at regular intervals throughout a 30 day storage period. All plates were incubated at 30°C.

Since the solubilization of the non-water soluble chitosan was quite time consuming, a water soluble chitosan, "chitosan hydrochloride" (Pronova Biopolymers Inc., Raymond, Washington, USA) was used in all further studies.

2.2.4. Effect of pH on the Antilisterial Activity of Chitosan

In this study, a water soluble chitosan powder (Pronova Biopolymers Inc., Raymond, Washington, USA) was used. Chitosan was added directly to the tryptic soy broth medium to give concentrations ranging from 0 to 0.20% (w/v). The pH of the broths was adjusted to 5, 6 and 7 using food-grade lactic acid or 1 M NaOH. The pH was measured using a previously calibrated pH meter (Model 220, Corning Glass Works, Corning, NY) with a gel filled polymer body combination electrode with Ag/AgCl reference (Fischer Scientific, model 13-620-104). The broths were autoclaved and cooled as described previously. The pH of control samples was monitored after autoclaving. The final pH of the broths were the same as the pH values obtained prior

to autoclaving (i.e., 5.0, 6.0 and 7.0 $\pm$0.1). Inoculation with *L.monocytogenes*, incubation and enumeration of broths over a 30 day storage period were as described in 2.2.2.

2.2.5. Antilisterial Effect of Chitosan in an Agar System

Chitosan hydrochloride was incorporated into TSA at concentrations of 0, 0.05, 0.10, 0.15 and 0.20% prior to autoclaving. The pH of the agar medium was adjusted to 6 or 7 using food grade lactic acid or 1M NaOH. The agar was autoclaved at 15 psi for 15 minutes (i.e., 121^{o}C), cooled to 50^{o}C, and then poured into petri dishes to give plates of uniform agar thickness (1 cm). This thickness was used to ensure that the water activity (a_w) of the media did not decrease significantly over prolonged storage and therefore interfere with growth. All plates were inoculated with 0.1 ml of a working suspension of $2x10^5$ cfu/ml *L.monocytogenes* using the spread plate technique to give a final inoculum level of $2x10^4$cfu/ml/plate. The plates were stored at 2^{o}C, and at 10 and 15^{o}C, i.e. conditions of mild temperature abuse, and monitored daily for visible signs of growth.

2.3. RESULTS AND DISCUSSION

2.3.1. Growth Curve of *L.monocytogenes*

Initial growth curves for *L.monocytogenes* in tryptose soya broth (TSB) at 5, 10 and 15°C are shown in Figure 7. The exponential growth phase of *L.monocytogenes* is faster at both 10 and 15°C, with numbers reaching 10^9 cfu/ml within 7 days. At 5°C, similar counts were reached after 20 days. At 5°C, *L.monocytogenes* reached the stationary phase at day 21, compared to day 7 or 8 for broths stored at 10 and 15°C respectively. The stationary phase lasted for approximately 35 days at both 10 and 15°C, while at 5°C the death phase commenced after only 9 days in the stationary phase. Further incubation studies showed that at 35°C (results not shown), counts of *L.monocytogenes* reached 10^9 cfu/ml within 18 hours. This study illustrates that *L.monocytogenes* has an optimum growth temperature of about 35°C. However, these results clearly indicate that *L.monocytogenes* is able to grow at low temperatures, i.e., it is a psychrotrophic pathogen, or at mild temperature abuse conditions. Therefore, low temperature alone cannot be relied upon to control the growth of *L.monocytogenes*. Alternative or additional methods of control in conjunction with low temperature must be used to control the growth of this food borne pathogen.

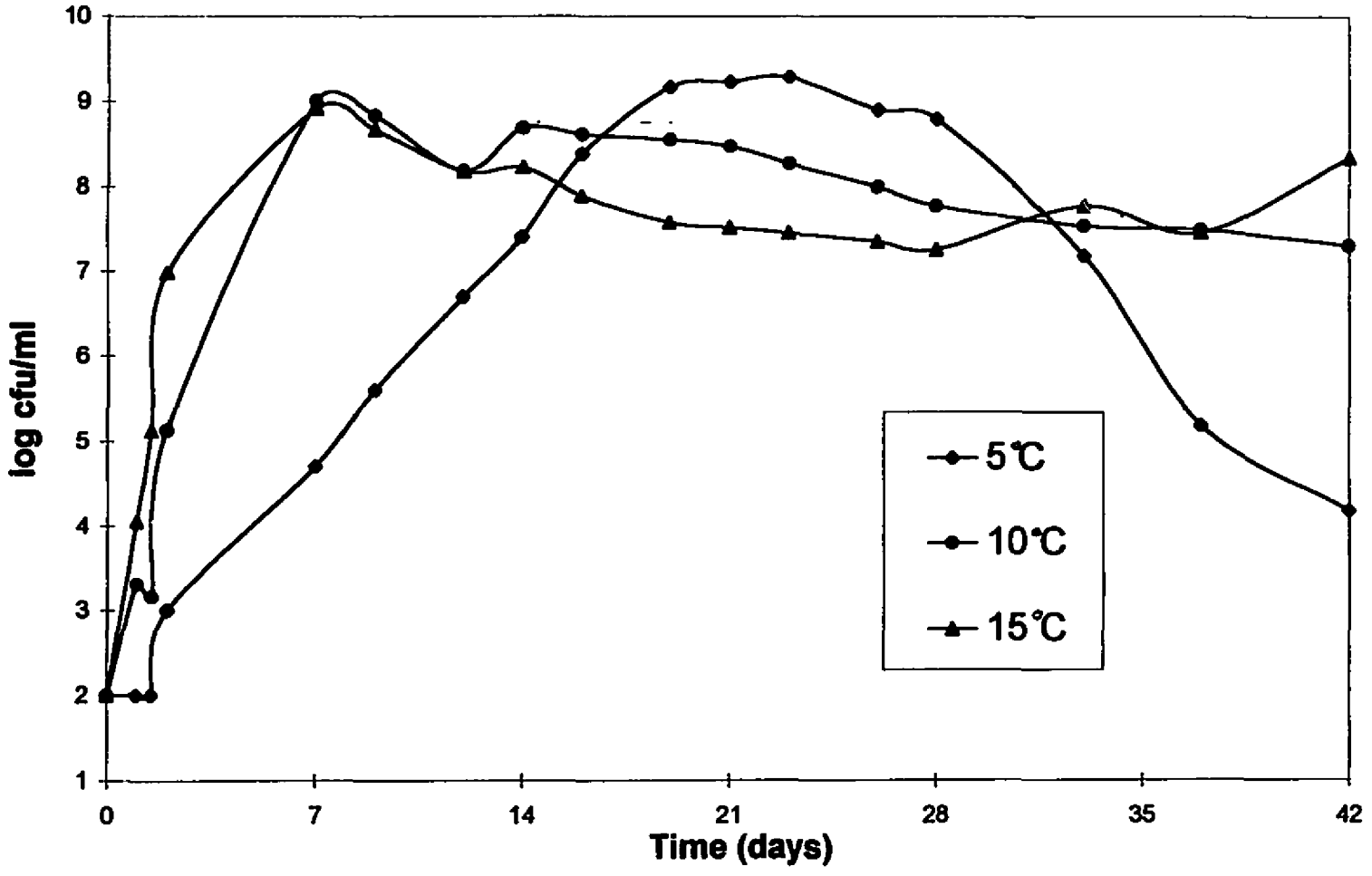


Figure 7: Growth curve of *Listeria monocytogenes* in tryptose soya broth at 5, 10 and 15°C

2.3.2. Antilisterial Activity of a Non-Water Soluble Chitosan

The effects of various concentrations (0-0.2% w/v) of non-water soluble chitosan on the growth of *L.monocytogenes* and *L.innocua* in tryptose soya broth at pH 7 and incubated at 30°C are shown in Figures 8 and 9 respectively. After 1-2 days, counts of *L.monocytogenes* reached 10^{10} cfu/ml in the control sample (0% chitosan), 10^9 cfu/ml in broths containing 0.05-0.15% chitosan and approximately 10^7cfu/ml in broths containing 0.2% chitosan. However, after 2 days counts began to decrease in all treatments. In control broths, counts decreased rapidly to approximately 10^5cfu/ml after 10 days and then began to gradually increase throughout the storage period reaching approximately 10^7 cfu/ml after day 30. Counts decreased more slowly in all broths containing chitosan and did not increase again throughout the storage period, indicating a possible residual antimicrobial effect of chitosan. A 3-4 log reduction in *L.monocytogenes* was observed in broths containing 0.2% chitosan after 5 days, and throughout the storage period (Figure 8).After 15 days, counts in broths containing 0.05% chitosan were similar to counts of control broths while in broths containing 0.1, 0.15 and 0.2% chitosan, there was a 1.5 to 2 log reduction in counts relative to the control.

Similar, but less dramatic trends, were observed for *L.innocua*, i.e., all counts increased to approximately 10^9 cfu/ml in all broths and then decreased gradually throughout storage. This decrease was most pronounced in broths containing 0.15 and 0.2 % chitosan. However, unlike *L.monocytogenes*, counts began to increase again throughout the storage period. At the stationary phase counts of 10^9cfu/ml were observed for control samples along with samples containing 0.05 and 0.1% chitosan. At the 0.15% chitosan level, a 3 log reduction was observed after 8 days, with numbers of *L.innocua* increasing after this point to 10^8 cfu/ml by the end of storage. At 0.2% chitosan, a 5 log reduction was observed after 8 days, followed by a slight increase in numbers and then reaching a 3 log reduction by the end of the storage period. This study shows that both *L.monocytogenes* and *L.innocua* would appear to exhibit a biphasic growth pattern in the presence of a non-water soluble chitosan, especially at lower concentrations of chitosan. It can be concluded from this study that levels of 0.2% or higher of non-water soluble chitosan are required to give moderate control of the growth of *L.monocytogenes* and *L.innocua* in tryptose soya broth at pH 7 for 30 days. Wang (1992) observed that *L.monocytogenes* was not inhibited by 2.5% chitosan

at pH 6.5 but was completely inhibited at pH 5.5 with chitosan concentrations of 0.5 to 2.5%. The differences in results may be attributed to the nature of the chitosan used and differences in environmental storage conditions of this study.

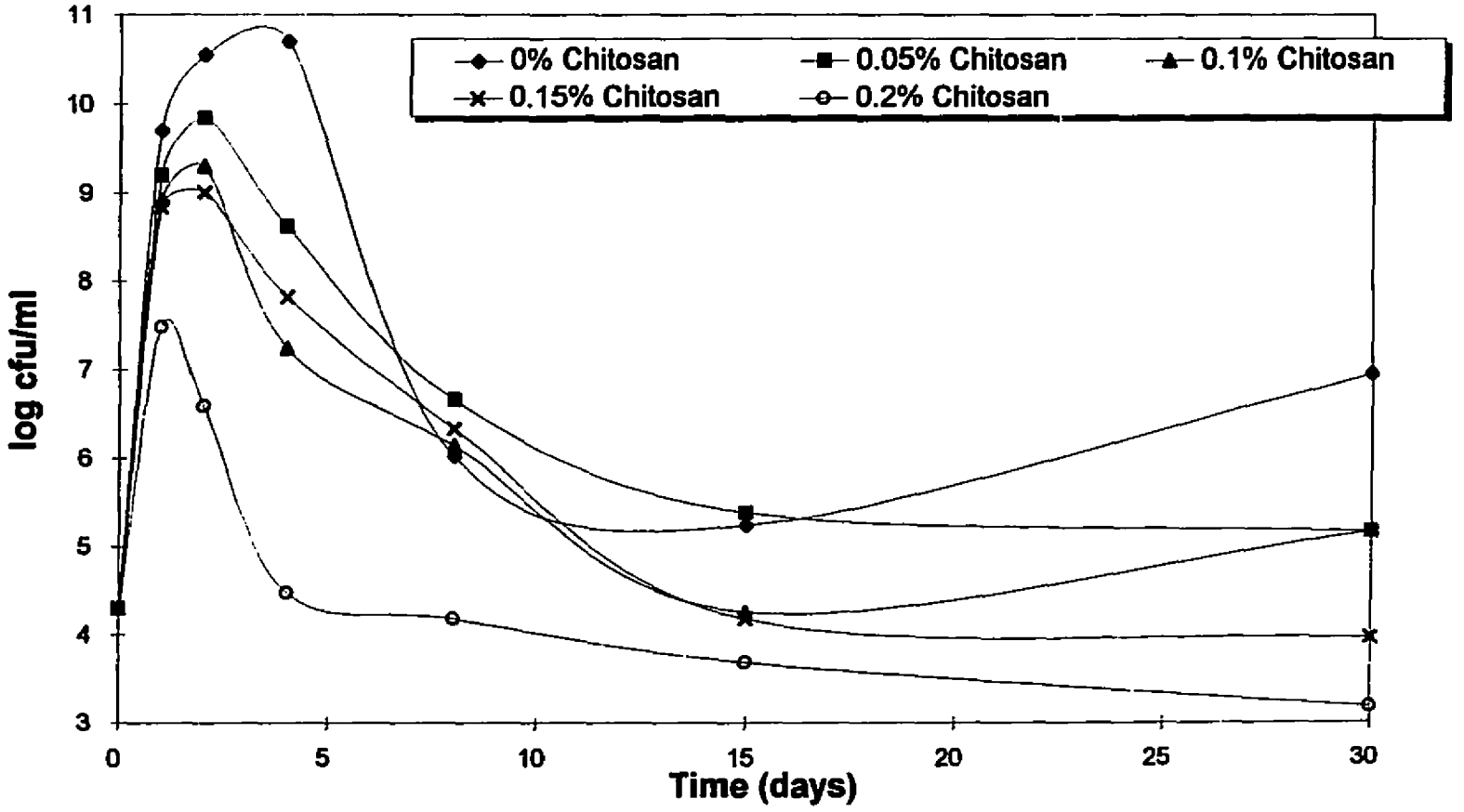


Figure 8: Effect of Non-Water Soluble Chitosan on *Listeria monocytogenes* incubated at 30°C

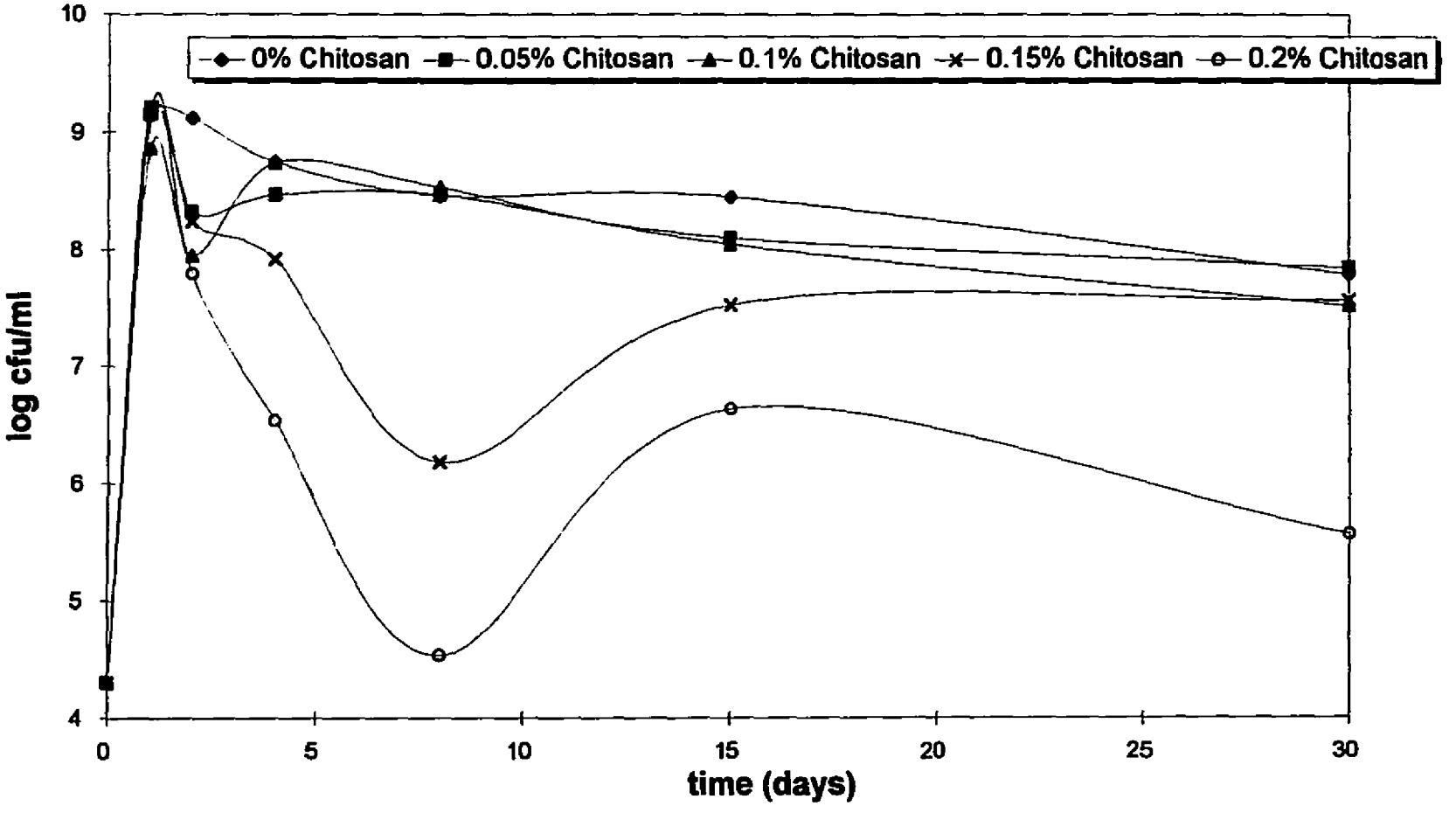


Figure 9: Effect of non-water soluble chitosan on *Listeria innocua* incubated at 30°C

2.3.3. Effect of pH on the Antilisterial Activity of Chitosan

The initial study on the effect of various concentrations of chitosan was carried out at pH 7 only. The intent of this study was to determine the effect of a range of pH values (5-7) on the antilisterial activity of a water soluble chitosan. The results of these studies are shown in Figures 10 and 11. Complete inhibition of *L.monocytogenes* was observed at pH 5 in control broths and at all chitosan concentrations, indicating that the inhibition was due to a pH effect and not to the presence of chitosan (results not shown). At pH 6 and 7, growth of *L.monocytogenes* was observed in control broths. Counts were approximately 1 log cycle less at pH 6 compared to pH 7, indicating a slight pH effect on growth. At pH 6, 0.05% chitosan and 0.1% chitosan increased the lag phase of *L.monocytogenes*. Only at higher concentrations (0.15 and 0.2%) was complete inhibition observed at pH 6.

At pH 7 (Figure 10) growth occurred at all chitosan concentrations tested. Counts increased from the initial inoculum level by 4 log cycles in broths containing 0.05% chitosan, by 3 log cycles in broths containing 0.1% chitosan and by 1.5 log cycles in broths containing 0.15 and 0.2% chitosan. In broths containing 0.2% chitosan, a 3 log reduction occurred by the third day of storage with numbers decreasing to $< 10^1$ cfu/ml by the end of the 30 day storage period. After 30 days of storage, samples at pH 6 had reached the death phase faster than those at pH 7. This study clearly shows that the antimicrobial activity of chitosan is pH dependent and that it acts in a similar manner to organic acids, i.e., sorbic acid, propionic acid. Again, Wang (1992) noticed a similar pH effect on the antilisterial activity of chitosan, with complete inhibition occurring at pH 5.5 in broths containing 0.5 to 2.5% chitosan. However, this author did observe growth in control broths at pH 5.5. For example, while counts of *L.monocytogenes* reached 10^7/cfu/ml after 8 days in control broths, these levels were not reached until day 15 in broths containing 0.05% chitosan. At slightly higher levels (0.1% chitosan) counts reached 10^7 only after 28 days.

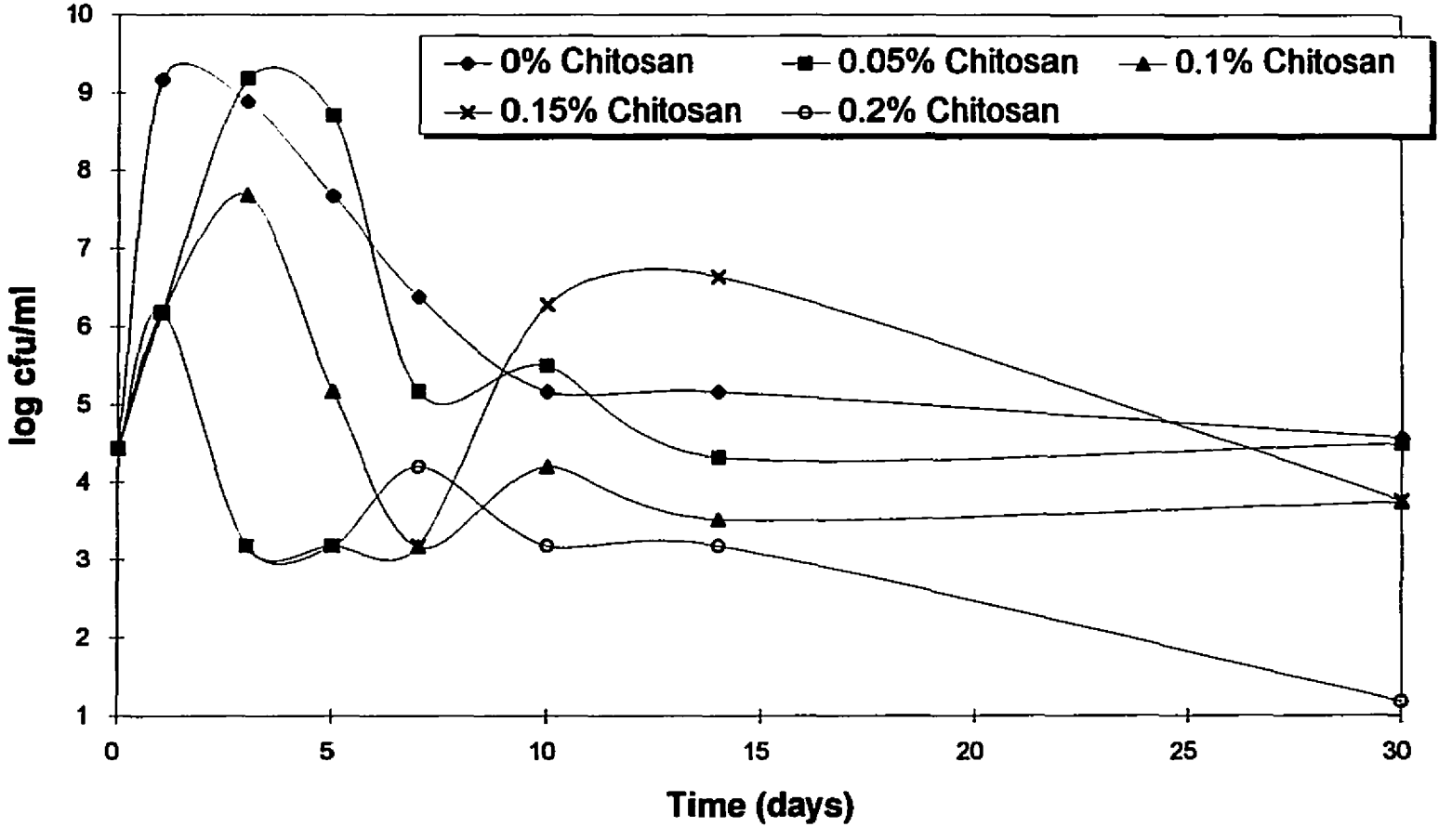


Figure 10: Effect of chitosan hydrochloride (pH 7) on the growth of *L.monocytogenes* incubated at 30°C

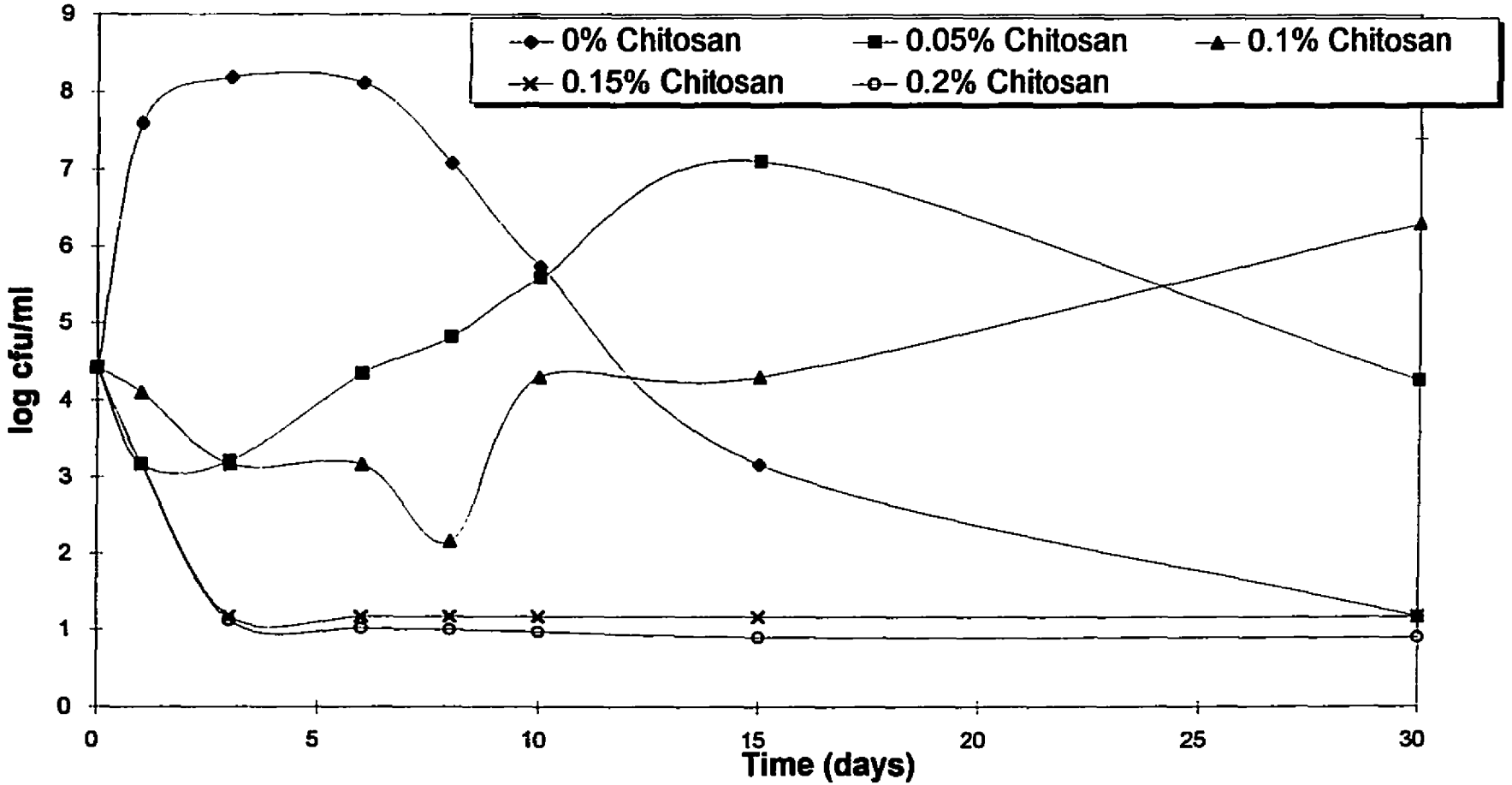


Figure 11: Effect of chitosan hydrochloride (pH 6) on the growth of *L.monocytogenes* incubated at 30°C

2.2.4. Antilisterial Effect of Chitosan in an Agar System

The combined effect of temperature, pH and chitosan concentration on the growth of *L.monocytogenes* was investigated in a model agar system (Figures 12 and 13). At 2°C and pH 6, growth of *L.monocytogenes* was inhibited for approximately 25 days in control plates. Addition of chitosan to plates delayed the onset of growth for a further 3 days at 2°C. At higher storage temperatures (10 and 15°C), colonies of *L.monocytogenes* were visible after 7 and 12 days respectively. At pH 6, a level of 0.2% chitosan was required to inhibit the growth of *L.monocytogenes* at 10 and 15°C for 23 and 19 days respectively compared to 23 and 7 days at pH 7 (Figures 12 and 13). Lower concentrations of chitosan did not inhibit *L.monocytogenes* in the model agar system to the same extent. Furthermore, the inhibition of *L.monocytogenes* is enhanced at lower storage temperatures, illustrating the importance of low storage temperature used in conjunction with this potential antimicrobial agent.

This study clearly indicates that chitosan has an antimicrobial effect in a solid medium on the growth of *L.monocytogenes*. This may be due to a specific antimicrobial effect of chitosan e.g., interference with cell membrane, or to a reduction in a_w caused by the dissolution of chitosan in the agar medium. Therefore, it could be added to a solid food substrate e.g. fermented meat products, ground meats and other minced or ground products where *L.monocytogenes* may pose a public health threat. Furthermore, the levels of chitosan used i.e., 0.15-0.2%, are comparable to levels of other preservatives used in foods e.g., sorbates, propionates. However, while chitosan is used as an antimicrobial agent in Japan, its GRAS status in North America is currently under scrutiny .

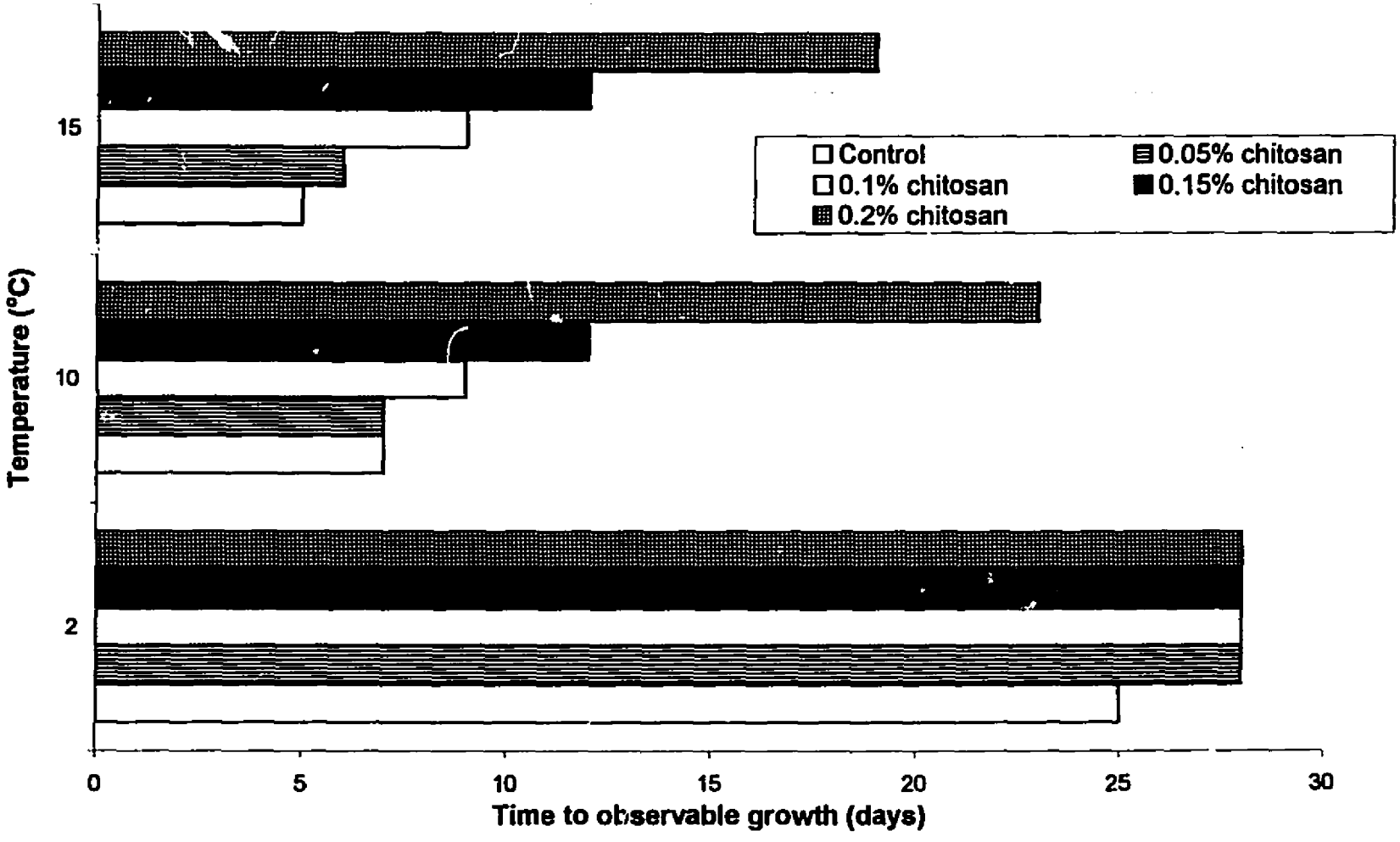


Figure 12: Effect of chitosan at pH 6 in a model agar system on the growth of *L.monocytogenes*

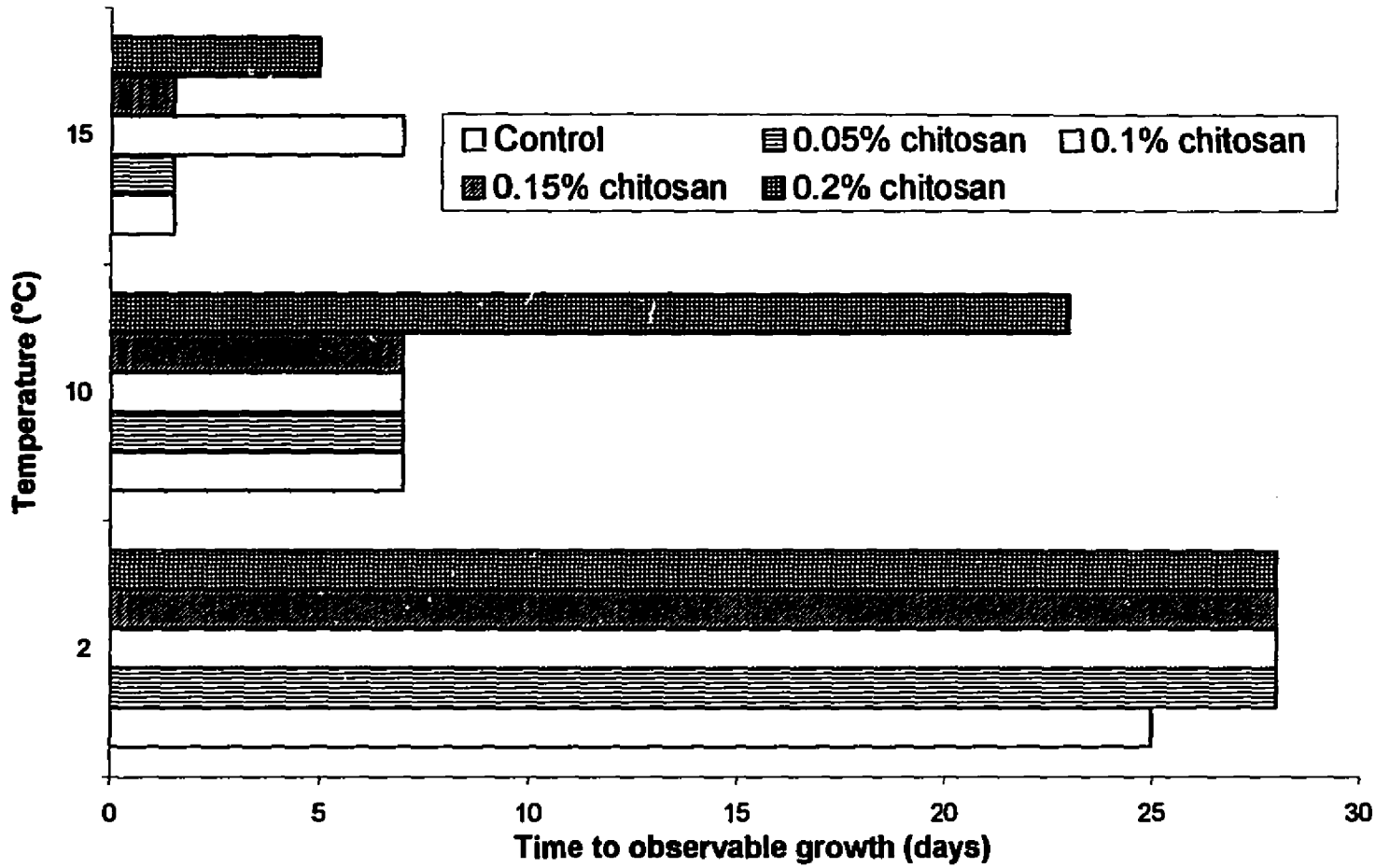


Figure 13: Effet of chitosan at pH 7 in a model agar system on the growth of *L.monocytogenes*

CHAPTER 3
MODIFIED ATMOSPHERE PACKAGING AND COMBINATION STUDIES

3.1. INTRODUCTION

There is a paucity of data available on the ability of *L.monocytogenes* to grow under modified atmospheres, especially in model systems. Furthermore, new methods of gas atmosphere modification involving oxygen absorbents and ethanol vapor generators are available on the market. Again, little is known about the effects of such methods of atmosphere modification to control the growth of *L.monocytogenes*.

Objectives

On the basis of the above comments, the objectives of this study were:

(i) To investigate the potential of various methods of atmosphere modification (gas packaging and oxygen absorbents) to control the growth of *L.monocytogenes* in a model agar system;

(ii)To determine the effect of MAP and chitosan in conjunction with each other on the growth of *L.monocytogenes* in a model agar system.

Based on these studies, an appropriate combination of chitosan and gas atmospheres could be investigated to extend the shelf life and safety of fresh pork.

3.2 MATERIALS AND METHODS

3.2.1. Microorganisms and Preparation of Inoculum

Storage, sub-culturing and preparation of *L.monocytogenes* inoculum was as described in section 2.2.1.

3.2.2. Effect of MAP on *Listeria monocytogenes*

To determine the effect of various gaseous conditions on the growth of *L.monocytogenes*, TSA plates were prepared as described previously in section 2.2.3, without the addition of chitosan. The pH of all plates was monitored. The plates were inoculated with $2x10^4$cfu/ml of *L.monocytogenes* as described previously and packaged in 210x210 mm Cryovac high barrier bags (Oxygen transmission rate: 3-6 cm^3/m^2/day at 4.4°C, 0%RH). Bags were flushed with levels of CO_2 ranging from 0 to 100% at 20% increments (balance N_2) using a Multivac chamber type, heat seal packaging machine (Model KM100-3M). A Smith's proportional gas mixer (Model 299-028, Tescom corp., Minneapolis, Minnesota) was used to give the desired proportions of gases in the package head space. In half of the packages, an oxygen absorbent sachet, Ageless type FX (Mitsubishi Gas Chemical Company Ltd., Japan) was incorporated prior to gas flushing to remove all traces of residual oxygen in the package head space. The gaseous combinations used in this study are shown in Table 5.

Table 5: Gas Mixtures used for 3.2.2.

Sample code (No Ageless)	$\%N_2$	$\%CO_2$	Sample code (With Ageless FX)
A	100	0	AF
B	80	20	BF
C	60	40	CF
D	40	60	DF
E	20	80	EF
F	0	100	FF

3.2.2.1. Headspace Gas Analysis

Samples were analyzed for changes in headspace gas at regular intervals throughout storage or until growth was observed. Gas samples were withdrawn using a 0.5 ml gas tight Pressure-lok syringe (Precision Sampling Corp., Baton Rouge, La.) through silicone seals attached to the package exterior. The gas samples were injected into a Varian gas chromatograph (Model 3400, Varian Canada Inc.) equipped with a thermal conductivity detector (TCD) and using Porapak Q (80-100 mesh) and Molecular Sieve 5A (80-100 mesh) columns in series (Supelco Canada Ltd). The carrier gas was helium set at a flow rate of 30 ml/min. The column oven was set at 80°C, the injector at 100°C and the detector filament at 150°C. Peaks were recorded using a Hewlett Packard integrator (Model 3390A, Hewlett Packard Co., Avondale, PA.).

3.2.3. Effect of Various Oxygen Absorbents on the Growth of *L.monocytogenes*

TSA plates (without chitosan) were inoculated with *L.monocytogenes* as described previously in 2.2.3. Plates were packaged in air in Cryovac bags each containing one of three types of oxygen absorbents: Ageless FX, Ageless SS and Ageless SE. All absorbents were obtained from the Mitsubishi Gas Chemical Company Ltd., Tokyo, Japan. The characteristics of each of these absorbents is summarized in Table 6. All packages were sealed with an impulse heat sealer and stored at 2°C, and at 10 and 15°C, i.e., conditions of mild temperature abuse. Plates were monitored daily for visible signs of growth.

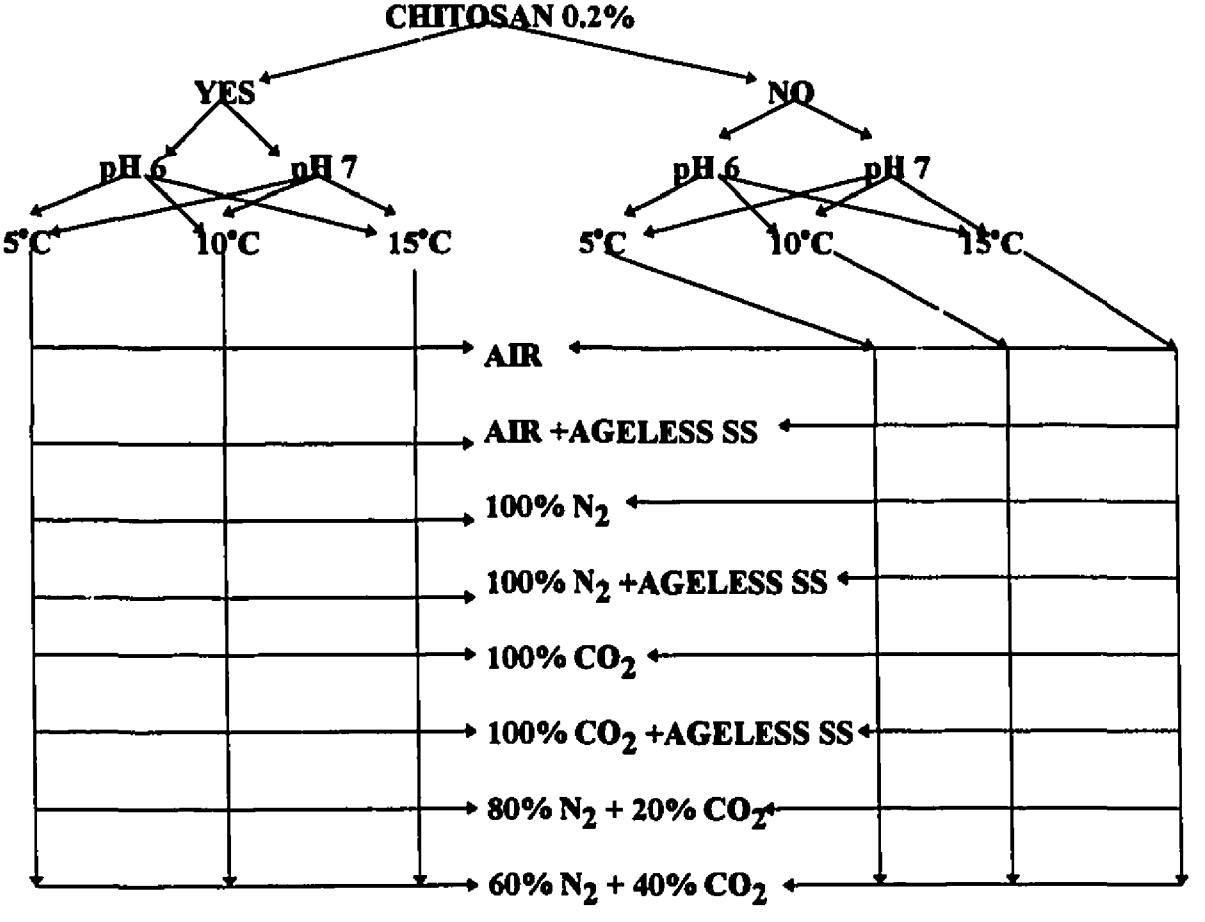
Table 6: Absorbents used in 3.2.3.

Absorbent type	Description
Ageless FX	Moisture dependent oxygen absorbent
Ageless SS	Self reacting oxygen absorbent, low temperature applications
Ageless SE	Moisture dependent oxygen absorbent and ethanol vapor generator

3.2.4. Combined Effects of Chitosan and MAP on *L.monocytogenes* in an Agar System

In this study, the combined effect of chitosan and MAP on the growth of *L.monocytogenes* was investigated in a model agar system. Modified Oxford agar (MOX) was obtained from Difco (Michigan, USA). The medium was prepared according to the manufacturer's directions, autoclaved, cooled to 50°C and poured into petri dishes. Half of the medium contained chitosan hydrochloride at a level of 0.2% added prior to autoclaving. The pH of the media was adjusted to 6.0 and 7.0 using 1M NaOH or food grade lactic acid. MOX was used in this study instead of TSA to distinguish growth since *L.monocytogenes* appears as black colonies on this medium due to the fermentation of esculin present. All MOX plates were inoculated with $2x10^4$ cfu/ml of *L.monocytogenes* by the spread plate technique and placed in Cryovac bags. Plates were packaged under various gas atmospheres as illustrated in Figure 14. Packaging was done as described in section 3.2.3.. All packages were stored at 5, 10 and 15°C and monitored for visible growth daily. Headspace gas analysis was done as described in section 3.2.3.1. when visible growth was observed.

Figure 14: Treatment scheme used for 3.2.4.



3.3. RESULTS AND DISCUSSION

3.3.1. Effect of MAP on *L.monocytogenes*

Initial studies were done to determine the effect of gas packaging, with and without an oxygen absorbent (Ageless FX), on the growth of *L.monocytogenes* on agar plates stored at mild temperature abuse conditions (Tables 7 and 8). The percentage of O_2 ,CO_2 and N_2 in the package headspace shown was the headspace gas composition of the packages when visible growth of *L.monocytogenes* was detected.

For plates packaged without an absorbent, colonies of *L.monocytogenes* were visible in control plates (air) after 7 days at 10°C. Low levels of CO_2 (approximately 20%) appeared to stimulate the growth of *L.monocytogenes,* with growth appearing within 5 days in all plates stored at 10°C. Several studies have reported the stimulatory effect of low levels of CO_2 on the growth of molds in cereals and lactic acid bacteria in vacuum and gas packaged beef (Lambert et al., 1991). More recently, Lambert at al. (1992) reported the stimulatory effect of low levels of CO_2 (20%) on the growth and toxin production by *Clostridium botulinum* in gas packaged pork.

When plates were packaged in higher CO_2 levels (approximately 40%) growth appeared at about the same time as control plates, while growth was delayed until day 9 for plates packaged in atmospheres of approximately >60% CO_2. For gas packaged plates without an absorbent, CO_2 levels of 70% or higher were required to inhibit the growth of *L.monocytogenes* at 10°C until 14 days. However, no additional control was achieved with CO_2 levels above 70%. Razavilar and Genigeorgis (1992) determined that 100% CO_2 was the most inhibitory modified atmosphere to delay the growth of *L.monocytogenes*. However, these authors did not compare other levels of CO_2.

Similar trends were observed for all gas packaged plates without an absorbent stored at 15°C. However, growth was detected within 3 to 5 days in all plates stored at 15°C, indicating once again the importance of low temperature to enhance the antimicrobial effect of CO_2 enriched atmospheres.

The combined effect of packaging in a CO_2 enriched atmosphere with an oxygen absorbent (Ageless FX) on the growth of *L.monocytogenes* at 10 and 15°C is shown in Tables 9 and 10 respectively. The addition of an oxygen absorbent delayed the growth of *L.monocytogenes* in control plates and in plates packaged with various levels of CO_2. This can be attributed to lower levels of O_2 in all packaged plates indicating an antimicrobial synergism between low O_2 and high CO_2 levels. However, similar trends were observed in this study with an absorbent, i.e., a stimulatory effect of lower CO_2 (20 to 40%) resulting in visible growth approximately 2 days prior to control plates and an inhibitory effect of higher CO_2 levels (60 to 100%) on the growth of *L.monocytogenes*. However, an interesting effect was observed for plates packaged in 100% N_2 with an oxygen absorbent and stored at 10 and 15°C. In these plates, growth was delayed for approximately the same time or longer than in plates packaged in 100% CO_2. This can be attributed to the low level of residual O_2 (i.e., $< 1\%$).

This initial study has shown that low levels of CO_2 (20 to 40%) appear to stimulate the growth of *L.monocytogenes*. For moderate control of *L.monocytogenes*, products need to be packaged in approximately 60-80% CO_2. For optimum control of *L.monocytogenes*, products need to be stored in either high CO_2 levels (70-100%) or very high N_2 levels (100%) in conjunction with an oxygen absorbent. To date, there have been no other studies carried out on the effects of oxygen absorbents on the growth of *L.monocytogenes*

Table 7: Effect of MAP on *L.monocytogenes* at 10^0C, no oxygen absorbent used

Sample	Time to visible Growth (days)	Headspace gas composition(%) CO_2	O_2	N_2
Control	7	15.2	17.3	67
A	8	.35	3.7	95.9
B	5	14.5	2.8	82.7
C	8	37	0.1	62.9
D	9	56	0.3	43.7
E	14	69	2.4	28.6
F	14	95.2	1.2	3.6

Table 8: Effect of MAP on *L.monocytogenes* at 15^0C, no oxygen absorbent used

Sample	Time to visible Growth (days)	Headspace gas composition(%) CO_2	O_2	N_2
Control	3	16.2	17.5	56.3
A	3	1.2	1.4	97.4
B	3	15.8	2.1	82.9
C	4	37.4	0.4	62.2
D	5	58.4	0.2	41.4
E	5	70.7	2.1	27.3
F	5	96.8	0.9	2.3

Table 9: Effect of MAP on *L.monocytogenes* at 10°C using an Ageless FX oxygen absorbent

Sample	Time to visible Growth (days)	Headspace gas composition(%) CO_2	O_2	N_2
Air+FX	12	25.5	0.3	74.2
AF	25	1.2	0.3	98.4
BF	10	8.4	0.4	91.2
CF	10	33.3	0.3	66.4
DF	15	42.5	0.2	57.3
EF	20	62.5	0.1	37.4
FF	25	94.2	0.1	5.7

Table 10: Effect of MAP on *L.monocytogenes* at 15°C using an Ageless FX oxygen absorbent

Sample	Time to visible Growth (days)	Headspace gas composition(%) CO_2	O_2	N_2
Air+FX	10	30.2	0.4	69.4
AF	18	1.4	0.3	98.3
BF	5	9.8	0.4	89.8
CF	6	35	0.3	64.7
DF	10	52.6	0.1	47.3
EF	12	75.2	0.2	24.6
FF	14	92.6	0.1	7.3

3.3.2. Effect of Various Oxygen Absorbents on the Growth of *L.monocytogenes*

The combined use of oxygen absorbents and CO_2 enriched atmospheres can be used effectively to control the growth of *L.monocytogenes*. However, this combination means additional costs to the processor. Therefore, preliminary studies were done to determine the effect of various oxygen absorbents alone to control the growth of this important pathogen. The effects of three oxygen absorbents, Ageless types FX, SS and SE and temperature on the growth of *L.monocytogenes* on TSA plates are shown in Table 11. In control plates, growth occurred within 25, 7 and 2 days at 2, 10 and 15^{o}C respectively. Addition of an oxygen absorbent, Ageless type SS delayed growth of *L.monocytogenes* for greater than 52 days at 2^{o}C. Ageless types SE and FX were not studied at this temperature (2^{o}C) since they are not designed to scavenge oxygen at refrigeration temperatures. However, the effects of oxygen absorbents on the growth of *L.monocytogenes* at higher temperatures (10 and 15^{o}C) is evident. Growth was inhibited for approximately 23 to 30 days with all absorbents stored at 10^{o}C. At higher storage temperatures, growth of *L.monocytogenes* was delayed for 12 days using Ageless FX and for 20 and 30 days using Ageless SS and SE respectively. The greater effect of Ageless type SE at higher storage temperatures can be attributed to the fact that this dual functional absorber not only absorbs oxygen but also generates ethanol vapor. While the level of ethanol vapor generated was not measured in this study, approximately 1% ethanol vapor is generated by Ageless SE (Mitsubishi Gas Chemical Corporation, Personal Communication). This study has shown that the use of absorbents alone can give similar or greater control over the growth of *L.monocytogenes* compared to combination of elevated levels of CO_2 and oxygen absorbents. The use of oxygen absorbents alone is therefore a simple, cost effective method of controlling the growth of *L.monocytogenes* even at mild temperature abuse conditions.

Table 11: Effect of Various Oxygen Absorbents on *L.monocytogenes*

Absorbent Type	Temperature (°C)		
	2	10	15
Air-Control	25*	7	2
Ageless FX	N/A	30	12
Ageless SS	>52	23	20
Ageless SE	N/A	30	30

* Values represent time to visible growth in days and are the means of three replicates

3.3.3. The Combined Effects of Chitosan, pH and MAP on *L.monocytogenes* in an Agar System.

To determine the effect of various hurdles or barriers on the growth of *L.monocytogenes*, studies were done on MOX agar plates at pH 6 and 7, with and without 0.2% chitosan in MOX agar and packaged under various modified atmospheres. The levels of pH and chitosan in the agar media and the packaging atmospheres used were selected on the basis of previous studies. The results of the combined effects of chitosan, pH and MAP are summarized in Table 12. Growth of *L.monocytogenes* was observed on air packaged plates within 3 to 12 days and within 3 to 14 days for plates packaged in 100% N_2 depending on pH and storage temperature. Packaging in 100% CO_2 delayed growth for 5 to 39 days with the greatest inhibitory effect being observed for plates stored at 5°C, i.e. temperatures at which CO_2 has a strong antimicrobial effect (Finne, 1982). Packaging in mixtures of gases, i.e. 20 to 40 % CO_2 , balance N_2, had little effect on *L.monocytogenes* with growth occurring at approximately the same time as plates packaged in air (control) or 100% N_2. These results confirm earlier work which showed that elevated levels of CO_2 (80 to 100%) were necessary to delay the growth of *L.monocytogenes* for an extended period of time. The addition of an oxygen absorbent (Ageless SS) to plates packaged in air, 100% N_2 or 100% CO_2 had a pronounced effect on the growth of *L.monocytogenes* (Table 12). These results confirm the role that residual oxygen may play on the growth of *L.monocytogenes* in plates packaged under modified atmospheres. There is a slight antimicrobial advantage gained when plates are packaged under 100% N_2 with an oxygen absorbent as opposed to air alone with an oxygen absorbent. The difference between the results of air packaged plates and those of plates packaged in 100%N_2 may be due to the scavenging rate of Ageless SS at low temperatures and the effect of the initial head space O_2 on the growth of *L.monocytogenes*.

The addition of chitosan (0.2%) to MOX agar plates resulted in approximately a 100% extension in time to visible growth of *L.monocytogenes* in plates packaged under modified atmospheres without an oxygen absorbent. The addition of an oxygen absorbent had a pronounced effect on the growth of *L.monocytogenes* (Table 12) particularly in air packaged plates with the growth being delayed for almost three months in plates stored at 5 and 10°C. However, at 15°C, the addition of 0.2% chitosan to plates stored under air and MAP conditions had little or no effect on the

time to visible growth. These results confirm previous studies which showed a synergistic effect between pH, chitosan, gaseous atmosphere and storage temperature on the growth of *L.monocytogenes*. Furthermore, the results confirm that an oxygen absorbent could be added to gas packaged products to reduce the residual O_2 level in the package headspace thereby enhancing the antimicrobial effect of modified atmosphere packaging.

Table 12: Effect of MAP, pH, Temperature and Chitosan on *L.monocytogenes*

	No Chitosan			0.2%Chitosan		
Sample	5°C	10°C	15°C	5°C	10°C	15°C
Air,pH6	12[a]	9	4	28	24	18
Air,pH7	11	7	3	28	23	7
Air+SS[b], pH6	30	14	12	82	75	14
Air+SS. pH7	45	12	10	80	75	12
100%N_2, pH6	14	11	6	31	27	20
100% N_2, pH7	11	8	3	28	22	8
100%N_2, pH6,+SS	56	56	14	111	82	28
100%N_2, pH7,+SS	56	25	18	111	40	23
100%CO_2, pH6	39	38	7	35	40	24
100%CO_2, pH7	28	14	5	32	31	9
100%CO_2, pH6+SS	82	50	19	111	82	25
100%CO_2, pH7, +SS	53	25	14	111	28	19
80%N_2,20%CO_2, pH6	16	14	5	26	25	19
80%N_2, 20%CO_2,pH7	14	5	3	25	21	7
60%N_2, 40%CO_2,pH6	13	12	3	17	18	6
60%N_2, 40%CO_2, pH7	10	8	4	12	10	5

a:Values represent time (days) to observable growth and are the means of three replicates

b: Ageless SS oxygen absorbent

CHAPTER 4
PORK CHALLENGE STUDIES

4.1. INTRODUCTION

MAP is a rapidly expanding technology being used for the preservation of foods including bakery products, meat, fish, poultry and snack food products (Smith et al, 1990). However, there is concern about the public health safety of these foods, particularly muscle foods, for the following reasons: (i) the normal aerobic spoilage microorganisms which would normally warn consumers of spoilage (by off odors and off colors) are inhibited, (ii) the growth of pathogens may be allowed or even stimulated, (iii) the emergence of psychrotrophic pathogens such as *L.monocytogenes* and (iv) the potential for temperature abuse (Farber, 1991). It is evident that refrigeration alone or in conjunction with MAP is inadequate and that additional barriers, such as chitosan, must be considered to prolong the shelf life and ensure the safety of MAP products, i.e., the hurdle concept. While minimal studies to date have been done on model agar/broth systems, challenge studies with *L.monocytogenes* in pork are essential to evaluate the public health safety of MAP pork subjected to mild temperature abuse storage conditions.

Objectives:

Based on previous results, the objectives of this study were:

(i) To determine the physical, chemical, microbiological and sensorial changes occurring in the packaged pork product.

(ii) To determine the effect of chitosan, MAP and storage temperature on the growth of *L.monocytogenes* in fresh pork loin;

4.2. MATERIALS AND METHODS

4.2.1. Microorganisms and Preparation of Inoculum:

Storage, sub-culturing and preparation of *L.monocytogenes* inoculum was as described in section 2.2.1

4.2.2. Preparation of Chitosan Dipping Solution

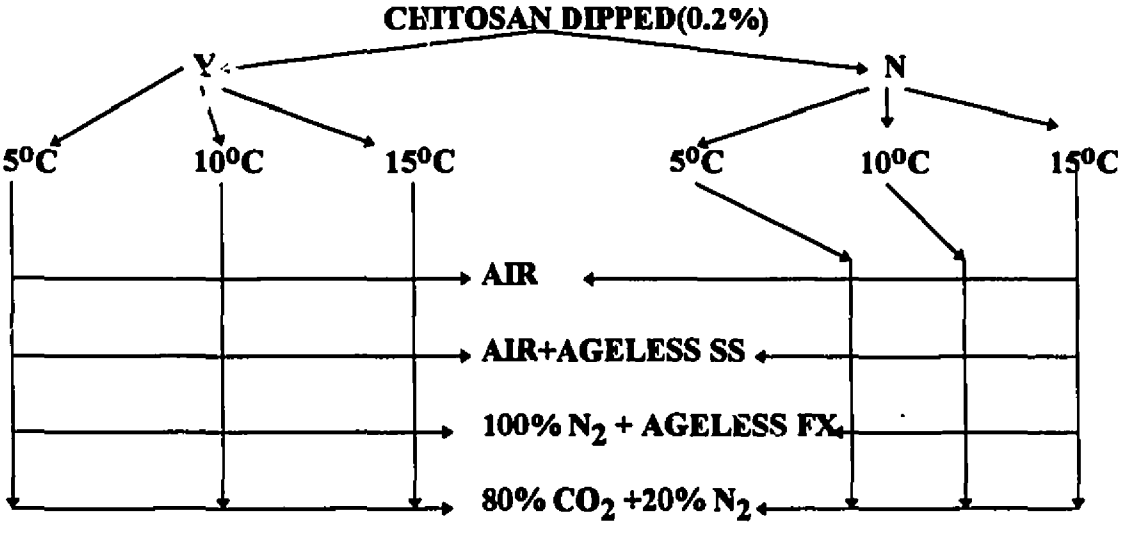
A 0.2% (w/v) dipping solution was prepared in 1 litre batches by adding 20g of chitosan hydrochloride powder to 980 ml of distilled deionised water. The dipping solution was stirred for one hour and the pH adjusted to 6.0 with 1.0M NaOH. The dipping solution was then autoclaved at 15 psi for 15 minutes (i.e., 121^{o}C) and then cooled. The pH was checked as described previously in section 2.2.3., and was aseptically readjusted to 6.0 if necessary with 1M NaOH or food grade lactic acid. The dipping solution was changed after dipping 10 pork samples. A control dipping solution of autoclaved distilled deionized water was used for control samples and this was also changed after every 10 samples.

4.2.3. Preparation of Pork

Fresh, trimmed, pork loins were obtained from "Les Viandes de l'Ile Perrot Inc.", (Ile Perrot, Quebec). The meat had been de-boned and pre-cut into samples of 100 g $\pm$ 10g loins. The samples were transported in a cooler to our laboratory at Macdonald Campus. Half of the pork samples were dipped in a 0.2% chitosan dipping solution (pH 6.0) for sixty seconds prior to inoculation, the remainder of the loins were dipped in a beaker containing distilled deionized water for 60 seconds. After dipping, the pork loin slices (one per bag) were placed in 210x210mm Cryovac barrier bags (OTR:3-6 cc/m^2/day at 4.4^{o}C and 0%RH). All samples were weighed in their bags prior to packaging and storage. The pork loins were then inoculated with 1.0 ml of a working suspension of *L.monocytogenes* (2x10^4 cfu/ml) at four different places on the meat sample to distribute the inoculum. The pork samples were then gently massaged in the bags to further distribute the cells. Sample preparation, dipping and inoculation were

carried out under a Purifier^{TM} Class II Safety Cabinet (Labconco, Model #36205-04, Labconco, Kansas, Missouri) equipped with a HEPA filter to ensure minimal contamination of samples and safety to the technicians. The samples were randomly assigned to one of the treatments as shown in Figure 15. Uninoculated control samples were also prepared in a similar manner. All treatments were carried out in triplicate.

Figure 15: Scheme of treatments used in pork challenge studies



4.2.4. Packaging

All inoculated and uninoculated pork samples were packaged in Cryovac barrier bags using a Multivac chamber type, heat seal packaging machine (Model KM100-3M). A Smith's proportional gas mixer (Model 299-028, Tescom Corp., Minneapolis, Minnesota) was used to give the desired proportions of O_2, CO_2 and N_2 in the package headspace. Residual oxygen levels were kept to a minimum in certain treatments using an oxygen absorbent sachet (Ageless type FX or SS) obtained from the Mitsubishi Gas Chemical Company Ltd., Tokyo, Japan.

4.2.5. Headspace Gas Analysis

Samples were analyzed for changes in headspace gas at regular intervals throughout the 28 day storage period (i.e., days 0,3,7,14,21 and 28 at 5 and 10°C and up to 14 days at 15°C). Gas samples were withdrawn using a 0.5 ml gas tight Pressure-lok syringe (Precision Sampling Corp., Baton Rouge, La.) Through silicone seals attached to the package exterior. The gas samples were injected into a Varian gas chromatograph (Model 3400, Varian Canada Inc.) equipped with a thermal conductivity detector (TCD) and using Porapak Q (80-100 mesh) and Molecular Sieve 5A (80-100 mesh) columns in series (Supelco Canada Ltd). The carrier gas was helium set at a flow rate of 30 ml/min. The column oven was set at 80°C, the injector at 100°C and the detector filament at 150°C. Peaks were recorded using a Hewlett Packard integrator (Model 3390A, Hewlett Packard Co., Avondale, PA.).

4.2.6. Sensory Analysis

At day 0 and at each subsequent sampling time (days 0,3,7,14,21 and 28 at 5 and 10°C and up to 14 days at 15°C), pork samples were evaluated subjectively for color and odor. All samples were evaluated in a random sequence. Fresh samples were used as controls at each sampling time.

4.2.6.1. Color Analysis

Samples were evaluated by the laboratory technicians in a random sequence for surface discoloration using a five point scale (1= No surface discoloration, 5= complete surface discoloration). A color chart from Agriculture Canada (publication 5180/B) served as a reference for the normal color of pork. A score of 3 or more was considered to be the upper limit of acceptability, implying that shelf life was terminated when this score was reached.

Color was also analyzed objectively for L* (lightness), a*(hue) and b* (chroma) color values using a Minolta Chroma Meter colorimeter (Model CR-100, Minolta Corp. Ramsey, New Jersey) with a luminant C (6774K) light source. The colorimeter was calibrated using a standard white reflector plate prior to measuring meat color.

4.2.6.2. Odor Analysis

Odor evaluation was carried out in a laminar flow hood (Purifier^{TM} Class II Safety Cabinet, Labconco, Model #36205-04, Labconco, Kansas, Missouri) using a five point scale (1= no off odor, 5= extreme off odor). Odor was assessed from a discreet distance by waving a small sample of air towards the laboratory technicians. A score of 3 or more was considered to be the upper limit of acceptability, implying that shelf life was terminated when this score was reached.

4.2.7. Microbiological Analysis

At each sampling day (0,3,7,14,21 and 28 at 5 and 10°C and up to 14 days at 15°C) bags were aseptically opened and a 30 gram sample was cut with a sterile knife from several different places on the meat slice and placed in a stomacher bag. To this, 270 ml of 0.1% peptone was added in the stomacher bag and the sample was blended in a Stomacher (Lab Blender 400, BA6021, Seward Medical, London) for 2 minutes. Dickson (1990) found that there were no differences in numbers of *Listeria* recovered by using either stomaching or blending and that peptone water recovered stressed cells as well as 2% trisodium citrate buffer. All subsequent dilutions were made from this 10^{-1} dilution using 0.1% peptone.

Total aerobic counts were determined by plating appropriate dilutions on tryptic soy agar (Difco) using the spread plate method. All plates were incubated at 30°C for 48 hours and were plated in duplicate. Lactic acid bacteria were enumerated using *Lactobacillus* MRS agar medium (MRS (DeMan, Rogosa and Sharpe) broth (Difco) plus 1.5% agar) by plating appropriate dilutions onto the agar using the spread plate technique. All plates were stored at 35°C for 48 hours and were plated in duplicate. *Pseudomonas* species were enumerated using *Pseudomonas* isolation agar (PIA, Difco) by plating the appropriate dilutions using the spread plate method. All plates were stored at 35°C for 48 hours and were plated in duplicate. *L.monocytogenes* was enumerated using LPM agar (Lithium Chloride Phenylethanol Moxalactum, Difco) prepared according to the manufacturer's directions and by plating the appropriate dilutions using the spread plate method. Warburton et al., (1992) showed that LPM agar

was comparable to Oxford agar and PALCAM agar in its ability to recover and enumerate stressed *L.monocytogenes* cells. All plates were stored at 30°C and were plated in duplicate and enumerated after 48 hours. In't Veld and Boer (1991) found that incubating for 48 hours on LPM agar gave more reproducible results as compared to incubating for only 24 hours. Confirmation tests were carried out on randomly selected colonies, i.e, Henry's illumination, hemolysis reaction on defibrinated horse blood agar, API Listeria strips, motility test and Gram stain reaction. Johnson and Lattuada, (1993) reported that the API *Listeria* system could readily differentiate between the *L.monocytogenes* and *L.innocua* species without the need for additional tests. These authors also reported that API *Listeria* strips were convenient, easy to use and reasonably priced and that reactions were not ambiguous and results were available within 24 hours. These results were in agreement with Dever at al. (1993). Hitchens et al. (1992) reported that there is no official method for the enumeration of *L.monocytogenes*. However, direct plate counts on LPM or MOX agar were acceptable method for enumeration.

The exudate loss from randomly selected samples was also analyzed for the presence of *Listeria monocytogenes* using LPM agar by using a spread plating technique in duplicate for all tested samples

4.2.8. Drip Loss

At each sampling time (days 0,3,7,14,21 and 28 at 5 and 10°C and up to 14 days at 15°C), individual bags were weighed upon removal of the pork slice. Individual samples had been pre-weighed prior to storage and thus a percent w/w value was obtained. i.e., the weight of the drip loss divided by the original sample weight.

4.2.9. pH Measurement

The pH of the pork samples was measured with a previously calibrated Corning pH meter (Model 2220, Corning Glass Works, Corning, NY) by placing the electrode into the 10^{-1} dilution of the pork samples after stomaching for 2 minutes, once all microbiological analysis was complete. Analysis was carried out at each sampling time (days 0,3,7,14,21 and 28 at 5 and 10°C and up to 14 days at 15°C).

4.3. RESULTS AND DISCUSSION

4.3.1. Introduction

In previous studies, the combined effect of chitosan and modified atmospheres on the growth of *L.monocytogenes* was investigated in model broth and model agar systems. Results of these initial studies clearly indicated that control of this pathogen was possible using a combination of 0.2% chitosan and MAP. However, little is known about their combined effect in a food system. Therefore, this present study examined the combined effect of a 0.2% chitosan dipping solution and MAP to control the growth of the indigenous spoilage bacteria and *L.monocytogenes* in fresh pork.

4.3.2. Physiochemical Analysis

4.3.2.1. Changes in Headspace Gas Composition

The changes in headspace gas composition for non-chitosan dipped pork stored at 5°C are shown in Figure 16. For air packaged samples, head space O_2 decreased to approximately 11% after 28 days with a concomitant increase in CO_2 to approximately 28% (Figure 16, A). This can be attributed to the action of meat respiratory enzymes and aerobic microorganisms which utilize O_2 and produce CO_2 (Gill, 1980).

For samples packaged with an Ageless oxygen absorbent (Figure 16B), headspace oxygen decreased to approximately 1% after 3 days of storage and remained unchanged throughout the storage period while head space CO_2 increased to approximately 20%. The increase in CO_2 in the non-chitosan dipped can again be attributed to meat tissue respiration, to the growth of aerobic and facultative microorganisms and possible heterofermentative strains of lactic acid bacteria.

For gas packaged samples ($80\%CO_2/20\%N_2$), headspace oxygen increased slightly from 1 to 25% throughout storage indicating slight leakage. Headspace CO_2 decreased slightly to approximately 60% due to possible dissolution in the meat tissue and then gradually increased throughout storage to approximately 80%. This increase can again be attributed to the growth of aerobic and facultative spoilage microorganisms in pork. Several studies have shown that *Pseudomonas* species can grow in low levels of O_2,

i.e., less than 1% even in the presence of elevated levels of CO_2 (De Vore and Solberg, 1974; Nottigham 1982).

For samples packaged in 100%N_2 with an Ageless FX oxygen absorbent, fluctuations in head space gas composition were similar to those for pork packaged with an Ageless SS absorbent, i.e., O_2 levels remained at very low levels throughout the 28 day storage period. However, after day 3, head space O_2 increased to approximately 8% indicating leakage and then decreased to less than 1% throughout storage. A similar increase in CO_2 production to approximately 15% was observed for this set of packaging conditions, i.e., similar to Ageless SS packaged samples.

Similar but more dramatic trends were observed for all non-chitosan dipped samples stored at 10 and 15°C (Figures 17 and 18 respectively). In all instances CO_2 production was greater at higher storage temperatures again emphasizing the importance of low temperature to control the growth of spoilage microorganisms in both air and MAP packaged pork.. Similar trends were observed by Enfors et al. (1979) and Blickstad et al. (1981) in pork samples packaged initially with air and stored at 2, 4, and 14°C.

The changes in headspace gas composition for chitosan dipped pork and packaged under various gaseous conditions and stored at 5, 10 and 15°C are shown in Figures 19, 20 and 21 respectively. In all cases, similar trends in headspace gas changes were observed for both chitosan dipped and non-chitosan dipped pork. This would infer that chitosan had no noticeable effect on the normal spoilage microorganisms of pork under the storage conditions investigated.

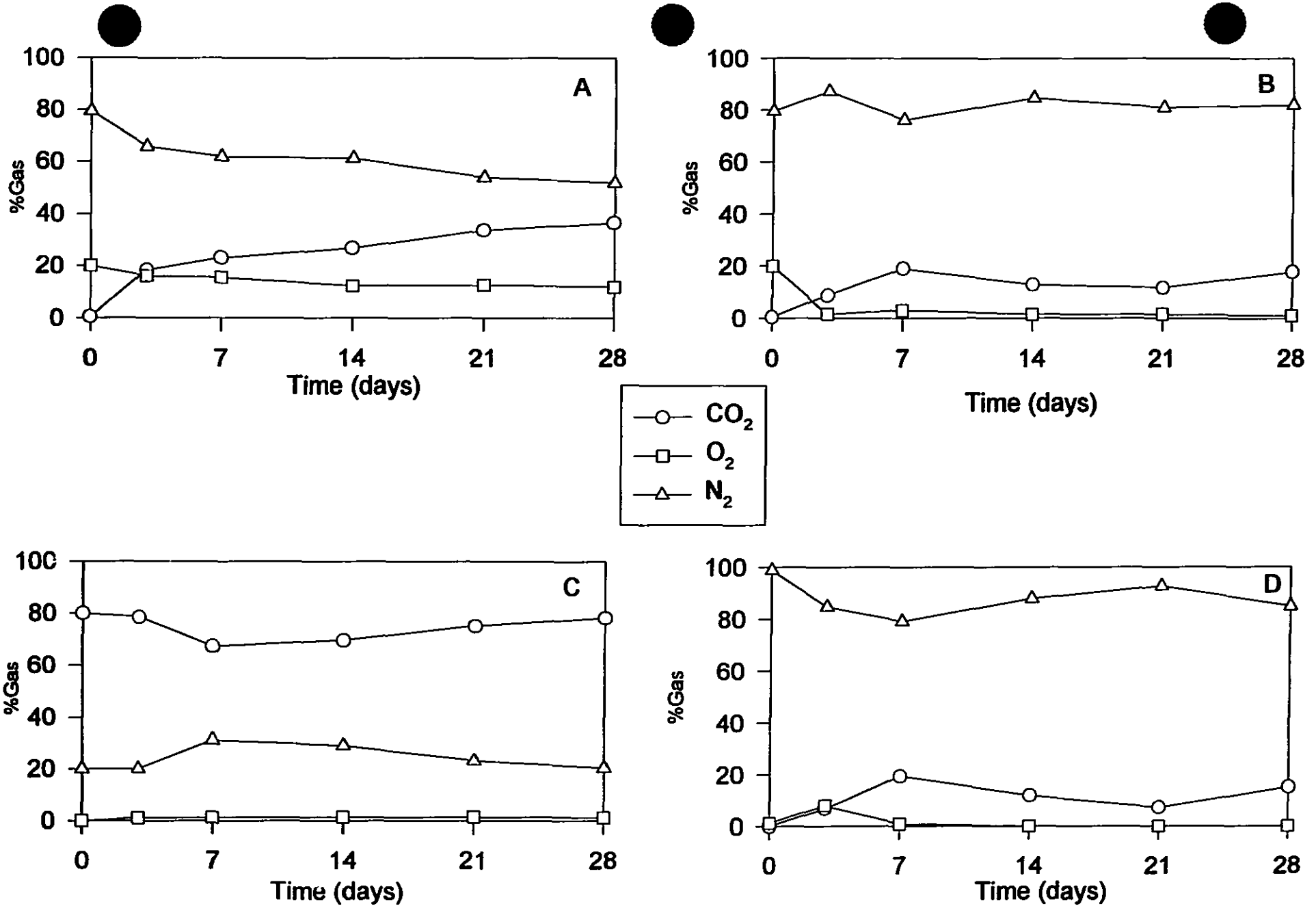


Figure 16: Changes in head space gas composition of non-chitosan dipped pork at 5°C and stored under (A)Air; (B)Air+Ageless SS; (C) 80%CO_2/20%N_2; and (D) 100%N_2+Ageless FX.

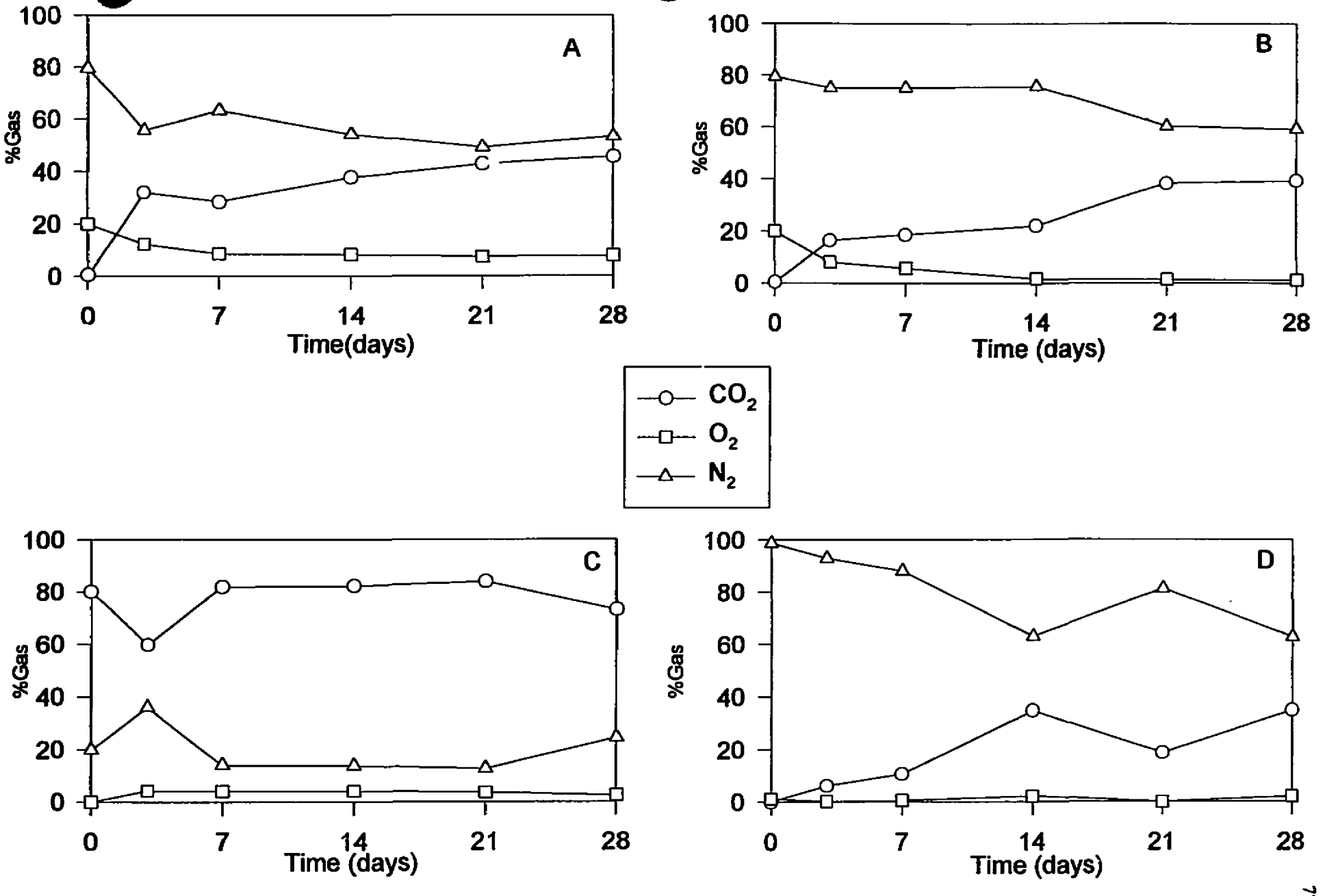


Figure 17: Changes in head space gas composition of non-chitosan dipped pork at 10°C and stored under: (A) Air; (B) Air+Ageless SS; (C) 80%CO_2/20%N_2; and (D)100%N_2+Ageless FX.

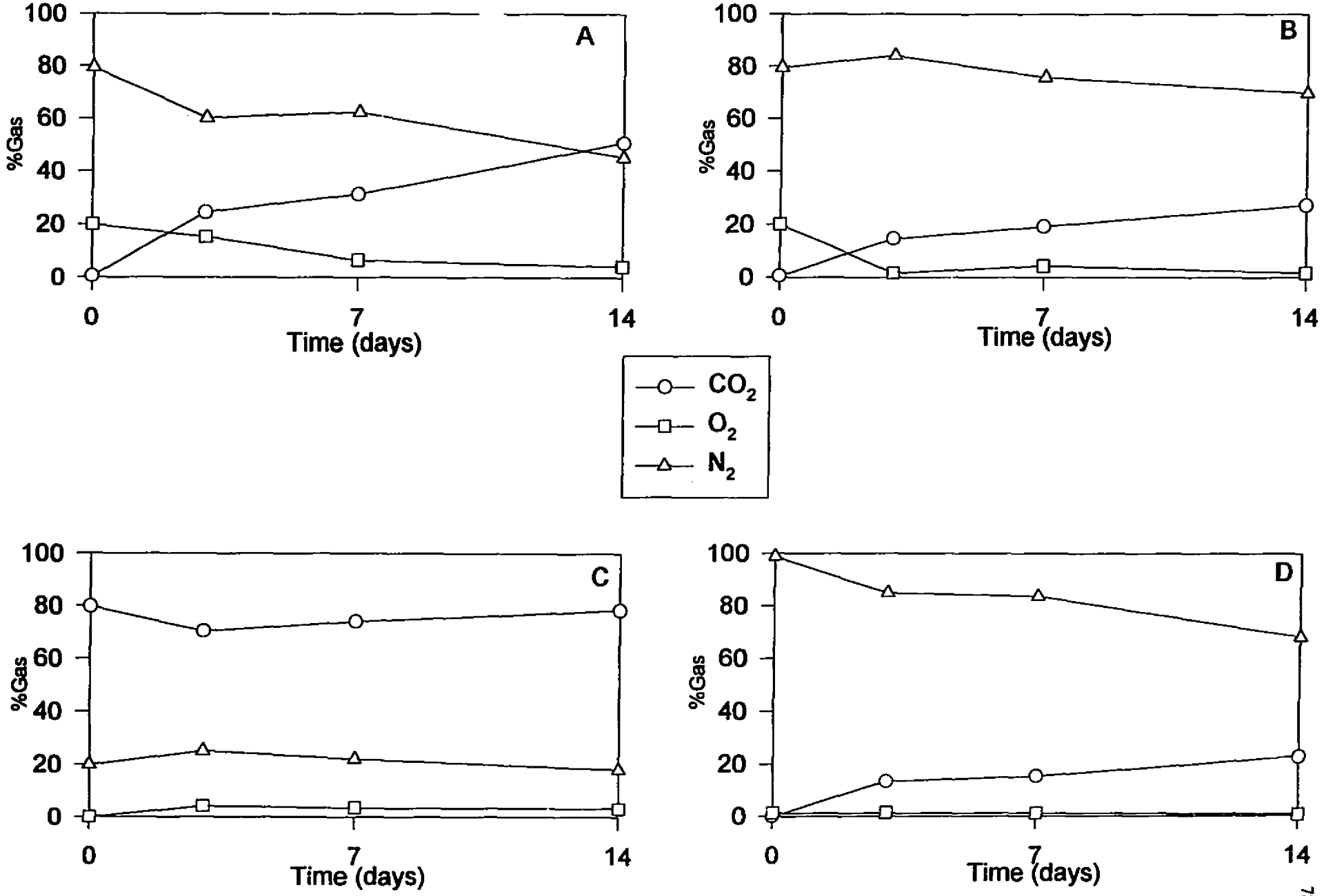


Figure 18: Changes in head space gas composition on non-chitosan dipped pork at 15°C and stored under: (A) Air; (B) Air+Ageless SS; (C)80%CO_2/20%N_2; and (D) 100%N_2+Ageless FX.

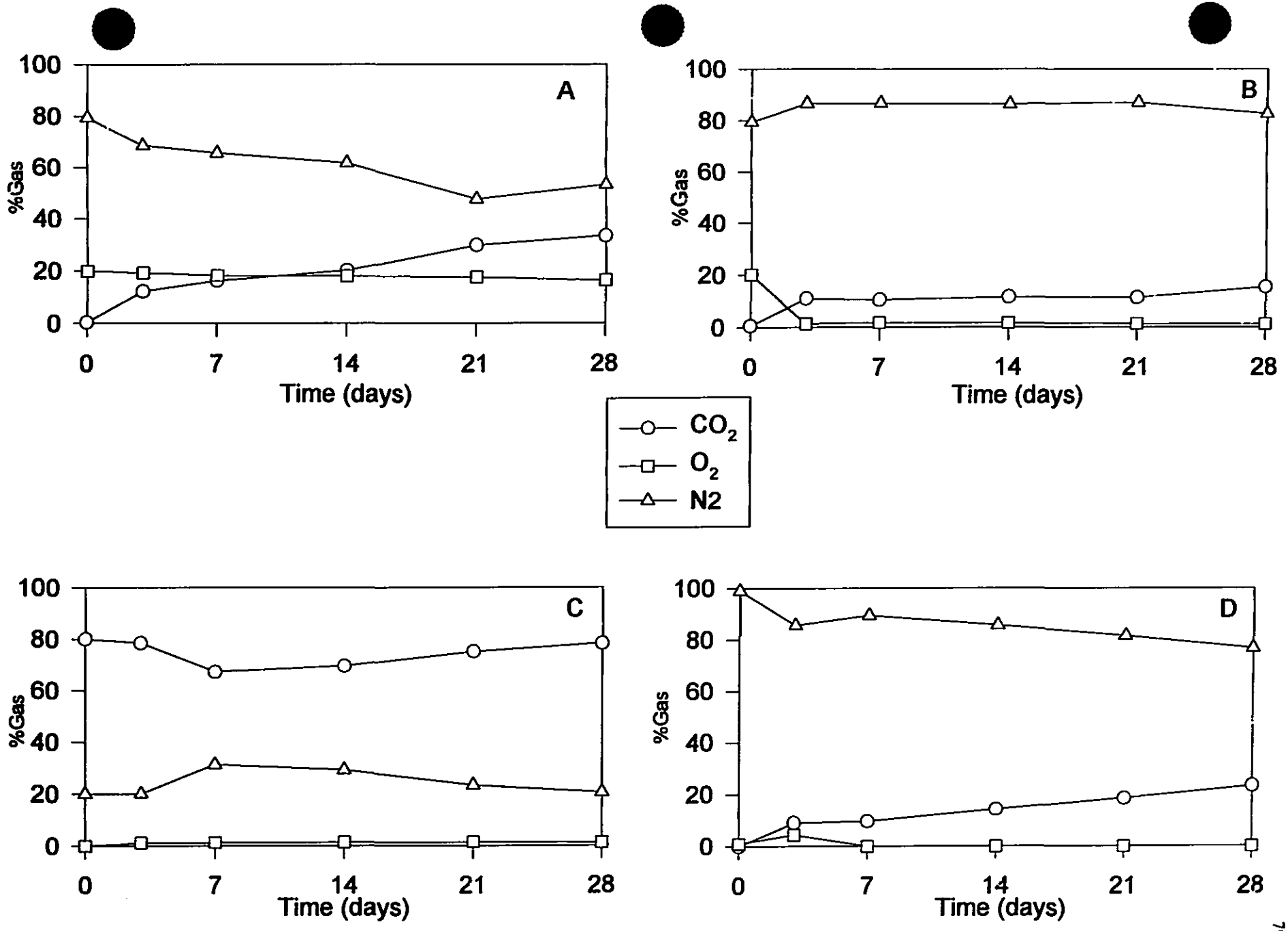


Figure 19:Changes in head space gas composition of chitosan dipped pork at 5°C and stored under (A)Air; (B) Air+Ageless SS; (C) 80%CO_2/20%N_2 ;and (D) 100%N_2+Ageless FX.

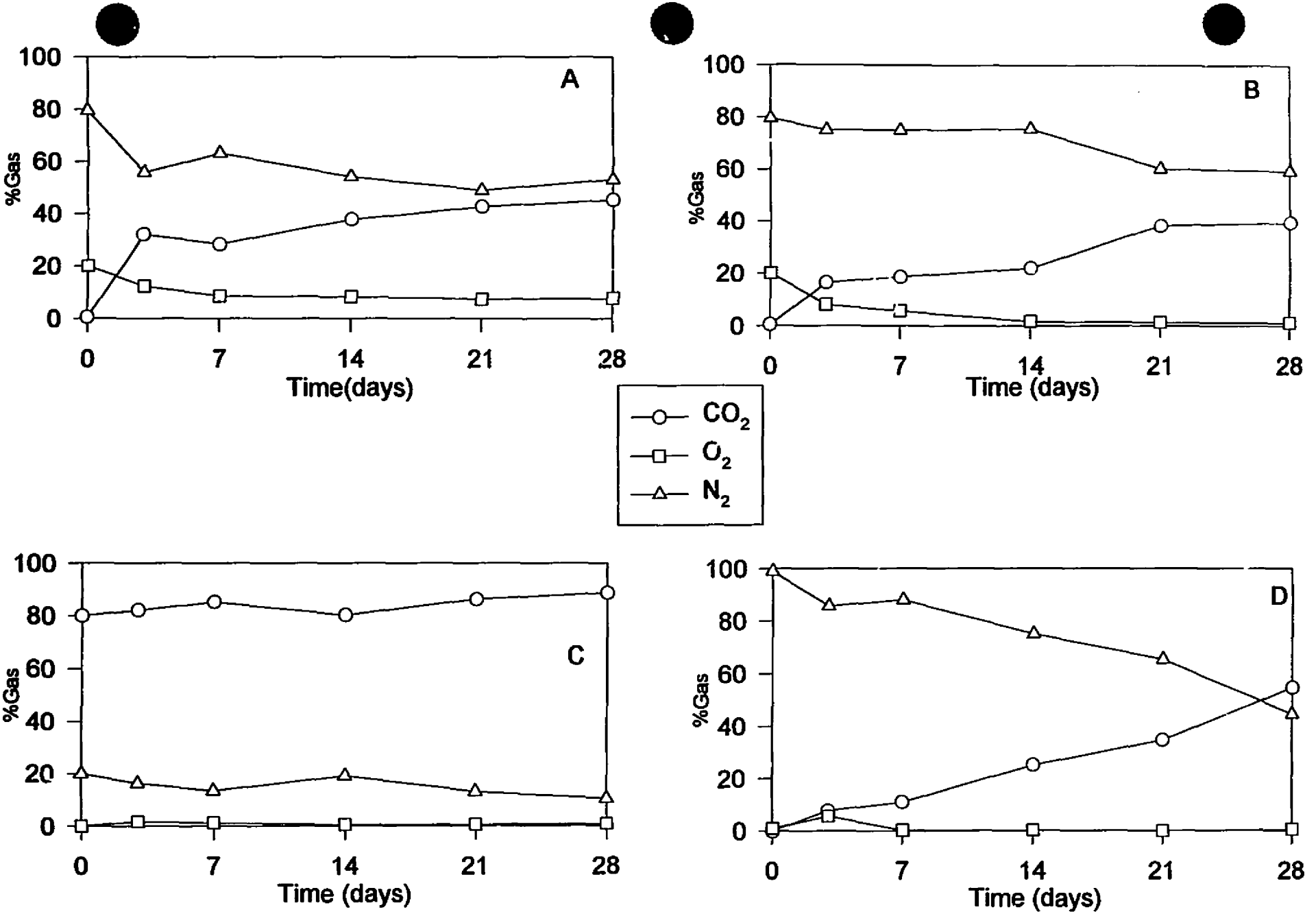


Figure 20: Changes in head space gas composition of chitosan dipped pork at 10°C and stored under: (A) Air; (B) Air+Ageless SS; (C) 80%CO_2/20%N_2; and (D) 100%N_2+Ageless FX.

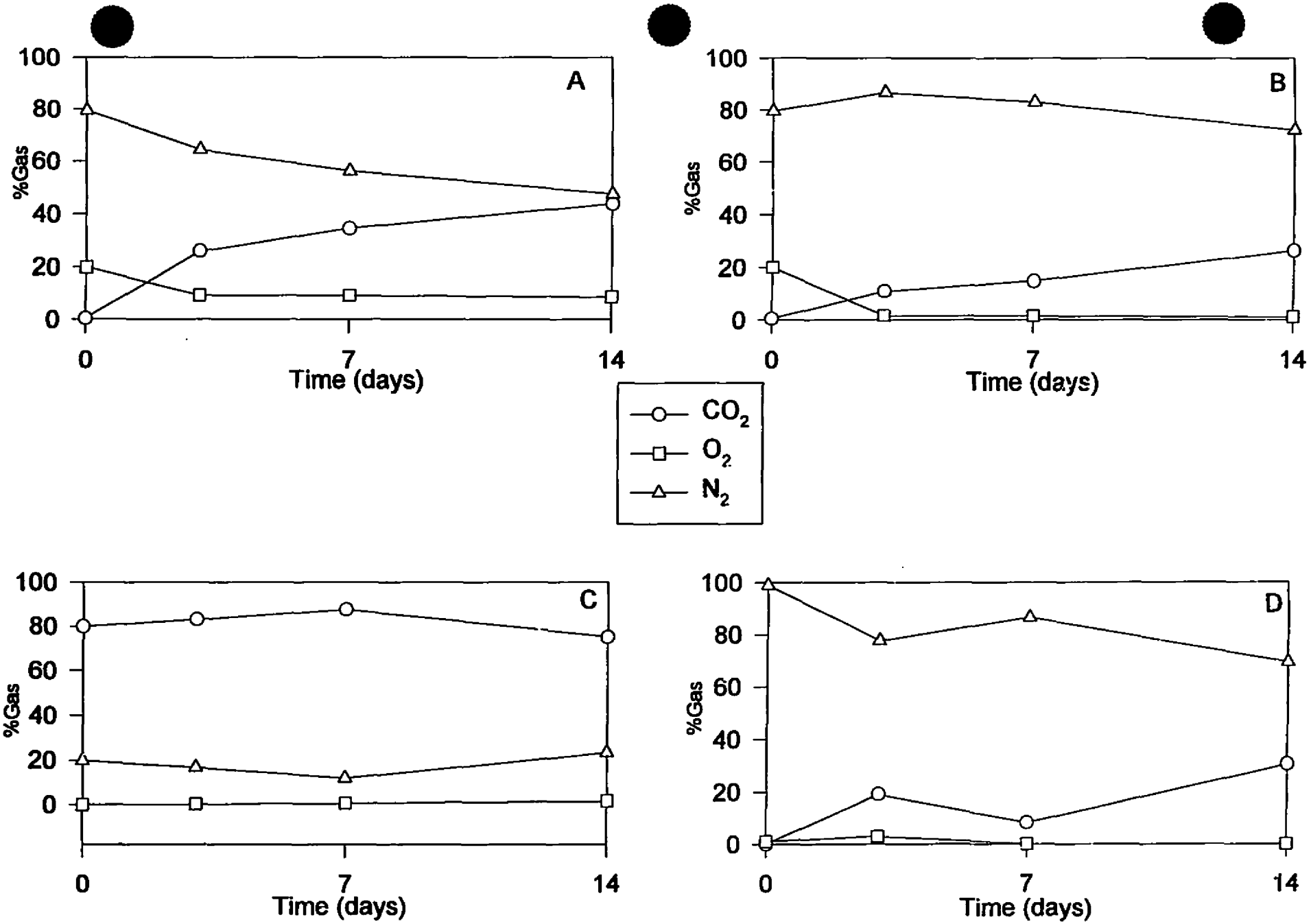


Figure 21: Changes in head space gas composition of chitosan dipped pork at 15°C and stored under: (A) Air; (B) Air+Ageless SS; (C) 80%CO_2/20%N_2; and (D)100%N_2+Ageless FX.

4.3.2.2. Changes in pH

Changes in pH for non-chitosan dipped and chitosan dipped pork are shown in Figure 25. Air packaged samples were generally of lower pH than MAP samples, particularly at the end of storage life. The lower pH in these samples can be attributed to a slightly higher level of headspace CO_2 and the dissolution of CO_2 in the aqueous phase of the product. The lower pH in the air packaged samples may also be attributed to the growth of lactic acid bacteria which are favored by a CO_2 enriched atmosphere. An interesting trend was observed in all MAP products, irrespective of dipping treatment. In these samples, pH decreased initially then increased towards the end of storage. This may be due to the lower levels of headspace CO_2 produced in products packaged with absorbents and a greater buffering effect of meat proteins. Another explanation could be a gradual permeation of headspace O_2 through the packaging film. Again, no noticeable differences in pH values were observed between non-chitosan dipped pork and chitosan dipped pork, irrespective of packaging conditions.

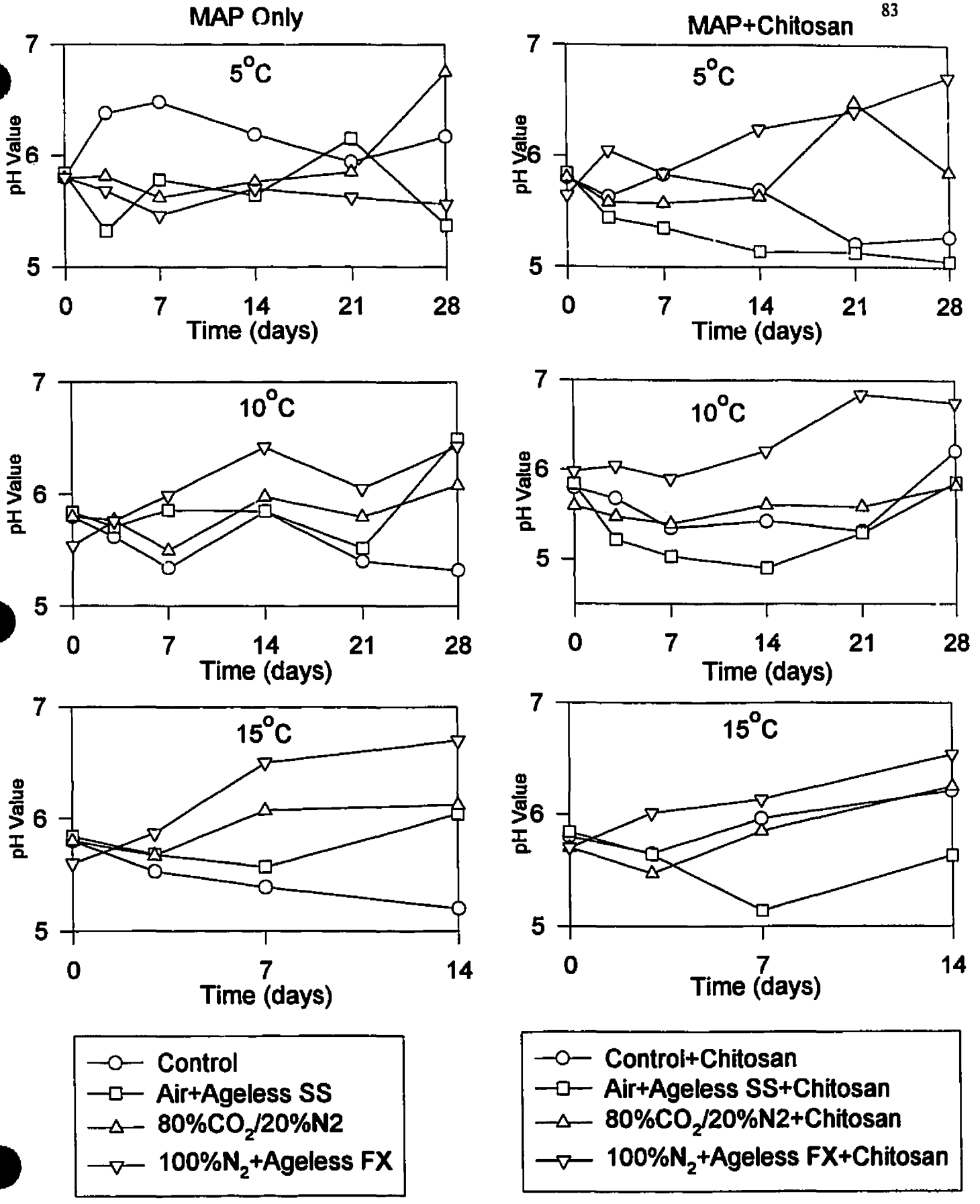


Figure 22: Changes in pH values of MAP pork stored at 5, 10 and 15°C.

4.3.2.3. Changes in Drip Loss

Changes in exudate or drip loss for non chitosan dipped and chitosan dipped packaged pork are shown in Figure 23. Drip loss either decreased and then increased throughout storage or vice versa. This could be attributed to changes in pH, i.e., the closer the pH to the isoelectric point of pork (6.2-6.5) the less the water holding capacity. conversely, as pH moved further away from the isoelectric point of meat proteins, the greater the water holding capacity of pork. Again, no observable differences were observed in drip loss between non-chitosan dipped and chitosan dipped pork.

4.3.2.4. Color Changes

Changes in the L* (lightness), a* (redness) and b* (yellowness) color values are shown in Figures 24, 25 and 26 respectively.

Generally, the L* values of samples increased slightly throughout storage indicating that the meat was becoming paler (Figure 24). Non-chitosan dipped pork samples packaged under MAP conditions were lighter than air packaged samples at 5oC. This can be attributed to a lower residual head space O_2 level (1-2%) compared to 11% in the air packaged samples. However, as temperature increased and the residual O_2 in air packaged samples decreased (4%) this trend was reversed. At 15°C, air packaged samples were paler than MAP samples. This can be attributed to the enhanced growth of spoilage microorganisms which favors the oxidation of myoglobin by reducing the O_2 tension at the meat surface (Seideman et al., 1984). Similar trends in L* values were observed for chitosan dipped pork (Figure 24). In some samples, chitosan dipped pork had higher L* values (i.e., paler) than non-dipped samples. This may be attributed to chitosan forming a thin film barrier at the meat surface thereby reducing the O_2 tension at the meat surface. In general, there was no noticeable difference between the L* values of non-dipped chitosan and chitosan dipped pork.

Changes in a* values (redness) are shown in Figure 25. Generally, a* values decreased throughout storage in both non-chitosan and chitosan dipped samples. However, the decreases in redness was less apparent in MAP samples than in air packaged products. Samples packaged in air with an Ageless SS oxygen absorbent maintained and even

increased their a* values throughout storage. This may be due to the conversion of myoglobin to a more stable bright red pigment (oxymyoglobin) due to low levels of residual O_2 in the package headspace. Control samples at 15°C also showed an increase in a* values. this can be attributed to an increase in reductase activity in the meat tissue at higher storage temperatures resulting in the conversion of any metmyoglobin to myoglobin. This, in time, would react with the residual oxygen (4%) converting it to the red oxymyoglobin pigment. Again, there was no observable difference between a* values in non-chitosan dipped or in chitosan dipped pork.

Changes in b* values (yellowness) for all dipping/packaging treatments are shown in Figure 24. Generally, b* values decreased and then increased throughout storage, i.e., samples were more yellow in color. Furthermore, chitosan dipped samples had higher b* values than non-chitosan dipped samples. Overall, samples with higher b* values had lower a* values and higher L* values and there appears to be no observable difference in these values for chitosan dipped and non-chitosan dipped samples.

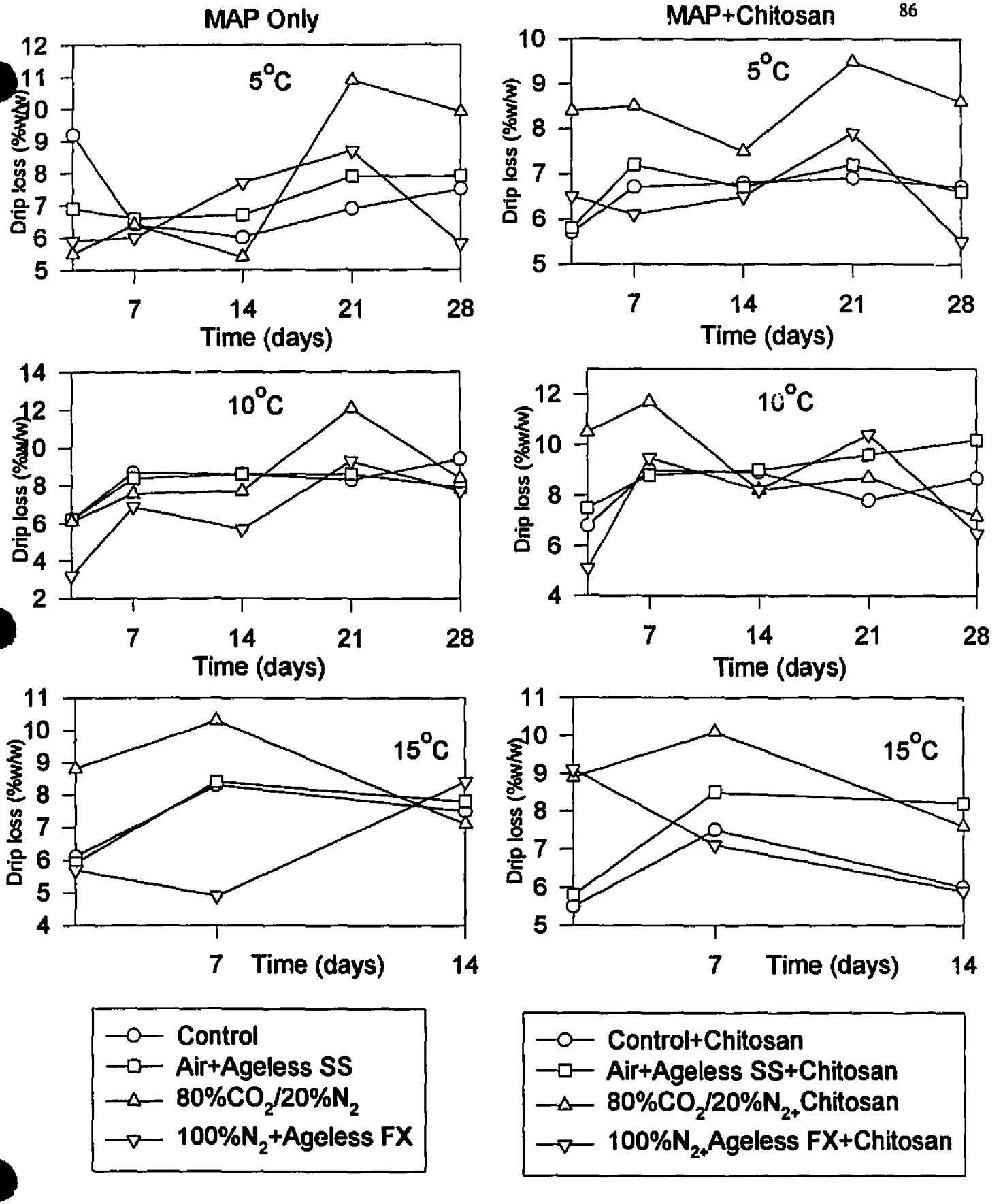


Figure 23: Changes in drip loss of MAP pork stored at 5, 10 and 15°C.

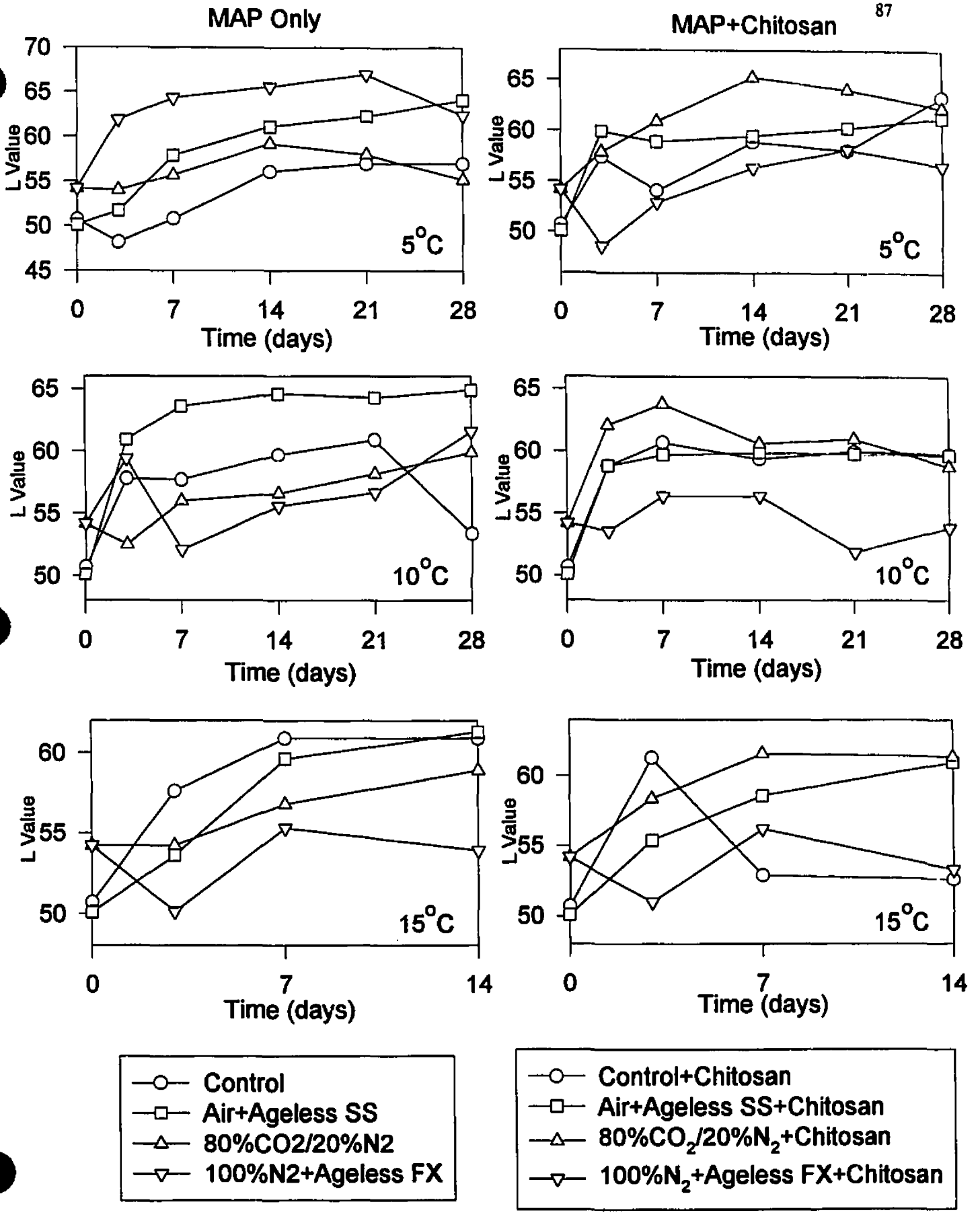


Figure 24: Changes in L* values of MAP pork stored at 5,10 and 15°C

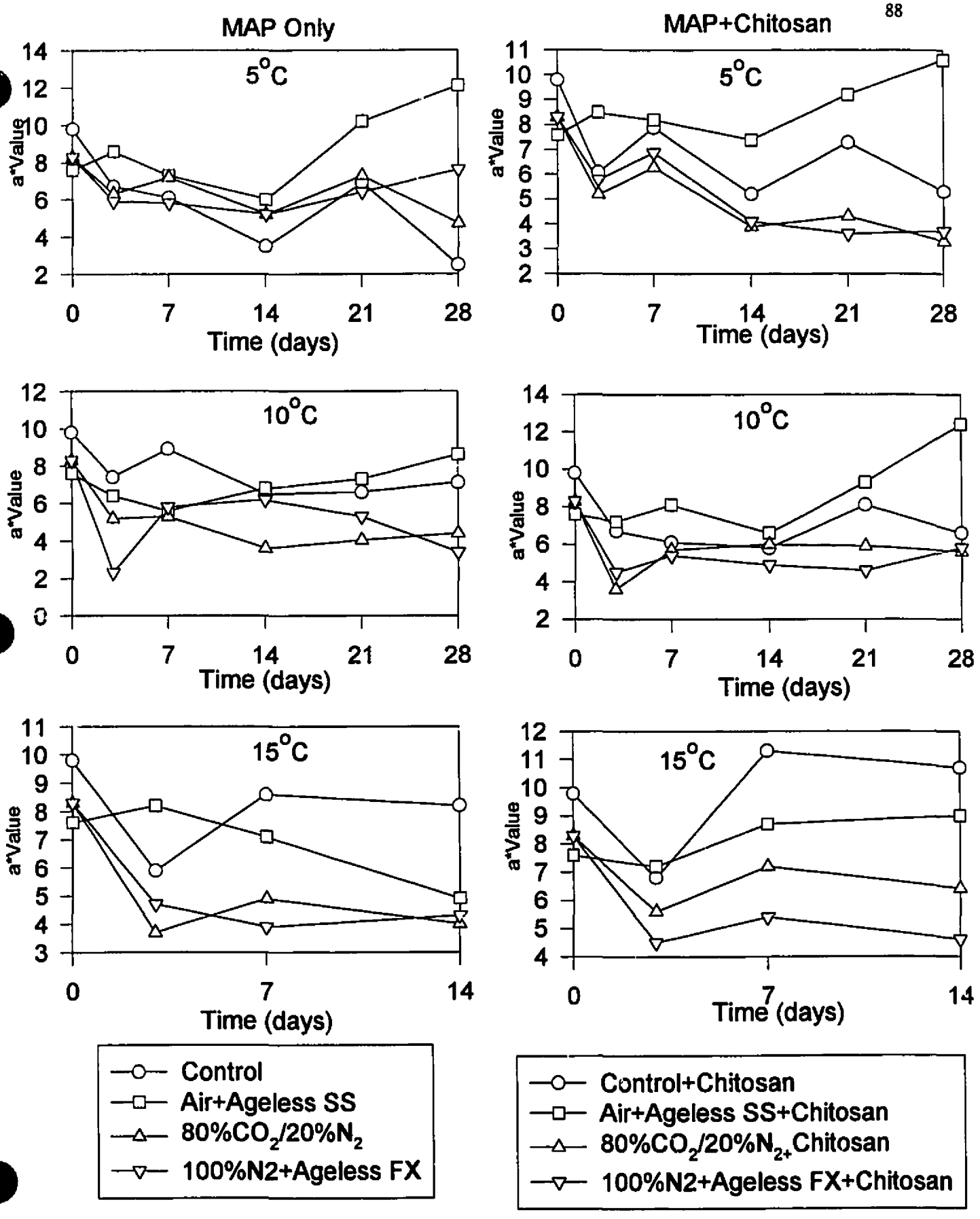


Figure 25:Changes in a* values of MAP pork stored at 5, 10 and 15°C

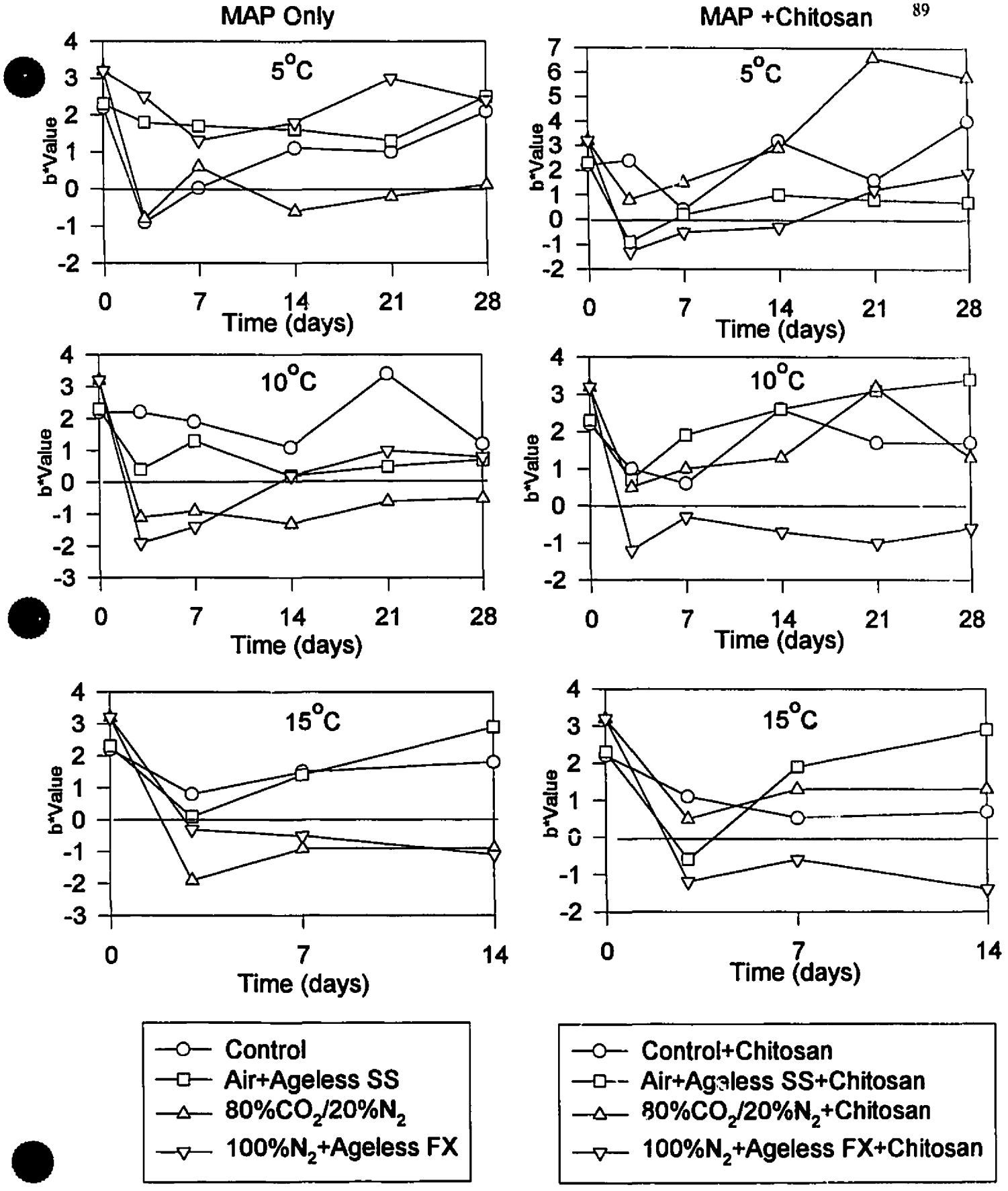


Figure 26:Changes in b* values of MAP pork stored at 5,10 and 15°C

4.3.3. Sensory Evaluation

4.3.3.1. Color

Changes in color scores (results not shown) and time until rejection based on a score of 3 out of 5 for all dipping/ packaging treatments are summarized in Figure 27 and 28.

For non-chitosan dipped pork, samples were rejected within 7-14 days at 10 and 15°C. However, at 5°C, most samples (with the exception of air packaged pork) were deemed acceptable after 21 to 25 days. However, from a sensory viewpoint, the effect of dipping pork in chitosan had a pronounced effect on shelf-life. Most MAP samples had an acceptable color even after 25 days especially at lower storage temperatures. These results indicate that both MAP and chitosan had a pronounced effect on color when viewed subjectively (through the eyes of the sensory panel). The rejection of control (air packaged) samples after 7 days is in agreement with the higher L* and b* values particularly at higher storage temperatures.

4.3.3.2. Odor

Changes in odor scores (results not shown) and time until rejection, based on a score of 3 out of 5 for all dipping/packaging treatments are summarized in Figures 29 and 30.

For non-chitosan dipped pork, air packaged samples were rejected after 3 to 7 days depending on the storage temperature. This is in agreement with higher microbial counts, increasing L* and b* values and higher color scores observed for these samples. Packaging under modified atmospheres increased the odor shelf life of pork, particularly at lower storage temperatures. Dipping in chitosan prior to packaging did not appear to have an appreciable effect on the odor shelf life of pork. Most chitosan dipped treatments had a similar shelf-life to non-chitosan dipped products (Figure 30).

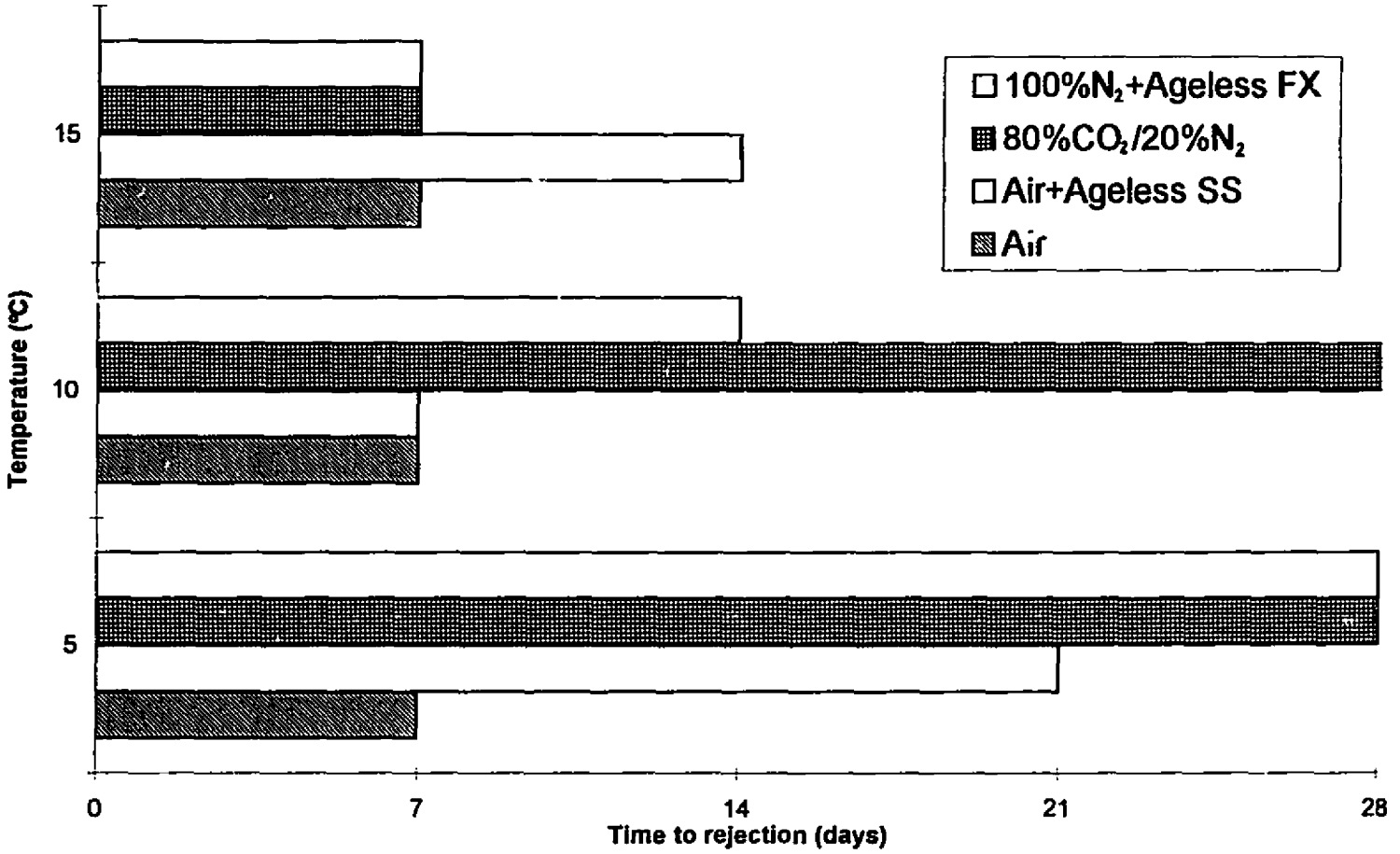


Figure 27: Effect of MAP alone on the time required to reject pork samples on the basis of color

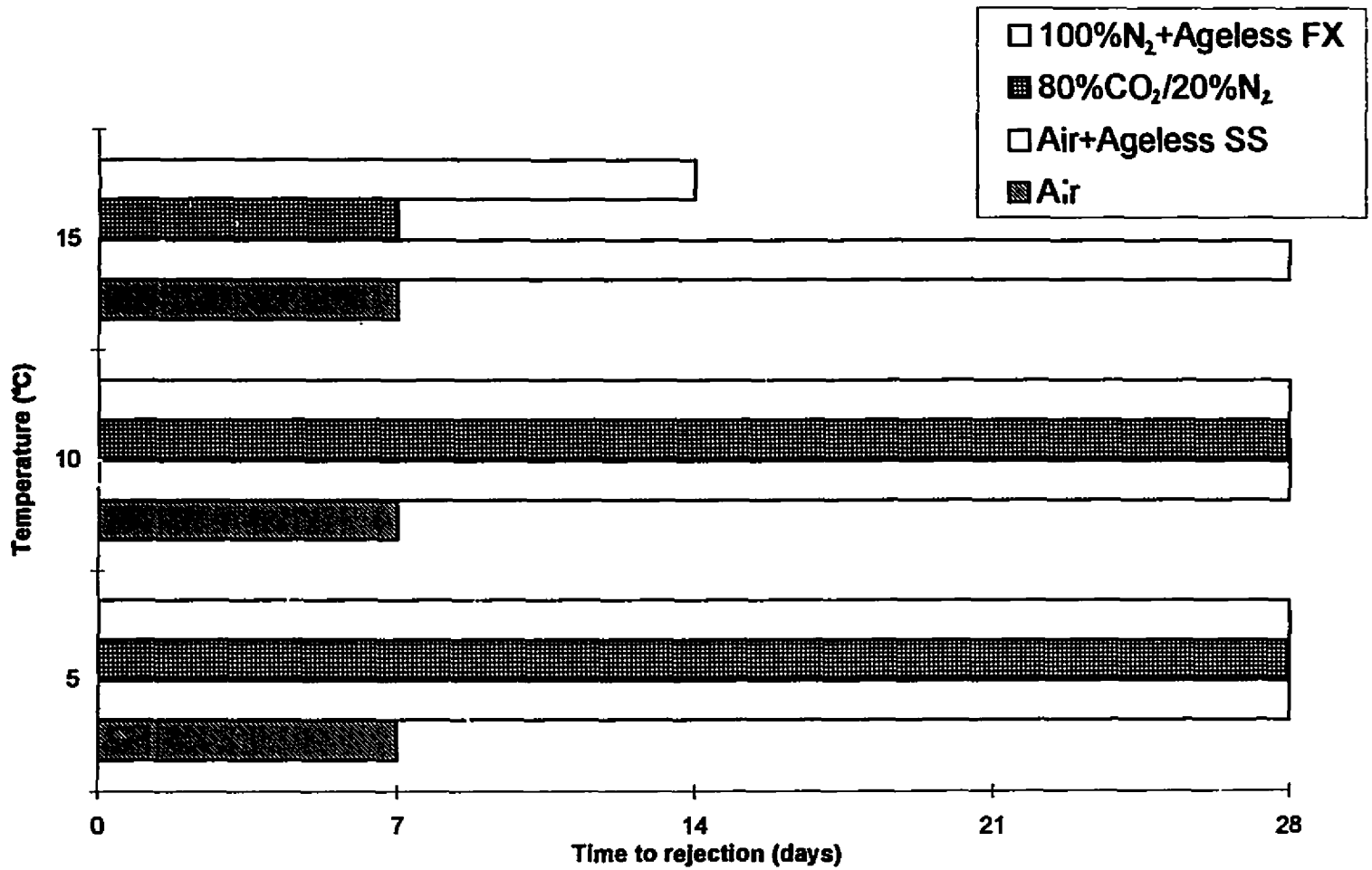


Figure 28: Effect of MAP and dipping in chitosan on the time required to reject pork samples on the basis of color

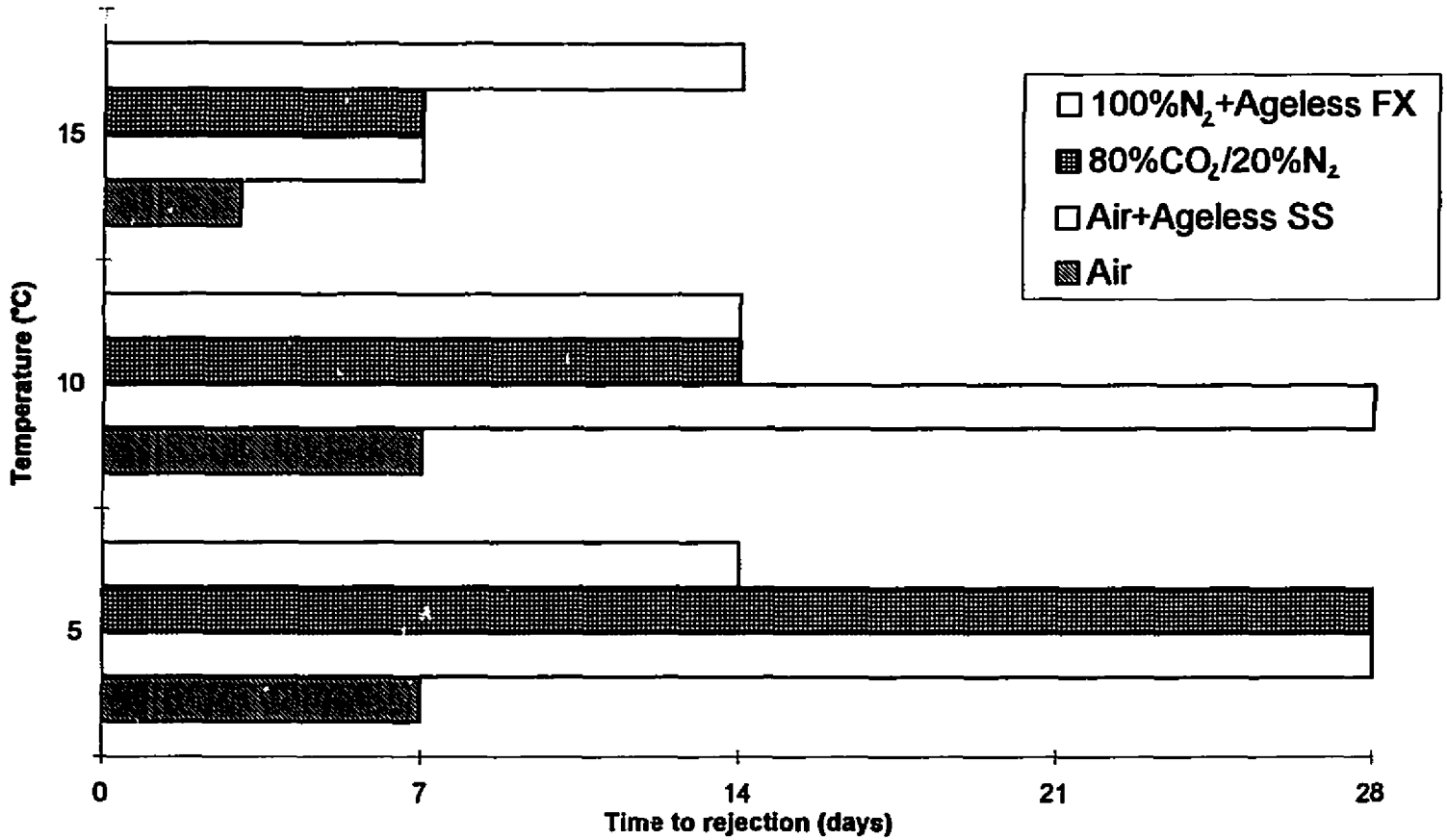


Figure 29: Effect of MAP alone on the time required to reject pork samples on the basis of odor

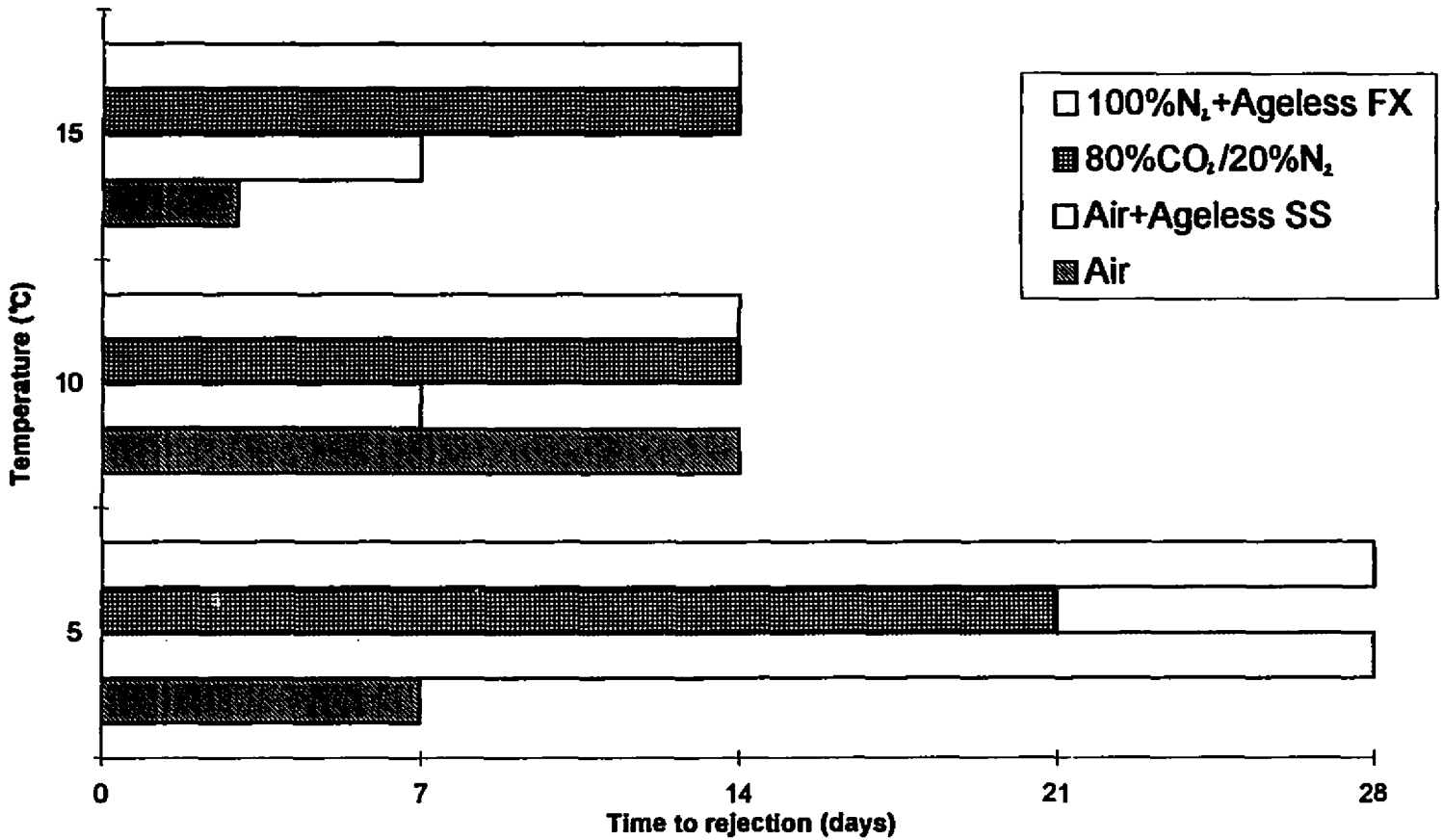


Figure 30: Effect of MAP and dipping in chitosan on the time required to reject pork samples on the basis of odor

4.3.4. Microbiological Changes

The microbiological changes for various dipping/packaging treatments stored at 5, 10 and 15°C are shown in Figures 31, 32, 33, 34, 35 and 37. Changes in non-chitosan dipped pork will be discussed first (Figures 32,33 and 34).

Initially, total aerobic counts increased more rapidly for air packaged samples than samples packaged under a modified atmosphere. For example, at 5°C, counts increased from an initial level of 10^4 cfu/g to 10^9 cfu/g after 3-7 days and remained at this level throughout the 28 day storage period (Figure 31) due to a depletion in head space O_2 and a build up of CO_2. For pork packaged under MAP conditions, aerobic counts either decreased or increased slightly throughout storage depending on the level of residual head space oxygen. However, counts never reached an unacceptable level (10^7 cfu/g) and at the end of the storage period, counts were about 2 to 4 log cycles less than air packaged samples at 5°C. A total number of 10^7 cfu/g of bacteria in meat is considered to be the upper limit of microbiological acceptability (Niemand et al., 1981).

Similar but more rapid trends were observed for pork stored at 10 and 15°C. However, counts at the end of the storage period were approximately 2 log cycles higher for all packaging conditions compared to those at 5°C. These observations emphasize the importance of strict temperature control to obtain the maximum beneficial effect on shelf life when packaging muscle foods under modified atmospheres.

The changes in *Pseudomonas* counts of pork samples stored at 5, 10 and 15°C are shown in Figure 32. For non-chitosan dipped pork at 5°C, *Pseudomonas* counts increased more rapidly in air packaged samples than in MAP samples. Again, this is due to the fact that a major proportion of the spoilage bacterial population of fresh pork is composed of *Pseudomonas* strains which are favored by aerobic conditions and low storage temperatures (Gill and Greer, 1993). In pork packaged in 80%CO_2 and 20%N_2 and in 100%N_2 with an Ageless FX oxygen absorbent, a lag time of about 3 days was observed followed by an active growth phase (Figure 32). *Pseudomonas* counts in these packaged samples were less than samples packaged in air or with Ageless SS. The results with Ageless SS were slightly surprising since counts were higher than expected. Again, this can probably be explained by package leakage and residual oxygen in the package head space. Similar trends were observed for samples stored at 10 and 15°C

(Figure 32). Even at higher storage temperatures *Pseudomonas* counts never reached unacceptable levels, particularly in MAP packaged pork.

Changes in lactic acid bacteria (LAB) counts for non-chitosan dipped pork and stored at 5, 10 and 15°C are shown in Figure 33. LAB counts increased in nearly all packaging conditions at all storage temperatures. Since LAB are facultative anaerobes, they soon become the predominant spoilage microorganism in chilled meat stored under anaerobic conditions (Gill and Greer, 1993). This is again apparent in Figure 33, where LAB counts were higher in all MAP samples than in air packaged pork since the gsaeous conditions, i.e., depleted O_2, elevated CO_2 are all conducive to the growth of LAB. While the counts of LAB reached 10^7 cfu/g after 28 days, these counts are not of major concern with respect to meat spoilage. Indeed, similar and even higher counts have been reported without adverse effect on the sensory attributes of these products (Lambert et al., 1991).

The effect of dipping pork in a 0.2% solution of chitosan for 60 seconds prior to packaging under various gaseous conditions on the growth of indigenous microorganisms (total aerobes, *Pseudomonas* and lactic acid bacteria) are shown in Figures 34, 35 and 36 respectively. Similar changes/fluctuations in counts were observed for chitosan dipped and non-chitosan dipped pork. At the end of the storage period there was no significant difference in total aerobic counts, *Pseudomonas* counts or lactic acid bacteria counts between the two dipping treatments. Indeed, *Pseudomonas* counts at 5°C and packaged under 100% N_2 with an Ageless oxygen absorbent were higher in chitosan dipped pork than non-chitosan dipped pork. However, these results may be explained through leakage, resulting in levels of O_2 conducive to the growth of *Pseudomonas* species even in the presence of elevated levels of N_2 and an oxygen absorbent. Therefore, dipping pork in a 0.2% chitosan solution in conjunction with MAP would appear to offer a limited extension in microbiological spoilage shelf life of pork over packaging under MAP conditions alone. These results are in agreement with Darmadji and Izumimoto (1994) who studied the effect of chitosan on the shelf-life of fresh ground beef. Only when higher levels of chitosan (0.5 to 1%) were used, was a significant decrease in spoilage bacteria and an extension in shelf life observed.

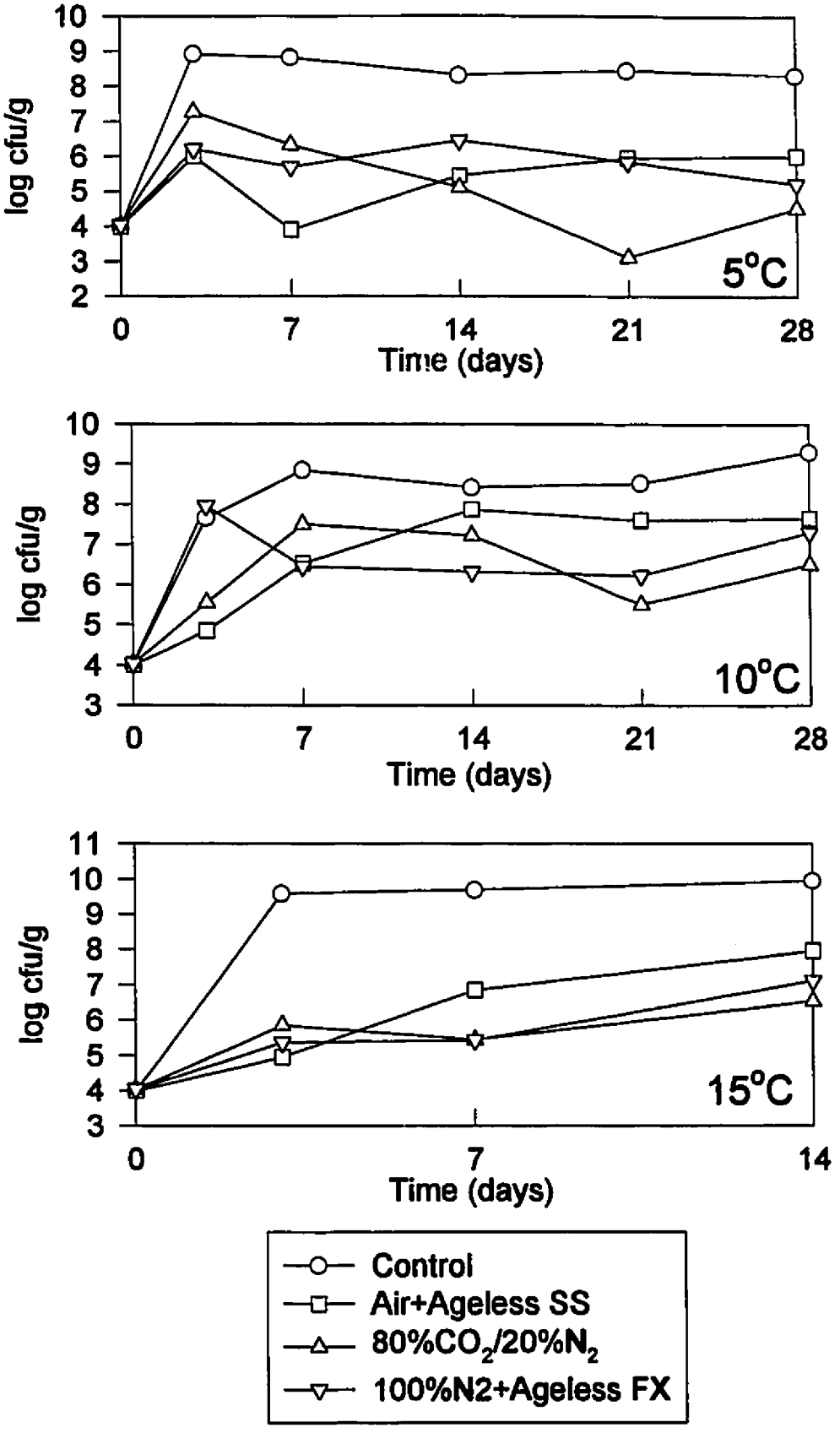


Figure 31: Changes in total aerobic counts of non-chitosan dipped pork stored at 5, 10 and 15°C

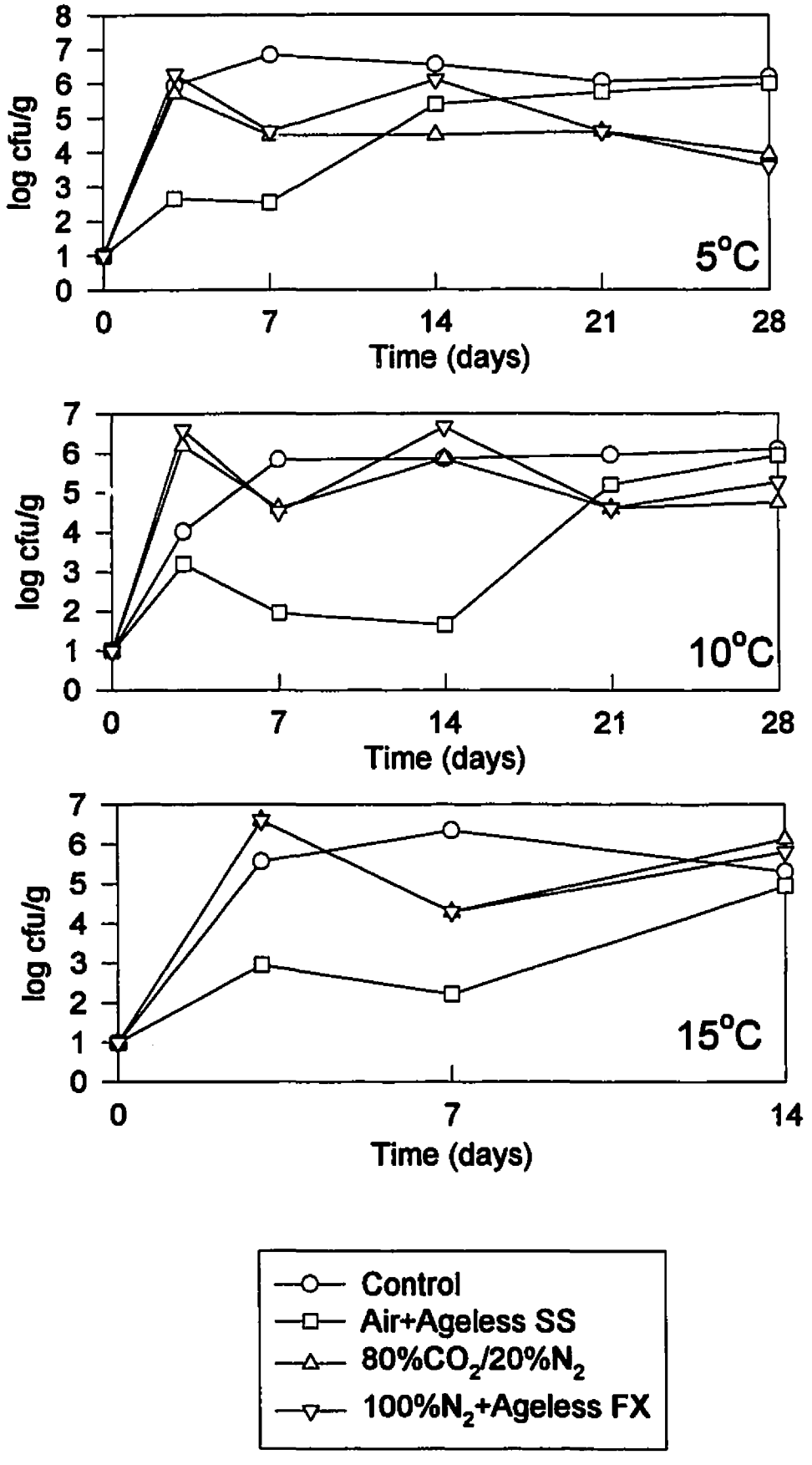


Figure 32: Changes in total *Pseudomonas* counts of non-chitosan dipped pork stored at 5,10 and 15°C

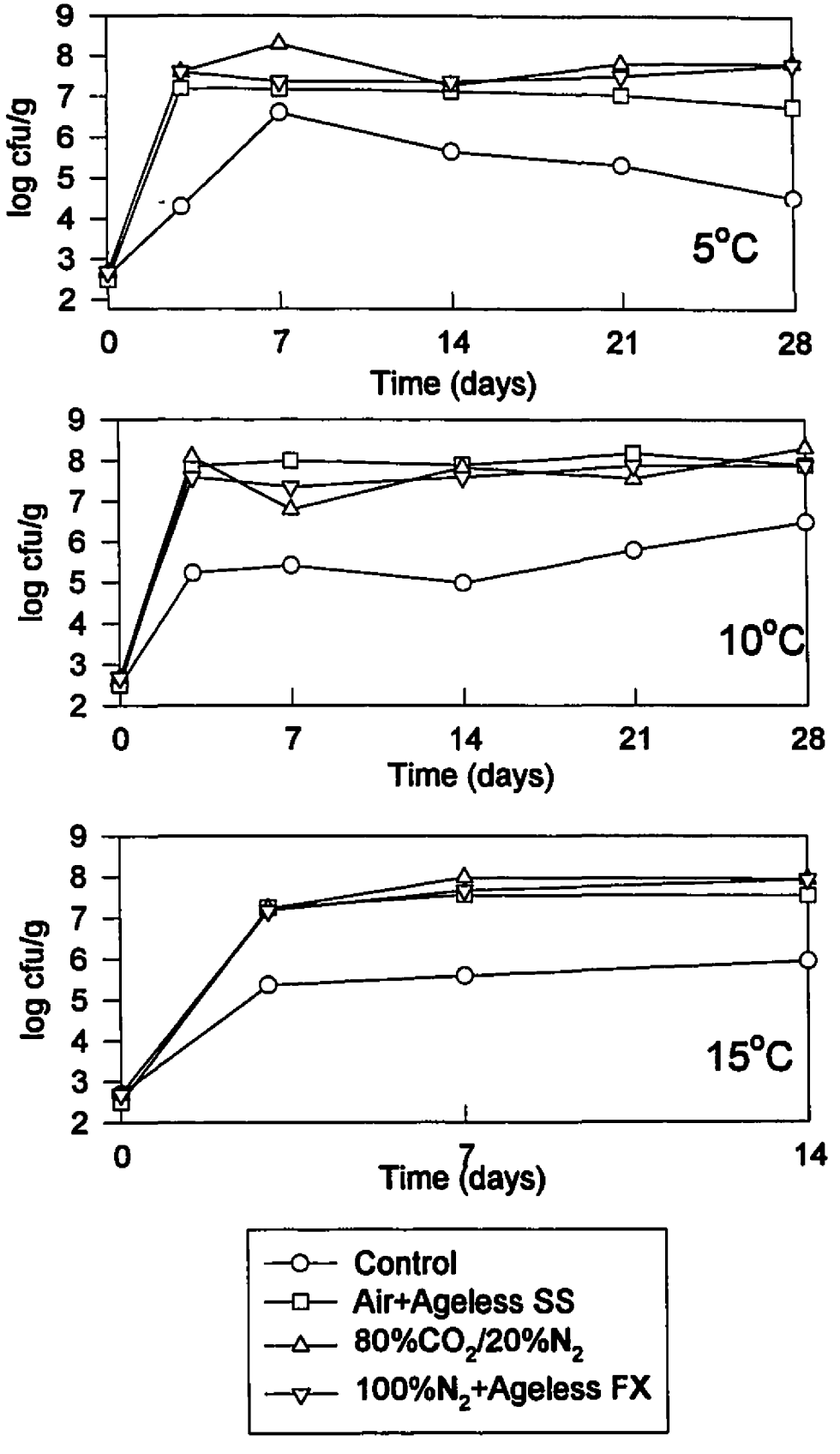


Figure 33: Changes in lactic acid bacteria counts of non-chitosan dipped pork stored at 5, 10 and 15°C

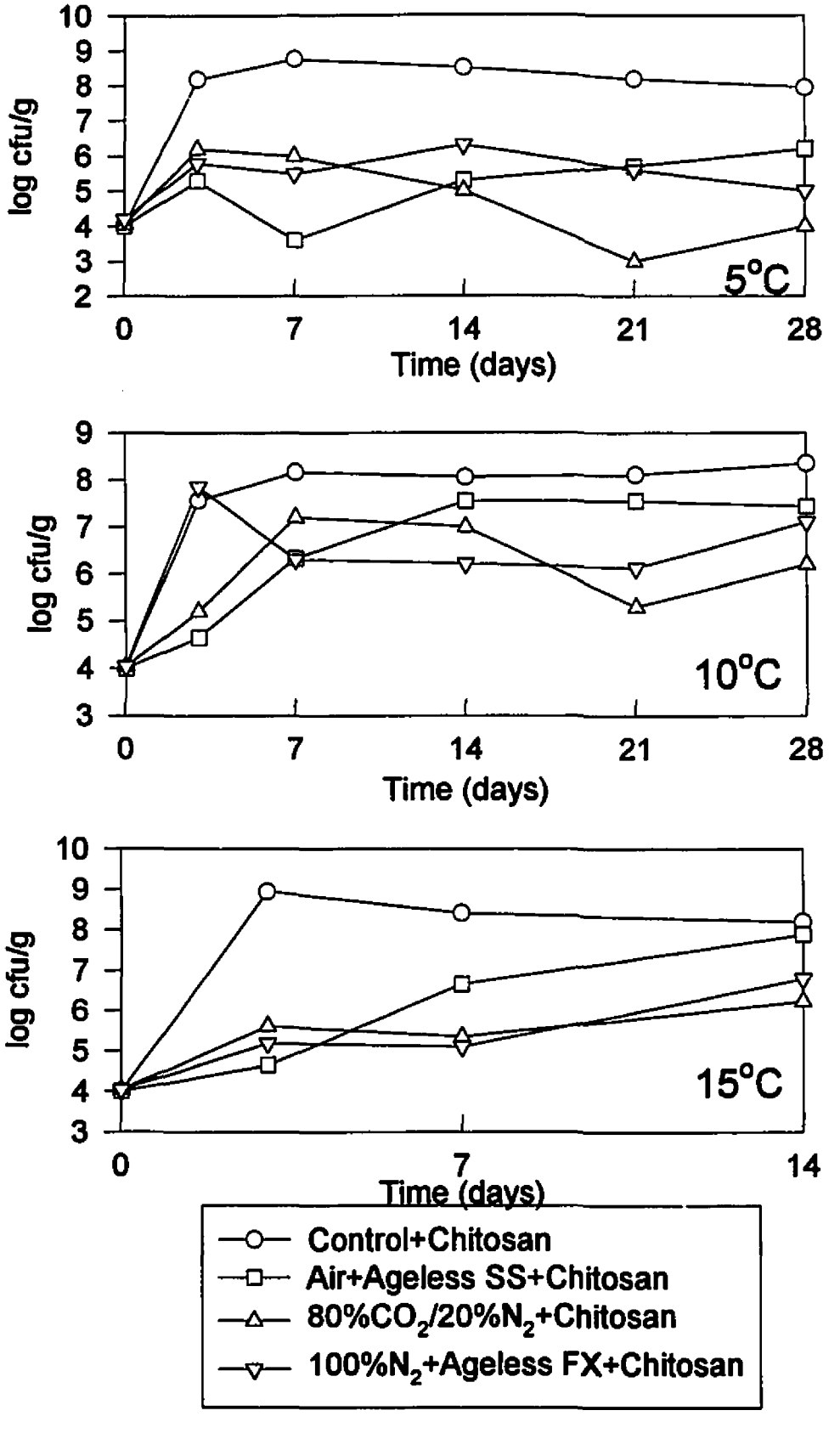


Figure 34: Changes in total aerobic counts of chitosan dipped pork stored at 5, 10 and 15°C

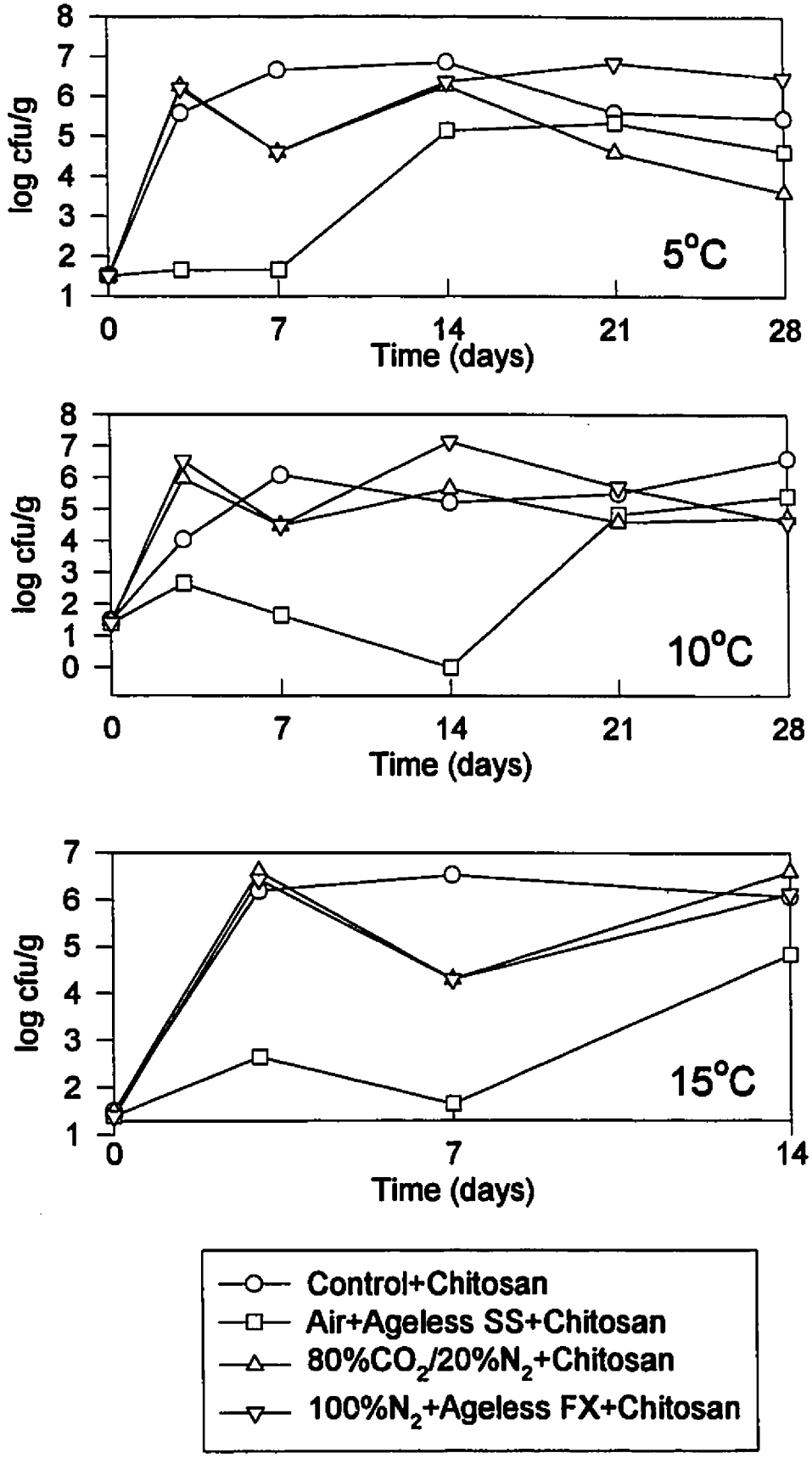


Figure 35: Total *Pseudomonas* counts of chitosan dipped pork stored at 5, 10 and 15°C.

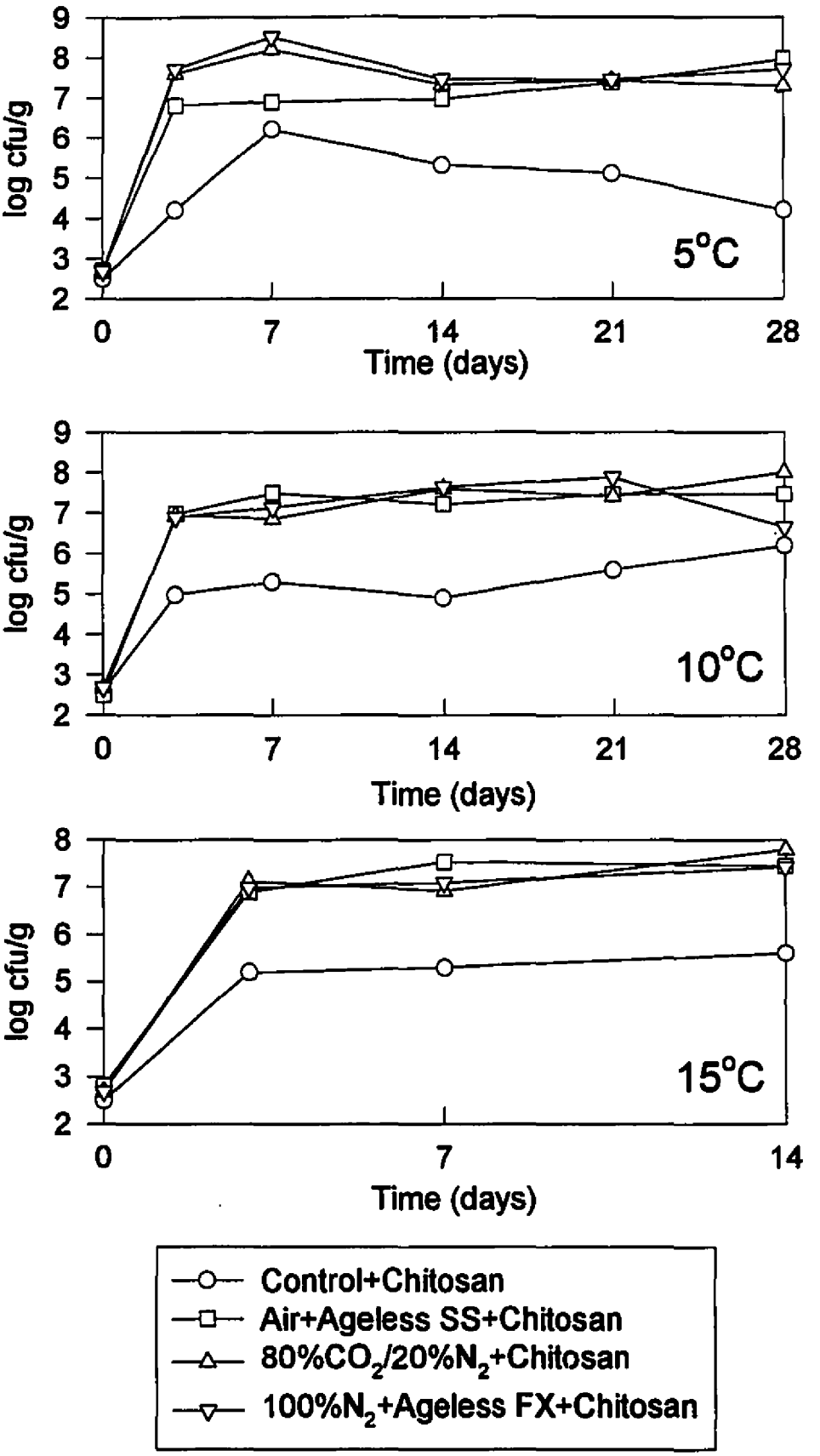


Figure 37: Total lactic acid bacteria counts of chitosan dipped pork stored at 5, 10 and 15°C

4.3.5. Shelf-Life

Based on this study, the estimated shelf life of all dipping packaging treatments at 5, 10 and 15°C is summarized in Table 13. The microbiological shelf life of pork was based on the time necessary to reach an aerobic plate count of 10^7 cfu/g (Ehioba et al., 1987). However, while there is disagreement with respect to the use of the total aerobic plate count as an indicator of spoliage, it was used in this study based on the observations of Lambert et al.(1991). The storage life of pork, estimated from the sensory evaluation scores for color and odor, i.e. two of the most important factors consumers associate with meat quality and freshness is shown in Table 13. The average time (in days) to reach either a color or odor score of 3 (rejection point) was used as an indicator of the shelf life. It is evident from Table 13, that shelf life of both non-chitosan dipped and chitosan dipped samples were very similar irrespective of packaging conditions. Thus, dipping in chitosan prior to packaging would appear to offer no advantage to the meat processor in terms of additional shelf life. This can best be obtained through packaging in a modified atmosphere in conjunction with low storage temperatures. However, MAP is no substitute for proper temperature control as shelf life decreases as products are subjected to mild temperature abuse conditions. However, even at 15°C, a 100 to 200% extension in shelf life is possible using some form of MAP technology, i.e., gas flushing or oxygen absorbents. Ageless SS did not perform as well as expected since it is designed to function at low temperature storage conditions. However, the time taken to scavenge headspace O_2 may have influenced the growth rate of *L.monocytogenes*. However, of concern is the fact that for products stored at 10 and 15°C, i.e., mild temperature abuse conditions, sensory shelf life was still acceptable although aerobic plate counts had reached high levels (10^7 cfu/g). Therefore, such MAP products may pose a public health hazard if contaminated with pathogenic bacteria which could reach similar dangerous levels, yet from a sensory viewpoint the product would still be acceptable to the consumer.

Table 13: Estimated Shelf-Life of Pork Loin at 5, 10 and 15°C for all Packaging Treatments Non-Chitosan Dipped

Packaging Conditions	Storage Temperature (°C)	Microbial[a] Shelf-Life (Days)	Rejection Point[b] Color (Days)	Rejection Point[b] Odor (Days)	Sensory[c] Shelf-Life (Days)
Air	5	3	7	7	7
Air+Ageless SS	5	>25	21	25	23
80%CO_2/20%N_2	5	>25	25	25	25
100%N_2+Ageless FX	5	>25	25	14	20
Air	10	3	7	7	7
Air+Ageless SS	10	14	7	25	16
80%CO_2/20%N_2	10	14	25	25	25
100%N_2+Ageless FX	10	25	14	14	14
Air	15	2	7	3	5
Air+Ageless SS	15	10	14	7	11
80%CO_2/20%N_2	15	14	7	7	7
100%N_2+Ageless FX	15	14	7	14	11

a: Time necessary to reach an aerobic plate count of 10^7 cfu/g

b: Time at which samples reached a color or odor score of 3 out of 5

c: The average time (days) to reach either a color or odor score of 3 out of 5

Table 14: Estimated Shelf-Life of Pork Loin at 5, 10 and 15°C for all Packaging Treatments Dipped in Chitosan

Packaging Conditions	Storage Temperature (°C)	Microbial[a] Shelf-Life (Days)	Rejection Point[b] Color (Days)	Rejection Point[b] Odor (Days)	Sensory[c] Shelf-Life (Days)
Air	5	3	7	7	7
Air+Ageless SS	5	>25	25	25	25
80%CO_2/20%N_2	5	>25	25	21	23
100%N_2+Ageless FX	5	>25	25	25	25
Air	10	3	7	14	11
Air+Ageless SS	10	14	7	25	16
80%CO_2/20%N_2	10	14	25	14	19
100%N_2+Ageless FX	10	3	25	14	19
Air	15	3	7	3	5
Air+Ageless SS	15	10	25	7	16
80%CO_2/20%N_2	15	14	7	14	11
100%N_2+Ageless FX	15	14	14	14	14

a: Time necessary to reach an aerobic plate count of 10^7 cfu/g

b: Time at which samples reached a color or odor score of 3 out of 5

c: The average time (days) to reach either a color or odor score of 3 out of 5

4.3.6. Challenge Studies with *L.monocytogenes*

Modified atmosphere packaging (MAP) of muscle foods has been gaining in popularity as a preservation technique for shelf life extension. Under conditions of low oxygen tension, growth of aerobic spoilage microorganisms is inhibited and shelf life is extended.

Recently, concerns have been expressed over the microbiological safety of MAP foods, particularly with regards to the possible growth of *L.monocytogenes*. These concerns are justified in view of (i) the ability of *L.monocytogenes* to grow under anaerobic conditions and at refrigerated temperatures; (ii) the inhibition of the normal spoilage microorganisms of meat which are the indicators of spoilage and (iii) the potential for temperature abuse.

Indeed, previous studies have shown that the sensory quality of fresh pork is still acceptable although numbers of spoilage microorganisms have reached high levels, i.e., 10^7cfu/g. If such a scenario is possible with pathogenic bacteria, particularly *L.monocytogenes*, then MAP pork poses a potential public health hazard to consumers.

4.3.6.1. Changes in *L.monocytogenes* counts in Pork

Changes in counts of *L.monocytogens* in non-chitosan dipped pork packaged under various gas atmospheres are shown in Figure 37. *L.monocytogenes* was not detected in any samples prior to inoculation studies. Therefore, changes in *L.monocytogenes* counts were solely due to the inoculum added on day 0. In all cases, counts of *L.monocytogenes* increased throughout storage. At 5°C, counts reached 10^7 cfu/g after 11 days in air packaged pork and after 12 days in pork packaged in air with an Ageless SS oxygen absorbent. For pork packaged under a CO_2 enriched atmosphere (80%CO_2/20%N_2) or with 100%N_2 and an Ageless FX oxygen absorbent, counts did not reach these unacceptable levels until 18 days and 27 days respectively. Similar trends were observed for pork stored at 10°C while at 15°C with counts reaching 10^7cfu/g after only 3-5 days irrespective of packaging conditions. These results show that MAP can be used to delay the growth of *L.monocytogenes* to unacceptable levels, particularly at refrigerated storage temperatures. However, when, the product is subjected to temperature abuse, MAP offers little advantage, with counts of

L.monocytogenes reaching unacceptable levels at approximately the same time as air packaged samples. These results once again emphasize the importance of strict temperature control if MAP is to be an effective barrier to control microbial growth.

However, when pork was dipped in a chitosan dipping solution prior to packaging under similar gaseous conditions, some interesting results were obtained as shown in Figure 38. While growth of *L.monocytogenes* occurred in all treatments, dipping in chitosan delayed its growth to unacceptable levels by approximately 100%. For example, in air packaged pork at 5°C, counts of *L.monocytogenes* reached 10^7 cfu/g after 21 days (compared to 11 days for non-chitosan dipped control) and after 24 days in pork packaged with Ageless SS (compared to 14 days in non-chitosan dipped control). Counts never reached these unacceptable levels in gas packaged pork (80%CO_2/20%N_2) or pork packaged in 100%N_2 with and Ageless FX oxygen absorbent. (Figure 38). Dipping in chitosan prior to packaging also offered additional control in pork stored at 10°C and 15°C indicating a synergistic effect between gas packaging and chitosan.

It is evident from both Figures 37 and 38 that MAP and refrigeration can delay the growth of *L.monocytogenes* to unacceptable levels. Optimum control can be achieved by packaging in a CO_2 enriched atmosphere or with 100% N_2 and an Ageless FX oxygen absorbent. Similar results are obtained for chitosan dipped pork. These results confirm previous agar studies which showed that growth of *L.monocytogenes* was delayed in plates containing 0.2% chitosan and packaged in 80%CO_2/20%N_2 or in 100% N_2 with an Ageless FX oxygen absorbent.

4.3.6.2. Changes in *L.monocytogenes* in Exudate Loss

Since there was considerable drip loss in all packaged pork, studies were done to determine levels of *L.monocytogenes* in this drip loss, results of which are shown in Figures 39 and 40. It is evident that while counts of *L.monocytogenes* increased in the exudate, they did not reach the levels found in the pork itself. The lower counts in the exudate may be due to the fact that there are insufficient nutrients in the exudate to support the growth of *L.monocytogenes*. Another possible explanation may be that the exudate contains natural substances that are inhibitory to the growth of *L.monocytogenes* or that the water soluble chitosan dissolves in the exudate and delays the growth of *L.monocytogenes*.

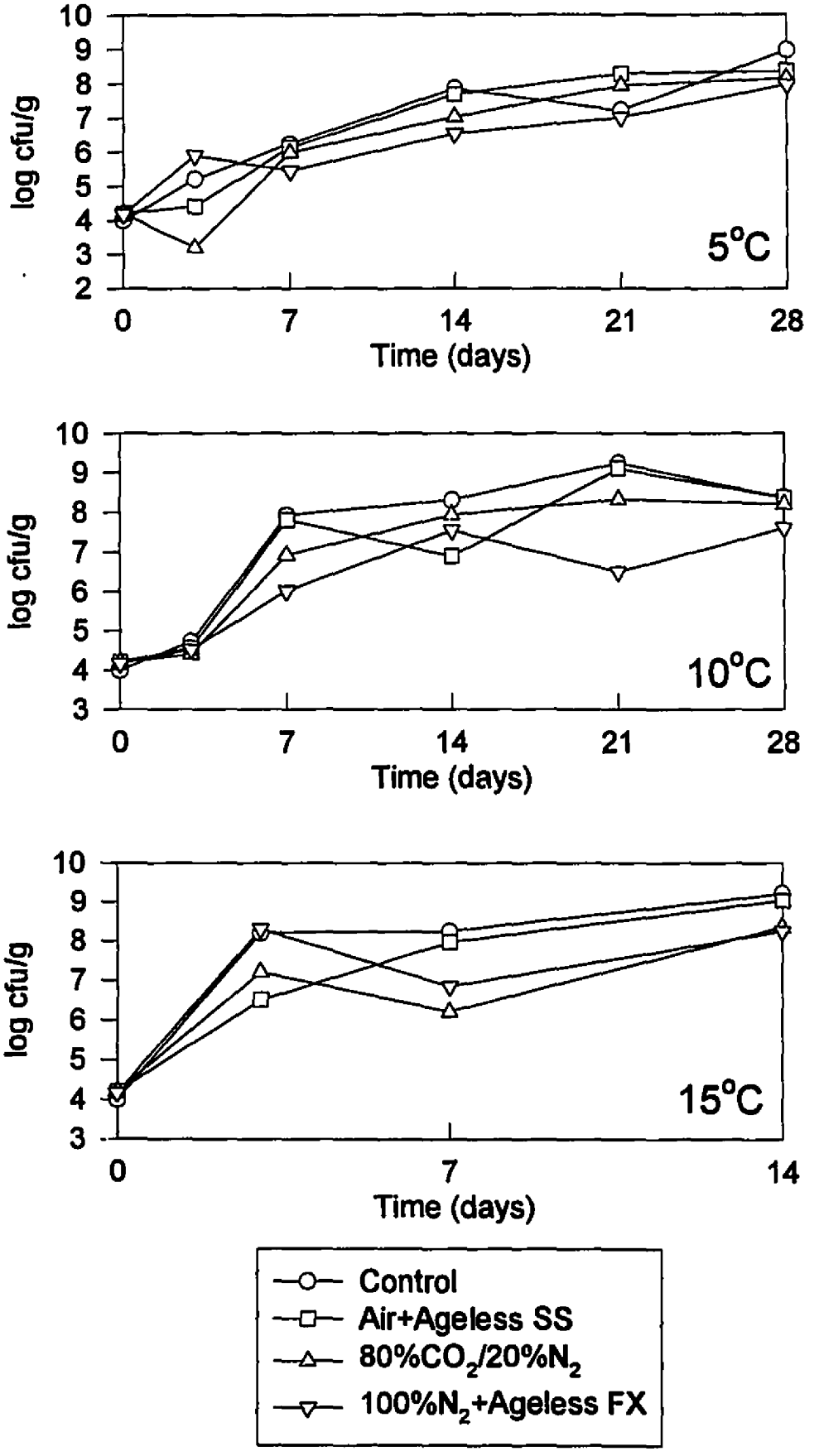


Figure 37: Changes in total *L.monocytogenes* counts of non-chitosan dipped pork stored at 5, 10 and 15°C

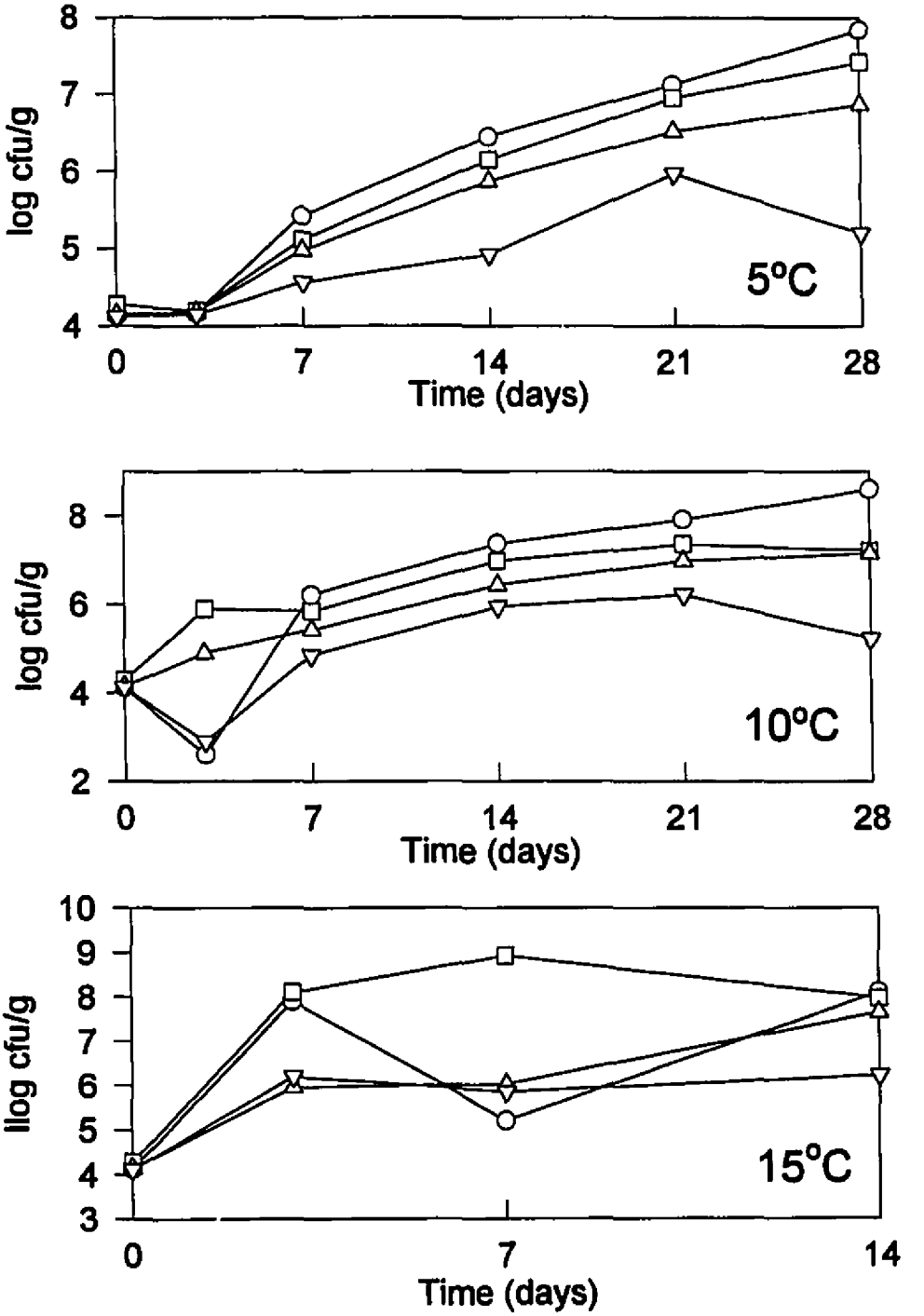


Figure 38:Changes in *L.monocytogenes* counts of chitosan dipped pork stored at 5, 10 and 15°C

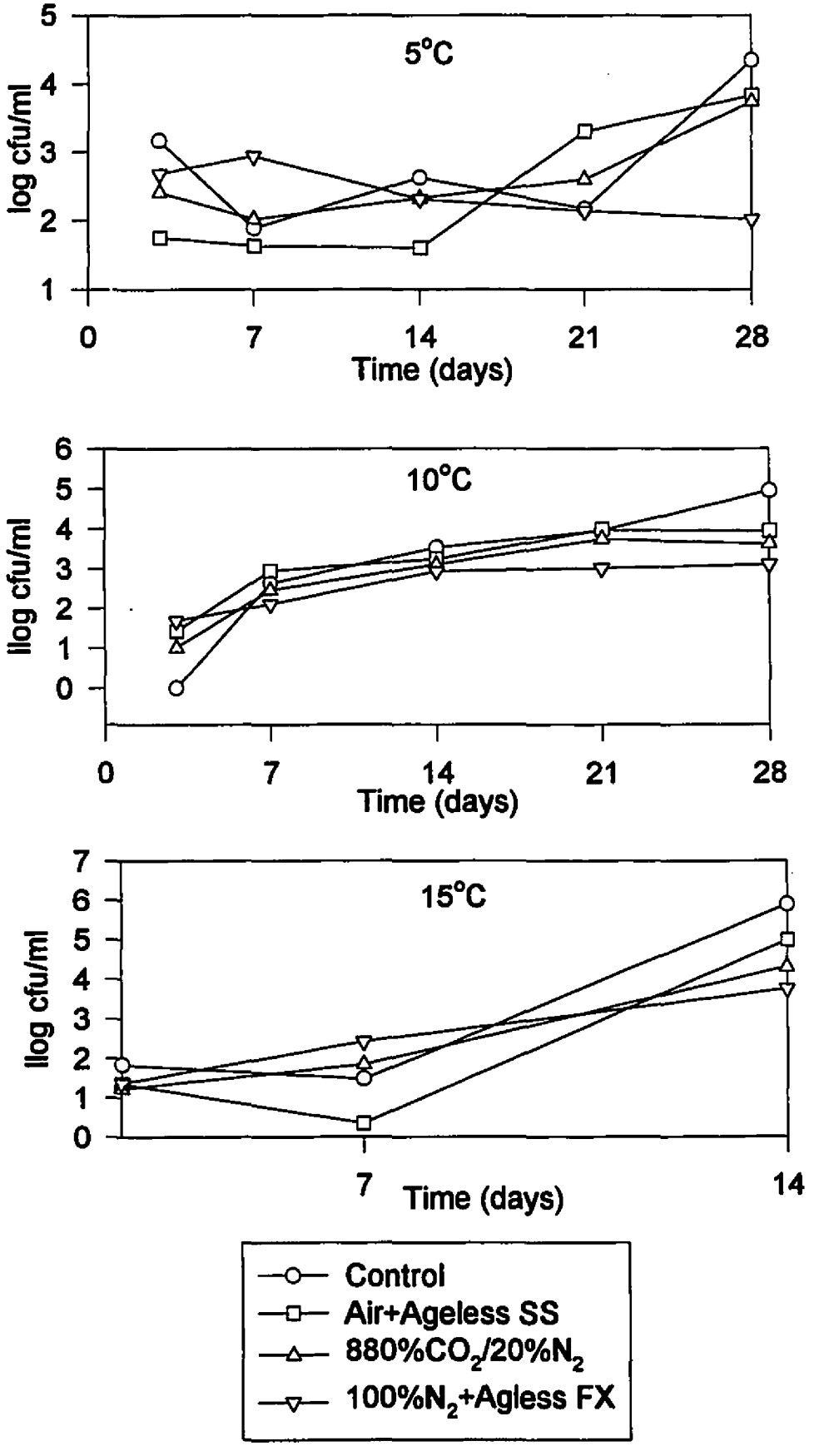


Figure 39: Changes in *L.monocytogenes* counts from drip loss of non-chitosan dipped pork stored at 5, 10 and 15°C

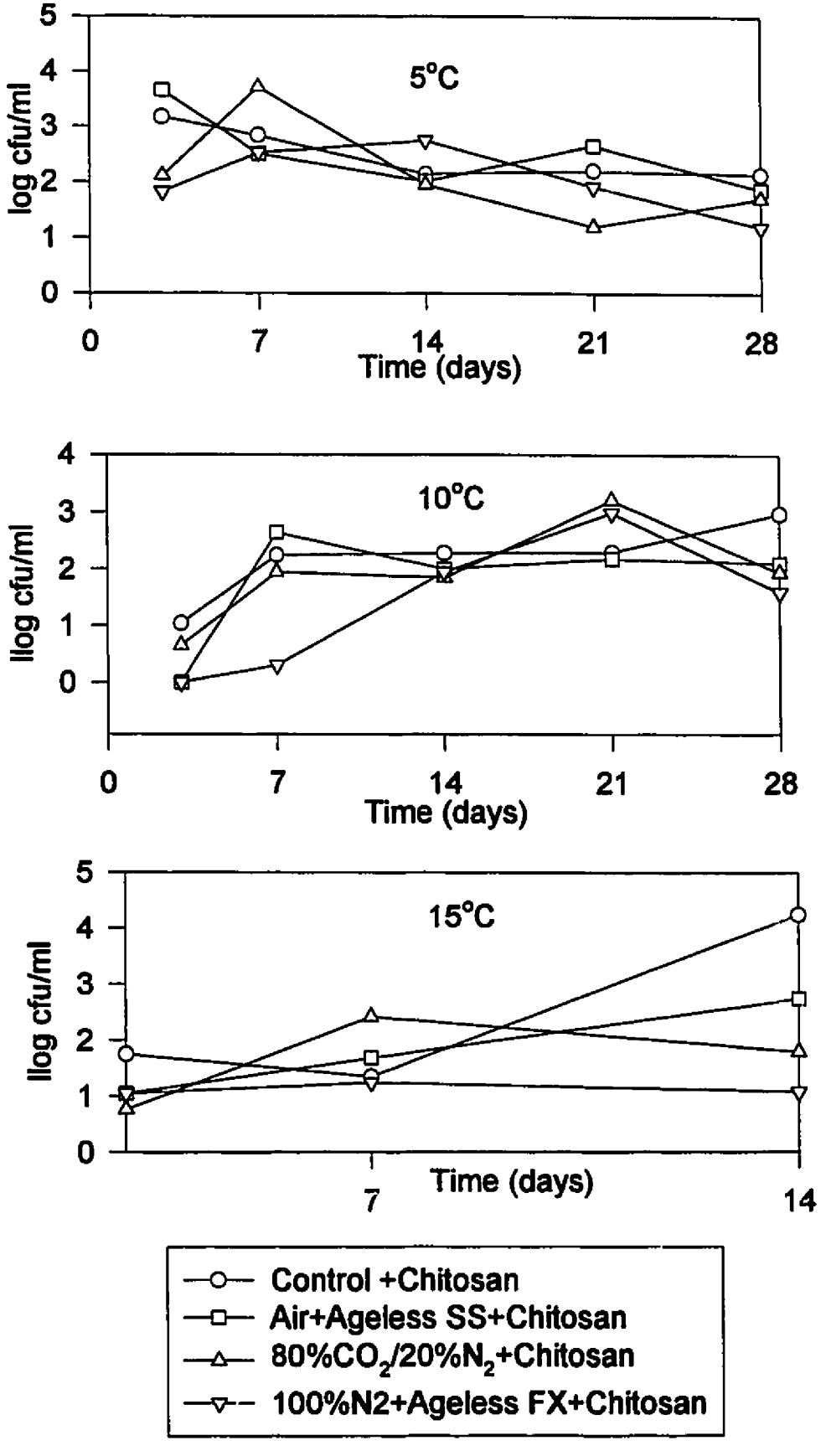


Figure 40: Changes in *L.monocytogenes* counts from drip loss of chitosan dipped pork stored at 5, 10 and 15°C

4.3.7. Shelf Life and Safety

When shelf life and safety data are merged, some interesting results are obtained as shown in Tables 15 and 16.

It is evident from Table 15 that for most treatments, sensory shelf life of non-chitosan dipped pork was still acceptable although counts of *L.monocytogenes* had reached unacceptable levels (approximately 10^7 cfu/g). Therefore, these results would appear to confirm opinions that MAP alone could pose a public health hazard with respect to the growth of *L.monocytogenes.* However, when pork is pre-dipped in a chitosan dipping solution prior to packaging, this trend would appear to be reversed, particularly at 5 and 10°C. At these storage temperatures, spoilage generally precedes growth of *L.monocytogenes* to unacceptable levels. However at 15°C, the synergistic effect of chitosan and MAP is reduced with levels of *L.monocytogenes* reaching unacceptable levels while sensory shelf life was still acceptable.

Table 15: Shelf Life vs. Safety for Non-Chitosan Dipped Pork.

Packaging Conditions	Storage Temperature (°C)	Sensory[a] Shelf-Life (Days)	Safety[b] Shelf-Life (Days)
Air	5	7	11
Air+Ageless SS	5	23	12
80%CO_2/20%N_2	5	25	18
100%N_2+Ageless FX	5	20	27
Air	10	7	7
Air+Ageless SS	10	16	7
80%CO_2/20%N_2	10	25	9
100%N_2+Ageless FX	10	14	14
Air	15	5	3
Air+Ageless SS	15	11	5
80%CO_2/20%N_2	15	7	4
100%N_2+Ageless FX	15	11	3

a: The average time (days) to reach either an odor or color score of 3 out of 5

b: Time (days) required for *L.monocytogenes* counts to reach unacceptable levels, 10^7cfu/g

Table 14: Shelf Life vs Safety for Pork Dipped in Chitosan

Packaging Conditions	Storage Temperature (°C)	Sensory[a] Shelf-Life (Days)	Safety[b] Shelf-Life (Days)
Air	5	7	21
Air+Ageless SS	5	25	24
80%CO_2/20%N_2	5	23	>28
100%N_2+Ageless FX	5	25	>28
Air	10	11	14
Air+Ageless SS	10	16	21
80%CO_2/20%N_2	10	19	28
100%N_2+Ageless FX	10	19	>28
Air	15	5	3
Air+Ageless SS	15	16	3
80%CO_2/20%N_2	15	11	12
100%N_2+Ageless FX	15	14	>14

a: The average time (days) to reach either an odor or color score of 3 out of 5

b: Time (days) required for *L.monocytogenes* counts to reach unacceptable levels, 10^7cfu/g

GENERAL CONCLUSION

Increasing consumer demands for minimally processed, MAP foods has placed an onus on meat processors to ensure the safety of such products. It has been demonstrated that temperature alone cannot be regarded as a reliable barrier since temperature abuse, particularly at the retail level, occurs readily. Therefore, additional barriers, such as antimicrobial dipping solutions, could achieve this additional control, particularly under temperature abuse conditions.

Preliminary studies clearly indicated that chitosan could be used effectively to control the growth of *L.monocytogenes* in a broth system. Further studies in an agar medium indicated that MAP alone or in conjunction with chitosan could also be used to control the growth of *L.monocytogenes* for a substantial time period.

However, when these combinations treatments were extrapolated to fresh pork, somewhat different results were obtained. While MAP alone or in combination with chitosan could be used to delay the growth of meat spoilage microorganisms and *L.monocytogenes*, the extension time observed was significantly less than the time observed in the agar system. This is perhaps to be expected since the meat system may have provided *L.monocytogenes* with nutrients conducive to its growth. Another possible reason for the observed differences in growth may be due to the nature of the substrate, i.e., agar vs. meat. In the latter system, *L.monocytogenes* had to compete with the indigenous spoilage microorganisms of meat. Indeed, these microorganisms may have enhanced the growth of *L.monocytogenes* by providing breakdown products of their own metabolism e.g., amino acids, which would enhance the growth of *L.monocytogenes* Another possible reason for the differences could be attributed to changes in headspace gas composition. Increasing concentrations of CO_2 may have actually stimulated the growth of *L.monocytogenes*. This has been demonstrated in previous studies with *L.monocytogenes* as well as *C.botulinum*. Despite these differences, this study has shown that MAP alone can be used to delay spoilage and the growth of *L.monocytogenes*. However, the results of this study show that the concerns about the growth of *L.monocytogenes* in MAP systems are justified since in nearly all

instance "toxigenesis" preceded spoilage, a potentially dangerous scenario. However, when pork was dipped in chitosan, this trend was reversed, i.e., spoilage generally preceded "toxigenesis" at lower storage temperatures, i.e., 5 and 10°C.

In conclusion, this study has shown that control of the psychrotrophic pathogen, *L.monocytogenes,* can be obtained through combinations of low temperature control, MAP and a 0.2% chitosan dipping solution. Optimum packaging conditions would appear to be 80%CO_2/20%N_2 or 100% N_2 and an Ageless FX oxygen absorbent. This study has also shown that chitosan offers the meat industry a simple, natural and inexpensive "antimicrobial" which could be used to delay the growth of *L.monocytogenes.*

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