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GROWTH AND SURVIVAL OF *SALMONELLA* IN MODIFIED ATMOSPHERE
PACKAGED RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) FILLETS

A Thesis
Presented for the
Master of Science
Degree
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Melissa Edlin Denton

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DEDICATION

This thesis is dedicated with love to my parents,
Mr. Harold E. Edlin and Mrs. Doris J. Edlin
and my husband, W. Neal Denton.

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ABSTRACT

The growth and survival of *Salmonella* in rainbow trout fillets stored under vacuum, aerobic conditions, or a modified atmosphere of carbon dioxide(CO₂):nitrogen(N₂) (60:40) with or without a carbonic acid dip treatment was evaluated at 3 and 10°C. In addition, the effect of these storage conditions on the growth of aerobic, psychrotrophic, lactic, and coliform bacteria was also examined.

The *Salmonella* inoculum level was approximately log 4 CFU/g trout, although direct plating resulted in initial counts of log 3.0 to 3.5 CFU/g. The growth of *Salmonella* at 10°C was significantly higher ($p < 0.05$) in trout held under vacuum and air packaging than in trout held under the CO₂-modified atmosphere. *Salmonella* numbers declined in the trout samples stored at 3°C, with significantly greater ($p < 0.05$) survival under air packaging than in vacuum and CO₂-modified atmosphere packaging. The growth of aerobic, psychrotrophic, lactic, and coliform bacteria in the trout fillets was significantly lower ($p < 0.05$) under the modified atmosphere of CO₂:N₂ (60:40) than air and vacuum packaging at 3 and 10°C with a shelf-life extension of approximately 3 days at 10°C and 9 days at 3°C. The carbonic acid dip had some initial effect upon the growth of aerobic and coliform bacteria at 10°C, although no extension of shelf life was evident.

TABLE OF CONTENTS

CHAPTER	PAGE
I. INTRODUCTION	1
II. REVIEW OF LITERATURE	3
Rainbow trout	3
Modified atmosphere packaging	5
Mechanism of action	5
Applications of MAP	6
Modified atmosphere packaging of fishery products	6
<i>Salmonella</i>	9
Microbiological characteristics	9
<i>Salmonella</i> as a pathogen in fishery products	9
Growth of <i>Salmonella</i> in MAP	10
Enrichment and isolation methods for <i>Salmonella</i>	12
III. MATERIALS AND METHODS	14
Trout samples and packaging	14
<i>Salmonella</i> cultures	15
Monitoring of samples	15
Microbial analysis	16
<i>Salmonella</i> detection and enumeration	16
Gas Analysis	17
Statistical Analysis	17
IV. RESULTS AND DISCUSSION	19
Microbiological measurements	19
Mesophilic aerobic bacteria	19
Psychrotrophic bacteria	22
Coliform bacteria	25
Lactic acid bacteria	28
<i>Salmonella</i>	28
Carbonic acid dip	34
pH measurements	35
Replication differences	35
Headspace gas analysis	38
V. CONCLUSIONS	46
REFERENCES	48
APPENDIXES	52
APPENDIX A. Statistical Tables	53
APPENDIX B. Preliminary Studies	67
VITA	77

LIST OF TABLES

TABLE	PAGE
1. Proximate composition of 100 g of the edible portion of raw rainbow trout	4
2. Extension of shelf-life of trout, salmon, and other species of fish by modified atmosphere packaging	8
3. Growth requirements for <i>Salmonella</i> and conditions of MAP-fish	11
A1. Anova table for aerobic plate count in rainbow trout fillets stored at 10°C	54
A2. Anova table for psychrotrophic count in rainbow trout fillets stored at 10°C	54
A3. Anova table for coliform count in rainbow trout fillets stored at 10°C	55
A4. Anova table for lactic acid bacteria count in rainbow trout fillets stored at 10°C . . .	55
A5. Anova table for <i>Salmonella</i> count in rainbow trout fillets stored at 10°C	56
A6. Anova table for pH of rainbow trout fillets stored at 10°C	56
A7. Mean separations between replicatons for bacterial counts ($\log_{10}$ CFU/g) in rainbow trout fillets stored at 10°C	57
A8. Mean separations between atmospheres for bacterial counts ($\log_{10}$ CFU/g) in rainbow trout fillets stored at 10°C	57
A9. Anova table for the effects of <i>Salmonella</i> inoculation on microbial counts at 10°C . . .	58
A10. Anova table for aerobic plate count in rainbow trout fillets stored at 3°C	59
A11. Anova table for psychrotrophic count in rainbow trout fillets stored at 3°C	59
A12. Anova table for coliform count in rainbow trout fillets stored at 3°C	60

A13.	Anova table for lactic acid bacteria count in rainbow trout fillets stored at 3°C . . .	60
A14.	Anova table for <i>Salmonella</i> count in rainbow trout fillets stored at 3°C	61
A15.	Anova table for pH of rainbow trout fillets stored at 3°C	61
A16.	Mean separations between replicatons for bacterial counts ($\log_{10}$ CFU/g) in rainbow trout fillets stored at 3°C	62
A17.	Mean separations between atmospheres for bacterial counts ($\log_{10}$ CFU/g) in rainbow trout fillets stored at 3°C	62
A18.	Anova table on the effects of <i>Salmonella</i> inoculation on microbial counts at 3°C . . .	63
A19.	Mean separations between replication for pH in rainbow trout fillets stored at 3 and 10°C	64
A20.	Mean separations between atmosphere for pH in rainbow trout fillets stored at 3 and 10°C	64
A21.	Anova table for headspace gas analysis of rainbow trout fillets packaged under an air atmosphere	65
A22.	Anova table for headspace gas analysis of rainbow trout fillets packaged under a CO ₂ :N ₂ (60:40) atmosphere	66
B1.	Recovery of <i>Salmonella</i> from rainbow trout fillets using enrichment methods	70
B2.	Evaluation of enrichment methods for recovery of <i>Salmonella</i> from rainbow trout fillets stored at 10°C	74

LIST OF FIGURES

FIGURE	PAGE
1. Growth of mesophilic aerobic bacteria in rainbow trout fillets either dipped in carbonic acid or icewater and held at 10°C under vacuum, air, or a gas mixture of CO ₂ :N ₂ (60:40)	20
2. Growth of mesophilic aerobic bacteria in rainbow trout fillets either dipped in carbonic acid or icewater and held at 3°C under vacuum, air, or a gas mixture of CO ₂ :N ₂ (60:40)	21
3. Growth of psychrotrophic bacteria in rainbow trout fillets either dipped in carbonic acid or icewater and held at 10°C under vacuum, air, or a gas mixture of CO ₂ :N ₂ (60:40)	23
4. Growth of psychrotrophic bacteria in rainbow trout fillets either dipped in carbonic acid or icewater and held at 3°C under vacuum, air, or a gas mixture of CO ₂ :N ₂ (60:40)	24
5. Growth of coliforms in rainbow trout fillets either dipped in carbonic acid or icewater and held at 10°C under vacuum, air, or a gas mixture of CO ₂ :N ₂ (60:40)	26
6. Growth of coliforms in rainbow trout fillets either dipped in carbonic acid or icewater and held at 3°C under vacuum, air, or a gas mixture of CO ₂ :N ₂ (60:40)	27
7. Growth of lactic acid bacteria in rainbow trout fillets either dipped in carbonic acid or icewater and held at 10°C under vacuum, air, or a gas mixture of CO ₂ :N ₂ (60:40)	29
8. Growth of lactic acid bacteria in rainbow trout fillets either dipped in carbonic acid or icewater and held at 3°C under vacuum, air, or a gas mixture of CO ₂ :N ₂ (60:40)	30

9.	Survival of <i>Salmonella</i> in rainbow trout fillets either dipped in carbonic acid or icewater and held at 10°C under vacuum, air, or a gas mixture of CO ₂ :N ₂ (60:40)	32
10.	Survival of <i>Salmonella</i> in rainbow trout fillets either dipped in carbonic acid or icewater and held at 3°C under vacuum, air, or a gas mixture of CO ₂ :N ₂ (60:40)	33
11.	pH of rainbow trout fillets either dipped in carbonic acid or icewater and held at 10°C under vacuum, air, or a gas mixture of CO ₂ :N ₂ (60:40)	36
12.	pH of rainbow trout fillets either dipped in carbonic acid or icewater and held at 3°C under vacuum, air, or a gas mixture of CO ₂ :N ₂ (60:40)	37
13.	Changes in gas composition (%) in packages of rainbow trout fillets either dipped in carbonic acid or icewater and held at 10°C under air	39
14.	Changes in gas composition (%) in packages of rainbow trout fillets either dipped in carbonic acid or icewater and held at 3°C under air	40
15.	Changes in gas composition (%) in packages of rainbow trout fillets either dipped in carbonic acid or icewater and held at 10°C under a gas mixture of CO ₂ :N ₂ (60:40)	42
16.	Changes in gas composition (%) in packages of rainbow trout fillets either dipped in carbonic acid or icewater and held at 3°C under a gas mixture of CO ₂ :N ₂ (60:40)	43
17.	Changes in gas composition (%) in empty packages held at 3°C under a gas mixture of CO ₂ :N ₂ (60:40)	44
B1.	Mean bacterial counts of rainbow trout fillets purchased at Knoxville area retail markets .	72

Chapter I

INTRODUCTION

In recent years, fish consumption has greatly increased in the United States. It has risen primarily because of heightened health awareness and increased availability of fresh fish in retail markets (Consumer Reports, 1992). One of the more popular fish for sport and commercial uses is rainbow trout (*Oncorhynchus mykiss*, formerly *Salmo gairdneri*) (Brannon, 1991). Although sales of food size trout have decreased annually since 1990 (\$64 million to \$53 million), the total pounds sold per year have remained relatively constant, illustrating a constant market for this product (Anonymous, 1993). Because fish in general have a neutral pH, low body temperature, and are high in protein, they are an excellent source for microbial growth, especially psychrotrophic spoilage organisms (Pedrosa-Menabrito and Regenstein, 1988; Reddy et al., 1992). For these reasons, fish, such as rainbow trout, tend to have a short shelf life and may be spoiled upon reaching the display case in the local retail market. There is certainly the need for research to extend the shelf life of fishery products.

One method under investigation is the utilization of a modified atmosphere storage system enriched with carbon dioxide (CO₂). Although modified atmosphere packaging has

been shown to significantly increase the shelf life of fresh fish (Reddy et al., 1992) and several species of trout and salmon (Brown et al., 1980; Banks et al., 1980; Barnett et al., 1987; Gray et al., 1983; Haard and Lee, 1982; Richter and Banwart, 1983; and Stier et al., 1981), additional research is needed to ensure the safety of these products with regard to pathogens (Farber, 1991).

One pathogen of concern is the genus *Salmonella*. Although salmonellae do not grow well at low temperatures (<5°C), they are facultative anaerobes and are capable of growing in the absence of oxygen (Jay, 1992), a condition which may occur in modified atmosphere packaging. *Salmonella* spp. are not endemic to the intestinal tract of fish, although fish may come in contact with this organism from fecal contamination in aquaculture systems or during processing (Kvenberg, 1991). In addition, the growth of this organism may reach a hazardous level in a modified atmosphere package before the product reaches spoilage level if strict temperature controls are not observed. Therefore it is important to monitor the growth of *Salmonella* in rainbow trout fillets under modified atmosphere packaging.

The objective of this project was to determine the relative effect of vacuum, air, and CO₂:N (60:40) atmosphere packaging in conjunction with a carbonic acid dip upon the growth of *Salmonella* and spoilage bacteria in rainbow trout fillets stored at 3 and 10°C.

Chapter II

REVIEW OF LITERATURE

Rainbow trout

Rainbow trout are members of the family Salmonidae. The recent change in scientific name from *Salmo gairdneri* to *Oncorhynchus mykiss* technically classifies this fish as a species of salmon rather than trout. The distinguishing characteristics of rainbow trout include a metallic blue color on the dorsal surface with silvery sides. This fish also has black spots on the dorsal and caudal fins, and along the back. Although rainbow trout are native to the streams of the Pacific Northwest, they have been introduced around the world where climate and water quality are acceptable (Stickney, 1991). The popularity of rainbow trout may be attributed to its adaptability to a variety of environments. It can be found in freshwater within a temperature range of 0 to 27°C. Rainbow trout are also very suitable for aquaculture production because the eggs have a short incubation period, the fish grow fast, and they have a variety of food sources (Brannon, 1991). Rainbow trout provide an excellent source of protein and also are low in saturated fat and carbohydrate (Table 1). Although sales of food-size trout have decreased since 1990 in the United States, the total pounds sold has remained relatively constant. Sales of this fish totaled \$53 million in 1993

Table 1. Proximate composition of 100 g of the edible portion of raw rainbow trout.

Component	Unit	Mean	Stand. error	No. of Samples
Water	g	71.48	0.924	26
Food energy	kcal kJ	118 494		
Protein (N x 6.25)	g	20.55	0.356	24
Total lipid	g	3.36	0.235	46
Saturated	g	0.649		
Unsaturated	g	2.234		
Carbohydrate	g	0.00		
Crude fiber	g	0.00		
Ash	g	1.30	0.020	23

Source: Exler, 1987.

reflecting a sizeable market for this product (Anonymous, 1993).

Modified atmosphere packaging

Mechanism of action. Modified atmosphere packaging (MAP) refers to a system in which the air inside a package is replaced with other gases. MAP can be used to control microbial growth, therefore extending the shelf life of the product. The replacement gases usually consist of higher concentrations of carbon dioxide (CO₂) which exhibit a bacteriostatic effect upon the microflora of the product. Proposed mechanisms of this action are reviewed by Lampila (1991). These include: (1) CO₂ inhibits the growth of aerobic organisms, which are the primary spoilage organisms, and causes a shift in microflora to more gram-positives such as the lactic acid bacteria, (2) hydration of CO₂ to carbonic acid may result in a reduced pH of the tissue, (3) CO₂ may interfere with the permeability of the bacterial cell membrane, and (4) increased levels of CO₂ may affect metabolic pathways and alter enzymatic activity of the microflora. Lampila (1991) suggested that the bacteriostatic effect was most likely attributed to a combination of these mechanisms. In addition, Daniels et al. (1985) has reviewed the effects of carbon dioxide and the mechanisms of action on microbial growth and the resulting quality of the food product. Since one of the proposed mechanisms involves the hydration of CO₂ to

carbonic acid, further investigation yielded significant differences in microbial growth between carbonic acid dipped, water dipped, and control treatments of unpackaged cod fillets (Daniels et al., 1986).

Applications of MAP. The technology of MAP has been investigated with many food products such as red meats, poultry, seafoods, cheese, prepared salads, and pasta. Farber (1991) reviewed the microbiological aspects of modified atmosphere packaging in relation to various food products. This review examined much of the information presently existing pertaining to MAP. In summary, the main advantages of using a MAP system include extended product quality and shelf-life, increased distribution area, and reduced economic loss. The disadvantages may include added processing costs, increased temperature control, products may require different types of packaging, and MAP may provide conditions for pathogenic growth. Farber (1991) stressed the importance of future research focusing toward food safety in MAP with regard to psychrotrophic pathogens, such as *Listeria monocytogenes*, *Aeromonas hydrophila*, and *Yersinia enterocolitica*, and mesophiles such as *Salmonella* and *Staphylococcus*.

Modified atmosphere packaging of fishery products. Several studies have been conducted to investigate the use of MAP for extending the shelf life of fishery products. These studies were extensively reviewed by Reddy et al.

(1992). Overall, the quality of fresh fishery products and the shelf-life were extended with the use of CO₂-MAP and a high barrier film in conjunction with refrigerated storage. MAP successfully prolonged the lag phase of spoilage bacteria when elevated levels of CO₂ (40-100%) were used. In addition, a number of research studies on different types of salmon and trout found using MAP was effective in extending shelf life of these products (Table 2). However, Reddy et al. (1992) expressed concern that MAP may allow foodborne pathogens to reach high populations before spoilage occurs.

Many gas mixtures have been used in shelf life studies of modified atmosphere packaged fishery products. The most commonly used contain CO₂ and N₂ while others may contain small percentages of O₂. As stated previously, the CO₂ acts to inhibit aerobic microbial activity by extending the lag phase of growth. This effect may be limited by the concentration of the gas, temperature, and the type of product. Along with these beneficial aspects, there may also be some undesirable effects. The CO₂ may dissolve into the fish flesh causing a decrease in pH, therefore resulting in reduced water holding capacity and excessive drip. In addition, the dissolved CO₂ may cause changes in organoleptic characteristics or collapse of the package. The N₂ is used as a filler gas to control concentration of other gases. It has been hypothesized that O₂ may be used

Table 2. Extension of shelf-life of trout, salmon, and other species of fish by modified atmosphere packaging.

Storage Temp., °C	Type of Fish	Atmosphere Tested	Conclusion	Source
4.0	Speckled trout Golden croaker	CO ₂ (100%)	CO ₂ atmosphere supported lactic growth while inhibiting spoilage bacteria.	Banks et al., 1980
1.7	Rainbow trout	CO ₂ :N ₂ (80:20)	Combination of refrigeration and MAP doubled shelf life of rainbow trout.	Barnett et al., 1987
4.5	Rockfish Silver Salmon	CO ₂ :air (20:80) and (40:60)	20% CO ₂ was not sufficient to inhibit growth of spoilage bacteria. Higher levels of CO ₂ provide longer shelf life.	Brown et al., 1980
1.1, 10	Perch Seatrout Croaker Bluefish	in ³ CO ₂ :1b fish (10:1) (20:1)	CO ₂ -MAP treatment extended lag phase of spoilage bacteria. MAP cannot be used to salvage an inferior product.	Gray et al., 1983
3.0	Atlantic Salmon	CO ₂ (100%) atm. pressure and 2.02 kPa	Although both conditions were effective in extending shelf life, the reduced pressure was more effective.	Haard and Lee, 1982
1.0	Ocean perch Sea trout	CO ₂ :N ₂ (80:20)	Observed extension of lag phase of spoilage bacteria.	Richter and Banwart, 1983

to retard the growth of anaerobic spoilage bacteria, although there is little evidence supporting this (Reddy et al., 1992). Various gas mixtures are recommended for different species of fish. For example, Cann et al. (1983), has recommended a gas mixture of CO₂:N₂ (60:40) for MAP of salmonoids and fatty fish such as herring or mackerel.

Salmonella

Microbiological characteristics. The organisms within the genus *Salmonella* are gram-negative, flagellated, facultative, nonsporeforming rods, which most commonly cause salmonellosis (acute enteritis) when significant numbers of appropriate strains are ingested. The genus *Salmonella* consists of approximately 2,200 serotypes which are pathogenic to humans, animals, or both. Salmonellae are classified into different serotypes on the basis of the antigens present on the cell. The optimum pH for growth is around neutrality (7.0), with a range of growth extending from a pH of 4.5 to 9.0. Although salmonellae grow best at temperatures from 32-35°C, growth has been reported as low as 5.3°C for certain strains and as high as 45°C (Jay, 1992).

Salmonella as a pathogen in fishery products.

Although salmonellae are not endemic to the intestinal tract of fish, crustaceans, or mollusks, these products may come in contact with salmonellae through fecal

contamination and processing. The primary habitat of *Salmonella* spp. is the intestinal tract of mammals, birds, amphibians, and reptiles; therefore, fecal contamination of aquaculture systems is highly possible from farm runoff, livestock, or wild and domestic birds (Kvenberg, 1991). One survey conducted on pond reared catfish in nine southeastern states indicated an overall incidence of *Salmonella* at 5% (Andrews et al., 1977). Another survey conducted by Wyatt et al. (1979) yielded an 8.4% occurrence of *Salmonella* in retail samples. Lewis (1975) investigated the retention of *Salmonella* by fresh-water and marine species of fish and found they may remain positive for 30 days after exposure to *Salmonella* in polluted waters. In the period from 1973-1987, the incidence of salmonellosis contracted from fishery products reported to the CDC included four outbreaks and 96 cases from finfish and four outbreaks and 105 cases from shellfish (Kvenberg, 1991).

Growth of *Salmonella* in MAP. A modified atmosphere packaged fish does provide an environment that is potentially capable of supporting growth of *Salmonella* if held at appropriate temperatures (Table 3). Silliker and Wolfe (1980) investigated the growth of *Salmonella* in MAP ground beef with a gas mixture of CO₂:O₂:N₂ (60:25:15). After 10 days at 10°C, the numbers of *Salmonella* in the air packs had increased 3 logs, whereas the MAP samples

Table 3. Growth requirements for *Salmonella* and conditions of MAP-fish.

	<i>Salmonella</i>	MAP-fish
O ₂ requirements	facultative anaerobe ^a	anaerobic conditions ^b
pH range	4.5-9.0 ^a	6.6-6.8 ^a
Temperature	5.3-45°C ^a	Storage temp.
Water activity	minimum 0.94 ^a	0.99 ^a

Sources: ^a Jay, 1992

^b Reddy et al., 1992

supported only a 0.5 log increase. In the samples held at 20°C, *Salmonella* grew equally well in the air packs as in the MAP samples. *Salmonella typhimurium* was unable to grow on beef steaks stored at 10°C under an atmosphere of CO₂:O₂(60:40) during a 9-day storage period; however, *Salmonella* numbers increased approximately 3 logs in air packs stored under similar conditions (Luiten et al., 1982). Baker et al. (1986) observed that a CO₂:air (80:20) mixture inhibited the growth of *Salmonella* in a meat culture and trypticase soy broth (TSB) at 2, 7, and 13°C when compared to air samples. The growth of *Salmonella* was inhibited on roast beef held under modified atmospheres containing CO₂:O₂:N₂ (75:various:balance) at 4°C, although *Salmonella* was able to recover and grow when samples were transferred to 12.8°C (Hintlian and Hotchkiss, 1987).

Enrichment and isolation methods for *Salmonella*. The methods for the isolation of *Salmonella* from foods are described in the "Bacteriological Analytical Manual" (Andrews et al., 1984), "Official Methods of Analysis" (AOAC, 1990), and in the "Compendium of Methods for the Microbiological Examination of Foods" (Flowers et al., 1992). The traditional culture method for the detection of *Salmonella* in foods consists of a pre-enrichment, enrichment, selective plating, biochemical tests, and serological confirmation, while rapid methods utilize ELISA

techniques (Van Poucke, 1990) and DNA probe technology (Fitts et al., 1983).

Developments in the use of the cultural methods include the use of an elevated temperature (41-43°C) for incubation of enrichment broth (Georgala and Boothroyd, 1965; Silliker and Gabis, 1974) and the addition of novobiocin to plating media (Tate et al., 1990) to enhance detection and recovery of *Salmonella* from foods. In addition, a new selective plating media, xylose lysine tergitol-4, increased recovery of *Salmonella* from poultry samples (Miller et al., 1991). James and Iyer (1972) investigated the recovery of *Salmonella* from seafoods and found dulcitol selenite and selenite cystine to be equally efficient as enrichments, while xyloselysine deoxycholate agar, brilliant green sulphadiazine agar, and brilliant green agar yielded better recovery of *Salmonella* than *Salmonella-Shigella* agar, although their study did not include all *Salmonella* selective media currently available.

Chapter III

MATERIALS AND METHODS

Trout samples and packaging

Rainbow trout were purchased as fillets from the Cross-Eyed Cricket Trout and Catfish farm (Lenoir City, TN). The fish were packed in ice during transport and held on ice within a 4°C storage room until time of packaging (approximately 18 hr). Prior to packaging, styrofoam trays (Linpac, Wilson, NC) with a rippled bottom were vacuum packaged to remove the majority of oxygen dissolved in the styrofoam. The trout were divided into two groups. The first group of fillets was soaked in carbonic acid (dry ice and distilled water, pH 4.6) for approximately 5 min. and allowed to drain approximately 30 sec. This group was further subdivided so that half were inoculated with *Salmonella*. The second group was rinsed in distilled ice water and further subdivided so that half were also inoculated with *Salmonella*.

Each fillet was placed on a styrofoam tray and vacuum packaged in a barrier film bag (BDF-2001 film, oxygen transmission rate 5.0 cc/m²/24hr., Cryovac, Duncan, SC). The atmospheres of air or CO₂:N₂ (60:40) were each injected into one-third of the packages through a syringe inserted in an adhesive tape septum, (Modern Control, Inc., Minneapolis, MN). The packages were distributed into three groups:

vacuum, air, and CO₂:N₂ (60:40). Three replications were performed following this packaging procedure and samples were held at 3°C with an additional three replications held at 10°C.

Salmonella cultures

All media used throughout this study was purchased from Difco Laboratories (Detroit, MI) unless otherwise stated. Three cultures of *Salmonella* (*S. typhimurium*, *S. enteritidis*, and *S. newport*) were purchased from the American Type Culture Collection (ATCC; Rockville, MD) and maintained on tryptic soy agar slants held at 4°C. A "cocktail" mixture was prepared for inoculation from a 24 h culture grown in tryptic soy broth. The combined culture was centrifuged at 5000 RPM for 10 min. at 4°C in a Biofuge 17R refrigerated centrifuge (Baxter Sci., Atlanta, GA.). The resulting pellet was resuspended in sterile Butterfield's diluent to yield approximately log 6 *Salmonella* CFU/mL. This inoculum level yielded log 4 *Salmonella* CFU/g fish considering the average weight of a rainbow trout fillet being approximately 100 g (weight of fish fillet, mean = 97.30 g ± 11.67 g, n = 26). One mL of the inoculum was spread over the flesh side of the fillet.

Monitoring of samples

Twelve packages were tested at each sampling day, consisting of an uninoculated and inoculated, dipped or not dipped fish packaged under each of the three atmospheric

treatments. Samples were tested every other day at 10°C and every three days at 3°C, with the sampling period extending until the fish reached spoilage (aerobic plate count reached log 8 CFU/g) (Stier et al., 1981). In addition, the pH of the uninoculated samples was recorded at each sampling day.

Microbial analysis

To obtain the microbial counts, approximately 20 to 25 g of the trout sample was added to Butterfield's diluent to yield a 1:10 dilution and mixed for 2 min. in a Stomacher Lab Blender, Model 400 (Steward; London, England). Serial dilutions were then performed to monitor aerobic plate counts using tryptic soy agar incubated at 32°C for 48 h, psychrotrophic counts using tryptic soy agar incubated at 7°C for 5 d, lactic acid bacteria counts using MRS lactobacilli agar (Becton Dickson Microbiology Systems, Cockeysville, MD) incubated at 32°C for 48 h, and coliform counts using violet red bile agar incubated at 32°C for 24h.

Salmonella detection and enumeration

Salmonella enumeration for inoculated and control samples was performed by directly plating the appropriate dilution from Butterfield's diluent onto XLT4 agar (Miller et al., 1991) and incubated at 35°C for 24 h. *Salmonella* detection for inoculated and control samples was performed by stomaching 25 g of the sample with 250 mL tetrathionate broth followed by incubation at 42°C for 24 h. The enrichment broth was then streaked for isolation on XLT4

with incubation at 35°C for 24 h. Presumptive *Salmonella* isolates were tested biochemically with triple sugar iron agar, lysine iron agar, urea agar, and serologically confirmed by agglutination with polyvalent 'O' antiserum (Becton Dickinson Microbiology Systems, Cockeysville, MD).

Gas Analysis

The headspace gases of packages containing uninoculated air and CO₂:N (60:40%) atmospheres were analyzed on each sampling day. The headspace gas analysis of vacuum packages was not performed. A 5 mL sample of the headspace gas was withdrawn under water using a 5 cc syringe. The headspace gas sample was then injected into a Hewlett Packard Model 5890A Series II Gas Chromatograph equipped with a TCD at 25°C. The gases were separated using a stainless steel CTRI column (Alltech Associates, Inc., Deerfield, IL) at a oven temperature of 25°C. Helium was used as the carrier gas at a flow rate of 40 mL/min. The percentages of oxygen, carbon dioxide, and nitrogen were determined from a standard curve obtained using Scotty I analyzed calibration gases.

Statistical Analysis

The microbial data were averaged over the three replications at each temperature to obtain mean scores and standard deviations. The General Linear Models procedure of SAS (1990) was used to analyze the main effects of atmosphere [vacuum, air, CO₂:N (60:40)], carbonic acid dip, storage time, storage temperature, and interactions. Mean

separations for the data were performed using the Student-Newman-Keuls test of SAS with a significance level of $p \leq 0.05$.

Chapter IV

RESULTS AND DISCUSSION

Microbiological measurements

Mesophilic aerobic bacteria. The effect of atmosphere on APC was found to be significant ($p < 0.05$) at 10 and 3°C (Tables A1 and A10). The mean APC for trout stored in air, vacuum, and the CO₂ mix packages was 7.3, 6.4, and 5.9 log CFU/g at 10°C and 6.1, 5.5, and 4.0 log CFU/g at 3°C, respectively (Tables A8 and A17). The mean APC values of each atmosphere were significantly different ($p < 0.05$) within each temperature.

The initial aerobic plate count (APC) on the rainbow trout fillets was between log 3 and log 4 CFU/g fish (Figures 1 and 2). During storage of samples held at 10°C, the APC increased to approximately log 8 CFU/g (spoilage reference point) in 3 days for air packages, 4 days for vacuum packages, and 6 days for gas mix CO₂:N₂ (60:40) packages. At 10°C the vacuum packaging delayed spoilage 1 day whereas the CO₂-MAP delayed spoilage 3 days. In the samples held at 3°C, the APC reached approximately log 8 CFU/g in 9 days for air packages, 12 days for vacuum packages, and 18 days for the CO₂ mix packages. At 3°C the vacuum packaging delayed spoilage 3 days and the CO₂-MAP packaging delayed spoilage 9 days (Figures 1 and 2). The bacteriostatic effect of the CO₂-modified atmosphere on APC

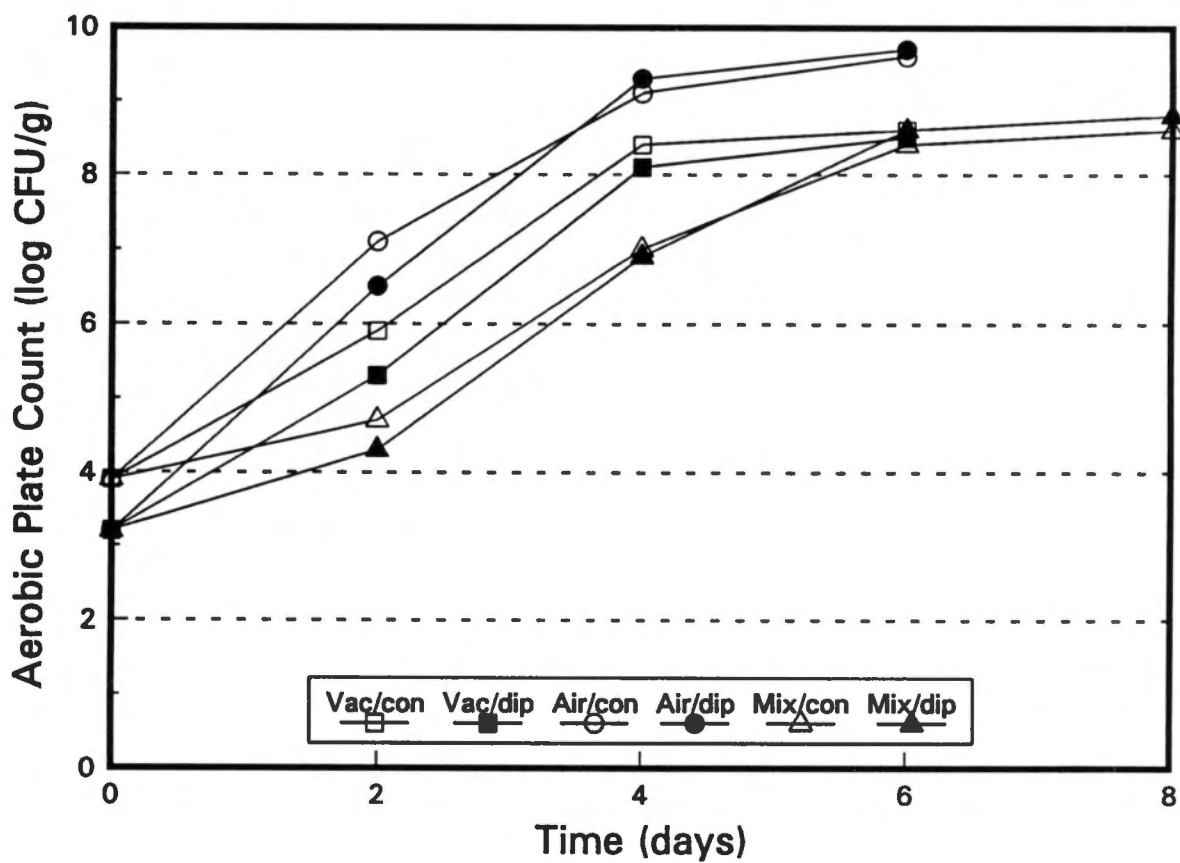


Figure 1. Growth of mesophilic aerobic bacteria in rainbow trout fillets either dipped in carbonic acid or icewater and held at 10°C under vacuum, air, or a gas mixture of CO₂:N₂ (60:40).

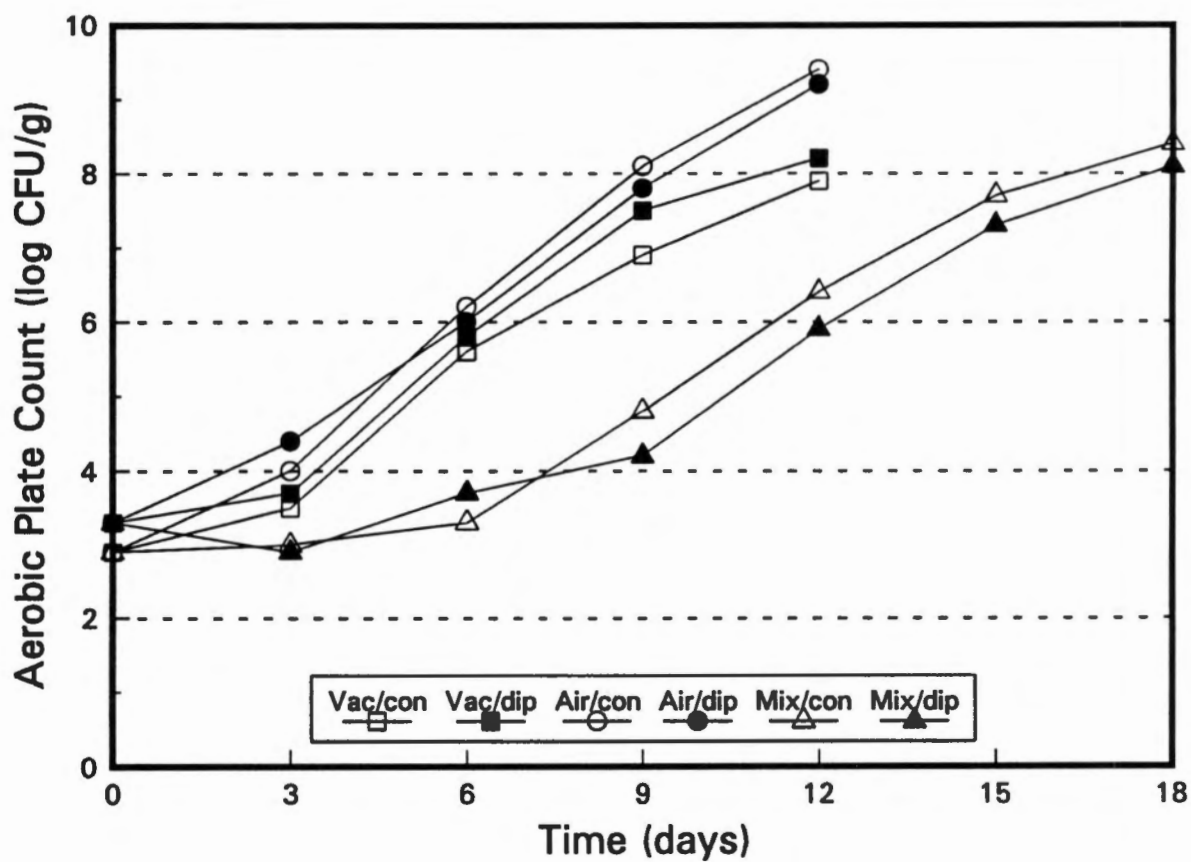


Figure 2. Growth of mesophilic aerobic bacteria in rainbow trout fillets either dipped in carbonic acid or icewater and held at 3°C under vacuum, air, or a gas mixture of CO₂:N₂ (60:40).

was evident during the first 1-2 days of storage at 10°C and during the first 6 days of storage at 3°C (Figures 1 and 2). This bacteriostatic effect can be attributed to the action of CO₂ since the vacuum packages were also low in O₂. These results indicate the importance of strict temperature control in combination with a modified atmosphere package.

The carbonic acid dip had a significant effect on APC at 10°C ($p < 0.05$) (Table A1). This is primarily due to reduced counts on day 0 which resulted in lower counts during the first 2 days of storage (Figure 1). In contrast, the carbonic acid dip did not have a significant effect upon APC at 3°C ($p > 0.05$) (Table A10).

Psychrotrophic bacteria. The growth of psychrotrophic organisms in rainbow trout fillets held at 10 and 3°C closely resembled that of the aerobic bacteria held under the same conditions (Figures 1-4), suggesting the aerobic population consisted primarily of psychrotrophs. Therefore, the atmosphere within the package had the same significant effects on psychrotrophic plate counts (PPC) at both temperatures ($p < 0.05$) (Tables A2 and A11). The initial PPC ranged between log 2 and 3 CFU/g but reached levels approximately equal to APC by day 2 at 10°C and day 3 at 3°C (Figures 3 and 4). The carbonic acid dip had no significant effect on PPC ($p > 0.05$) at either 10 or 3°C (Tables A2 and A11).

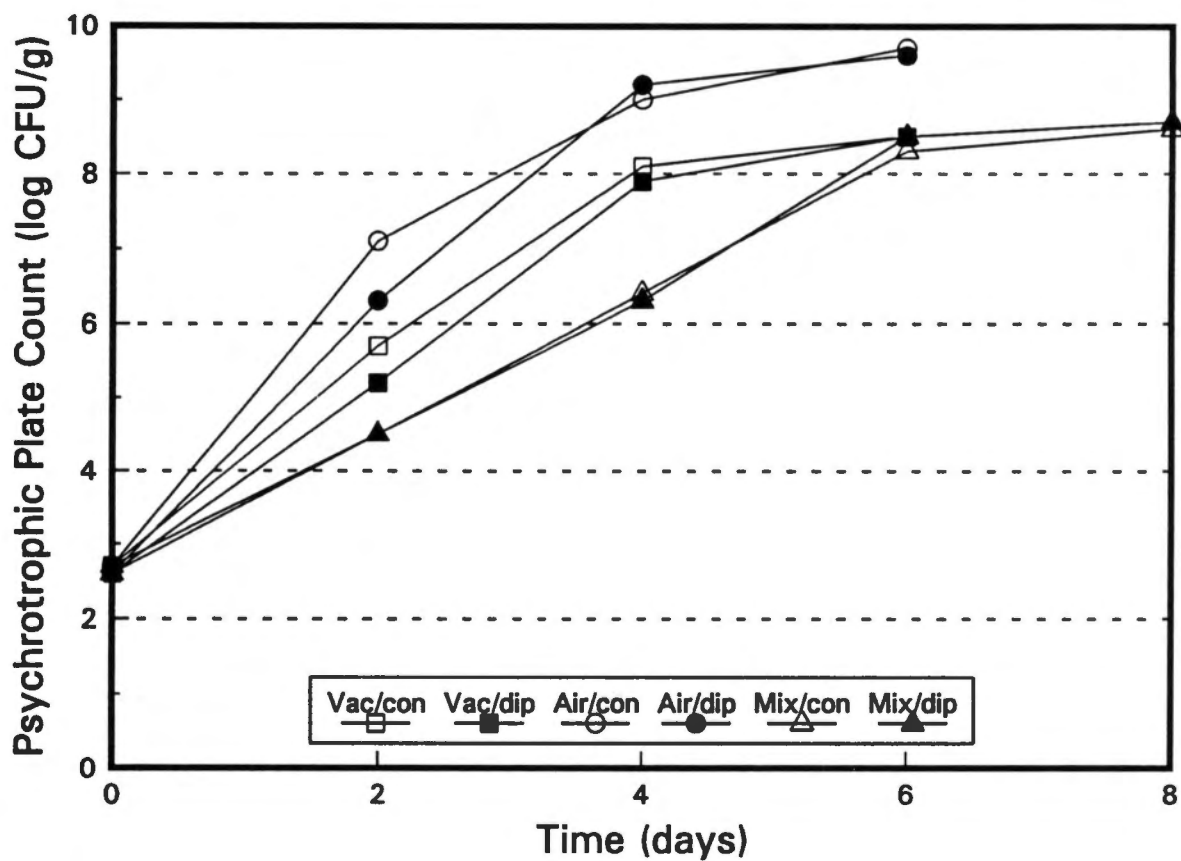


Figure 3. Growth of psychrotrophic bacteria in rainbow trout fillets either dipped in carbonic acid or icewater and held at 10°C under vacuum, air, or a gas mixture of CO₂:N₂ (60:40).

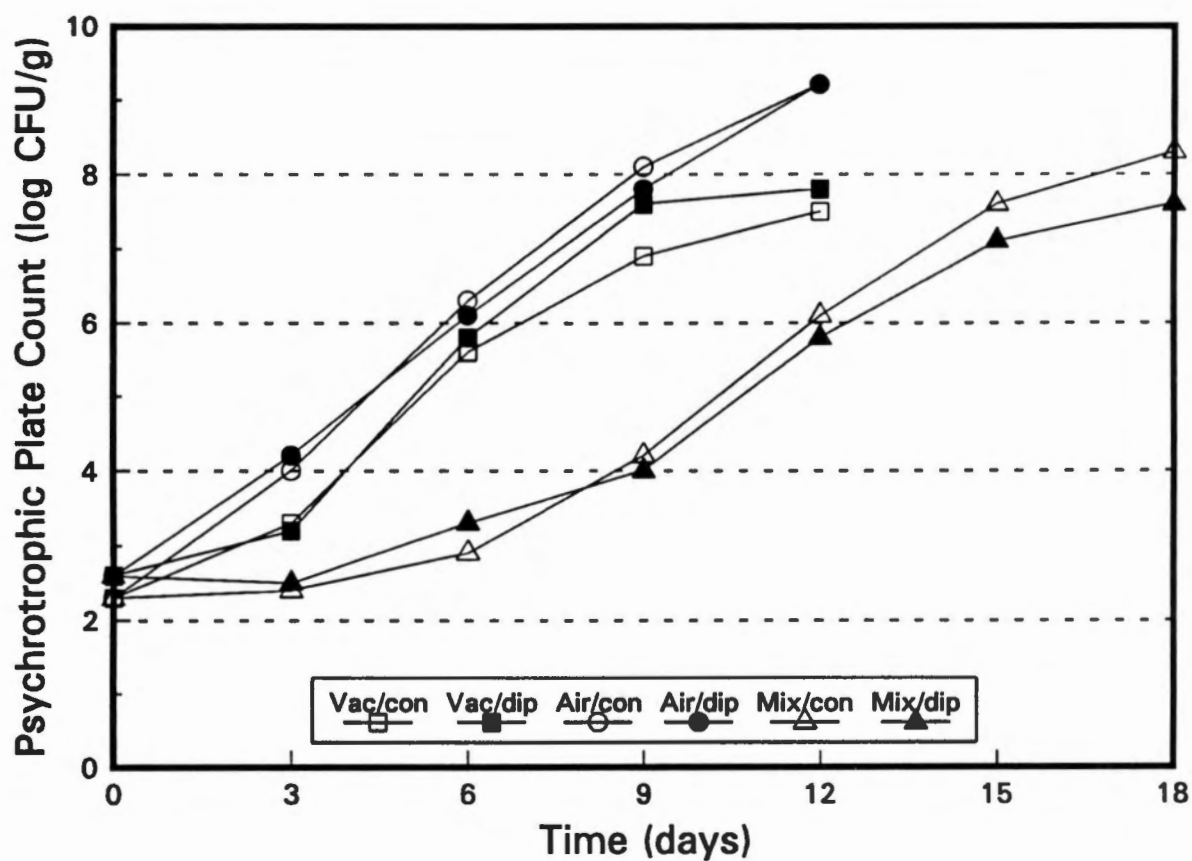


Figure 4. Growth of psychrotrophic bacteria in rainbow trout fillets either dipped in carbonic acid or icewater and held at 3°C under vacuum, air, or a gas mixture of CO₂:N₂ (60:40).

Coliform bacteria. The atmosphere of the package had a significant effect upon the growth of coliforms in trout samples at 10 and 3°C ($p < 0.05$) (Tables A3 and A12). The mean coliform counts of each atmosphere were significantly different ($p < 0.05$) (Tables A8 and A17) with the CO₂ mix gas having the lowest mean coliform count and air packages having the highest mean coliform count (Figures 5 and 6).

The initial levels of coliform bacteria ranged between log 1 and 2.5 CFU/g in the rainbow trout fillets and increased over storage time at 10 and 3°C (Figures 5 and 6). Coliform levels reached log 8.1, 7.7, and 5.0 CFU/g by day 4 at 10°C in undipped fish held under air, vacuum, and CO₂ gas packaging, respectively (Figure 5). Coliform levels in undipped fish stored at 3°C reached log 7.4, 5.6, and 2.8 CFU/g by day 12 in air, vacuum, and CO₂ gas packaging, respectively (Figure 6). The bacteriostatic effect of the CO₂ gas mixture upon coliform bacteria was evident during the first two days of storage at 10°C, whereas the same effect was evident during the first nine days of storage at 3°C (Figures 5 and 6). The carbonic acid dip had a significant effect upon the growth of coliform bacteria at 10°C ($p < 0.05$) (Table A3), although this effect was more evident during the first two days of storage (Figure 5). In contrast, the carbonic acid dip had no significant effect upon the growth of coliform bacteria at 3°C ($p > 0.05$) (Table A12).

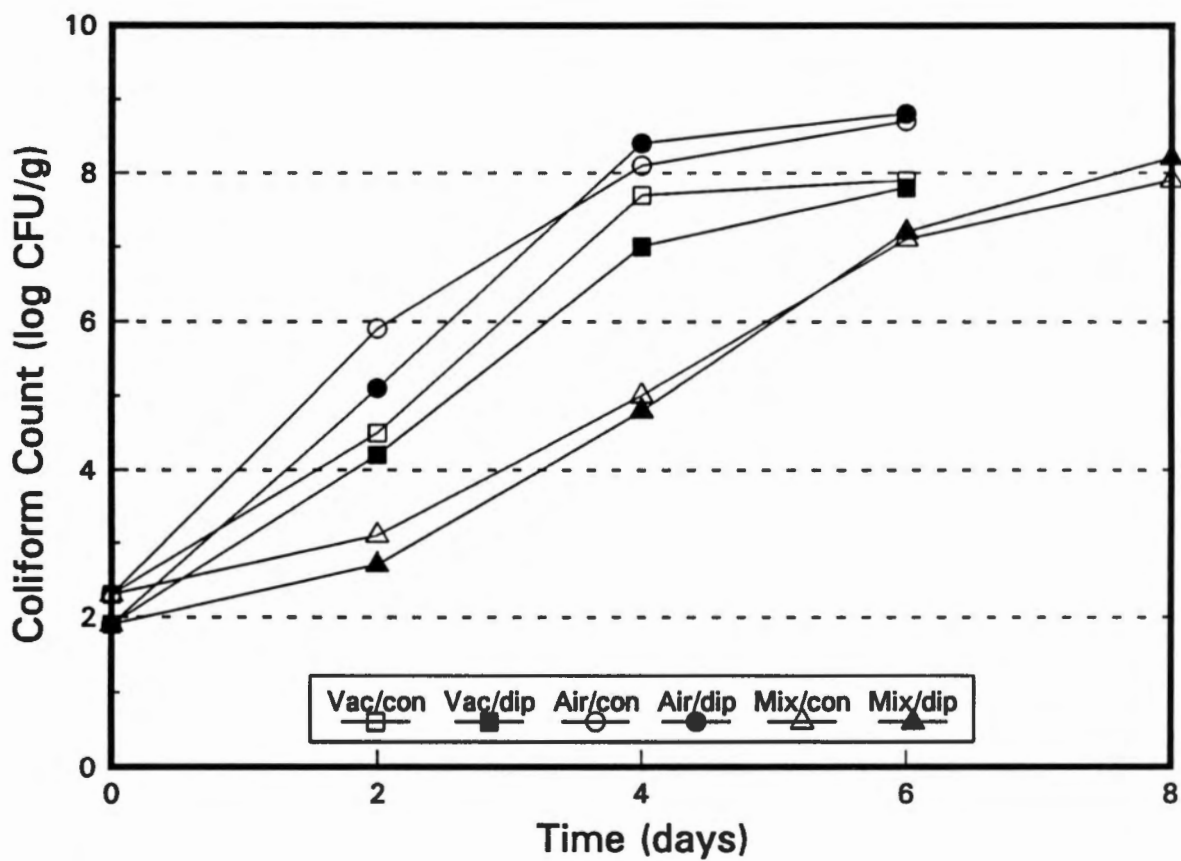


Figure 5. Growth of coliforms in rainbow trout fillets either dipped in carbonic acid or icewater and held at 10°C under vacuum, air, or a gas mixture of CO₂:N₂ (60:40).

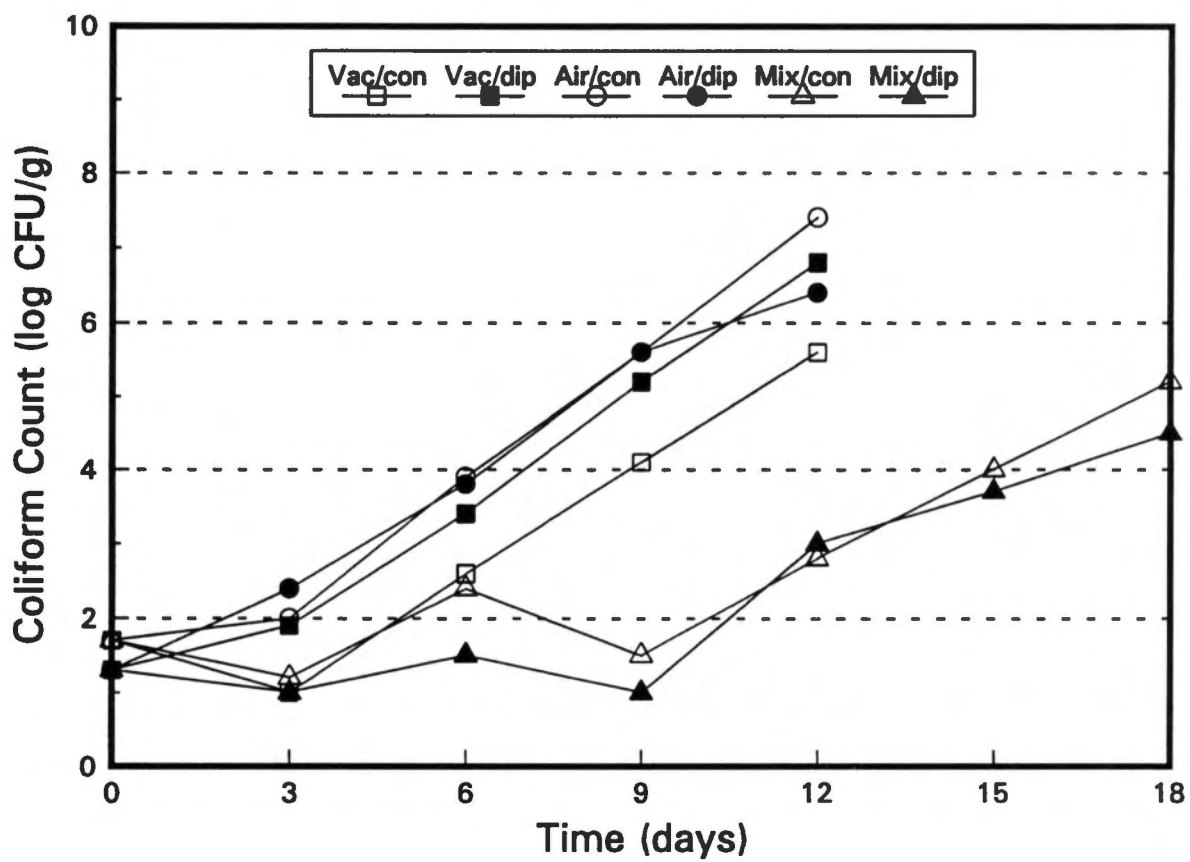


Figure 6. Growth of coliforms in rainbow trout fillets either dipped in carbonic acid or icewater and held at 3°C under vacuum, air, or a gas mixture of CO₂:N₂ (60:40).

Lactic acid bacteria. Atmosphere had a significant effect on the growth of lactic acid bacteria at 10 and 3°C ($p < 0.05$) (Tables A4 and A13). The mean lactic counts for fish packaged under air and vacuum were significantly higher ($p < 0.05$) than the mean lactic counts for fish packaged under the CO₂ gas mixture (Tables A8 and A17). The lactic acid bacterial count on the rainbow trout fillets initially ranged from log 1.5 to 2.5 CFU/g. At 10°C, the level of lactic acid bacteria in the trout samples reached approximately log 8 CFU/g by day 6 in all treatments. At 3°C the number of lactic acid bacteria in undipped trout packaged under air, vacuum, and CO₂ gas reached approximately log 6.0 CFU/g by day 12 (Figures 7 and 8). The bacteriostatic effect of the CO₂ was evident during the first day of storage at 10°C and during the first 3 days at 3°C. This was expected since lactic acid bacteria are more resistant to CO₂ than gram-negatives (Jay, 1992). The carbonic acid dip had no significant effect upon the growth of lactic acid bacteria in the trout at 10°C ($p > 0.10$) (Table A4). In contrast, it did have a significant effect at 3°C ($p < 0.10$) with lactic counts higher in fish that had been dipped in carbonic acid. This may be attributed to the higher counts on day 0 that persisted throughout storage (Figure 8).

Salmonella. *Salmonella* was not detected in any of the uninoculated rainbow trout fillets by direct plating or

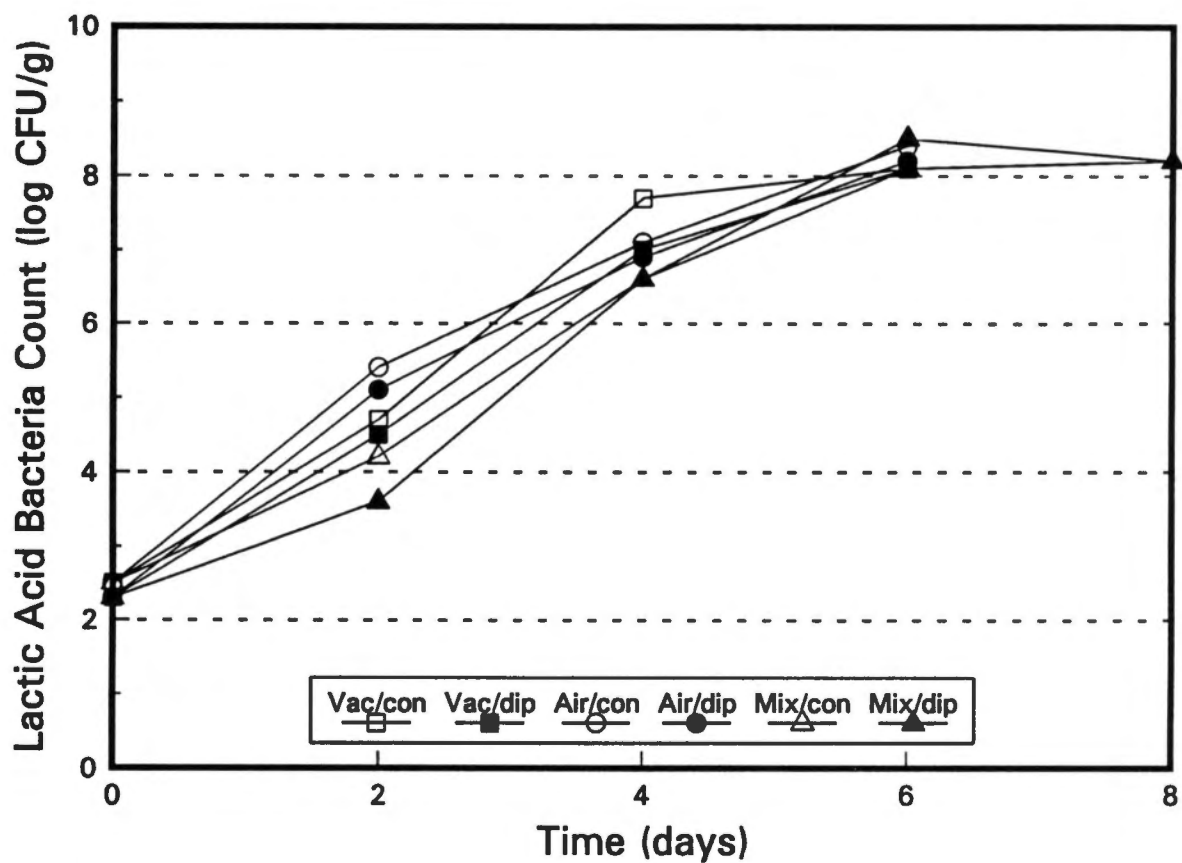


Figure 7. Growth of lactic acid bacteria in rainbow trout fillets either dipped in carbonic acid or icewater and held at 10°C under vacuum, air, or a gas mixture of CO₂:N₂ (60:40).

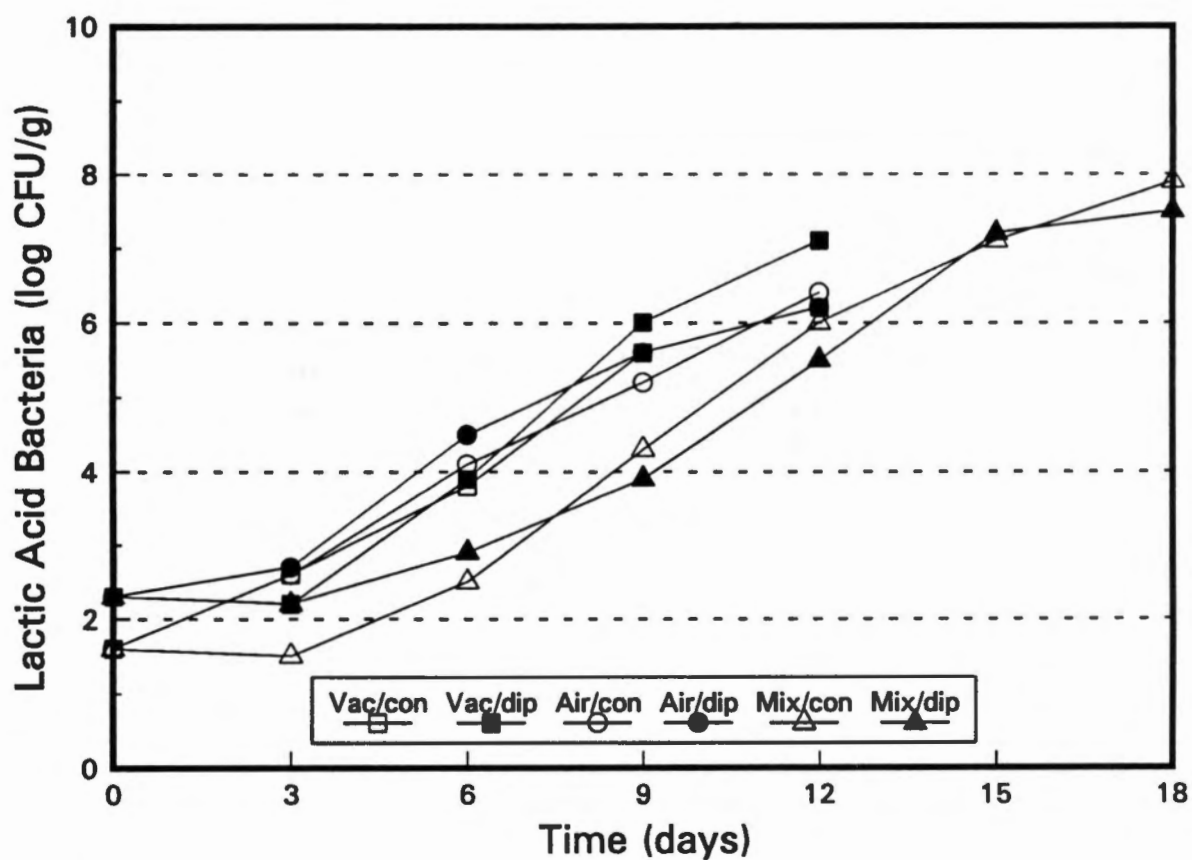


Figure 8. Growth of lactic acid bacteria in rainbow trout fillets either dipped in carbonic acid or icewater and held at 3°C under vacuum, air, or a gas mixture of CO₂:N₂ (60:40).

enrichment. Initial detection of *Salmonella* by direct plating of inoculated samples ranged from log 3.0 to 3.5 CFU/g, although the inoculum level was approximately log 4.0 CFU *Salmonella*/g rainbow trout fillet (Figures 9 and 10). *Salmonella* numbers increased slightly over time in packages held at 10°C, ranging between log 5.0 and 6.0 CFU/g for samples packaged under vacuum and air, and log 4.0 CFU/g in packages held under the CO₂ gas mixture by day 6 (Figure 9). *Salmonella* numbers declined approximately 1.5 log cycles during storage at 3°C (Figure 10).

The type of atmosphere did have a significant effect on the growth of *Salmonella* ($p < 0.05$) at 10 and 3°C (Tables A5 and A14). The mean *Salmonella* counts in air and vacuum packages (log 4.2 and 4.0 CFU/g) were significantly higher ($p < 0.05$) than *Salmonella* counts in the CO₂ gas packages (log 3.3 CFU/g) held at 10°C (Table A8). In samples held at 3°C, mean *Salmonella* counts were significantly lower ($p < 0.05$) in vacuum and CO₂ gas packages (log 2.6 and 2.7 CFU/g) than in air packages (log 2.9 CFU/g) (Table A17). These results indicated that a gas mixture of CO₂:N₂ (60:40) did not enhance the growth of *Salmonella* in rainbow trout at 10 or 3°C when compared to spoilage bacteria (Figures 1-6), although concern has been expressed that mixed gases containing CO₂ may favor the growth of *Salmonella* since it is a facultative anaerobe.

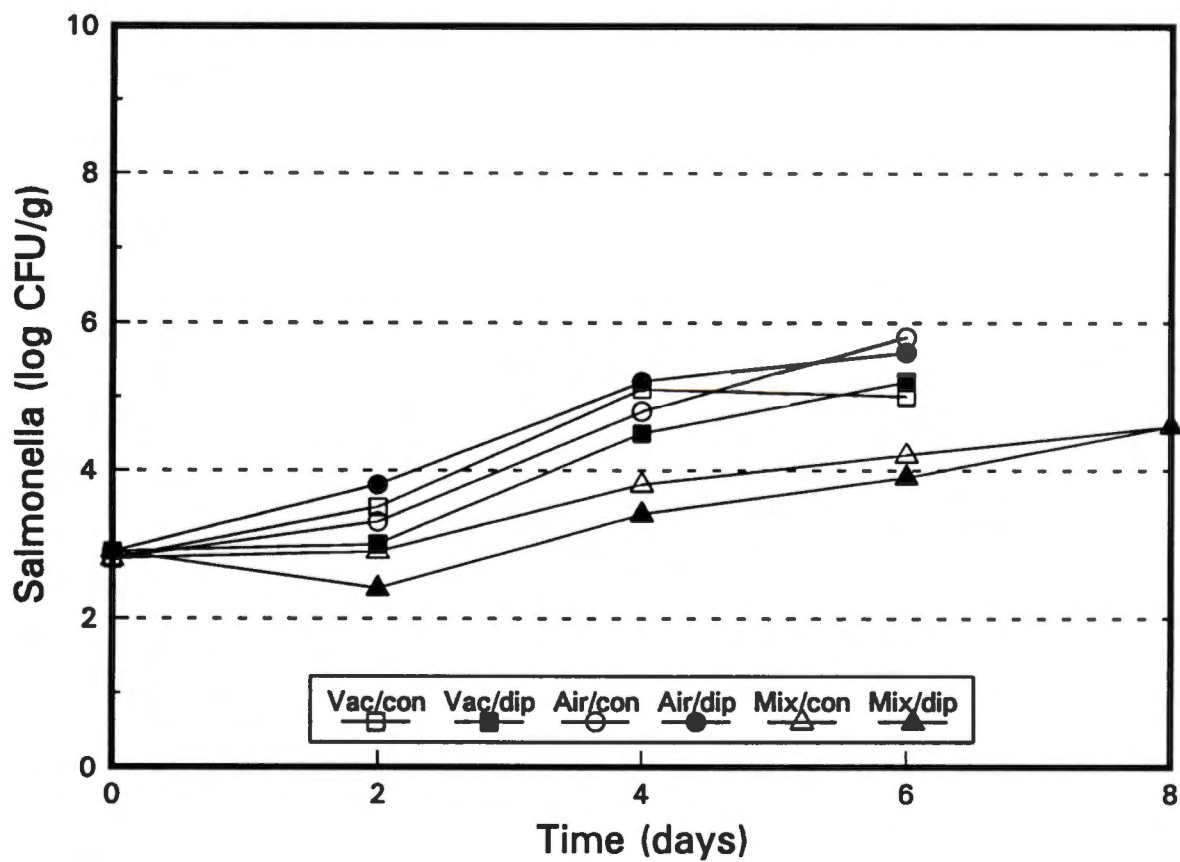


Figure 9. Survival of *Salmonella* in rainbow trout fillets either dipped in carbonic acid or icewater and held at 10°C under vacuum, air, or a gas mixture of CO₂:N₂ (60:40).

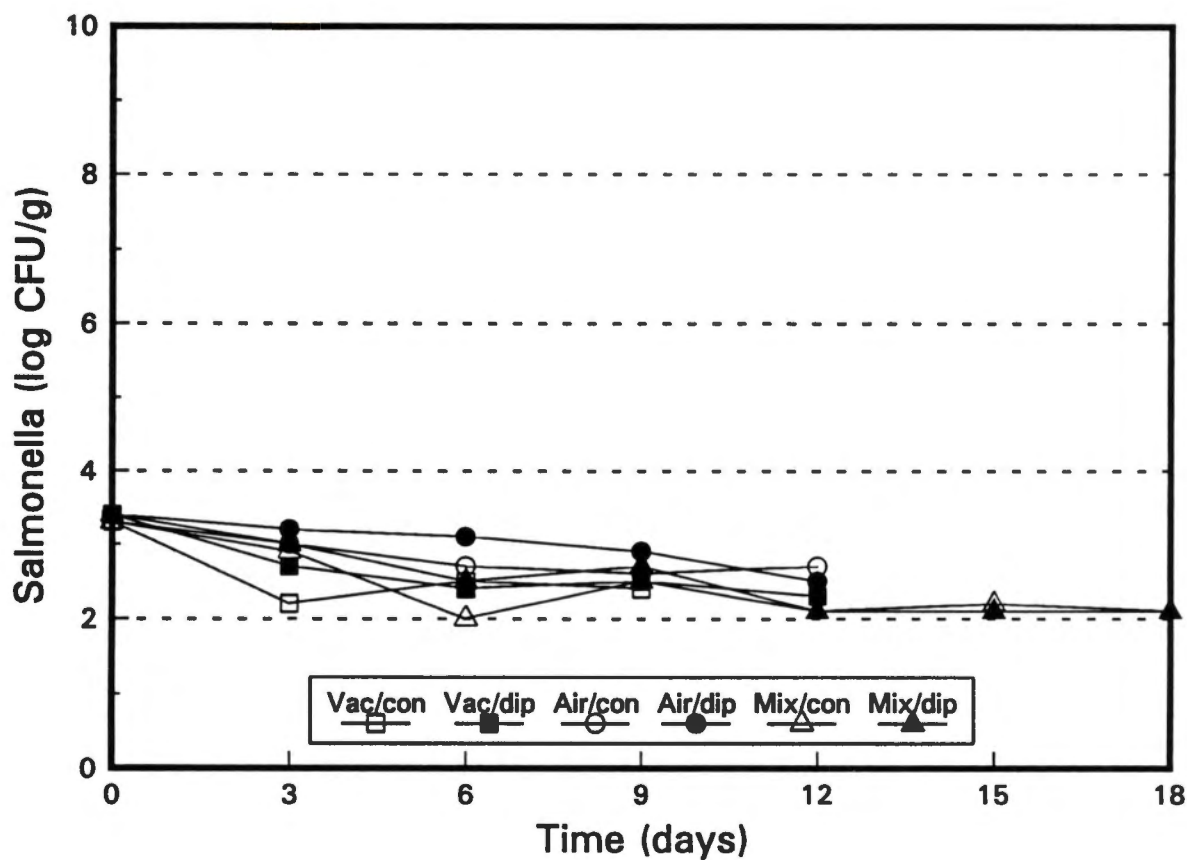


Figure 10. Survival of *Salmonella* in rainbow trout fillets either dipped in carbonic acid or icewater and held at 3°C under vacuum, air, or a gas mixture of CO₂:N₂ (60:40).

The carbonic acid dip had a significant effect upon the growth of *Salmonella* in trout samples at 3°C ($p < 0.05$) (Table A14), although no effect was evident ($p > 0.05$) at 10°C (Table A5). *Salmonella* did not have a significant effect ($p > 0.05$) upon the growth of other microflora on the rainbow trout fillets at 10 or 3°C (Tables A9 and A18). Therefore, the aerobic, coliform, psychrotrophic, and lactic counts reflect only values of uninoculated samples.

Carbonic acid dip

The carbonic acid dip was found to be effective in retarding the initial growth of aerobic bacteria ($p < 0.05$) (Table A1) and coliforms ($p < 0.05$) (Table A3) at the 10°C storage temperature. The effect of the carbonic acid dip at 10°C was seen primarily during the first two days of storage, suggesting an initial bacteriostatic effect (Figures 1 and 5). The initial bacteriostatic effect supports the theory that carbon dioxide may form carbonic acid on the tissue surface to exhibit a bacteriostatic effect. Although in this case, the carbonic acid dip did not have a significant effect on pH ($p < 0.05$) (Table A6) and an extension of shelf life was not evident. These results do not agree with those found by Daniels et al. (1986), where a carbonic acid dip was comparable to a CO₂ modified atmosphere and extended shelf life of unpackaged cod fillets between 7 and 21 days with sensory evaluations being fair to marginal.

pH measurements

Initial measurements of the pH of the rainbow trout fillets ranged between 6.4 and 6.5 and remained relatively constant for samples stored under air and vacuum. The pH of the samples packaged with the CO₂ gas mixture declined initially, but then remained relatively constant thereafter (Figures 11 and 12). The type of atmosphere did have a significant effect upon pH at 10 and 3°C ($p < 0.05$) (Tables A6 and A15). The pH of the fish stored under the CO₂ mix (pH = 6.26) was significantly lower ($p < 0.05$) than the pH of fish stored under air or vacuum (pH = 6.43 and 6.39) at 3°C. For packages stored at 10°C, the pH of the fish packaged under the CO₂ mix (pH = 6.25) was significantly lower ($p < 0.05$) than in air packages (pH = 6.40), with the pH of vacuum packaged fish (pH = 6.31) not significantly different ($p > 0.05$) from fish stored under air or the CO₂ mix (Table 20). Barnett et al. (1987) observed similar pH measurements on modified atmosphere packaged rainbow trout and attributed this to the effect of dissolved CO₂ absorption on the surface of the flesh of the trout.

Replication differences

There was a significant difference between replications of the coliform and lactic plate counts ($p < 0.05$) (Tables A3 and A4) at the 10°C storage temperature. In addition, significant differences were also observed among replications in all microbial counts and pH measurements at

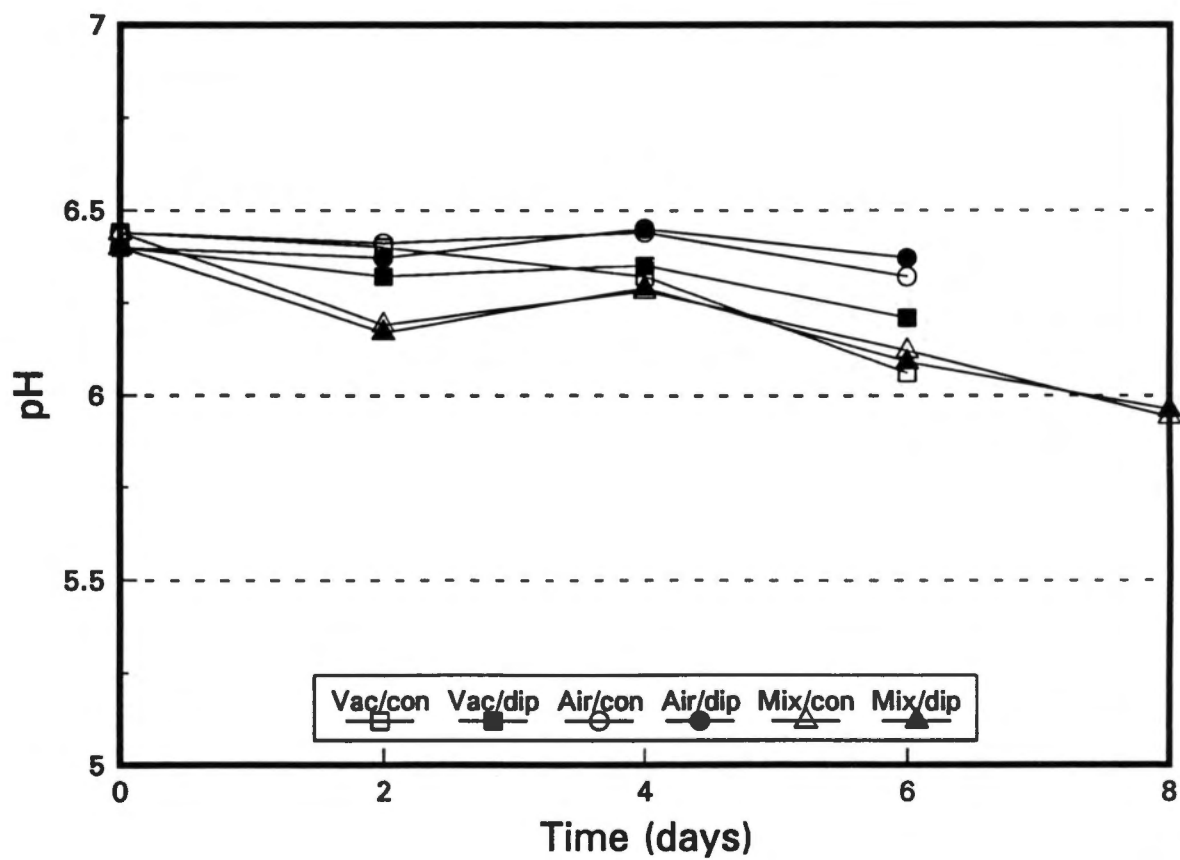


Figure 11. pH of rainbow trout fillets either dipped in carbonic acid or icewater and held at 10°C under vacuum, air, or a gas mixture of CO₂:N₂ (60:40).

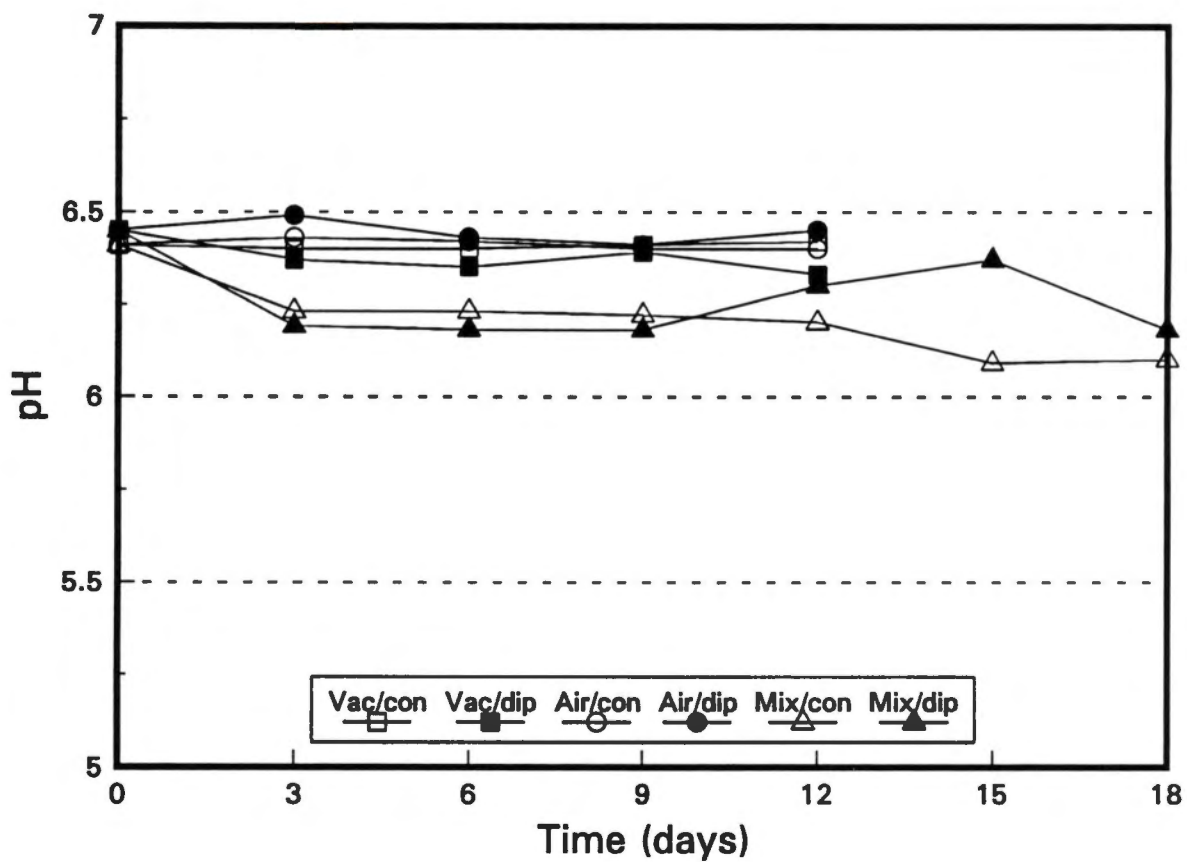


Figure 12. pH of rainbow trout fillets either dipped in carbonic acid or icewater and held at 3°C under vacuum, air, or a gas mixture of CO₂:N₂ (60:40).

the 3°C storage temperature ($p < 0.05$) (Tables 10-15). Mean separations of the microbial counts according to replication are shown in Tables A7 and A16 and pH measurements are shown in Table 19. A general pattern observed was that replications two and three were significantly different than replication one ($p < 0.05$). This may be attributed to sampling across a seasonal change, using a different group of fish for each replication, and replications two and three were closer together in time than replication one.

Headspace gas analysis

The composition of the air initially measured in the packages of rainbow trout consisted of approximately 22% O₂, 0.8% CO₂, and the balance N₂. Over storage time at 10°C, a decline in O₂ was observed along with an increase in CO₂ (Figure 13). Similar results were also observed in air packages stored at 3°C (Figure 14), although gas composition was approaching the same concentrations at day 12 as the 10°C samples were approaching at day 6. These days also corresponded to the APC reaching spoilage level at each temperature (Figures 1 and 2). These results were expected since microbial action and chemical reactions would utilize oxygen and carbon dioxide would be produced (Labuza et al., 1992).

The composition of the CO₂ gas mixture initially measured in the packages of rainbow trout fillets consisted of 57% CO₂ and 0.8% O₂, and the balance N₂. Levels of CO₂

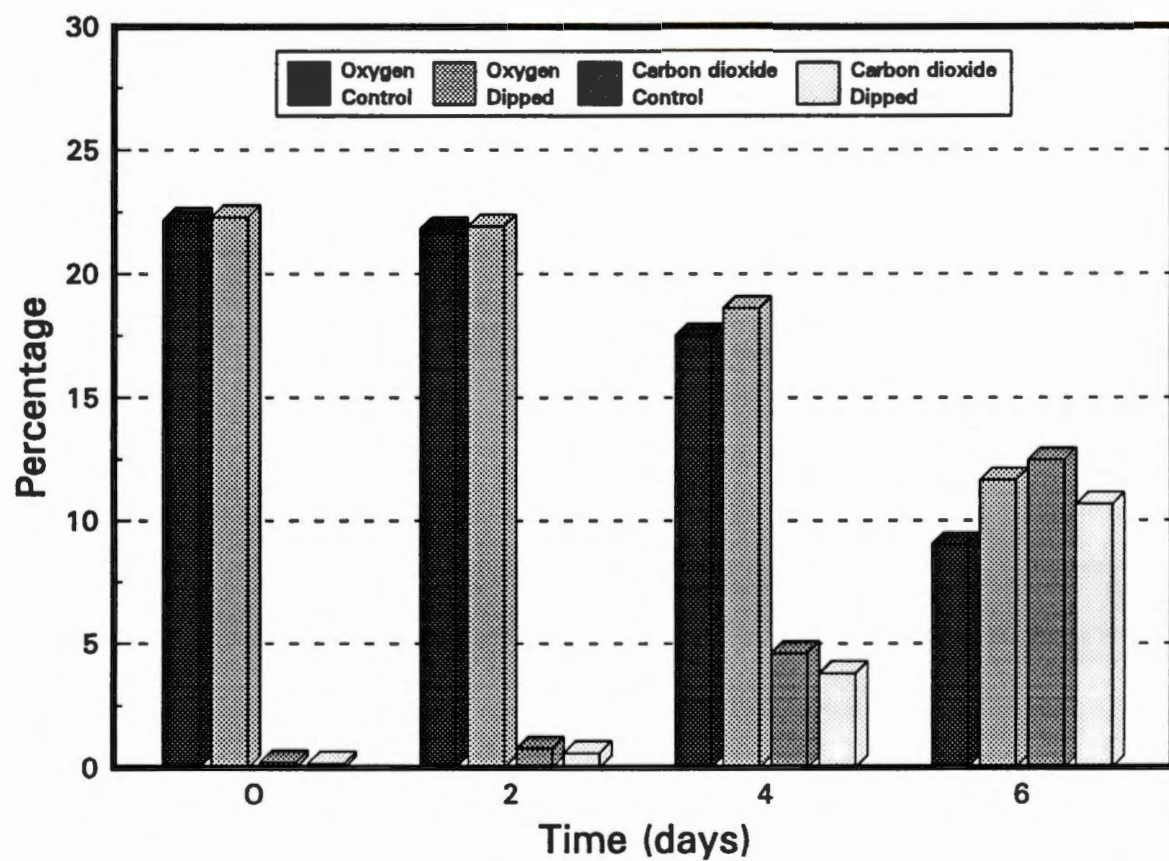


Figure 13. Changes in gas composition (%) in packages of rainbow trout fillets either dipped in carbonic acid or icewater and held at 10°C under air.

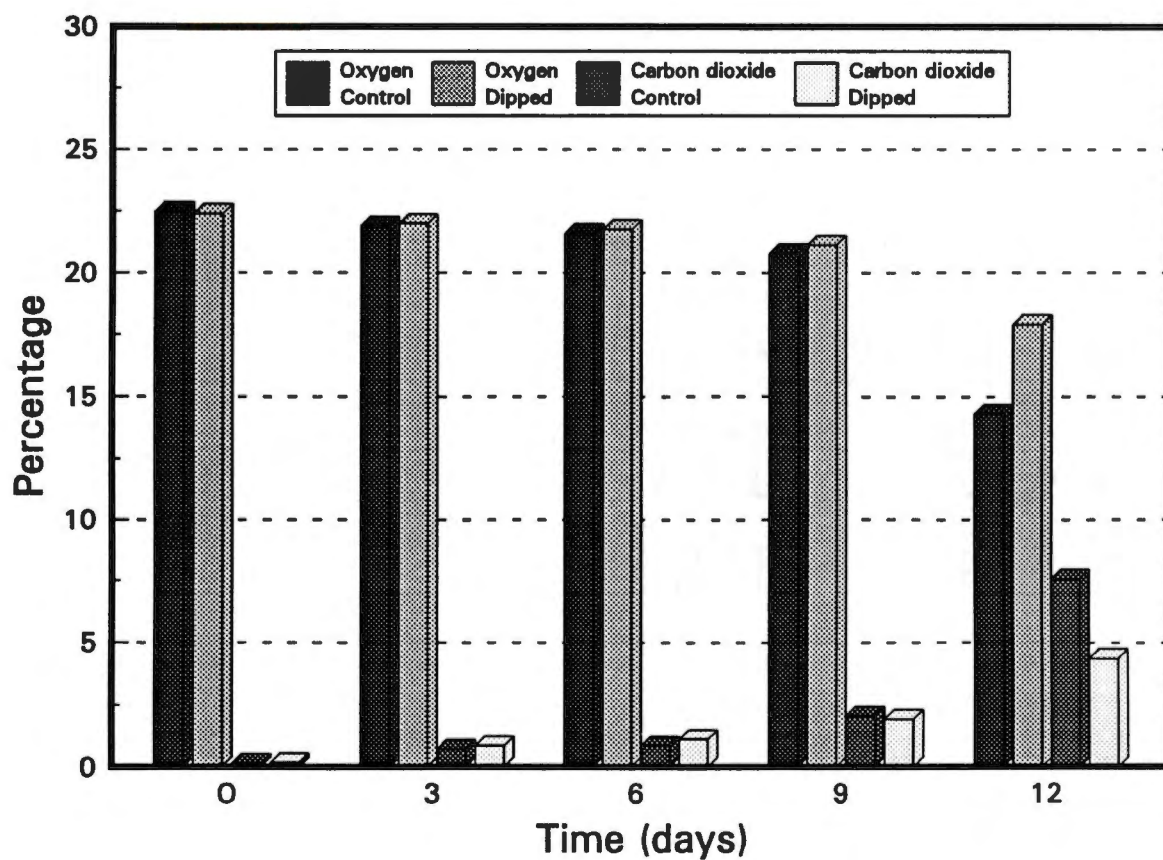


Figure 14. Changes in gas composition (%) in packages of rainbow trout fillets either dipped in carbonic acid or icewater and held at 3°C under air.

decreased slightly over time reaching 53% by day 6 at 10°C and 51% by day 12 at 3°C with O₂ levels initially increasing slightly, but remaining relatively constant thereafter (Figures 15 and 16). The slight decrease (4-6%) in CO₂ may be attributed to absorption in the fish flesh and the tray and the increase in O₂ may be attributed to oxygen in the fish flesh and the tray dissolved into the atmosphere of the package. The headspace gas analysis of the vacuum packages was not performed primarily because this was a very difficult procedure to perform without drawing liquid into the syringe. In addition, the vacuum was maintained throughout storage with no indications of leakage.

The stability of the package was demonstrated with the headspace analysis of an empty package filled with CO₂:N₂ (60:40). The initial CO₂ gas mixture consisted of 58.8% CO₂, undetectable O₂, with the balance N₂. Initially, levels of CO₂ decreased slightly and O₂ increased slightly, but gas levels remained relatively constant thereafter reaching 52.3% CO₂ and 2.8% O₂ by day 13 (Figure 17). These results indicated that the changes in gas composition were due to interaction between the atmosphere in the package and the tray, therefore demonstrating the stability of the package relative to the environment.

The carbonic acid dip had a significant effect ($p < 0.05$) upon the level of O₂ and CO₂ in the headspace of fish packaged under an air atmosphere at 10 and 3°C (Table A21),

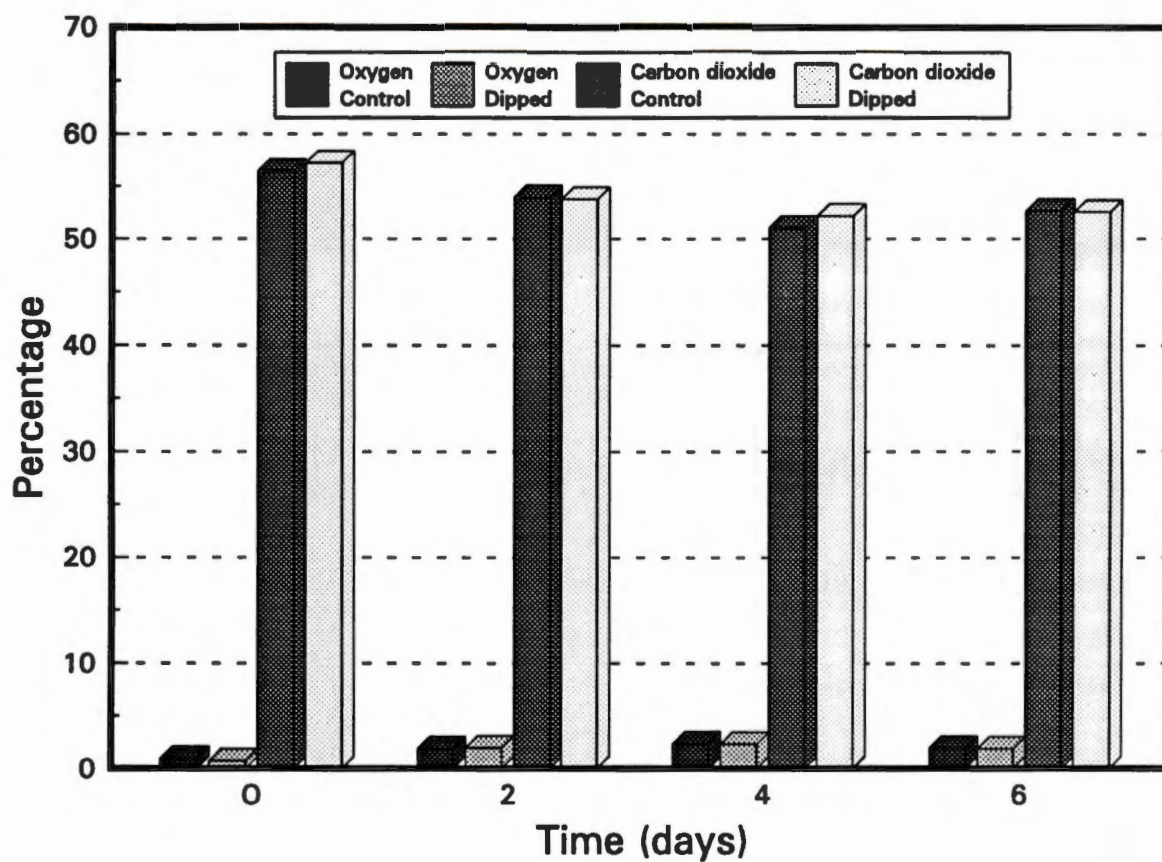


Figure 15. Changes in gas composition (%) in packages of rainbow trout fillets either dipped in carbonic acid or icewater and held at 10°C under a gas mixture of CO₂:N₂ (60:40).

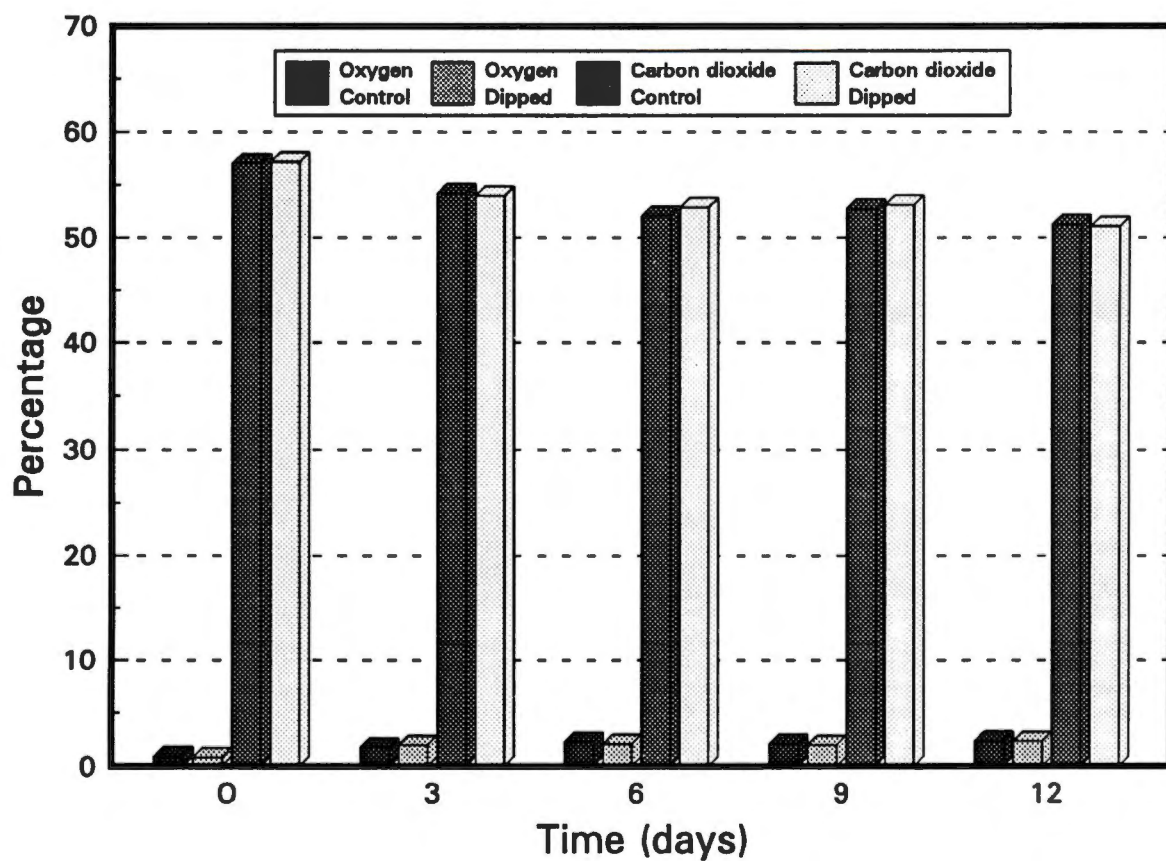


Figure 16. Changes in gas composition (%) in packages of rainbow trout fillets either dipped in carbonic acid or icewater and held at 3°C under a gas mixture of CO₂:N₂ (60:40).

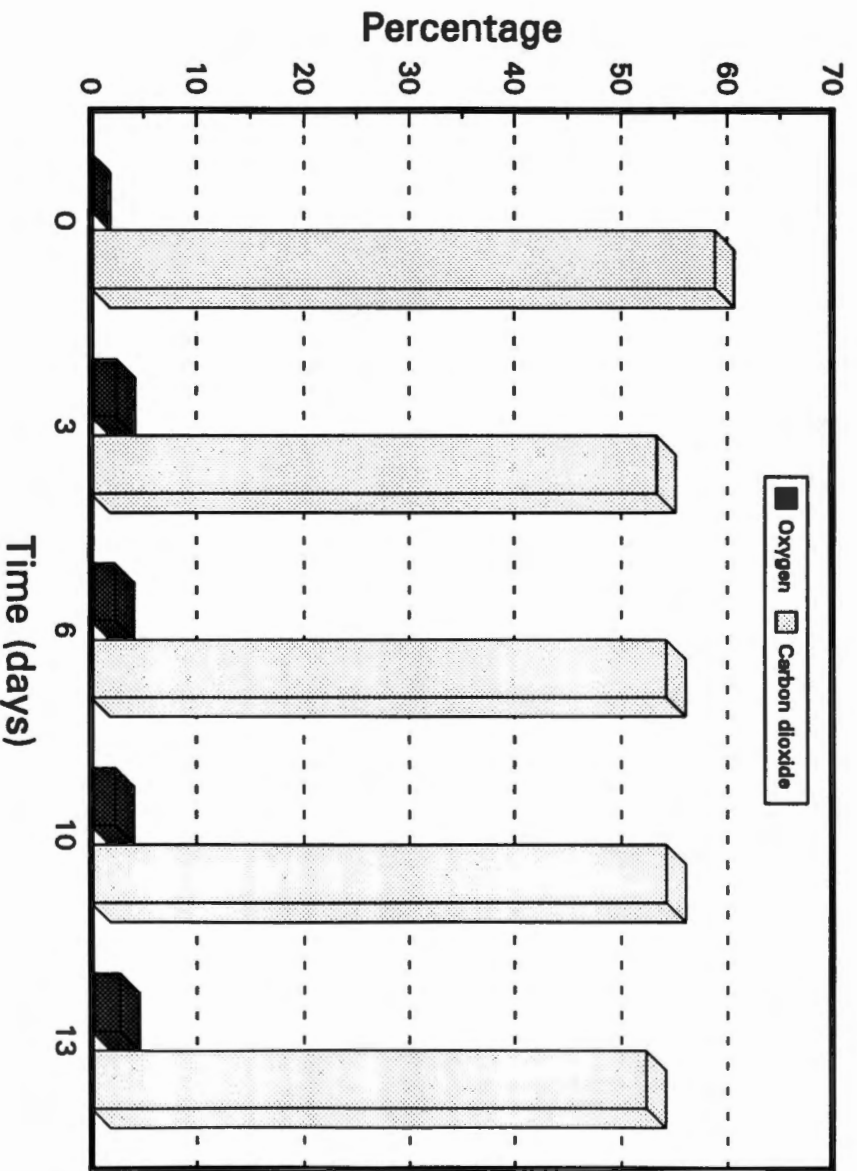


Figure 17. Changes in gas composition (%) in empty packages held at 3°C under a gas mixture of CO₂:N₂ (60:40).

with the effect more pronounced at the end of the storage time when the fish had reached spoilage. The dipped samples maintained higher levels of oxygen and lower levels of carbon dioxide (Figure 13 and 14). The carbonic acid dip had no significant effect ($p > 0.05$) upon the levels of CO_2 and O_2 in the headspace of trout packaged under the CO_2 gas mixture (Table A22).

Chapter V

CONCLUSIONS

The shelf life of fresh rainbow trout was effectively extended with the use of a modified atmosphere packaging system consisting of CO₂:N₂ (60:40) in conjunction with refrigeration. The extension of shelf life was accomplished by prolonging the lag phase of bacterial growth, an effect which was enhanced with a decrease in temperature. Although a carbonic acid dip was effective in initially retarding microbial growth in some instances, an extension of shelf life was not evident. The number of *Salmonella* increased slightly under the CO₂-atmosphere at 10°C, whereas numbers declined under the same conditions at 3°C, emphasizing the need for strict temperature control. In addition, the CO₂-MAP did not enhance the growth of *Salmonella* in comparison to spoilage bacteria. Even though *Salmonella* did not grow under the CO₂-modified atmosphere at 3°C, viable cells were recovered from each inoculated fish indicating the ability of this organism to survive under MAP conditions. These results stress the need for precautionary measures to be taken to avoid contamination of rainbow trout with *Salmonella* during production and processing.

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APPENDIXES

APPENDIX A
Statistical Tables

Table A1. Anova table for aerobic plate count
in rainbow trout fillets stored at
10°C.

Source	DF	Mean Square	Prob > F
Atmosphere	2	11.9068	0.0001
CO ₂ dip	1	1.2272	0.0304
Day	3	103.2837	0.0001
Replication	2	0.3601	0.2430
Atm*Dip	2	0.0610	0.7827
Atm*Day	6	12.2205	0.0001
Dip*Day	3	0.6998	0.0476
Error	52	0.2477	

Table A2. Anova table for psychrotrophic count
in rainbow trout fillets stored at
10°C.

Source	DF	Mean Square	Prob > F
Atmosphere	2	13.1523	0.0001
CO ₂ dip	1	0.1742	0.3628
Day	3	134.2779	0.0001
Replication	2	0.3623	0.1836
Atm*Dip	2	0.0647	0.7325
Atm*Day	6	2.3234	0.0001
Dip*Day	3	0.1386	0.5740
Error	50	0.2065	

Table A3. Anova table for coliform count in rainbow trout fillets stored at 10°C.

Source	DF	Mean Square	Prob > F
Atmosphere	2	22.0954	0.0001
CO ₂ dip	1	1.3068	0.0354
Day	3	123.6850	0.0001
Replication	2	1.9017	0.0024
Atm*Dip	2	0.0585	0.8122
Atm*Day	6	3.4413	0.0001
Dip*Day	3	0.2331	0.4821
Error	52	0.2800	

Table A4. Anova table for lactic acid bacteria count in rainbow trout fillets stored at 10°C.

Source	DF	Mean Square	Prob > F
Atmosphere	2	1.2829	0.0203
CO ₂ dip	1	0.4835	0.2138
Day	3	120.5379	0.0001
Replication	2	16.2454	0.0001
Atm*Dip	2	0.0568	0.8307
Atm*Day	6	0.7696	0.0322
Dip*Day	3	0.1687	0.6486
Error	52	0.3051	

Table A5. Anova table for *Salmonella* count in rainbow trout fillets stored at 10°C.

Source	DF	Mean Square	Prob > F
Atmosphere	2	6.4626	0.0001
CO ₂ dip	1	0.2006	0.4302
Day	3	18.2948	0.0001
Replication	2	2.5885	0.0008
Atm*Dip	2	0.3185	0.3735
Atm*Day	6	0.8863	0.0197
Dip*Day	3	0.0791	0.8615
Error	52	0.3173	

Table A6. Anova table for pH of rainbow trout fillets stored at 10°C.

Source	DF	Mean Square	Prob > F
Atmosphere	2	0.1426	0.0083
CO ₂ dip	1	0.0003	0.9206
Day	3	0.1618	0.0014
Replication	2	0.0714	0.0812
Atm*Dip	2	0.0015	0.9433
Atm*Day	6	0.0271	0.4364
Dip*Day	3	0.0109	0.7533
Error	52	0.0271	

Table A7. Mean separations between replicatons for bacterial counts ($\log_{10}$ CFU/g) in rainbow trout fillets stored at 10°C.

Rep.	Aerobic	Coliform	Psy.	Lactic	Sal.
1	6.5 ^a	5.6 ^a	6.3 ^a	5.9 ^a	4.1 ^a
2	6.6 ^a	5.1 ^b	6.3 ^a	4.6 ^b	4.0 ^a
3	6.4 ^a	5.1 ^b	6.0 ^a	6.1 ^a	3.5 ^b

Within each column, means with the same letter are not significantly different ($p \leq 0.05$) using the Student-Newman-Keuls mean separation test.

Table A8. Mean separations between atmospheres for bacterial counts ($\log_{10}$ CFU/g) in rainbow trout fillets stored at 10°C.

Atm.	Aerobic	Coliform	Psy.	Lactic	Sal.
Air	7.3 ^a	6.2 ^a	7.0 ^a	5.7 ^a	4.3 ^a
Vacuum	6.4 ^b	5.4 ^b	6.2 ^b	5.6 ^a	4.0 ^a
CO ₂ mix	5.9 ^c	4.3 ^c	5.5 ^c	5.3 ^b	3.3 ^b

Within each column, means with the same letter are not significantly different ($p \leq 0.05$) using the Student-Newman-Keuls mean separation test.

Table A9. Anova table for the effects of *Salmonella* inoculation on microbial counts at 10° C.

Count	Source	DF	Mean Square	Prob > F
Aerobic	Micro	2	0.0140	0.9691
	Day	4	98.3217	0.0001
	Error	71	0.4445	
Coliform	Micro	2	0.3258	0.6418
	Day	4	112.1610	0.0001
	Error	71	0.7300	
Psychrotrophic	Micro	2	0.0858	0.8447
	Day	4	99.4755	0.0001
	Error	68	0.5071	
Lactic	Micro	2	0.0978	0.7154
	Day	4	99.9413	0.0001
	Error	71	0.2907	

Table A10. Anova table for aerobic plate count
in rainbow trout fillets stored at
3°C.

Source	DF	Mean Square	Prob > F
Atmosphere	2	34.8008	0.0001
CO ₂ dip	1	0.1361	0.4733
Day	4	70.7608	0.0001
Replication	2	2.3848	0.0003
Atm*Dip	2	0.3441	0.2754
Atm*Day	8	3.5784	0.0001
Dip*Day	4	0.2092	0.5300
Error	66	0.2617	

Table A11. Anova table for psychrotrophic count
in rainbow trout fillets stored at
3°C.

Source	DF	Mean Square	Prob > F
Atmosphere	2	44.1463	0.0001
CO ₂ dip	1	0.1690	0.4887
Day	4	82.3775	0.0001
Replication	2	1.6213	0.0129
Atm*Dip	2	0.1630	0.6286
Atm*Day	8	4.3997	0.0001
Dip*Day	4	0.0443	0.9722
Error	66	0.3486	

Table A12. Anova table for coliform count in rainbow trout fillets stored at 3°C.

Source	DF	Mean Square	Prob > F
Atmosphere	2	41.2284	0.0001
CO ₂ dip	1	0.0360	0.8133
Day	4	46.6984	0.0001
Replication	2	13.6648	0.0001
Atm*Dip	2	2.5333	0.0239
Atm*Day	8	6.1976	0.0001
Dip*Day	4	0.3771	0.6720
Error	66	0.6406	

Table A13. Anova table for lactic acid bacteria count in rainbow trout fillets stored at 3°C.

Source	DF	Mean Square	Prob > F
Atmosphere	2	7.1688	0.0001
CO ₂ dip	1	1.7921	0.0716
Day	4	59.2896	0.0001
Replication	2	4.3608	0.0007
Atm*Dip	2	0.0301	0.9452
Atm*Day	8	0.9229	0.1084
Dip*Day	4	0.2718	0.7294
Error	66	0.5343	

Table A14. Anova table for *Salmonella* count in rainbow trout fillets stored at 3°C.

Source	DF	Mean Square	Prob > F
Atmosphere	2	0.9163	0.0005
CO ₂ dip	1	0.5138	0.0320
Day	4	2.6082	0.0001
Replication	2	1.4503	0.0001
Atm*Dip	2	0.0121	0.8932
Atm*Day	8	0.2243	0.0485
Dip*Day	4	0.0941	0.4814
Error	66	0.1070	

Table A15. Anova table for pH of rainbow trout fillets stored at 3°C.

Source	DF	Mean Square	Prob > F
Atmosphere	2	0.2387	0.0001
CO ₂ dip	1	0.0002	0.8953
Day	4	0.0284	0.0413
Replication	2	0.1293	0.0001
Atm*Dip	2	0.0077	0.4930
Atm*Day	8	0.0170	0.1462
Dip*Day	4	0.0031	0.8844
Error	66	0.0108	

Table A16. Mean separations between replicatons
for bacterial counts ($\log_{10}$ CFU/g)
in rainbow trout fillets stored at
3°C.

Rep.	Aerobic	Coliform	Psy.	Lactic	Sal.
1	5.6 ^a	3.8 ^a	5.2 ^a	4.3 ^a	3.0 ^a
2	5.1 ^b	2.5 ^b	4.8 ^b	3.5 ^b	2.5 ^b
3	5.1 ^b	2.8 ^b	4.8 ^b	3.8 ^b	2.7 ^c

Within each column, means with the same letter are not significantly different ($p \leq 0.05$) using the Student-Newman-Keuls mean separation test.

Table A17. Mean separations between atmospheres
for bacterial counts ($\log_{10}$ CFU/g) in
rainbow trout fillets stored at 3°C.

Atm.	Aerobic	Coliform	Psy.	Lactic	Sal.
Air	6.1 ^a	4.0 ^a	6.0 ^a	4.1 ^a	2.9 ^a
Vacuum	5.5 ^b	3.4 ^b	5.3 ^b	4.1 ^a	2.6 ^b
CO ₂ mix	4.0 ^c	1.7 ^c	3.6 ^c	3.3 ^b	2.7 ^b

Within each column, means with the same letter are not significantly different ($p \leq 0.05$) using the Student-Newman-Keuls mean separation test.

Table A18. Anova table on the effects of *Salmonella* inoculation on microbial counts at 3° C.

Count	Source	DF	Mean Square	Prob > F
Aerobic	Micro	2	0.1136	0.8984
	Day	6	54.8038	0.0001
	Error	93	1.0589	
Coliform	Micro	2	0.6227	0.6921
	Day	6	23.1998	0.0001
	Error	93	1.6858	
Psychrotrophic	Micro	2	0.7222	0.5570
	Day	5	85.5257	0.0001
	Error	88	1.2261	
Lactic	Micro	2	1.6395	0.1499
	Day	6	55.5629	0.0001
	Error	93	0.8464	

Table A19. Mean separations between replication
for pH in rainbow trout fillets
stored at 3 and 10°C.

Replicaton	pH - 3°C	pH - 10°C
1	6.35 ^a	6.32 ^a
2	6.43 ^b	6.37 ^a
3	6.30 ^a	6.26 ^a

Within each column, means with the same letter are not significantly different ($p \leq 0.05$) using the Student-Newman-Keuls mean separation test.

Table A20. Mean separations between atmosphere
for pH in rainbow trout fillets
stored at 3 and 10°C.

Atmosphere	pH - 3°C	pH - 10°C
Air	6.43 ^a	6.40 ^a
Vacuum	6.39 ^a	6.31 ^{a,b}
CO ₂ mix	6.26 ^b	6.25 ^b

Within each column, means with the same letter are not significantly different ($p \leq 0.05$) using the Student-Newman-Keuls mean separation test.

Table A21. Anova table for headspace gas analysis of rainbow trout fillets packaged under an air atmosphere.

Gas	Temp., °C	Source	DF	Mean Square	Prob > F
O ₂	10	CO ₂ dip	1	5.7820	0.0310
		Day	3	182.8648	0.0001
		Rep	2	2.3552	0.1210
		Day*Rep	6	0.6987	0.5808
		Trt*Day	3	2.1175	0.1333
		Error	8	0.8465	
CO ₂	10	CO ₂ dip	1	3.0716	0.0702
		Day	3	165.6289	0.0001
		Rep	2	1.2272	0.2354
		Day*Rep	6	0.4696	0.6802
		Trt*Day	3	0.9553	0.3235
		Error	8	0.7042	
O ₂	3	CO ₂ dip	1	5.0840	0.0084
		Day	4	39.7773	0.0001
		Rep	2	0.3405	0.5123
		Day*Rep	8	2.1456	0.0149
		Trt*Day	4	3.6252	0.0044
		Error	10	0.4757	
CO ₂	3	CO ₂ dip	1	2.4883	0.0336
		Day	4	32.588	0.0001
		Rep	2	0.7041	0.2291
		Day*Rep	8	1.7818	0.0170
		Trt*Day	4	3.2962	0.0036
		Error	10	0.4109	

Table A22. Anova table for headspace gas analysis of rainbow trout fillets packaged under a CO₂:N₂ (60:40) atmosphere.

Gas	Temp., °C	Source	DF	Mean Square	Prob > F
O ₂	10	CO ₂ dip	1	0.0155	0.4583
		Day	3	2.3886	0.0001
		Rep	2	0.7251	0.0002
		Day*Rep	6	0.0537	0.1629
		Trt*Day	3	0.0271	0.4183
		Error	8	0.0255	
CO ₂	10	CO ₂ dip	1	0.9923	0.2819
		Day	3	31.2694	0.0001
		Rep	2	9.7292	0.0030
		Day*Rep	6	0.6237	0.5742
		Trt*Day	3	0.6532	0.4927
		Error	8	0.7455	
O ₂	3	CO ₂ dip	1	0.0208	0.6364
		Day	4	1.9220	0.0001
		Rep	2	2.2958	0.0001
		Day*Rep	8	0.4375	0.0104
		Trt*Day	4	0.0397	0.7677
		Error	10	0.0875	
CO ₂	3	CO ₂ dip	1	0.2412	0.6088
		Day	4	30.0776	0.0001
		Rep	2	7.9990	0.0053
		Day*Rep	8	6.3657	0.0024
		Trt*Day	4	0.2691	0.8639
		Error	10	0.8641	

APPENDIX B
Preliminary Studies

Preliminary Studies

Objectives

The objectives of the preliminary work were: (1) to conduct studies on the recovery of *Salmonella* from inoculated rainbow trout using currently available pre-enrichments, enrichments, and selective media, (2) to conduct microbial analysis of rainbow trout at the retail level for spoilage bacteria and *Salmonella* in rainbow trout, and (3) to examine the enumeration methods for *Salmonella* in trout over an extended storage time.

Recovery studies

Trout samples and preparation. All media used the preliminary study was purchased from Difco Laboratories (Detroit, MI) unless otherwise stated. The fish were caught at the Cross Eyed Cricket Trout and Catfish Farm, packed on ice, and brought to the laboratory. The fish were cut into 10 g samples and inoculated with approximately 10 CFU *Salmonella*/g fish (see *Salmonella Cultures*, p. 17). This low level was used to simulate natural contamination conditions.

Enrichment evaluation. The samples were then placed in lactose broth and M-9 broth (Gomez et al., 1973) with incubation at 35°C for 24 h. One mL of each sample was then transferred into 9 mL tetrathionate broth, selenite cystine broth, and Rappaport-Vassiliadis broth (Vassiliadis, 1983)

and incubated for 24 h at 35°C. Each of the enrichment broths were streaked for isolation onto brilliant green agar (BGA), bismuth sulfite agar (BSA), xylose lysine deoxycholate agar (XLD), and Hektoen enteric (HE) agar with incubation for 24 h at 35°C. Typical colonies were subjected to confirmation tests (Andrews, 1984).

Results and discussion. *Salmonella* isolates were recovered using each combination of pre-enrichment, enrichment, and selective plating, (Table B1), demonstrating no significant advantage of using one method over another. *Salmonella* was recovered on each of the different plating media, although they were different in the relative ease in selecting presumptive *Salmonella* colonies. XLD yielded a high number of atypical colonies and HE had a high number of swarming colonies. BGA and BSA both gave *Salmonella* colonies which were easier to identify and were therefore selected for use in subsequent experiments.

Retail survey

Trout samples and preparation. Rainbow trout were purchased from Knoxville, TN, area retail markets during the period from December 1992 to May 1993. The samples were immediately stored on ice after purchase and brought the laboratory for analysis.

Microbial analysis. Counts of aerobic bacteria and coliforms were determined (see *Microbial analysis*, p. 16).

Table B1. Recovery of *Salmonella* from rainbow trout fillets using enrichment methods.

Pre-enrichment						
Selective Plating	Lactose Broth			M-9 Broth		
	Selective enrichment					
	TT	SC	RV	TT	SC	RV
BGA	+	+	+	+	+	+
BSA	+	+	+	+	+	+
XLD	+	+	+	+	+	+
HE	+	+	+	+	+	+

TT - tetrathionate broth

SC - selenite cystine broth

RV - Rappaport-Vassiliadis

(+) - Confirmed *Salmonella*

Salmonella detection was performed by stomaching approximately 25 g of the trout sample with 250 mL tetrathionate broth and selenite cystine broth followed by incubation at 35°C for 24 h. The enrichment broth was then streaked for isolation onto BGA and BSA and incubated at 35°C for 24 h. Suspect *Salmonella* colonies were further tested for confirmation (Andrews, 1984).

Results and discussion. Of the seven retail markets tested for the microbial quality of rainbow trout, B and C had the highest mean aerobic plate counts of log 6.6 and 6.7 CFU/g and the highest mean coliform counts of log 4.1 and 4.0 CFU/g respectively. Retail market D and G had the lowest aerobic plate counts of log 5.1 and 3.6 CFU/g and the lowest coliform counts of log 1.3 and 1.8 CFU/g respectively. (Figure B1). The 74 samples had an overall mean aerobic plate count of log 6.2 CFU/g fish (range - log 2.4 to 8.6 CFU/g fish) with 28% exceeding log 7 CFU/g. The overall mean coliform count was log 3.2 CFU/g fish (range - log <1.0 to 6.9 CFU/g fish) with 32% exceeding log 4 CFU/g. Fish with an aerobic plate count of approximately log 8 CFU/g was found to correspond with spoilage according to Stier et al. (1981), where spoilage was determined by a combination of microbial counts, color degradation, and evidence of proteolysis or strong off odors ranging from putrid to slightly oxidized. Although no samples were confirmed *Salmonella* positive, 32% were presumptive

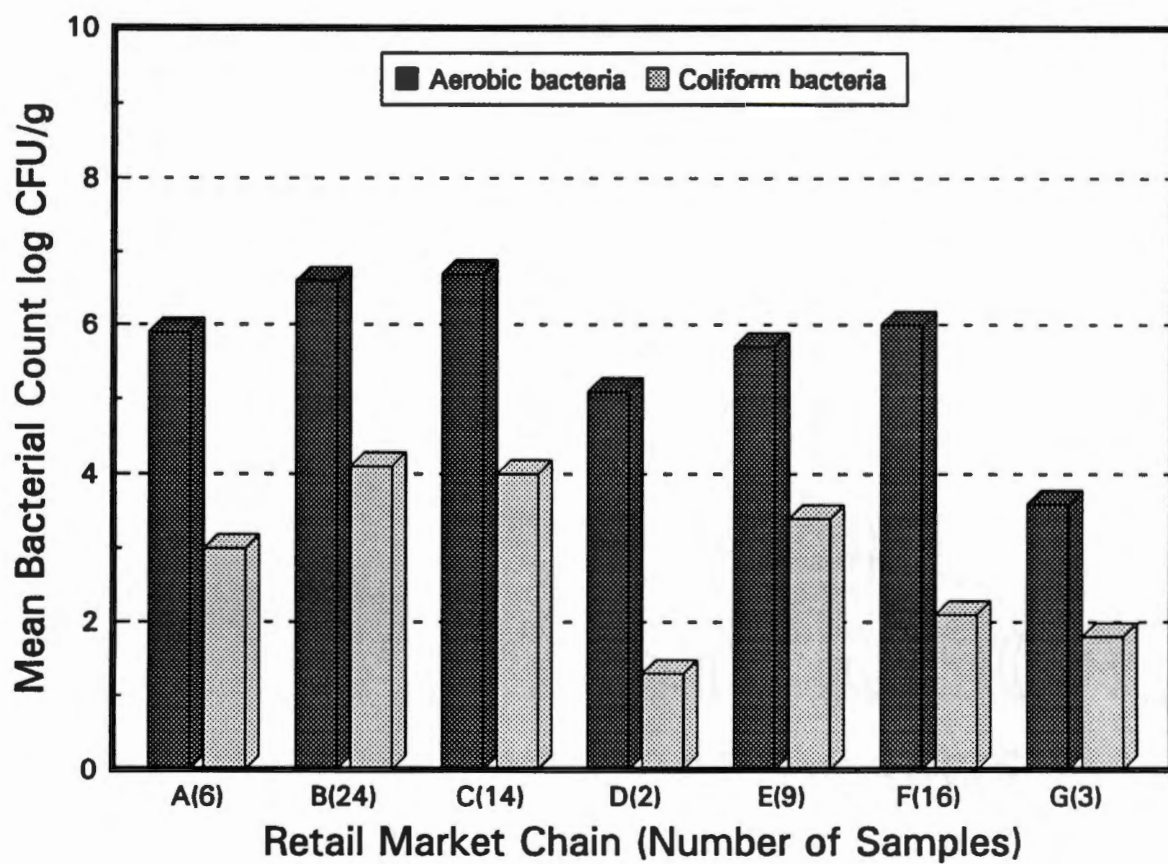


Figure B1. Mean bacterial counts of rainbow trout fillets purchased at Knoxville area retail markets.

positive. This isolate was suspected to be *Providencia*, which "Bergey's Manual of Determinative Bacteriology" (Penner, 1984) also suggests selenite cystine broth and tetrathionate broth for enrichment.

Salmonella enumeration in MAP during extended storage

Trout preparation, inoculation, and sampling. Sample preparation and packaging was conducted as stated in *Trout samples and packaging*, p. 14. Exceptions included the following: (1) the carbonic acid dip treatment was not performed, (2) the experiment was conducted only at 10°C, and (3) the inoculum level was adjusted to be approximately log 2 *Salmonella* CFU/g fish.

Salmonella detection for inoculated and control samples was performed using the same method as in the retail market survey stated above. In addition, *Salmonella* enumeration was performed by directly plating the appropriate dilution onto BGA and BSA from an original 1:10 dilution of sample and Butterfield's diluent.

Results and discussion. *Salmonella* isolates were recovered from samples packaged under vacuum and air using tetrathionate broth up to day 4 and up to day 8 for those packaged under a gas mixture of CO₂:O₂ (60:40). In contrast, when using selenite cystine broth, *Salmonella* isolates were only recovered on day 0 from samples packaged under vacuum and air and up to day 2 for the mixed gas samples (Table B2). These results indicated that the

Table B2. Evaluation of enrichment methods for recovery of *Salmonella* from rainbow trout fillets stored at 10°C.

Day	Vacuum		Air		CO ₂ :N ₂ (60:40)	
	TT	SC	TT	SC	TT	SC
0	+	+	+	+	+	+
2	+	-	+	-	+	-
4	+	-	+	-	+	-
6	-	-	-	-	+	-
8	nt	nt	nt	nt	+	-

TT - tetrathionate broth
 SC - selenite cystine broth
 nt - not tested

selenite cystine broth either did not sufficiently inhibit competitive microflora as their numbers increased or was too toxic for the *Salmonella* to grow with increasing levels of competitive microflora over time. Therefore, only tetrathionate broth was used in future experiments.

The enumeration of *Salmonella* on BSA and BGA by direct plating yielded results in which controls were not visibly different from inoculated samples, resulting in a number of false positive *Salmonella* isolates. These results indicated the need for further evaluation of selective media for direct plating of *Salmonella*.

Evaluation of xylose lysine tergitol-4 (XLT-4).

Samples were prepared as in the previous extended storage survey except only an air atmosphere was examined at 10°C, with sampling only on day 4. Appropriate dilutions, using Butterfield's diluent, were directly plated onto XLT-4 (Miller et al., 1991). Counts were not recorded because the primary concern was the percentage of false positive results. After incubation for 24 h at 35°C, the uninoculated samples yielded no typical *Salmonella* colonies although other growth was evident. The inoculated samples yielded typical *Salmonella* growth along with other atypical colonies. Ten typical *Salmonella* colonies were subjected to confirmation testing (Andrews, 1984), with all 10 resulting in positive confirmation. These results indicated that although XLT-4 was not selective enough to inhibit all non-

Salmonella growth, it was successful in differentiating between *Salmonella* and non-*Salmonella* growth of the cultures used in rainbow trout fillets. Therefore XLT-4 was used in future experiments involving direct plating for *Salmonella*.

VITA

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