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Growth of Yersinia enterocolitica and spoilage microorganisms on fresh pork and minimally processed broccoli packaged in modified atmospheres

Peter William Bodnaruk

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I am submitting herewith a dissertation written by Peter William Bodnaruk entitled "Growth of Yersinia enterocolitica and spoilage microorganisms on fresh pork and minimally processed broccoli packaged in modified atmospheres." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Science and Technology.

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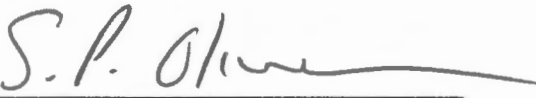Vice Provost and Dean of the Graduate School

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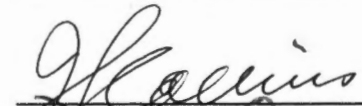
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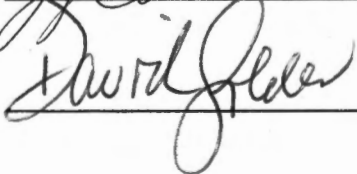
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Growth of *Yersinia enterocolitica* and Spoilage Microorganisms on Fresh

Pork and Minimally Processed Broccoli Packaged in Modified Atmospheres

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Peter William Bodnaruk

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ABSTRACT

Growth and survival of spoilage microflora and pathogenic *Yersinia enterocolitica* were investigated on fresh pork and minimally processed fresh broccoli packaged in modified atmospheres (MA). Phenotypic characteristics of *Y. enterocolitica* were used to detect presence of the virulence plasmid after exposure to each packaging and storage regime. These included Congo red uptake, calcium dependence, and autoagglutination in methyl red-Vogues Proskauer broth and tissue culture medium.

Fresh broccoli was inoculated with 3 to 4 log CFU/g *Y. enterocolitica* immersed in a salt solution, packaged in air, MA and under vacuum and stored at 4 and 10°C. The packaging material used was PD941 (Cryovac), a low barrier multilayered film. *Y. enterocolitica* populations were monitored over 15 days at 4°C and 12 days at 10°C. Loss of the virulence plasmid in *Y. enterocolitica* isolates was examined using phenotypic markers at the completion of each storage period. Populations of *Y. enterocolitica* increased in untreated and salt-treated broccoli during storage at 4 and 10°C. Salt treatment of broccoli did not inhibit growth of *Y. enterocolitica* in each packaging treatment. The virulence plasmid of *Y. enterocolitica* was detected in 13-22% of isolates following salt treatment and storage on minimally processed broccoli.

The microbiological quality of salt-treated minimally processed broccoli packaged in PD941 (Cryovac) film and stored at 4 and 10°C in MA was studied

by examining the fate of spoilage bacteria. Packaged broccoli, stored at 10°C, spoiled more rapidly than broccoli stored at 4°C. Fresh untreated broccoli packaged in air and MA maintained a crisp green appearance for 9 days at 4°C and 6 days at 10°C. Vacuum packaged fresh broccoli had an extended shelf-life of up to 15 days at 4°C. Vacuum packaged salt-treated broccoli showed signs of deterioration within 3 days of packaging, while salt-treated broccoli packaged in air and MA began to deteriorate within 3 to 6 days at 4 and 10°C.

Slices of high (pH>6.0) and normal (pH<5.8) pH pork striploin were inoculated with *Y. enterocolitica*, vacuum packaged or packaged under 100% CO_2 in B900 (Cryovac) barrier bags, and stored at 4°C. Numbers of *Y. enterocolitica* on the lean surface of high pH pork slices increased approximately 3.0 log CFU/cm^2 when vacuum packaged and stored at 4°C for 30 days. Storage of inoculated normal pH pork in 100% CO_2 resulted in *Y. enterocolitica* remaining in lag phase throughout the storage period. The virulence plasmid of *Y. enterocolitica* was maintained in 10-21% of isolates for the duration of storage on vacuum and CO_2 packaged pork.

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CHAPTER I

INTRODUCTION

Modified atmosphere (MA) packaging of foods in conjunction with refrigerated storage is well established in certain sectors of the food industry (Jones, 1989). Benefits of MA packaged foods are increased shelf-life, reduced economic loss, decreased distribution costs and a higher quality product (Farber, 1991). Although there are several benefits to packaging foods in MA, there also exist problem areas and various economic disadvantages such as a specialized equipment requirement. Safety is one of the principal areas of concern regarding MA packaged foods. Advances in MA packaging technology have resulted in a lengthened storage life for some foods, increasing the likelihood of pathogen growth.

The incentive for manufacturing MA foods arises from consumer demands for fresh, chilled, preservative-free products (Farber, 1991). Ready-to-eat foods, such as minimally processed fruit and vegetables, are becoming increasingly popular commodities (Demetrakakes, 1996; Dornblaser, 1995). Fresh fruit and vegetables contain a varied microflora and can become contaminated with pathogens during growth, harvesting, postharvest handling, processing and distribution (Beuchat, 1996). *Yersinia enterocolitica*, a psychrotrophic pathogen, is distributed widely in the environment. Several types, both pathogenic and avirulent, have been isolated from a variety of

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foods including prepared raw vegetables. Pathogenic strains of *Y. enterocolitica* maintain virulence through possession of an unstable virulence plasmid (Kwaga and Iversen, 1991). As yet, there have been no yersiniosis outbreaks reported due to the consumption of contaminated raw vegetables. Nonetheless, presence of this pathogen on raw produce raises concern for the potential of minimally processed vegetables to serve as vehicles for *Y. enterocolitica* infection.

Traditionally, salt has been used for flavor and as a preservative in foods. Recently, salt has been proposed as a possible processing tool in minimally processed vegetables (Lazarides, 1994). Few studies have documented changes in microflora of fresh vegetables treated with salt. Since broccoli is a popular vegetable, eaten both raw and cooked, and as little information is available regarding the microbial quality of the salt-treated product, this research was designed to investigate growth and survival of pathogenic *Y. enterocolitica* and spoilage microorganisms on minimally processed broccoli. The influence of MA packaging, salt treatment and temperature was investigated and the changes in virulence phenotype of *Y. enterocolitica* following storage in a MA was examined.

The shelf-life of fresh meat has been extended by using MA packaging technology (Lambert et al., 1991). Currently, the most common packaging type for fresh meat in retail outlets is overwrapped trays. A growing trend toward centralized prepackaging of retail portions of meat in MA is evident in

European countries and is currently under consideration by several companies in the United States (Nunes, 1995). *Y. enterocolitica* is frequently isolated from pigs and pork products (Feng and Weagant, 1994). Studies show that *Y. enterocolitica* is capable of growth and survival on refrigerated MA packaged meats (Doherty et al., 1995; Gill and Reichel, 1989; Hudson et. al., 1994). Concern has been expressed regarding the presence of *Y. enterocolitica* on MA packaged fresh meats. However, no study has shown changes in pork microflora in the presence of *Y. enterocolitica* and the fate the *Y. enterocolitica* virulence plasmid following passage through a pork packaging and storage regime. The aim of the second aspect of this research was to investigate growth and survival of pathogenic *Y. enterocolitica* on fresh pork as influenced by ultimate meat pH and MA packaging and to examine changes in virulence of the organism following storage in a MA.

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CHAPTER II

REVIEW OF LITERATURE

MINIMALLY PROCESSED VEGETABLES

Minimally processed vegetables are products that consist of live tissue that has been slightly modified from its fresh-condition but still maintains a like-fresh character (Wiley, 1994). Modification of fresh vegetables can occur by using one or a combination of several treatments. These include washing, slicing, peeling, shredding or treatment with a preservative agent. Initial preparation of minimally processed vegetables is followed usually by packaging in a modified atmosphere (MA) then storage in a reduced temperature environment. Each of these treatments adds value to the raw product and facilitates convenient consumption and distribution to the consumer (King and Bolin, 1989).

The primary aims of minimally processed vegetable producers are to extend the shelf-life of the vegetable so that it remains safe and wholesome (Day, 1992). Two basic factors associated with the extension of shelf-life of minimally processed vegetables, are the physiology of live vegetative tissue and control of the growth of spoilage and pathogenic microorganisms (King and Bolin, 1989).

Respiration in vegetables is a complex phenomenon involving the breakdown of energy sources such as carbohydrates and organic acids,

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resulting in the production of heat (Day, 1992). Depending on the type of respiration, the end-products may vary. Aerobic respiration leads to production of carbon dioxide (CO_2) and water while anaerobic respiration products include ethanol, acetaldehyde and various organic acids (Day, 1992). The metabolic activity of minimally processed vegetables varies with each product type, processing and storage conditions (Gorris, 1994). Any processing operation such as slicing or cutting, disrupts the cellular structure of the vegetable leading to increased respiration and enzyme activity (Shewfelt, 1994). As minimally processed vegetables have an active metabolism, an increase in respiratory and metabolic activity will compromise quality and consequently shorten shelf-life.

Microbial growth on minimally processed vegetables is affected by several factors. These include the number and types of microorganisms present on the vegetable, the ability of the vegetable to support microbial growth, the extent of tissue damage due to processing, storage temperature and surrounding gas atmosphere (Brocklehurst et al., 1987; Hotchkiss and Banco, 1992). Each factor plays a role in determining the kind of microorganisms that will survive and grow (Brackett, 1987). A variety of microorganisms can grow on minimally processed vegetables. If allowed to grow without restriction, spoilage organisms such as pectinolytic bacteria will cause breakdown of tissue resulting in deterioration and a shorter shelf-life. Preservation methods for extending shelf-life of minimally processed

vegetables, such as refrigeration and MA packaging, can improve their stability during storage and distribution.

PRESERVATION OF MINIMALLY PROCESSED FRESH VEGETABLES

Spoilage of minimally processed vegetables is a result of microbial degradation as well as degradation of physical characteristics such as color, texture and flavor (Huxsoll and Bolin, 1989). Efforts to extend product shelf-life have incorporated several treatments to limit microbial growth. The combination of these treatments for the preservation and safety of foods is referred as the "hurdles concept" (Scott, 1989). In minimally processed vegetables several hurdles are assigned for each vegetable type depending on the ultimate quality requirements (Huxsoll and Bolin, 1989).

Storage temperature

Low temperature storage can be used as a hurdle assisting in the inhibition of microbial growth. Temperature control during processing, packaging and storage of fresh vegetables is an important factor affecting quality of minimally processed vegetables. At chilled temperatures (0 to 5°C), vegetable respiration rates are lowered significantly and the growth of some spoilage and some pathogenic microorganisms is inhibited (Day, 1992).

Modified atmosphere packaging

Vacuum or MA packaging may be used as a hurdle to extend the shelf-life of minimally processed vegetables by inhibiting microbial growth and reducing the rate of vegetable respiration (Zagory and Kader, 1988). Atmospheres can be created actively, by packaging the vegetable in a polymeric film and gas flushing with a desired atmosphere, or passively by packaging the vegetable in a hermetically sealed package and relying on the vegetable's respiration to generate the atmosphere (Day, 1992). The aim of MA packaging, whether active or passive, is to establish an equilibrium MA (EMA) and to prevent formation of ethylene essential to yellowing processes (Berrang et al., 1990; Day, 1992). An EMA is established when the vegetable's respiration rate equals rates of oxygen (O_2) and CO_2 transmission through the package (Day, 1992). The EMA established within a package is dependent on an individual vegetable's respiration rate and other factors such as storage temperature and relative humidity (Zagory and Kader, 1988).

Each vegetable type packaged in a MA has tolerance limits for O_2 and CO_2 levels (Day, 1992). These limits are dependent on produce type, storage temperature, maturity, physiological condition and whether the vegetable has been exposed to any previous treatment. Packaging minimally processed vegetables outside these atmospheric limits can result in physiological damage, development of off-flavors and can affect adversely product quality (Huxsoll and Bolin, 1989).

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When adopting a particular MA for vegetable packaging, consideration must also be assigned to the atmospheric effects on the microflora. The three main gases used in MA packaging of foods are CO_2, O_2 and nitrogen (N_2). The effect of CO_2 on microorganisms is related to its ability to interfere with cellular metabolism through alteration of intracellular pH and inhibition of enzyme systems (Daniels et al., 1985; Lambert et al., 1991a). Effects of CO_2 on bacteria vary depending on the concentration, organisms present and storage temperature (Daniels et al., 1985). Microorganisms exhibiting greatest sensitivity to CO_2 are Gram-negative bacteria, while lactic acid bacteria and anaerobes show resistance (Jay, 1992). Oxygen normally stimulates growth of aerobic bacteria and can restrict growth of obligate anaerobes (Farber, 1991). Nitrogen has little effect on microorganisms and is used mainly as a filler or to displace O_2 assisting in inhibition of aerobic bacteria.

Films used for MA packaging of minimally processed vegetables have requirements for gas permeability, water vapor transmission, mechanical strength, sealability and transparency (Day, 1992). The type of package used depends on the particular vegetable and its packaging requirements for preservation of quality.

Water activity

Water activity (Aw) reduction can be used as a hurdle to increase the shelf stability of minimally processed vegetables (Huxsoll and Bolin, 1989).

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Fresh fruit and vegetables are high moisture commodities and have Aw values between 0.97 and 1.00 (Day, 1992). As the Aw of minimally processed vegetables is high, growth of pathogens and spoilage microorganisms can continue without inhibition (Jay, 1992). To inhibit growth of some microorganisms and increase their sensitivity to other inhibitors and inhibitory conditions, a process known as osmotic dehydration can be used (Scott, 1989). Osmotic dehydration of minimally processed vegetables involves reduction of surface Aw by removal of product water through direct contact with a hypertonic medium, for example, a high concentration sugar or salt solution (Lazarides, 1994). When osmotic dehydration is used exclusively for minimally processed vegetable preservation, flavor defects such as excessive saltiness or sweetness have been associated with dehydrated products (Huxsoll and Bolin, 1989).

MINIMALLY PROCESSED VEGETABLES - SAFETY ISSUES

Due to the proximity of vegetables to the soil and the possibility of irrigation with contaminated waters, food poisoning bacteria are of concern on minimally processed vegetables (King and Bolin, 1989). Cutting, slicing or peeling destroys integrity of the protective exterior of vegetables and provides microorganisms with access to internal tissue (Shewfelt, 1994). Shelf-life extension of minimally processed vegetables, through MA packaging and refrigeration, is an area of concern due to increased health risks associated

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with pathogenic microorganisms (Hotchkiss and Banco, 1992). Modified atmosphere packaging technology extends the edible shelf-life of minimally processed vegetables and could allow also sufficient time for psychrotrophic pathogens, such as *Listeria monocytogenes* and *Yersinia enterocolitica*, to grow (Hotchkiss and Banco, 1992). In situations where temperature abuse of minimally processed vegetables packaged for retail sale is a possibility, pathogens such as *Salmonella* spp., *Staphylococcus aureus* and *Bacillus cereus* may be a potential food poisoning problem (Gorris and Peppelenbos, 1992; Ronk et al., 1989). Where a gas flush system failure may occur, anaerobic pathogens such as *Clostridium botulinum* may have an opportunity to proliferate and produce toxin (Farber, 1991; Gorris and Peppelenbos, 1992). Under normal vegetable processing conditions, contamination with pathogens alone is unlikely to cause foodborne disease (Hotchkiss and Banco, 1992). The vegetable, its physiological state and storage conditions must be suitable for pathogen growth to increase the chance for a foodborne disease occurrence.

PACKAGING MINIMALLY PROCESSED BROCCOLI

Broccoli (*Brassica oleracea* L.) is considered one of the most perishable vegetables and requires storage temperatures around 0°C to maintain quality (Rij and Ross, 1987). Its visual and organoleptic qualities are largely dependent on storage conditions. Broccoli is classified as a vegetable

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with a high respiration rate. At a storage temperature of 10°C whole heads of broccoli respire at 59 mL CO_2/kg/hr, while cut florets respire at 78 mL CO_2/kg/hr (Ballantyne, 1989). The difference in respiration rates between cut florets and whole heads is due mainly to the amount of cut surface in each product (Rushing, 1990).

An effective atmospheric environment for MA packaged broccoli has been investigated. Factors such as gas and water vapor permeability of the packaging material, respiration rate and tolerance of the product to CO_2 and O_2 are important for ensuring a suitable product. Several authors investigated broccoli stored under various MA treatments at different storage temperatures in packaging material of both high and low gas permeability (Ballantyne et al., 1988; Liebermann and Hardenburg, 1954; Lipton and Harris, 1974; Makhlouf et al., 1989). Problems with product quality as a result of MA storage were primarily in the development of off-odor compounds, for example methanethiol, changes in turgidity, yellowing, soft rots, mold and opening of flower buds (Forney et al., 1991; Makhlouf et al., 1989).

Several authors have reported their findings regarding optimum storage conditions for broccoli (Ballantyne et al., 1988; Barth et al., 1993; Bastrash et al., 1993; Lipton and Harris, 1974; Makhlouf et al., 1989). Leshuk and Saltveit (1990) listed the beneficial O_2 and CO_2 concentrations for packaged broccoli as 1 to 3% and 5 to 10%, respectively. If packaged in an EMA that maintains these levels, injury caused by an excess of CO_2 or

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deficient levels of O_2 will be minimized.

PRESERVATION OF FRESH MEATS

Several factors influence shelf-life of fresh meats. These include storage temperature, availability of O_2, moisture, indigenous enzymes and light (Lambert et al., 1991a). Meat color, odor, texture and flavor are all affected by these factors to a degree. Spoilage of meat is most often due to the action of a mixed population of microorganisms (Jay, 1992). Variations in storage temperature and conditions, such as vacuum and MA packaging, have allowed meat spoilage to be retarded and resulted in an extended shelf-life for fresh meats.

Meat spoilage

Fresh meat is a nutritionally rich environment capable of supporting the growth of a variety of microorganisms (Blickstad and Molin, 1983). The initial microflora of a carcass taken from the slaughter line is diverse. Organisms best suited to the environmental conditions will compete successfully with others and dominate the spoilage flora. The major spoilage bacteria of meat are Gram-negative bacteria, including aerobic mesophiles, psychrotrophs and facultative anaerobes (Gill and Newton, 1978). Gram-positive bacteria such as *Lactobacillus* spp. and *Brochothrix thermosphacta* have been found also in high numbers on fresh meat and can contribute to meat spoilage depending

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on packaging conditions (Egan and Shay, 1982).

Meat composition influences spoilage flora and the pattern of meat spoilage (Lambert et al., 1991a). During development of postmortem rigor, several biochemical changes occur in meat that affect microbial growth. In normal pH meat, some glycogen is converted to lactate reducing meat pH to 5.8 or below (Jay, 1992). Spoilage of post-rigor meat of normal pH is delayed due to the reserve supply of glycogen (Gill and Newton, 1979). Spoilage bacteria present on meat deplete the available glycogen before consuming amino acids and creating associated spoilage odors. Where meat has a high ultimate pH (>6.0), residual glycogen levels are low, and consequently, there are lower levels of lactate. Due to a limited supply of muscle glycogen, amino acids are metabolized and spoilage becomes evident at lower bacterial population levels.

Modified atmosphere packaged meats

Methods for long term preservation of fresh meat include refrigeration, irradiation and packaging under a MA or vacuum. Of the various methods available, refrigeration is the most important (Lambert et al., 1991a). Modified atmosphere storage of meats reduces microbial growth, retards enzymatic spoilage and assists in extending meat shelf-life (Farber et al., 1990). Packaging fresh meat in a MA involves surrounding the meat in an environment where gaseous components are something other than air

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(Farber, 1991). A major factor limiting shelf-life of fresh meat is the presence of atmospheric O_2 (Lambert et al., 1991a). The presence of O_2 stimulates growth of aerobic spoilage and pathogenic bacteria and has a detrimental effect on meat color over long term storage (Farber, 1991; Penney and Bell, 1993).

Two methods used commonly to eliminate O_2 from packaged meats are vacuum packaging and gas flushing with CO_2. Vacuum packaging of fresh meat is used widely in the meat industry and is the most common method of modifying the internal package environment (Lambert et al., 1991a). Packaging and storage of fresh meat in a gas other than air has been the subject of much investigation (Farber, 1991).

Effects of various mixtures of O_2, CO_2 and N_2 on microbial populations and meat color have been studied extensively. Oxygen can be used in MA packaging of meat to help maintain myoglobin in its oxygenated form and preserve the red color. The main function of N_2 in a gas mix is to act as filler gas and prevent package collapse. Carbon dioxide is used in a MA gas mix for its bacteriostatic properties (Farber, 1991). As CO_2 is highly soluble in water and oils, when applied to meat in rigid packs, it is absorbed by muscle and fat tissues until an equilibrium is reached (Gill, 1988). Use of high concentrations of CO_2 (20 to 100%) in MA meat packaging has been shown to retard microbial growth, particularly pseudomonads on beef, pork and chicken (Blickstad and Molin, 1983; Grau et al., 1985).

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Gram-negative spoilage organisms are inhibited by concentrations of 20% CO_2 or greater, while Gram-positive organisms are usually resistant to inhibition (Farber, 1991; Gill, 1988; McMullen and Stiles, 1991). The specific method by which CO_2 exerts its bacteriostatic effect is unknown although it is believed that CO_2 alters intracellular pH of bacterial cells and disrupts enzymic activity (Lambert et al., 1991a). Inhibitory effects of CO_2 on microorganisms are dependent on several factors. These include types of microorganisms present on the meat, concentration and partial pressure of CO_2, headspace volume, microbial growth phase and storage temperature (Farber, 1991; Lambert et al., 1991a). To exploit the maximum antimicrobial effect of CO_2, MA packaged meats are kept at chilled temperatures as the solubility of CO_2 increases with decreasing temperature (Gill, 1988).

Case-ready meats

Preparation of case-ready meats involves centralized prepackaging of retail sized meat portions in modified atmospheres (Nunes, 1995). Several variations in package design have been utilized in case ready-meat production. These include vacuum skin packaging, barrier trays with barrier-lidded materials and master packaging, where multiple pouches packaged with gas permeable film are enclosed in a large barrier bag. Case-ready technology will not improve the quality of fresh meats but will provide economic benefits due to centralized packaging and shelf-life extension. The

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growing consumer trend focused on self service retailing has encouraged companies in the United States to consider the concept of case-ready meats as a feasible option for distribution and sale of fresh meat.

MICROBIOLOGICAL SAFETY OF
MODIFIED ATMOSPHERE PACKAGED MEATS

Concern has been expressed about the safety of MA packaged meats with regard to foodborne pathogens (Farber et al., 1990). Normal meat microflora are used by consumers as indicators of meat spoilage. By using MA packaging, spoilage can be retarded and the associated organoleptic changes inhibited, thereby concealing possible spoiled meats.

Despite favorable growth conditions for strict anaerobes such as *C. botulinum* (non-proteolytic), there is little concern over the product's safety regarding this pathogen (Hauschild et al., 1985). There are several reasons why *C. botulinum* receives little attention in MA packaged meats, the main one being that spoilage and putrefactive changes precede toxigenesis (Hauschild et al., 1985; Lambert et al., 1991b). Therefore, organoleptic deterioration in MA packaged meats due to contamination with sporulating *C. botulinum* would give retailers and consumers ample warning that the product was unsafe for consumption (Hauschild et al., 1985).

In recent years attention has focused on *Aeromonas hydrophila*, *Listeria monocytogenes* and *Yersinia enterocolitica* as potential hazards in MA

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packaged meats since these organisms are capable of growth at 0 to 5°C (Jay, 1992). The magnitude of the health risk posed by the presence of these pathogens on MA packaged food, such as meats, increases the risk for foodborne illness outbreaks (Palumbo, 1986).

YERSINIA ENTEROCOLITICA

Y. enterocolitica was first recognized as human pathogen in the 1930's (Jay, 1992). In recent years there has been an increase in the reported incidence of *Y. enterocolitica* infections. In the United States there are up to 20,000 cases of yersiniosis per year (Feng and Weagant, 1994). Infections are caused mainly by ingestion of fecally contaminated food or water, although many infections are the result of animal-to-human transmission (Boyd and Hoerl, 1981).

Characteristics

Y. enterocolitica is a Gram-negative, facultatively anaerobic, rod-shaped, psychrotrophic bacterium, occurring singly or arranged in small clusters (Feng and Weagant, 1994). Biochemical features of *Y. enterocolitica* that allow it to be distinguished from other members of the *Enterobacteriaceae* are listed in Table 2-1.

Table 2-1. Biochemical characteristics of *Yersinia enterocolitica*

Reaction	*Y. enterocolitica*
Lysine	-
Arginine	-
Ornithine	+
Motility at	
22-26°C	+
35-37°C	-
Urea	+
Mannitol	+
Sorbitol	+
Cellobiose	+
Adonitol	-
Inositol	±
Sucrose	+
Rhamnose	-
Raffinose	-
Melibiose	-
Simmons citrate	-
Vogues-Proskauer	±
Indole	±
Salicin	±

(Weagant et al., 1992)

Infections caused by *Y. enterocolitica* can present a broad range of symptoms dependent on factors such as age and physical condition of the host (Prpic and Hughes, 1989). Cases of gastroenteritis and pseudoappendicitis appear most frequently in childhood and adolescence, whereas cases of acute abdominal disorders, diarrhea and arthritis occur mainly in adults (Palumbo, 1986). The most common symptoms associated with yersiniosis are abdominal pain accompanied by fever (Feng and Weagant, 1994). Other symptoms such as self-limiting watery diarrhea, headache and vomiting may also be associated with the illness. Gastrointestinal symptoms usually subside after two or three days, although symptoms can persist in a milder form for one to three weeks. More severe complications that can develop in some patients are an ulcerative enterocolitis that may become chronic and persist for several months. When *Y. enterocolitica* invades the lymph system, symptoms of fever and abdominal pain can be confused with acute appendicitis. Several incidences of unnecessary appendectomies performed on yersiniosis patients have been reported in past outbreaks (Palumbo, 1986).

Pathogenesis

The pathogenicity of *Y. enterocolitica* is complex and has not been fully established (Feng and Weagant, 1994). Virulence in *Y. enterocolitica* varies according to biotype and possession of a 40-50 megadalton virulence

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plasmid (Prpic et al., 1985). Multiple factors encoded by both chromosomal and plasmid genes appear to be involved in the organism's ability to cause disease. These pathogenic factors include invasion genes, an endotoxin, an enterotoxin, iron-regulated proteins and plasmid-encoded virulence factors.

The *Y. enterocolitica* endotoxin is not a major factor in foodborne illness, although it is significant in nosocomial infections such as endotoxic shock from contaminated blood supplies (Feng and Weagant, 1994). The *Y. enterocolitica* enterotoxin is a heat stable, thermoregulated toxin produced by both pathogenic and nonpathogenic serotypes. The enterotoxin has been detected only *in vitro* and is produced only at incubation temperatures between 4 and 30°C. Consequently, its role in foodborne illness is still under investigation (Prpic and Hughes, 1989).

Iron is essential for *Y. enterocolitica* growth and pathogenicity. Where growth conditions are iron-deficient, *Y. enterocolitica* can produce several chromosomally encoded iron-regulated proteins. The precise role of iron-regulated proteins in *Y. enterocolitica* pathogenicity has not been determined although it is believed they are involved in the uptake of iron during infection (Feng and Weagant, 1994).

Cellular invasion genes are present in pathogenic and nonpathogenic strains of *Y. enterocolitica*. Two chromosomally located genetic loci that encode cellular invasion properties have been identified; these are the *inv* (invasion) and *ail* (attachment invasion locus) genes (Feng and Weagant,

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1994). Genetic studies have found that the presence of these two genes is associated closely with disease-causing *Y. enterocolitica*, whereas avirulent, noninvasive varieties have a nonfunctional *inv* gene and do not carry the *ail* gene. The role of invasion genes is thought to enable *Y. enterocolitica* to penetrate intestinal walls and to allow colonization.

The virulence plasmid of *Y. enterocolitica* encodes several virulence-related determinants such as autoagglutination, serum resistance, reduced ability to grow in low calcium environments and production of surface antigens and outer membrane proteins (Prpic and Hughes, 1989). These properties are expressed at 37°C and not at 25°C. Surface antigens, particularly the V antigen, are believed to facilitate survival of *Y. enterocolitica* within macrophages, while other antigens provide resistance to the bacteriocidal effects of serum. Although details concerning the pathogenesis of *Y. enterocolitica* have not been defined fully, a likely depiction of the infection process proposed by Prpic and Hughes (1989) is shown in Table 2-2.

Epidemiology

Y. enterocolitica has been isolated from humans throughout the world, although geographic differences in frequency and distribution are evident (Cover and Arber, 1989). Infections with *Y. enterocolitica* are important in cool temperate countries, whereas yersiniosis is uncommon in the tropics (Prpic and Hughes, 1989).

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Table 2-2. Pathogenesis of infection with *Yersinia enterocolitica*

Stage of infection	Virulence attribute	Possible mechanism(s)	Location of genes
1. Colonization	Adhesion	Hydrophilic surface; surface fibrillae; motility	Chromosome and virulence plasmid
2. Invasion	Stimulation of parasite mediated endocytosis	Specific surface antigens	Chromosome
3. Proliferation	Resistance to phagocytosis	Hydrophobic surface; outer membrane proteins	Virulence plasmid
	Survival within phagocytes	V antigen; outer membrane proteins	Virulence plasmid
	Resistance to killing by serum	Hydrophobic surface; outer membrane proteins	Virulence plasmid

(Prpic and Hughes, 1989)

Y. enterocolitica has been isolated from a variety of animals, including domestic pets, wild animals, pigs, cattle and sheep, where pigs are recognized as the main reservoir of the organism (Cover and Arber, 1989). *Y. enterocolitica* has been isolated also from inanimate reservoirs such as lakes, streams, soil and vegetables. Documented transmission of *Y. enterocolitica* to humans has occurred mainly by consumption of contaminated foods, water and milk (Cover and Arber, 1989; Feng and Weagant, 1994). The ability of *Y. enterocolitica* to grow at low temperature allows enrichment of the pathogen in cold environments such as water and refrigerated food and milk. Several outbreaks of yersiniosis recorded in the United States over the past two decades were associated with pasteurized milk, nonchlorinated drinking water and pork products (Feng and Weagant, 1994).

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CHAPTER III

EFFECT OF PACKAGING ATMOSPHERE AND pH ON THE VIRULENCE AND GROWTH OF *YERSINIA ENTEROCOLITICA* ON PORK STORED AT 4°C

ABSTRACT

Growth of pathogenic *Yersinia enterocolitica* was investigated on high (pH>6.0) and normal (pH<5.8) pH pork packaged in modified atmospheres and stored at 4°C. Modified atmospheres used in the study were vacuum packaging and saturated CO_2. Pork was packaged in a high gas barrier packaging film and examined over a 30 day period. Phenotypic characteristics were used to detect presence of the virulence plasmid of *Y. enterocolitica* after exposure to the pork packaging and storage regime. These included Congo red uptake, calcium dependence and autoagglutination in methyl red Vogues-Proskauer broth and tissue culture medium. Numbers of *Y. enterocolitica* on the lean surface of high pH pork slices increased approximately 3.0 log CFU/cm^2 when vacuum packaged and stored at 4°C for 30 days. Storage of inoculated normal pH pork in 100% CO_2 resulted in *Y. enterocolitica* remaining in the lag phase over the storage period. Virulence in *Y. enterocolitica* was maintained for the duration of storage on vacuum and CO_2 packaged meats.

INTRODUCTION

Refrigerated storage (0 to 4°C) is one of the most important methods of preservation used in the meat industry (Lambert et al., 1991). When coupled with modified atmosphere (MA) packaging, spoilage of fresh meat can be retarded by inhibiting spoilage flora and retarding enzymatic degradalton (Young et al., 1988). As aerobic spoilage organisms usually warn consumers of meat spoilage, MA packaging may inhibit spoilage flora and may allow growth of psychrotrophic pathogens such as *Yersinia enterocolitica* and *Listeria monocytogenes* (Farber, 1991). Centralized prepackaging of meat in MA has been proposed as an effective method for retail sale (McMullen and Stiles, 1991). Production of pre-cut, MA packaged fresh meat that is ready for retail sale appears to be a current trend in the North American meat industry (Nunes, 1995). Vacuum or MA packaging meat of normal (pH<5.8) and high (pH>6.0) pH may lengthen the shelf-life when compared with aerobic storage, but may also increase the risk of foodborne illness (Farber, 1991). *Y. enterocolitica* frequently is associated with pigs and pork products and can be transmitted to humans by consumption of raw, undercooked or recontaminated processed meats (Manu-Tawaih et al., 1993; Rodriguez et al., 1994).

Pathogenic strains of *Y. enterocolitica* harbor a 40-50 megadalton (MDa) plasmid that confers virulence on the infecting organism (Prpic et al., 1985). Loss of the plasmid results in loss of virulence characteristics (Bhaduri

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and Turner-Jones, 1993). Both virulent and avirulent strains of *Y. enterocolitica* have been isolated from foods (Stern, 1982). Effects of consuming avirulent varieties may be no more hazardous than consuming nonpathogenic coliform bacteria. The stability of the virulence plasmid has been investigated under various environmental conditions. Bodnaruk and Golden (1996) reported that *Y. enterocolitica* is capable of retaining virulence after exposure to reduced pH. Bhaduri and Turner-Jones (1993) attempted to assess the stability of the virulence plasmid in *Y. enterocolitica* under various anaerobic conditions found in food processing. No plasmid loss with virulent *Y. enterocolitica* in logarithmic and stationary phase was observed when exposed to a vacuum or to an atmosphere of 94% CO_2: 6% H_2 for 24 h at 28°C.

The objective of this study was to investigate growth and survival of pathogenic *Y. enterocolitica* on MA packaged fresh pork of normal and high pH stored at 4°C and to examine possible changes in virulence following exposure of *Y. enterocolitica* to a food processing regime. Normal and high pH meat was chosen due to the differences in rates of spoilage when packaged without O_2 (Gill and Newton, 1979).

MATERIALS AND METHODS

Bacterial strains

Three strains of *Y. enterocolitica*, YE133 (serotype O:8), YE228 (serotype O:3) and YE321 (serotype O:20) (obtained from Peter Feng, United States Food and Drug Administration, Washington, DC), were used for inoculation studies. Stock cultures were stored on trypticase soy agar (TSA; BBL, Cockeysville, MD) slants at 4°C.

Preparation of inocula

Pure cultures of *Y. enterocolitica* were grown for 48 h at 25°C in trypticase soy broth (TSB; BBL, Cockeysville, MD). Cultures were pooled and added to 1 L of 0.1% peptone water (PW; Difco, Detroit, MI) to give an approximate final concentration of 10^6 CFU/mL. The diluted culture of *Y. enterocolitica* was used for meat inoculation.

Preparation of meat samples

Pork striploins (*M. longissimus dorsi*) were obtained from a local supplier. High and normal pH muscles were selected based on lean tissue color. Normal pH pork has a pale pink color while high pH pork has a dark red appearance (Wilson et al., 1981). All pH values were checked in the laboratory using a Corning 350 pH/ion analyzer (Corning, NY) by direct application of a glass electrode to the muscle. Muscle tissue was trimmed of

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fat and divided into steaks approximately 1.5 cm thick. Pork steaks were inoculated with *Y. enterocolitica* by immersion for 30 sec in the diluted pure culture. This gave a surface inoculum level of approximately 10^3 CFU/cm^2. Uninoculated control slices of pork were dipped in sterile water. Steaks were allowed to dry for 10 min on sterile racks.

Meat packaging

The packaging film used was B-900 (Cryovac, Duncan, SC), a multilayered polyolefin with gas barrier properties (O_2 transmission rate - 3-6 mL/m^2/24 h at 4.4°C and 0% RH). A Multivac gas flushing machine (Koch, Kansas City, MO) was used to achieve the desired atmosphere. Two packaging atmospheres were used, vacuum and 100% CO_2 (MG Industries, Valley Forge, PA). Slices of inoculated and uninoculated pork were either vacuum packaged or packaged in 100% CO_2.

Storage and sampling

Pork loins were tested for the presence of *Y. enterocolitica* at the time of packaging. Packaged pork samples were stored at 4°C. On each sampling day the gas composition of CO_2 packages was determined using a Hewlett Packard gas chromatograph, model 5890-series II (Hewlett Packard, Wilmington, DE) equipped with a thermal conductivity detector (TCD) and a stainless steel CTRI column (Alltech Associates, Inc., Deerfield, IL). Column

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and TCD temperatures were set at 25°C, the quantity of sample injected was 5 mL and the total run time was 3 min. Helium was used as the carrier gas at a flow rate of 40 mL/min. No gas composition analysis was made on vacuum packaged pork slices because drip present in the package interfered with sample collection.

Using a cork borer, 4 samples (each 3.5 cm^2 x ca. 0.5 cm deep) were taken from the lean surface at 5-day intervals throughout the 30-day storage period. The lean tissue samples were blended with 0.1% PW in a Model 400 Stomacher Lab Blender (Steward, London, UK) for 1 min. Aliquots (0.1 mL) of serial dilutions were surface plated onto appropriate solid media for bacterial enumeration and identification. Inoculated pork slices were sampled for *Y. enterocolitica* only, while uninoculated pork slices were sampled for normal spoilage flora. The media and conditions for enumeration and isolation of microorganisms were as follows: *Y. enterocolitica*, cefsulodin irgasan novobiocin agar (CIN; BBL, Cockeysville, MD) incubated at 30°C for 24 to 48 h; aerobic mesophiles, tryptone yeast extract soy glucose agar (TYSG; Grau et al., 1985) incubated at 25°C for 72 h; lactic acid bacteria, De man, Rogosa, Sharpe agar (MRS; Oxoid, Basingstoke, UK) incubated at 25°C for 72 h; *Brochothrix thermosphacta*, streptomycin thallous acetate actidione agar (STAA; Gardner, 1966) incubated at 25°C for 72 h and Gram-negative bacteria, peptone agar (PEPA; Grau, 1983) incubated at 25°C for 48 h.

Identification of microorganisms

Bacterial colonies on CIN agar were identified as *Y. enterocolitica*, using motility and biochemical tests (Weagant et al., 1992). Gram-positive, catalase negative colonies selected from MRS agar were presumed to be lactic acid bacteria. Oxidase negative colonies selected from STAA were identified as *B. thermosphacta* (Gardner, 1966). To determine the count of Gram-negative bacteria, colonies picked from PEPA were tested for Gram reaction, using the method of Buck (1982). Colonies counted on TYSG were recorded as an aerobic plate count (APC).

Virulence testing

Isolates of *Y. enterocolitica* were tested for virulence before inoculation onto pork and after storage. The reliability of indicator tests for virulence has been reported by Kwaga and Iversen (1991) and Prpic et al. (1985). As no individual virulence-associated characteristic is a reliable single indicator of virulence, several testing methods were used (Kwaga and Iversen, 1991). These included Congo red uptake and calcium dependence (CRMOX) (Riley and Toma, 1989), autoagglutination (AA) in methyl red Vogues-Proskauer media (MR-VP) (Weagant et al., 1992) and AA in tissue culture media RPMI-1640 (Laird and Cavanaugh, 1980).

Statistical analysis

The General Linear Models (GLM) procedure was used to analyze main effects and interactions of packaging atmospheres, storage time and lean tissue pH on differences in mean microbial numbers (SAS Institute, Inc., 1990). Three replicates were conducted for all experiments.

RESULTS

Meat examined prior to inoculation was found to be free of *Y. enterocolitica*. At 4°C, *Y. enterocolitica* grew on high pH pork slices, reaching 5.8 log CFU/cm^2 after 30 days storage on vacuum packaged pork and 4.8 log CFU/cm^2 on pork packaged under CO_2 (Figure 3-1). Normal pH pork supported growth of *Y. enterocolitica* when vacuum packaged, reaching 5.2 log CFU/cm^2 after 25 days storage at 4°C. *Y. enterocolitica* packaged in CO_2 on normal pH pork survived in an extended lag phase, maintaining a level of approximately 3 log CFU/cm^2.

Lactic acid bacteria became the dominant component of the flora on uninoculated pork after 30 days storage at 4°C, reaching between 6.8 and 7.6 log CFU/cm^2 (Figure 3-2). Lactic acid bacteria increased 4.8 to 4.9 log CFU/cm^2 on normal pH pork, whereas on high pH pork a 3.0 to 3.7 log CFU/cm^2 increase was shown.

A large component of the aerobic mesophile count was lactic acid bacteria, reaching between 7 and 8 log CFU/cm^2 after 20 days storage in all

Figure 3-1. Growth of *Yersinia enterocolitica* on the lean surface of high and normal pH pork stored at 4°C under conditions of vacuum and saturated CO_2 atmosphere packaging.
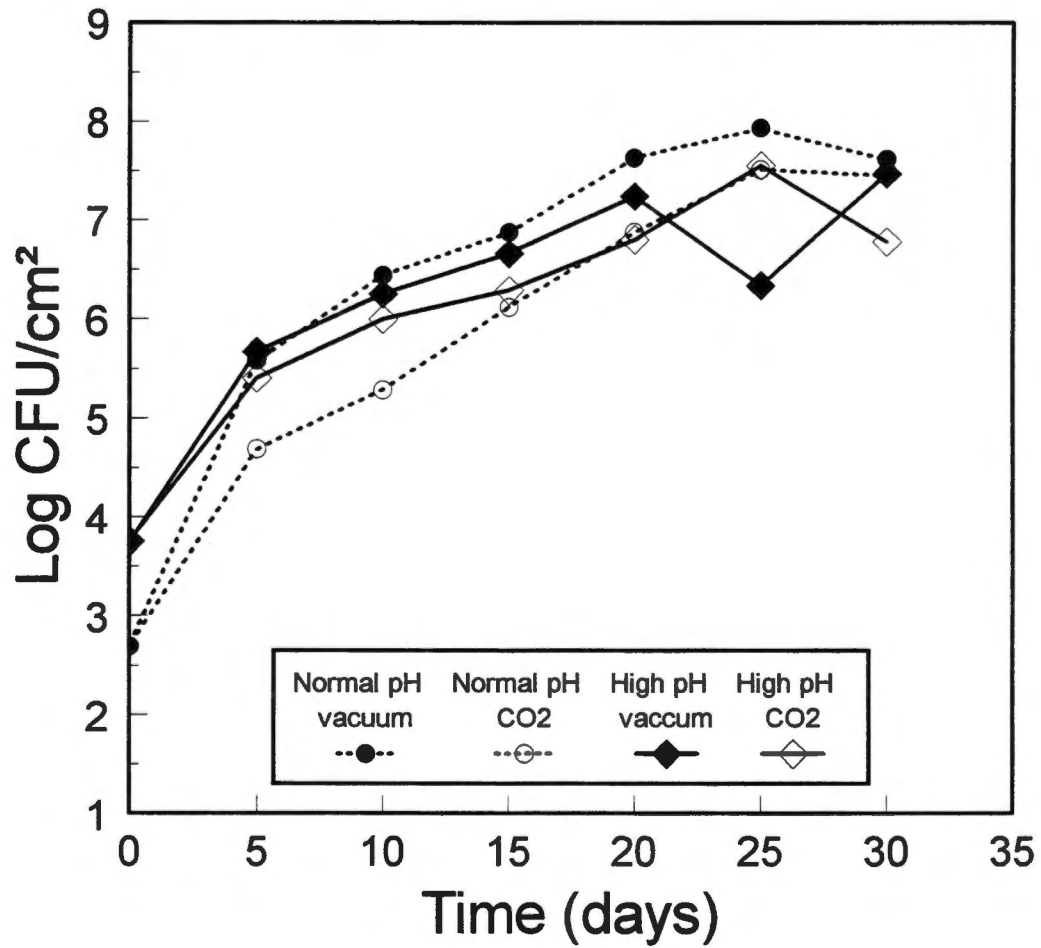
Figure 3-2. Growth of lactic acid bacteria on the lean surface of high and normal pH pork stored at 4°C under conditions of vacuum and saturated CO_2 atmosphere packaging.

packaging treatments (Figure 3-3). Numbers of aerobic mesophiles increased more rapidly on vacuum packaged normal pH pork, increasing approximately 3.0 log CFU/cm^2 within 5 days of storage at 4°C.

B. thermosphacta grew on vacuum and CO_2 packaged high pH pork, increasing between 0.6 and 1.0 log CFU/cm^2 over the 30-day storage period. Numbers of B. thermosphacta were significantly ($P<0.05$) higher on high pH pork than on normal pH pork (Table A-1). No increase in growth could be detected on vacuum or CO_2 packaged normal pH pork (Figure 3-4).

Gram-negative bacteria reached populations in excess of 6.0 log CFU/cm^2 on vacuum packaged meat (Figure 3-5). Meat packaged under CO_2 supported a Gram-negative bacterial count of around 5.0 log CFU/cm^2 after 30 days storage. Numbers of Gram-negative bacteria on high pH pork were consistently ($P<0.05$) higher than corresponding normal pH pork treatments vacuum packaged or packaged in CO_2 (Table A-1).

Gas analysis data showed that the percentage of residual O_2 in CO_2 packaged bags for inoculated and uninoculated pork increased slightly over time, but an atmosphere in excess of 99% CO_2 was maintained over 30 days storage at 4°C (Figure 3-6). Indicator tests for virulence confirmed that a portion of the Y. enterocolitica population was capable of retaining the virulence plasmid following inoculation on pork, packaging in MA and storage at 4°C for 30 days (Table 3-1).

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Table 3-1. Effect of packaging conditions on the stability of *Yersinia enterocolitica* virulence plasmid following 30 days storage at 4°C.

Pork packaging conditions	Total number of colonies selected from CIN[a] agar	No. (%) of colonies AA[b] in MR-VP[c]	No. (%) of colonies AA in RPMI-1640[d]	No. (%) of CRMOX+[e] colonies
Normal pH vacuum	41	10 (25)	12 (29)	12 (29)
Normal pH 100% CO_2	44	14 (32)	15 (34)	13 (30)
High pH vacuum	55	16 (29)	17 (31)	16 (29)
High pH 100% CO_2	60	19 (32)	21 (35)	17 (28)

[a] Cefsulodin irgasan agar

[b] Autoagglutination

[c] Methyl red Vogues-Proskauer broth

[d] Tissue culture medium RPMI-1640

[e] Congo red binding, calcium dependence positive
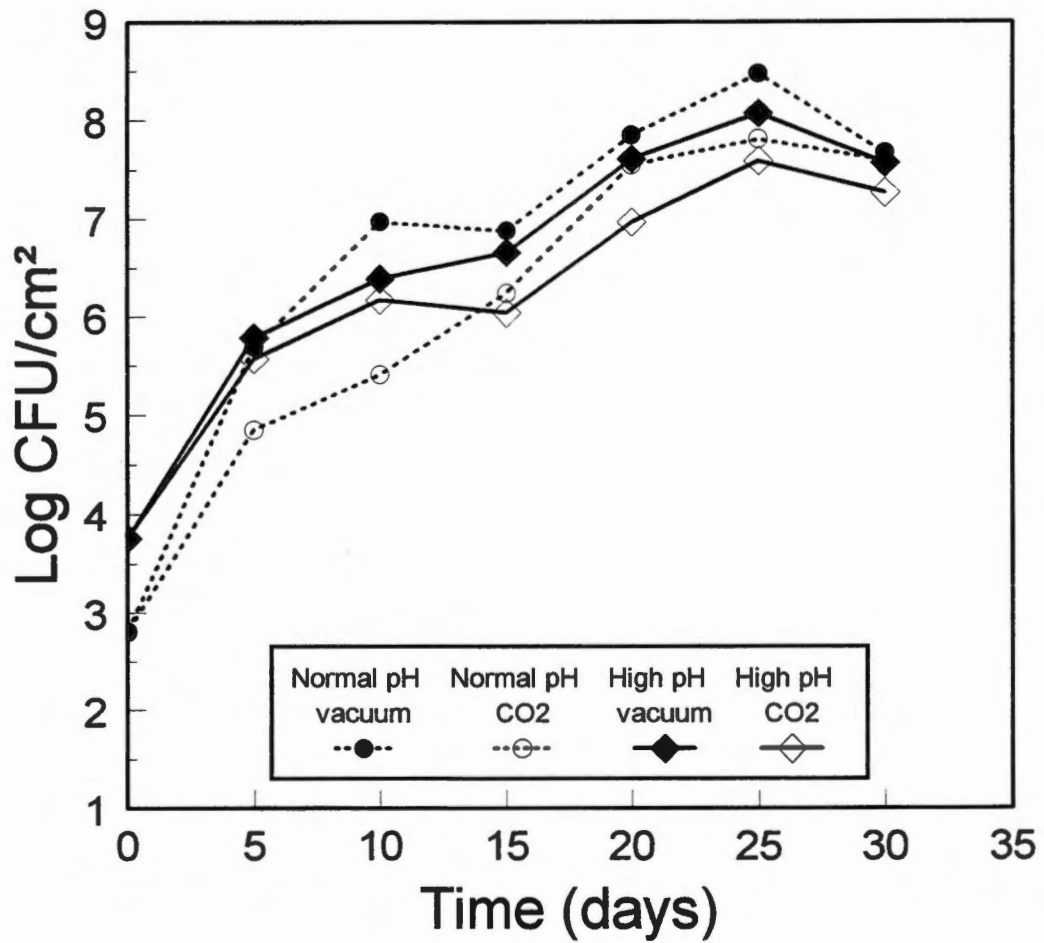
Figure 3-3. Aerobic plate count on the lean surface of high and normal pH pork stored at 4°C under conditions of vacuum and saturated CO_2 atmosphere packaging.
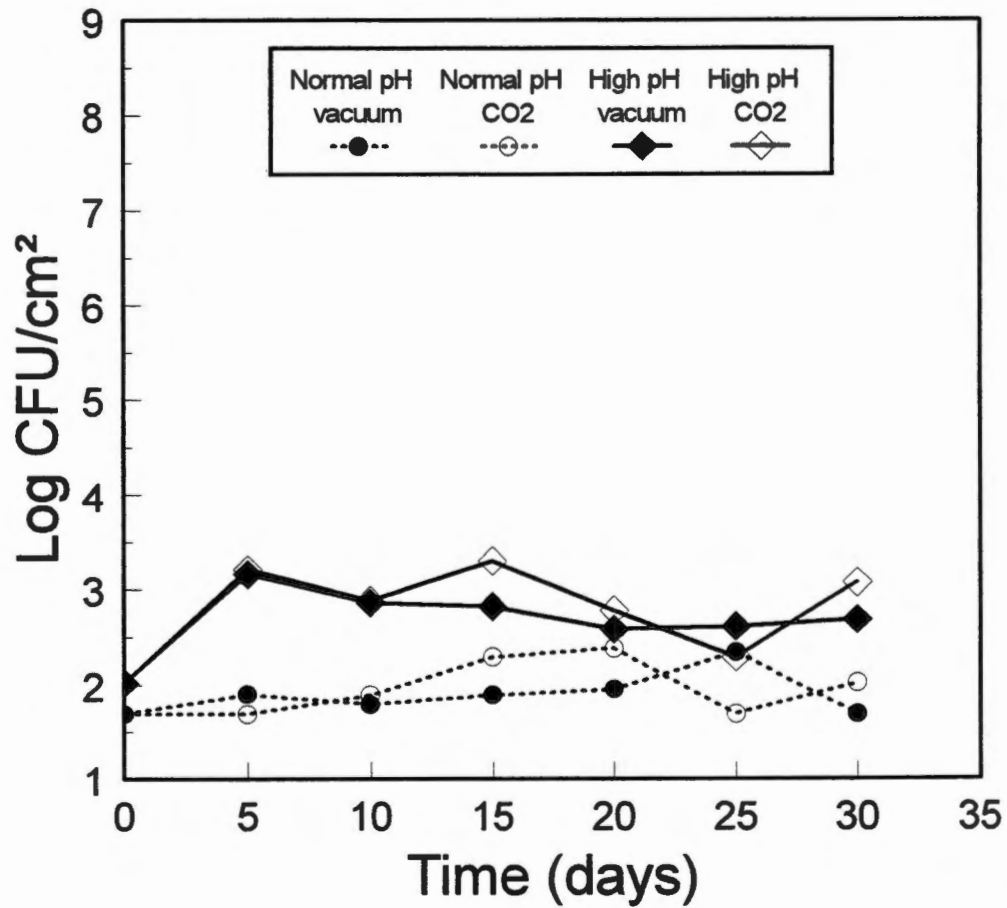
Figure 3-4. Growth of *Brochothrix thermosphacta* on the lean surface of high and normal pH pork stored at 4°C under conditions of vacuum and saturated CO_2 atmosphere packaging.
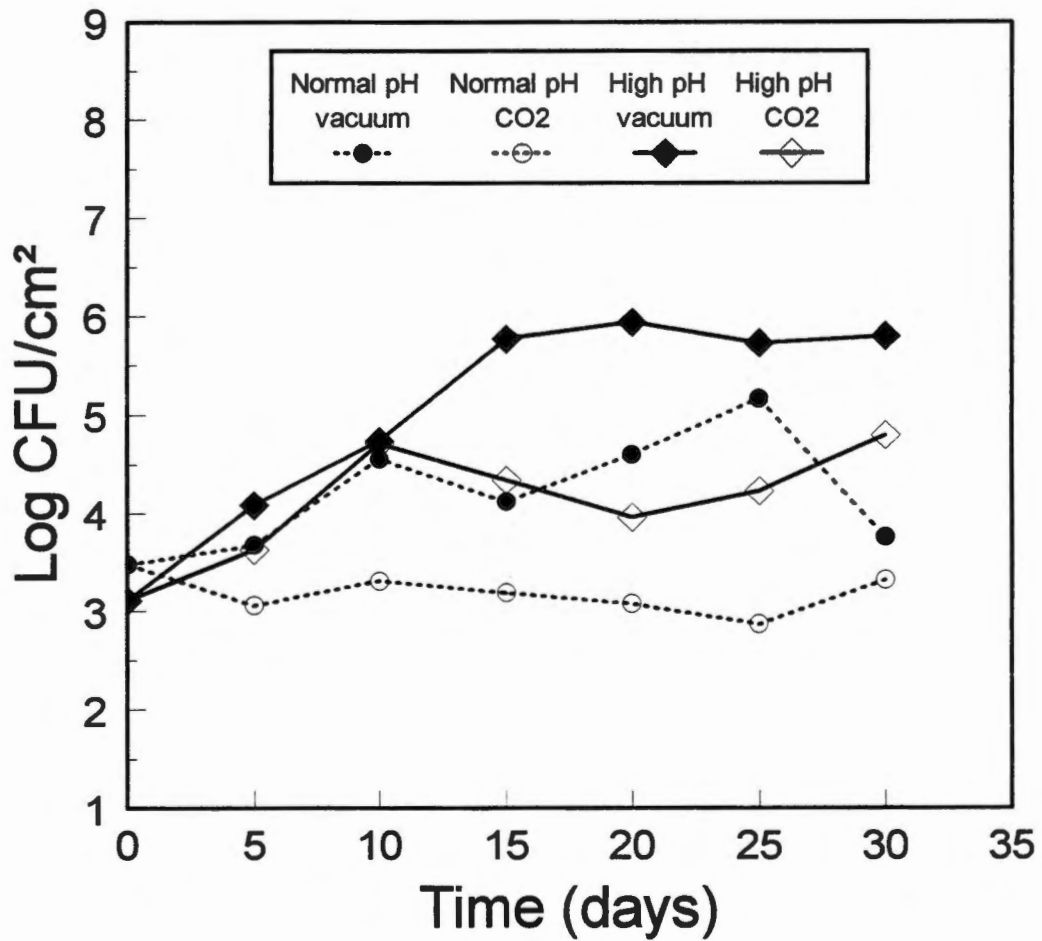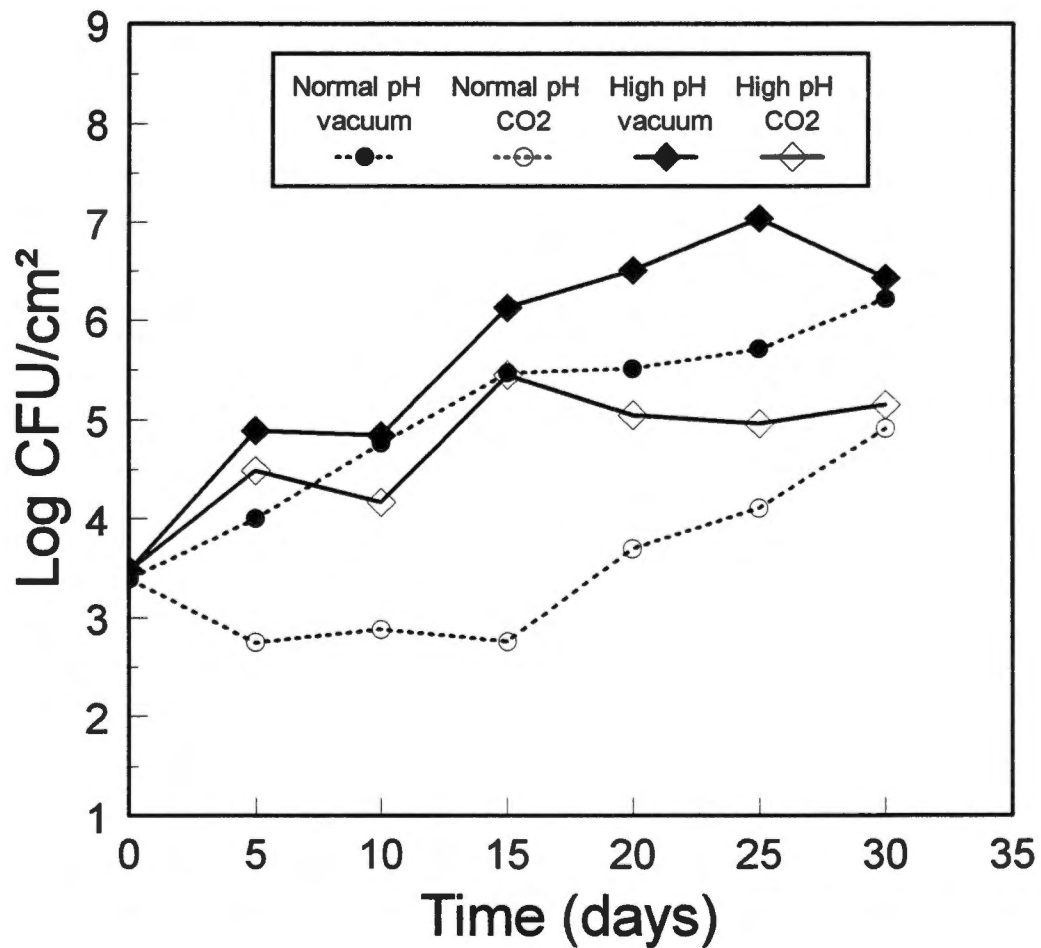
Figure 3-5. Gram-negative bacteria count on the lean surface of high and normal pH pork stored at 4°C under conditions of vacuum and saturated CO_2 atmosphere packaging.
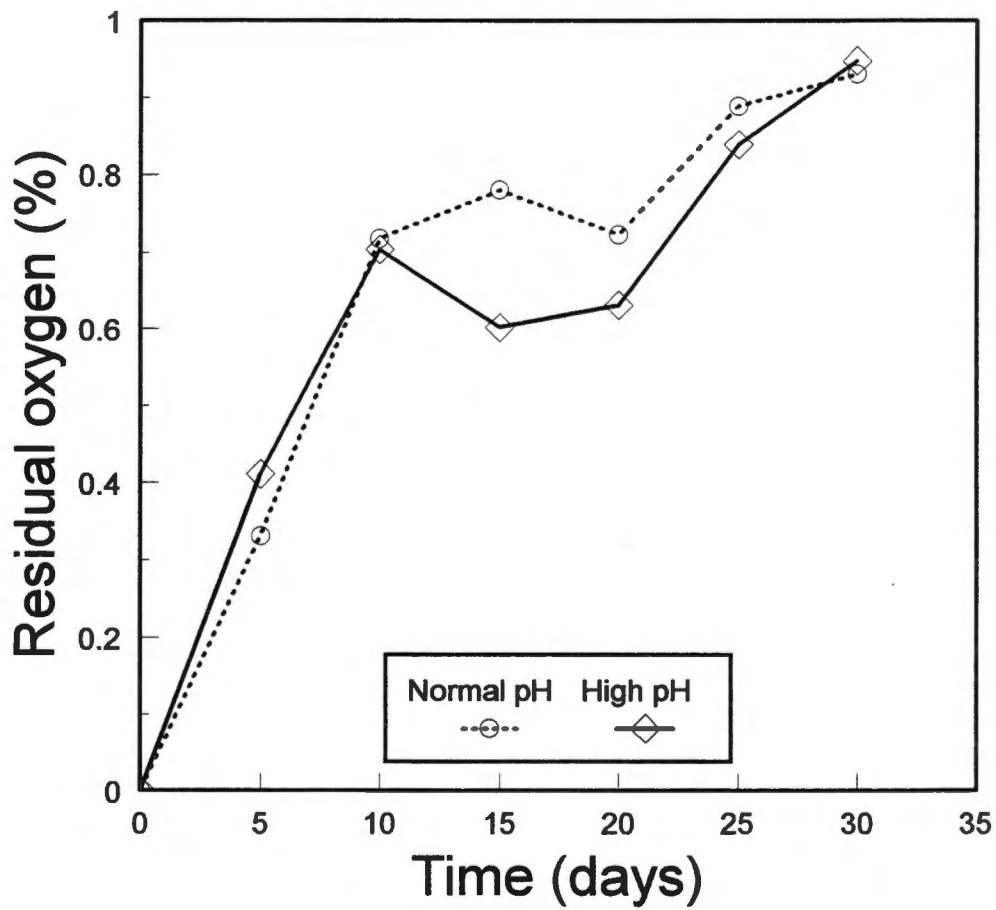
Figure 3-6. Residual oxygen levels of CO_2 packaged high and normal pH pork stored at 4°C.

DISCUSSION

Except for normal pH pork packaged in CO_2, *Y. enterocolitica* grew in all packaging treatments at 4°C. Several authors have reported the growth of *Y. enterocolitica* in vacuum packaged meats (Doherty et al., 1995; Gill and Reichel, 1989; Manu-Tawaih et al., 1993). Exposure to 100% CO_2 is believed to increase the lag phase and decrease the growth rate of *Y. enterocolitica* (Farber, 1991). However, Gill and Reichel (1989) observed growth of *Y. enterocolitica* on high pH beef stored under 100% CO_2 at 5 and 10°C. Doherty et al. (1995) reported almost a 4 log CFU/g increase of *Y. enterocolitica* on lamb pieces packaged in 100% CO_2 during a 28-day storage period at 5°C. It is evident that *Y. enterocolitica* may be a potential problem on MA packaged meats. Results of the present study show that normal pH pork packaged in 100% CO_2 and stored at 4°C provides an environment that suppresses growth of *Y. enterocolitica*. Packaging high pH pork in either vacuum or 100% CO_2 does not inhibit *Y. enterocolitica* growth. Lactate production in post rigor muscle is responsible for the decrease in pH observed in meat. In muscle of pH 5.55, lactate concentrations of 115 to 145 mM have been measured, whereas for muscle of pH 6.1 concentrations of lactate between 65 and 105 mM have been recorded (Grau, 1981). As lactate is an effective inhibitor of *Y. enterocolitica* growth, increased numbers observed on high pH meat is likely due to lower lactate concentrations (Little et al., 1992). Numbers of *Y. enterocolitica* on vacuum packaged high pH pork increased more rapidly compared to other treatments.

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This may have been due to a less inhibitory environment created by lower lactate concentrations and to reduced CO_2 concentrations.

Gram-negative bacteria grew most rapidly on vacuum packaged high and normal pH pork. Packaging pork slices in a high concentration of CO_2 had an inhibitory effect on Gram-negative populations, particularly in normal pH pork. Further inhibition of Gram-negative bacteria may have been imposed by the high numbers of lactic acid bacteria present on the CO_2 packaged pork. A similar effect was noted by Gill and Penney (1988) on CO_2 packaged beef.

Carbon dioxide is used in MA packaging to retard bacterial growth by increasing the lag phase and generation time (Daniels et al., 1985). Several investigators found that 100% CO_2 does not exert an inhibitory effect on lactic acid bacteria (Blickstad and Molin, 1983; Rousset and Renerre, 1991). Our results also indicated packaging atmosphere had no significant effect ($P>0.05$) on growth of lactic acid bacteria. Meat pH had a significant effect ($P<0.05$) on growth with the greatest mean increase in lactic acid bacteria occurring on both CO_2 and vacuum packaged normal pH pork (Figure 3-2).

The meat spoilage organism, *B. thermosphacta*, grew to significantly ($P<0.05$) higher levels on vacuum and 100% CO_2 packaged high pH pork (Figure 3-4, Table A-1) as compared with other treatments. Growth of *B. thermosphacta* on beef is controlled by a combination of meat pH and availability of oxygen (Campbell et al., 1979). When packaged in the absence of oxygen, *B. thermosphacta* is able to grow on high pH beef while growth is inhibited on

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normal pH beef. Our results indicate that a similar trend may be evident on vacuum and CO_2 packaged pork.

Virulence in *Y. enterocolitica* is encoded by virulence genes that increase the pathogenic potential of the organism. Characteristic of pathogenic *Y. enterocolitica* is the presence of a 40-50 MDa plasmid that carries essential virulence genes (Prpic et al., 1985). Culturing at 37°C and prolonged storage of *Y. enterocolitica* can result in loss of the virulence plasmid and hence loss of virulence (Bhaduri and Turner-Jones, 1993; Kwaga and Iversen, 1991). Data in Table 3-1 show that a proportion of virulent *Y. enterocolitica* isolates can retain their virulence plasmid following passage through a pork packaging and storage regime. These results suggest that virulent *Y. enterocolitica* on MA packaged pork have the potential for maintaining their virulence properties even when grown under adverse conditions such as in a high CO_2 atmosphere.

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CHAPTER IV

EFFECT OF MODIFIED ATMOSPHERE AND SALT TREATMENT ON GROWTH OF SPOILAGE BACTERIA ON MINIMALLY PROCESSED BROCCOLI STORED AT 4 OR 10°C

ABSTRACT

Immersion of fresh broccoli in a saturated sodium chloride (NaCl) solution may improve shelf-life through a reduction in surface water activity and reduced respiration. The microbiological quality of salt-treated minimally processed broccoli stored at 4 and 10°C in modified atmospheres (MA) was studied by examining the fate of spoilage bacteria. Results from this investigation indicate that use of salt as a limiting factor to inhibit spoilage bacteria and extend shelf-life of minimally process broccoli while maintaining its fresh-like quality is ineffective. Packaged broccoli, stored at 10°C, spoiled more rapidly than broccoli stored at 4°C. Fresh untreated broccoli packaged in air and MA maintained a crisp green appearance for around 9 days at 4°C and 6 days at 10°C, while vacuum packaged fresh broccoli had an extended shelf-life of up to 15 days at 4°C. Vacuum packaged salt-treated broccoli showed signs of deterioration within 3 days of packaging, while salt-treated broccoli packaged in air and MA began to deteriorate within 3 to 6 days at 4 and 10°C.

INTRODUCTION

Storage of harvested fresh vegetables depends on several factors to maintain quality. These include harvesting at an optimum stage of growth, hygiene, handling, appropriate storage temperature, vegetable respiration rate, pH, water activity (Aw) and biological structure (Day 1992; Kader et al., 1989). Fruit and vegetables, postharvest, are living material capable of respiration (Ballantyne, 1989). The respiration rate of a vegetable is considered a useful indicator of potential storage life. Generally, a rapid respiration rate is associated with a high level of deterioration. Broccoli is a vegetable with a high respiration rate.

Application of the "hurdles concept" for minimally processed vegetable preservation involves limiting microbial growth by using a combination of inhibitory treatments (Scott, 1989). Several authors have investigated broccoli stored under various modified atmospheres (MA) at different storage temperatures in packaging film of both high and low gas permeability (Ballantyne et al., 1988; Lipton and Harris, 1974; Makhlouf et al., 1989). Problems with product quality as a result of MA storage were primarily in the development of off-odor compounds, for example methanethiol, changes in turgidity, yellowing, soft rots and mold and opening of flower buds (Forney et al., 1991; Makhlouf et al., 1989).

Increased carbon dioxide (CO_2) level with reduced oxygen (O_2) in MA packaged broccoli may decrease deterioration during storage (Ballantyne et al.,

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1988; Lebermann et al., 1968; Makhlouf et al., 1989). O'Donnell (1995) reported that the optimum atmospheric storage conditions for broccoli was a MA of 1 to 2% O_2 and 5 to10% CO_2. Incorporation of NaCl into the preservation regime of minimally processed refrigerated broccoli may assist in prolonging shelf-life. Immersion of fresh produce in a salt solution is a method of reducing surface Aw of a vegetable of high osmotic pressure (Torreggiani, 1993; Wiley, 1994). Water flows out of the vegetable and soluble solids present in the osmotic solution migrate through cellular membranes (Biswal and Bozorgmehr, 1992; Torreggiani, 1993). Use of a salt treatment in conjunction with MA packaging may lower the surface Aw of minimally processed broccoli and reduce the potential for growth of spoilage microorganisms (Wiley, 1994). As the minimum Aw for microbial growth is approached, sensitivity to other inhibitors and inhibitory conditions increases (Scott, 1989).

With the advent of minimally processed vegetables and their increased acceptance and availability, prolonged storage through minimizing deterioration is of prime importance. Objectives of this investigation were to study growth and survival of spoilage microorganisms on minimally processed broccoli prepared with a salt treatment and stored under conditions of air, MA and vacuum packaging at 4 and 10°C.

MATERIALS AND METHODS

Broccoli and storage conditions

Fresh broccoli heads shipped on ice were purchased from a local supplier. Broccoli was graded and cut into florets and stored at 4°C prior to packaging. Broccoli florets were divided into two treatment groups for packaging, salt-treated and untreated broccoli.

Salt treatment

A 40-L saturated NaCl solution was used to prepare salt-treated broccoli. Broccoli florets were immersed in the saturated NaCl solution for 21 min to achieve a final salt concentration of 1%. Salt concentration in the broccoli was determined with AOAC (1990) method 50.029.

Packaging materials and methods

The packaging film used was PD-941 (Cryovac, Duncan, SC), a multilayered film for packaging produce with high respiration rates (O_2 transmission rate - 16544 mL/m²/24 h). A Multivac (Koch, Kansas City, MO) gas flushing machine was used to achieve the desired atmosphere. Each broccoli treatment was packaged in air, MA and a vacuum. A standard gas mixture of $CO_2:O_2:N_2$ (10:5:85) (MG Industries, Valley Forge, PA) was used for packaging florets in a MA. Approximately 250 g of each broccoli treatment were weighed into separate PD-941 bags for storage and sampling.

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Storage and sampling

Packaged samples were stored in the dark at 4 and 10°C. Broccoli samples were taken at 3-day intervals during the storage period. On each sampling day the gas composition of the package was determined using a Hewlett Packard gas chromatograph, model 5890 - series II (Hewlett Packard, Wilmington, DE) equipped with a thermal conductivity detector and a stainless steel CTRI column (Alltech Associates, Inc., Deerfield, IL). Column and TCD temperatures were set at 25°C, the quantity of sample injected was 5 mL and the total run time was 3 min. Helium was used as the carrier gas at a flow rate of 40 mL/min. Vacuum packaged salt-treated broccoli was not sampled for gas analysis since excessive liquid present in the package interfered with sample collection.

Isolation and enumeration of microorganisms

Samples (25 g) were removed from each PD-941 bag and placed into sterile stomacher bags with 225 mL of 0.1% peptone water and blended for 1 min using a Model 400 Stomacher Lab Blender (Steward, London, UK). Serial dilutions of the broccoli slurry were surface plated (0.1 mL) onto appropriate solid media for enumeration. Media and conditions for enumeration and isolation of microorganisms were as follows: mesophilic aerobes, TSA (BBL, Cockeysville, MD) incubated at 25°C for 72 h; lactic acid bacteria (LAB), De

man, Rogosa, Sharpe agar (MRS; BBL, Cockeysville, MD) incubated at 25°C for 72 h; yeasts and molds, oxytetracycline glucose yeast extract agar (OGYE; Atlas, 1993) incubated at 25°C for 5 days; coliform bacteria, Mackonkey No.3 (MAC; Unipath, Basingstoke, UK) incubated at 35°C for 48 h; psychrotrophic bacteria, TSA (BBL, Cockeysville, MD) incubated at 7°C for 10 days; pectinolytic bacteria, pectin agar media (PAM; Bowen and Kominos, 1979) incubated at 25°C for 24 - 48 h.

Identification of microorganisms

Gram-positive, catalase negative colonies selected from MRS were assumed to be LAB. Lactose fermenting colonies capable of growth at 35°C isolated from MAC were assumed to be coliforms. On PAM, pectinolytic bacteria were identified by colonial depressions produced in the solid medium (Bowen and Kominos, 1979). Colonies on OGYE were examined microscopically to confirm the presence of yeasts.

Statistical analysis

The General Linear Models (GLM) procedure was used to analyze main effects and interactions of packaging atmosphere, storage time and salt treatment on differences in mean microbial numbers (SAS Institute, Inc., 1990). When significant, means were separated using the Least Square Means procedure.

RESULTS

Microbial analyses

Packaging atmosphere and salt treatment of broccoli had no effect (P>0.05) on LAB populations at 4°C (Table A-2). However, populations of LAB were higher (P<0.05) on salt-treated broccoli than on untreated broccoli in each of the test atmospheres when stored at 10°C (Figures 4-1 and 4-2). Population changes of LAB were greater (P<0.05) on vacuum packaged broccoli stored at 10°C than with other packaging treatments. On untreated vacuum packaged broccoli, LAB numbers increased 3.2 log CFU/g, while on salt-treated vacuum packaged broccoli LAB numbers increased 5.6 log CFU/g.

Aerobic mesophile populations on untreated broccoli increased approximately 1 to 2 log CFU/g at 4°C while 10°C storage resulted in a 2 to 3 log CFU/g increase (Figure 4-3). Population changes of aerobic mesophiles were higher (P<0.05) on salt-treated broccoli stored at 4 and 10°C (Table A-2). On salt-treated broccoli, numbers of aerobic mesophiles increased approximately 3.5 log CFU/g by day 9 at 10°C and 3 log CFU/g by day 12 at 4°C in all packaging atmospheres (Figure 4-4). Aerobic mesophiles on vacuum packaged broccoli stored at 4°C experienced longer lag phase than on other packaging treatments resulting in a reduced final population. Growth of aerobic mesophiles on vacuum packaged salt-treated broccoli was significantly (P<0.05) lower than other packaging treatments.

Changes in psychrotroph numbers were higher (P<0.05) on salt-treated

Figure 4-1. Lactic acid bacteria count for untreated broccoli stored at 4 or 10°C under conditions of air, modified atmosphere (MA) and vacuum packaging.

Figure 4-2. Lactic acid bacteria count for salt treated broccoli stored at 4 or 10°C under conditions of air, modified atmosphere (MA) and vacuum packaging.

Figure 4-3. Aerobic mesophile count for untreated broccoli stored at 4 or 10°C under conditions of air, modified atmosphere (MA) and vacuum packaging.

Figure 4-4. Aerobic mesophile count for and salt treated broccoli stored at 4 or 10°C under conditions of air, modified atmosphere (MA) and vacuum packaging.

broccoli than on untreated broccoli when stored at 4 and 10°C (Figures 4-5 and 4-6, Table A-2). On salt-treated broccoli, psychrotroph populations increased 3.5 to 4 log CFU/g for each packaging treatment, whereas numbers increased 2 to 2.5 log CFU/g for untreated broccoli packaged in air and MA. Growth of psychrotrophs on vacuum packaged salt-treated broccoli stored at 4°C was lower (P<0.05) than other packaging atmospheres.

Growth of pectinolytic bacteria was apparent in all atmospheres on untreated broccoli stored at 4 and 10°C, achieving between a 2 to 3 log CFU/g increase, although no differences (P<0.05) were observed between each packaging treatment (Figure 4-7, Table A-2). On salt-treated vacuum packaged broccoli stored at 4°C, pectinolytic bacteria remained in lag phase for 9 days, whereas pectinolytic bacteria growing in air and MA experienced shorter lag phase lasting between 0 and 3 days (Figure 4-8). Packaging atmospheres did not influence growth of pectinolytic bacteria on salt-treated broccoli stored at 10°C.

Salt treatment of broccoli did not influence yeast populations during storage at 10°C (Figures 4-9 and 4-10, Table A-2). Yeasts present on vacuum packaged salt-treated broccoli at 4°C experienced lag phase that lasted at least 9 days. Mold growth remained constant on salt and untreated broccoli stored at 4 and 10°C in all packaging atmospheres (Figures 4-11 and 4-12). Mold populations on salt-treated broccoli packaged in MA, air and vacuum remained in lag phase lasting around 9 days at 4°C and 12 days at 10°C.

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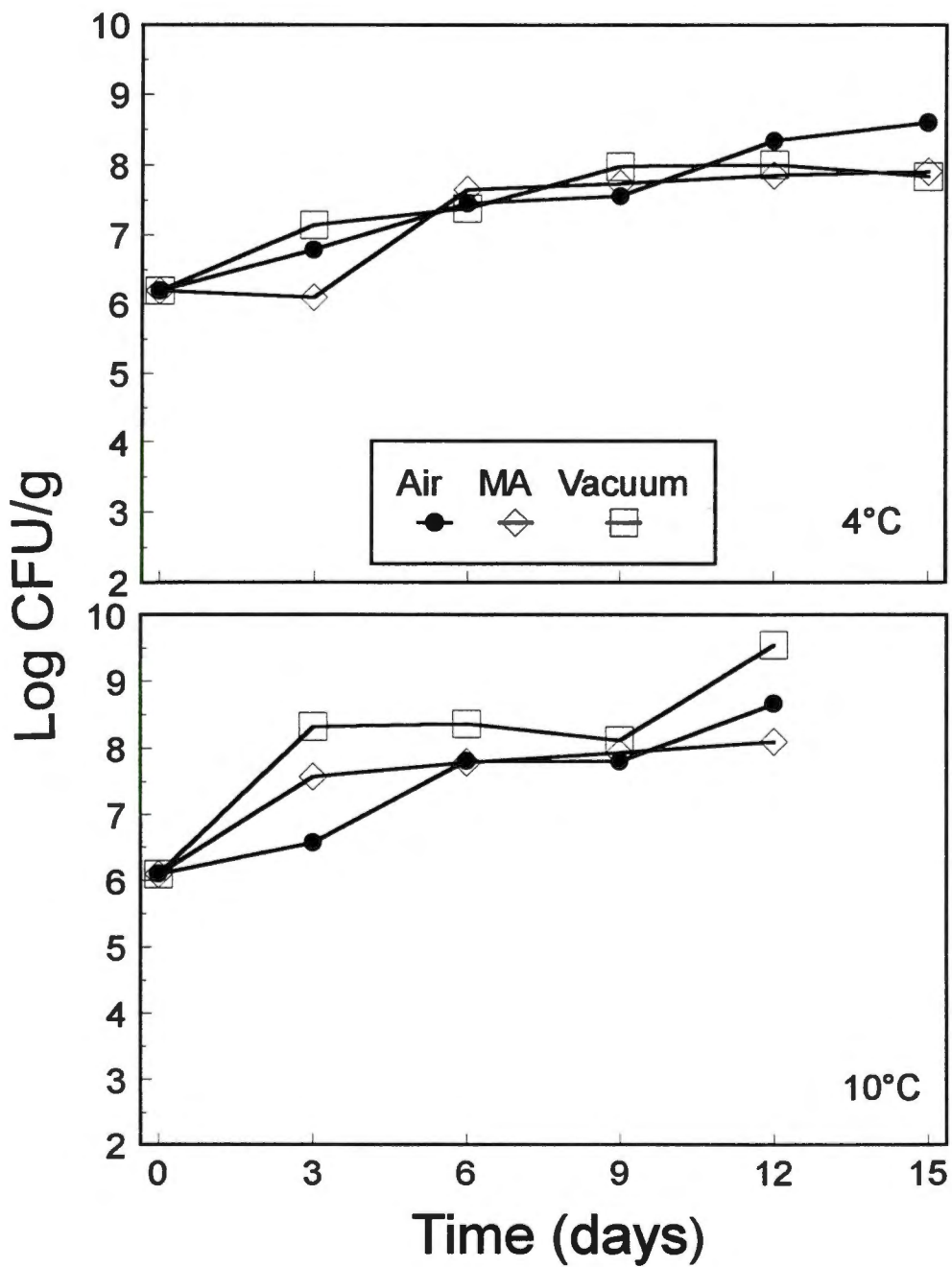
Figure 4-5. Psychrotroph count for untreated broccoli stored at 4 or 10°C under conditions of air, modified atmosphere (MA) and vacuum packaging.
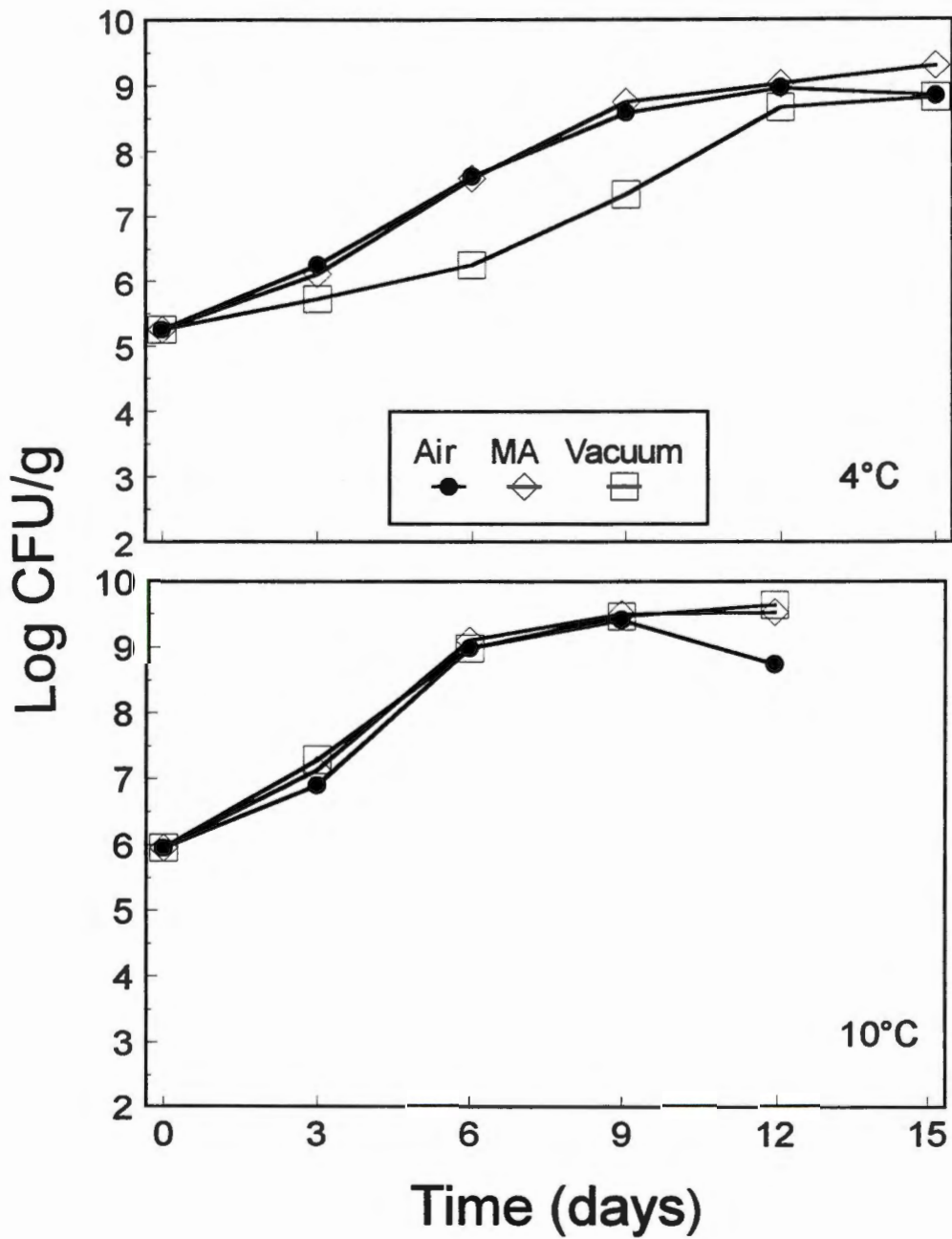
Figure 4-6. Psychrotroph count for salt treated broccoli stored at 4 or 10°C under conditions of air, modified atmosphere (MA) and vacuum packaging.
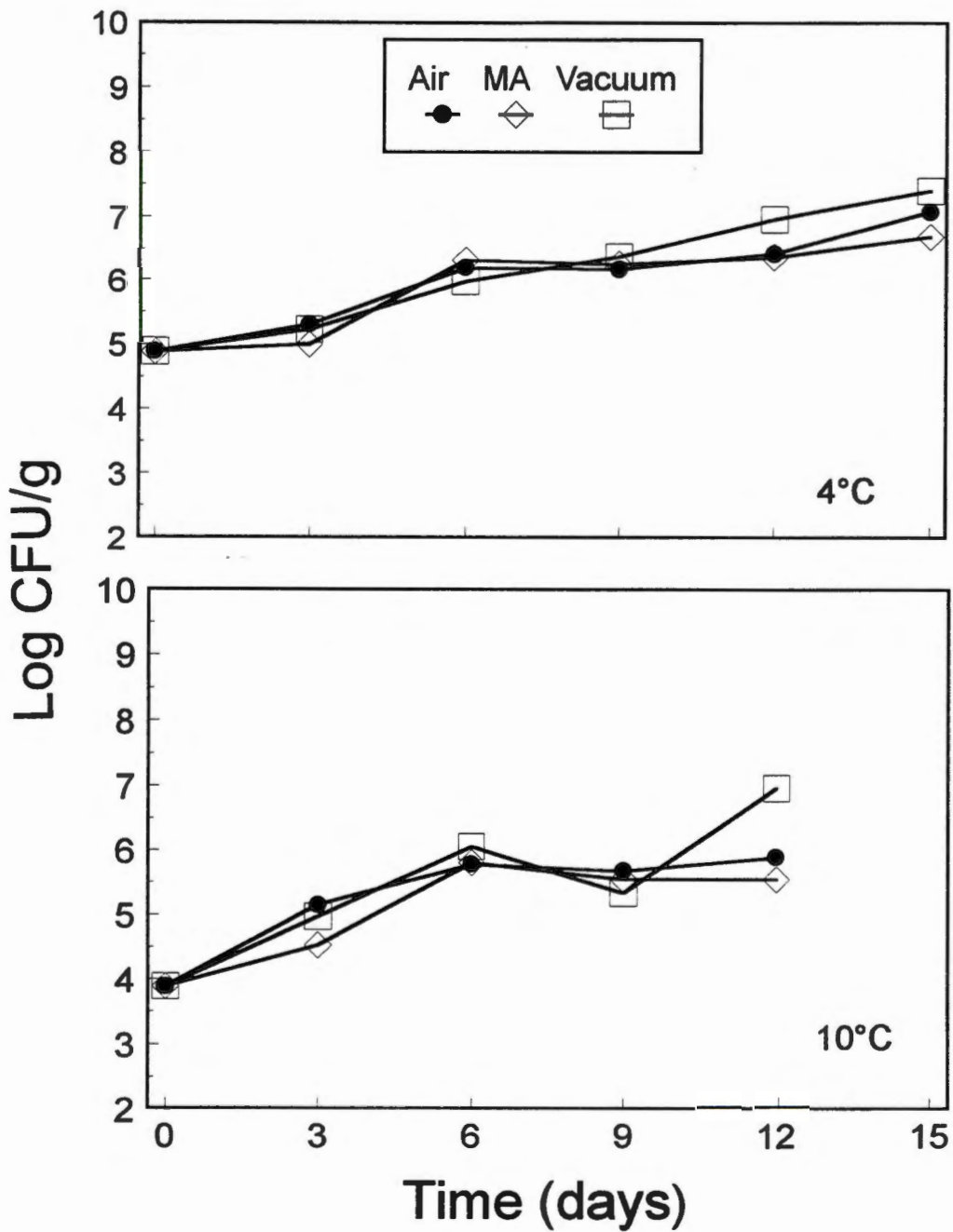
Figure 4-7. Pectinolytic bacteria count for untreated broccoli stored at 4 or 10°C under conditions of air, modified atmosphere (MA) and vacuum packaging.
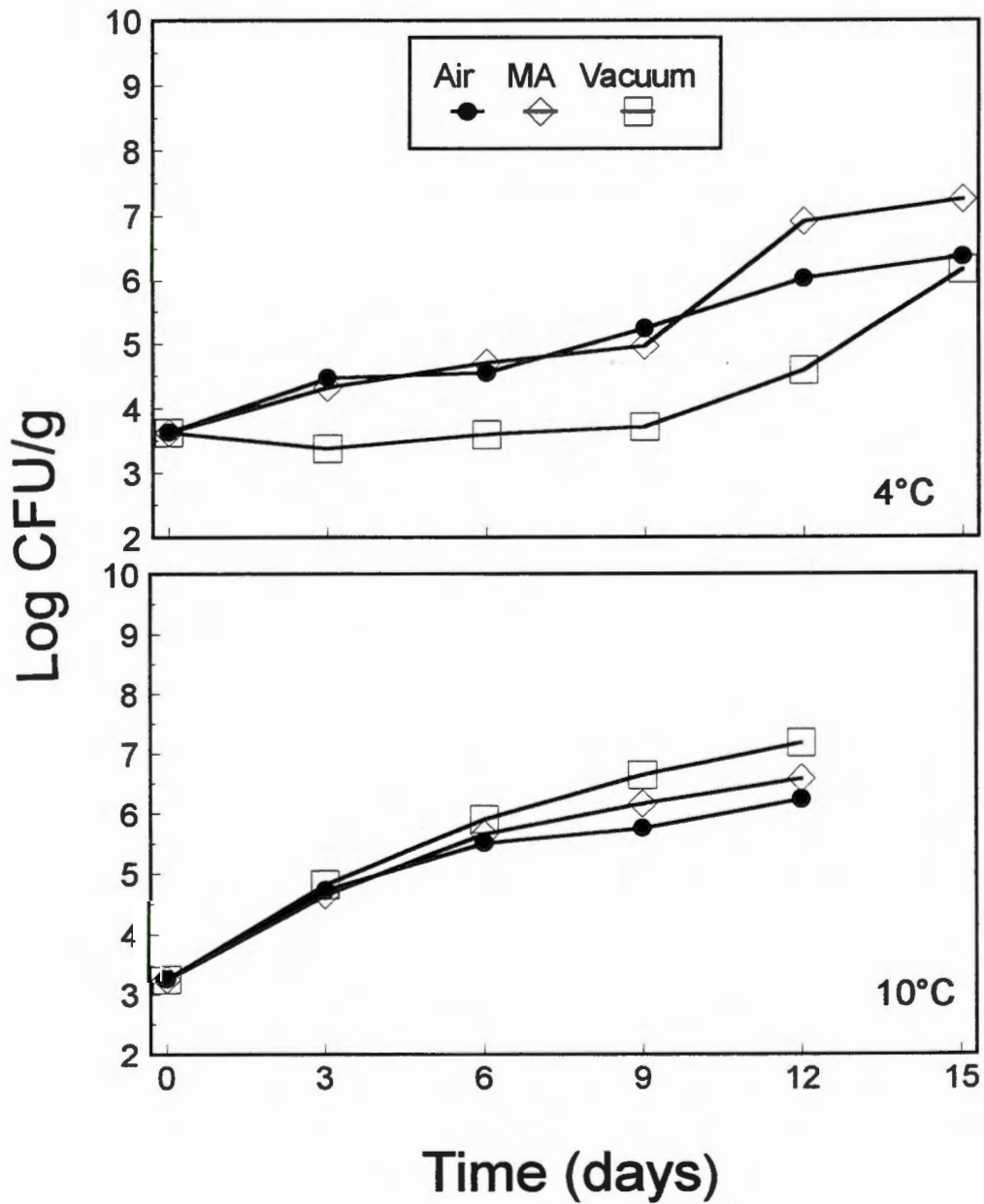
Figure 4-8. Pectinolytic bacteria count for salt treated broccoli stored at 4 or 10°C under conditions of air, modified atmosphere (MA) and vacuum packaging.
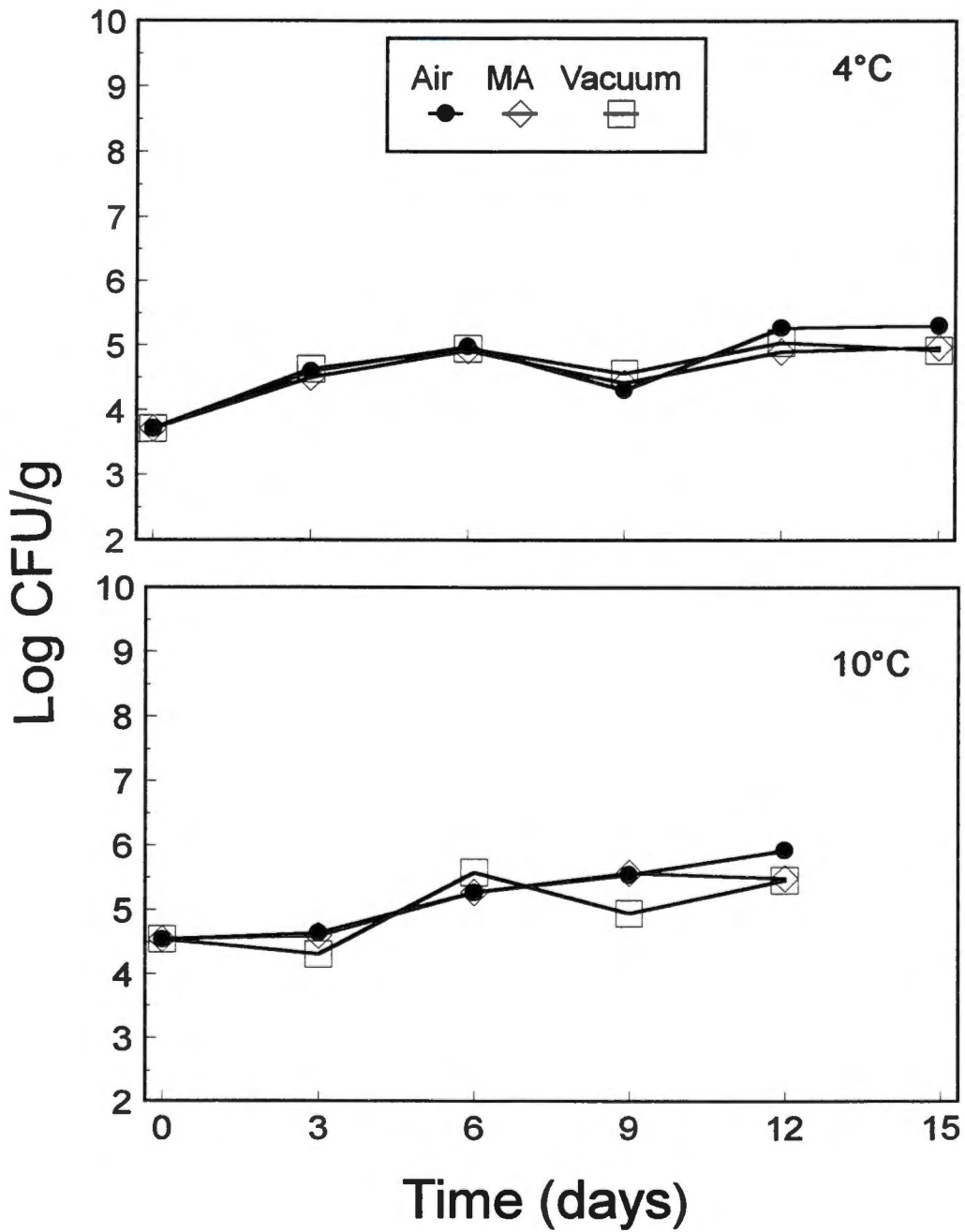
Figure 4-9. Yeast count for untreated broccoli stored at 4 or 10°C under conditions of air, modified atmosphere (MA) and vacuum packaging.
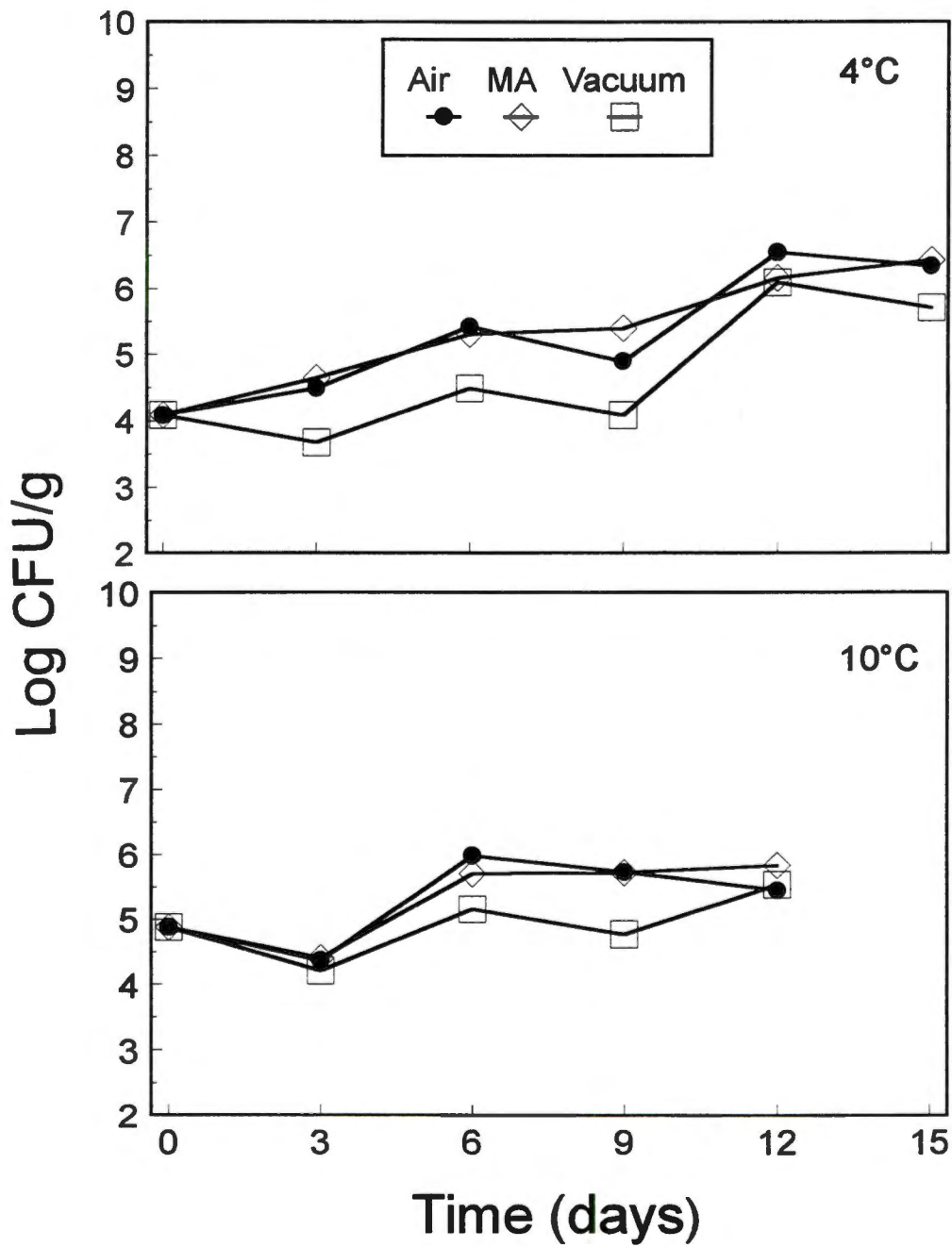
Figure 4-10. Yeast count for salt treated broccoli stored at 4 or 10°C under conditions of air, modified atmosphere (MA) and vacuum packaging.
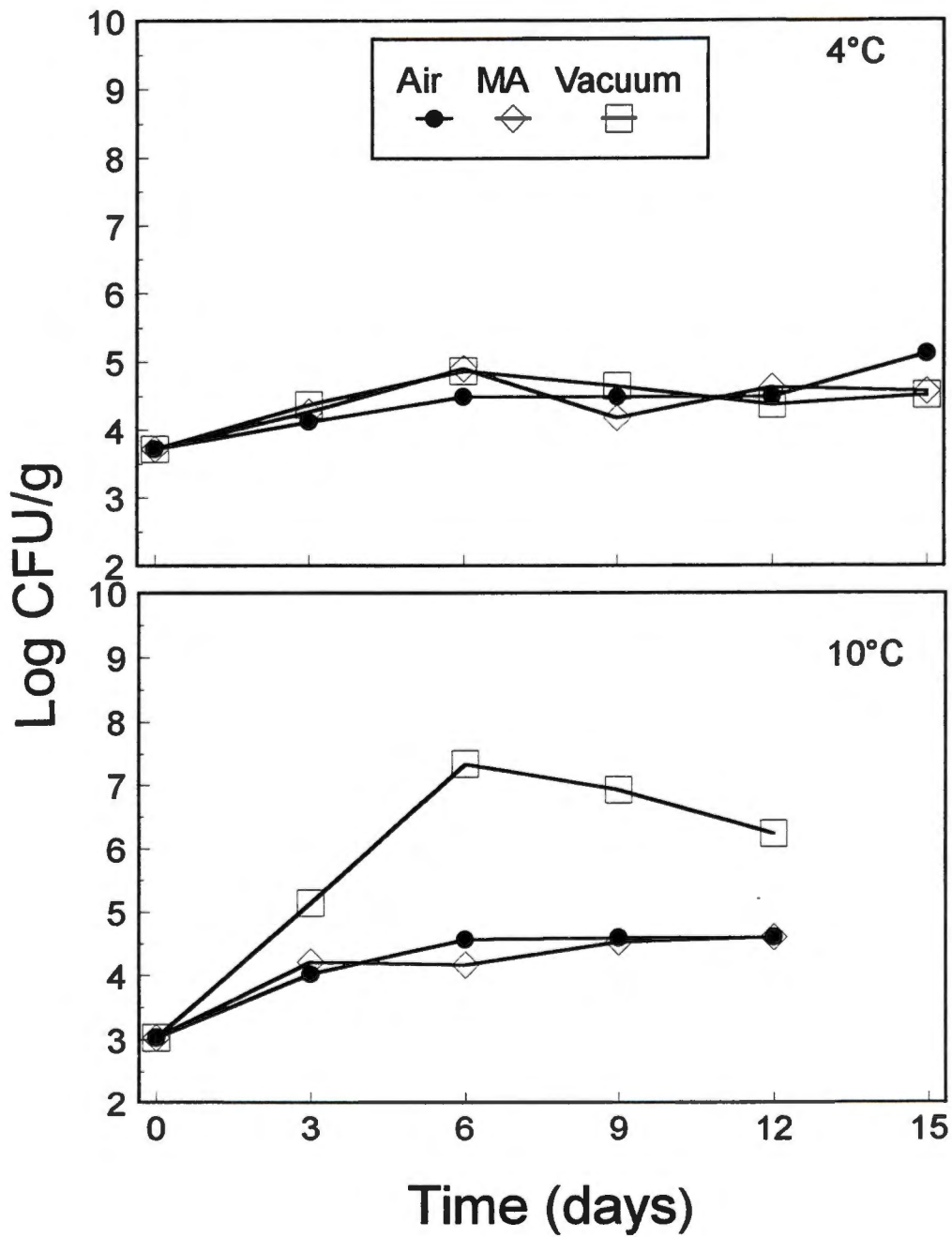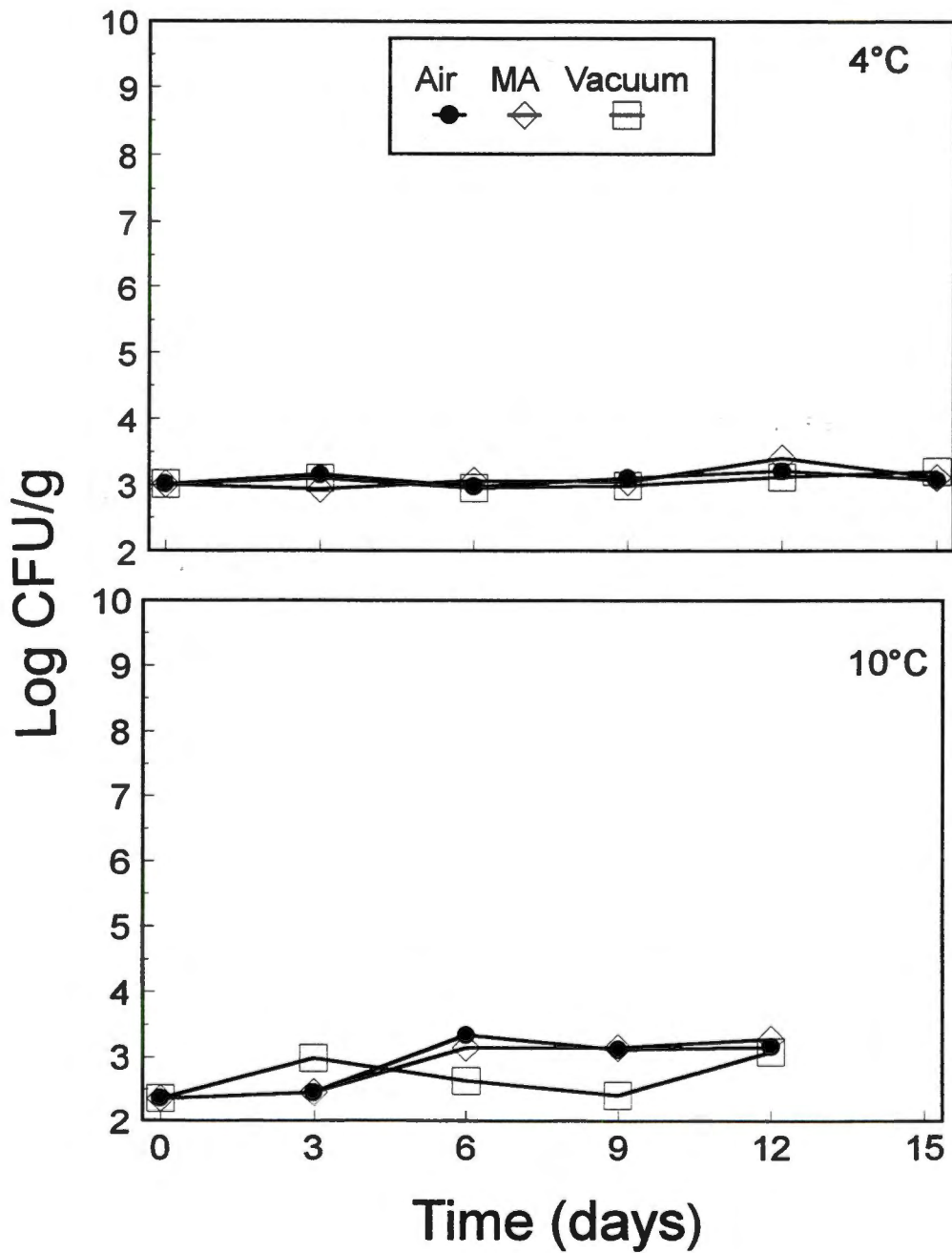
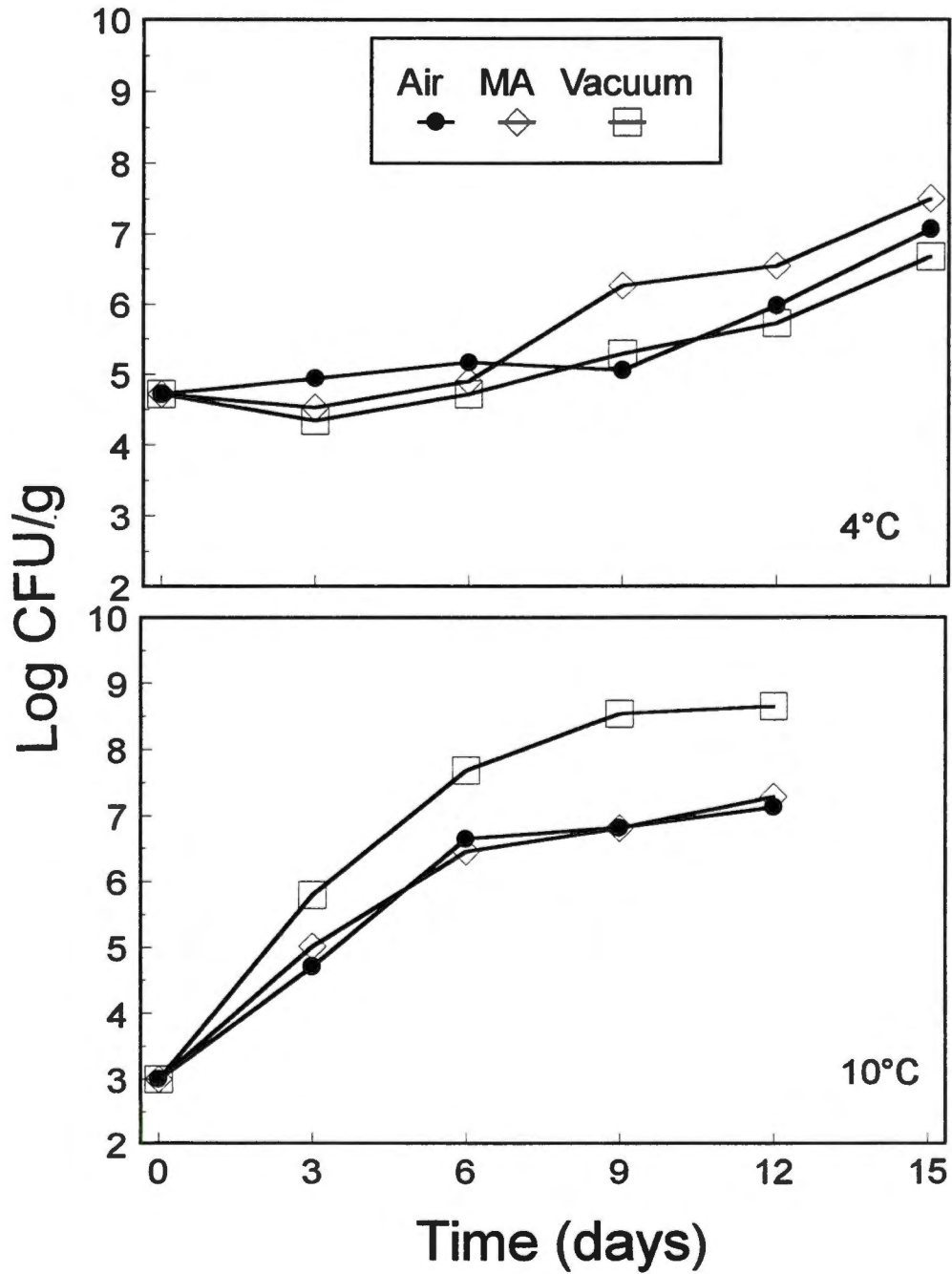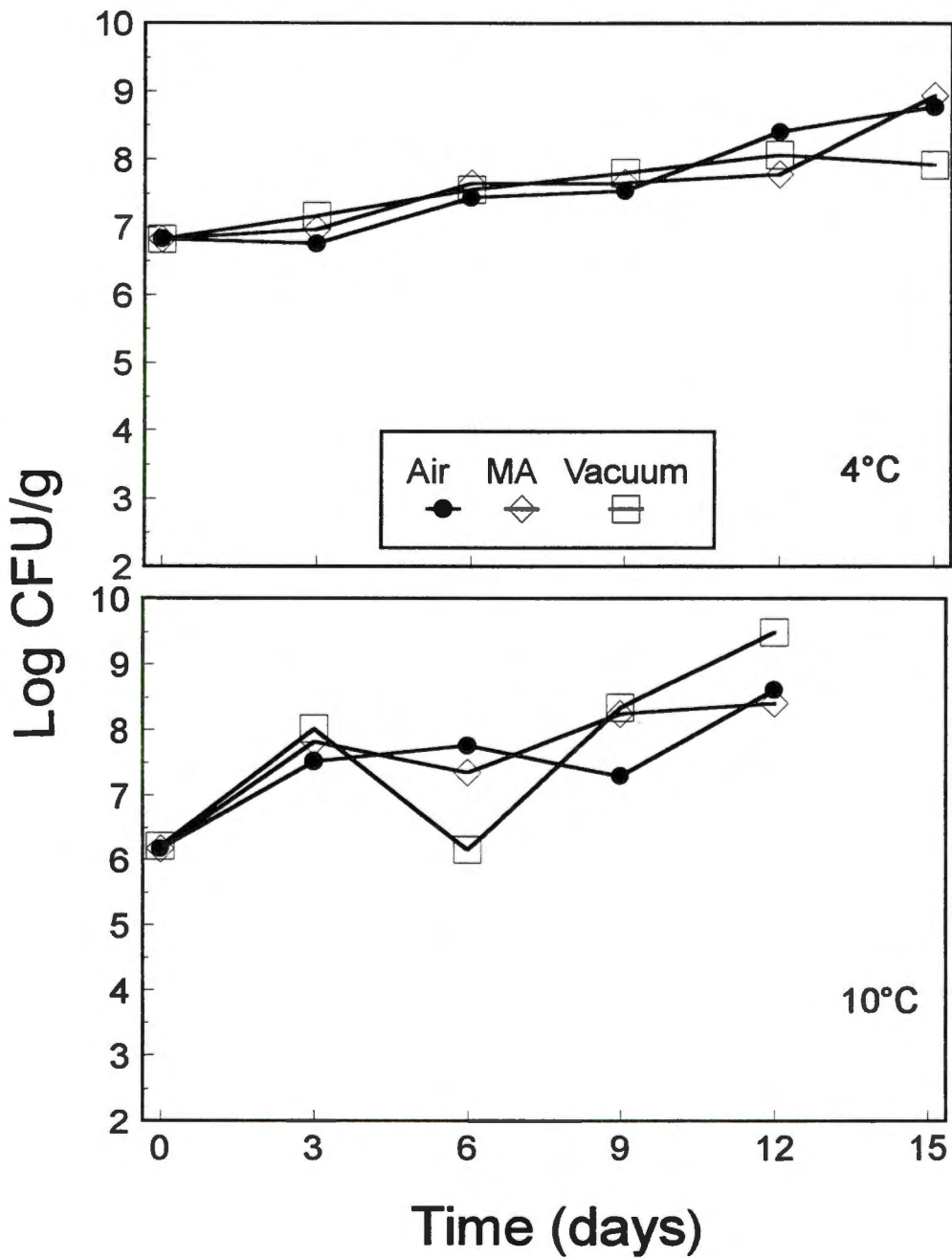Figure 4-11. Mold count for untreated broccoli stored at 4 or 10°C under conditions of air, modified atmosphere (MA) and vacuum packaging.

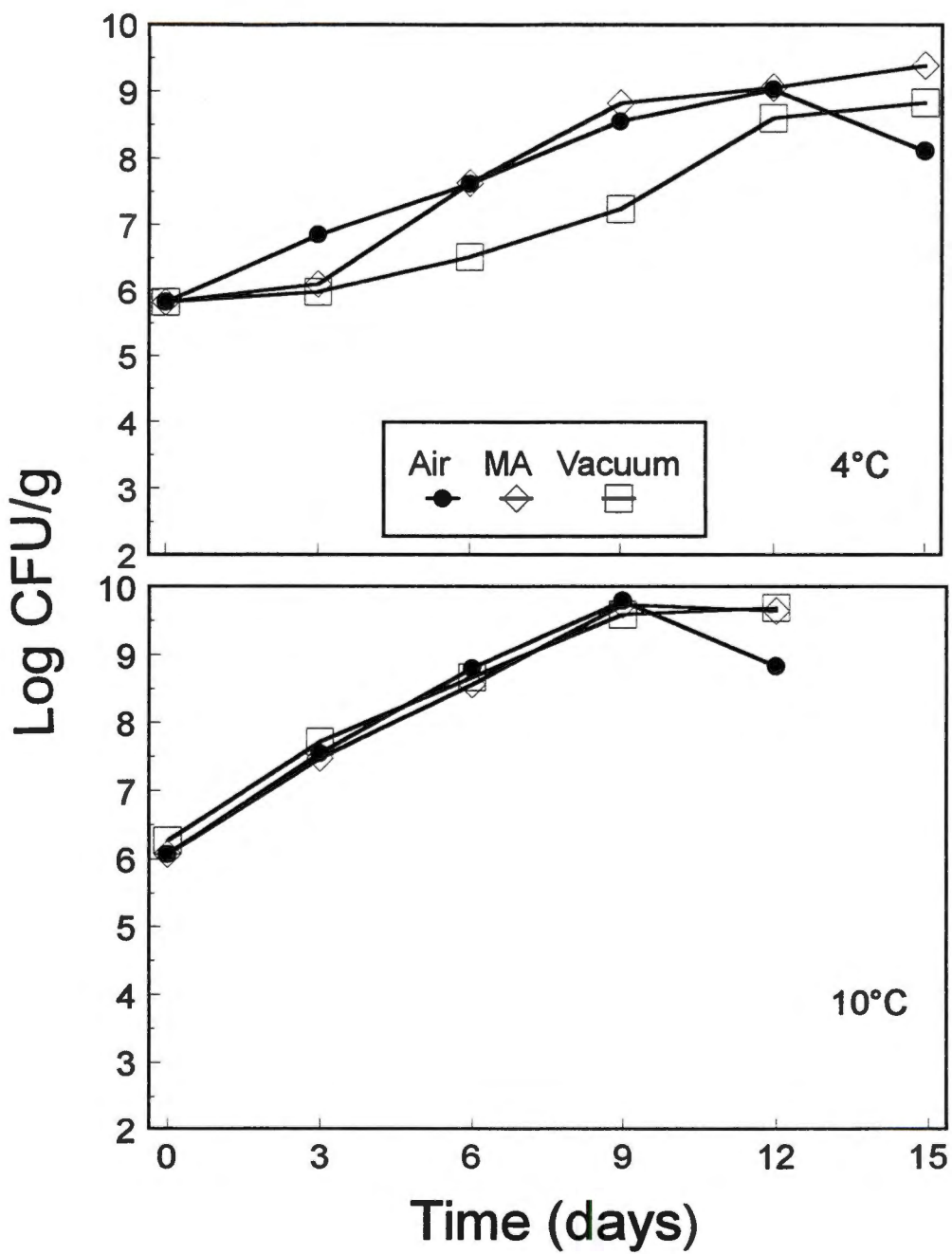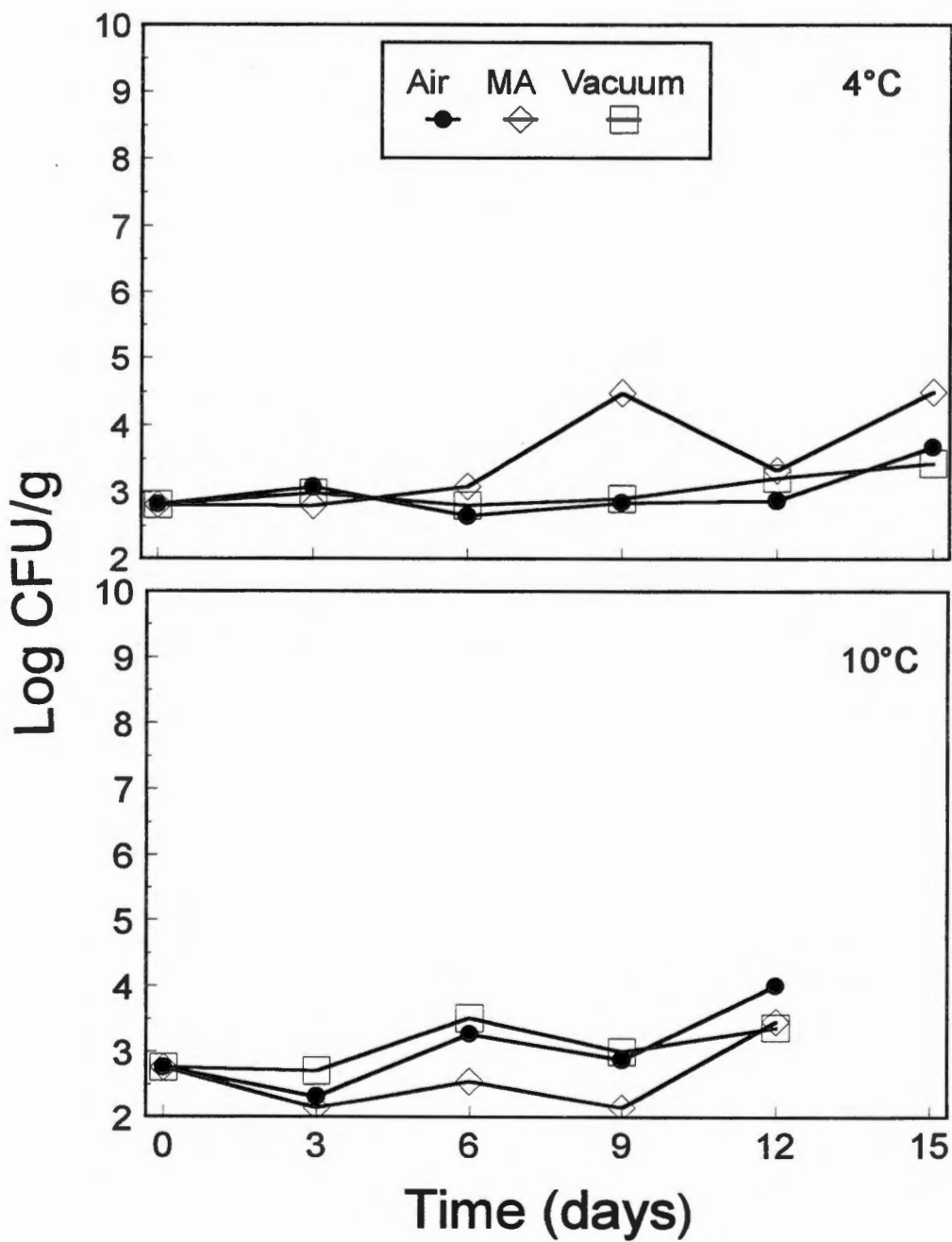Figure 4-12. Mold count for salt treated broccoli stored at 4 or 10°C under conditions of air, modified atmosphere (MA) and vacuum packaging.

Initially, coliforms were detected at low levels on broccoli stored at 4°C but could not be detected on either untreated and salt-treated broccoli after 3 days. Coliform growth at 10°C on salt-treated broccoli was higher (P<0.05) than growth on untreated broccoli for each packaging atmosphere (Table A-2). The initial coliform count for broccoli was approximately 3 log CFU/g (Figure 4-13). In untreated broccoli, coliforms increased between 1.8 and 2.6 log CFU/g by day 9 but by day 12 numbers had begun to decrease. In salt-treated broccoli, coliform numbers increased between 4 and 5.5 log CFU/g by day 9 before reaching stationary phase. Coliforms on vacuum packaged salt-treated broccoli grew more rapidly than those packaged in air and MA.

Headspace gas analysis

Headspace gas composition of vacuum, MA and air packaged broccoli changed substantially within the first 3 days of storage at 4 and 10°C. At 4°C, broccoli packaged under MA experienced increased O_2 for both salt-treated and untreated broccoli (Figure 4-14, Table A-3). Oxygen content was higher (P<0.05) for untreated broccoli. Levels of CO_2 in MA packaged broccoli decreased from approximately 10% to between 1 and 3% within 3 days. Carbon dioxide concentrations in both salt-treated and untreated broccoli were not different (P>0.05). When packaged in air, salt-treated broccoli had a higher (P<0.05) CO_2 level and lower O_2 level than untreated broccoli (Table A-4).

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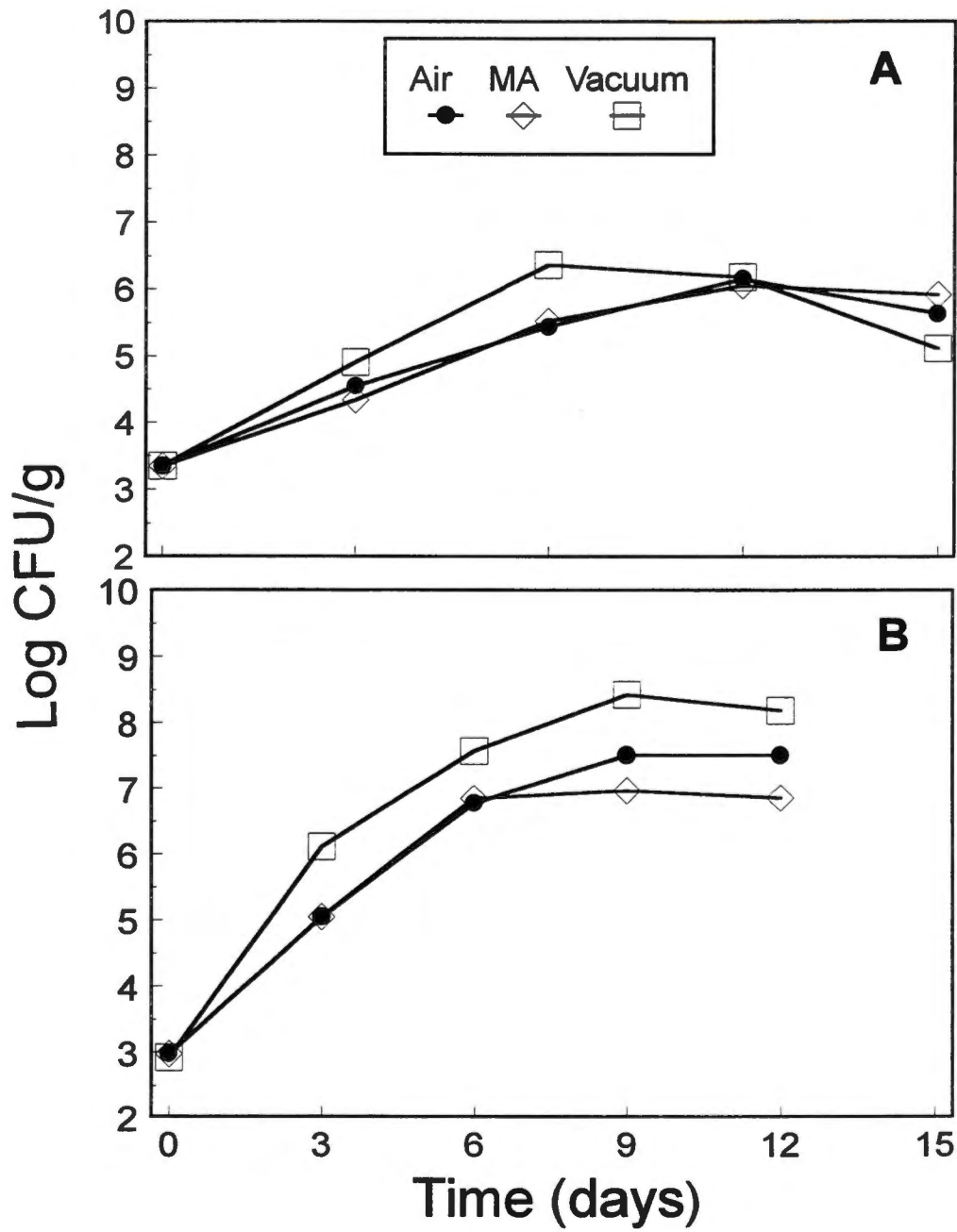
Figure 4-13. Coliform count for untreated (A) and salt treated (B) broccoli stored at 10°C under conditions of air, modified atmosphere (MA) and vacuum packaging.
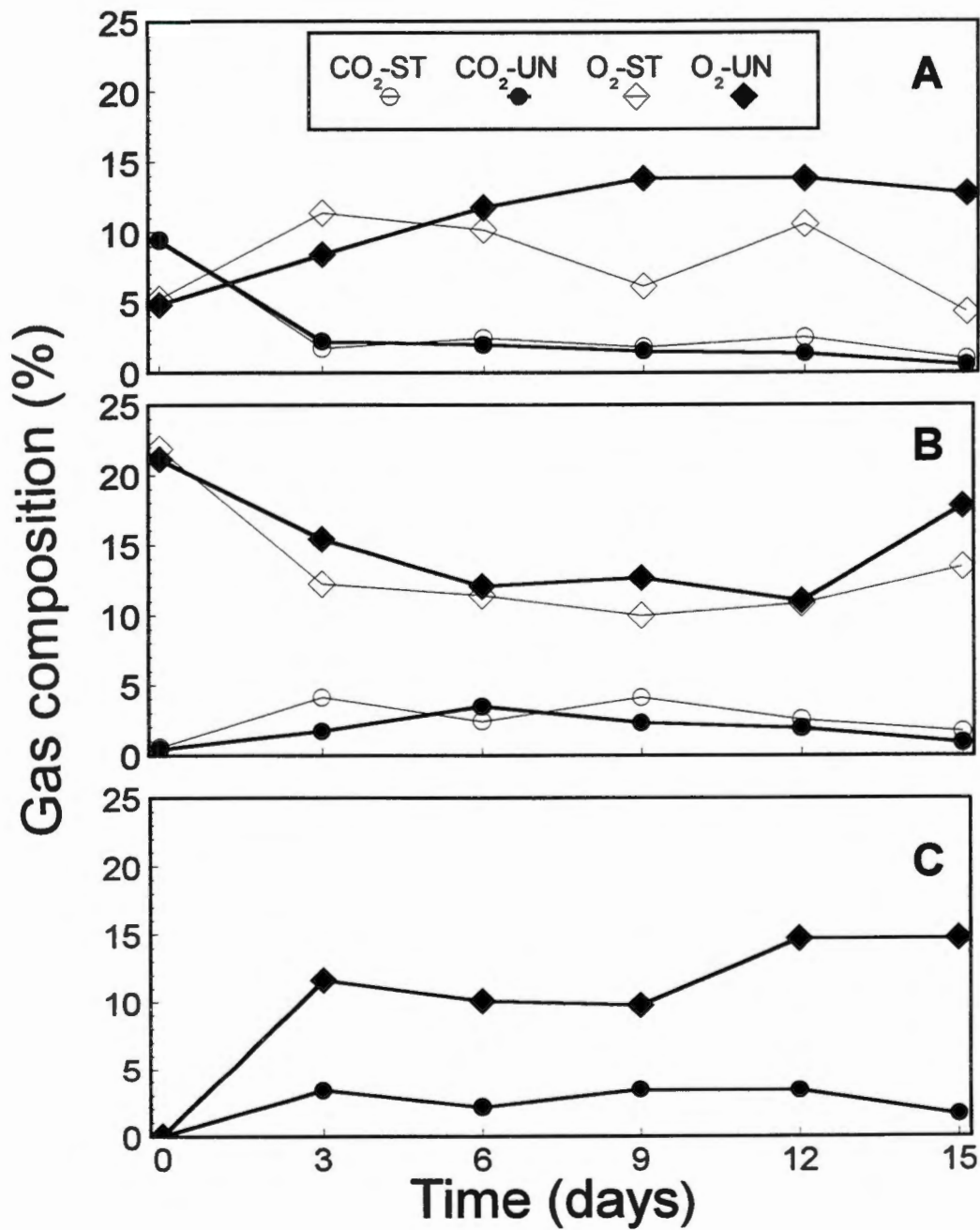
Figure 4-14. Headspace CO_2 and O_2 concentrations for (A) modified atmosphere (MA), (B) air and (C) vacuum packaged untreated (UN) and salt treated (ST) broccoli stored at 4°C.

At 10°C, untreated broccoli packaged in MA exhibited O_2 levels reaching approximately 7% at completion of storage, whereas salt-treated broccoli experienced an O_2 decrease (Figure 4-15). Oxygen content was higher (P<0.05) for untreated broccoli in MA at 10°C. Levels of CO_2 in MA packaged broccoli decreased from approximately 10% to around 4 to 5% within 3 days. Carbon dioxide concentrations in both salt-treated and untreated broccoli were not different (P>0.05). Salt-treated broccoli packaged in air had a higher (P<0.05) CO_2 level and lower O_2 level than untreated broccoli.

Vacuum packaged broccoli stored at 4 and 10°C maintained package integrity throughout storage, but due to packaging material permeability an equilibrium MA was established within the floret spaces of untreated broccoli. Gas analysis revealed that the equilibrium MA reached in untreated broccoli stored at 4°C was between 10 and 15% O_2 and 2 and 3% CO_2. The equilibrium MA established in broccoli stored at 10°C reached CO_2 levels between 5 and 7% and O_2 levels between 6 and 10%. Gas analysis was not conducted on salt-treated broccoli since excessive liquid present in the package interfered with sample collection.
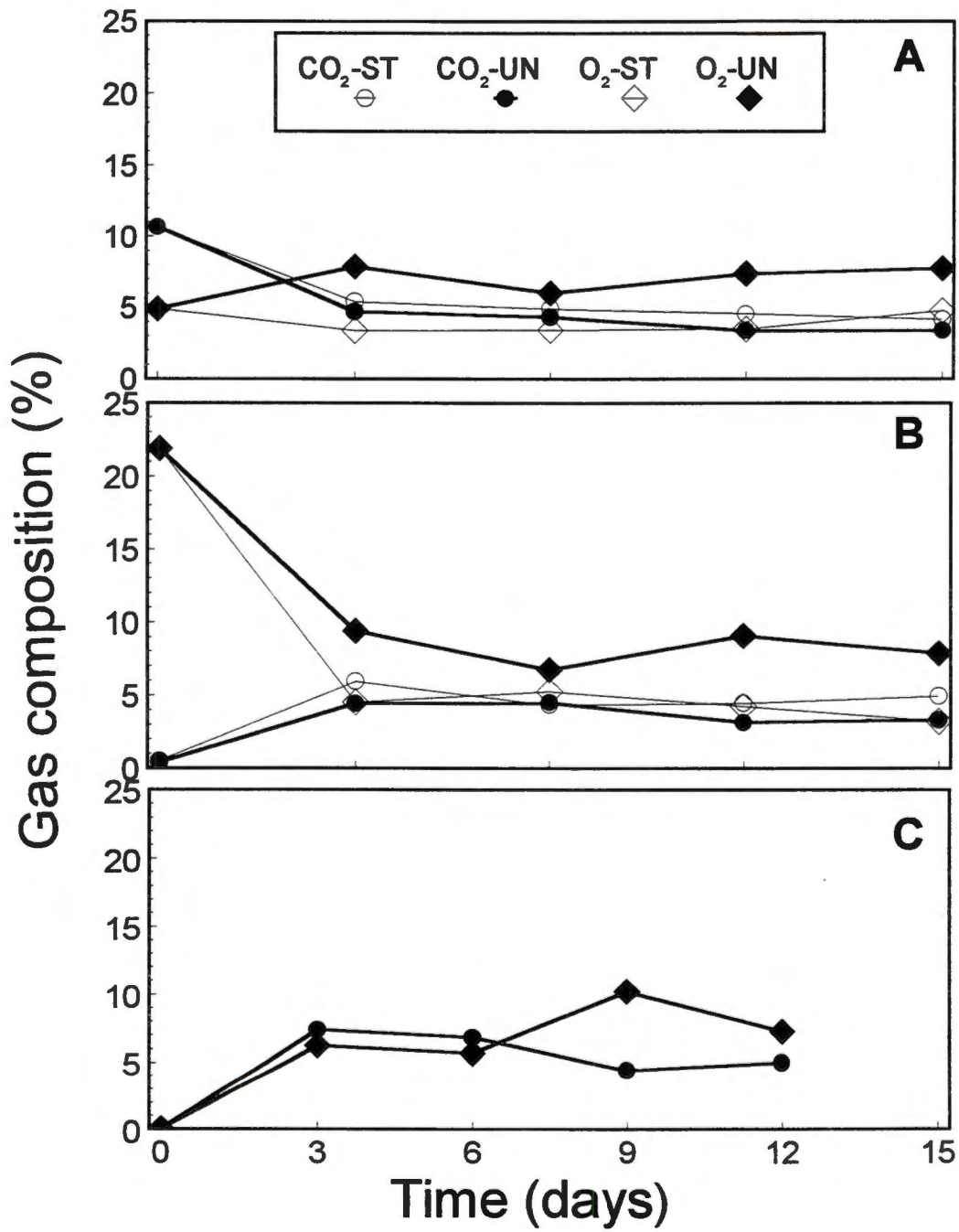
Figure 4-15. Headspace CO_2 and O_2 concentrations for (A) modified atmosphere (MA), (B) air and (C) vacuum packaged untreated (UN) and salt treated (ST) broccoli stored at 10°C.

DISCUSSION

Results show that the microflora of fresh broccoli consists of a large number of spoilage microorganisms. These data agree with results reported by Brackett (1989), indicating the presence of 5 to 6 log CFU/g of aerobic bacteria on fresh broccoli. Fresh broccoli has high water and nutrient contents and a neutral pH and can support growth of a diverse microflora (Brackett, 1994). The most frequently isolated genera of bacteria include *Pseudomonas*, *Erwinia*, *Enterobacter*, *Bacillus* and coryneforms. The microflora present on fresh broccoli is affected by factors such as its proximity to the soil, environmental conditions immediately before or during harvest and processing conditions. As broccoli was shipped on ice, it is likely that chilled storage conditions provided a selective advantage for psychrotrophic bacteria.

Salt treatment of broccoli increased LAB numbers in all packaging treatments at 4 and 10°C. As LAB are able to tolerate salt concentrations of 15.9% in food products such as pickles, salt treatment of broccoli would most likely have a selective effect (Jay, 1992). Storage of untreated broccoli at 4 and 10°C resulted in a minimal increase in numbers of LAB except in the case of vacuum packaged broccoli at 10°C where numbers increased 4 log CFU/g within 6 days. The reason for this increase is unclear but may be due to reduced competition from aerobic bacteria, thereby, allowing rapid LAB growth. Results show that aerobic mesophile numbers increased approximately 2 log CFU/g in vacuum packaged untreated broccoli by day 6 at 10°C, indicating growth

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conditions were more favorable for LAB. Gas concentrations in vacuum, air and MA packaged broccoli are similar, although the gas volume to product volume (GV:PV) ratio in vacuum packaged broccoli is low compared to air and MA packaged product. The difference in GV:PV coupled with a higher storage temperature may offer LAB advantageous growth conditions.

Numbers of pectinolytic bacteria increased in each packaging treatment during the storage period contributing to the breakdown and spoilage of broccoli. However, salt-treated broccoli stored at 4°C offered an inhibitory environment for pectinolytic bacteria resulting in a 9-day lag phase. The predominant pectinolytic bacteria present on fresh vegetables are *Erwinia carotovora* and fluorescent *Pseudomonas* spp. (King and Bolin, 1989). These two types of organisms are psychrotrophic and pseudomonads generally will outgrow *Erwinia* spp. (Brackett, 1994). Although pectinolytic bacteria present on broccoli were not identified, it is likely that the combination of low GV:PV ratio, salt treatment and low temperature were principal factors that contributed to the longer lag phase experienced.

Yeast and mold populations on broccoli remained at relatively low levels when stored at 4 and 10°C. The reason for fungal growth inhibition is that few fungi spoil vegetables at refrigeration temperatures and the presence of CO_2 in packages may have had an inhibitory effect (Brackett, 1994). Salt treatment of broccoli did not affect significantly ($P>0.05$) yeast and mold counts during storage at 4 and 10°C.

Coliforms did not grow in any of the broccoli packaging treatments during storage at 4°C. Coliforms were detected at low levels initially suggesting that they were a minor component of the normal surface flora of broccoli. As the rate of coliform growth in foods stored below 5°C is reduced, the chief limiting factor in determining the fate of the coliform population was storage at 4°C (Jay, 1992). Storage at 10°C resulted in enhanced coliform growth for untreated and salt-treated broccoli.

Packaging film PD941 (Cryovac, Duncan, SC) is designed for produce with high respiration rates. High O_2 and CO_2 transmission rates of the film allowed an equilibrium MA to be established in packaged broccoli assisting in shelf-life extension. Air and MA packages containing salt-treated broccoli had lower O_2 and higher CO_2 contents at 4 and 10°C. Reduced O_2 and raised CO_2 levels indicate salt-treated broccoli had a lower respiration rate than untreated broccoli (Wu and Salunkhe, 1976).

Results from this investigation indicate that use of salt as a limiting factor to inhibit spoilage bacteria and to extend shelf-life of minimally processed broccoli while maintaining its fresh-like quality is ineffective. Lowering storage temperature is an effective means for reducing respiration, ethylene production and growth of spoilage flora on broccoli (Brackett, 1994; Corcuff et al., 1996). Packaged broccoli, stored at 10°C, spoiled more rapidly than broccoli stored at 4°C. Fresh untreated broccoli packaged in air and MA maintained a crisp green appearance for around 9 days at 4°C and 6 days at 10°C while vacuum

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packaged fresh broccoli had an extended shelf-life of up to 15 days at 4°C. Improved shelf-life of vacuum packaged broccoli is believed to be due to a delay in senescence caused by ethylene inhibition (Wu and Salunkhe, 1976). Vacuum packaged salt-treated broccoli showed signs of deterioration within 3 days of packaging, while salt-treated broccoli packaged in air and MA began to deteriorate within 3 to 6 days at 4 and 10°C. Storage at 1°C can lengthen the shelf-life of broccoli up to 3 weeks by reducing physiological damage and reducing the microbial load (Brackett, 1989; Makhlouf et al., 1989). Temperature abuse of MA products is widespread in distribution channels and storage of broccoli at 4 and 10°C can be used to simulate conditions used in retail display and distribution (Brody, 1996). It is obvious from this study that salt treatment of minimally processed packaged broccoli offered no preservation advantage when compared with untreated broccoli packaged in the same manner.

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CHAPTER V

EFFECT OF MODIFIED ATMOSPHERE AND SALT TREATMENT ON GROWTH OF *YERSINIA ENTEROCOLITICA* ON MINIMALLY PROCESSED BROCCOLI STORED AT 4 OR 10°C.

ABSTRACT

Effects of storage in modified atmospheres (MA) and salt treatment on survival and growth of *Yersinia enterocolitica* on fresh broccoli were investigated. Broccoli was inoculated with pathogenic strains of *Y. enterocolitica*, treated with NaCl, and stored at 4 and 10°C under conditions of MA, air and vacuum packaging. *Y. enterocolitica* populations were monitored over 15 days at 4°C, and 12 days at 10°C. Presence of the virulence plasmid in *Y. enterocolitica* isolates was determined by evaluation of phenotypic characteristics at the completion of each storage period. Populations of *Y. enterocolitica* increased in untreated and salt-treated broccoli during storage at 4 and 10°C. Salt treatment of broccoli did not inhibit growth of *Y. enterocolitica* in each packaging treatment. The virulence plasmid of *Y. enterocolitica* was detected in 13 to 22% of isolates following salt treatment and storage on minimally processed broccoli.

INTRODUCTION

Consumer demand for convenience foods has resulted in changes in production and handling practices of fresh vegetables. Production of minimally processed vegetables as ready-to-eat salads has substantially increased in the past four years (Demetrakakes, 1996; Dornblaser, 1995). The variety of products includes single or mixed vegetables intended for cooking and prewashed ready-to-eat "salad kits."

Techniques for shelf-life extension of minimally processed vegetables include use of refrigeration in combination with various modified atmosphere (MA) gas mixtures. Usually, MA packaging of vegetables involves use of a lower oxygen (O_2) concentration and a higher carbon dioxide (CO_2) concentration than is normally found in air (Berrang et al., 1989). Metabolic activities of MA packaged vegetables result in continued consumption of O_2 accompanied by production of water, CO_2 and heat (Lee at al., 1991). The reduction in O_2 and accumulation of CO_2 can impede microbial growth and retard quality deterioration (Ballantyne, 1989; Kader et al., 1989). Temperature control is a significant factor in shelf-life extension of minimally processed vegetables. Cooling packaged minimally processed vegetables retards microbial growth, vegetable respiration and other chemical processes such as oxidation and browning of cut surfaces. Treatment with a hypertonic medium such as a high concentration sugar or salt solution has been proposed as a valuable processing tool for minimally processed fruit and vegetables (Lazarides, 1994). This method

relies on water activity (Aw) reduction through moisture removal for microbial inhibition (Wiley, 1994).

Yersinia enterocolitica is a Gram-negative, psychrotrophic, facultative anaerobe. Several varieties of *Y. enterocolitica*, both pathogenic and avirulent, have been isolated from unpasteurized milk, non-chlorinated drinking water, fresh vegetables, raw or rare meats and raw shellfish (Beuchat, 1996; Brocklehurst et al., 1987; Stern, 1982). *Y. enterocolitica* is transmitted to humans through ingestion of contaminated food and water; the minimum infective dose for humans is not known (Cover and Arber, 1989; Farber, 1991). *Y. enterocolitica* can tolerate up to 5% NaCl in growth media and has been reported to grow at temperatures between 0 and 44°C (Doyle, 1988; Feng and Weagant, 1994). Pathogenic strains of *Y. enterocolitica* harbor a 40-50 megadalton plasmid that confers virulence on the infecting organism (Prpic et al., 1985). Loss of the plasmid results in loss of virulence characteristics. Effects of consuming avirulent varieties may be no more hazardous than consuming nonpathogenic coliform bacteria. Bhaduri and Turner-Jones (1993) attempted to assess the stability of the virulence plasmid in *Y. enterocolitica* under various anaerobic conditions found in food processing. Their results indicated no plasmid loss with virulent *Y. enterocolitica* in logarithmic and stationary growth phases when organisms were exposed to a vacuum or to an atmosphere of 94% CO_2: 6% hydrogen (H_2) for 24 h at 28°C.

Several aspects of processing and storage protocol of minimally

processed vegetables may lend themselves to potential contamination with psychrotrophic pathogens such as *Y. enterocolitica*. For example, postharvest handling can result in contamination, while refrigeration for extended periods may ensure proliferation of the pathogen. As little data has been reported regarding the growth of *Y. enterocolitica* on raw vegetables, the aim of this study was to investigate growth and survival of pathogenic *Y. enterocolitica* on minimally processed broccoli. Broccoli was prepared using a salt treatment and was stored under several MA conditions at 4 and 10°C. Changes in the virulence of pathogenic *Y. enterocolitica* was also studied to determine if the pathogen could be rendered avirulent following exposure to a food processing regime.

MATERIALS AND METHODS

Broccoli and storage conditions

Fresh broccoli heads shipped on ice were purchased from a local supplier. Broccoli was graded and cut into florets and stored at 4°C prior to packaging. Broccoli florets were divided into two treatment groups for packaging, salt-treated and untreated broccoli and were inoculated with *Y. enterocolitica* as described below.

Bacterial strains

Three strains of *Y. enterocolitica*, YE133 (serotype O:8), YE228 (serotype O:3) and YE321 (serotype O:20) (obtained from Peter Feng, United States Food and Drug Administration, Washington, DC), were used for inoculation studies. Stock cultures were stored on trypticase soy agar (TSA; BBL, Cockeysville, MD) slants at 4°C.

Inoculation of broccoli florets with Y. enterocolitica

Broccoli florets were inoculated with *Y. enterocolitica* prior to salt treatment. Pure cultures of each *Y. enterocolitica* strain were grown for 48 h at 30°C in trypticase soy broth (BBL, Cockeysville, MD). The 3 cultures were added in equal proportions to 30 L of 0.1% peptone water to give an approximate final mixed population of 10^6 cells/mL. Broccoli florets were dipped into the diluted *Y. enterocolitica* suspension, held for 1 min and allowed to drain for 5 to 10 min before salt treatment.

Salt treatment

A 40 L saturated NaCl solution was used to prepare salt-treated broccoli. Broccoli florets were immersed in the saturated NaCl solution for 21 min to achieve a final salt concentration of 1%. Salt concentration in the broccoli was determined using AOAC (1990) method 50.029.

Packaging materials and methods

The packaging film used was PD-941 (Cryovac, Duncan, SC), a multilayered film for packaging produce with high respiration rates (oxygen transmission rate - 16544 mL/m²/24 h). A Multivac (Koch, Kansas City, MO) gas flushing machine was used to achieve the desired atmosphere. Each broccoli treatment was stored under conditions of MA, air and vacuum packaging. A standard gas mixture of $CO_2:O_2:N_2$ (10:5:85) (MG Industries, Valley Forge, PA) was used for packaging florets in a MA. Approximately 250 g of each broccoli treatment were weighed into separate PD-941 bags for storage and sampling.

Storage and sampling

Packaged samples were stored in the dark at 4 and 10°C and were taken for analysis at 3-day intervals throughout the storage period. On each sampling day, gas composition of the package was determined using a Hewlett Packard gas chromatograph, model 5890 - series II (Hewlett Packard, Wilmington, DE) equipped with a thermal conductivity detector and a stainless steel CTRI column (Alltech Associates, Inc., Deerfield, IL). Vacuum packaged salt-treated broccoli was not sampled for gas analysis since excessive liquid present in the package interfered with sample collection.

Isolation and enumeration of microorganisms

Samples (25 g) were removed from each PD-941 bag and placed into

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sterile stomacher bags with 225 mL of 0.1% peptone water and blended for 1 min using a Model 400 Stomacher Lab Blender (Steward, London, UK). Serial dilutions of the broccoli slurry were surface plated (0.1 mL) onto cefsulodin irgasan novobiocin agar (CIN; BBL, Cockeysville, MD) for *Y. enterocolitica* and incubated at 30°C for 24 to 48 h for enumeration. Colonies on CIN medium were confirmed as *Y. enterocolitica* using motility and biochemical tests (Weagant et al., 1992).

Virulence testing

Isolates of *Y. enterocolitica* were tested for virulence before and after exposure to salt-treated packaged broccoli. Virulence testing methods included Congo red uptake and calcium dependence (Riley and Toma, 1989), autoagglutination in methyl red Voges-Proskauer (MR-VP) medium (Weagant et al., 1992), and autoagglutination in tissue culture medium RPMI-1640 (Laird and Cavanaugh, 1980).

Statistical analysis

Three replications of all experiments were done. The General Linear Models (GLM) procedure was used to analyze main effects and interactions of packaging atmosphere, storage time and salt treatment on differences in mean microbial numbers (SAS Institute, Inc., 1990).

RESULTS

Broccoli examined prior to inoculation was found to be free of *Y. enterocolitica*. Growth of *Y. enterocolitica* was higher($P<0.05$) on salt-treated broccoli than on untreated broccoli at 4 and 10°C (Figures 5-1, 5-2, 5-3 and 5-4, Table A-2). *Y. enterocolitica* grew on salt-treated broccoli under each of the packaging atmospheres, achieving a 3.2 to 4.5 log CFU/g increase in cell numbers at 4°C, and 3.5 to 4.5 log CFU/g increase at 10°C. *Y. enterocolitica* populations detected on untreated broccoli were higher ($P<0.05$), when vacuum packaged, than on broccoli packaged in air or MA at 4°C. At 10°C, changes in *Y. enterocolitica* populations were higher ($P<0.05$) for vacuum packaged broccoli than for air packaged broccoli. In salt-treated broccoli, no packaging regime was significantly ($P>0.05$) different at either 4 or 10°C with respect to *Y. enterocolitica* growth.

Headspace gas composition of MA and air packaged broccoli changed substantially within the first 3 days of storage. When packaged under MA, O_2 increased for both salt-treated and untreated broccoli at 4°C. Oxygen content was higher ($P<0.05$) for untreated broccoli at 10°C (Table A-5). Levels of CO_2 in MA packaged broccoli decreased from approximately 10% to between 1 and 3% at 4°C and to between 5 and 6% at 10°C within 3 days. Carbon dioxide concentrations in salt-treated and untreated broccoli were not significantly ($P>0.05$) different in air or MA packages at either storage temperature. When packaged in air, CO_2 levels in salt-treated and untreated broccoli were not
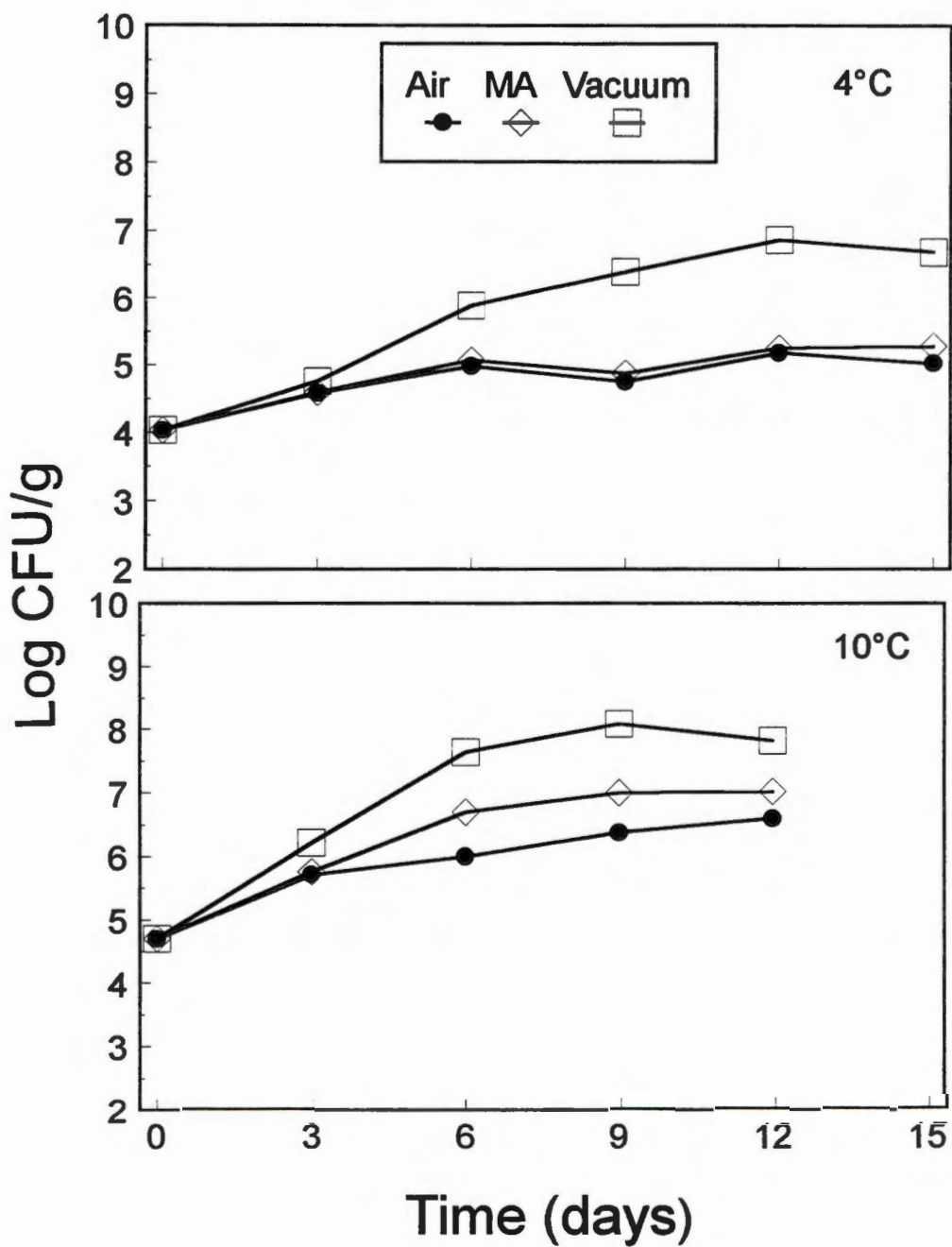
Figure 5-1. *Yersinia enterocolitica* count for untreated broccoli stored at 4 or 10°C under conditions of air, modified atmosphere (MA) and vacuum packaging.
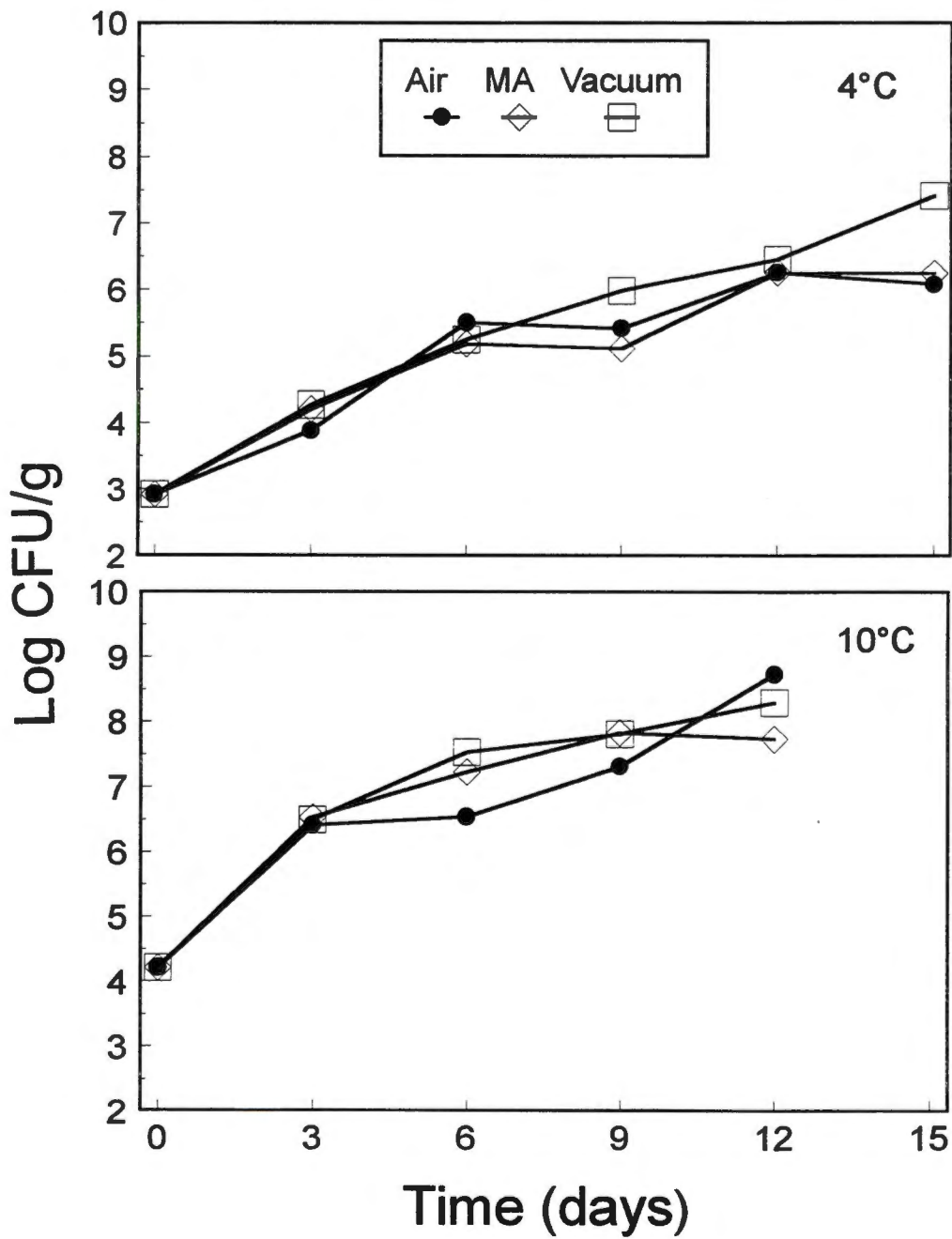
Figure 5-2. *Yersinia enterocolitica* count for salt treated broccoli stored at 4 or 10°C under conditions of air, modified atmosphere (MA) and vacuum packaging.
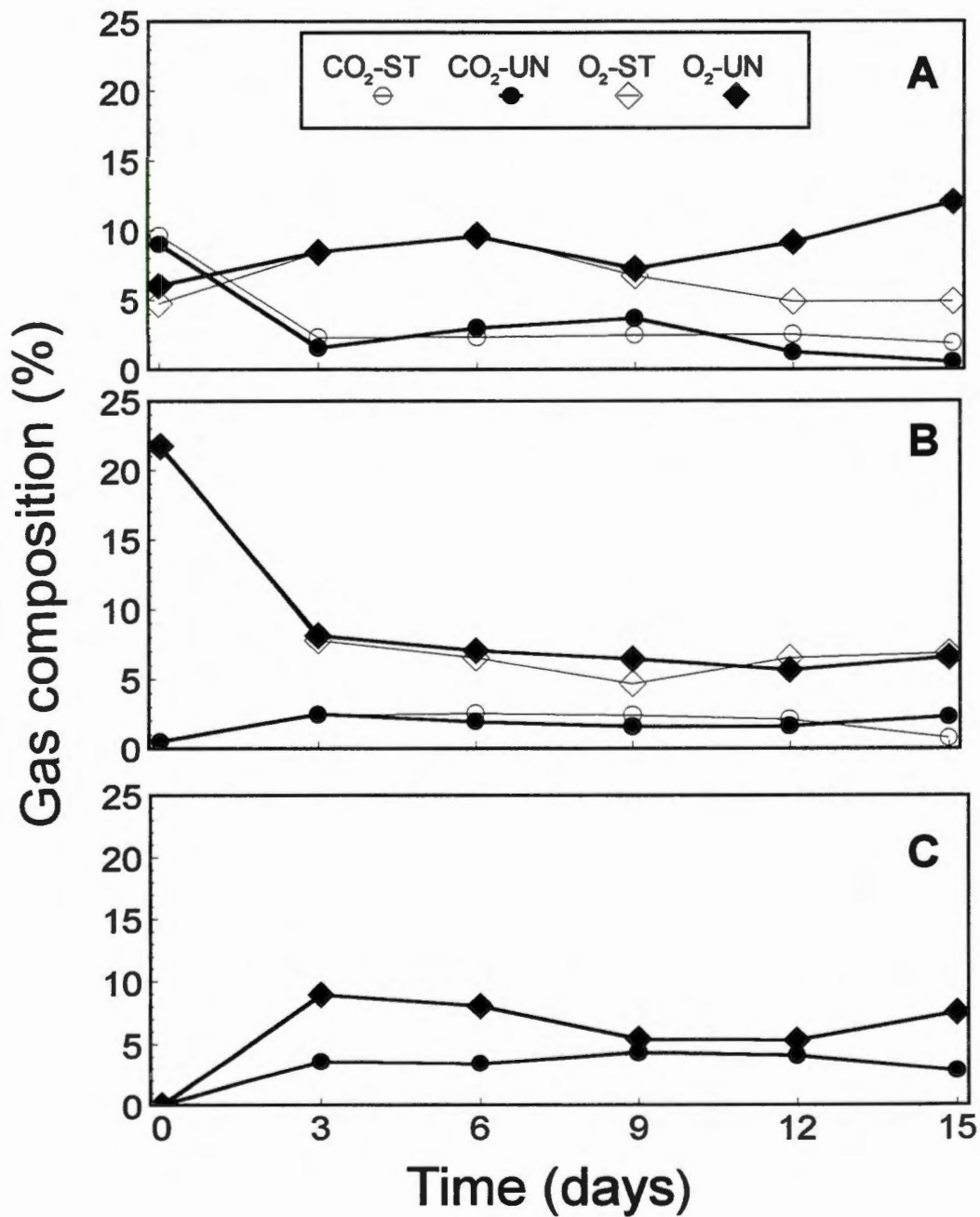
Figure 5-3. Headspace CO_2 and O_2 concentrations for (A) modified atmosphere (MA), (B) air and (C) vacuum packaged untreated (UN) and salt treated (ST) broccoli inoculated with *Yersinia enterocolitica* and stored at 4°C.
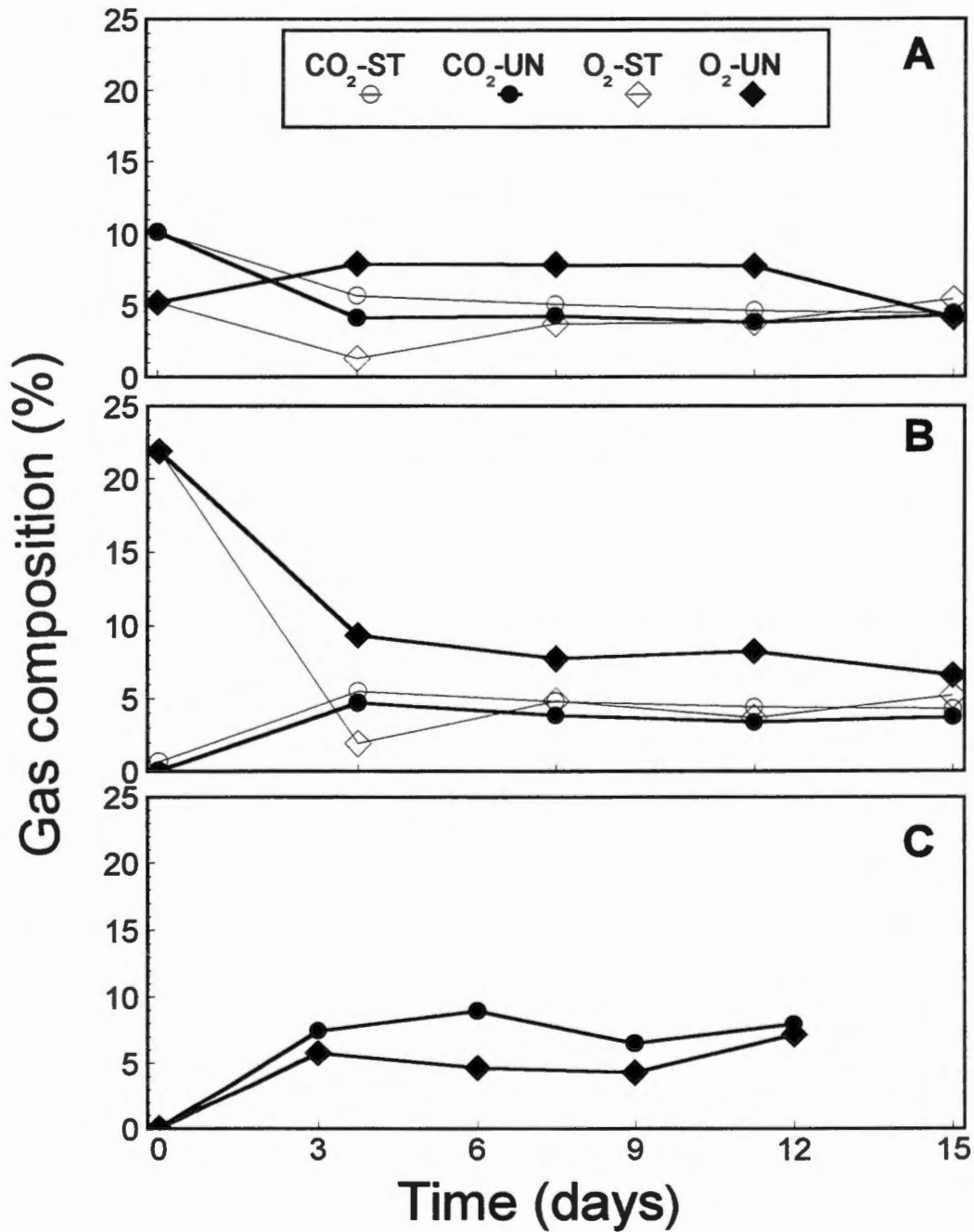
Figure 5-4. Headspace CO_2 and O_2 concentrations for (A) modified atmosphere (MA), (B) air and (C) vacuum packaged untreated (UN) and salt treated (ST) broccoli inoculated with *Yersinia enterocolitica* and stored at 10°C.

different (P>0.05), while O_2 levels in broccoli stored at 10°C were higher (P<0.05) in untreated broccoli (Table A-6). Vacuum packaged broccoli maintained package integrity throughout storage, but due to the gas permeability of the packaging material, an equilibrium MA was established within floret spaces. Gas analysis revealed that the equilibrium MA reached in vacuum packaged untreated broccoli was between 6 and 9% CO_2 at 10°C, and between 3 and 5% CO_2 at 4°C, with the O_2 level remaining between 5 and 10% for both storage temperatures. Gas analysis was not done on salt-treated broccoli since excessive liquid present in the package interfered with sample collection.

Indicator tests for virulence confirmed that 13 to 22% of virulent *Y. enterocolitica* isolates were capable of retaining the virulence plasmid following exposure to salt treatment and storage at 4 or 10°C under conditions of MA, air and vacuum packaging (Table 5-1).

DISCUSSION

Packaging in MAs and salt treatment of broccoli did not inhibit growth of *Y. enterocolitica* at 4 or 10°C. *Y. enterocolitica* grew equally well on salt-treated broccoli in all atmospheres, increasing more than 3 log CFU/g at 4°C and approximately 4 log CFU/g at 10°C over the specified storage periods. In vacuum packaged untreated broccoli, *Y. enterocolitica* numbers increased more rapidly than on product packaged in air or MA. Other studies evaluating growth of *Y. enterocolitica* in MA conditions have dealt mainly with meats and also

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Table 5-1. Effect of packaging conditions on the stability of the *Yersinia enterocolitica* virulence plasmid following 15 days storage at 4°C and 12 days storage at 10°C on fresh and salt-treated broccoli packaged in modified atmospheres.

Storage Temperature	Packaging conditions	Total number of colonies selected from CIN[a] agar	No. (%) of colonies AA[b] in MR-VP[c]	No. (%) of colonies AA in RPMI-1640[d]	No. (%) of CRMOX+[e] colonies
4°C	UN[f]-air	60	10 (17)	10 (17)	10 (17)
	UN-MA[g]	60	10 (17)	11 (18)	10 (17)
	UN-vacuum	60	11 (18)	12 (20)	12 (20)
	ST[h]-air	60	11 (18)	11 (18)	11 (18)
	ST-MA	60	9 (15)	10 (17)	9 (15)
	ST-vacuum	60	11 (18)	10 (17)	11 (18)
10°C	UN-air	60	9 (15)	10 (17)	9 (15)
	UN-MA	60	11 (18)	11 (18)	9 (15)
	UN-vacuum	60	11 (18)	11 (18)	11 (18)
	ST-air	60	8 (13)	12 (20)	9 (15)
	ST-MA	60	10 (17)	10 (17)	12 (20)
	ST-vacuum	60	12 (20)	11 (18)	13 (22)

[a] Cefsulodin irgasan agar

[b] Autoagglutination

[c] Methyl red Vogues-Proskauer broth

[d] Tissue culture medium RPMI-1640

[e] Congo red binding, calcium dependence positive

[f] Untreated broccoli

[g] Modified atmosphere

[h] Salt-treated broccoli

found that this organism grew to higher levels under vacuum conditions when compared with gas flushed packages (Doherty et al., 1995; Gill and Reichel, 1989; Hudson et al., 1994). The reason for increased growth under vacuum in meats and in this study is unclear. The MA established in the vacuum package was in the form of gas trapped within floret buds and stalks. The observed increase in *Y. enterocolitica* growth in vacuum packages possibly was due to the extremely low gas volume to product volume ratio in vacuum packaged broccoli (Day, 1992). *Y enterocolitica* on the outer surface of broccoli was not subjected to the equilibrium MA established within the broccoli florets and, therefore, exposure to effects of gases was minimal resulting in greater population increases.

Results of this investigation indicate that use of salt as a limiting factor to inhibit *Y. enterocolitica* and to extend the shelf-life of minimally processed broccoli is ineffective. The influence of salt on growth of *Y. enterocolitica* has been reported using bacteriological media and processed meats (Stern et al., 1980; Nielsen and Zeuthen, 1985). Stern et al. (1980) reported growth of *Y. enterocolitica* in brain heart infusion broth containing 5% (w/v) salt at 3 and 25°C. Nielsen and Zeuthen (1985) showed that *Y. enterocolitica* could grow at 5°C on vacuum packed cooked meats containing 4.5% salt. These data suggest that salt conditions that commonly inhibit Enterobacteriaceae under refrigeration are not adequate to inhibit *Y. enterocolitica* (Stern et al., 1980). In this study, immersion of broccoli florets in a saturated salt solution for 21 min did not inhibit

growth of *Y. enterocolitica* throughout storage in MA conditions.

Virulent *Y. enterocolitica* can dissociate into virulent and avirulent clones after growth at elevated temperatures due to loss of the virulence plasmid (Wolf-Watz, 1987). Studies show that the virulence plasmid is stable in *Y. enterocolitica* following passage through a food system or exposure to pH and salt extremes (Bhaduri and Mertz, 1989; Bodnaruk and Golden, 1996; Hellmann and Heinrich, 1985). In this study, indicator tests for virulence confirmed that *Y. enterocolitica* strains retained their virulence factor throughout processing and storage at 4 and 10°C. Results in Table 5-1 show that virulence in *Y. enterocolitica* is stable when subjected to salt treatment coupled with air, vacuum or MA packaging. Indicator tests showed that 13 to 22% of *Y. enterocolitica* isolates detected at the completion of broccoli storage maintained their virulence phenotype.

Extending the shelf-life of fresh produce without considering microbial growth patterns can present problems. Salt treatment of broccoli and packaging in MA conditions was not sufficient to prevent growth of *Y. enterocolitica*. Due to the potential for rapid growth of *Y. enterocolitica* on broccoli, more effective means for controlling pathogen growth on minimally processed MA packaged broccoli are needed.

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CHAPTER VI

CONCLUSION

Growth of *Y. enterocolitica* on CO_2 and vacuum packaged high pH pork increased rapidly compared with growth on normal pH pork packaged under the same conditions. On normal pH pork packaged in 100% CO_2, *Y. enterocolitica* remained in the lag phase for the duration of storage, whereas vacuum packaging allowed for substantial growth. These results suggest that refrigerated MA packaged high pH pork does not provide an environment that is sufficiently inhibitory to suppress growth of *Y. enterocolitica*. To reduce the risk of proliferation of *Y. enterocolitica* on fresh MA packaged pork, packaging of retail cuts or primals should be confined to normal pH meat in a gas atmosphere that has inhibitory properties, such as 100% CO_2.

Y. enterocolitica grew on minimally processed broccoli regardless of treatment. At 10°C, population increases were more rapid than increases at 4°C. Salt treatment of broccoli increased salt content and reduced respiration rate, but did not inhibit microbial growth. Results showed that growth of *Y. enterocolitica* and some spoilage organisms was enhanced by salt treatment, effectively reducing the shelf-life of the product. Packaging system (air, MA or vacuum) generally did not influence growth of spoilage microorganisms, although it appeared that vacuum packaged untreated broccoli was less inhibitory to *Y. enterocolitica* when compared with air or MA packaging.

The variety of MA packaged products in North America has increased in recent years. Proactive research on microbiological safety of some of these products provides information necessary for producers and regulatory agencies to ensure the safety of these new additions to grocery store shelves.

APPENDIX

Table A-1. Analysis of variance (GLM) of microbial counts (log CFU/cm^2) on pork packaged in modified atmospheres and stored at 4°C.

Factor	Source	df	Mean square	Prob>F
Y. enterocolitica	Atmosphere	1	19.2209	0.0001
	Day	6	2.8583	0.0001
	Meat pH	1	33.4766	0.0001
	Replication	2	0.7489	0.2063
	Atm*Day	6	1.3920	0.0124
	Atm*Meat pH	1	0.0224	0.8264
	Day*Meat pH	6	1.4586	0.0095
	Error	58	0.4617	
B. thermosphacta	Atmosphere	1	0.1612	0.3504
	Day	6	0.6634	0.0038
	Meat pH	1	4.8672	0.0001
	Replication	2	0.3996	0.1202
	Atm*Day	6	0.3078	0.1387
	Atm*Meat pH	1	0.0189	0.7484
	Day*Meat pH	6	0.4561	0.0313
	Error	60	0.1820	
Gram-negative	Atmosphere	1	19.6330	0.0001
bacteria	Day	6	7.8585	0.0001
	Meat pH	1	6.0536	0.0051
	Replication	2	0.3472	0.6183
	Atm*Day	6	0.6113	0.5346
	Atm*Meat pH	1	0.7410	0.3133
	Day*Meat pH	6	0.4804	0.6738
	Error	60	0.7166	
lactic acid	Atmosphere	1	2.5515	0.1049
bacteria	Day	6	26.1988	0.0001
	Meat pH	1	22.4440	0.0001
	Replication	2	4.7791	0.0092
	Atm*Day	6	0.4747	0.8027
	Atm*Meat pH	1	1.1340	0.2768
	Day*Meat pH	6	1.0690	0.3529
	Error	60	0.9413	
aerobic	Atmosphere	1	4.1398	0.0575
mesophiles	Day	6	31.2169	0.0001
	Meat pH	1	16.3417	0.0003
	Replication	2	2.8428	0.0846
	Atm*Day	6	0.2438	0.9686
	Atm*Meat pH	1	0.3936	0.5527
	Day*Meat pH	6	0.6797	0.7169
	Error	60	1.1041	

Table A-2. Analysis of variance (GLM) of differences in microbial counts(log CFU/g) on broccoli stored at 4 and 10°C under conditions of air, modified atmosphere and vacuum packaging.

Factor	Source	4°C			10°C		
		df	Mean square	Prob>F	df	Mean square	Prob>F
Y.enterocolitica	Day	5	18.8464	0.0001	4	30.2299	0.0001
	Atmosphere	2	5.4854	0.0001	2	2.9934	0.0074
	Salt treatment	1	33.0893	0.0001	1	18.0275	0.0001
	Replication	2	0.4821	0.2175	2	6.8262	0.0001
	Atm*Day	10	0.6534	0.0331	8	0.5689	0.4400
	Salt treat*Atm	2	1.0379	0.0402	2	1.2078	0.1262
	Salt treat*Day	5	2.4187	0.0001	4	1.5050	0.0401
	Error	80	0.3100		66	0.5654	
aerobic	Day	5	16.652	0.0001	4	25.0306	0.0001
mesophiles	Atmosphere	2	1.3038	0.0287	2	0.6877	0.1172
	Salt treatment	1	24.2062	0.0001	1	11.6352	0.0001
	Replication	2	0.0089	0.9747	2	0.0729	0.7912
	Atm*Day	10	0.3352	0.4887	8	0.2922	0.4891
	Salt treat*Atm	2	0.7831	0.1140	2	0.2312	0.4787
	Salt treat*Day	5	2.2587	0.0001	4	3.0309	0.0001
	Error	80	0.3509		66	0.3104	

Table A-2. continued

Factor	Source	4°C			10°C		
		df	Mean square	Prob>F	df	Mean square	Prob>F
lactic acid	Day	5	6.6964	0.0001	4	38.9295	0.0001
bacteria	Atmosphere	2	0.4726	0.2335	2	17.0782	0.0001
	Salt treatment	1	0.2241	0.4045	1	42.8352	0.0001
	Replication	2	1.9625	0.0033	2	8.7366	0.0001
	Atm*Day	10	0.1550	0.8945	8	1.5504	0.0074
	Salt treat*Atm	2	0.6447	0.1393	2	0.6890	0.2792
	Salt treat*Day	5	2.8963	0.0001	4	4.5043	0.0001
	Error	80	0.3190		66	0.5296	
psychrotroph	Day	5	24.2506	0.0001	4	27.8495	0.0001
	Atmosphere	2	0.9992	0.0210	2	1.8252	0.0649
	Salt treatment	1	25.6766	0.0001	1	9.6040	0.0002
	Replication	2	0.4577	0.1627	2	1.9056	0.0578
	Atm*Day	10	0.2829	0.3380	8	0.3812	0.7779
	Salt treat*Atm	2	1.6707	0.0019	2	0.6724	0.6266
	Salt treat*Day	5	2.7522	0.0001	4	2.7967	0.5913
	Error	80	0.2464		66	0.6399	

Table A-2. continued

Factor	Source	4°C			10°C		
		df	Mean square	Prob>F	df	Mean square	Prob>F
pectinolytic	Day	5	16.6529	0.0001	4	22.5322	0.0001
bacteria	Atmosphere	2	2.0687	0.0029	2	1.0713	0.2339
	Salt treatment	1	0.1488	0.5025	1	10.6495	0.0003
	Replication	2	0.0476	0.8651	2	35.8832	0.0001
	Atm*Day	10	0.2669	0.6165	8	0.3659	0.8466
	Salt treat*Atm	2	4.4194	0.0001	2	0.2977	0.6635
	Salt treat*Day	5	1.2684	0.0035	4	1.3535	0.1249
	Error	80	0.3281		66	0.7212	
yeasts	Day	5	8.6803	0.0001	4	4.9774	0.0001
	Atmosphere	2	1.1676	0.0098	2	0.7711	0.0736
	Salt treatment	1	0.2591	0.3002	1	1.5080	0.0244
	Replication	2	0.2446	0.3629	2	12.6545	0.0001
	Atm*Day	10	0.1461	0.7983	8	0.1817	0.7415
	Salt treat*Atm	2	1.2481	0.0073	2	0.1337	0.6266
	Salt treat*Day	5	1.4961	0.0001	4	0.2002	0.5913
	Error	80	0.2383		66	0.2839	

Table A-2. continued

Factor	Source	4°C			10°C		
		df	Mean square	Prob>F	df	Mean square	Prob>F
molds	Day	5	0.9105	0.0001	4	2.5181	0.0001
	Atmosphere	2	0.7944	0.0066	2	0.4060	0.2502
	Salt treatment	1	2.2016	0.0002	1	2.1529	0.0079
	Replication	2	0.5274	0.0333	2	5.0066	0.0001
	Atm*Day	10	0.2582	0.0861	8	0.2259	0.6153
	Salt treat*Atm	2	0.6761	0.0134	2	0.7809	0.0732
	Salt treat*Day	5	0.6474	0.0015	4	0.4439	0.1989
	Error	80	0.1484		66	0.2869	
coliform[a]	Day	-	-	-	4	42.6804	0.0001
bacteria	Atmosphere	-	-	-	2	2.3850	0.1331
	Salt treatment	-	-	-	1	44.6054	0.0001
	Replication	-	-	-	2	15.1639	0.0001
	Atm*Day	-	-	-	8	0.3120	0.9729
	Salt treat*Atm	-	-	-	2	1.1630	0.3683
	Salt treat*Day	-	-	-	4	3.5596	0.0211
	Error	-	-		66	1.1469	

[a]Coliform bacteria not detected on packaged broccoli stored at 4°C

Table A-3. Analysis of variance (GLM) for headspace gas analysis of salt-treated and untreated broccoli packaged in a modified atmosphere and stored at 4 or 10°C.

Gas	Source		4°C			10°C	
		df	Mean square	Prob>F	df	Mean square	Prob>F
CO_2	Day	5	61.8419	0.0001	4	48.7235	0.0001
	Salt treatment	1	0.9078	0.3080	1	3.2761	0.1052
	Replication	2	2.3683	0.0799	2	2.2104	0.1692
	Salt treat*Day	5	0.4247	0.7660	4	0.2981	0.8954
	Error	22	0.8336		17	1.1185	
O_2	Day	5	37.0602	0.0025	4	2.6152	0.7451
	Salt treatment	1	77.7247	0.0031	1	64.2698	0.0030
	Replication	2	15.7546	0.1298	2	43.5656	0.0034
	Salt treat*Day	5	30.3835	0.0068	4	5.4073	0.4311
	Error	22	7.0222		17	5.3684	

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Table A-4. Analysis of variance (GLM) for headspace gas analysis of salt-treated and untreated broccoli packaged in air and stored at 4 or 10°C.

Gas	Source	4°C			10°C		
		df	Mean square	Prob>F	df	Mean square	Prob>F
CO_2	Day	5	6.9170	0.0076	4	24.5561	0.0001
	Salt treatment	1	5.6453	0.0766	1	5.5987	0.0035
	Replication	2	7.5447	0.0212	2	1.0425	0.1508
	Salt treat*Day	5	2.2422	0.2732	4	1.1274	0.1010
	Error	22	1.6354		18	0.4952	
O_2	Day	5	96.6748	0.0007	4	294.696	0.0001
	Salt treatment	1	26.6961	0.1903	1	75.7476	0.0001
	Replication	2	38.3836	0.0949	2	5.4907	0.1190
	Salt treat*Day	5	5.8662	0.8426	4	7.8010	0.0304
	Error	22	14.6156		18	2.2864	

Table A-5. Analysis of variance (GLM) for headspace gas analysis of salt-treated and untreated broccoli inoculated with *Yersinia enterocolitica* packaged in modified atmosphere and stored at 4 or 10°C.

Gas	Source	4°C			10°C		
		df	Mean square	Prob>F	df	Mean square	Prob>F
CO_2	Day	5	54.1997	0.0001	4	37.3718	0.0001
	Salt treatment	1	1.1732	0.3033	1	3.1263	0.1674
	Replication	2	1.3810	0.2906	2	1.9805	0.2937
	Salt treat*Day	5	1.6376	0.2153	4	0.5824	0.8146
	Error	22	1.0561		17	1.5029	
O_2	Day	5	13.5136	0.0662	4	1.9834	0.8154
	Salt treatment	1	42.8959	0.0108	1	53.9247	0.0048
	Replication	2	1.8030	0.7253	2	8.7642	0.2110
	Salt treat*Day	5	12.8994	0.0765	4	16.0439	0.0425
	Error	22	5.5323		17	5.1336	

Table A-6. Analysis of variance (GLM) for headspace gas analysis of salt-treated and untreated broccoli inoculated with *Yersinia enterocolitica* packaged in air and stored at 4 or 10°C.

Gas	Source	4°C			10°C		
		df	Mean square	Prob>F	df	Mean square	Prob>F
CO_2	Day	5	2.9254	0.0001	4	23.3305	0.0001
	Salt treatment	1	0.0246	0.7575	1	3.2013	0.0583
	Replication	2	2.7172	0.0005	2	0.4853	0.5490
	Salt treat*Day	5	1.1016	0.0065	4	0.2538	0.8580
	Error	22	0.2521		18	0.7827	
O_2	Day	5	223.299	0.0001	4	305.509	0.0001
	Salt treatment	1	5.3269	0.1009	1	79.5441	0.0004
	Replication	2	0.7088	0.6815	2	7.5716	0.1954
	Salt treat*Day	5	12.1734	0.0006	4	12.4866	0.0487
	Error	22	1.8165		18	4.2288	

VITA

Peter Bodnaruk was born in Penrith, New South Wales, Australia on October 29, 1961. He received his Bachelor of Science degree in microbiology and genetics from the University of New England, Armidale, Australia in April 1984. In February 1985 he received a Graduate Diploma in Medical Technology from the Western Australian Institute of Technology and in August 1988 he received his Master of Applied Science degree in microbiology from Curtin University of Technology, Perth, Australia. He obtained employment as a Microbiologist with the New South Wales Department of Health in Sydney for two years, then moved to London and worked in Pathology at Queen Charlottes and Chelsea Hospital. During his stay in London he also worked for the Leatherhead Food Research Association as a research scientist in the rapid methods microbiology laboratory. He returned to Australia in 1990 and obtained a position as Experimental Scientist with the Commonwealth and Scientific Industrial Research Organisation (CSIRO), Division of Food Science and Technology, Brisbane, Australia. He moved to the United States in 1993, commenced Ph.D. studies in Food Science and Technology and graduated in December 1996 and is presently employed as Director of Microbiology with Armour Swift-Eckrich in Downers Grove, Illinois.