EFFECT OF CONTROLLED ATMOSPHERE STORAGE, MODIFIED ATMOSPHERE PACKAGING AND GASEOUS OZONE TREATMENT ON THE SURVIVAL CHARACTERISTICS OF *SALMONELLA* ENTERITIDIS AT CHERRY TOMATOES

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ELIF DAS

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Prof. Dr. Canan ÖZGEN
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

Prof. Dr. Levent BAYINDIRLI
Head of the Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

Prof. Dr. Alev BAYINDIRLI
Co-Supervisor

Assoc. Prof. Dr. Candan GÜRAKAN
Supervisor

Examining Committee Members

Prof. Dr. Faruk BOZOGLU (METU, FdE) _____

Assoc. Prof. Dr. Candan GÜRAKAN (METU, FdE) _____

Prof. Dr. Alev BAYINDIRLI (METU, FdE) _____

Prof. Dr. Cumhur ÇÖKMÜS (A.U., Biology) _____

Assist. Prof. Dr. Hami ALPAS (METU, FdE) _____

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Elif DAS

Signature :

ABSTRACT

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GASEOUS OZONE TREATMENT
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SALMONELLA ENTERITIDIS AT CHERRY TOMATOES

Das, Elif

M.Sc. Department of Food Engineering
Supervisor: Assoc. Prof. Dr. Candan GÜRAKAN
Co. Supervisor: Prof. Dr. Alev BAYINDIRLI

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In recent years, outbreaks of infections associated with raw and minimally processed fruits and vegetables have been reported. Possible sources for contamination are irrigation water, manure, wash water, handling by workers and contact with contaminated surfaces. Pathogens can occur on raw and minimally processed produce at populations ranging from 10^3 to 10^9 CFU/g and able to survive and sometimes grow under various storage conditions. The objective of this study was to analyse the growth/survival of *Salmonella* Enteritidis at spot-inoculated or stem-injected cherry tomatoes during passive modified atmosphere packaging (MAP), controlled atmosphere (CA) and air storage at 7 and 22°C. Low density polyethylene (LDPE) with a package size of 10x10 cm^2 for 25±2 g tomatoes was used for MAP storage in which the gas composition equilibrated to 6% O_2/ 4% CO_2 and a carbon dioxide incubator was used for CA storage in which the CO_2

level was monitored and maintained as 5% through the term of storage at 7 and 22°C. During the research, the effect of ozone treatment (5-30 mg/L ozone gas for 0-20 min) was also considered for surface sanitation. The results demonstrate that *S*.Enteritidis can survive and/or grow during the storage of tomatoes depending on the location site of the pathogen on fruit, suspension cell density and storage temperature. During MAP, CA and air storage, *S*.Enteritidis with initial population of 7.0 $\log_{10}$ CFU/tomato survived on tomato surfaces with an approximate decrease of 4.0-5.0 $\log_{10}$ CFU/tomato in population within the storage period; however, in the case of initial population of 3.0 $\log_{10}$ CFU/tomato, cells died completely on day 4 during MAP storage and on day 6 during CA and air storage. The death rate of *S*.Enteritidis on the surfaces of tomatoes that were stored in MAP was faster than that of stored in air. Storage temperature was effective on the survival of *S*.Enteritidis for the samples stored at ambient atmosphere; cells died completely on day 6 at 7°C and on day 8 at 22°C. Stem scars provided protective environments for *Salmonella;* an approximate increase of 1.0 $\log_{10}$ CFU/tomato in stem-scar population was observed during MAP, CA and air storage at 22°C within the period of 20 days. Cells survived with no significant change in number at 7°C. The development of the microbial association in tomatoes was dominated by lactic acid bacteria (LAB). The pH values of the tomatoes changed approximately from 4.0 to 3.0 during the storage period. LAB grew well under all atmospheric conditions with or without the presence of *S*.Enteritidis. Gaseous ozone treatment has bactericidal effect on *S*.Enteritidis, inoculated on the surface of the tomatoes. 5 mg/L ozone gas treatment was not effective. 30 mg/L ozone gas treatment affected surface color.

Key Words: MAP, ozone gas, *Salmonella*, tomatoes.

ÖZ

KONTROLLÜ ATMOSFER DEPOLAMA,
MODIFIYE ATMOSFER PAKETLEME VE
GAZ OZON UYGULAMASININ CHERRY DOMATESLERDE
SALMONELLA ENTERITIDIS ÜZERINE ETKISI

Das, Elif
Yüksek Lisans, Gida Mühendisligi Bölümü
Tez Yöneticisi: Doç. Dr. Candan Gürakan
Yardimci Tez Yöneticisi: Prof. Dr. Alev Bayindirli

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Son yillarda, taze ve minimum islem görmüs meyve ve sebzelerle olusan hastaliklar üzerine çalismalar yapilmaktadir. Sulama suyu, gübre, yikama suyu, isçiler ve bulasik yüzeylerle temas olasi kontaminasyon kaynaklaridir. Patojenler, 10^3 ile 10^9 CFU/g arasinda degisen sayilarda taze ve minimum islem görmüs meyve ve sebzelerde bulunabilmekte, canli kalabilmekte ve bazen çesitli depolama kosullarinda çogalabilmektedir. Bu çalismada, yüzey inokulasyonu ve sap-enjeksyonu yapilmis cherry domateslerdeki *Salmonella* Enteritidis'in, 7 ve 22°C de, modifiye atmosfer paketleme (MAP), kontrollü atmosfer (CA) ve havada depolama sirasinda, canliligi ve/veya çogalmasinin izlenmesi amaçlanmistir. Gaz kompozisyonunun 6% O_2/ 4% CO_2 degerlerine dengelendigi MAP depolamada, 25±2 g domates için paket büyüklügü 10x10 cm^2 olacak sekilde düsük yogunluklu polietilen (LDPE), CA depolamada ise 7 ve 22°C'de CO_2 miktari %5 olarak ayarlanmis karbondioksit inkübatörü kullanilmistir. Çalisma sirasida, yüzey sanitasyonu için ozon uygulamasinin etkisi (5-30 mg/L ozon

gazi 0-20 dak için) de incelenmistir. Sonuçlar, *S*.Enteritidis'in meyve üzerinde bulundugu konuma, hücre yogunluguna ve depolama sicakligina bagli olarak domateslerin depolanmasi sirasinda canli kalabildigini ve/veya çogalabildigini göstermektedir. MAP, CA ve havada depolama sirasida baslangiçtaki bakteri sayisi 7.0 $\log_{10}$ CFU/tomato oldugunda, patojen, depolama süresince hücre sayisinda yaklasik 4.0-5.0 $\log_{10}$ CFU/tomato kadar bir azalmayla birlikte domates yüzeyinde canli kalabilmistir; ancak, baslangiç bakteri sayisinin 3.0 $\log_{10}$ CFU/tomato olmasi durumunda, MAP depolama sirasida 4. günde, CA ve havada depolama sirasinda ise 6. günde hücreler tamamen ölmüstür. *S*. Enteritidis'in ölümü modifiye atmosferde depolanan domateslerin yüzeylerinde, havada depolanankilere göre daha hizli gerçeklesmistir. Havada depolanan örneklerde, depolama sicakligi, *S*.Enteritidis'in canliligi üzerine etkili olmustur; 7°C'de hücreler 6. günde, 22°C'de ise 8. günde tamamen ölmüstür. Sap yerleri bakteriye koruyucu ortam saglamislardir; domateslerin 22°C'de MAP, CA ve havada depolanmasi sirasinda, 20 günlük depolama süresi içerisinde, sap yerlerine enjekte edilen bakteri sayisinda yaklasik 1.0 $\log_{10}$ CFU/tomato kadar bir artis gözlenmistir. Domateslerdeki mikrobiyal floranin olusumunda laktik asit bakterilerinin (LAB) baskin oldugu görülmüstür. Depolama sirasida domateslerin pH degerleri yaklasik 4.0'ten 3.0'e düsmüstür. Tüm atmosferik kosullarda, *S*.Enteritidis varliginda ya da yoklugunda, LAB çogalmistir. Gaz ozon uygulamasinin *S*.Enteritidis üzerinde öldürücü etkisi vardir. 5 mg/L gaz ozon uygulamasi etkili bulunmazken, 30 mg/L gaz ozon uygulamasi domateslerde yüzey rengini etkilemistir.

Anahtar Kelimeler: MAP, gaz ozon, *Salmonella,* domates

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TABLE OF CONTENTS

LIST OF TABLES

TABLE Page

LIST OF FIGURES

FIGURE Page

CHAPTER 1

INTRODUCTION

Fruits and vegetables constitute a vital part of the human diet, with their per capita consumption rate steadily increasing in recent years. Traditionally, fruits and vegetables have been regarded as microbiologically safer than other unprocessed foods, such as meat, milk, eggs, poultry and seafood.

In recent years, outbreaks of infections associated with raw and minimally processed fruits and vegetables have occurred with increased frequency (De Roover, 1998). Factors thought to influence this increase include globalization of the food supply including importation of produce from countries with lower sanitation standards, the inadvertent introduction of pathogens from new geographical areas, the development of new virulence factors by microorganisms, decreases of immunity among certain segments of the population, changes in raw fruit and vegetable processing and eating habits (Hedberg et al., 1994; Beuchat, 1996; Altekruse et al., 1997). Documented illnesses have been linked to bacteria, parasites and viruses (Beuchat, 1996; NACMCF, 1999; Tauxe et al., 1997) and have involved many types of fruits and vegetables, including tomatoes (Hedberg et al., 1999; Wood et al., 1991), lettuce, alfalfa sprouts, parsley, scallions and cantaloupe, as well as unpasteurized apple and orange juice. Most outbreaks with identified etiology were of bacterial origin; *Salmonella* spp. were most commonly reported (Table 1).

Table 1. Pathogens most commonly associated with fresh or minimally processed produce-associated outbreaks and the food items that have been linked epidemiologically to outbreaks caused by these pathogens

Pathogen	Fresh produce	References
Bacterial etiology		
Bacillus cereus	Sprouts	Portnoy et al., 1976
C.botulinum	Chopped garlic	St.Lois et al., 1988
Enterotoxigenic *E.coli* (ETEC)	Carrots	CDC, 1994
E.coli O157:H7	Apple cider	Besser et al., 1993
L.monocytogenes	Lettuce	Ackers et al., 1996 Mermin et al., 1996
	Cabbage	Schlech et al., 1983
Salmonella spp.	Tomatoes	CDC,1993 Wood et al., 1991
	Sprouts	Mahon et al., 1996 Van Beneden et al., 1996
	Water melon	Blostein, 1991 Gayler et al., 1955
	Cantaloupe	Ries et al., 1990 CDC, 1991
	Orange juice	Cook et al., 1966
	Apple cider	CDC, 1975
Shigella spp.	Lettuce	Davis et al., 1988 Frost et al., 1995 Martin et al., 1986
	Scallions	Cook et al., 1995
Viral etiology		
Hepatitis A virus	Lettuce	Rosenblum et al., 1990
	Tomatoes	Williams et al., 1995
Norwalk/Norwalk-like virus	Melon	Iversen et al., 1987
Parasitic etiology		
Cyclospora cayetanensis	Raspberries	CDC, 1996
Cryptosporidium parvum	Apple cider	Millard et al., 1994

Ref: Tauxe, et al., 1997.

In farm-to-table production, processing and distribution chain, there are various possible points of contamination of fruits and vegetables with disease causing microorganisms. These include irrigation water, manure, wash water, handling by workers and contact with contaminated surfaces (Beuchat and Ryu, 1997; Tauxe et al., 1997). It is essential that interventions be developed to prevent or minimize contamination of raw produce and remove pathogens prior to consumption. To date, however, none of the chemical or physical treatments presently authorized by regulatory agencies for use to disinfect raw produce, can be relied on to eliminate all types of pathogens from the surface or internal tissues (Beuchat, 1998). One of the keys to enable the selection of appropriate intervention steps to reduce populations of pathogenic microorganisms on fruits and vegetables is to identify the sources of contamination and to characterize the ecology of pathogens as it is affected by agronomic and processing practices (Beuchat, 1998; Brackett, 1999; Buchanan, 1999).

Pathogens such as *Listeria, Clostridium, Salmonella* and other *Enterobacteriaceae* are carried by ruminants in their gastrointestinal tracts and are shed in feces (Alterkruse et al., 1997; Beuchat, 1996; Hosek et al., 1997), so might be present in vegetables owing to highly contaminated soil, water and natural fertilisers to processing and handling (Francis et al., 1999; Gorris LGM, 1994).

Microorganisms can occur on raw and minimally processed produce at populations ranging from 10^3 to 10^9 CFU/g (Francis et al., 1999; Nguyen-the and Carlin, 2000). Washing with tap water is a currently recommended means for reducing microbial contamination on raw fruits and vegetables. Although washing produce in water may remove some soil and other debris, it can not be relied upon to completely remove microorganisms and may result in cross-contamination of food preparation surfaces, utensils and other food items (Beuchat, 1992; Beuchat, 1998; Beuchat and Ryu, 1998; Brackett, 1992).

Salmonella serotypes are estimated to cause approximately 1.5 million cases of foodborne infection each year in the United States, with 15.000 hospitalizations and 500 deaths (Mead et al., 1999). Foods of animal origin such as poultry, eggs, meat and dairy products have been historically recognized as vehicles of *Salmonella*. However, salmonellosis has also been associated with consumption of tomatoes (CDC, 1993; Tauxe et al., 1997; Wood et al., 1991 and Hedberg et al., 1999), seed sprouts (Mahon et al., 1997; O'Mahony et al., 1990; Van Beneden, 1996), watermelons (Blostein, 1991; CDC, 1979; Gayler, 1955), cantaloupes (CDC,1991; Ries, 1990) and unpasteurized apple cider (CDC, 1975) and orange juice (Cook, 1996). Rude et al. (1984) cultured *Salmonella* from 4 of 50 vegetables examined in the United States. In Spain, Garcia-Villanova-Ruiz et al. (1987) cultured *Salmonella* from 7.5% of the market vegetables examined.

Consumption of raw tomatoes was epidemiologically linked to 176 cases of *Salmonella enterica* serotype Javina infections in Illinois, Michigan, Minnesota and Wisconsin in 1990 (Wood et al., 1991). In 1993, tomatoes were identified as the vehicle for a multistate outbreak of *S.enterica serotype* Montevideo infection (CDC, 1993). More recently, *S.enterica* serotype Baildon was implicated in an outbreak with diced tomatoes in geographically seperate areas of the United States (Tauxe et al., 1997).

Zhuang et al. (1995) described conditions influencing survival and growth of *S.enterica* serotype Montevideo on the surfaces of intact tomatoes. Rapid growth occurred in chopped ripe tomatoes (pH 4.1±0.1) at ambient temperature; *S.enterica* serotype Enteritidis, Infantis and Typhimurium were reported to grow in fresh-cut tomatoes (pH 3.99 to 4.37) at 22 and 30°C (Asplund and Nurmi, 1991). Wei et al. (1995) reported that *S.enterica* serotype Montevideo is able to multiply on wounded and cut tomatoes. The acidic pH (4.2 to 4.39 for ripe tomatoes and 4.33 to 4.52 for green tomatoes) did not completely inhibit growth. Weissinger (2000) reported that, *S.enterica* serotype Baildon can grow in diced tomatoes (pH 4.40±0.1); 0.79 $\log_{10}$

CFU/g increased to 5.32 $\log_{10}$ CFU/g and 7.00 $\log_{10}$ CFU/g within 24 h at 21 and 30°C, respectively. Ercoloni and Casolari (1966) demonstrated possible internalization of bacteria into tomato fruits by spraying tomato blossoms with a suspension of a plant pathogen, *Xanthomonas campestris*. Typical symptoms of disease were observed on tomato leaves one month after inoculation. The bacterium was isolated from the centers of fruits that didn't show external symptoms of infection. A pathogenic isolate of *Erwinia carotovora* was injected into the centers of healthy cucumber fruits attached to the wine without causing disease (Meneley et al., 1975). However, the bacterium was detected in the internal tissues of fruits harvested from the inoculated plants. Samish et al. (1962) studied 10 fruits and vegetables grown on different farms and found that bacteria, mostly gram-negative ones, representatives of the *Pseudomonadaceae* and the *Enterobacteriaceae*, can occur within healthy raw fruit tissues.

Fresh fruits and vegetables are raw agricultural commodities, fresh produce of many sorts can be expected to harbor microorganisms, including on occasion some that are pathogenic (Beuchat, 1996) (Table 2). To a great extent, the microflora of fresh produce reflects the species present in the harvest environment (Nguyen-the and Carlin, 1994) and because they generally do not go any processing to eliminate pathogenic microorganisms and because of the lack of lethal treatment during production and processing, the difficulty of eliminating the risk associated with the consumption of uncooked fresh produce increases. Therefore, methods to effectively reduce or eliminate pathogenic microorganisms (e.g. ozone treatment) and the storage conditions (e.g. controlled atmosphere storage or modified atmosphere packaging) including the temperature and atmosphere of the environment have great importance in the safety of the produce.

Table 2. Examples of bacterial pathogens isolated from fresh produce

Country	Pathogen	Fresh produce	Reference
Egypt	*Salmonella* *Shigella*	Salad greens	Saddik et al.,1985
England	*Salmonella*	Bean sprouts	O'Mahony et al.,1990
France	*Yersinia* *enterocolitica*	Vegetables	Catteau et al.,1985 Darbas et al.,1985
Iraq	*Salmonella*	Vegetables	Al-Hindawi et al.,1979
Italy	*Salmonella*	Fennel Lettuce	Ercolani,1976 Fantasia et al.,1994
Netherlands	*Salmonella*	Cauliflower Lettuce Eggplant Sprouts Peppers	Tamminga et al.,1978
Spain	*Salmonella*	Vegetables	Ruiz et al.,1987
Surinam	*Salmonella*	Chili	Tamminga et al.,1978
Thailand	*Salmonella*	Bean sprouts	Sartanu et al.,1993

Ref: Tauxe, et al., 1997.

Storage of fresh fruits and vegetables prolongs their usefulness. The principal goal of storage is to preserve the commodity in its most useable form for the consumer. The extension of storage life and the improvement of quality of fresh fruits and vegetables can be supplied by harvesting at proper maturity, control of post-harvest diseases, chemical treatments, refrigeration, controlled and modified atmospheres. The main goals of storage are to (1) slow the biological activity of fruits and vegetables (2) slow the growth of microorganisms (3) reduce transpirational losses.

Modified atmosphere packaging (MAP) and controlled atmosphere (CA) storage techniques to reduce the oxygen around the food are largely used for the preservation of fresh produce. There have been great technological advances in this area of preservation, particularly as it refers to improving the quality and shelf-stability of highly perishable food products, such as produce. However, when using these technologies, careful attention

must be paid to the effect on the survival and growth of pathogenic organisms (U.S. FDA, Center for Food Safety and Applied Nutrition, 2001). MAP may slow the rate of deterioration of the produce but could also provide sufficient time for human pathogens to grow, rendering the product unsafe while still edible (Hotchkiss and Banco, 1992).

The safety and stability of foods depend on the microorganisms initially present being unable to overcome various adverse factors, both extrinsic and intrinsic to the food, and multiply. Modification of the atmosphere surrounding the food may provide one condition or 'hurdle' that helps to restrict the growth of microorganisms. Another 'hurdle' can be provided by storage at low temperatures (< 4°C). The combination of chill temperatures and MAP generally results in a more effective and safer storage regime and longer shelf life (Leistner, 1995). Intrinsic and extrinsic factors, influencing shelf life of MAP products are listed in Table 3. Use of MAP does not eliminate the necessity for proper, safe manufacturing procedures and the need for careful handling at all stages from factory to table.

Table 3. Factors influencing shelf life of MAP products

Intrinsic factors	Extrinsic factors
a_w	Temperature control at all stages
pH	Hygienic processing including HACCP
Microbial flora	Raw material quality
(a) Initial	Finished product
(b) After processing	i.e. add of ingredients in package
(c) Developing	Time of packaging
	Initial and final gas composition
Available nutrients	Permeability of packaging film
Concentration and type	Gas to product ratio
of preservative agent	Gas purity
Redox potential	Pack design
Presence of naturally occurring	i.e. circulation of gases
anti-microbial compounds	
Presence of spores	

Ref: Church, 1993

Since fruits and vegetables are still alive and therefore respiring when harvested and processed, there are many factors that affect the post-harvest shelf life extension and the quality of fresh produce and the success of MAP (U.S. FDA, Center for Food Safety and Applied Nutrition, 2001). The primary factors, which influence the quality and shelf life of such commodities are minimizing mechanical injuries, proper sanitation, optimum temperature and relative humidity conditions during marketing. Secondary factor is to control the respiration, which includes the modification of surrounding atmosphere (Exama et al., 1993). Chilling slows the deterioration of stored foods but if the atmosphere surrounding the product is also modified to reduce oxygen concentration, the shelf life is increased considerably because of further reduction in the rate of chemical oxidation by oxygen and in the growth of aerobic microorganisms. These are major causes of food spoilage (Parry, 1993).

1.1. Modified Atmosphere Packaging

MAP is the alteration of the gaseous environment produced as a result of respiration or by the addition and removal of gases from small sized food packages to manipulate the levels of O_2, CO_2, N_2, C_2H_4. Passive MAP, produced as a result of respiration, is created naturally in sealed package by permitting the product and the package to react normally. The packaging material transmits oxygen, carbon dioxide and water vapor, thus causing further changes in the gaseous environment different from its initial condition. Active MAP is produced by addition or removal of gases to achieve an atmospheric composition different than that of normal air around the food. MAP is an attempt to retard physiological processes and also minimize microbial infections in order to maintain optimum quality and extend the shelf life of foods.

MAP was first recorded in 1927 as an extension of shelf life of apples by storing them in atmospheres with reduced oxygen and increased carbon dioxide concentrations. In the 1930s, it was used as modified atmosphere storage to transport fruits in the holds of ships and increasing the carbon dioxide concentration surrounding beef carcasses transported long distances was shown to increase shelf life by up to 100% (Davies, 1995). However, the technique was not introduced commercially for retail packs until the early 1970s in Europe. In the UK, Marks and Spencer introduced MAP in meat in 1979; the success of this product led, two years later, to the introduction of MAP bacon, fish, sliced cooked meats and cooked shellfish. Other food manufacturers and supermarket chains followed, resulting in a sharply increased availability of MAP food products reflecting the increase in consumer demand for longer shelf life foods and less use of preservatives. MAP techniques are now used on a wide range of fresh or chilled foods, including raw and cooked meats and poultry, fish, fresh pasta, fruit and vegetables, and more recently, coffee, tea and bakery products (Philips, 1996).

1.1.1 Advantages and Disadvantages of Modified Atmosphere Packaging

Depleted O_2 and/or enriched CO_2 levels can reduce respiration, delay ripening, decrease ethylene production and sensitivity, retard textural softening, slow down compositional changes associated with ripening, reduce chlorophyll degradation and enzymatic browning, alleviate physiological disorders and chilling injury, maintain color and preserve vitamins of fresh produce, thereby resulting an extension in shelf life and improvement in quality. However, exposure of fresh produce to O_2 and CO_2 levels outside the limits of tolerance for a particular commodity, can initiate anaerobic respiration with the production of undesirable odors and flavors, as well as cause other physiological disorders. Besides, there is a visible added cost as special equipment and training is required and different gas formulations are needed for each produce (Day, 1993; Farber, 1991).

Recommended atmospheres used in MAP products are listed in Table 4. Some characteristics and optimum storage conditions of whole fruits and vegetables are listed in Table 5.

Table 4. Atmospheres used in MAP products

Product	Atmosphere
White fish	40% CO_2 ; 30% O_2 ; 30% N_2
Fatty fish	40-60% CO_2 ; 40-60% N_2
Bacon	20-35% CO_2 ; 65-80% N_2
Cooked poultry	30% CO_2 ; 70% N_2
Poultry	100% CO_2
	25-30% CO_2 ; 70-75% N_2
	20-40% CO_2 ; 60-80% O_2
	60-75% CO_2 ; 5-10% O_2 ; 20% N_2
Cured meat	20-50% CO_2 ; 50-80% N_2
Fresh meat	30% CO_2 ; 30% O_2 ; 40% N_2
	15-40% CO_2 ; 60-85% O_2
Cheese	0-70% CO_2 ; 0-30% N_2
Bakery	100% N_2
	100% CO_2
	20-70% CO_2 ; 20-80% N_2
Fruits/vegetables	3-8% CO_2 ; 2-5% O_2 ; 87-95% N_2

Ref: Farber, 1991; Church, 1993

Table 5. Some characteristics and optimum storage conditions of whole fruits and vegetables for MAP

Commodity	Respiration Rate (at 5°C, mgCO_2 /kg/h)	Tolerance		Optimum		Recommended storage temperature	Approximate storage life
		Max CO_2 (%)	Min O_2 (%)	CO_2 (%)	O_2 (%)		
Apple	5-10	2-5	1-2	1-3	1-2	0-3	2-11 m
Apricot	10-20	2	2	2-3	2-3	0-5	-
Cherry	10-20	15	2	10-12	3-10	0-5	-
Peach	10-20	5	2	3-5	1-2	0-5	-
Strawberry	20-40	15	2	15-20	5-10	0-5	-
Asparagus	> 60	14	5	10-14	Air	1-5	21 d
Broccoli	> 60	10	1	5-10	1-2	0-5	2-3 m
Brussels sprouts	40-60	5	2	5-7	1-2	0-5	2-3 m
Cabbage	10-20	5	2	3-6	2-3	0-5	6-12 m
Carrot	10-20	5	5	3-4	5	0-5	4-5 m
Cauliflower	20-40	5	2	2-5	2-5	0-5	2-3 m
Lettuce (leaf)	10-20	2	2	0	1-3	0-5	3-4 wks
Mushrooms	> 60	15	1	5-15	3-21	0-5	3-4 d
Bell peppers	10-20	2	3	0	3-5	8-12	2-3 wks
Spinach	> 60	15	-	10-20	air	0-5	2-3 wks
Tomatoes	10-20	2	3	3-5	3-5	10-15	2 wks
Potato	5-10	-	-	none	none	4-12	-
Onion	5-10	-	-	0	1-2	0-5	8 m

Ref: Day, 1993; Exama et al., 1993; Moleyar and Narasimham, 1994.

1.1.2. Methods for Creating Modified Atmosphere Conditions

Modified atmospheres can be created either passively by the commodity or intentionally through active packaging.

1.1.2.1. Active Modification

In order to avoid uncontrolled levels of O_2, CO_2 and C_2H_4 that can be injurious, active modification can be applied in a number of ways. It can be done by pulling a slight vacuum and replacing the package atmosphere with the desired gas mixture. This mixture can be further adjusted through the use of absorbers or adsorbers in the package to scavenge these gases.

1.1.2.2. Passive Modification

Modified atmospheres can passively evolve within an hermetically sealed package as a consequence of a commodity's respiration, i.e. O_2 consumption and CO_2 evolution. The rate of change of the composition of the modified atmosphere will depend largely on the packaged product and the permeability of the packaging material. If a commodity's respiration characteristics are properly matched to film permeability values, then a beneficial modified atmosphere can be passively created within a package. It is important not to select the films of insufficient permeability because of the hazard of creating anaerobic conditions and/or injuriously high levels of CO_2 (Day, 1993; Floros, 1990).

1.1.3. Packaging Materials

Most packs for MAP products are made from one or more of four polymers: polyvinylchloride (PVC), polyethylene terephthalate (PET), polyethylene (PE) and polypropylene (PP) depending on the features desired for the intended use. The choice of packaging material destined to protect the food product during the storage should not ignore the physical and chemical properties of the packaged food. Packaging of the food product is an operation, which aims the prevention of all kinds of degradation that render it unsuitable for consumption or of a lower sensorial value (Mathlouthi and Leiris, 1990).

Polymeric films are increasingly used for conditioning food products under controlled atmosphere, since they preserve the initial environment in such a way that the intrinsic properties of food products are unaffected by prolonged storage periods. Recently, the packaging of horticultural commodities in polymeric films with specific gas permeabilities in combination with low temperature storage has increased.

The main characteristics to consider when selecting packaging materials for MAP of fruits and vegetables are gas permeability, water vapor transmission rate, mechanical properties, type of package, transparency, sealing reliability and microwaveability (Day, 1993).

There are several groupings in MAP films. Table 6 will indicate the necessity to utilize complementary properties to provide the features particularly desired for the intended use (Greengrass, 1993).

Table 6. Basic film barriers

Films	Water vapor Transmission (g/m^2.day) at 38°C and 90% RH	Gas permeability (cm^3/m^2.day. atm at 25°C)		
		Oxygen	Nitrogen	Carbon dioxide
Polyester, oriented	25-30	50-130	15-18	180-390
Polyester, oriented PVdC coated	1-2	9-15	-	20-30
Nylon-6	84-3100	40	14	150-190
Nylon-6,6	45-90	78	6	140
Nylon-11	5-13	500	52	2000
Polyurethane(polyester)	400-600	800-1500	600-1200	7000-25000
Polystyrene, oriented	100-125	5000	800	18000
APET	40-50	110-130	-	-
CPET	Permeabilities change according to level of crystallinity. For each 1% change in crysallinity, there is a 1.5% improvement in transmission rate.			
EVOH	16-18	3-5	-	-
Rigid PVC	30-40	150-350	60-150	450-1000
Plasticised PVC	15-40	500-3000	300-10000	1500-4600
PVdC-PVC copolymer (saran)	1.5-5.0	8-25	2-2.6	50-150
Polyacrylonitrile	78	12	3	17
Polyethylene, LD	18	7800	2800	42000
Polyethylene, HD	7-10	2600	650	7600
Polypropylene, cast	10-12	3700	680	10000
Polypropylene, oriented	6-7	2000	400	8000
Polypropylene, oriented PVdC coated	4-5	10-20	8-13	35-50
Polybutylene	8-10	5000	-	-
Ionomer	25-35	6000	-	6000
Ethylene-vinyl acetate	40-60	12500	4900	50000

Ref: Greengrass, 1993

When the packaged item respires, it consumes O_2 and generates CO_2. Thus the O_2/CO_2 ratio continues to decrease until it reaches a level at which permeation of the two gases through the package, O_2 in and CO_2 out, balances the rate of generation and consumption of the two gases. In the ideal case, a film with adjustable O_2 and CO_2 permeation rates could be used to establish within the package any desired steady state O_2 /CO_2 ratio; this ideal ratio will be different for every produce item. To achieve something

close to the desired O_2 /CO_2 ratio range in the package, the film permeability characteristics should be such that its CO_2 permeability is three to five greater than its oxygen permeability. These requirements are best obtained by low density polyethylene (LDPE) and polyvinyl chloride (PVC), and for this reason these films are the most widely used for wrapping produce (Jenkins and Harrington, 1991).

Polyethylene is one of the most important packaging materials of the present time. It is a hydrocarbon polymer with the nominal formula $-(CH_2-CH_2)_n-$. The simplest of all monomers as far as chemical structure concerned is ethylene. Commercial polyethylenes are produced with a variable amount of branching within this nominally linear polymer. HDPE has the least branching and as a result the greatest thermal stability and lowest permeability. LDPE has the advantage of maximum flexibility, low cost and widely used for packaging of foods in bags or as an over wrap.

LDPE is the most widely used polyethylene (PE) version which has good tensile strength, burst strength, impact resistance and tear strength, retaining its strength down to -60°C. While it is an excellent barrier to water vapor, it is not a good barrier to gases (Robertson, 1993). In addition, because of its very good heat sealing properties, it is widely used as the food contact and sealable layer in laminated packages.

LDPE films are generally used for packaging foods. This material is suitable for apple, carrots and tomatoes, which have low respiration rate but not suitable for fruits and vegetables which have high respiration rates, such as strawberry. Classification of selected fruits and vegetables according to their respiration rates is shown in Table 7.

1.1.4. Effects of Modified Atmosphere Packaging on Overall Quality of Fresh Fruits and Vegetables

Maintaining the quality of a food product during storage is mainly due to the inhibition of growth of spoilage microorganisms and in most cases the conditions chosen are those that reduce microbial growth. Table 3 shows the main factors considered when an atmosphere is decided upon, although A Hazard Analysis and Critical Control Points (HACCP) evaluation should also be used to asses the potential safety risks of using MAP techniques for each product (Davies, 1995).

Table 7. Classification of selected fruits and vegetables according to respiration rate*

Class	Respiration rate at 10°C in air (ml CO_2 kg^{-1} h^{-1})	Commodity
Low	< 10	Onion Cabbage Celery (white) Cucumber Tomato Lettuce (Kordaat)
Medium	10-20	Carrots (whole, peeled) Parsnip Potatoes (whole, peeled) Mango Cabbage Lettuce (Kloek) Melon Turnip
High	20-40	Cauliflower Brussels sprouts Strawberries Blackberries Asparagus Celery Leek
Very high	40-60	Spinach Broad beans Sweetcorn Raspberries Asparagus (blended) Eggplant Fennel
Extremely high	> 60	Carrots (Juilienne-cut) Mushrooms (sliced) Broccoli Bean

Ref: Day, 1993; Robertson, 1993

* Unless stated, produce is whole and unprepared.

Fresh fruits and vegetables are metabolically active for long periods after harvesting due to both endogenous activity, such as respiration, and external factors, such as physical injury, microbial flora, water loss and storage temperature (Kader et al., 1989). Respiration may result in anaerobiosis being quickly established if the produce is sealed in an impermeable film with low initial oxygen concentrations. Subsequently, anaerobic respiration of produce will be initiated at very low oxygen concentrations resulting in the accumulation of ethanol, acetaldehyde and organic acids and deterioration in organoleptic properties. Rates of respiration are influenced by the initial gas concentration so that, for example, reducing the oxygen concentration to 2% and increasing the carbon dioxide concentration to approximately 5% results in more than a 10-fold reduction in respiration rate of vegetables such as broccoli florets (Zagory and Kader, 1988). The initial gas composition must be determined for each fruit or vegetable product since too little oxygen or too much carbon dioxide results in irregular ripening, browning and other changes in organoleptic properties (Kader et al., 1989). Maintenance of color is important and in red peppers, MAP has been shown to increase carotenoid retention and reduce browning (Lee et al., 1992).

In extending shelf life, consumer acceptability is a prime consideration. For example ‘Lapins’ sweet cherries stored under modified atmosphere, showed a loss of flavour after 1 week, of texture after 2 weeks but no further changes over 8 weeks, and off-flavours were present at week 8. However, acceptability remained high for 4-6 weeks (Meheriuk et al., 1995). The acceptability of fruits and vegetables to the consumer is related to the growth of an aerobic spoilage microflora so that the inhibitory effect of increasing carbon dioxide concentrations increases the shelf life of vegetables such as cauliflower, broccoli and asparagus by approximately 7 days (Berrang et al., 1990).

1.1.5. Effects of Modified Atmosphere Packaging on Microbial Growth

The major goal of MAP is to reduce the growth rate of microorganisms, which cause the product to become organoleptically unacceptable. However, pathogens do not cause organoleptic changes in many cases (Yam and Lee, 1995). MAP can delay senescence in vegetables and therefore lessen susceptibility to pathogens.

1.1.5.1. Spoilage Organisms

The commonly encountered microflora of fruits and vegetables are *Pseudomonas* spp., *Erwinia herbicola*, *Flavobacterium*, *Xanthomonas*, *Enterobacter agglomerans*, lactic acid bacteria such *as Leuconostoc mesenteroides* and *Lactbacillus* spp., and molds and yeasts (Nguyen-the and Carlin, 1994). Although this microflora is largely responsible for the spoilage of fresh produce, it can vary greatly for each product and storage conditions. Temperature can play a large role in determining the outcome of final microflora found on refrigerated fruits and vegetables, leading to a selection for psychrotrophs and a decrease in number of mesophilic microorganisms. Cabbage deteriorated at the same rate at 7°C and 14°C; however at 7°C, the reduction in the total microbial load was significant (King et al., 1976). Similar phenomena have been reported for shredded chicory salads (Nguyen-the and Prunier, 1989) and shredded carrots (Carlin et al., 1989), where the total counts of mesophilic flora decreased with temperature. Low temperature storage not only decreases the growth rate of food borne pathogens but also increases the inhibitory effects of MAP by increasing the solubility of CO_2 in the liquid phase surrounding the food.

Storage of broccoli under 10% CO_2 and 5% O_2 inhibited growth of normal aerobic spoilage bacteria at 1°C (Kerbel et al., 1993). The effect of MAP on lactic acid bacteria can vary depending on the type of produce

packaged. The increased CO_2 and decreased O_2 concentrations used in MAP generally favour the growth of lactic acid bacteria. This can expedite the spoilage of produce sensitive to lactic acid bacteria, such as lettuce, chicory leaves and carrots (Nguyen-the and Carlin, 1994). The effect of MAP on yeasts is negligible, however, molds are aerobic microorganisms and therefore CO_2 can cause growth inhibition at concentrations as low as 10% (Molin, 2000), although the effect is not fungicidal (Littlefield et al., 1996). CO_2 levels > 10% or O_2 levels < 1% are needed to suppress fungal growth (Zagory and Kader, 1988).

1.1.5.2. Pathogenic Organisms

Psychrotrophic pathogens are *Clostridium botulinum* (non proteolytic), *Yersinia enterocolitica, Listeria monocytogenes, Aeromonas hydrophila*. Pathogens capable of growing between 5-12°C are *Salmonella* spp., *Clostridium perfinges, Bacillus cereus*. Pathogen capable of growing between 31-41°C is *Camplobacter jejuni*. It appears that some of the clostridia and camplobacter species may be able to survive better in modified atmosphere as compared to an air atmosphere. In addition, *L.monocytogenes, Y.enterocolitica* and *A.hydrophila* appear capable of growing at chill temperatures in certain modified atmosphere. The growth of *L.monocytogenes* and *A.hydrophila* appear capable of growing at chill temperatures at certain modified atmospheres in various food commodities, and the enhanced survival of anaerobic spores and *C.jejuni* under certain gas atmospheres were reported (Farber, 1991; Day, 1990).

There are many steps involved along the whole farm to fork produce chain and, therefore, and many points of potential microbial contamination (NACMCF, 1999). Pre-harvest contamination of fresh produce can occur through the use of non-pasteurized manure for fertilization, fecal contamination by indigenous or domestic animal species as well as agricultural workers, contaminated irrigation water and general human

handling. During harvest and post-harvest, critical points for contamination include contaminated water or ice, human handling, animals, contaminated equipment or transportation vehicles, cross-contamination and inefficient process of the product that fails to remove substantial levels of bacteria (NACMCF, 1999). Therefore, MAP produce is vulnerable from a safety standpoint because modified atmospheres may inhibit organisms that usually warn consumers of spoilage, while the growth of pathogens may be encouraged. Also, slow growing pathogens may further increase in numbers due to the extension of shelf life. Currently, there is concern with the psychrotrophic foodborne pathogens such as *L.monocytogenes, Y.enterocolitica* and *A.hydrophila,* as well as non-proteolytic *C.botulinum,* although clearly a number of other microorganisms, especially *Salmonella* spp., *E.coli* O157:H7 and *Shigella* spp., can be potential health risks when present on MAP produce (U.S. FDA, Center for Food Safety and Applied Nutrition, 2001).

Organisms such as *Salmonella, Shigella, E.co*li, and various enteric viruses, such as hepatitis A, have been implicated in produce outbreaks, and therefore, there is concern about their behaviour under modified atmosphere conditions (Zagory, 1995; Amanatidou et al., 1999; Bidawid et al., 2001). Fernandez-Escartin, (1989) tested the ability of three strains of *Shigella* to grow on the surface of fresh-cut papaya, jicama and watermelon and reported that populations increased significantly when the inoculated product was left at room temperature for 4-6 h. *Shigella* is not a part of normal flora associated with produce but can be on as contaminants by infected food handlers, contaminated manure and irrigation water.

In agar-based study, to investigate the effects of high (80-90%) O_2 and moderate (10-20%) CO_2 concentrations on food borne pathogens at 8°C, Amanatidou et al. (1999) noted little inhibitory action against a number of pathogens. All pathogens were able to grow in air; however, *S*.Typhimurium grew slowly. 10-20% CO_2 was inhibitory to *S*.Enteritidis; however,

S.Typhimurium, *L*.monocytogenes and non-pathogenic *E.coli* were unaffected or stimulated. Only when high O_2 (90%) and moderate CO_2 levels were used, did consistently strong inhibition of *S.*Enteritidis and *E.coli* occur. Kakiomenou et al. (1998) however, found that *S*.Enteritidis numbers decreased on both carrots and lettuce when stored under 5% CO_2 , 5.2% O_2 and 89.9% N_2 . *Salmonella* Typhimurium and *L.monocytogenes* actually had an increased growth rate at these concentrations. In general, *E.coli* O157:H7, *Salmonella* Hadar and *S*.Typhimurium were only inhibited by CO_2 levels that cause damage and spoilage to the produce (Amanatidou et al., 1999; Francis et al., 1999).

There has been a noticeable increase in the consumption of fresh fruits and vegetables in the last two decades, and more consumers are choosing the less labor-intensive fresh-cut produce. There has been a parallel rise in the number of produce linked foodborne outbreaks, but not linked to fresh-cut produce packaged under MAP. However, vigilance with respect to the safety of these products must be maintained (U.S. FDA, Center for Food Safety and Applied Nutrition, 2001).

1.2. Controlled Atmosphere Storage

High carbon dioxide and low oxygen storage have been applied in ancient times. The earliest use of controlled-modified atmosphere storage may be attributed to the Chinese. Litchi fruits were transported from Northern China to Southern China in sealed clay pots to which fresh leaves and grass were added by ancient Chinese. During the two-week journey, respiration of the fruits, leaves and grass generated a high carbon dioxide and low oxygen atmosphere in the pots and delayed ripening of the litchis (Salunkhe et al., 1991). Effect of atmosphere on ripening of fruits was determined in the early 1800s by Jacques Berard, a chemist at Montpellier Institute in France. Then several further studies were made in the United States to research the effects of atmosphere on ripening of fruits (Salunkhe et al., 1991). The scientific

beginning of controlled and modified atmosphere storage was in England by Kidd and West in the 1920s (Ryall and Lipton, 1979). Since its beginning, the studies on controlled atmosphere storage literature has grown enormously.

Controlled atmosphere storage can be defined as the storage of a commodity under an atmosphere that is different than air. The atmosphere is monitored and maintained through the term of storage. Usually controlled atmosphere storage of vegetables employs a lower O_2 concentration and higher CO_2 concentration than normally found in air. Ideally, the rate of respiration of vegetables held under CA storage is lower than it would be under air (Shewfelt, 1986). CA storage is particularly effective when vegetables are held under refrigeration. Such a system is exceptionally valuable for preserving high market value produce such as cauliflower, asparagus (Bohling and Hansen, 1980; Platenius, 1943) and broccoli (Lebermann et al., 1968).

Under controlled atmospheric conditions, the atmosphere is modified from that of the ambient atmosphere, and these conditions are maintained throughout the storage. MAP uses the same principles as CA storage; however, it is used on smaller quantities of produce and the atmosphere is only initially modified (Moleyar and Narasimham, 1994; Zagory, 1995)

1.3. Gases Used in Modified Atmosphere Packaging and Controlled Atmosphere Storage

O_2, CO_2 and N_2, are most often used in MAP/CA Storage (Parry, 1993; Phillips, 1996). Other gases such as nitrous and nitric oxides, sulphur dioxide, ethylene, chlorine (Philips, 1996), as well as ozone and propylene oxide (Parry, 1993) have been suggested and investigated experimentally. However, due to safety, regulatory and cost considerations, they have not been applied commercially. These gases are combined in three ways for use in modified atmospheres: inert blanketing using N_2, semi-reactive blanketing

using CO_2 /N_2 or O_2 / CO_2 /N_2 or fully reactive blanketing using CO_2 or CO_2 /O_2 (Parry, 1993; Moleyar and Narasimham, 1994).

1.3.1. Oxygen

Normally, the concentration of O_2 in a pack is kept very low (1-5%) to reduce the respiration rates of fruits and vegetables (Lee et al., 1995). Reducing the rate of respiration by limiting O_2 prolongs the shelf life of fruits and vegetables by delaying the oxidative breakdown of the complex substrates, which make up the product. Also O_2 concentrations below 8% reduce the production of ethylene, a key component of ripening and maturation process. However, at extremely low O_2 levels (that is, < 1%), anaerobic respiration can occur, resulting in tissue destruction and the production of substances that contribute to off-flavors and off-odors (Lee et al., 1995; Zagory, 1995), as well as potential for growth of foodborne pathogens such as *Clostridium botulinum* (Austin et al., 1998). Therefore, the recommended percentage of O_2 in a modified atmosphere for fruits and vegetables falls between 1 and 5% (Table 5).

1.3.2. Nitrogen

Nitrogen has three uses in MAP: displacement of O_2 to delay oxidation, retardation of the growth of aerobic spoilage organisms and action as a filler to maintain package conformity (Parry, 1993).

1.3.3. Carbon dioxide

Of the three major gases used in MAP, CO_2 is the only one that has significant and direct antimicrobial activity. A number of theories have been suggested to explain this antimicrobial effect. Theories to explain the antimicrobial action of CO_2 have been summarized by Farber (1991):

- Alteration of cell membrane function including effects on nutrient uptake and absorption;
- Direct inhibition of enzymes or decreases in the rate of enzyme reactions;
- Penetration of bacterial membranes leading to intracellular pH changes;
- Direct changes to the physico-chemical properties of proteins

The inhibitory action of CO_2 has differential effects on microorganisms. Thus, while aerobic bacteria such as pseudomonads are inhibited by moderate levels of CO_2 (10-20%), microorganisms such as lactic acid bacteria can be stimulated by CO_2 (Carlin et al., 1989; Amanatidou et al., 1999). Furthermore, pathogens such as *C.perfringens, C.botulinum* and *L.monocytogenes* are minimally affected by CO_2 levels below 50% and there is concern that by inhibiting spoilage microorganisms, a food product may appear edible while containing high numbers of pathogens that may have multiplied due to a lack of indigenous competition (Farber, 1991; Zagory, 1995; Phillips, 1996).

1.4. Ozone Treatment in Food Industry

Pathogenic outbreaks and incidents have greatly increased the concerns about the safety of fruits and vegetables. The use of chemicals to enhance the safety of fresh and processed fruits and vegetables is of great interest to the food industry. However, the most widely used chemicals, chlorine (200 ppm) or chlorine dioxide (1 to 5 ppm) in water, achieve 1 to 2 log reduction of microorganisms on fruits and vegetables (Brackett, 1992; Beuchat, 1992; Beuchat, 1999). To attain 5 log reduction recommendation set by the Food and Drug Administration (FDA) for selected commodities is a big challenge. Furthermore, the concerns about the residual by-products of chlorine in foods are increasing. Therefore, the food industry is in search of disinfectants that are effective against common and emerging pathogens and

safe to use in many specific applications of food processing, in other words, high effective sanitizers with minimum residual by-product need to be developed. Ozone may be such a good alternative sanitizer to chlorine for surface sanitation of fresh fruits and vegetables (Han et al., 2002; Yousef et al., 1999).

Even though ozone is new for the U.S., it has been utilized in European countries for a long time. In the U.S. ozone application in food industry has not been widely used; however, the U.S. Food and Drug Administration granted generally recognized as safe (GRAS) status for use of ozone in bottled water in 1982 (FDA, 1982). Ozone use was approved by the U.S. Department of Agriculture for reconditioning recycled poultry chilling water in 1997. After a year of reviewing the worldwide database on ozone, an expert panel in 1997 decreed that ozone was a GRAS substance for use as a disinfectant or sanitizer for foods when used in accordance with good manufacturing practices (Graham, 1997). Since the U.S. Food and Drug Administration did not object to the expert panel's findings, ozone has now been approved for use as a disinfectant or sanitizer in foods and food processing in the United States (USDA, 1997).

Up to the beginning of this century, ozone had been tested for the preservation of food and food ingredients such as milk, meat products, gelatin, casein, and albumin. Hill and Rice (1982) noted that ozone was applied for the purification and artificial aging of alcoholic beverages including wine and spirits, disinfection of brewing and cider manufacturing practices and odor control. However, most known applications dealt with treatment of drinking water (Bryant et al., 1992) and municipal and industrial waste water (Stover and Jarnis, 1981). Ozone applications in food industry are mostly related to decomposition of product surface and water treatment. Ozone has been used with success to inactivate contaminant microflora on meat, poultry, eggs, fish, fruits and vegetables and dry foods. The gas also is useful in detoxification and elimination of mycotoxins and pesticide residues from

some agricultural products. Excessive use of ozone, however, may cause oxidation of some ingredients on food surface. This usually results in discoloration and deterioration of food flavor (Yousef et al., 1999).

According to the Code of Federal Regulations (USDA, 1997), there must be at least 60% reduction in total microorganisms and similar reduction in coliforms, *E.coli,* and *Salmonella* spp. Treating fruits and vegetables with ozone has been used to increase shelf life (Norton et al., 1968; Rice et al., 1982). Treatment of apples with ozone resulted in lower weight loss and spoilage (Bazarova, 1982). An increase in shelf life of apples and oranges by ozone has been attributed to the oxidation of ethylene and to the removal of other metabolic products by ozone (Horvath et al.,1985). However, inactivation of spoilage microorganisms on fruits, without a doubt, contributed to this shelf life extension. Fungal deterioration of blackberries and grapes was decreased by ozonation of the fruits (Beuchat, 1992; Barth et al., 1995; Sarig et al., 1996).

In vegetables, the advances of ozone were similar to those experienced in fruit processing. Onions have been treated with ozone during storage. Mold and bacterial counts were greatly decreased without any change in chemical composition and sensory quality (Song et al., 2000). Shredded lettuce in water bubbled with ozone gas had decreased bacterial content (Yousef et al., 1999). Ozone has been used experimentally as a substitute for ethylene oxide for the decontamination of whole black peppercorns and ground black pepper resulted in slight oxidation of volatile oil constituents but ozone had no significant effect on the volatile oils of whole peppercorns. Because ozonation successfully reduced microbial loads and did not cause significant oxidation of the volatile oils in whole black peppercorns, this method was recommended for industrial treatment of the spice (Zhao and Cranston, 1995). Carrots have been treated with gaseous ozone and 50% reduction in daily growth rates of fungi was reported (Liew and Prange, 1994).

Several patents for preservation of fruits and vegetables by ozone technology are currently available. Cantelli (1988) developed a method based on holding the produce in a sealed container while maintaining an electrical discharge that forms ozone and nitrogen oxides, at concentrations of 0.05 ppm and 0.5 ppm respectively. Karg (1990) obtained a patent for sterilization of heavily contaminated foods such as herbs, spices, fruits and vegetables by ozone treatment. His process comprises an initial conditioning phase, treatment with gas mixture containing ozone, and elimination of residual ozone. Mitsuda et al. (1991) patented a method to sterilize foods such as fish, fruits, vegetables and beef in a processing room, packing receptacles, or a refrigerator using a gas mixture that includes O_3 , CO_2 and/or N_2 .

Ozone (O_3) is a strong antimicrobial agent. High reactivity, penetrability, and spontaneous decomposition to a non-toxic product (i.e., O_2) make ozone a viable disinfectant for ensuring the microbial safety for food products. Ozone, in the gaseous or aqueous phases, is effective against the majority of microorganisms tested by numerous research groups (Yousef et al., 1999). The bactericidal effects of ozone have been documented on a wide variety of organisms, including Gram positive and Gram-negative bacteria as well as spores and vegetative cells (Yousef et al., 1999) (Table 8). Relatively low concentrations of ozone and short contact time are sufficient to inactivate bacteria, molds, yeasts, parasites and viruses. However, rates of inactivation are greater in ozone-demand free systems than when the medium contains oxidizable organic substances. Susceptibility of microorganisms also varies with the physiological state of the culture, pH of the medium, temperature, humidity and presence of additives (e.g., acids, surfactants and sugars).

There are many advantages of using ozone as a potent antimicrobial agent in food and other industries. It is potentially useful in decreasing the microbial load and the level of toxic organic compounds. Ozone converts

many nonbiodegradable organic materials into biodegradable forms. The molecule decomposes spontaneously to oxygen; thus, using ozone minimizes the accumulation of inorganic waste in the environment (Horvath et al., 1985). The high oxidizing power and spontaneous decomposition also make ozone a viable disinfectant for ensuring the microbial safety and quality of food products (Yousef et al., 1999).

Table 8. Inactivation of bacteria by ozone

Bacterium	Inact. (log_{10})	Treat. time (min)	Conc. (mg/L)	pH	T (°C)	Medium	Reactor type	Reference
B.cereus	> 2.0	5	0.12		28	O_3 demand-free water		Broadwater et al., 1973
B.cereus (spores)	> 2.0	5	2.29		28	O_3 demand-free water		Broadwater et al., 1973
E.coli	4.0	1.67	0.23-0.26	7	24	O_3 demand-free water	Cont. flow	Farooq et al.,1983
E.coli	3.0	19	2.2	7.5	16	Raw wastewater	Cont. flow	Joret et al., 1982
E.coli	2.0	0.1	0.53	6.8	1	Phosphate buffer	Batch	Fetner et al., 1956
Salmonella Enteritidis	1.0	0.25	8% (wt/wt)		25	Broiler carcass	Ozone gas	Ramirez et al., 1994
*S.*Typhimurium	4.3	1.67	0.23-0.26	7	24	O_3 demand-free water	Cont. flow	Farooq et al.,1983
S.aureus	> 2.0	0.25		7	25	Phosphate buffer	Batch (bubble)	Burleson, 1975

Ref. Yousef et al, 1999.

1.4.1. Ozone Generation

When used in the industry, ozone is usually generated at the point of application and in closed systems. The corona discharge method has been used most widely to produce large amounts of ozone. When a high-voltage alternating current is applied across a discharge gap in the presence of air or oxygen, it excites oxygen electrons and thus induces splitting of oxygen molecules. Atoms from split oxygen combine with other oxygen molecules to form ozone, O_3. Ozone production varies depending on voltage, current

frequency, dielectric material property and thickness, discharge gap, and absolute pressure within the discharge gap. To optimize ozone production, an efficient heat removal system is essential. Dried air is passed through a high-voltage current along the discharge gap, thus, converting oxygen into ozone at concentrations up to 4% by weight. The use of pure oxygen is recommended over dried air to maximize the yield of ozone (Rosen, 1972). If air is passed through the generator as a feed gas, 1-3% ozone can be produced; however, using pure oxygen allows yields to reach up 6% ozone (Rice et al., 1981). Ozone gas can not be stored since ozone spontaneously degrades back to oxygen atoms (Kogelschatz, 1988; Wickramanayaka, 1991; Coke, 1993).

1.4.2. Measurement of Ozone

The analytical methods for the determination of ozone can be grouped into physical, physicochemical and chemical methods. Physical methods are based on measuring particular ozone properties, such as the intensity of absorption in the UV, visible or infrared region of the spectrum. The physicochemical methods measure physical effects of ozone reaction with different reagents; such effects include heat of the reaction. Chemical methods measure the quantity of the reaction products that are released when ozone reacts with an appropriate reagent (e.g. KI or HI) or the reduction in the molecular weight of a polymer. These methods differ in sensitivity and accuracy (Adler and Hill, 1950).

1.4.3. Antimicrobial Action of Ozone

Ozone destroys microorganisms by the progressive oxidation of vital cellular components. The bacterial cell surface has been suggested as the primary target of ozonation. Two major mechanisms have been identified in ozone destruction of the target organisms (Victorin, 1992): first mechanism is that ozone oxides sulfhydryl and amino acids of enzymes, peptides and

proteins to shorter peptides. The second mechanism is that, ozone oxides polyunsaturated fatty acids to acid peroxides (Victorin, 1992). Ozone degradation of the cell envelope unsaturated lipids result in cell disruption and subsequent leakage of cellular contents. Double bond of unsaturated lipids are particularly vulnerable to ozone attack. In Gram negative bacteria, the lipoprotein and lipopolysaccharide layers are the firs sites of destruction, resulting in increases in cell permeability and eventually cell lysis (Yousef et al., 1999). Chlorine selectively destroys certain intracellular enzyme systems; ozone will cause widespread oxidation of internal cellular proteins causing rapid cell death (Mudd, et al., 1969; Hinze, et al., 1987; Takamoto et al., 1992; Yousef et al., 1999). Cellular death can also occur due to the potent destruction and damage of nucleic acids. Thymine is more sensitive to ozone than cytosine or uracil. Ozone also destroys viral RNA and alters polypeptide chains in viral protein coats (Yousef et al., 1999).

1.4.4. Limitations of Ozone

Excessive use of ozone may cause surface oxidation of food (Rice at al., 1982). Fournaud and Lauret (1972) detected discoloration and undesirable odors in ozone-treated meat. Ozone also changes the surface color of some fruits and vegetables such as peaches (Bediani et al., 1996), carrots (Liew and Prange, 1994) and broccoli florets (Lewis et al., 1996). Studies showed that ozone decreased ascorbic acid in broccoli florets (Zhuang et al., 1996) and thiamin content in wheat flour (Naitoh et al., 1989). Ozone had a negative effect on the sensory quality of other commodities such as grains (Naitoh et al., 1988), ground spices (Zagon et al., 1992), milk powder (Ipsen, 1989) and fish cake (Chen et al., 1987) due to the lipid oxidation. However, other researches reported that, ozone treatment improved the sensory quality in beef and eggs (Bailey et al., 1996; Dondo et al., 1992) and did not alter the sensory quality of some fruits and vegetables significantly (Baranovskaya et al., 1979; Kute et al., 1995; Lewis et al., 1996).

In spite of ozone's pleasant odor at low concentrations, 0.1 mg/L is objectionable to all normal humans because of irritation to the nose, throat and eyes (Witheridge and Yaglou, 1939). Scott and Lesher (1963) reported as little as 0.02 to 0.04 mg/L can be detected by man, and prolonged exposure to a concentration of 1.00 mg/L, or greater, can cause death. Thorp (1950) indicated that with an hour exposure, symptomatic irritant, toxic and irreversible lethal effects can be induced by ozone concentrations of 2, 4, 15 and 95 ppm, respectively. The toxic effects of ozone upon inhalation are manifested in the lungs (Borek and Mehlman, 1983; Goldstein, 1979).

Although in low concentrations ozone is not an extremely toxic gas, at high concentration, ozone may be fatal to humans. After 1-2 h exposure to ozone (0.65 ppm), dogs exhibited rapid breathing whereas long-term (4-6 weeks) ozone exposure (0.2 ppm) to young rats exhibited lung distension (Barlett et al., 1974). It was found that 0.2 ppm and higher concentrations of ozone can cause varying degrees of damage to the respiratory tract, depending on exposure length (Schwartz et al., 1976).

1.5. Aim of the study

In recent years, documented illnesses, especially associated with *Salmonella* and fresh produce have occurred with increased frequency (De Roever, 1998). For that reason, there have been several studies on *S*.Enteritidis at fruits and vegetables. These studies widely focus on the risk of contamination of *Salmonella* to the fresh produce during pre-harvest period (Wood et al., 1991; Samish et al., 1963; Guo et al., 2002; Guo et al., 2001; Lin and Wei, 1997) and rarely, the growth/survival of these bacteria under different storage atmospheres such as MAP, VP, CA on fresh or minimally processed produce such as diced tomatoes (Drosinos et al., 1999), shredded carrots, cabbage and lettuce (Kakiomenou et al., 1998; Finn et al., 1997), etc. The studies also coincide at a common point, which is the ability

of these bacteria to survive and/or grow in low-pH produce such as tomatoes (Asplund et al., 1991; Tassou et al., 1996.

There have been also studies discussing the effective disinfection techniques used on fresh fruits and vegetables to inactivate the pathogens such as *Salmonella,* which have high possibility of presence on the produce. The studies commonly focus on the produce sanitizers (Harris et al., 2001), chlorine treatment (Weissinger, 2000; Wei et al., 1995; Pirovani et al., 2000), lactic acid-hydrogen peroxide (Venkitanarayanan et al., 2002), lactoperoxidase-thiocyanate-hydrogen peroxide (Touch et al., 2004) and ozone application. When specialized to ozone application on fresh produce, it is seen that mostly aqueous solution of ozone with or without bubbles and rarely gaseous ozone application have been studied.

Eggs (Davies et al., 2003; Whistler et al., 1989), apples (Bazarova, 1982), blackberries (Barth et al., 1995), lettuce (Kim et al., 1998), onions and potatoes (Faitel'berg-Blank et al., 1979), carrots (Liew and Prange, 1994), green peppers (Han et al., 2002) have been treated with gaseous ozone to analyse the effect of ozone on molds and bacteria, including *Salmonella*. However, there have been no study on treatment of cherry tomatoes with ozone gas to inactivate *Salmonella.* Similarly, no study about the effect of MAP and CA storage on *Salmonella* Enteritidis inoculated to cherry tomatoes has been reported.

Thus, our main aim in this study is to analyse the growth/survival characteristics of *S*.Enteritidis during passive MAP and CA storage of cherry tomatoes at 7 and 22°C with/without ozone treatment.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Cherry Tomatoes

Cherry tomatoes were supplied by a local vegetable market. The tomatoes were 3±1 cm in diameter and 4±1 g in weight (n=100).

2.1.2. Polymeric Material for Modified Atmosphere Packaging

Low Density Polyethylene (LDPE) was used for the packaging material. LDPE film was manufactured by Plasan A.S. (Ankara). The density of LDPE is 0.92 g/cm^3 with a film thickness of 0.05 mm.

2.1.3. Bacterial culture-Inoculation dip

Pure culture of *Salmonella* Enteritidis PT4 E10, which was isolated from egg was used throughout the study. This bacterial strain was kindly provided by Uludag University, Department of Microbiology, Bursa. Phage typing of E10 was previously confirmed by the Central Public Health Laboratory (CPHL), Laboratory of Enteric Pathogens, Colindale, United Kingdom and was maintained on tryptic soy agar-yeast extract (TSA-YE, Oxoid) at 4°C. A loop of TSA-YE working stock was inoculated into 10 ml of tryptic soy broth-yeast extract (TSB-YE, Oxoid), incubated at 37°C for 24 h and harvested by centrifugation (MSE Mistral, 1000; 1500 x *g* for 15 min). The pellet was washed twice with 0.1% sterile peptone water and

resuspended in it to give a final suspension of $2x10^8$ CFU/ml. This suspension was diluted with 0.1% sterile peptone water and three stock cultures with cell densities of $2x10^8$ CFU/ml (8.3 $\log_{10}$ CFU/ml), $2x10^6$ CFU/ml (6.3 $\log_{10}$ CFU/ml) and $2x10^4$ CFU/ml (4.3 $\log_{10}$ CFU/ml) were obtained.

2.1.4. Tomato Juice

Forty red cherry tomatoes were washed under running tap water, then with distilled water and wiped with paper towels. They were cut into small pieces and homogenized at high speed for 3 min in a sterile blender. After the contents were poured onto two layers of cheesecloth, the juice was squeezed into a sterile beaker. The tomato juice (pH=4) was autoclaved at 121°C and refrigerated at 4°C.

2.2 Methods

2.2.1. Inoculation, packaging, storage

Cherry tomatoes were washed under running tap water, then with sterile distilled water and wiped with paper towels, then were divided into three groups.

The first group was used to study the attachment and infiltration of *S.*Enteritidis onto/into tomatoes that were placed in contact with moist cotton layer which was inoculated with the pathogen in the following procedure; sterile cotton moistened with 250 ml 0.1% sterile peptone water and 50 ml stock culture of the cell density 8.3 $\log_{10}$ CFU/ml and 4.3 $\log_{10}$ CFU/ml, was spread in 5-cm cotton layer in polypropylene trays, to supply a moist cotton layer, containing 7.6 $\log_{10}$ CFU/ml (high inoculum dose) and 3.6 $\log_{10}$ CFU/ml (low inoculum dose)

S.Enteritidis cells in peptone water, respectively (A simulation of contaminated irrigation water in the field). Tomatoes were placed on the inoculated moist cotton layer with the stem scar facing downward and firmly pressed to assure good contact with the inoculated layer. Negative controls were placed on sterile moistened cotton layer. The trays containing tomatoes were stored at 22±2°C for up to 14 days. Each experiment described here and thereafter was repeated three times.

The second group of tomatoes was used to determine the survival characteristics of *S.*Enteritidis on the surfaces of the tomatoes which were spot-inoculated and then stored at three different atmospheric conditions, at 7±2°C and 22±2°C. Stock culture (50µl) with cell densities 8.3 and 4.3 $\log_{10}$ CFU/ml was deposited in several small drops (spot inoculation) within a 1 cm-diameter circle near the blossom end of each tomato so that each tomato contained 7.0 $\log_{10}$ CFU/tomato (high inoculum dose) and 3.0 $\log_{10}$ CFU/tomato (low inoculum dose), respectively. The spot-inoculated tomatoes were then dried in a laminar flow hood for 1 h at 22°C to allow the attachment of the cells on the tomato surfaces. Negative controls consisted of tomatoes on which 50µl sterile tap water was applied instead of cell suspension. Spot-inoculated tomatoes were divided into 3 subgroups.

First subgroup of inoculated tomatoes (25±2 g) was packed in LDPE packages, which were sealed by portable heat sealer. The samples were stored at 7±2°C and 22±2°C for up to 20 and 10 days, respectively.

Second subgroup of inoculated tomatoes was put in polypropylene trays and placed in carbon dioxide incubator for CA storage. The samples were stored at 7±2°C and 22±2°C for up to 20 and 10 days, respectively.

Third subgroup of inoculated tomatoes was put in polypropylene trays and stored in air. The samples were stored at 7±2°C and 22±2°C for up to 20 and 10 days, respectively.

The third group of cherry tomatoes weighing all equal, was used to determine the survival characteristics of *S.*Enteritidis in the stem scars of the tomatoes which were inoculated through their stem scars and then stored at three different atmospheric conditions at 7±2°C and 22±2°C. Stock culture (50µl) with cell densities 8.3 and 4.3 $\log_{10}$ CFU/ml was applied to the stem scars of each tomato so that each tomato contained 7.0 $\log_{10}$ CFU/tomato (high inoculum dose) and 3.0 $\log_{10}$ CFU/tomato (low inoculum dose), respectively. Two different ways in which inoculation to the stem scars of the tomatoes were achieved. One was to inject into with a sterile syringe and the other was to pipette onto the stem scars. Negative controls consisted of tomatoes to which 50µl sterile tap water was applied instead of cell suspension. Inoculated tomatoes were divided into three subgroups.

First subgroup of inoculated tomatoes (25±2 g) was packed in LDPE packages, which were sealed by portable heat sealer. The samples were stored at 7±2°C and 22±2°C for up to 20 and 10 days, respectively.
Second subgroup of inoculated tomatoes was put in polypropylene trays and placed in carbon dioxide incubator for CA storage. The samples were stored at 7±2°C and 22±2°C for up to 20 and 10 days, respectively.
Third subgroup of inoculated tomatoes was put in polypropylene trays and stored in air. The samples were stored at 7±2°C and 22±2°C for up to 20 and 10 days, respectively.

2.2.2. Modified Atmosphere Packaging

The size of the package was 10x10 cm^2 for 25±2 g cherry tomatoes. The packages were sealed by portable heat sealer. The inoculated cherry tomatoes were packed and then stored at 7±2°C and 22±2°C for up to 20 and 10 days, respectively.

During the storage, the samples were analysed on each day of the storage period for the changes in populations of *S*.Enteritidis, which was inoculated to the tomatoes, lactic acid and total viable bacteria taking place in normal microbial flora of the tomatoes and also the pH values. The same experiments were repeated for the unpacked (air stored) control samples. Besides these, the package interior CO_2. and O_2 composition was determined.

2.2.3. Controlled Atmosphere Storage

A carbon dioxide incubator (Sanyo, MCO 175) with inner dimensions of 490W x 505D x 690 H/mm was used to create controlled atmosphere (CA) in which, CO_2 concentration and temperature of the medium can be monitored and maintained as the desired value (Appendix A).

Inoculated cherry tomatoes in polypropylene trays with dimensions of 120W X 80D X 40 H/mm were placed on the shelves of the incubator. 15 inoculated cherry tomatoes were placed in one polypropylene tray. In this study, tomatoes were stored in CA containing 5% CO_2. The samples were stored at 7±2°C and 22±2°C for up to 20 and 10 days, respectively.

During the storage, the samples were analysed on each day of the storage period for the changes in populations of *S*.Enteritidis which was inoculated to the tomatoes, lactic acid and total viable bacteria taking place in normal microbial flora of the tomatoes and also the pH values. The same experiments were repeated for the air stored control samples. Besides these, the internal gas atmosphere was withdrawn by a syringe through the inside air sampling outlet of the incubator to determine the chamber interior O_2 composition (Appendix A) during the storage by a gas chromatograph.

2.2.4. Carbon dioxide and Oxygen Analyses

The concentration of oxygen and carbon dioxide in the packages and the oxygen concentration in CA chamber were analysed at certain time intervals during the storage. Internal gas atmosphere in the packages was withdrawn by means of a gas tight syringe. The samples of 1 ml were injected into the gas chromatograph (Shimadzu, GC-14A) and the concentrations of CO_2. and O_2 were measured. The same method was applied to measure the O_2 concentration in the CA chamber by injecting 1 ml of internal gas atmosphere into the gas chromatography.

CO_2 concentration was determined with Propaq-Q column and O_2 concentration was measured by using Molecular Sieve 5A column.

2.2.5. Ozone Gas Treatment

Ozone gas was generated from a laboratory corona discharge ozone generator using oxygen, with a working voltage of 220 volt, 50 Hz which is based on high frequency and water cooling principles (Opal, OG4 Model). Ozone gas treatment was carried out in a 1 L glass jar where the spot-inoculated cherry tomatoes were placed in.

In this study, the concentration of the generated ozone gas was measured by using a standard iodometric titration method in which KI (0.5 % wt) is used as the absorbtion solution and $Na_2S_2O_3$ (0.1 N) as the titrate and was recorded as mg/L.

To allow the attachment of the *S*.Enteritidis cells on the surfaces of the spot-inoculated tomatoes before the treatment with gaseous ozone, one group of tomatoes were dried in laminar flow at 22°C for 1 hour and the other group for 4 hours. Afterwards, these groups of spot-inoculated tomatoes were treated with 5, 10, 20 and 30 mg/L ozone gas at 22±2°C.

During the ozone gas treatment, the samples were analysed on certain time intervals of the treatment period for the changes in populations of *S*.Enteritidis, which was spot-inoculated onto the surfaces of the tomatoes.

2.2.6. Microbiological Analyses

2.2.6.1. Analysis of Survival of *S.*Enteritidis in Distilled water, Tryptic Soy Broth and Tomato Juice

Stock cultures with cell densities 4.3, 6.3 and 8.3 $\log_{10}$ CFU/ml were added separately in 0.5 ml aliquots to 20 ml of distilled water, tryptic soy broth (TSB) and tomato juice in 125 ml Erlenmeyer flasks so that, initially, all the three media contained 3.0 CFU/ml (low inoculum dose), 5.0 CFU/ml (medium inoculum dose) and 7.0 CFU/ml (high inoculum dose) *S*.Enteritidis cells in peptone water, respectively. Within the incubation period of 24 h at 37°C, aliquots (1 ml) were removed from each medium, serially diluted in 0.1% sterile peptone water and plated (0.1 ml in duplicate) on xylose lysine deoxycholate agar (XLD, Oxoid) for bacterial enumeration. The plates were incubated at 37°C for 24 h.

2.2.6.2. Analysis of Ability of Attachment and Infiltration of *S.*Enteritidis onto/into Tomatoes

Tomatoes from each treatment were analysed on 0, 1, 2, 4, 6, 8, 10 and 14 days.

To analyse the ability of attachment of *S*.Enteritidis onto tomato surfaces and the change in population of this bacterium within the storage period, on each day, one tomato that had been in contact with inoculated moist cotton layer, was placed in a stomacher bag with 20 ml of 0.1% sterile peptone water and was hand rubbed for 2 min. The wash fluid was serially

diluted and plated (0.1 ml in duplicate) on XLD Agar. Plates were incubated at 37°C for 24 h before presumptive colonies were counted.

To analyse the ability of infiltration of *S.*Enteritidis into tomato tissues through stem scars and the change in population of this bacterium within the storage period, on each day of the storage period, one tomato that had been in contact with inoculated moist cotton layer, was surface-disinfected by immersion in 100 ml of 70% ethanol for 3 min and then was left to dry in a laminar flow hood at room temperature for 1 h before the enumeration of *S.*Enteritidis at subsurface locations. This ethanol treatment was applied to the tomatoes to disinfect the surfaces of the tomatoes and to be able to count the *Salmonella* cells, which passed into the inner tissues of the tomatoes while the tomatoes were stored in contact with inoculated moist cotton layer. Surface-disinfected tomato was then placed in a stomacher bag with 20 ml of 0.1% sterile peptone water and homogenised with a stomacher (Laboratory type, 400) for 60 s at room temperature. Decimal dilutions were plated (0.1 ml in duplicate) on XLD agar for *S*.Enteritidis cell enumeration. Plates were incubated at 37°C for 24 h before presumptive colonies were counted.

2.2.6.3. Analysis of Survival of *S*.Enteritidis Inoculated on Tomato Surfaces

After spot-inoculation of *S*.Enteritidis onto the surfaces of the tomatoes and storage at different atmospheric conditions, on each day of the storage period, one spot-inoculated tomato was placed in a stomacher bag with 20 ml of 0.1% sterile peptone water and hand rubbed for 2 min. The wash fluid was serially diluted and plated (0.1 ml in duplicate) on XLD agar for *S*.Enteritidis cell enumaration. Plates were incubated at 37°C for 24 h before presumptive colonies were counted.

2.2.6.4. Analysis of Survival of *S*.Enteritidis Inoculated to Stem Scars of Tomatoes

After proper inoculation of *S*.Enteritidis to the stem scars of the tomatoes and storage at different atmospheric conditions, on each day of the storage period, one inoculated tomato was placed in a stomacher bag with 20 ml of 0.1% sterile peptone water and homogenised with a stomacher (Laboratory type, 400) for 60 s at room temperature. Decimal dilutions were plated (0.1 ml in duplicate) on XLD agar for *S*.Enteritidis cell enumeration. Plates were incubated at 37°C for 24 h before presumptive colonies were counted.

2.2.6.5. Enumeration of Initial Count of Lactic Acid Bacteria and Total Viable Bacteria on/in Tomatoes and Analysis of Change in Population

To determine the population of the lactic acid bacteria (LAB) and total viable count (TVC) in the tomatoes, on each day of the storage, one uninoculated tomato was placed in a stomacher bag with 20 ml of 0.1% sterile peptone water and homogenised with a stomacher (Laboratory type, 400) for 60 s at room temperature. The same experiment was repeated for the stem injected tomatoes. Additionally, for determination of the microbial count, especially LAB just on the surface of the tomatoes, one tomato was placed in 20 ml of 0.1% peptone water in a sterile stomacher bag and hand rubbed for 2 min then decimal dilutions of peptone wash water were plated (0.1 ml in duplicate) on the following agar media for the isolation of particular groups of bacteria.

- Plate count agar (PCA, Oxoid) for TVC, incubated at 25°C for 3 days.
- De man rogosa, sharp medium (MRS, Oxoid) for LAB, overlayed with the same medium and incubated at 25°C for 4 days under anaerobic conditions.

2.2.6.6. Gaseous Ozone Treatment on Inoculated Tomatoes

Spot-inoculated tomatoes were dried in a laminar flow hood for 1 or 4 hours at 22°C to allow the attachment of the cells on the surfaces of the tomatoes and afterwards gaseous ozone treatment was applied. At certain time intervals (0, 5, 10, 15, 20 min) of the treatment period, one inoculated tomato was placed in a stomacher bag with 20 ml of 0.1% sterile peptone water and homogenised with a stomacher (Laboratory type, 400) for 60 s at room temperature. Decimal dilutions were plated (0.1 ml in duplicate) on XLD agar for *S*.Enteritidis cell enumeration. Plates were incubated at 37°C for 24 h before presumptive colonies were counted.

2.2.7. pH

pH was measured on each day of the storage period for the pressed tomato by pH meter (SenTix, WTW, Germany).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Survival of *S.*Enteritidis in Distilled Water, Tryptic Soy Broth and Tomato Juice

The activity of *S*.Enteritidis in distilled water and tryptic soy broth (TSB) and the survival pattern of the pathogen in tomato juice are shown in Figure 1. As expected, *Salmonella* cells grew rapidly in TSB (Figure 1-a) and were just able to survive in distilled water (Figure 1-b) at selected inoculum doses. However, these bacteria at a concentration of 3.0 $\log_{10}$ CFU/ml in tomato juice died after 25 hours, while at higher concentrations, cells survived although they decreased in number (Figure 1-c). Thus, the initial bacterial numbers and medium composition affected the survival of *Salmonella* cells.

Similarly, it was reported that *S.*Montevideo cells grew rapidly in TSB at any inoculum dose. However, these bacteria at a concentration of $\leq$ 5.2 $\log_{10}$ CFU/ml in Butterfield's buffer and tomato juice died rapidly, while in distilled water the death rate was slower (Wei et al., 1995).

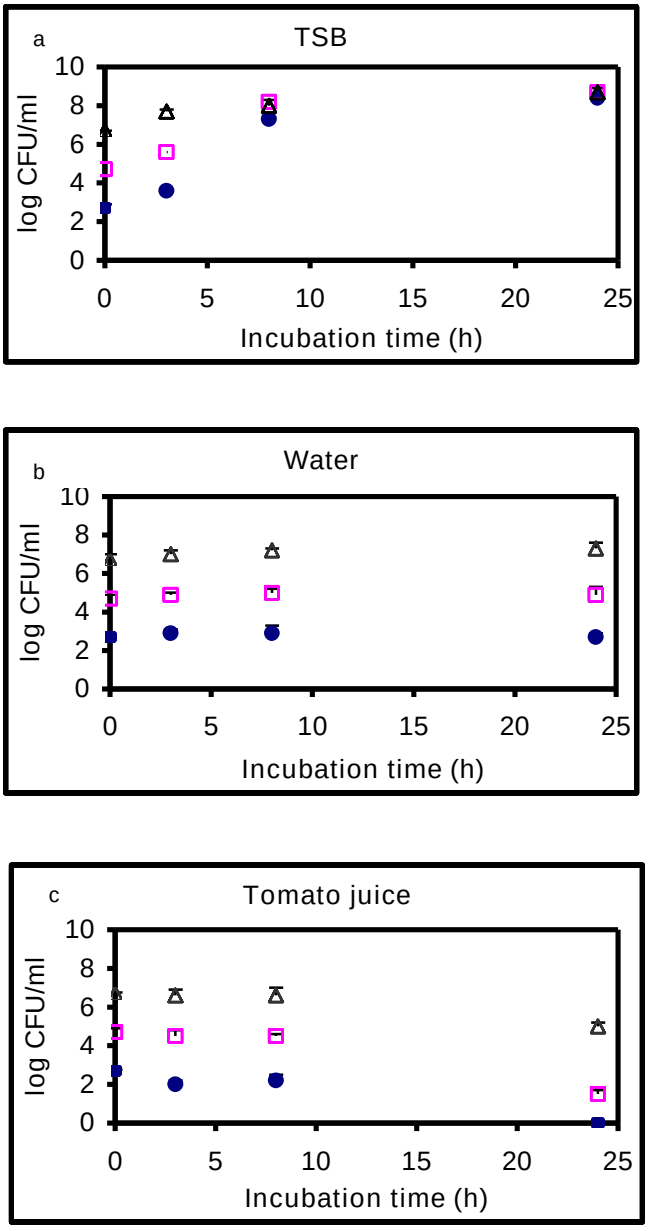


Figure 1. Time related changes in *S*.Enteritidis cell numbers in TSB, water and tomato juice (l low inoculum dose; medium inoculum dose; p high inoculum dose)

3.2. Ability of Attachment and Infiltration of *S*.Enteritidis onto/into Tomatoes

Contamination of tomatoes with microorganisms can occur during fruit development and harvesting, in other words, during pre-harvest and post-harvest stages. It is often difficult to identify with certainty the source of microbial contamination for fresh produce. It is not currently known what proportion of produce may become contaminated with water used in agricultural or processing facility operations. However, research has shown that the use of contaminated irrigation water can increase the frequency of pathogen isolation from harvested produce (Norman et al., 1953). In 1990 and 1993, two outbreaks involving 300 cases in four states of U.S.A attributed to *Salmonella* species, were linked to consumption of fresh tomatoes (Wood et al., 1991).

Samish et al. (1963) studied the distribution of bacteria within healthy tomato fruits and observed that tomatoes from farms using overhead irrigation contained bacteria more frequently and in larger populations than did tomatoes from farms using furrow irrigation. Overhead irrigation may produce a high-humidity microclimate that is more favorable for the attachment and the growth of some bacterial species on the surfaces of tomatoes.

In our experiment, to study the ability of attachment of *S*.Enteritidis onto tomatoes and the ability of infiltration of this bacterium into tomato tissues through stem scars, we created a medium with a potential hazard of contaminated water. We observed the growth/survival characteristics of *S*.Enteritidis on tomato surface and in tomato tissues besides the ability of attachment and infiltration of this pathogen onto/into tomato at 22±2°C.

3.2.1. Attachment of *S*. Enteritidis onto Tomatoes

The ability of attachment of *S*.Enteritidis cells onto the surfaces of the tomatoes, which were stored in contact with inoculated moist cotton layer and the growth/survival characteristics of this bacterium are shown in Figure 2.

S.Enteritidis cell enumeration following 1 h storage on day 0 revealed that 5.0 $\log_{10}$ CFU of *S*.Enteritidis cells attached onto the surface of one tomato upon contact with moist cotton layer containing 7.6 $\log_{10}$ CFU/ml of *S*.Enteritidis cells in peptone water. This count was 2.5 $\log_{10}$ CFU per tomato in the case of contact of the tomatoes with moist cotton layer containing 3.6 $\log_{10}$ CFU/ml of *S*.Enteritidis cells in peptone water. There was an increase of approximately 2.0 $\log_{10}$ CFU/tomato during the first four days of storage in both cases and counts remained constant between days 4 and 10. *S*.Enteritidis was not detected on negative controls.

In a similar study of Guo et al. (2002), it was reported that *Salmonella* survived at high numbers in moist soil for at least 45 days and count of the pathogen on the tomatoes which were in contact with inoculated moist soil was approximately 5.5 $\log_{10}$ CFU/tomato on day 0 just after 10 min and concluded that cells survived with an increase of 2.0 $\log_{10}$ CFU/tomato in population within the 10 days of the storage period. Watkins and Sleath (1981) demonstrated that *Salmonella* in sewage sludge applied to agricultural soils died within five weeks whereas *Listeria monocytogenes* survived for 8 weeks. Microorganism on flooded croplands may remain in soil for months or years (Beuchat et al., 1997).

3.2.2. Infiltration of *S*.Enteritidis into Tomatoes

The ability of infiltration of *S.*Enteritidis into the tissues of the tomatoes, which were stored in contact with inoculated moist cotton layer and the growth/survival characteristics of this bacterium are shown in Figure 3.

Guo et al. (2001) reported that complete surface disinfection is reached as a result of immersing *Salmonella* inoculated tomatoes into 70% ethanol for 2 min. Thus, in this part of the experiment, in order to disinfect the surfaces of the tomatoes and be able to determine the population of the pathogen remaining in tomato tissues, we immersed the tomatoes which were stored in contact with inoculated moist cotton layer, in 70 % ethanol for 3 min. Afterwards, the number of cells infiltrated into the tissues through stem scars were determined.

S.Enteritidis cell enumeration following 1 h storage on day 0 revealed that approximately 2.5 $\log_{10}$ CFU/tomato cells infiltrated into one tomato through its stem scar upon contact with moist cotton layer containing 7.6 $\log_{10}$ CFU/ml of *S*.Enteritidis cells in peptone water. This count was 1.0 $\log_{10}$ CFU/tomato in the case of contact of the tomatoes with moist cotton layer containing 3.6 $\log_{10}$ CFU/ml of *S*.Enteritidis cells in peptone water. There was an increase of 1.0 $\log_{10}$ CFU/tomato during the first four days of the storage in both cases and counts remained constant between days 4 and 10. *S*.Enteritidis was not detected on negative controls.

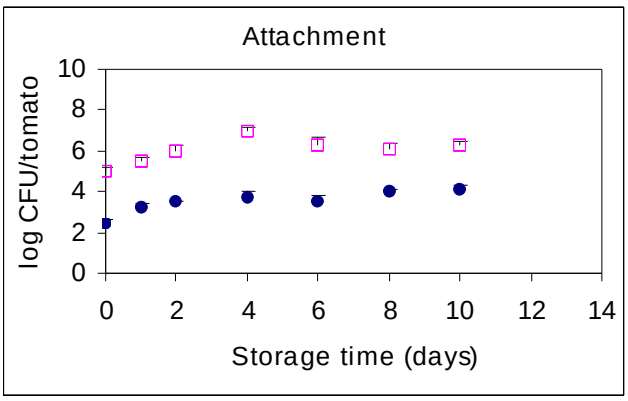


Figure 2. Population of *S*.Enteritidis on the surface of the tomatoes that were placed in contact with inoculated cotton layer during the storage. (˜ low inoculum dose; ¢ high inoculum dose)

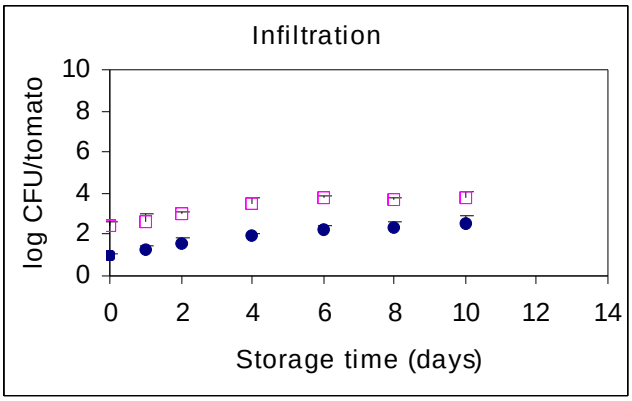


Figure 3. Population of *S*.Enteritidis in the tissues of the tomatoes that were placed in contact with inoculated cotton layer during the storage. (˜ low inoculum dose; ¢ high inoculum dose)

Similarly, Lin and Wei (1997) also observed that the distribution of *Salmonella* Montevideo on the pulp surfaces of tomatoes cut with a knife was related to the inoculum dose applied to the stem scar. *Salmonella* Montevideo colonies were clustered in the stem scar regions of tomatoes receiving a low inoculum level; colonies were more widely dispersed on the cut pulp of tomatoes receiving a higher inoculum level. Zhuang et al. (1995) reported that populations of *Salmonella* on dip-inoculated tomatoes are largest in the stem scar tissue. Other researches have described the infiltration of fruits with pathogenic bacteria. The greatest extent of infiltration of apples with *E.coli* O157:H7 was observed by Buchanan (1999) to be consistently associated with the outer-core region, followed by the skin and the pulp. These studies and our observations on the infiltration of *Salmonella* into tomatoes support contention that pre-harvest contact of produce with contaminated water or soil exacerbates problems associated with post-harvest removal or inactivation of pathogens.

At the end of these studies, it was concluded that S.Enteritidis is able to attach and infiltrate on/in tomatoes during storage in contact with contaminated water. Once attached to tomato fruits, *Salmonella* either survives or slowly dies (Guo et al., 2002).

Thus, as the further studies of this experiment, different storage conditions with three different atmospheres; air, modified atmosphere and controlled atmosphere and with two different temperatures; 7±2°C and 22±2°C, were supplied to analyse the effects of these storage techniques on survival of *S.*Enteritidis which was used to inoculate cherry tomatoes either by injecting stems with inoculum or inoculating surfaces with it.

There is a similar study of Guo et al. (2001) in which the fate of salmonellae applied to tomato plants was investigated; different *Salmonella* serotypes were used to inoculate tomato plants before and after fruits set, either by injecting stems with inoculum or brushing flowers with it and it was

reported that, *Salmonella* may attach and remain viable during fruit development, thus serving as routes or reservoirs for contaminating ripened fruit and concluded as, although *Salmonella* is a human pathogen, it has ability to survive on or in tomato fruits throughout the course of plant growth, flowering, fruit development and maturation. During plant growth, phytopathogens can penetrate the plant surface through natural openings such as stomata or leaf hydathodes through wounds (Agrios, 1997). Some bacteria enter blossoms through the nectarthodes or nectaries, which are similar to hydathodes. However, bacteria enter plants most often through wounds, and less frequently through natural openings. Plant pathogens may grow briefly on or in wounded tissue before advancing into healthy tissue (Agrios, 1997). Injection of *Salmonella* into tomato stem may introduce the pathogen into xylem, which has the principal role of transporting water and nutrients from the root to the extremities of the plant. Additionally, in the secondary xylem, the axial and ray parenchyma store nutrients and water (Bowes, 1996), which sustain viability of plants, and possibly, promote survival of human pathogenic bacteria. The presence of epiphytal flora within tissue of fruits and vegetables through various pathways was reported by Samish et al. (1962). By examining eight internal locations of tomatoes, they observed that bacteria are unevenly distributed in the fruit, and entry may be from the stem scar tissue through the core and into the endocarp.

3.3. Gas Composition of Modified Atmosphere Packages and Controlled Atmosphere

Passive MAP method using LDPE film was used in this study for packaging of the inoculated tomatoes. The initial gas composition during MAP storage was that of the ambient air, containing 20% O_2 and 80% CO_2.

Figure 4-a and Figure 4-b summarise the gas composition during MAP storage at 7±2°C and 22±2°C, respectively. As shown in the figures,

MAP storage atmosphere of cherry tomatoes equilibrated to approximately 6% O_2 / 4% CO_2 at 7±2°C and 22±2°C.

CA was obtained in a carbon dioxide incubator in which the CO_2 level was maintained and monitored as 5% at 7±2°C and 22±2°C throughout the storage. The incubator oxygen composition was 20% initially. Change in O_2 in the incubator was summarised in Appendix A.

Farber (1991) and Church (1993) reported that for fruits and vegetables, recommended atmosphere used in MAP is 3-8% CO_2 / 2-5% O_2. Similarly, Day et al. (1990) also reported that, a modified atmosphere containing 3-8% CO_2 was shown to extend the shelf life of a wide variety of fruits and vegetables. Day (1993), Exama et al. (1993), Moleyar et al. (1994), specialised the optimum storage conditions of fruits and vegetables for MAP and reported that 3-5% CO_2 / 3-5% O_2 , 10-15°C are optimum conditions for the storage of tomatoes.

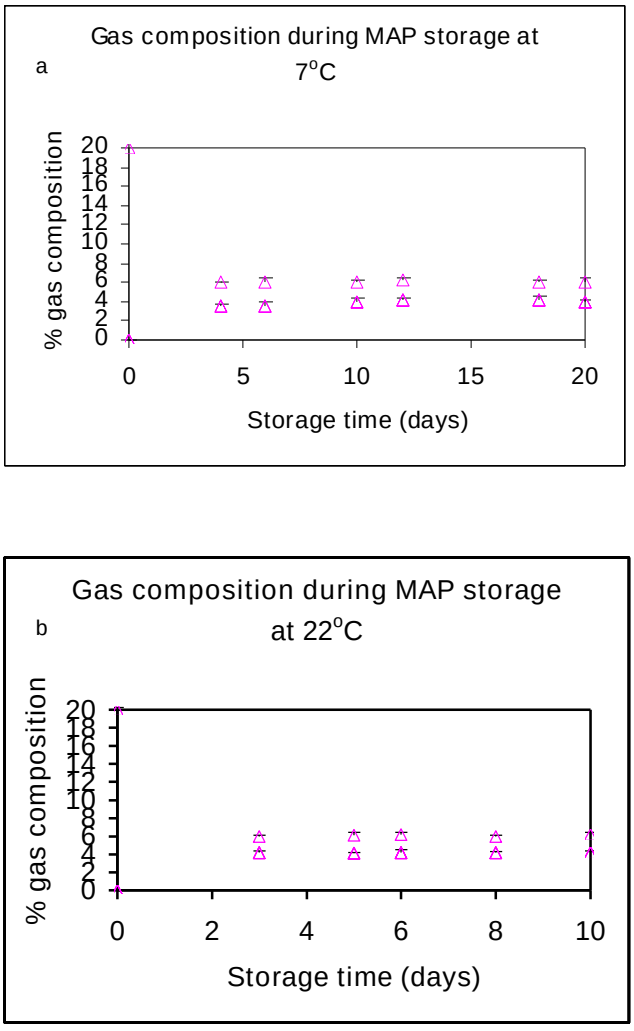


Figure 4. Gas composition during MAP storage of tomatoes stored at 7 and 22°C. (p carbon dioxide; oxygen)

Additionally, Day (1993), Floros (1990) reported that, if a commodity's respiration characteristics are properly matched to film permeability values, then a beneficial modified atmosphere can be passively within a package and added that LDPE is widely used packaging material. Similarly, Robertson (1993) reported that LDPE films are suitable for packaging of apple, carrots and tomatoes which have low respiration rates but not suitable for fruits and vegetables which have high respiration rates such as strawberry.

3.4. Survival of *S*.Enteritidis Inoculated on Tomato Surfaces at Different Atmospheric Conditions

3.4.1. Air

Figure 5-a-1 and Figure 5-b-1 summarise the survival of *S*.Enteritidis on inoculated tomatoes which were stored in air at 7±2°C and 22±2°C, respectively.

When suspension containing 3.0 $\log_{10}$ CFU/ml *S*.Enteritidis cells in peptone water was used for spot-inoculation of the surface of each tomato, cells died completely at the end of day 6 of the storage at 7±2°C in air (Figure 5-a-1); it was the day 8 of the storage period at 22±2°C (Figure 5-b-1) with a slower death rate. *Salmonella* enrichment broth (TSB-YE) was used to verify the results when no colonies were observed on plates. However, in the case of spot-inoculation of the tomatoes with the pathogen suspension containing 7.0 $\log_{10}$ CFU/ml *S*.Enteritidis cells in peptone water, the pathogen managed to survive on the surface of the tomatoes with a significant decrease of 4.5 $\log_{10}$ CFU/tomato at 7±2°C and approximately 4.0 log CFU/tomato in population at 22±2°C at the end of day 20 and day 10 of the storage, respectively. *S*.Enteritidis was not detected on the surfaces of the negative controls.

Similarly, Guo et al. (2002) demonstrated that, after storage of *Salmonella* inoculated tomatoes at 20°C in air for 1 day, a decrease of approximately 1 log CFU/tomato occurred; the population gradually decreased by an additional 3 logs between days 1 and 14.

Also, Wei et al. (1995) reported that the inoculum dose and the inoculation site as well as the medium used to prepare the suspensions affected the survival of *Salmonella* Montevideo cells; when the bacteria were inoculated at low doses (2.85 to 3.86 $\log_{10}$ CFU/ml) in distilled water onto tomato skin, the cells died almost completely following overnight storage; however, *S*.Montevideo survived on tomato skin for 2 to 3 days when the concentration in distilled water was increased to 9.48 $\log_{10}$ CFU/ml and the tomatoes were stored at 20 or 25°C.

3.4.2. MAP

Figure 5-a-2 and Figure 5-b-2 summarise the survival of *S*.Enteritidis on inoculated tomatoes which were stored in MAP at 7±2°C and 22±2°C, respectively.

When suspension containing 3.0 $\log_{10}$ CFU/ml *S*.Enteritidis cells in peptone water was used for spot-inoculation of the surface of each tomato, cells died completely at the end of day 4, regardless of the storage temperature (Figure 5-a-2 and Figure 5-b-2). However, in the case of spot-inoculation of the tomatoes with the pathogen suspension containing 7.0 $\log_{10}$ CFU/ml *S*.Enteritidis cells in peptone water, the pathogen managed to survive on the surface of the tomatoes with a significant decrease of 5.0 $\log_{10}$ CFU/tomato at 7±2°C and approximately 4.5 log CFU/tomato in population at 22±2°C at the end of day 20 and day 10 of the storage, respectively. *S*.Enteritidis was not detected on the surfaces of the negative controls.

Similarly, it was reported that *S.*Enteritidis numbers decreased on fresh fruits when stored under 5 % carbon dioxide and 6 % oxygen (Kakiomenou et al., 1998)

3.4.3. CA Storage

Figure 5-a-3 and Figure 5-b-3 summarise the survival of *S*.Enteritidis on inoculated tomatoes which were stored in CA at 7±2°C and 22±2°C, respectively.

When suspension containing 3.0 $\log_{10}$ CFU/ml S.Enteritidis cells in peptone water was used for spot-inoculation of the surface of each tomato, cells died completely at the end of day 6 regardless of the storage temperature (Figure 5-a-3 and Figure 5-b-3). However, in the case of spot-inoculation of the tomatoes with the pathogen suspension containing 7.0 $\log_{10}$ CFU/ml S.Enteritidis cells in peptone water, the pathogen managed to survive on the surface of the tomatoes with a significant decrease of 5.0 $\log_{10}$ CFU/tomato at 7±2°C and approximately 4.5 log CFU/tomato in population at 22±2°C at the end of day 20 and day 10 of the storage, respectively. *S*.Enteritidis was not detected on the surfaces of the negative controls.

It was reported that, at chilled temperatures, respiration rates are significantly lowered and the growth of spoilage and food-poisoning microorganisms are restricted (Day, 1993).

At the end of these studies, it was concluded that, the death rate of *S*.Enteritidis on the surfaces of tomatoes that were stored in modified atmosphere was faster than that of stored in air or CA. The initial microbial contamination dose affected the survival of *S*.Enteritidis. Storage temperature was effective on the survival of *S*.Enteritidis for the samples stored at ambient atmosphere.

Limiting oxygen in the package environment of the samples was the key point of the fast death rate of the *Salmonella* cells on the tomato surfaces, when compared to controlled atmosphere and air stored samples. Oxygen concentration below 8% reduce the production of ethylene, a key component of the ripening and the maturation process, therefore susceptibility of the fruits and vegetables to microorganisms is reduced (Lee et al., 1995).

In our experiment, we used a spot inoculation procedure, which allows a known population of *Salmonella* applied, whereas Zhuang et al. (1995) and Beuchat and Brackett (1991) dipped tomatoes into a cell suspension. The latter procedure may result in cells becoming lodged in tissue areas, imposing conditions that enhance the survival and growth of cells during subsequent storage (Guo et al., 2002).

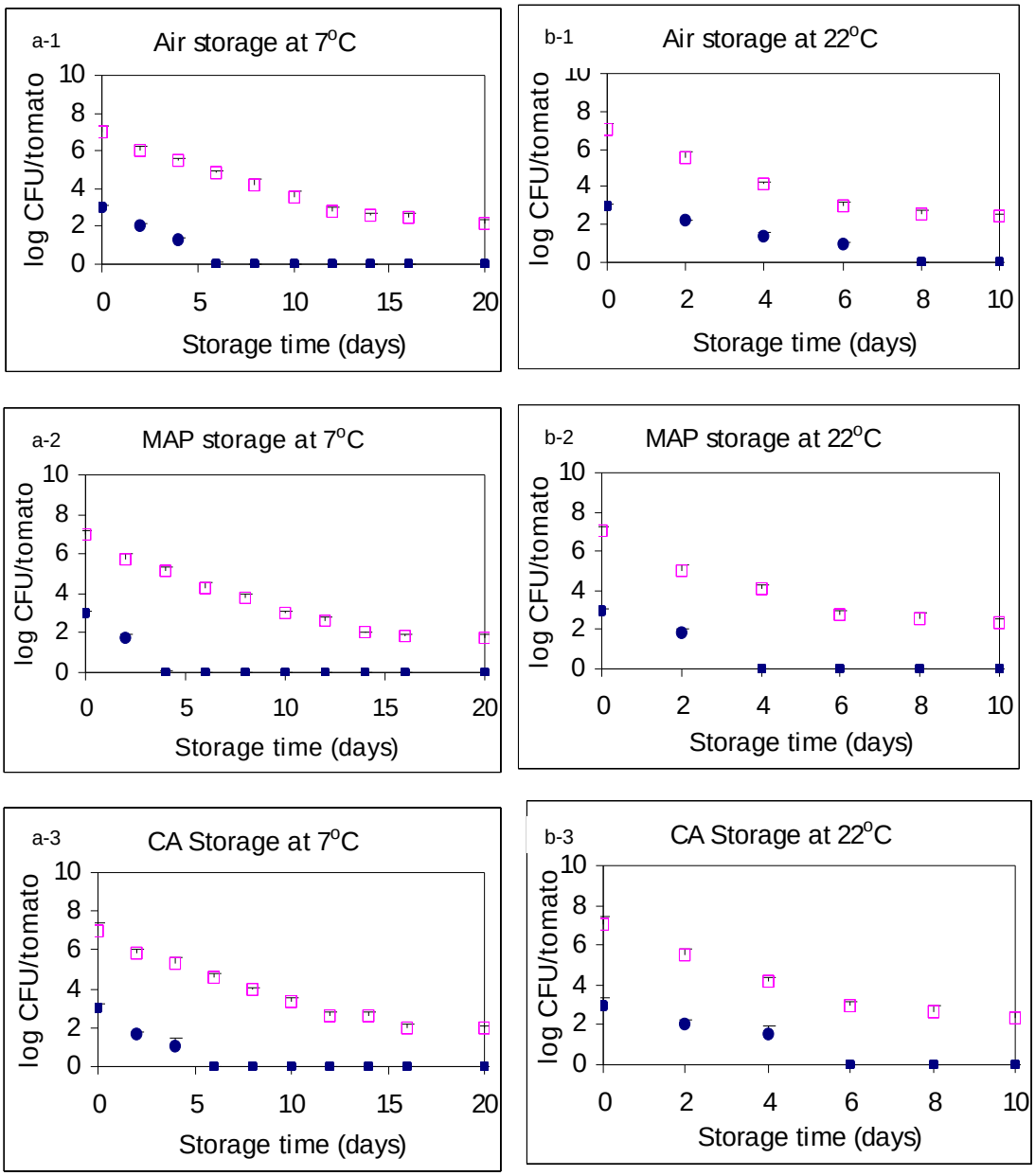


Figure 5. Survival characteristics of *S*.Enteritidis inoculated on the surface of the tomatoes stored different atmospheres at 7 and 22°C. (˜ low inoculum dose; ¢ high inoculum dose)

3.5. Survival of *S*.Enteritidis Inoculated to Stem Scars of Tomatoes at Different Atmospheric Conditions

3.5.1. Air

Two different ways in which inoculation to the stem scars of the tomatoes were achieved. One was to inject into and the other was to pipette onto the stem scars. Figure 6-a-1 and Figure 6-b-1 summarise the survival of *S*.Enteritidis at inoculated tomatoes which were stored in air at 7±2°C and 22±2°C, respectively.

When suspension containing 3.0 $\log_{10}$ CFU/ml *S*.Enteritidis cells in peptone water was injected into the stem scar for sub-inoculation of each tomato, growth of the pathogen was observed with an approximate increase of 1.0 $\log_{10}$ CFU/tomato in population at 22±2°C (Figure 6-a-1 and Figure 6-b-1). In the case of inoculation by pipetting, as seen in the same figures, a decrease of 2.0 $\log_{10}$ CFU/tomato at 7±2°C and approximately, 1.0 $\log_{10}$ CFU/tomato at 22±2°C was demonstrated.

When suspension containing 7.0 $\log_{10}$ CFU/ml *S*.Enteritidis cells in peptone water was injected into the stem scar for sub-inoculation of each tomato, growth of the pathogen was observed with an approximate increase of 1.0 $\log_{10}$ CFU/tomato at 7±2°C and 2.0 $\log_{10}$ CFU/tomato in population at 22±2°C was observed (Figure 6-a-1 and Figure 6-b-1). In the case of inoculation by pipetting, as seen in the same figures, a decrease of 1.5 $\log_{10}$ CFU/tomato at 7±2°C and approximately, 1.0 $\log_{10}$ CFU/tomato at 22±2°C was demonstrated. *S*.Enteritidis was not detected on the surfaces of the negative controls.

3.5.2. MAP

Figure 6-a-2 and Figure 6-b-2 summarise the survival of *S*.Enteritidis at inoculated tomatoes which were stored in MAP at 7±2°C and 22±2°C, respectively.

When suspension containing 3.0 $\log_{10}$ CFU/ml *S*.Enteritidis cells in peptone water was injected into the stem scar for sub-inoculation of each tomato, growth of the pathogen was observed with an approximate increase of 1.0 $\log_{10}$ CFU/tomato in population at 22±2°C (Figure 6-a-2 and Figure 6-b-2). In the case of inoculation by pipetting, as seen in the same figures, a decrease of 2.0 $\log_{10}$ CFU/tomato at 7±2°C and approximately, 1.0 $\log_{10}$ CFU/tomato at 22±2°C was demonstrated just as in the stem scars of the air stored tomatoes (Figure 6-a-1 and Figure 6-b-1).

When suspension containing 7.0 $\log_{10}$ CFU/ml *S*.Enteritidis cells in peptone water was injected into the stem scar for sub-inoculation of each tomato, growth of the pathogen was observed with an approximate increase of 1.0 $\log_{10}$ CFU/tomato in population at 22±2°C (Figure 6-a-2 and Figure 6-b-2). In the case of inoculation by pipetting, as seen in the same figures, a decrease of 2.0 $\log_{10}$ CFU/tomato at 7±2°C and approximately, 1.0 $\log_{10}$ CFU/tomato at 22±2°C was demonstrated, similar with the results of air stored samples. *S*.Enteritidis was not detected on the surfaces of the negative controls.

3.5.3. CA Storage

Figure 6-a-3 and Figure 6-b-3 summarise the survival of *S*.Enteritidis at inoculated tomatoes which were stored in CA at 7±2°C and 22±2°C, respectively.

When suspension containing 3.0 $\log_{10}$ CFU/ml *S*.Enteritidis cells in peptone water was injected into the stem scar for sub-inoculation of each tomato, growth of the pathogen was observed with an approximate increase of 1.0 $\log_{10}$ CFU/tomato in population at 22±2°C (Figure 6-a-3 and Figure 6-b-3). In the case of inoculation by pipetting, as seen in the same figures, a decrease of 2.0 $\log_{10}$ CFU/tomato at 7±2°C and approximately, 1.0 $\log_{10}$ CFU/tomato at 22±2°C was demonstrated just as in the stem scars of the air stored and modified atmosphere packed tomatoes.

When suspension containing 7.0 $\log_{10}$ CFU/ml *S*.Enteritidis cells in peptone water was injected into the stem scar for sub-inoculation of each tomato, growth of the pathogen was observed with an approximate increase of 1.0 $\log_{10}$ CFU/tomato in population both at 7±2°C and 22±2°C (Figure 6-a-3 and Figure 6-b-3). In the case of inoculation by pipetting, as seen in the same figures, a decrease of 2.0 $\log_{10}$ CFU/tomato at 7±2°C and approximately, 1.0 $\log_{10}$ CFU/tomato at 22±2°C was demonstrated similar with the results of air stored and modified atmosphere packed samples. *S*.Enteritidis was not detected on the surfaces of the negative controls.

Similarly, Wei et al. (1995) reported that the bacteria suspended in distilled water when added to tomato stem scars remained alive for 7 days although a time-related decrease in bacterial numbers occurred.

The results further show that *S*.Enteritidis can survive and/or grow during the storage of tomatoes depending on the location site of the pathogen on the fruit, load of contamination and the temperature of the storage. Stem scars provide protective environments for *Salmonella*; growth was observed with an approximate increase of 1.0 $\log_{10}$ CFU/tomato in stem-scar injected population at 22±2°C, regardless of the storage atmosphere.

Similarly, Kakiomenou et al. (1998) studied the influence of initial head spaces of air, 4.9% CO_2/2.1% O_2 /93% N_2 and 5.0% CO_2/5.2% O_2 /89.8% N_2

on *S*.Enteritidis and *L.monocytogenes,* and on microbial association with shredded carrots and lettuce at 4°C and reported that in both vegetables numbers of *S*.Enteritidis decreased but the bacterium survived during the storage in both of the packaging system and concluded that packaging in modified atmosphere does not necessarily signify an additional hurdle for growth of *S*.Enteritidis and *L.monocytogenes* compared with conventional packaging in aerobic conditions.

It was also reported that, although MAP is known to extend the shelf life of raw vegetables, such treatments were also known to increase the possibility of pathogenic bacteria reaching large numbers (Beuchat, 1999)

Drosinos, E.H. et al. (1999) similarly demonstrated that there was a decline trend of *S*.Enteritidis in modified atmosphere packaged diced tomatoes stored at 4°C while it survived during the storage at 10°C and concluded that *S*.Enteritidis can not grow in low pH with modified atmosphere storage at refrigeration temperatures (e.g. 4°C) but it may constitute a risk when a temperature abuse is produced in commercial chain.

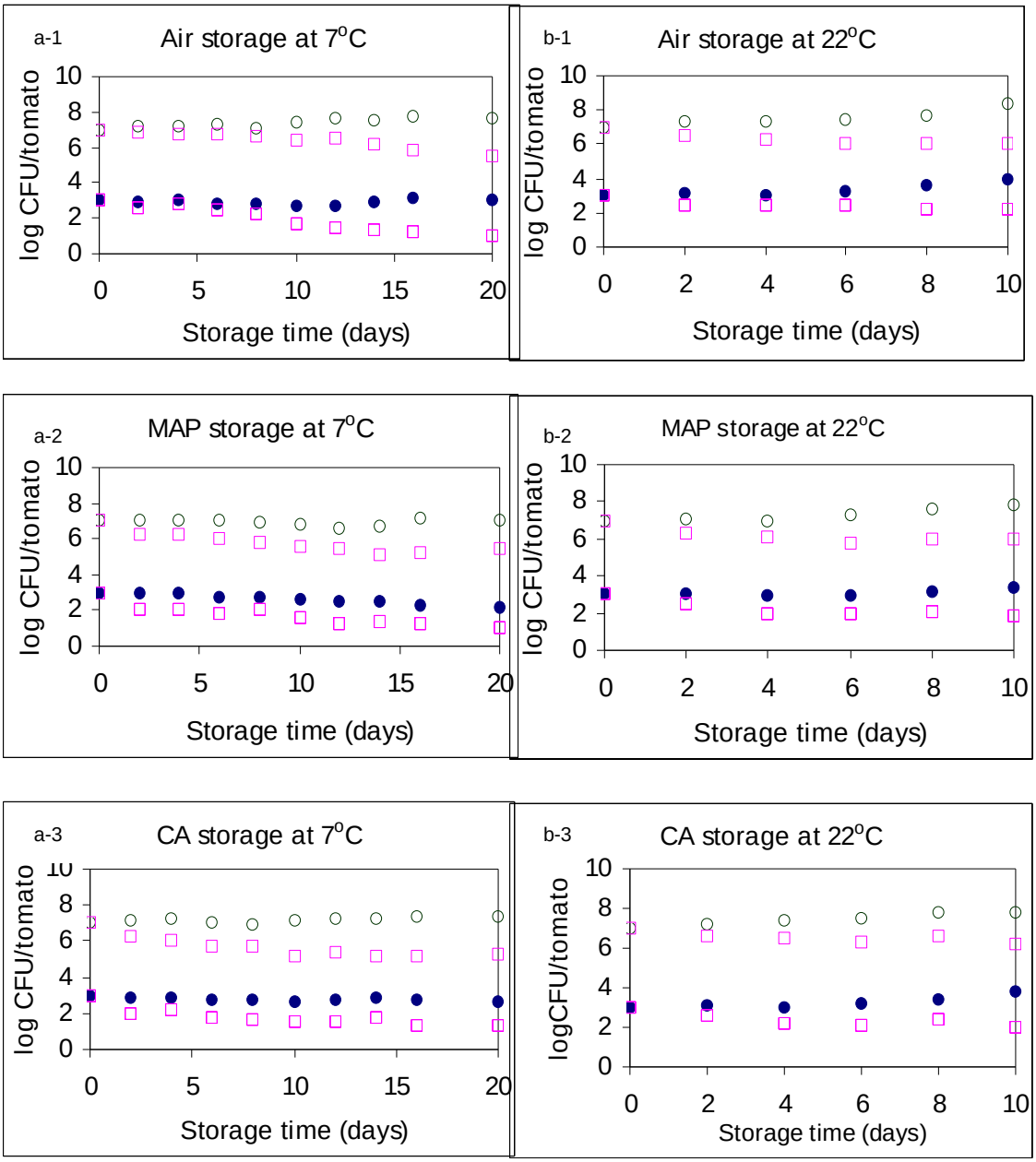


Figure 6. Survival characteristics of *S.*Enteritidis for stem scars-inoculated tomatoes stored at different atmospheres at 7 and 22°C. (• high dose injection to the stem scar; £ high dose pipetted on the stem scar; ˜ low dose injection to the stem scar; ¢ low dose pipetted on the stem scar)

3.6. Initial Count of Lactic Acid Bacteria and Total Viable Bacteria on/in the Tomatoes and Change in Population.

Figure 7-a and Figure 7-b summarise the initial count and change in population of lactic acid bacteria (LAB) and total viable count (TVC) in the tomatoes which were stored in air, CA and MAP at 7±2°C and 22±2°C, respectively. As demonstrated in the figures, the initial count of LAB present in the tomatoes was 4.5 $\log_{10}$ CFU/tomato. However, no colony of LAB was detected on the surface of the tomatoes. Nutrient broth was used for enrichment to verify the result. The development of the microbial association on tomatoes was dominated by LAB, those the population size obtained by MRS. This population was almost equal to that obtained with PCA.

In our study, additionally, to be also able to analyse the fate of lactic acid bacteria with the co-existence and not of *S*.Enteritidis, we observed the growth characteristics of lactic acid bacteria both in uninoculated (negative controls) and inoculated tomatoes. As inoculated tomatoes, we used the stem-injected in other words, sub-inoculated samples, for this aim. The reason of using especially sub-inoculated tomatoes but not spot-inoculated ones in this part of the study was that, since LAB were not detected on the surface of the tomatoes, it would have been impossible to analyse the effect of *S*.Enteritidis on the fate of lactic acid bacteria in the case of using spot-inoculated tomatoes. The results suggested that the initial count and the growth patterns of LAB both in uninoculated (Figure 7-a and Figure 7-b) and inoculated tomatoes (not shown) were same. These bacteria grew well regardless of the presence of *S*.Enteritidis, atmosphere and the temperature of the storage.

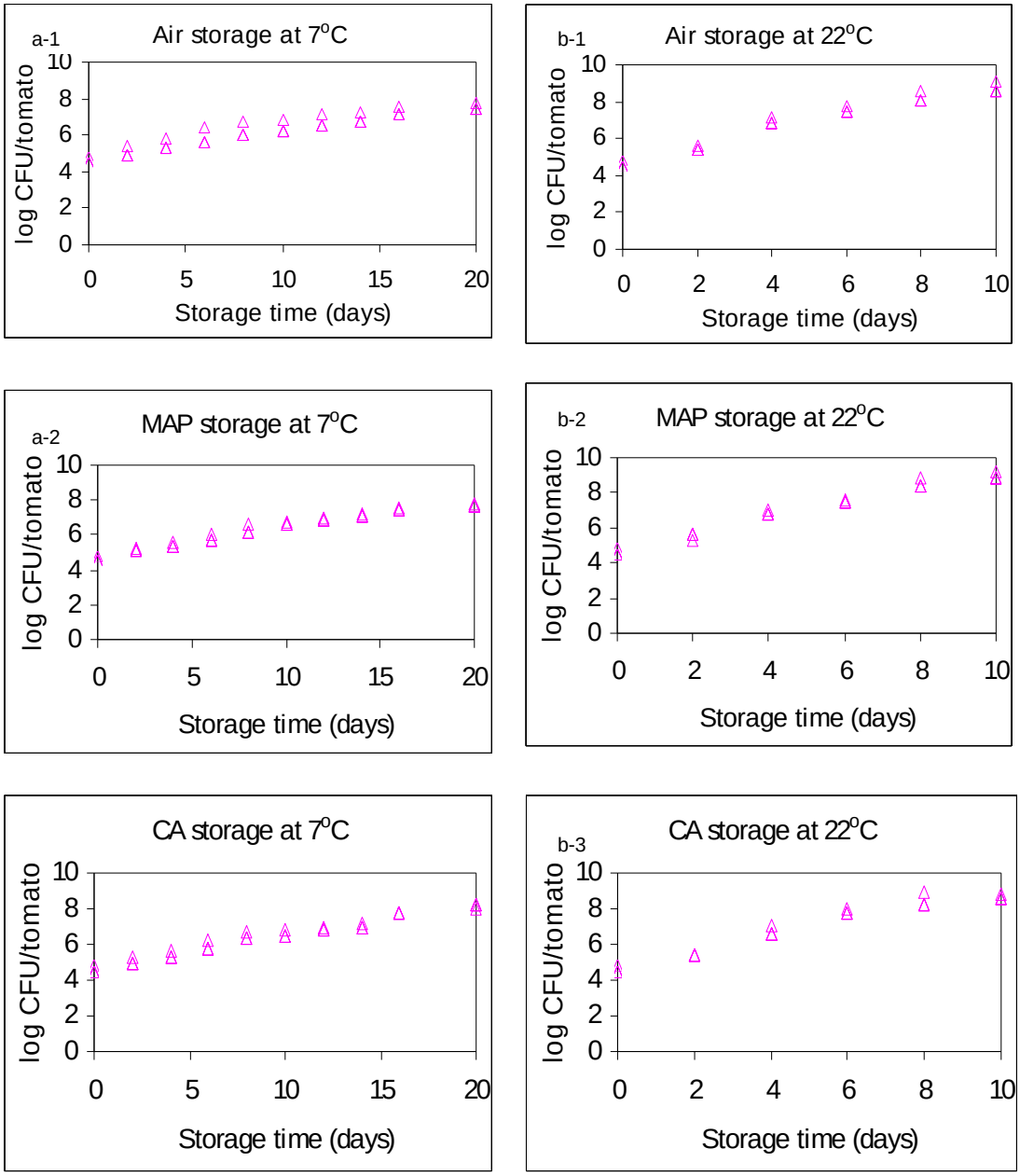


Figure 7. Change in population of LAB (p) and TVC ( ) in the normal microflora of tomatoes stored at different atmospheres at 7 and 22°C.

Similarly, Kakiomenou et al. (1998) also studied the influence of initial head spaces of air, 4.9% CO_2/2.1% O_2 /93% N_2 and 5.0% CO_2/5.2% O_2 /89.8% N_2 on *S*.Enteritidis and *L.monocytogenes,* and on microbial association with shredded carrots and lettuce at 4°C and reported that the lactic acid bacteria grew well under all three atmospheric conditions with or without the presence of *S*.Enteritidis and added that the survival of the latter was not affected by *Lactobacillus* sp.

Similarly, Enfors and Molin (1978) reported that LAB are quite resistant to the antimicrobial effects of CO_2. On the other hand, Drosinos, et al (1999) reported that during the storage of the tomatoes that were cut into slices in different atmospheric conditions, the increase of lactic, acetic, formic and propionic acid changed pH values and added that concentrations of lactic acid and propionic acid were higher in samples stored under 5% CO_2/95% N_2 conditions than in samples stored under aerobic.

It was concluded in our study that, LAB in the tomatoes grew well regardless of the storage atmosphere (Figure 7-a and 7-b), decreasing the pH values of the tomatoes from 4.0 to 3.0 (Figure 9-a and Figure 9-b) and the survival of *S*.Enteritidis was not inhibited (Figure 6-a and Figure 6-b) by the low pH range of the cherry tomatoes. Similarly, it was reported by Chung, et al. (1970) that *S.*Enteritidis*, S.*Infantis and *S.*Typhimurium were capable of growing in chopped cherry tomatoes, these microorganisms together with many other *Salmonella* spp., were able to grow at low pH (3.99 to 4.37) under certain conditions (Asplund, et al., 1991).

It was also reported that, *Salmonella* spp. can survive in low-pH foods such as vegetables stored under VP/MAP conditions at chill temperature (Kakiomenou et al., 1998; Finn et al., 1997; Tassou, et al., 2002)

Survival of *S.*Enteritidis in low pH products such as tomato may be attributed to its adaptation by increasing its capacity for pH homeostasis

(McLaggan et al., 1997). Similar results have been reported with low pH products with other Enterobacteriaceae (AbdulRauf et al., 1993; Maipa et al., 1993; Massa et al., 1997; Kakiomenou et al., 1998). Since the effect of pH of vegetables would allow the growth of commensal bacteria (Lund, 1992), the prevention of growth of *S*.Enteritidis can be possibly attributed to the inability of this pathogen to compete successfully with lactic acid bacteria (Gibbs, 1987) either due to bacteriocin production (Vescono et al., 1995) or due to their specific metabolic products such as lactic and acetic acids, produced from these organisms during storage (Kakiomenou et al., 1996).

As a result of a similar study that support the survival of *Salmonella* in low pH products such as tomato, Mahon et al. (1997) reported that, citric acid is the predominant acid in tomato fruit, and the pH of the pulp of most cultivars is below 4.5. More than 90% of the weight of tomato fruits is water. As tomato fruits develop, the amount of sucrose decreases while the starch and reducing sugars increase which would favor nutrient availability for growth of *Salmonella*. Growth of serotypes Anatum, Senftenberg and Tennessee at pH 4.05 under otherwise ideal conditions has been reported (Chung et al., 1970).

Tomato stems and fruits are subject to mechanical injury in the field and during post-harvest handling, which make them more susceptible to infiltration of bacteria. In contaminated agricultural environments e.g., soil and water, *Salmonella* may attach to or enter tomato fruits and survive for an extended time. In our study, different storage conditions with different atmospheres and temperatures were supplied to analyse the effects of these storage techniques on survival of *S.*Enteritidis which was used to inoculate cherry tomatoes either by injecting stems with the inoculum or inoculating surfaces with it and was concluded that, *S*.Enteritidis constitutes a risk by managing to survive and sometimes grow depending on the location site of the pathogen on the fruit, load of contamination and the temperature of the storage.

These results clarifies the fact that, interventions need to be applied, firstly, to eliminate contamination of tomato fruits with *S.*Enteritidis by preventing or minimizing its contact with tomato plants and fruits at all points from the farm to the consumer and secondly, in the case of presence of contamination, to inactivate this pathogen. A zero-tolerance for *Salmonella* spp., VTEC, C*ampylobacter* spp., and pathogenic *Y.enterocolitica* is recommended for ready-to-eat vegetables (Odumeru, et al., 1997). In the lack of interventions, contamination of tomato fruits with *S*.Enteritidis will result in an increased risk of human infection. As the final experiment in this study, gaseous ozone treatment was applied on inoculated tomatoes to analyse the effect on inactivation of the bacterium, which could be achieved neither by controlled atmosphere storage nor by modified atmosphere packaging.

3.7. Effect of Gaseous Ozone Treatment on *S*.Enteritidis

Previously, one group of spot-inoculated tomatoes were left in the laminar flow hood for 1 hour and the other for 4 hours to allow the *S*.Enteritidis cells attach on the surfaces, which were afterwards treated with gaseous ozone. Figure 8 (a, b, c and d) summarise the effect of gaseous ozone treatment of 5-20 mg/L on *S*.Enteritidis.

When suspension containing 7.0 $\log_{10}$ CFU/ml *S*.Enteritidis cells in peptone water was used for spot-inoculation of the surface of each tomato, 2 log reduction in population of the pathogen was observed (Figure 8-b) as a result of ozone gas (10 mg/L or 0.75 % wt/wt) treatment for 5 min and cells died completely after 20 min. In the case of the other group of tomatoes, which were dried in the laminar flow hood for just 1 hour after spot-inoculation to allow the cells attach on the surface, a faster death rate was observed when compared to the ones on which the cells were left to attach for 4 hours (Figure 8-b).

When suspension containing 3.0 $\log_{10}$ CFU/ml *S*.Enteritidis cells in peptone water was used for spot-inoculation of the surface of each tomato, 3 log reduction in population of the pathogen was observed, following an ozone gas (10 mg/L) treatment for 10 min, cells died completely (Figure 8-a).

Thus, it was concluded in our study that, a faster death rate of *S*.Enteritidis cells was observed on the tomatoes which were left in the laminar flow hood for 1 hour to allow the cells attach on the surface, when compared to the ones on which the cells were left to attach for 4 hours. The degree of attachment of the microorganism with food carries a great importance on the effectiveness of the bactericidal effect of ozone.

Similarly, Yousef et al. (1999) reported that inactivation of microflora on food by ozone depends greatly on the nature and composition of food surface, the type and load of microbial contaminant, and the degree of attachment or association of microorganisms with food.

As shown in Figure 8-c, when suspension containing 7.0 $\log_{10}$ CFU/ml *S*.Enteritidis cells in peptone water was used for spot-inoculation of the surface of each tomato, approximately 1 log reduction in population of the pathogen was observed, following an ozone gas (5 mg/L or 0.4% wt/wt) treatment for 5 min, and the cells were not completely inactivated within 20 min treatment (Figure 8-c). However, in the case of using an ozone gas of concentration 20 mg/L (1.5 % wt/wt), 7 log reduction in population of the pathogen was observed after 15 min, cells died completely (Figure 8-d).

When ozone gas of concentration of 30 mg/L (2.25% wt/wt) was applied on the tomatoes which were spot-inoculated by a suspension containing 7.0 $\log_{10}$ CFU/ml *S*.Enteritidis cells in peptone water, 1 log reduction in the population of the pathogen was observed within 30 seconds and complete death of the cells occurred within 5 min of the ozone treatment

(not shown). However, a color change was seen on the ozone-applied tomatoes.

As a result of a similar study, Ramirez et al. (1994), concluded as, when broiler skin was inoculated with *Salmonella* Enteritidis and exposed to an ozone-air mixture (8% wt/wt) for 15 s, approximately 1 log reduction in population of the pathogen was observed.

Similarly, Kaess and Weidemann (1968) demonstrated that the count of *Pseudomonas* spp. and *C.scotti* on contaminated beef decreased significantly at $>$ 2 μg/L gaseous ozone and added that the color of the muscle surface treated with $<$ 0.6 μg/L ozone did not differ from that of the control treatment. Kaess and Weidemann (1968) also reported that ozone has been tested in the process of tenderizing meats to control surface microflora (*Pseudomonas* spp., spores, *Salmonella* spp., *Staphylococcus* spp.) and added that ozone in a gas mixture at 0.1 mg/L and RH of 60 to 90% were required in the tenderizing room to inactivate bacteria but higher concentrations of ozone were required to inhibit molds.

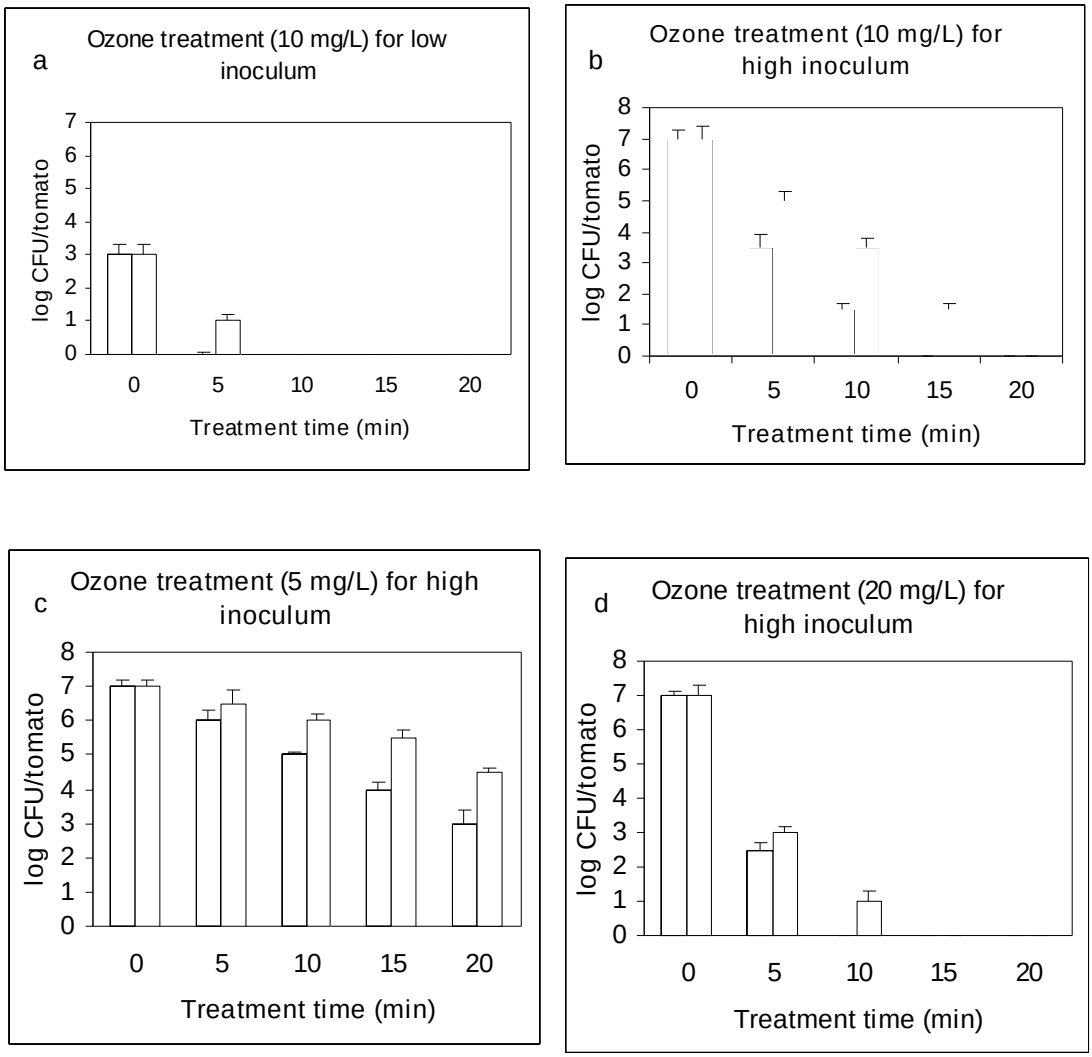


Figure 8. Effect of gaseous ozone treatment on *S*.Enteritidis inoculated on the surface of the tomatoes. (attachment time of the cells on tomatoes after inoculation: ¢ 1 h; £ 4 h)

Additionally, ozone has been tested for disinfecting hatchery, hatching eggs, poultry chiller water, poultry carcass and contaminated eggs. As an example, cultures of *Staphylococcus, Streptococcus* and *Bacillus* species previously isolated from poultry hatcheries and culture collections of *E.coli, P.fluorescens, Salmonella* Typhimurium, *Proteus* species and *A.fumigatus* were spread-plated on open Petri plates and exposed to ozone gas in a prototype laboratory poultry setter by Whistler and Sheldon (1989). Whistler and Sheldon (1989) concluded that, ozone treatment (1.5 to 1.65%, wt/wt) decreased microbial populations by > 4 to 7 logs for bacteria and > 4 logs in the case of fungi. They also evaluated ozone as a disinfectant against natural contaminants on hatching eggs reporting that microbial counts significantly decreased (> 2.5 logs) on the shell of eggs that were ozone gas treated (2.83%, wt/wt) for 2 hours.

Bailey et al. (1996) also reported that, ozone decreased the aerobic plate counts and *Salmonella* in hatching cabinet air samples by 75 to 99%.

In an other study of ozone gas application, Bazarova (1982) stored apples in a specially constructed stainless steel chamber at 0 to 1°C and 90 to 95% RH with ozone gas being admitted daily for 4 h at 5 to 6 μg/L and concluded that ozone treatment reduced weight loss and spoilage incidence in apples.

Ozone at 0.1 to 0.3 ppm in atmosphere during blackberry storage suppressed fungal development for 12 days at 2°C and did not cause observable injury or defects (Barth et al., 1995). Grapes exposed for 20 min to ozone (8 mg/L) had considerably reduced counts of bacteria, fungi and yeasts (Sarig at al., 1996). Fungal decay following cold storage of the grapes was reduced and shelf life increased by the ozone treatment. Horvath et al., (1985) attributed the increase of the shelf life of apples and oranges to the oxidation of ethylene and to the removal of other metabolic products by

ozone. However, inactivation of spoilage microorganisms on fruits, without a doubt, contributed to this shelf life extension.

As an example study on vegetables, Faitel'berg-Blank et al.(1979) stored onions and potatoes in wooden chambers covered with polyethylene film in which ozone (0.2 μg/L) was produced for 8 h/day on 5 days/weekend concluded that ozone treatment decreased oxygen uptake, catalase and peroxidase activities and had a marked inhibitory effect on the growth of surface microorganisms. Similarly, Baranovskaya et al. (1979) used ozone in the industrial storage of potatoes, onions and sugar beets, maintaining ozone concentration at 3 mg/L with temperature within 6 to 14°C and RH at 93 to 97% and showed that bacteria and mold counts were very low for treated samples, whereas chemical composition and sensory quality did not change appreciably. Prange et al. (1997) presented ozone as an alternative to chlorpropham (isopropyl-*N*-[3-chlorophenyl] carbamate as a sprout control agent for Russet Burbank potatoes in Canada.

In an other ozone gas application study, carrots, inoculated with pathogenic fungi, *Botrytis cinerea* and *Sclerotinia sclerotiorum* were exposed to a gas mixture containing 0 to 60 mg/L ozone at a flow rate of 0.5 L/min for 8 h daily for 28 days (Liew and Prange, 1994) and it was concluded that a 50% reduction in daily growth rates for both fungi was obtained at 60 mg/L ozone. However, carrot respiration rate, electrolyte leakage, and total colour differences increased with an increase in ozone concentration.

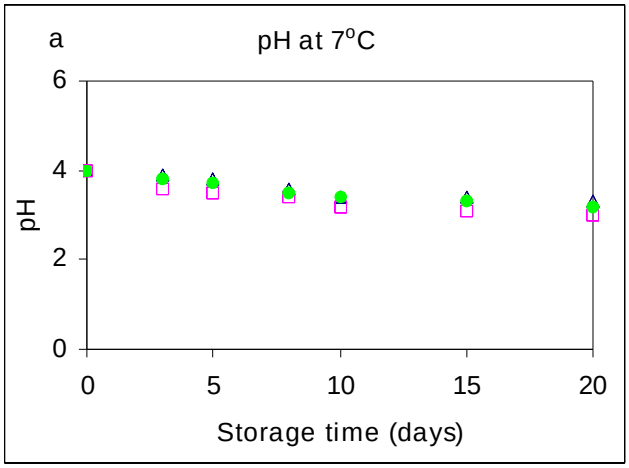
Kim et al. (1993) investigated the effects of treating kimchi ingredients (cabbage, hot pepper powder, garlic, ginger, green onion and leak) with ozone gas (6 mg/L/s for 60 min) on the vitamin content and sensory properties of this product besides the bacterial count and concluded that ozone treatment eliminated 80 to 90% of the total bacterial population in garlic and ginger and improved sensory properties of kimchi.

Han et al. (2002) reported that, more than 5 log reductions of *E.coli* O157:H7 on green peppers after the treatments with 7 mg/L ozone for 20 and 40 min, under 85% RH and at 22°C. Similarly, Zhao and Cranston (1995) reported that, 3 to 6 log reduction of *E.coli, Salmonella* spp., and *Staphylococcus aureus* on black pepper could be achieved by passing ozonized air (6.7 mg/L; 6 L/min) through black pepper for 60 min.

In our study, it was shown that ozone gas has a bactericidal effect on *S*.Enteritidis and is a potential effective sanitizer for cherry tomatoes, which may meet the 5 log reduction recommendation set by FDA.

3.8. pH

The change in pH of cherry tomatoes that were stored in different atmospheres and temperatures are demonstrated in Figure 9 (a and b). pH varied in the range of 4.0 and 3.0 during the storage period.



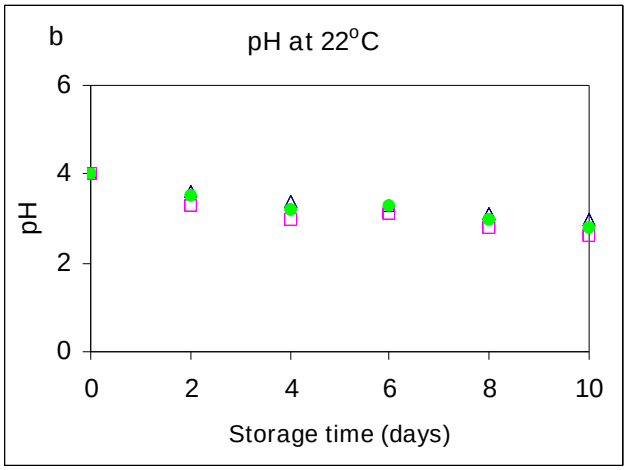


Figure 9. Change in the pH of the tomatoes stored at different atmospheres at 7 and 22°C. (¢ MAP storage; ˜ CA storage; p Air storage)

CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

Salmonella Enteritidis is able to attach on and infiltrate into the tomatoes during storage in contact with contaminated water.

Storage atmosphere and temperature have great importance on safety of the produce. Modifying the storage atmosphere is a largely used technique to inhibit the survival of microorganisms on food. However, *S*.Enteritidis can survive and/or grow during the MAP and CA storage of tomatoes, depending on the location site of the pathogen on the fruit, load of contamination and the storage temperature. Stem scars provide protective environments for *Salmonella*; growth occurs at room temperature within the storage period, regardless of the storage atmosphere.

Limiting oxygen and increased carbon dioxide in modified atmospheres do not demonstrate an inhibition effect on the survival of *S*.Enteritidis on fresh produce. For that reason, an intervention step such as gaseous ozone treatment must be included before the MAP and CA storage for the safety of the produce.

Lactic acid bacteria (LAB), taking place in the normal microflora of tomatoes grow well under all atmospheric conditions with or without the presence of *S*.Enteritidis. pH values of tomatoes changed from 4.0 to 3.0 during the storage period.

Ozone gas has bactericidal effect on *S*.Enteritidis and is a potential effective sanitizer for fresh produce, which may meet the 5 log reduction

recommendation set by FDA. However, high concentrations of ozone gas cause surface color change and may result in organoleptic and nutritional loss. That is why, besides food safety, the effect of ozone treatment on organoleptic and nutritional quality of produce must be studied.

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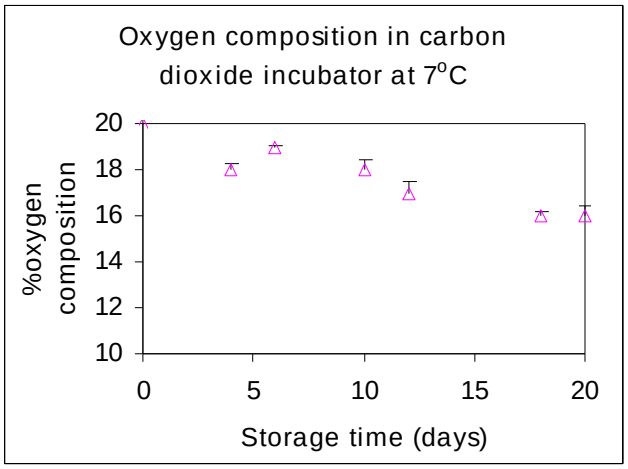
APPENDIX A

CARBON DIOXIDE INCUBATOR (MCO 175)

Name and Function of Parts

1. Door
2. Inner Door
3. Regulating Legs
4. Shelves
5. Shelf Supports
6. Fan
7. Water Supplying Inlet, Draining Outlet
8. Tank
9. Control Panel
10. Motor Compartment
11. Drain Pan
12. Inside Air Sampling Outlet
13. CO_2 Pipe Connector
14. Earth Terminal
15. Door Switch
16. Water Level Indicator
17. Humidifying Pan



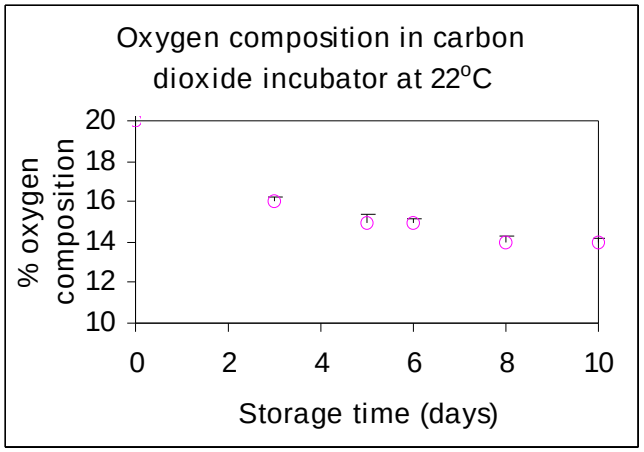


Figure 10. Changes in the oxygen composition in carbon dioxide incubator. (at 7 °C and • at 22°C)