

**Survival of *Listeria monocytogenes* in Fruit Juices
During Refrigerated and Temperature-Abusive Storage**

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ABSTRACT

Survival of *Listeria monocytogenes* in apple, orange, red grape, and white grape juice was evaluated. A six-strain cocktail of *L. monocytogenes* was used to inoculate (approx. 7 log cfu/ml) fruit juices, which were stored at 4, 10 and 24° C for up to 61 days. Inoculated red grape juice was stored for up to 5 hours only. Samples were withdrawn at appropriate intervals, neutralized with 1.0 N NaOH, serially diluted in 0.1% peptone water, and surface plated onto Tryptic Soy Agar + 0.6% Yeast Extract (TSAYE) and Modified Oxford Agar (MOX), followed by incubation at 32° C for 48 hours. When *L. monocytogenes* was no longer detected by direct plating, samples were enriched for *L. monocytogenes* using *Listeria* Enrichment Broth (LEB), followed by isolation on MOX. *L. monocytogenes* remained viable in white grape, apple, and orange juices for up to 12, 24 and 61 days, respectively. Over time, recovery of *Listeria* on TSAYE versus MOX was not significantly different ($P>0.05$), indicating that limited acid-injury developed during storage. The results of this study demonstrate the ability of *L. monocytogenes* to survive in apple, orange, and white grape juices during refrigerated and abusive storage conditions. Therefore, measures to prevent or eliminate *L. monocytogenes* in the fruit juice-processing environment are necessary to ensure the safety of juice products for public consumption.

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TABLE OF CONTENTS

SECTION	PAGE
ABSTRACT	ii
ACKNOWLEDGEMENTS	iii
TABLE OF CONTENTS.....	iv
LIST OF FIGURES	vi
CHAPTER I:	
INTRODUCTION	1
Objective	3
References.....	4
LITERATURE REVIEW	6
I. Foodborne Pathogens	6
A. <i>Listeria monocytogenes</i>	7
1. Characteristics.....	7
2. Reservoirs and Disease Sources	7
3. Illness	8
4. Prevalence and Dosage	9
5. Foods.....	10
6. <i>Listeria</i> Outbreaks	10
7. Factors Affecting Growth of <i>Listeria</i>	11
a. Temperature, Acid and pH.....	11
b. Refrigeration, Freezing and Chilled Storage Effects	12
8. Host Defenses.	14
9. Acid Tolerance Response	15
a. Organic Acids.....	15
b. Internal pH Homeostasis.....	17
c. Stress Protein Synthesis	18
d. Virulence.....	18
e. Virulence Factors and Functions.....	19
f. <i>Listeria monocytogenes</i> Zero-Tolerance	20
B. <i>Escherichia coli</i> O157:H7.....	21
C. <i>Salmonella</i> species	22
D. <i>Cryptosporidium parvum</i>	24
II. Fruit Juices	24
A. Apple Cider	25
1. Apples	25
2. Processing.....	25
3. FDA Inspection Apple Cider Manufacturers.....	27

B. Grape Juice.....	28
1. Grapes	28
2. Processing.....	29
C. Orange Juice.....	29
III. Processing.....	30
A. Pasteurization	30
B. Alternatives	31
IV. Prevention and Control Measures.....	33
A. Contamination Sources	33
B. Cleanliness	34
C. Niches.....	34
V. Regulations	35
A. Juice HACCP	35
B. Definition of Juice.....	37
VI. References.....	38

CHAPTER II:

SURVIVAL OF <i>LISTERIA MONOCYTOGENES</i> IN FRUIT JUICES DURING REFRIGERATION AND TEMPERATURE-ABUSIVE STORAGE.....	47
INTRODUCTION	48
MATERIALS & METHODS	50
Strains and preparation of inoculum.....	50
Confirmatory testing.....	50
Inoculation of fruit juices.....	51
Bacteriological analysis	52
Non-neutralized sample comparison.....	53
Statistical analysis.....	53
RESULTS & DISCUSSION.....	54
CONCLUSION.....	79
REFERENCES	81
APPENDIX	84
VITAE	85

LIST OF FIGURES

Table 1	Final Sampling Day Giving Positive Results for Detection of66 <i>Listeria monocytogenes</i>
Figure 1	Fate of <i>Listeria monocytogenes</i> during refrigerated storage (4°C) in67 pasteurized red grape juice as determined by recovery on Tryptic Soy Agar supplemented with Yeast Extract (TSAYE) and Modified Oxford Agar (MOX). Limit of detection: 1 log cfu/ml. n=3
Figure 2	Fate of <i>Listeria monocytogenes</i> during abusive-refrigerated storage68 (10°C) in pasteurized red grape juice as determined by recovery on Tryptic Soy Agar supplemented with Yeast Extract (TSAYE) and Modified Oxford Agar (MOX). Limit of detection: 1 log cfu/ml. n=3
Figure 3	Fate of <i>Listeria monocytogenes</i> during ambient storage (24°C) in69 pasteurized red grape juice as determined by recovery on Tryptic Soy Agar supplemented with Yeast Extract (TSAYE) and Modified Oxford Agar (MOX). Limit of detection: 1 log cfu/ml. n=3
Figure 4	Fate of <i>Listeria monocytogenes</i> during refrigerated storage (4°C) in70 pasteurized apple cider as determined by recovery on Tryptic Soy Agar supplemented with Yeast Extract (TSAYE) and Modified Oxford Agar (MOX). Limit of detection: 1 log cfu/ml. n=3
Figure 5	Fate of <i>Listeria monocytogenes</i> during abusive-refrigerated storage71 (10°C) in pasteurized apple cider as determined by recovery on Tryptic Soy Agar supplemented with Yeast Extract (TSAYE) and Modified Oxford Agar (MOX). Limit of detection: 1 log cfu/ml. n=3
Figure 6	Fate of <i>Listeria monocytogenes</i> during ambient storage (24°C) in72 pasteurized apple cider as determined by recovery on Tryptic Soy Agar supplemented with Yeast Extract (TSAYE) and Modified Oxford Agar (MOX). Limit of detection: 1 log cfu/ml. n=3
Figure 7	Fate of <i>Listeria monocytogenes</i> during refrigerated storage (4°C) in73 pasteurized white grape juice as determined by recovery on Tryptic Soy Agar supplemented with Yeast Extract (TSAYE) and Modified Oxford Agar (MOX). Limit of detection: 1 log cfu/ml. n=3
Figure 8	Fate of <i>Listeria monocytogenes</i> during abusive-refrigerated storage74 (10°C) in pasteurized white grape juice as determined by recovery on Tryptic Soy Agar supplemented with Yeast Extract (TSAYE) and Modified Oxford Agar (MOX). Limit of detection: 1 log cfu/ml. n=3

Figure 9	Fate of <i>Listeria monocytogenes</i> during ambient storage (24°C) in75 pasteurized white grape juice as determined by recovery on Tryptic Soy Agar supplemented with Yeast Extract (TSAYE) and Modified Oxford Agar (MOX). Limit of detection: 1 log cfu/ml. n=3
Figure 10	Fate of <i>Listeria monocytogenes</i> during refrigerated storage (4°C) in76 pasteurized orange juice as determined by recovery on Tryptic Soy Agar supplemented with Yeast Extract (TSAYE) and Modified Oxford Agar (MOX). Limit of detection: 1 log cfu/ml. n=3
Figure 11	Fate of <i>Listeria monocytogenes</i> during abusive-refrigerated storage77 (10°C) in pasteurized orange juice as determined by recovery on Tryptic Soy Agar supplemented with Yeast Extract (TSAYE) and Modified Oxford Agar (MOX). Limit of detection: 1 log cfu/ml. n=3
Figure 12	Fate of <i>Listeria monocytogenes</i> during ambient storage (24°C) in78 pasteurized orange juice as determined by recovery on Tryptic Soy Agar supplemented with Yeast Extract (TSAYE) and Modified Oxford Agar (MOX). Limit of detection: 1 log cfu/ml. n=3

INTRODUCTION

Consumer demand for fresh fruit juice (i.e., unpasteurized) is ever increasing with the notion that juice processing is detrimental to the quality and nutritional value of the product (Yuste et al., 2002). Prior to the past two decades, the food industry and consumers were rather unconcerned about the microbial safety of juices due to the low pH (e.g. 3.8) of such products. It was generally accepted that high acid beverages and foods (pH < 4.6) prevented survival and growth of pathogens and added hurdles, such as preservatives and/or refrigeration, were utilized to prevent spoilage. However, a number of foodborne outbreaks that occurred during the 1990s were associated with the consumption of unpasteurized fruit juices (Datta and Benjamin, 1997).

The juices most commonly associated with foodborne disease outbreaks are unpasteurized orange juice and apple cider. Unpasteurized apple cider has been implicated as the vehicle of infection in numerous outbreaks of foodborne illness from the acid tolerant pathogen *E. coli* O157:H7, as well as *Salmonella* spp. and *Cryptosporidium parvum* (MMWR, 1996 and 1997).

The FDA estimates that there are between 16,000 and 48,000 cases of juice-related illnesses each year in the United States (USDHHS, 2001). Due to recent outbreaks, the FDA has mandated Hazard Analysis Critical Control Point (HACCP) regulations for juice processors in the U.S. Juice HACCP regulations require that processors treat juices in a manner that will result in a 5 log cfu/ml reduction in populations of the “pertinent” pathogen in the juice being processed. Retail-only processors, i.e., those processors who sell their product directly to consumers, are allowed to post the following warning in place of demonstrating the 5-log reduction performance standard:

“WARNING: This product has not been pasteurized and, therefore, may contain harmful bacteria which can cause serious illness in children, the elderly, and persons with weakened immune systems” (USFDA, 2002b).

Although, to date, *L. monocytogenes* has not been implicated in any cases of juice-borne illness, it has been isolated (Sado et al., 1998) in an unpasteurized apple juice (pH 3.78) and an apple/raspberry juice blend (pH 3.75). *Listeria monocytogenes* has been isolated from unpasteurized milk, ice cream, soft (Mexican-style) cheeses, smoked fish, turkey luncheon meats, hot dogs, as well as other Ready-To-Eat (RTE) foods such as fruits and vegetables (MMWR, 1985, 1992, 1998a, 1998b, 2000, 2001 and 2002a).

The Centers for Disease Control and Prevention (CDC) estimates that in the United States, 76 million persons will contract a foodborne illness each year (CDC, 2003), with *L. monocytogenes* infections causing an estimated 2500 of these cases, resulting in approximately 500 deaths each year (MMWR, 2000). Though most reported listeriosis in the U.S. is isolated and sporadic, when outbreaks do occur, they are particularly severe with a high mortality rate. Especially vulnerable populations for listeriosis are pregnant women and their fetuses, the elderly and the immune-compromised (MMWR, 1992 and 2003a). *Listeria monocytogenes* does not usually cause illness in healthy adults when consumed at doses below 10^2 cfu/g of food ingested (Hitchins, 1996).

Listeria has been classified as a human pathogen for over 70 years, yet it did not become a pathogen of concern in food products until the 1980's. It is often described as a hardy microorganism, as it can survive quite well in minimal conditions. Additionally, its psychrotrophic nature makes it a microorganism of concern in certain refrigerated products

(USFDA, 2003).

Listeria monocytogenes is a successful pathogen in terms of its ability to withstand acidic environments. Acid tolerance, though the mechanism is still not fully understood, makes *L. monocytogenes* survival possible in low pH foods and beverages, passage through the stomach, and engulfment by phagosomes and internalization by macrophages (Cotter et al., 2000). The acid tolerance of *L. monocytogenes* is maintained through adaptation or achieved through mutation, where external pH signals genetic regulators to synthesize stress proteins. This creates a homeostatic environment allowing the neutrophilic pathogen to survive. The pH limit, which *L. monocytogenes* can resist is dependent upon the food/beverage composition, the strain type and the phase of growth (Phan-Thanh et al., 2000).

OBJECTIVE

The objective of this research was to determine the survival of *Listeria monocytogenes* in artificially contaminated fruit juices during refrigerated and abusive storage temperatures.

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LITERATURE REVIEW

I. Foodborne Pathogens

According to the Centers for Disease Control (CDC) an estimated 76 million persons will contract a foodborne illnesses each year in the United States (CDC, 2003). In 1996, FoodNet began surveillance of *Escherichia coli* O157:H7, *Salmonella*, *L. monocytogenes*, *Campylobacter*, *Vibrio*, *Shigella* and *Yersinia enterocolitica*, based on laboratory diagnosed cases. A year later they added Hemolytic Uremic Syndrome (HUS, kidney failure occurring primarily in young children), *Cryptosporidium parvum* and *Cyclospora cayetanensis* to the surveillance list. Upon comparison of FoodNet's 1996-2001 surveillance data, there appears to be a general trend toward a substantial reduction in the number of foodborne illnesses caused by *L. monocytogenes*. The National Health Objective's goals are to reduce key pathogens' prevalence rates from the current 2002 numbers to those set for 2010. A few of the bacteria for which reduction goals (per 100,000 people) have been set are: *E. coli* O157:H7 from 1.73 to 1.00, *Salmonella* from 16.10 to 6.80, and *L. monocytogenes* from 0.27 to 0.25. However, current prevalence points toward an increased number of cases of foodborne illness mediated by foodborne pathogens such as *Salmonella* and *E. coli* O157:H7. These numbers indicate that increased efforts (i.e., research and educational awareness) are needed to reduce future incidences of these illnesses associated with particular pathogens (CDC, 2003).

According to 2002 FoodNet data reported, the most commonly laboratory-diagnosed foodborne illnesses were caused by: *L. monocytogenes*/101, *E. coli* O157:H7/647, HUS/44, *Salmonella* spp./6028 and *Cryptosporidium parvum*/541 (CDC, 2003). Year to year variation in reported incidence are skewed by large outbreaks, a suspect high number of unreported foodborne illnesses, and increasing acquisition of pathogens through non-food routes (i.e., water,

transmission via person-to-person contact and direct animal exposure). FoodNet only surveys a few states that combine to account for approximately 13% of the U.S. population, and in consideration of the factors mentioned, the predicted numbers of foodborne illness cannot be fully generalized to all persons in the U.S. (CDC, 2003).

A. *Listeria monocytogenes*

For over seventy years, *L. monocytogenes* has been recognized as a human pathogen. However, it has only been within the last two decades that this microorganism has been recognized as a foodborne pathogen. Since the 1980s research on this bacterium has intensified greatly (O' Driscoll et al., 1997; Gombas et al., 2003).

1. Characteristics

Listeria monocytogenes is a Gram-positive, small, rod-shaped, facultative anaerobe that is motile by means of a single flagellum. This pathogen is beta-hemolytic when cultured on blood agar. *Listeria monocytogenes* is both microaerophilic and a facultative anaerobe. Even though it is a non-sporulating bacterium, the microorganism is quite hardy in that it is somewhat acid, heat, freezing, drying and halotolerant (USFDA, 2003). *Listeria monocytogenes* is also a neutrophile and psychrotroph (O' Driscoll et al., 1997).

2. Reservoirs and Disease Sources

Listeria monocytogenes has a large number of reservoirs in nature and in industrial environments. A few of these reservoirs include: plants used for food, sewage, soil, feces and silage (Gray, 1960; Gray and Killinger, 1966; Blenden and Szatalowicz, 1967; Welshimer, 1968; Ralovich, 1984). Similarly to other foodborne pathogens, such as *Salmonella* Typhimurium, *L. monocytogenes* has been isolated in as many as 10% of asymptomatic humans (Slutsker and Schuchat, 1999; USFDA, 2003) and also in animals (Kathariou, 2002). It has been isolated in

over 17 species of fish, birds, shellfish (USFDA, 2003) and domesticated pets and agricultural animals.

3. Illness

Listeria monocytogenes causes an estimated 2500 foodborne infections each year, in the U.S., of which approximately 500 result in death (MMWR, 2000). Listeriosis rarely occurs in healthy adults, especially in cases of ingestion at a dose of less than 10^3 cfu/g or ml food or beverage (Tompkin, 2002). A few notable outbreaks of listeriosis in young, previously healthy (non-pregnant) adults include: 39 people attending a private dinner in Italy became ill from rice salad (Salamina et al., 1996), 16 people from Los Angeles, California attending a catered party became ill from pre-cooked sliced turkey (Frye et al., 2002), and in Illinois, 45 people attending a picnic became ill from chocolate milk consumption (Dalton et al., 1997). Not only is the infective dosage of *Listeria* unknown, but it is also believed that other factors such as the food type, strain source and host susceptibility greatly affect the outcome of the disease (Farber and Peterkin, 1991; Liberti et al., 1996). Despite the disease rate (i.e., about 1 to 9 cases per 1,000,000 persons/year) that accounts for only about 0.02% of all foodborne illnesses in the U.S., listeriosis accounts for about 28% of the deaths resulting from foodborne illness (Tompkin, 2002). Foods containing >1000 *L. monocytogenes* cfu/g and consumed by immune-compromised individuals (e.g., pregnant women, fetuses, the elderly, AIDS, diabetes, cancers, ulcerative colitis, etc.), may lead to infection and result in abortion, flu-like symptoms, pneumonia, meningoenzephalitis, septicaemia, endocarditis and urethritis (Marth, 1988; Giannuzzi and Zaritzky, 1996). Death results in approximately 30 to 80% of cases in susceptible populations (Farber and Peterkin, 1991; USFDA, 2003).

The incubation period of *L. monocytogenes* infection may be from 12 hours to a few weeks. Onset of listeriosis is characterized by flu-like symptoms with fever and gastroenteritis, and results from invasion of stomach epithelial cells. Upon further intracellular infection, when the host defense macrophages are defeated, *L. monocytogenes* infection may reach the bloodstream where leukocyte compromise occurs. If *L. monocytogenes* mediated illness is detected early, the infected patient may be treated with penicillin, ampicillin, or trimethoprim-sulfamethoxazole. These treatments are especially useful for parental survival of pregnant women (USFDA, 2003).

4. Prevalence and Dosage

It is unreasonable to believe that *L. monocytogenes* can be completely eradicated from all food processing environments. According to the FDA and USDA, *L. monocytogenes* is present in as much as 5% of RTE foodstuffs (Hitchins, 1996; Levine et al., 2001; Tompkin, 2002; Tompkin et al., 1999). A prevalence of this percentage means that the general public consumes *L. monocytogenes* in millions of food products each year. This estimate is further corroborated by the research of Hitchins (1996) who calculated that consumers in the U.S. are exposed to *Listeria* approximately once every 3 to 4 days, averaging 100 times each year. He concluded that most doses ingested are less than 10^3 cfu/25g to account for low frequency of cases observed. Tienungoon et al. (2000) reported that other countries (i.e., Canada, Germany, the Netherlands and France) consider *L. monocytogenes* levels below 10^3 cfu/25g to be acceptable in foods, with the requirement that storage conditions will not allow the pathogen to grow to unacceptable levels during the determined shelf-life of the food (Tienungoon et al., 2000).

5. Foods

Listeria monocytogenes has been implicated in outbreaks that were associated with consumption of such foods as raw vegetables, milk (MMWR, 1998a), cheese (Mexican-style soft) (MMWR, 1985, 1992 and 2001), coleslaw (Conner et al., 1986), lunch meat (MMWR, 1992) and hot dogs (MMWR, 1998b). Other foods found to contain *Listeria* through retail sampling studies include: deli salads, bagged salads, smoked seafood and seafood salads (Gombas, et al., 2003). Due to its high mortality rate and publicity of recent large outbreaks, *L. monocytogenes*, as well as the broad range of foods associated with listeriosis, research has intensified on the study of *L. monocytogenes* survival in high-risk foods.

6. Listeria Outbreaks

The first documented outbreak of foodborne listeriosis (serotype 1/2a) occurred in Halle, Germany in 1945 (Kathariou, 2002). World-wide, there are three main serotypes of *L. monocytogenes*, i.e., 1/2a, 1/2b and 4b, that account for 89 to 96% of laboratory-confirmed foodborne cases of listeriosis in humans (Tompkin, 2002).

There have been numerous outbreaks of listeriosis in the past few decades. A noteworthy outbreak occurred in Canada where sheep manure contaminated with *L. monocytogenes* was used to fertilize cabbage fields (Gray and Killinger, 1966). In a similar incident, manure was presumably the source of cabbage contamination in Nova Scotia, and coleslaw was identified as the food source (Kathariou, 2002). Outbreaks associated with meats include: in 1987 in the state of Pennsylvania, raw hot dogs and undercooked chicken were implicated (USFDA, 2003; MMWR, 1992); in 1998, 40 cases spanning 10 states resulted in 4 deaths from hot dogs produced by Bil Mar Food's (MMWR, 1998b); in 2000 there were 29 cases spread across 10 states with 7 deaths from Cargill Turkey Product's deli turkey meat (MMWR, 2000); and finally,

in 2002 there were 46 lab-confirmed cases in 8 states, which resulted in 10 deaths, due to Pilgrim's Pride Food's turkey deli meat, where 27.4 million pounds of the product was recalled (MMWR, 2002a).

Other extremely high-risk foods for *Listeria* contamination include Hispanic soft cheeses that are typically made from unpasteurized milk. In California during 1985, there were 86 cases of listeriosis, resulting in 29 deaths most of which were stillbirths, from Jalisco Product's Mexican-style fresh cheese. Due to this incident, the FDA began monitoring domestic and imported cheeses (MMWR, 1985; USFDA, 2003). In the year 2000 an outbreak occurred North Carolina in which 12 cases resulted in 5 stillbirths. The food source was determined to be from a non-commercial, homemade, Mexican-style fresh soft cheese produced from contaminated raw milk sold by a local dairy farm. The product was unlabeled and sold door-to-door and in local markets. Due to the severity of the outbreak, the sale of raw milk products, by dairy farms to noncommercial processors, were halted by North Carolina health authorities. Further measures were taken to educate store owners regarding the illegality of sales of unregulated dairy products (MMWR, 2001).

The CDC conducted an investigation of Listerial incidence over a span of two years in a populace of 18 million Americans, covering in 5 states. Results revealed an incidence of 7.4 cases of listeriosis per one million people. Three hundred and one cases were identified with a mortality rate of 23%, of which 33% were pregnant women, newborns or fetuses. Additionally, in homes of people with listeriosis, 64% of the refrigerators tested contained at least one *L. monocytogenes* adulterated food item (MMWR, 1992).

7. Factors Affecting Growth and Survival

a. Temperature, Acid and pH

Ranges for *Listeria* growth are: water activity 0.90-0.92, pH 4.0 to 9.6, and temperatures of 0 to 45° C, of which, most are codependent on one another (Sorrells et al., 1989; George et al., 1996; Tienungoon et al., 2000; Yuste and Fung, 2002). In terms of pH growth, studies performed by George et al., (1996) found that in lab broth media, minimum growth of *L. monocytogenes* occurred at 1° C at pH 5.4. Yet, minimum pH for growth at temperatures 4, 8 and 12° C was pH 5.0 and at 20° C was pH 4.3, respectively, over 50 days. In other research, it was discovered that at 30° C cottage cheese (pH 5.2), unclarified cabbage juice (pH 5.0) and TSBYE (pH 4.39) supported *L. monocytogenes* growth (Ryser, et al., 1985; Conner et al., 1986; George et al., 1988).

b. Refrigeration, Freezing and Chilled Storage Effects

Studies conducted to determine if refrigerated growth of *L. monocytogenes* causes an increase (Gray and Killinger, 1966; Czuprynski et al., 1989; Stephens et al., 1991), decrease (Buncic and Avery, 1995; Buncic et al., 1996) or no change (Buncic and Avery, 1995; Buncic et al., 1996) in virulence (Buncic and Avery, 1996) of the microorganisms have been contradictory. In a study by Dykes (1999), *L. monocytogenes* cells stored in favorable nutrient conditions, over extended chilled storage (4 weeks), showed sub-lethal injury with repair and renewed virulence when placed at optimum temperature. *Listeria monocytogenes* cultured in minimal nutrients during long-term refrigerated storage (4 weeks) had visible signs of damage. Cell damage included structural changes to the cell with separation of the cell wall and membrane, which lead to the formation of pores and allowed leakage of proteins and nucleic acids. This was also accompanied by cytoplasmic shrinkage (where granular density developed a stringy texture), a decreased growth rate and reduced virulence potential. It was reported that strain type, nutrients, temperature and storage time determine sublethal injury recovery rates.

Oyarzabal and associates (2003) recently studied *L. monocytogenes* survival in apple (pH 3.7), orange (pH 3.7), pineapple (pH 3.6) and white grape (pH 3.6) juice concentrates and banana (pH 5.5) puree. Concentrates were inoculated with greater than or equal to 10^3 cfu/g *L. monocytogenes*, *E. coli* O157:H7 or *Salmonella* and stored at -23°C , the National Food Processors Association's recommended transport temperature for juice concentrates. All three pathogens, which were previously acid-adapted (pH 4), were recovered in all 5 fruit concentrates after the 12 week sampling period. Even though results were highly variable between samples and replications, *L. monocytogenes* survived better overall than *E. coli* or *Salmonella* in all concentrates except the banana puree. The psychrotrophic and mesophilic nature of *Listeria* versus that of mesophilic only *E. coli* and *Salmonella* may have attributed to its greater survival rate (Oyarzabal et al., 2003). When considering why the low temperatures nor concentrated $^{\circ}\text{Brix}$ did not reduce the survival of these pathogens more drastically, it was determined that survival may have been achieved if the microbes were encapsulated and protected in pure ice crystals and not in direct contact with the acidic concentrate itself during frozen storage (Nogueira et al., 2003).

In a related study, Nogueira et al. (2003) utilized the same three acid-adapted pathogens, but evaluated in cranberry (pH 2.0-2.2), lemon (pH 1.8-2.0), and lime (pH 2.2) concentrates. The study was performed to test transport and storage of concentrates at low temperatures before final packaging, to see if these conditions introduced the risk of post-concentration contamination. A 5 log cfu/ml reduction was achieved when the juices were thermally concentrated to give Brix ranging from 45° to 55° . At least a 5 log cfu/ml reduction of *Salmonella* occurred at -23°C , in *E. coli* at -11°C and in *L. monocytogenes* at 0°C . In lemon and lime concentrates, *L. monocytogenes* was reduced by 5 log cfu/ml by 6 hours in nearly all

temperatures tested. But, *Listeria* was sporadically detected after 24 hours at -11° C. Also, in the cranberry concentrate *Listeria* was not reduced by 5 log cfu/ml until after 6 hours (due to one positive sample). After the fruit juice concentrates were inoculated with *L. monocytogenes*, within one hour, up to an 8 log cfu/ml cfu reduction was noted. Additionally, enrichments were sporadically positive for *L. monocytogenes* up to two weeks after inoculation of all three fruit juice concentrates. Since all the concentrates utilized contained inherent antimicrobial properties, in the case of post-processing contamination, pathogens would be inactivated; therefore, further control measures (i.e., re-pasteurization) would be unnecessary (Nogueira et al., 2003).

8. Host Defenses

Once *L. monocytogenes* cells have been consumed, the initial defenses of the host are acidic gastric juices (pH 2.5) (Cotter et al., 2001) and competition with natural microflora (Gahan and Hill, 1999). Reports have linked the use of antacids with increased risk of listeriosis, possibly due to an increase in stomach pH (Kathariou, 2002). Next, *Listeria* must face volatile fatty acids, a product of sugar fermentation (Gahan and Hill, 1999). If *L. monocytogenes* survives the bile containing and low oxygen environment (Gahan and Hill, 1999) of the intestinal tract, it may then invade epithelial cells (Vasquez-Boland et al., 2001). At this point *L. monocytogenes* is engulfed by macrophages and internalized by phagosomes (De Chastellier and Berche, 1994; Gahan and Hill, 1999), where the pH ranges from 4.4 to 5.7 (Bassoe and Bjerknes, 1985). Survival in the acidic environment of the phagosome allows for further invasion and colonization of surrounding healthy tissue (Conte et al., 2000). If *Listeria* survives these defenses, it may go on to infect secondary organs including the placenta and may transfer to the fetus (Kathariou, 2002). Once in the phagosome, the listerial cells are exposed to various

bactericidal (i.e., low pH environments, lysosomal and granular peptides and oxidative products such as hydrogen peroxide and superoxide radicals) (Gahan and Hill, 1999), which may be combated with the production of superoxide-dismutase (De Chastellier and Berche, 1994; Vasconcelos and Deneer, 1994; Conte et al., 2002).

9. Acid Tolerance Response

Listeria monocytogenes is an opportunistic pathogen which contains a complex sensing system, whose mechanism is not completely clear (Cotter et al., 2000). The system enables the pathogen to resist the harsh conditions of such host defenses as the stomach and phagosomes, in addition to altering virulence characteristics (Gahan and Hill, 1999). This complex is known as the Acid Tolerance Response (ATR), and incorporates various levels of sensing and response through protein synthesis and regulation of virulence factors (Gahan et al., 1996).

a. Organic Acids

The addition of preservatives, such as organic acids, to foods and beverages have a long used method history in the food industry to deter bacterial growth, whether spoilage or pathogenic (O' Driscoll et al., 1996). Typically, strong inorganic acids are not as effective as weak organic acids of the same pH (Phan-Thanh et al., 2000). It has been determined that organic acids have a greater antimicrobial effect on listeriae due to the undissociated form of the acids at acidic pH in foods (Banwart, 1979; Lueck, 1980). Inorganic acids, such hydrochloric acid, are nearly completely dissociated in aqueous solutions. The antimicrobial mode of action is primarily attributed to the undissociated acid concentration, rather than hydrogen ions. The undissociated form, when present in the non-charged state, has a greater capacity to penetrate bacterial cells than the dissociation products. Undissociated acids permeate the cellular membrane via porins or permeases. Since, cell membranes exhibit a low permeability to H⁺,

once organic acids are inside the cell they dissociate, H⁺ cannot diffuse out, and a drastic decline in internal pH (pH_i) results (Phan-Thanh et al., 2000). The bacteriostatic effect is best with higher pK_a's and low pH. Utilizing the Henderson-Hasselbalch equation (Banwart, 1979), the pK_a dissociation constant, the A⁻ unprotonated concentration (dissociated), and the H protonated concentration (undissociated) can be calculated: {pH = pK_a + log [A⁻] / [HA]} (Conner et al., 1990).

The ability of *L. monocytogenes* to survive acidic pH conditions contributes to its success as a foodborne pathogen (Cotter, et al., 2000). *Listeria monocytogenes*, as well as *E. coli* O157:H7 and *Salmonella* spp., have significant acid adaptability and tolerance, which must be taken into consideration in the food and beverage industry when establishing processing procedures and HACCP plans. The ATR of these pathogens has been shown to improve survival and proliferation in foods and beverages. The ATR of *Listeria* is important for intracellular survival (Cotter et al., 2000).

When *Listeria* cells are exposed to a sub-lethal pH (4.8-5.5) (Davis et al., 1996), acid adaptation and survival occurs at a normally lethal pH (3.5); therefore, enhancing resistance to the traditional low pH (<4.6) hurdle of acidic foods. In addition to demonstration of acid tolerance by early stationary phase cells, Gahan and Hill (1999) reported that *Listeria* also acquires acid tolerance upon phagosomal encasement. The phenomenon of acid tolerance has been studied for many years, but primarily on *E. coli* and *Salmonella*, not *Listeria* (Phan-Thanh et al., 2000).

It is known that log-phase *L. monocytogenes* is sensitive to acidic pH, yet through the ATR *L. monocytogenes* can increase its acid tolerance to nearly the level of acid tolerant mutants, which are derived from lengthy or repeated exposure to sub-lethal acidic conditions

(Jenkins et al., 1988; Davis et al., 1996; O' Driscoll et al., 1997). The ATR may also lead to secondary cross-protection through general stress protein synthesis, leading to additional enhancement of survival and increased virulence in sub-optimal conditions (Sanchez-Garcia, 1995; Phan-Thanh et al., 2000).

In studies performed by Phan-Thanh and others (2000), it was shown that optimal acid adaptation of mid-exponential listerial cells was conferred after 2-3 hours of challenge, with persistence in tolerance for several weeks if stored at 4° C. This ATR through adaptation was comparable to that of the stationary-phase acid tolerance (Kroll and Patchett, 1992; Davis et al., 1996; Gahan et al., 1996). In this study it was also determined that the effectiveness of the ATR was dependent on strain type and the acidification medium utilized, where organic acids yielded a more destructive response (lowered pH_i) versus that of inorganic acids.

In the food industry, preservatives (in the form of organic acids) are commonly used in both foods and beverages (Datta and Benjamin, 1997). Since it has been shown that *Listeria monocytogenes*, as well as other pathogens, are adaptable to these acidic (previously assumed safe) conditions, processors may need to adjust methods to compensate for pathogens' ATR.

b. Internal pH Homeostasis

As Hill et al. (1995) states, “the term ‘pH homeostasis’ is used to describe the ability of an organism to maintain its internal cellular pH at a value close to neutrality despite fluctuations in external pH (pH_o) conditions.” The neutrophilic *L. monocytogenes* desires to sustain a pH_i of 6-8, regardless of the pH_o. If the differential between the pH_i and pH_o is too great, the bacterium will die (McDonald et al., 1991). *Listeria monocytogenes* incorporates multiple unclear (Booth, 1985; Bearson et al., 1997; O' Driscoll et al., 1997) yet presumably elaborate systems for maintaining pH_i homeostasis. Datta and Benjamin (1997) surmise that other survival strategies

include: release of protons through the membrane-bound pump, actions of low proton permeability in the cell membrane itself, and increasing the buffering capacity of the cytoplasm, all of which may help in the stabilization of internal pH. In studies performed by Phan-Thanh et al. (2000), *L. monocytogenes* LO28 had a different pH_i than that of the EGD strain when exposed to the same pH_o , inferring that each may contain unique cell membrane attributes.

c. Stress Protein Synthesis

O' Driscoll and others (1997) found that through protein analysis, unique acidification media provide different (yet somewhat overlapping) stress protein synthesis patterns. In other studies performed, researchers wanted to prove that *de novo* protein synthesis of the ATR is key to stabilizing pH_i , through the use of chloramphenicol, to prevent synthesis (Davis et al., 1996; O' Driscoll et al., 1996). This addition showed that cells were unable to create a homeostatic pH and were therefore inactivated. The research of Davis and others (1996), determined that 23 protein patterns were altered, with only 11 being induced, while the other 12 were repressed. O' Driscoll and associates (1997) discovered in similar studies that 53 proteins in *L. monocytogenes* LO28 were modified due to acid adaptation. This information leads to the notion that stress response proteins are newly created, have up-regulation induction and repressive down-regulation of constitutive proteins. Ironically, despite finding unique protein patterns (O' Driscoll et al., 1997) no difference in acid tolerance could be noted, only that of which was due to different strains and media.

d. Virulence

Researchers have determined that in mouse models, *L. monocytogenes* acid-tolerant mutants display magnified levels of pathogenicity (O' Driscoll et al., 1996), whereas acid sensitive cells show decreased virulence characteristics (Portillo et al., 1993; Wilmes-

Reisenberg et al., 1996; Merrell and Camilli, 1999). Conversely, other studies have found that acid (adapted) tolerant *L. monocytogenes* exhibited no change in virulence when exposed to acid conditions (Gahan and Hill, 1999). The researchers attribute the retention of virulence in *L. monocytogenes* to inherent pH tolerance upon entry into the macrophage, which negated any adaptation advantages.

e. Virulence Factors and Functions

Listeria monocytogenes, as well as *E. coli* O157:H7 and *Salmonella* species, are able to increase pathogenicity when exposed to sub-lethal acidic conditions (Mekalanos, 1992; Conte et al., 2000). In terms of virulence factors and the genes associated with these functions, though still unclear, a more complete picture is emerging through molecular studies. In mouse models, if the LisRK operon is mutated (Gahan and Hill, 1999) or the ClpC-ATPase stress protein is inhibited (Ripio et al., 1998), decreased virulence results, producing a reduced capacity for acid resistance (Conte et al., 2000) and escape and replication of *L. monocytogenes* from macrophages (Rouquette et al., 1996).

When instigated under stressful *in vivo* circumstances, the PrfA regulatory system promotes the expression of virulence factors (listeriolysin O and hemolysin, the ActA protein, lecithinase operon products, internalins and phosphatidylinositol-specific phospholipase C) (Mengaud et al., 1991; Freitag et al., 1993) and is instrumental in listerial phagosomal survival (Gahan and Hill, 1999).

The gene *inlA* encodes internalin, which aids in the invasion of epithelial cells (Conte et al., 2002). *Hyl* encodes LLO, while *plc A* and *B* encode phospholipases, both of which aid in listerial phagosomal escape (Klarsfeld et al., 1994; Bubert et al., 1999). *PlcA* and *hyl* have balanced regulatory expression on pH_i to assure proper LLO and *PlcA* manufacture, thereby

creating an agreeable environment for survival, escape and spread (Dramsi et al., 1996; Vasquez-Boland et al., 2001; Conte et al., 2002). PlcA hydrolyzes phosphatidylinositol residues in the cytoplasm of the double membraned phagosome, while LLO, a cytolysin, forms pores in the membrane enabling escape. The actA gene is also under the control of prfA regulation (Chakraborty et al., 1992) through encoding in the LLO gene cluster region. The actin of the host is utilized in collaboration with the ActA protein (Kathariou, 2002), allowing actin-based intracellular motility of listerial cells to escape and invade other cells (Conte et al., 2002).

f. *Listeria monocytogenes* Zero-Tolerance

Under current regulations, any processing plant environmental samples or products themselves containing *L. monocytogenes*, are considered by the USDA Food and Safety Inspection Service (FSIS) to be adulterated and strongly recommends that these food items be recalled (Tompkin, 2002). According to both USDA and FDA law, there is a zero-tolerance policy pertaining to the detection of *L. monocytogenes* in beverage and foodstuffs. Despite the fact that *Listeria* is an ubiquitous organism and practically impossible to completely eradicate from processing environments, in reality this zero-tolerance policy cannot easily be attained. FSIS recognizes that *Listeria* is “reasonably likely to occur,” and even requirements for HACCP plans cannot fully contain the event of post-processing contamination (Tompkin, 2002). In agreement with the widespread nature of *Listeria*, Chen et al. (2003) have reported that a survey of over 31,000 ready-to-eat (RTE) retail food samples was performed, which showed an overall prevalence rate of 1.82% for these foods.

An example of the over bearing strictness of this law could be seen in the 1998 recall of 1.8 million pounds of sliced turkey luncheon meat and frankfurters. In this incident, the company in question tested positive for *L. monocytogenes* on a processing plant contact surface,

but not in the finished product. Despite, no cases of listeriosis being reported or laboratory confirmed, the entire line/lot of product was forced to be recalled causing negative sensationalism and loss of much profit for said company. Another disadvantage of product testing is that if a product is found to be positive, no information will have been gained to indicate what the mode of contamination was or how to prevent further occurrences (Tompkin, 2002).

In terms of the public and their safety, *Listeria* should not be a black and white issue. *L. monocytogenes* tolerances (recalls) should be based on the concentration, the number of cells present per gram of food or milliliter of beverage, with sampling frequency dependent upon consumer risk (as in RTE foods) (Tompkin, 2002). In the end, the beverage and food processing industry must rely heavily on prerequisite plans from farmer (GAPs) to processor (GMPs, SSOPs and HACCP programs) to aid in the battle for consumer safety.

B. *Escherichia coli* O157:H7

Escherichia coli is a Gram-negative, rod-shaped, mesophile (Sumner, 2002). The pathogen is extremely acid tolerant making it problematic in unpasteurized apple ciders and juices. It is a natural inhabitant of cattle intestines, causing much alarm in the ground meat industry and orchard environment, where run-off from adjacent cattle grazing areas may contaminate orchard fruit (Anonymous, 2001).

The infectious dose for *E. coli* O157:H7 is unknown, yet it appears to be very low (<1000 cells). The onset of symptoms, cramps and diarrhea, is typically within 24 to 48 hours. The disease can last 2-8 days and escalate to the telltale, grossly, bloody diarrhea and possible vomiting, even in healthy individuals (hemorrhagic colitis). If the infection leads to complications, the very young may develop hemolytic uremic syndrome (HUS, renal failure) or

the elderly may acquire thrombotic thrombocytopenic purpura (TTP, purplish blood clots), both of which have high mortality rates (USFDA, 2003).

In 1996 multiple outbreaks occurred due to unpasteurized apple juice and cider consumption. In Connecticut, the juice source was from a retail outlet with 8 cases (1 with HUS and 1 with TTP) and in the Western U.S. there were 66 cases, with the death of a child from HUS, also from a commercial brand juice (MMWR, 1997). Additionally in 1996, 9 U.S. states and British Columbia recalled Odwalla brand apple juice due to 45 cases, 12 of which developed HUS, with over 3 U.S. states and British Columbia having *E. coli* O157:H7 contaminated juice products (MMWR, 1996).

C. *Salmonella* species

Salmonella species contain over 2400 serotypes and like *E. coli*, it is a non-sporulating, Gram-negative rod, with flagellar motility, but it appears to have a higher infectious dose, more along the lines of 10^5 cfu/g or ml. This mesophilic pathogen is one of the leading causes of foodborne illness (Sumner, 2002).

Salmonella causes gastroenteritis, which is characterized by diarrhea, cramps, vomiting, dehydration and some fever. Symptoms are caused by the invasion of epithelial cells of the small intestine, leading to inflammation. This infection has a 6 to 48 hour onset, with a duration of 1 to 7 days depending on acuteness. In more severe chronic cases the infection may lead to bacteremia, meningitis, septicaemia, osteomyelitis, and abscesses. Following recovery, a human or animal may be an asymptomatic carrier of the bacterium, shedding it in feces for months (MMWR, 1999; Sumner, 2002; USFDA, 2003).

One of the reasons *Salmonella* is so prevalent in our food industry, besides its vast array of antibiotic genes and high heat resistance, the microorganism can be carried by swine, mice,

water, soil, lizards, insects, fowl, and humans alike giving it many opportunities to invade our food (Sumner, 2002).

Salmonella has been involved in a broad variety of food and juiceborne outbreaks over the past century. A notorious source of *Salmonella* besides poultry products is that of unpasteurized milk. As stated in the Morbidity and Mortality Weekly Report (2003b) during 1972-2000, a total of 58 raw milk-associated outbreaks were reported to the CDC, of which 17 (29%) were caused by *Salmonella* spp. Despite scientific knowledge that raw dairy products are a definite source of pathogens, such as *Salmonella* spp. and *L. monocytogenes*, 27 states still allow the sale of raw milk. Raw milk and its dairy products are similar to apple cider in that consumers perception of unprocessed beverages/foods are more nutritional, hence the continued efforts of organizations to allow the sale of raw milk to the public (directly from the farm).

Several outbreaks have been associated with unpasteurized orange juice containing *Salmonella*. In 20 states and 3 Canadian provinces in 1999 there were 423 illnesses resulting in 1 death. In 6 western states in 2000 there were 88 cases of *Salmonella* Enteritidis (USDHHS, 2001). The second largest *Salmonella* outbreak associated with unpasteurized orange juice was from commercially distributed Sun Orchard juice products. In 15 states and 2 Canadian provinces in 1999, there were 207 laboratory-confirmed cases of *Salmonella* serotype Muenchen reported, with an additional 91 cases still under investigation when this Morbidity and Mortality Weekly Report was posted (1999). Other juice related salmonellosis outbreaks include that of apple cider in 1974, where drop apples from a manure-fertilized orchard were used to make the product (MMWR, 1997).

D. *Cryptosporidium parvum*

Cryptosporidium parvum is an obligate intracellular parasitic protozoan. This single-celled animal is only metabolically active and able to reproduce during residence inside a host in the form of an oocyst. This parasite typically contaminates animals (wild, domesticated and agricultural). The hosts will shed these oocysts in their feces, leading to the infection of other humans and animals. This organism is susceptible to heat, drying and UV treatment and somewhat to freezing and frozen storage conditions. On the other hand, *Cryptosporidium*, in sporocysts form, is extremely resistance to most sanitizers (i.e., chlorine, bleach, iodine and sodium hydroxide substances) (USFDA, 2003).

Most cases of cryptosporidiosis involve contaminated fertilization of fresh fruits and vegetables or unsanitary infected food handlers preparing RTE meals. The cryptosporidiosis is characterized by acute (<10 infective organisms) intestinal, tracheal, pulmonary and muscle tissue complications. The tracheal disease leads to coughing; whereas, the intestinal form has symptoms of watery diarrhea, cramping and dehydration. Both of these are usually self-limiting in 2 days to 4 weeks (Anonymous, 2001; USFDA, 2003).

Cryptosporidiosis outbreaks are typically seen from improperly cooked pork and waterborne sources. An outbreak listed in the CDC's Morbidity and Mortality Weekly Report (1997) reported that in 1996 in New York, 20 cases (plus 11 suspected cases) resulted from contaminated apple cider and in 1993 *C. parvum* contaminated apple cider from the use of windfallen apples from an orchard adjacent to a cattle grazing area.

II. Fruit Juices

Consumer demand for freshly-squeezed juices (i.e., fresh, unpasteurized) is on the increase. As Yuste and others (2002) have said, research of alternatives in juice processing

methods has been stimulated due to consumer appeal for novel, natural and fresh-like beverages that are both safe and have improved nutritional and sensory characteristics.

A. Apple Cider

1. Apples

Early English settlers originally introduced apple trees to America. There are approximately 2500 known varieties of apples in the world, of which the U.S. grows about 100 varieties. However, 15 varieties account for over 90% of apples harvested in the U.S., with the top seven being: Red Delicious, Golden Delicious, Fuji, Granny Smith, Gala, Rome and McIntosh. The United States has about 7500 apple growers in 36 states, with the top six producers being: Washington, Michigan, California, New York, Pennsylvania and Virginia. The U.S. is the second largest producer of apples behind China. In fact, in the U.S., apple orchards were listed (2001) as covering the third most acres (430,200) of land after that of orange orchards and grape vineyards. Of these apples harvested, approximately 57% are eaten as fresh fruit and 41% are processed into apple products. Cider and apple juice accounts for 20% of the processed apples (Anonymous, 2002).

2. Processing

In the orchards, apple maturity is determined by the skin color, firmness and amount of sugar present. Once the apples are deemed harvest-worthy, most are picked by hand rather than mechanically. When the apples are crated and shipped to the processing facility, the raw fruit is visually inspected for its sanitary condition and apparent integrity. At this point, ideally, the fruit is brushed to remove external dirt, leaves and stems, followed by a water wash (flumed) to remove pesticides and insect filth. Occasionally cider producers will add chlorine-based sanitizers to the wash water to help control the microbial loads from further contaminating

recirculated water. After brushing, cleaning and sanitizing steps are performed, the fruit is conveyed to extractors (e.g., choppers, grinders or presses) where the juice is removed from the apple flesh. The size of the processor determines if the juice is immediately treated and bottled or if the raw-product will be held in a storage tank (non-continuous operation) until the appropriate amount of juice is pressed. Depending on the product type, further filtering, clarification or preservation of the juice may be needed. At this point, processors operating under new HACCP regulations would need to utilize an approved treatment to result in at least a 5-log reduction of *E. coli* O157:H7 in their product, whether by traditional heat pasteurization or new alternatives such as UV treatment, prior to addition of preservatives and bottling (Anonymous, 2003b).

There is no standard of identification for apple cider, differentiating it from apple juice. Cider is normally considered to be an opaque brownish colored fluid containing apple solids with a pH of around 3.3 to 4.1 (Mattick and Moyer, 1983). Fresh apple cider is a ready-to-drink product, which normally receives no processing to inactivate microorganisms during manufacturing.

Enhanced flavor, aroma and color are some of many reasons cider producers give to promote consumption of unpasteurized cider over its heat-treated counterpart, apple juice. Furthermore, the capital cost of pasteurization equipment (\$20,000-30,000) would put many smaller cider producers out of business (Higgins, 2002). These reasons are factors in the drive to find lower cost alternatives, such as UV light treatment, where heat is not a component in the process and where costs typically range from \$10,000-15,000 (Kozempel et al., 1998; Majchrowicz, 1999; Brown, 2001).

3. FDA Inspection of Apple Cider Manufacturers

The FDA performed an “Inspection of Fresh, Unpasteurized Apple Cider Manufacture” facilities in 1997. The report summary (USFDA, 1999b) stated that 237 sites were inspected in 32 states. According to the report, approximately 83% of processors grow their own apples. The typical annual volumes of cider sold per year varied greatly between 250 to 500,000 gallons. Sales mostly depended on the size of the company, with 53% selling 10,000 or less gallons of cider per year. The ratio of onsite and personally owned retail sites versus other local retail distribution establishments was 55:45 and over 84% was sold within their home state (USFDA, 1999b).

The FDA (USFDA, 1999b) determined that upon receipt of apples, 60% of processors did not perform a washing step. Fortunately, surveys revealed that before chopping apples for pressing, about 91% of processors culled poor quality apples.

Contaminants, whether pathogenic, spoilage or other substances on or in the apples, are released into the juice during pressing (Yuste et al., 2002). Immediately prior to pressing: 14% received no washing, 2% were not washed, but were dry-brushed, 22% were washed only (typically spray-washed, but sometimes float-tanked or flumed), 41% were both washed and brushed, and 21% were washed, brushed and sanitized with a chlorine solution prior to pressing. Additionally, 27% of the processors inspected used the preservatives potassium sorbate (63%) and sodium benzoate (27%) (USFDA, 1999b).

Pathogenic cider contaminants normally can be found in asymptomatic animal carriers such as agricultural (e.g., cattle, sheep, etc.) or wild grazing (e.g., deer, etc.) animals. Other sources of contamination are insects, rodents, birds, domestic animals and worker. Additionally, apples can be contaminated from manure treatments, unclean wash waters, and press clothes,

which are infrequently changed or cleaned. Also, since most cider producers store pressed juice in large vats, one compromised lot may result in contamination over several other batch-combined tanks (USFDA, 1999b).

Of the 237 cider processors surveyed, 72% admitted that either agricultural-animals grazed on adjacent properties or deer were found to freely graze in the orchards. During the inspections, it was noted that 33% of the firms were not using equipment in a sanitary manner, particularly when it came to press clothes and overall processing area cleanliness. Not surprisingly, 41% of the processing areas had open passageways, if not three-walled warehouses, and open finished product vats without protection from contamination via insects, birds and other animals. It was reported that about 25% of the 237 cider producers had deficiencies in employee hygiene, including smoking and eating in the processing area, inadequate toilet and handwashing facilities, and unsuitable attire (USFDA, 1999b).

Other sources of apple contamination, that are usually not detected through culling or remedied with external washing, have been demonstrated in research where micro-holes in the skin or entrance through the blossom end allows microbes to migrate through the healthy apple flesh. When contamination occurs, the only effective treatment allowed to reduce pathogens is to pasteurize the product with heat, or use an approved alternative such as ultraviolet light (UV) (USFDA, 1999b).

B. Grape Juice

1. Grapes

Grapes are one of the top two fruits in the United States, as determined according to vineyard acreage (Anonymous, 2003a). Grapes typically have a ripeness peak in October, which is determined by skin color, firmness, sugar content and titratable acidity. Harvests are 99%

mechanical, despite their fragile nature, grapes are vibrated from their stems into troughs, which direct the fruit to boxes. After harvest, grapes are transported to the processing plant where they are federally inspected to ensure that the standards of identity for color and sugar solids content are met.

2. Processing

According to the Concord Grape Association (Anonymous, 2003a), approved grapes are conveyed to a destemming hopper where paddles push the grapes through agitating cylinders containing grape-size holes, which lead to a collecting trough. Next, the grapes and their juices are pumped into receiving tanks and a heat treatment is applied. Unlike apple cider, heat processes are desired in grapes, since it brings out the rich color and full flavor of the fruit. This heat-treated slurry is then pumped into hot, agitating collection tanks for transfer to dejuicers, which screen out seeds, skin, and pulp, allowing the juice to flow into another collecting tank where it is heated to 185° F to inactivate pathogenic or spoilage organisms. Following heat treatments, the juice is chilled in refrigerated tanks to near the freezing point of the juice. Finally, the juice proceeds through two additional pasteurization steps, pre- and post-bottling, to ensure safety and shelf-stability. Remnants (seeds, skins and pulp) are sometimes utilized as vineyard mulch; therefore, grape processing leaves no waste products (Anonymous, 2003a).

C. Orange Juice

In Florida, orange crops, depending on the variety, bloom in the early spring and mature during the winter months. Ripeness is determined through acidity and °Brix testing to judge when to harvest the fruit. Unlike grapes, 98% of Florida orange orchards are harvested manually with traditional wooden ladders and canvas-pick sacks. Once the tree-ripened citrus is harvested, the fruit is shipped to processing plants where the oranges are conveyed through wash flumes,

culled, graded and separated according to size. Orange juice quality is determined by acid-to-sugar ratio tests where the °Brix is normally between 8.5 and 10.0 (Townsend, 2000) and the pH ranges from 3.4 to 4.0 (MMWR, 1999). Next the citrus peel is pricked to extract oils that have a detrimental effect on juice flavor, followed by juice extraction. Screens then remove seeds, which can be used with the peel in cattle feed. Depending on the level of pulp-turbidity, finishing screens further remove pulp. Juice is categorized as “Not From Concentrate” or “Frozen Concentrated.” Frozen concentrated orange juice is vacuum-evaporated, pasteurized and chilled to about 10° F, until reconstituted with filtered, chilled water. Recovered essence oils can be re-added as flavor enhancers. All finished products are typically piped to tank farms where juice is stored until shipment to packagers or blending processors (Townsend, 2000).

III. Processing

A. Pasteurization

Pasteurization is considered by some to be detrimental to the flavor, aroma, color and nutritional qualities of apple cider, orange juice and other juice beverages. Additionally, pasteurization requires heat energy, which leads to higher costs and, according to some, reduced juice quality. In addition to sensory changes in the juice, heat can induce non-enzymatic browning through Maillard reactions of carbohydrates, amino acids and proteins in juices like apple cider. Heat treatment, followed by storage, may result in a loss of phenolic compounds in grape juices and ascorbic acid (Vitamin C) in orange juice (Anonymous, 2003c).

There are two primary methods of thermal pasteurization, one is full pasteurization of 76 to 99° C for a few seconds up to one minute; whereas, the other technique is a lighter pasteurization of 66 to 75° C for about only 10 to 16 seconds. The full pasteurization is utilized in the production of shelf-stable juices while lighter pasteurization is typically used for juices

that are intended to be refrigerated (Alwazeer et al., 2002). The primary function of pasteurization is to eliminate pathogens, whereas the secondary goal is to inactivate spoilage microorganisms. Another role for this treatment is the stabilization of cloud in beverages such as orange juice or apple cider by inactivating the enzymes pectinmethylesterase and polyphenoxylase. Regardless of the desire for minimum thermal processing by producers (to reduce costs) and consumers (due to nutrition and flavor concerns) alike, all pasteurization must meet standards to ensure that pathogens are inactivated. Ultimately the pasteurization method chosen must be sufficient to inactivate 5 logs of *Salmonella* spp., *E. coli* O157:H7, or *L. monocytogenes* in accordance with FDA HACCP regulations (Shearer et al., 2002).

B. Alternatives

Currently, there are a number of efforts aimed at developing processing alternatives that meet the recent FDA HACCP regulations for a 5-log reduction in the pertinent pathogen in juices. A few of these alternative technologies include: high-hydrostatic pressure, electric resistance (ohmic) heating, ultrafiltration, carbon dioxide, aseptic packaging, electromagnetic fields, pulsed light, pulsed electric field, ozone, irradiation, freezing and thawing, ultraviolet light, hot water rinses of apples, antimicrobial preservatives, etc. (Friedman et al., 1987; Chaundry et al., 1998; USFDA, 1999b).

The FDA allows UV light (under food additives regulations) as an approved technique for the 5-log reduction of pertinent pathogens in juice. An added benefit of UV processed juice is that it may also double to triple the shelf-life of refrigerated juices as compared to untreated, unpasteurized juice. Most UV juice processing machines require a thin film flow (approximately 0.00003 inches thick) to allow for uniform exposure of the juice to UV light. More recent UV technologies are being developed to allow treatment of more viscous and pulpy-solid containing

juices such as carrot blends and home-style orange juice (Higgins, 2002). But in terms of labeling, UV-treated juice products cannot be labeled with the terms “fresh” or “pasteurized.” To do so would constitute misbranding according to section 403a of the Food, Drug and Cosmetic Act (USFDA, 1999b).

In recent studies, it has been shown that on a consumer panel, there was no significant difference detected in the fresh versus UV treated cider samples; whereas, thermally pasteurized cider was much less preferred in terms of flavor and color (Choi, 2001). In other studies performed, it was discovered that UV treated juice had a slight decrease in °Brix over a week of storage, but when treated with ozone the cider had significantly lower °Brix and sedimentation occurred (Choi, 2001).

When the effects of carbon dioxide application on juices was studied, it was reported that CO₂ does inactivate pathogens (Haas et al., 1989; Lin et al., 1994; Isenschmid et al., 1995), especially when used in conjunction with high pressure treatment. This combination allowed for the time and temperature of processing to be reduced versus that of pasteurization, with minimized nutrient degradation (Ballestra et al., 1996).

Erkman (2000) tested the survival of *L. monocytogenes* cells at 35° C in carrot (pH 5.65), orange (pH 3.98), apple (pH 3.58) and peach (pH 3.35) juices. In peach juice, *L. monocytogenes* was reduced by half a log within 4 hours, but in orange and apple juice 8 hours was needed to achieve an equivalent reduction in *Listeria*. Carrot juice showed no reduction in the populace of *L. monocytogenes* during the 12 hour testing period. In a study performed by Jordan et al. (2001), *L. monocytogenes* cells in apple juice (pH 3.5) held at 4° C for 24 hours showed < 0.5 log cfu/ml reduction, but when cells were treated with high pressure (250-300 MPa) a significant inactivation of the cells was seen during the storage period. This lead to the conclusion that

pressure and acidic environments vastly increase the rate of injury in cells. Additionally, no *Listeria* cells were detected in apple, orange or tomato juice with treatments of 300 MPa pressure followed by 24 hour storage at 4° C or after pressurization alone at 350 MPa, depicting a 5-log reduction (Jordan et al., 2001).

IV. Prevention and Control Measures

A. Contamination Sources

Many sources of pathogenic contamination may be prevented with simple yet effective use of control measures. Water is an important reservoir of microorganisms. Juice processing water should be potable, with regular testing, particularly if water is recirculated. Re-use of processing water may lead to a build up of nutrients and therefore microbial load, which can be remedied with monitored levels of antimicrobials or sanitizing substances such as chlorine compounds. Manure is another likely origin of pathogenic bacteria. Most orchards do not utilize raw manure, but those who do should use treated manure with application only during pre-growing/harvesting months. One of the most problematic causes of contamination of fruit with pathogens is that of animals which may be carriers of foodborne disease causing pathogens. When agricultural animals are kept in grazing areas adjacent to or in the line of rain run-off or when wild animals freely roam the orchards, the pathogens they carry (naturally in their intestines and spread through the fecal route) may contaminate the fruit. Following basic guidance of GAPs directs growers to ensure that no agricultural animals graze on adjacent fields, wild animals do not roam the orchards, and domestic animals also do not have access to the area. In terms of animals, one may also include that of pests, where a control program should be in place. Pests may be rodents, birds, lizards or insects and should be blocked from not only the orchard, but especially the produce storage and processing areas (USFDA, 1998).

B. Cleanliness

In addition GAPs including the use of treated manure, potable water and the blockade of animals and pests, other guidelines exist such as Good Manufacturing Practices (GMPs) and Sanitation Standard Operation Procedures (SSOPs) (Keller et al., 2002). Once the fruit has been harvested, the inspection process should emphasize the use of good quality fruits, which will lessen the likelihood of batch contamination; culling and removal of damaged and poor quality fruit is appropriate. When inside the storage and packing facility, clean bins, pallets and temperature-regulated storage conditions should be maintained at appropriate levels (USFDA, 1998).

As produce is readied for processing, the fruit should be thoroughly cleaned. Between batches, all equipment should be washed and sanitized, especially cider press clothes (Keller et al., 2002). Juice should be refrigerated, bottled and sealed, as soon as possible with minimal standing time in tanks. Also, no matter the packaging material, it needs to be maintained in a secure and clean environment as well (Anonymous, 2001).

Another source of contamination is that of the workers. Employee hygiene must be strictly enforced. These practices include hand-washing technique, placement of toilet facilities in orchards and processing areas, use of gloves and disinfectants, proper attire, and surveillance of illness since it has been shown that ill workers shed pathogens and viruses in their feces, which can lead to the contamination of foods and beverages (USFDA, 1998).

C. Niches

Processing areas have what are called “niches” in there environment; removing or inactivating all contaminants by cleaning and sanitizing properly is practically impossible in these sites. These niches become reservoirs of disease causing microbes, which can disperse into

the food or beverage product during processing. Niches may include places such as: worn rubber seal, switches, metal-on-metal parts, insulation, conveyor rollers, floor drains, etc. These niches may also harbor biofilms, which allow bacteria to adhere to surfaces trapping nutrients and promoting survival (Tompkin, 2002).

V. Regulations

A. Juice HACCP

The FDA estimates that there are between 16,000 to 48,000 cases of illness each year due to juiceborne contamination (USDHHS, 2001). These statistics led the FDA to begin working on a plan to improve juice safety. In 1997 the FDA consulted with the National Advisory Committee on Microbiological Criteria for Foods, held public meetings, opened a comment forum, and published a “Notice of Intent to Develop a HACCP Program, Interim Warning Statement, and Educational Program.” In 1997 the FDA, in the Federal Register, issued a voluntary request to the juice industry asking them to label their fresh, unpasteurized juices with a warning. Following that statement only 18% of processors utilized labels (USFDA, 1999a; USFDA, 1999b). These actions resulted in the January 2001 FDA final rule announcement in the Federal Register (66 FR 6138), where fruit and vegetable juice processors (and repackagers) must utilize a HACCP program during processing (21 CFR 120) (USDHHS, 2001; USFDA, 2002a).

Large and small processors must have complied by January of 2002 and 2003, respectively; whereas, very small firms still have until January 20th, 2004 to abide by these new regulations. The definition of “very small” businesses is defined as having “annual sales of less than \$500,000, total annual sales greater than \$500,000 but total food sales of less than \$50,000, or operations that employ fewer than an average of 100 full-time equivalent employees and sell

fewer than 100,000 units of juice in the U.S.” as stated by the Inspecting Topics for Apple Cider website (Anonymous, 2001). Until all the FDA HACCP implementations have been met and all facility inspections are completed, (very small) processors must continue to use the previously required warning label statement (USDHHS, 2001).

Federally mandated HACCP systems are already implemented in the meat, poultry and seafood industry. The FDA defines HACCP as “a science-based analysis of potential hazards, determination of where the hazards can occur in processing, implementing control measures at points where hazards can occur to prevent problems, and rapid corrective actions if a problem occurs.” A key part of HACCP plans are that of record keeping and verification processes. Under these new juice HACCP regulations, processes utilized by juice producers must be approved to attain a 5-log reduction, as compared to untreated juice, in the “pertinent” or most resistant pathogen to that particular juice product, including juice and juice ingredients in a beverage (21 CFR 120.1(a)). There are a few exemptions to the HACCP requirement, producers of shelf stable juice, retail businesses where juice is made and sold on-site, and those that already utilize thermal pasteurization to their products. Exceptions to the new HACCP regulations include, processors that make shelf-stable juices/concentrates utilizing a single thermal processing step meeting 3-A Accepted Practices (603-06) and retail establishments, such as juice bars, that make, package and sell directly to consumers on-site or through Internet sales (Anonymous, 2001; USDHHS, 2001).

In July of 1998, the FDA published in the Federal Register (63 FR 37030) the final ruling requiring a warning statement on unpasteurized juices, as a measure towards furthering public safety until HACCP principles could be instilled. As of September 1999, all fruit and vegetable juices that do not have an approved 5-log pathogen reduction treatment, were required to label

the product with a specific warning in addition to complying with mandated HACCP regulations by their company size and pre-determined date (USFDA, 1999b). “WARNING: This product has not been pasteurized and, therefore, may contain harmful bacteria that can cause serious illness in children, the elderly, and persons with weakened immune systems” (USFDA, 2002b). “The warning label on fresh vegetable juice has made a bigger impression on consumers than the warning label on cigarette packages. Our volume has dropped drastically because of that label,” says Dominic Marlia, California Day-Fresh’s quality assurance manager (Higgins, 2002).

GAPs by the grower are not considered to be part of HACCP controls for a juice processor (66 FR 6138 at 6166). For most fruits and vegetables (except citrus), the entire 5-log reduction treatment, whether one step or a combination of results, must be performed on the expressed juice at a singular facility. In terms of citrus fruit, a processor may utilize a surface treatment as part of the 5-log reduction goal, on undamaged, tree-picked, culled, washed and cleaned citrus fruits only (66 FR 6138 at 6171) (USFDA, 1999b; USFDA, 2002a).

B. Definition of Juice

New FDA HACCP regulations for juices include those sold as such or used as an ingredient in beverages. Their definition of juice is “the aqueous liquid expressed or extracted from one or more fruits or vegetables, purees of the edible portions of one or more fruits or vegetables or any concentrates of such liquid or puree.” Items related to fruit juices but not included in these HACCP rulings are fruit flavored candies containing juice as an ingredient, carbonated beverages, which are not defined as a juice (though the juice flavoring itself would fall under HACCP regulations), juice-flavored coffees and teas, pulp not used within another beverage product, citrus oils, and juice concentrates that are not intended for beverages (sweeteners and flavorings) (USFDA, 1999b).

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**Survival of *Listeria monocytogenes* in Fruit Juices
During Refrigerated and Temperature-Abusive Storage**

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INTRODUCTION

Consumers today are concerned with nutritional value, taste, aroma and the cost of juice products (Yuste et al., 2002). In the United States, juice is a multi-billion dollar industry, that relies heavily on fruit orchard farmers' Good Agricultural Practices, Sanitation Standard Operating Procedures, and Good Manufacturing Practices, to produce high quality juice products (Keller et al., 2002). Most juices today, whether shelf-stable or refrigerated are thermally pasteurized to achieve at least a 5-log reduction in the pertinent pathogen of the juice being processed (Shearer et al., 2002).

Traditional pasteurization is expensive (~\$20,000) and beyond the fiscal ability of many small fruit juice processors (Higgins, 2002). Some consumers and processors believe that pasteurization is detrimental to juice quality. Therefore, juice-processing technologies that may serve as an alternative to thermal pasteurization are being investigated. Methods, such as UV light, high-pressure and use of antimicrobials, are currently under investigation as to whether they can provide juice with a 5-log reduction in pertinent pathogens without losing nutritional value or the desired sensory characteristics of unpasteurized juice (USFDA, 1999b).

Escherichia coli O157:H7 and *Salmonella* are recognized as the pertinent pathogens in apple cider/apple juice and orange juice products, respectively. Previous to the 1990s, processors relied on the high acidity, refrigerated temperatures and inherent or added organic acids and preservatives present in the juice to inhibit pathogen survival or growth (Datta and Benjamin, 1997). However, certain pathogenic bacteria have evolved acid-adaptive mechanisms that allow them to tolerate the acidic environment of juices through the typical shelf-life of the products (Cotter et al., 2000).

Even though *Listeria monocytogenes* has not been implicated in any outbreaks of juice-

borne disease, the pathogen has been isolated from unpasteurized apple juice (pH 3.78) and an apple/raspberry juice blend (pH 3.75) (Sado et. al., 1998). Additionally, *Listeria* has recently been implicated in several foodborne disease outbreaks associated with Ready-To-Eat foods such as turkey luncheon meat, smoked fish, hot dogs, soft cheeses and raw milk (MMWR, 1985, 1992, 1998a, 1998b, 2000, 2001 and 2002a). Furthermore, listeriosis is a severe disease with a 30-40% mortality rate, among pregnant women and their fetuses, the very young, elderly or immune-compromised individuals. Listeriosis may result in flu-like symptoms, abortion, pneumonia, endocarditis, bacteremia, septicemia, meningitis, and death (Farber and Peterkin 1991; USFDA, 2003).

This study was conducted to show that *L. monocytogenes* is capable of survival during refrigerated storage of fruit juice. Further research is needed in order to determine the risk posed by *L. monocytogenes* in fruit and vegetables.

MATERIALS & METHODS

Strains and preparation of inoculum –

Six strains of *Listeria monocytogenes* (D43, V7, Scott A, LCDC, Brie and ATCC 19115) were used in this study. All cultures are held in the Virginia Polytechnic Institute and State University culture collection.

Cultures of each strain were grown separately in Tryptic Soy Broth (Difco; Becton-Dickinson; Sparks, MD) supplemented with 0.6% Yeast Extract (TSBYE) at 32° C and transferred at 24-h intervals. Broth cultures of each strain were combined to obtain a mixed culture containing equal proportions of the six strains. Equal portions of the individual strains were mixed and centrifuged (10,000 x g, 10 min). The spent culture medium was decanted, and the cell pellet was resuspended in 0.1% Bacto Peptone water (PW) (Difco; Becton-Dickinson; Sparks, MD) prior to inoculation of juices, giving a cell suspension of approximately 9 log cfu/ml.

Confirmatory Testing –

Each strain was grown separately in TSBYE at 32° C with three consecutive 24-h interval transfers, streaked onto Modified Oxford Agar (MOX) (Difco; Becton-Dickinson; Sparks, MD) and incubated for 48-h at 32° C. A typical isolated colony from each MOX plate was transferred by streaking, to a new MOX plate, which was then incubated at 32° C for 48-h. Gram staining was performed at 24-h to confirm the presence of small, rod-shaped, Gram-positive bacteria. Motility testing was performed by inoculating each strain using the stab method into a tube containing Sulfide Indole Motility Medium and was observed for umbrella-shaped growth

following incubation at ambient temperature (~20° C) for 24-h. Finally, *Listeria* API (BioMerieux; Hazelwood, MO) tests were performed on each strain with incubation at 32° C for 24-h followed by administration of test reagents according to manufacturer directions.

Inoculation of fruit juices –

Shelf-stable, pasteurized red grape (pH 2.21; Brix 15.7°) and white grape juices (pH 3.15; Brix 16.0°) and refrigerated, pasteurized orange juice (pH 3.85; Brix 11.7°) were purchased from a local grocery store. Shelf-stable, pasteurized, unfiltered apple cider (pH 3.64; Brix 11.2°) was purchased from a small, local cider producer. The red and white grape juices were stored at room temperature, while the orange juice and apple cider were stored at a refrigerated temperature (4° C) prior to experimentation.

Upon opening each new juice container, °Brix (Riechart ABBE Mark II Plus refractometer Model 10480; Pittsburgh, PA) and pH (Fisher Accumet® pH meter 10 with Accumet probe; Pittsburgh, PA) measurements were performed. Final °Brix and pH measurements were also taken at the final sampling of each replication, at the respective storage temperature.

Two days prior to *L. monocytogenes* inoculation, 500 ml of each juice was transferred to a sterile 500 ml Wheaton screw-top bottle, containing a sterile stir bar. Each juice was stored at the appropriate testing temperature (4° C or 24° C; Low Temperature Incubator 815 by Precision Scientific; Chicago, IL or 10° C; Fisher Scientific Low Temperature Incubator Model 307; Pittsburgh, PA), respectively. On Day zero, juices were inoculated with 5 ml of the mixed *L. monocytogenes* cell suspension and stirred (Allied Fisher Scientific Thermix® Stirrer, Model 220; Pittsburgh, PA) for 2 min prior to sampling. The initial concentration of *L. monocytogenes*

in each inoculated juice was approximately 7 log cfu/ml.

Bacteriological analysis –

A sample (5 ml) was withdrawn prior to inoculation; concentrations of background microflora were determined from each new juice bottle. The sample was neutralized (pH 6.5 to 7.5) with 1 N NaOH (Fisher Scientific; Fairlawn, NJ) and serially diluted in 0.1% PW. A portion of each neutralized, uninoculated sample was surface plated (0.1 ml) onto Tryptic Soy Agar (Difco; Becton-Dickinson; Sparks, MD) supplemented with 0.6% Yeast Extract (TSAYE) and MOX, as controls to determine the presence of background microflora.

Samples (5 ml) were withdrawn at intervals according to the experimental design for this study (Appendix). Red grape juice was sampled at 1-h intervals for up to 5-h (4, 10 and 24° C), apple cider every other day for up to 12 days (4, 10 and 24° C), white grape juice every other day (4 and 10° C) and daily (24° C) for up to 24 days, and orange juice every fourth day (4 and 10° C) and every other day (24° C) for up to 61 days.

At each sampling period, a sample (5 ml) was withdrawn, neutralized (pH 7) with 1 N NaOH, serially diluted in 0.1% PW, and surface plated (0.1 ml) onto TSAYE and MOX. All samples were plated in duplicate and incubated for 48-h at 32° C (Precision Scientific Gravity Convention Incubator Model 4L; Chicago, IL). All media were prepared according to manufacturer specifications.

When surface-plated growth of *L. monocytogenes* was shown to be less than 1 log cfu/ml, serial dilutions were no longer utilized and neutralized samples (10 ml) were placed in *Listeria* Enrichment Broth (40 ml) (LEB) (Difco; Becton-Dickinson; Sparks, MD) and incubated at 32° C for 24-h. Following enrichment, a loop full of LEB sample was streaked for isolation onto MOX

and incubated at 32° C for 48-h. When three consecutive enrichment samples were negative for growth on MOX, sampling of that particular juice/temperature combination was discontinued.

Non-neutralized sample comparison –

In addition to neutralized samples, three days of each overall sampling period (white grape juice and apple cider Days 0, 2 and 10; orange juice Days 0, 2 and 12) were selected to perform non-neutralized studies. An extra sample (5 ml), non-neutralized, was serially diluted and surface plated (0.1 ml) onto TSAYE and MOX to compare recovery of *L. monocytogenes*. All samples were plated in duplicate and incubated for 48 h at 32° C.

Statistical analysis –

All experiments were performed in triplicate. Recovery of *L. monocytogenes* by direct plating was statistically analyzed using the mixed procedure (PROC MIXED) of SAS version 8.1 (SAS Institute, Cary, NC). The experimental design was a randomized block design, with nested treatment arrangement, and repeated measures with sampling blocked on replication. Means were separated using Least Square Means; significant differences are defined at $P < 0.05$.

RESULTS & DISCUSSION

The juices utilized in this study were pasteurized; therefore, they were expected to have low initial background microflora. Neither background microflora (aerobic, non-fastidious mesophiles) nor *L. monocytogenes* were detected (i.e., populations were < 10 cfu/ml) in the four juices when surface plated on TSAYE. If detectable growth was found on the control plates the juice was considered contaminated and was not utilized for experimentation. However, if growth of microflora occurred during incubation of inoculated juices this data was disregarded except when spoilage dates were compared.

There were no statistically significant changes observed in the pH or °Brix values of any juices during the duration of the study. The red grape juice was sampled over a period of 0 to 5 hours. For the red grape juice, the initial and final pH was 2.21 and the beginning and ending °Brix values were 15.7. The white grape juice had an average initial pH of 3.15 and °Brix of 16.0 with a final pH 3.14 and °Brix of 15.8. The initial and final pH of apple cider were 3.64 and 3.57, respectively. For the apple cider, the initial and final °Brix values were 11.2 and 10.9. The beginning and ending pH for the orange juice was 3.85 and 3.80, respectively, and the initial and final °Brix values were 11.7 and 11.3, respectively. Likewise, other researchers such as Roering et al. (1999) found no significant changes in degrees Brix or pH values during incubation of juice. In their experiment, the survival of *L. monocytogenes* (initial inoculum: 7 log cfu/ml) was compared in preservative-free apple cider (pH 3.3-3.5) during storage at 4 and 10° C for up to 21 days.

In the current study, initial inoculum levels of *L. monocytogenes* in each juice were approximately 7.0 log cfu/ml, with a limit of detection of 1.0 log cfu/ml due to the plating

scheme implemented. Consequently, populations that were undetectable via direct plating represented an ~ 6 log cfu/ml reduction.

Regardless of juice type or incubation temperature, *L. monocytogenes* recovery was not influenced by media, showing no statistical difference between media (TSAYE and MOX) ($P>0.05$). Therefore, no appreciable sublethal acid injury of *L. monocytogenes* was detected. Although not statistically significant, differences between recovery on TSAYE and MOX for red grape juice (Figures 1-3) and apple cider (Figures 4-6) are notable. *Listeria monocytogenes* populations in red grape juice, as determined by plating onto TSAYE, were consistently higher with regard to average log cfu/ml than MOX by approximately 1.5 log cfu/ml. For apple cider, TSAYE was better for recovery than MOX by approximately 2 log cfu/ml over the same sampling period. For white grape juice (Figures 7-9) and orange juice (Figures 10-12), recovery on TSAYE and MOX were similar ($P>0.05$). Despite the lack of statistical difference, the somewhat higher recovery rate of *Listeria* on TSAYE versus MOX may suggest practical microbiological importance, where a small portion of the cells did develop acid injury.

In terms of detection of *L. monocytogenes*, traditional microbiological techniques require much time (e.g. approximately a week). Recommended incubation periods, in addition to enrichment periods cause crucial delay in reporting of results. Not only is time an issue, so is the use of non-selective versus selective media. If both media are utilized, acid injury may be determined. If only a selective media (such as MOX) is used, there is increased risk of false negative results and viable but injured cells remaining in the product, that may recover under certain conditions, present a health threat. All of these factors must be considered when evaluating a product or environmental sampling program in industry settings.

Non-neutralization studies were conducted (at the start of each new repetition) to

determine if there was any significant difference between plating inoculated juices at their respective pH or at a neutral pH. Over the entire sampling period, three samples were additionally duplicated (at Day 0, 2 and 10 in white grape juice (Figures 7-9) and in apple cider (Figures 4-6); Days 0, 4 and 12 for orange juice (Figures 10-12)) and sampled identically, with the exception that they were not neutralized with NaOH. This comparison was conducted to determine if sampling methodology influenced population estimates. According to the statistical interactions, there were no significant differences in acidic versus neutralized sample plating ($P>0.05$).

As expected, the first order variable, time, was significant in all cases independently, i.e., all juices at all temperatures tested had statistically significant log cfu/ml reduction in *L. monocytogenes* over time ($P<0.05$). For the juices (white grape juice, apple cider and orange juice) (Figures 4-12), the average log cfu/ml reduction was also statistically significant for the different sampling periods. Based on each individual temperature for all juice types, the nested interaction of juice within time was statistically significantly different ($P<0.0001$), but the first order variables, for the 10 and 24° C juices were not significantly different in the main (neutralized) study ($P>0.05$), yet all were in the non-neutralized study ($P<0.05$), in terms of number of sampling days required to achieve undetectable levels.

When comparing each juice at its respective storage temperature over the entire sampling period, *L. monocytogenes* populations in red grape juice (Figures 1-3) decreased by 3.5-5.5 log cfu/ml after 5 hours as determined on TSAYE and MOX. *Listeria monocytogenes* counts in white grape juice (Figures 7-9) were reduced by >6.5 log cfu/ml on both media used over a 4 (24° C) to 12 day (4 and 10° C) sampling period. Populations of *L. monocytogenes* in apple cider (Figures 4-6) decreased by >5.0-6.5 log cfu/ml on TSAYE and MOX at 4, 10 and 24° C, over 14,

24 and 12 testing days, respectively. *Listeria monocytogenes* populations in orange juice (Figures 10-12) were shown to have been reduced by >6.5 log cfu/ml on both media over a 44 (10 and 24° C) to 54 day (4° C) sampling period.

After storage for 5 h, *L. monocytogenes* populations in red grape juice at 4° C (Figure 1) decreased by approximately 5.5 and 5.0 log cfu/ml as determined on TSAYE and MOX, respectively. Over the 12 days sampling period, *L. monocytogenes* in white grape juice at 4° C (Figure 7) decreased by 6.5 log cfu/ml on both media used. *Listeria* populations in apple cider at 4° C (Figure 4) decreased by >5.0 log cfu/ml on TSAYE and MOX over 14 days. *L. monocytogenes* populations in orange juice at 4° C (Figure 10) decreased by >6.5 log cfu/ml on both media over a 54 day sampling period.

Listeria monocytogenes populations in red grape juice at 10° C (Figure 2) decreased by 5.25 and 4.75 log cfu/ml after 5 h as determined on TSAYE and MOX, respectively. *Listeria* in white grape juice at 10° C (Figure 8) decreased by >6.5 log cfu/ml on both media used, over the 12 day testing period. Populations of *L. monocytogenes* in apple cider at 10° C (Figure 5) decreased by >6.0 log cfu/ml on both TSAYE and MOX over 24 days. *Listeria* populations in orange juice at 10° C (Figure 11) decreased by >6.5 log cfu/ml on both media utilized at 10 days, with a <1.5 log cfu/ml stationary recovery (averaged over three repetitions) over a 44 day sampling period before reaching the detection limit.

During storage, *L. monocytogenes* populations in red grape juice at 24° C (Figure 3) decreased by 3.5 log cfu/ml after 5 h as determined on TSAYE and MOX. *Listeria monocytogenes* in white grape juice at 24° C (Figure 9) decreased by >6.5 log cfu/ml on both media over the 4 day sampling period. *Listeria* populations in apple cider at 24° C (Figure 6) decreased by >6.5 log cfu/ml on TSAYE and MOX over 12 testing days. Populations of *L.*

monocytogenes in orange juice at 24° C (Figure 12) decreased by >6.5 log cfu/ml on both media over a 44 day sampling period.

Generally, inactivation of *L. monocytogenes* in the fruit juices was influenced by storage temperature, following the order: 24° C > 10° C > 4° C, with the exception of apple cider (Figures 4-6) where inactivation followed the order: 24° C > 4° C > 10° C. Survival of *L. monocytogenes* in juices incubated at 4 and 10° C was significantly better than at 24° C (P<0.05). This finding is similar to reports on *Salmonella* and *E. coli* O157:H7 that revealed greater survival of these pathogens in juices at temperatures near refrigeration than at higher temperatures (Williams et al., 2001).

Sorrells et al. (1989) found that in acidified media broth, *L. monocytogenes* survived better at 10° C than at 25° C, a finding that supports the current study. Although unable to grow at 4° C, the psychrotrophic *L. monocytogenes* reportedly grew at 35° C and 25° C. The typical optimum incubation temperature for *Listeria* is approximately 32° C. The results of this study depict enhanced death rates at ambient temperature (24° C) versus that of refrigeration (4 and 10° C). Conner et al. (1990) research showed that inoculating *L. monocytogenes* into TSBYE acidified with different organic or inorganic acids to pH 4.0-6.0 and incubating at 30 or 4° C, revealed differences in inhibitory pH, depending on acid type: acetic acid pH 4.5, citric acid pH 4.0, hydrochloric acid pH 4.0, lactic acid pH 4.5 and propionic acid pH 5.0. In other studies performed by the same group, *L. monocytogenes* was inactivated at a much higher rate at 30° C, than at 10° C, but it did not grow at either temperature at low pH. The results of the current study correlates with the work of Conner and others (1990), where viable cells of *L. monocytogenes* were recovered over a greater storage period at 10° C versus 30° C.

Depending upon the incubation temperature of a bacteriological medium acidified with

hydrochloric acid, the minimum pH for *L. monocytogenes* growth ranges from 4.39 to 5.23 (George et al., 1988; Roering et al., 1999). Roering and associates (1999) reported the survival of *L. monocytogenes* in both pasteurized and unpasteurized preservative-free apple cider (pH 3.3-3.5), during storage at 4 and 10° C, at up to 21 days. *Listeria* populations decreased below the plating detection limit (1 log cfu/ml) within 2 days under all conditions tested (Roering et al., 1999). In other studies performed by Roering et al. (1999), survival of *L. monocytogenes* in pasteurized versus unpasteurized cider stored at 4 and 10° C and inoculated with approximately 6 log cfu/ml, resulted in undetectable counts by Day 2 in both ciders. However, the 4 and 10° C unpasteurized cider showed sporadic recovery of *L. monocytogenes*, by direct plating or enrichment, throughout the 21 and 7 day storage periods, respectively. Pasteurized cider stored at 4 and 10° C had no viable *L. monocytogenes* cells after 7 and 2 days, respectively (Roering et al., 1999).

In studies by Yuste and Fung (2002), *L. monocytogenes* inoculated (~4.5 log cfu/ml) into apple juice (pH 3.7), plated on TSA and stored at 5 and 20° C, was not detectable at Days 3 and 1 of sampling, respectively. Considerable acid injury was detected on MOX at Day 1 and Hour 1. When Yuste and Fung (2002) performed a similar experiment, but with the juice adjusted to pH 5.0, the Day 7 testing threshold counts of *L. monocytogenes* were reduced at 5° C at Day 3 to 7 from 4.12 to 2.78; whereas, in the 20° C juice, growth was seen at Day 1 on, with Day 7 having 5.85 log cfu/ml. Their *L. monocytogenes* experiment utilizing pH 3.7 apple juice is contradictory to the current study, with *L. monocytogenes* surviving in apple cider (pH 3.6) for 14 days at 4° C and 12 days at 24° C. These observations may be due to the particulate matter in the unfiltered cider used for the current study, possibly acting as protection for the cells.

Overall, results of the current study were more similar to Yuste et al. (2002), as they

employed similar conditions to the Yuste and Fung (2002) experiment protocol, but studied the slightly more acid tolerant *E. coli* O157:H7. When apple juice inoculated with *E. coli* O157:H7 (~4.2 cfu/ml) was stored at 5 and 20° C, *E. coli* survived until approximately Day 14. In the current study, *L. monocytogenes* in apple cider decreased by >5.0 and >6.5 log cfu/ml in 14 and 12 days, respectively. In another study, it was reported that apple cider allowed the survival of *E. coli* O157:H7 for 21 days (Miller and Kaspar, 1994) at 4° C. Researchers Uljas and Ingham (1998) reported that *E. coli* O157:H7 was more likely to survive in acidic beverages at refrigeration rather than ambient temperatures.

The orange juice utilized in the current study was an “original-style” that contained a normal amount of pulp. This orange juice also contained added ascorbic acid (Vitamin C). The pulp may provide a favorable environment for *Listeria* attachment. Alternatively, the inherent ascorbic acid in the orange juice (pH 3.8) may have aided in the acid-adaptation of *Listeria*, thereby developing a temporary acid tolerance.

Overall, *L. monocytogenes* decreased at the slowest rate in orange juice, at 4 and 10°C (Figures 10 and 11) incubation temperatures, allowing a much longer survival of *L. monocytogenes* than at 24°C (Figure 12). A greater than 5 log cfu/ml reduction of *L. monocytogenes* was observed at Day 12 for the 24°C incubation temperature, and Days 40 and 56 for the 10 and 4°C incubation temperatures, respectively.

Parish (1998) reported that *Listeria* does not proliferate in orange juice of a pH < 4.4. Parish and Higgins (1989) showed that *Listeria* populations in orange juice stored at 4° C were reduced by almost 6 log cfu/ml in 25 days (pH 3.6) and 43 days (pH 4.0). They reported that in addition to storage temperature, pH is key in determining the duration of Listerial survival. Whereas, in the current study, *L. monocytogenes* populations declined by > 6.5 log cfu/ml over a

54 days of sampling. In other studies, it was reported that *L. monocytogenes* Scott A at 30° C grew at pH 4.7. Sorrells et al. (1989) reported growth of *L. monocytogenes* at 10° C at a minimum of pH 4.4. When George et al. (1996) analyzed *L. monocytogenes* in laboratory media (with acetic or lactic acid) over a period of 50 days at 20° C, it reportedly grew at pH 4.3 but not pH 4.0. At 4, 8 and 12° C there was growth at pH 5.0 but not at pH 4.5 and finally at 1° C there was reported to be growth at pH 5.4 but not pH 5.0 during the same 50 day study to predict growth models (George et al., 1996).

Listeria monocytogenes did not grow in any of the juices at the conditions tested. Based on each individual juice type and all three storage temperatures, all the juices were very significantly different ($P < 0.0001$) in terms of the nested temperature within time interaction. *Listeria monocytogenes* populations were reduced in all juices at all storage temperatures; however, the rate of reduction was influenced by the pH of each juice type and storage temperatures.

In the white grape juice, at 4° C (Figure 7) and 10° C (Figure 8) *L. monocytogenes* entered the death phase around Day 9; whereas, at 24° C (Figure 9) average death phase began at Day 4. The statistics show that for the different temperatures the log cfu/ml reduction is significantly different for different sampling times ($P < 0.0001$). For the apple cider, *L. monocytogenes* populations reached the limit of detection at Days 24, 12 and 14 for temperatures 4, 10 and 24° C (Figures 4-6), respectively. In spite of the difference in reduction rates among temperatures, the average log cfu/ml reduction was not significantly different for the different sampling times ($P > 0.05$) in apple cider. Lastly, *L. monocytogenes* populations in orange juice began to rapidly decrease towards the detection limit around Day 12 reaching undetectable levels at Day 44 for the 24° C juice (Figure 12). However, *L. monocytogenes* was unrecoverable via

direct plating at 54 and 44 days storage for 4° C (Figure 10) and 10° C orange juice (Figure 11), respectively. In the orange juice, the three storage temperatures were significantly different, as the average log cfu/ml reduction was different over the sampling period. Overall, *L. monocytogenes* decreased at the slowest rate in orange juice at 4 and 10°C (Figures 10 and 11) incubation temperatures, allowing a much longer survival of *L. monocytogenes* than at 24°C (Figure 12). A greater than 5 log cfu/ml reduction of *L. monocytogenes* was observed at Day 12 for the 24°C incubation temperature, and Days 40 and 56 for the 10 and 4°C incubation temperatures, respectively.

When enrichments were performed on white grape juice, apple cider and orange juice samples (Table 1), there was found to be a large difference in *L. monocytogenes* survival (enrichment times) in apple cider (24° C) and orange juice (24° C) only; whereas the remaining juices had consistent enrichment times. No enrichments were performed on red grape juice samples due to the rapid inactivation of *L. monocytogenes* (i.e., less than 6 hours). Enrichments were performed until three consecutive samples streaked onto MOX were negative for typical *L. monocytogenes* growth. For all the white grape juice repetitions, at all temperatures tested, there were a consistent number of enrichment days (four). In white grape juice, no sporadic recovery of Listerial cells was seen at any storage temperatures once populations reached the detection limit (i.e., 1 log cfu/ml). Similar enrichment consistency was observed in orange juice with days of enrichment ranging from 4 to 10, with the exception of the first repetition of 24° C orange juice. This sample was enriched 65 days (sampled 111 days) until the study was finalized before the juice plating revealed three sequential enrichments resulting in no growth, due to the sample being depleted (less than 4 ml remaining). As for the apple cider, the enrichments were not agreeable in terms of temperature or repetition. Enrichment was conducted from 4 to 20 days,

with a notable deviation in the first repetition of the 24° C apple cider being enriched 69 days (sampled 82 days) prior to reaching three successive results of no growth before the study was concluded (Table 1).

Background microflora, such as spoilage yeast, lactic acid bacterium, and molds were not initially detectable in the control juices. In this study, the red grape juice did not spoil or become contaminated during its brief use. None of the juices (white grape juice, apple cider and orange juice) had spoilage at 4° C. The third repetition of the white grape juice at 10° C spoiled two days prior to enrichment in LEB. Spoilage appeared as particulate growth throughout with multi-colored mold at the surface of the juice. All three repetitions of 24° C white grape juice spoiled three days before enrichment began. As for apple cider, the first 24° C repetition spoiled three days before the start of enrichment, where the juice separated with unfiltered solids collecting on the bottom, a serum center, and a whitish growth on top, followed later by the formation of a mold mat. Both repetitions one and three of the 10° C apple cider also spoiled. Repetition three showed signs of spoilage the day enrichment began, with no visible mold, but a separated juice body with precipitate at the bottom of the container. The first repetition of the 10° C apple cider spoiled differently in that this contamination did not occur until almost three weeks into the enrichment period of testing. When considering the orange juice spoilage, there was no separation, mold or floating particles, but there was a color change from yellow-orange to orangish-brown. This occurred in repetition one of the 10° C juice two days prior to enrichment. Also, discoloration was seen in all three duplications of the 24° C orange juice. In the first repetition color change was noticed approximately a month prior to the start of enrichment, while the other two duplications had browning only slightly more than a week after enrichment had begun. This browning reaction may be due to an ascorbic oxidation reaction.

Lactic acid bacteria, especially *Lactobacillus plantarum* and *Leuconostoc spp.*, and yeast such as *Saccharomyces cerevisiae* and *Candida spp.*, are acid-tolerant and have been reported to spoil citrus juices before and after pasteurization. Even though yeast is typically the most adaptive spoilage organism in juice, lactic acid bacteria usually are predominant in number. Industry's problematic issues with spoilage organisms primarily pertain to shelf-life time-frames and production of undesirable, buttermilk-like off flavors, which can occur in juices containing relatively low spoilage populations. This spoilage contamination may result in a fermented flavor (byproduct of organic acids and ethanol), production of diacetyl, bulging of the juice container from CO₂ presence, all leading to decreased shelf-life and juice quality (Alwazeer et al., 2002).

In some juice conditions, the spoilage organisms may have had a protective benefit or an inhibitory action towards the inoculated *Listeria* cells. The inherent background microflora of foods and beverages is typically expected to have a competitive advantage over pathogenic contaminants. As summarized by Samelis et al. (2001), this struggle for balance may affect intrinsic factors (i.e., pH, A_w, nutrient uptake and by-product manufacture) of both microorganism forms, whether through suppression, no affect, or enhancement of survival and proliferation.

Orange juice and apple cider contain organic compounds such as organic acids, sugars and pectic substances, which may react with the pathogen causing a delay in inactivation, depending upon the storage temperature and pH of the juice. Several researchers (Davis et al., 1996; Phan-Thanh et al., 2000; O' Driscoll et al., 1997) have reported that studies involving acid adaptation and/or acid tolerant mutants are shown to have an increased virulence enabled through the Acid Tolerance Response (ATR) mechanisms of pathogens such as *L.*

monocytogenes, *E. coli* O157:H7 and *Salmonella spp.* Roering et al. (1999) studied enteric, pathogenic bacterial survival in the low pH environment of simulated gastric fluid (SGF; pH 1.5) at 37° C to mimic actual stomach conditions and determine the survival rates. When incubated in SGF, *S. Typhimurium* DT104 and *L. monocytogenes* were inactivated within 5 and 30 minutes (5.5- 6.0 log cfu/ml reduction), respectively. However, within 2 hours *E. coli* O157:H7 decreased by only 1.6 - 2.8 log cfu/ml (Roering et al., 1999). All factors of juice (inherent spoilage organisms, composition, pH, storage-temperature and processing treatment) and the pertinent, enteric, pathogenic bacteria particular to the individual juice should be taken into consideration in respect to providing the public with safe beverages for consumption.

Table 1**Final Sampling Day
Giving Positive Results for *Listeria monocytogenes***

Juice Temperature/Type	Replication 1	Replication 2	Replication 3
4° C Apple Cider	Day 16	Day 23	Day 20
10° C Apple Cider	Day 38	Day 35	Day 22
24° C Apple Cider	Day 19	Day 66	Day 12
4° C White Grape	Day 12	Day 12	Day 12
10° C White Grape	Day 10	Day 10	Day 10
24° C White Grape	Day 3	Day 3	Day 3
4° C Orange Juice	Day 61	Day 51	Day 51
10° C Orange Juice	Day 42	Day 40	Day 40
24° C Orange Juice	Day 111+	Day 14	Day 10

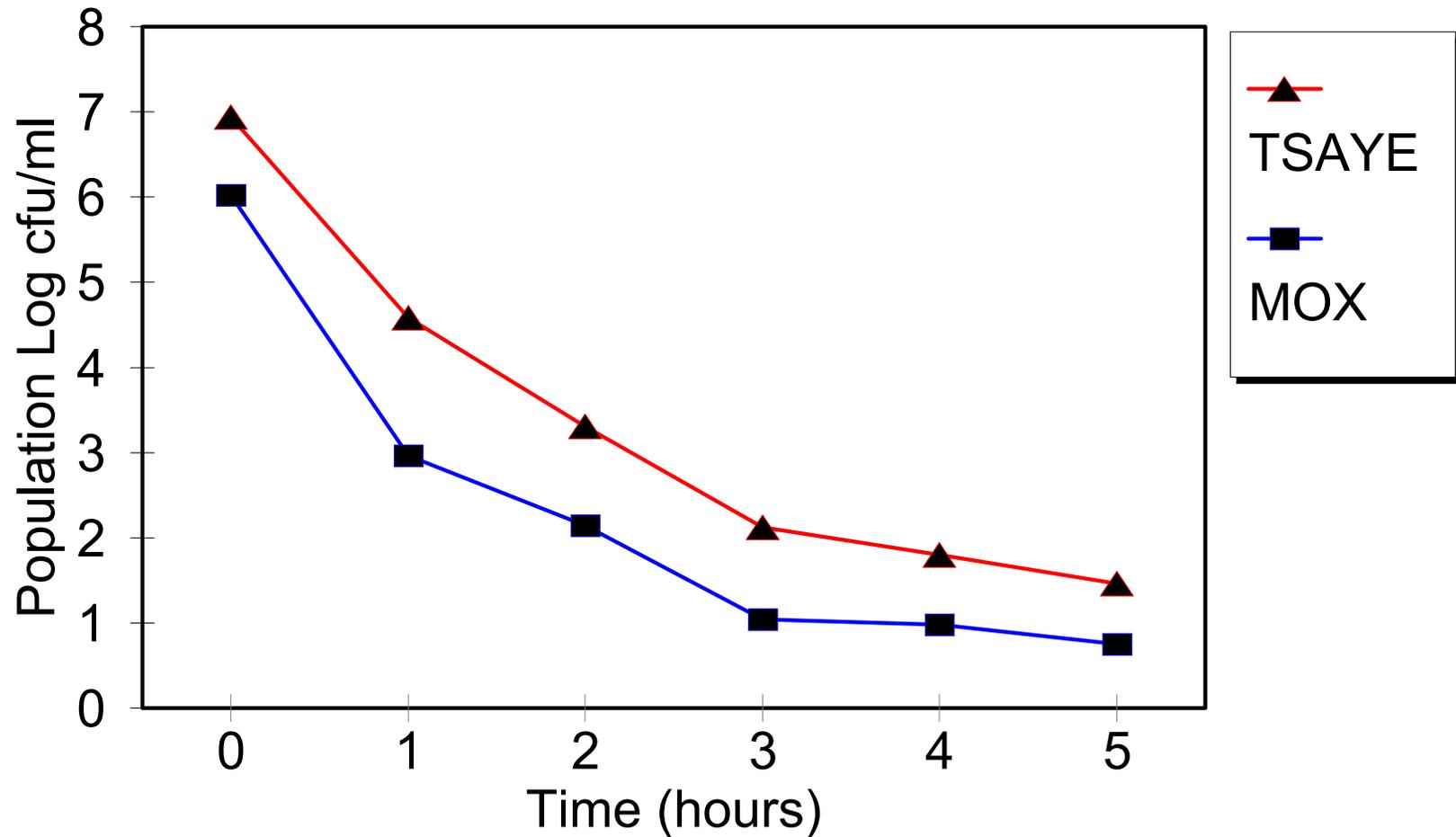


Figure 1: Fate of *Listeria monocytogenes* during refrigerated storage (4°C) in pasteurized red grape juice as determined by recovery on Tryptic Soy Agar supplemented with Yeast Extract (TSAYE) and Modified Oxford Agar (MOX). Limit of detection: 1 log cfu/ml. n=3

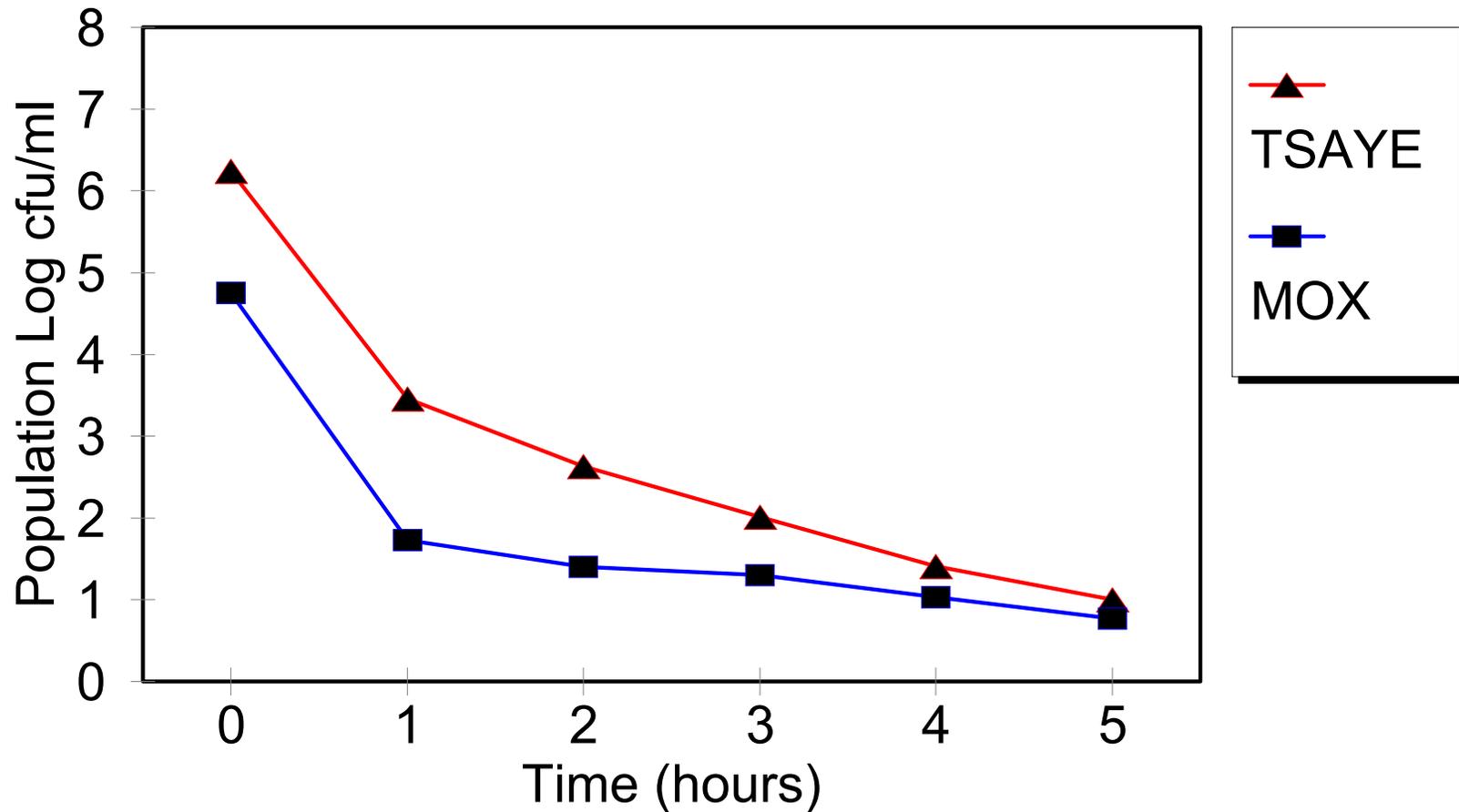


Figure 2: Fate of *Listeria monocytogenes* during abusive-refrigerated storage (10°C) in pasteurized red grape juice as determined by recovery on Tryptic Soy Agar supplemented with Yeast Extract (TSAYE) and Modified Oxford Agar (MOX). Limit of detection: 1 log cfu/ml. n=3

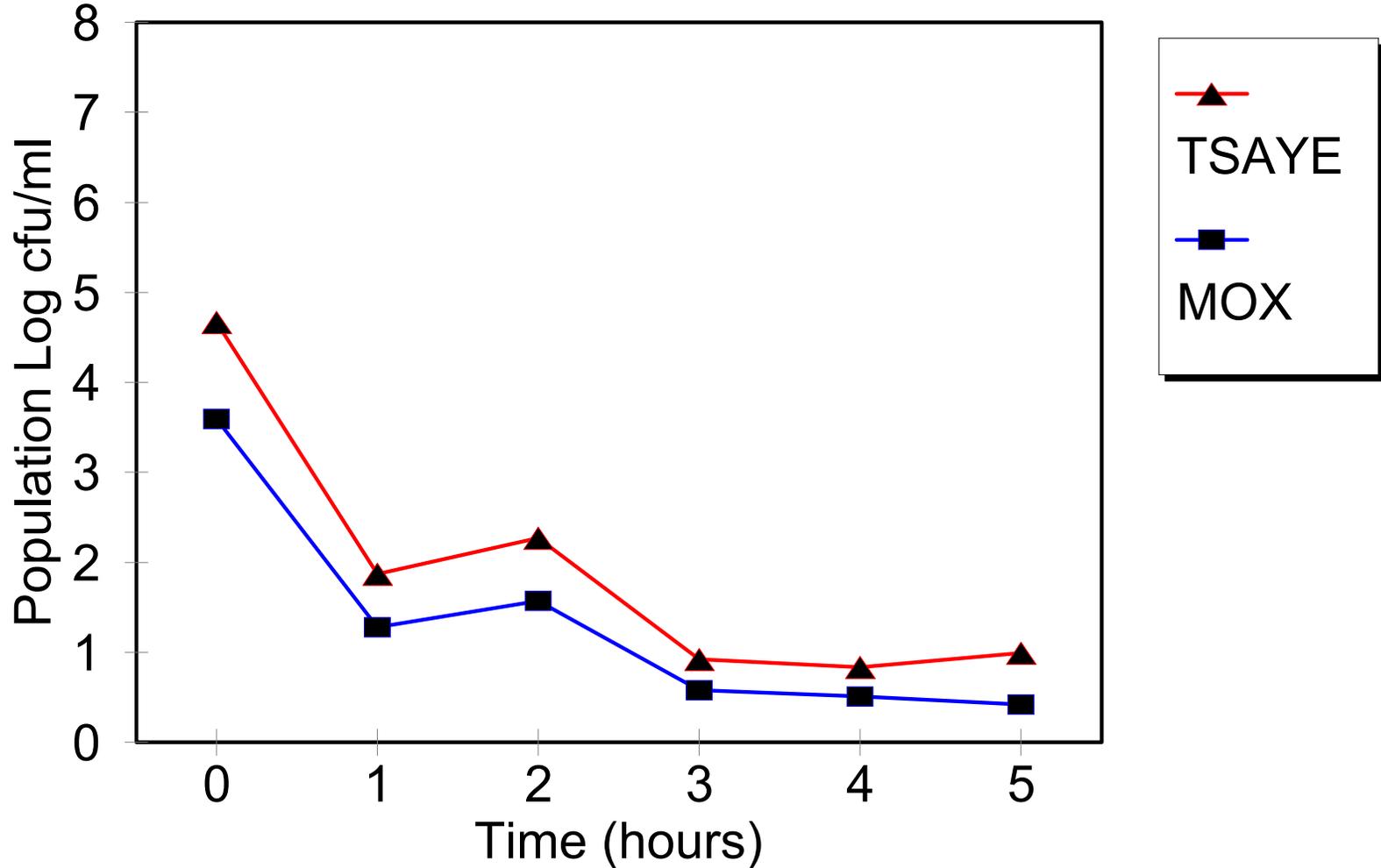


Figure 3: Fate of *Listeria monocytogenes* during ambient storage (24°C) in pasteurized red grape juice as determined by recovery on Tryptic Soy Agar supplemented with Yeast Extract (TSAYE) and Modified Oxford Agar (MOX). Limit of detection: 1 log cfu/ml.

n=3

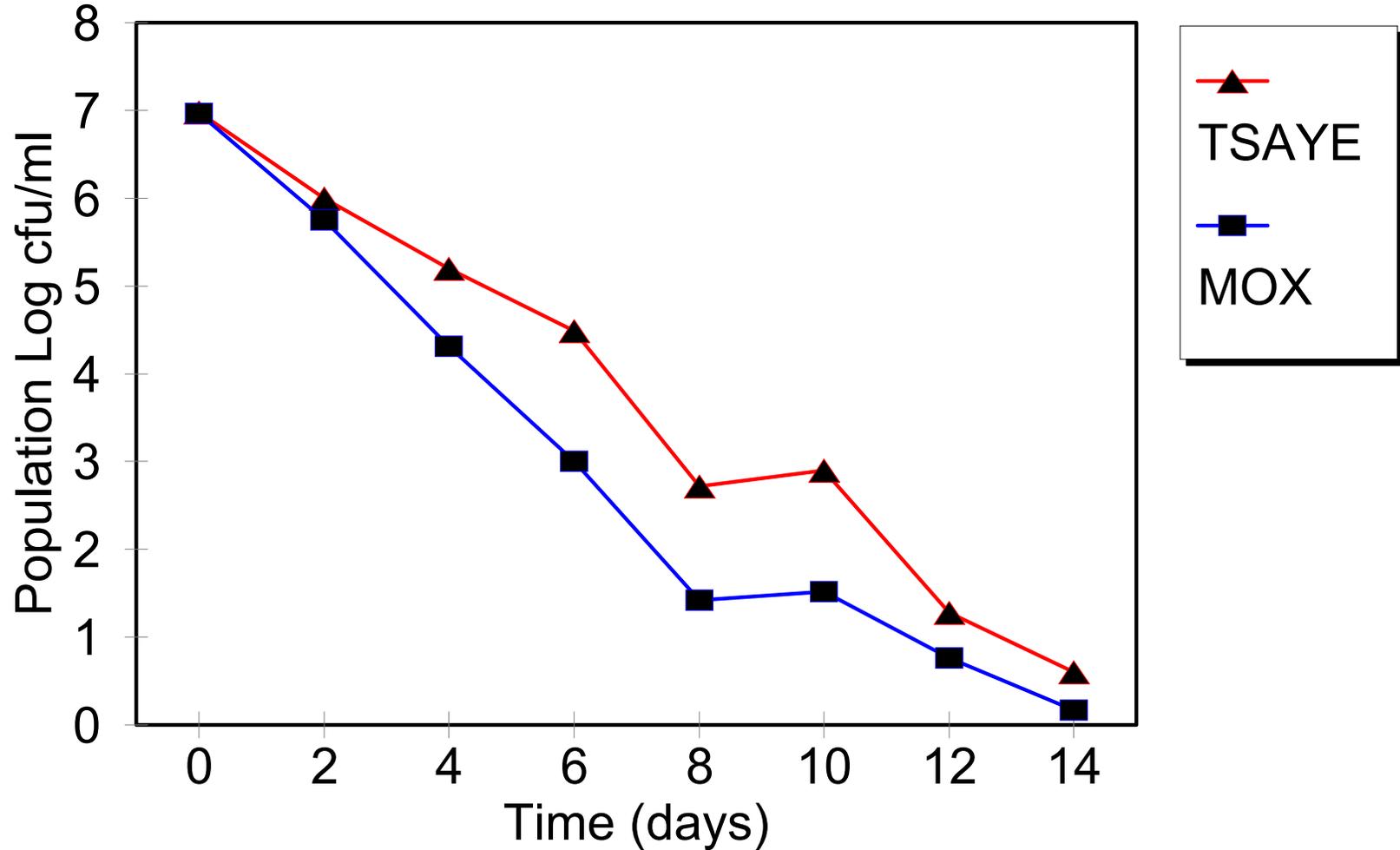


Figure 4: Fate of *Listeria monocytogenes* during refrigerated storage (4°C) in pasteurized apple cider as determined by recovery on Tryptic Soy Agar supplemented with Yeast Extract (TSAYE) and Modified Oxford Agar (MOX). Limit of detection: 1 log cfu/ml.

n=3

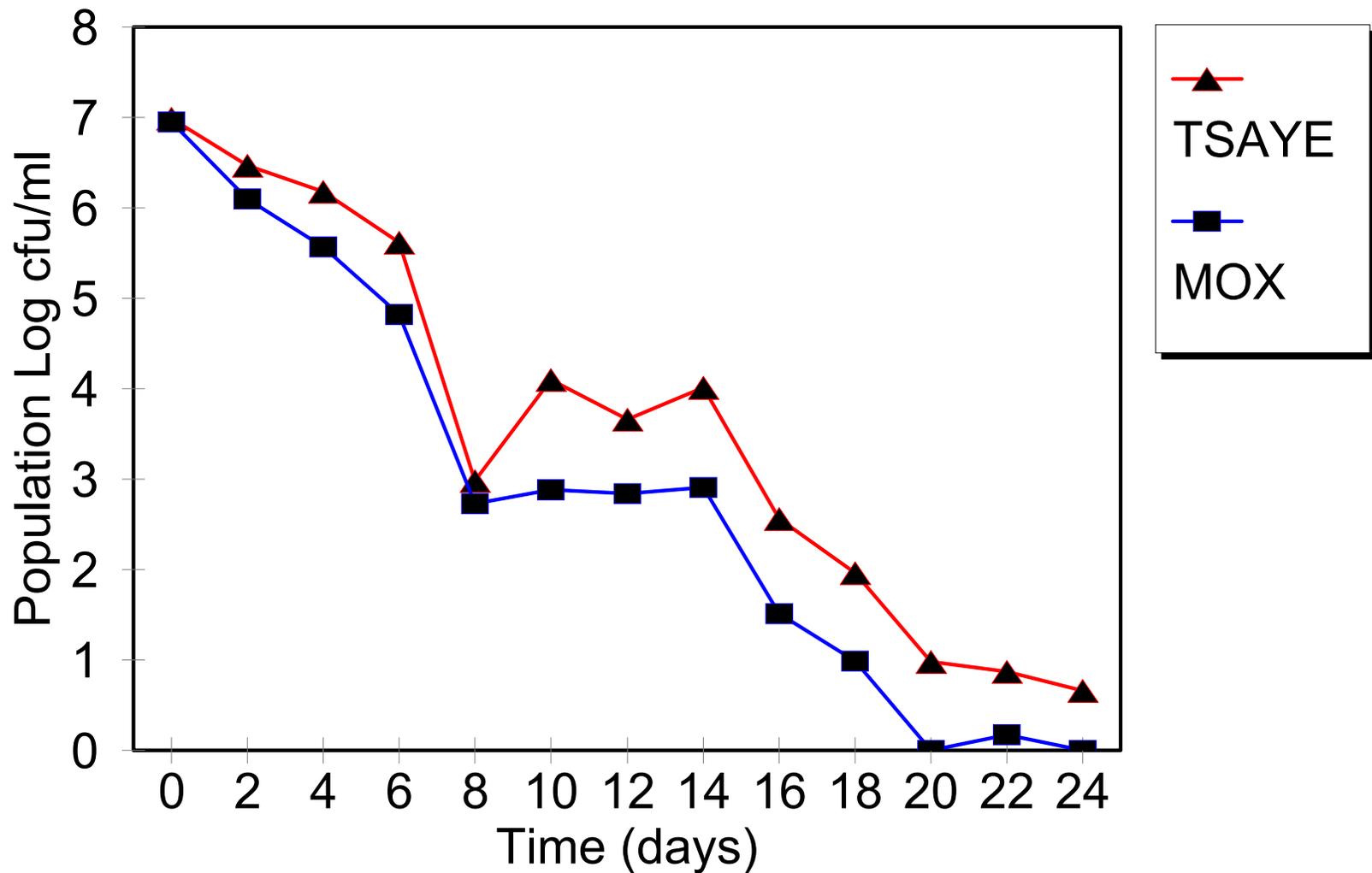


Figure 5: Fate of *Listeria monocytogenes* during abusive-refrigerated storage (10°C) in pasteurized apple cider as determined by recovery on Tryptic Soy Agar supplemented with Yeast Extract (TSAYE) and Modified Oxford Agar (MOX). Limit of detection: 1 log cfu/ml. n=3

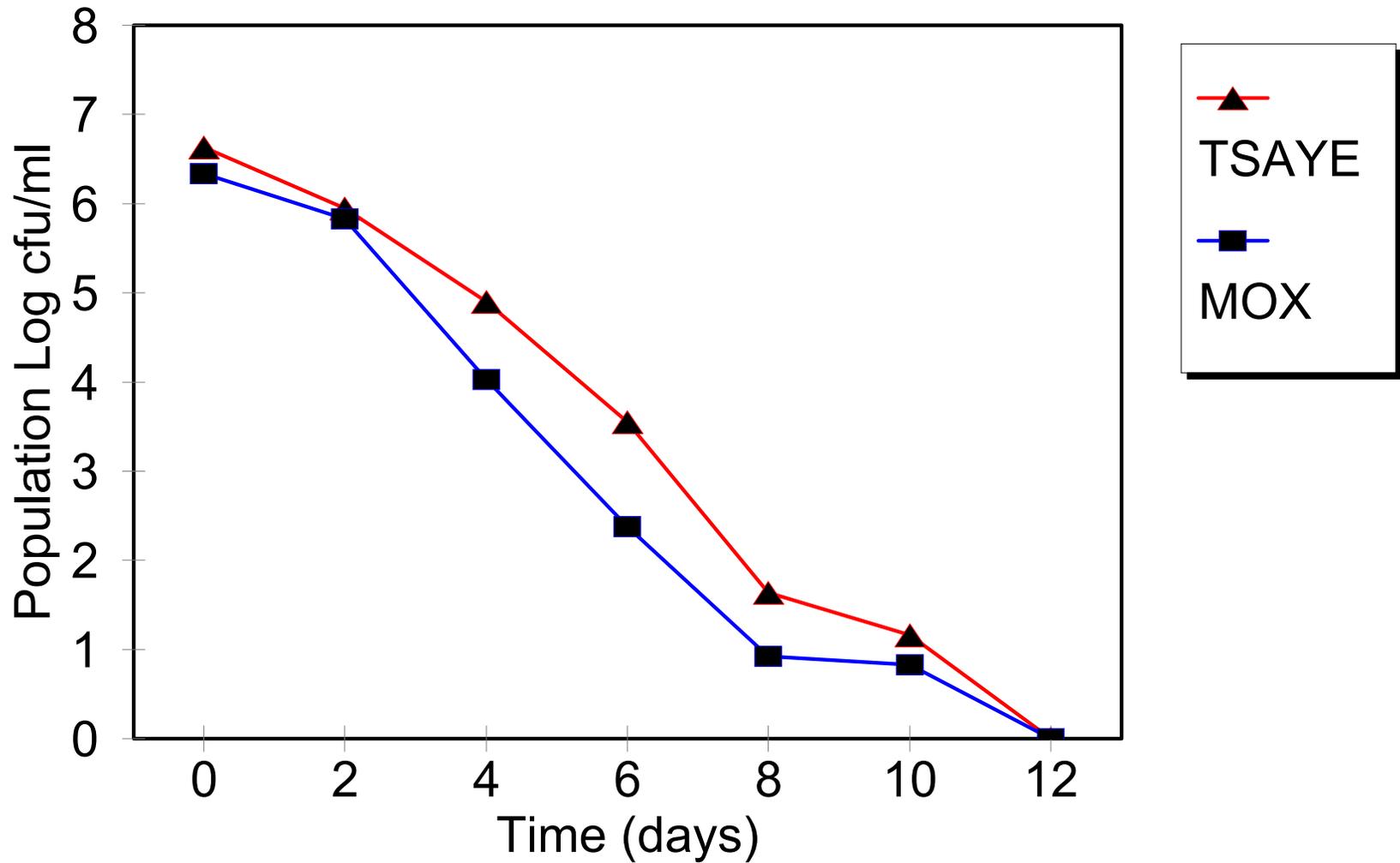


Figure 6: Fate of *Listeria monocytogenes* during ambient storage (24°C) in pasteurized apple cider juice as determined by recovery on Tryptic Soy Agar supplemented with Yeast Extract (TSAYE) and Modified Oxford Agar (MOX). Limit of detection: 1 log cfu/ml. n=3

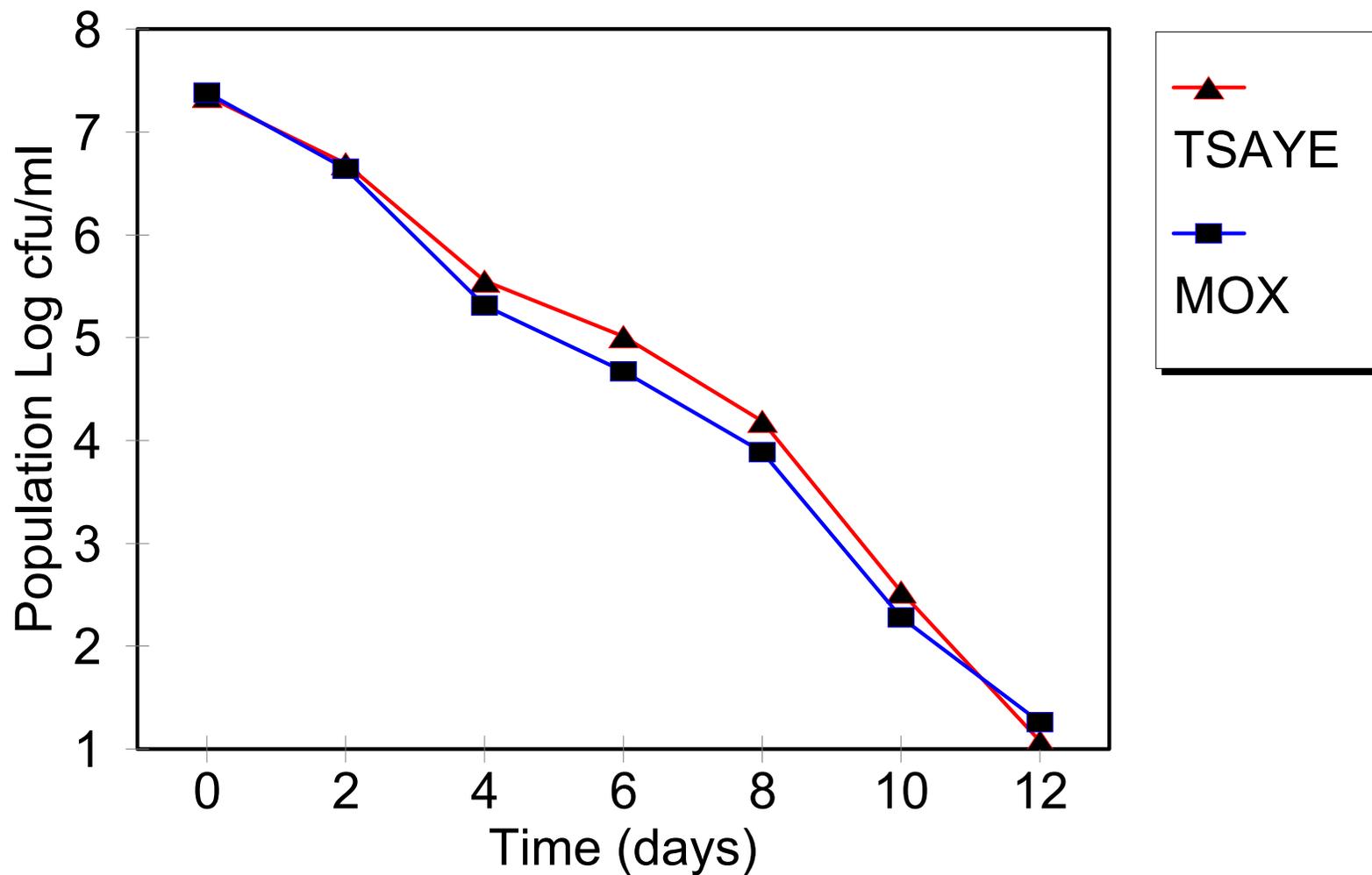


Figure 7: Fate of *Listeria monocytogenes* during refrigerated storage (4°C) in pasteurized white grape juice as determined by recovery on Tryptic Soy Agar supplemented with Yeast Extract (TSAYE) and Modified Oxford Agar (MOX). Limit of detection: 1 log cfu/ml. n=3

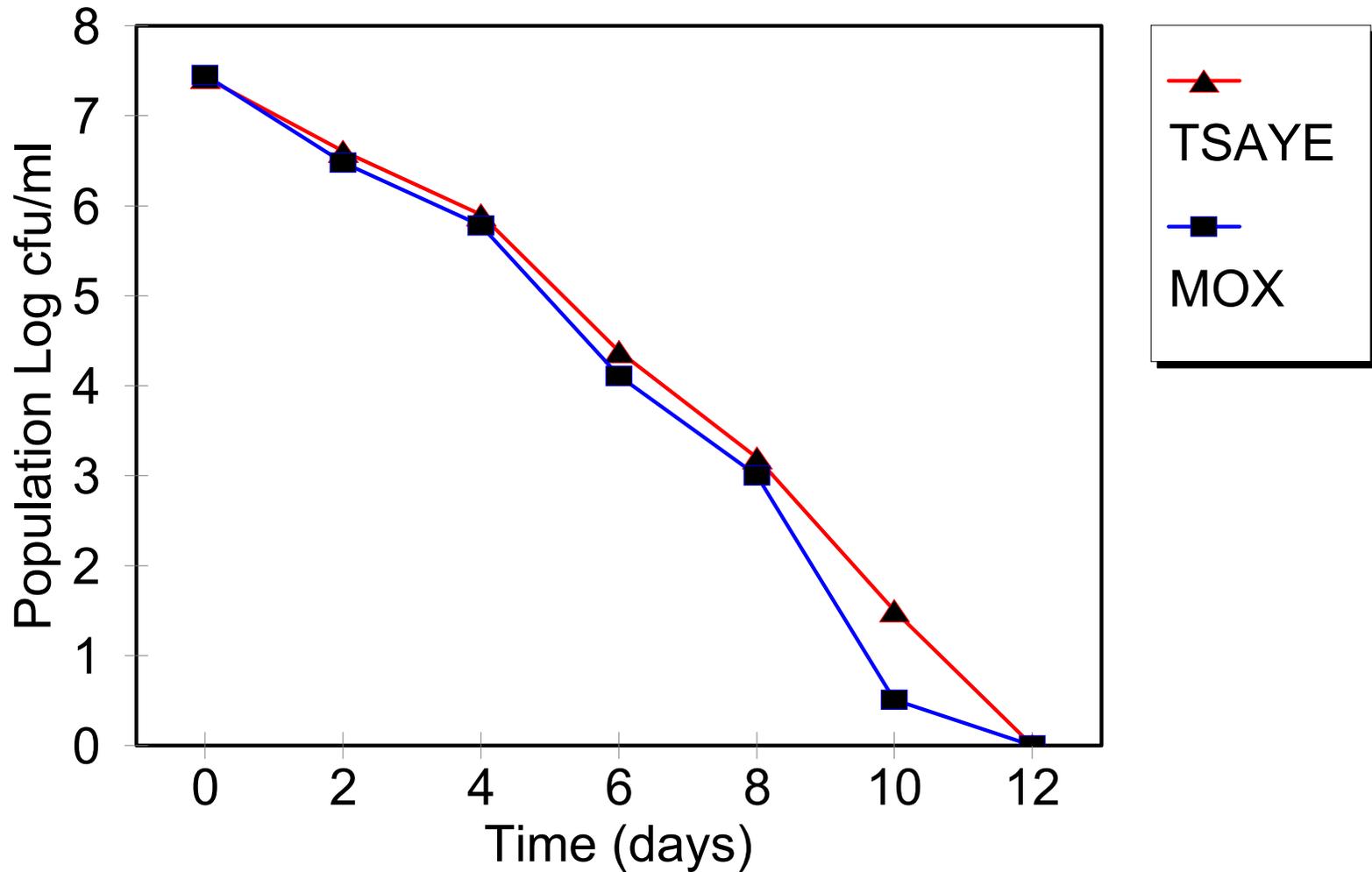


Figure 8: Fate of *Listeria monocytogenes* during abusive-refrigerated storage (10°C) in pasteurized white grape juice as determined by recovery on Tryptic Soy Agar supplemented with Yeast Extract (TSAYE) and Modified Oxford Agar (MOX). Limit of detection: 1 log cfu/ml. n=3

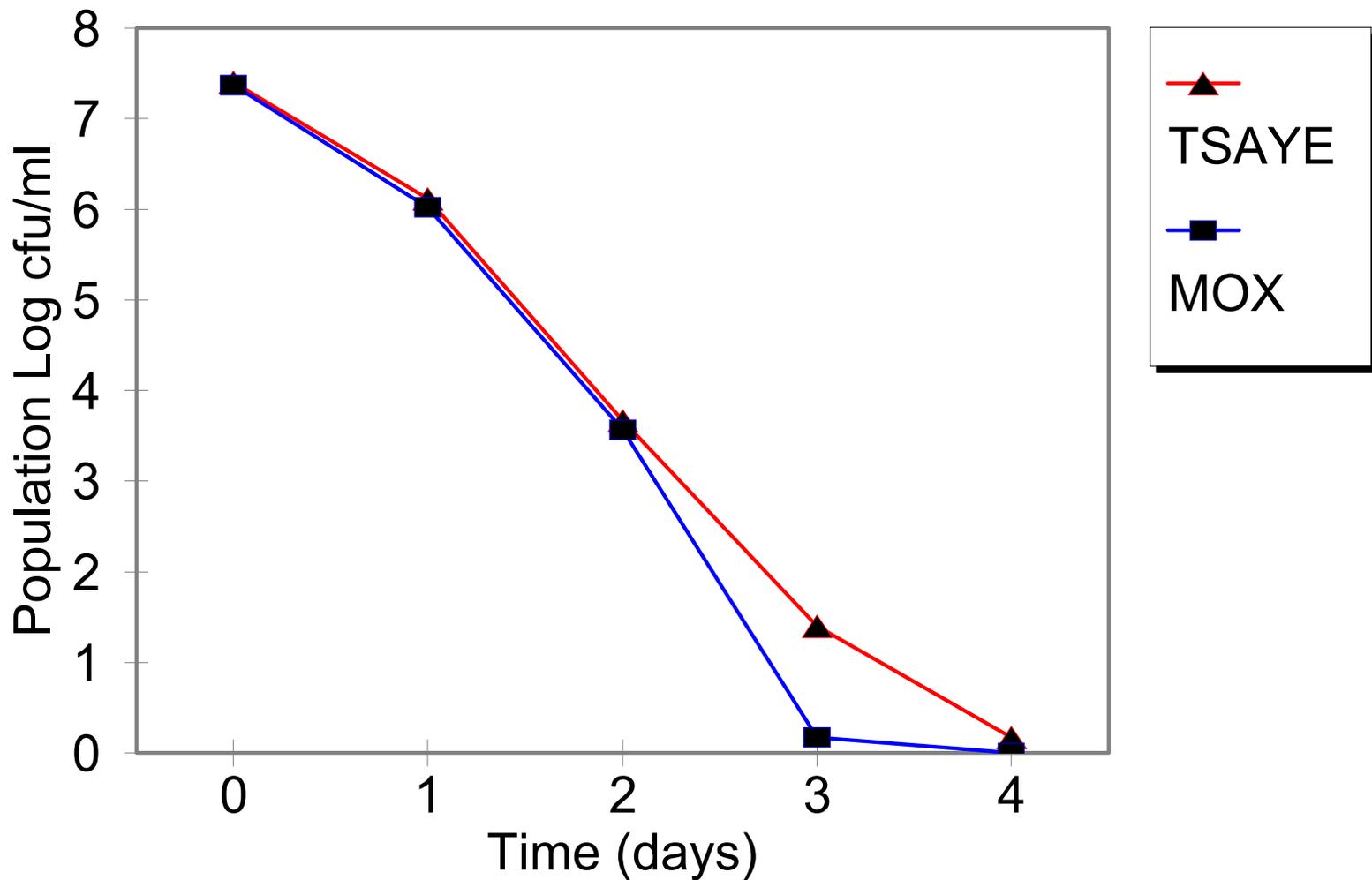


Figure 9: Fate of *Listeria monocytogenes* during ambient storage (24°C) in pasteurized white grape juice as determined by recovery on Tryptic Soy Agar supplemented with Yeast Extract (TSAYE) and Modified Oxford Agar (MOX). Limit of detection: 1 log cfu/ml. n=3

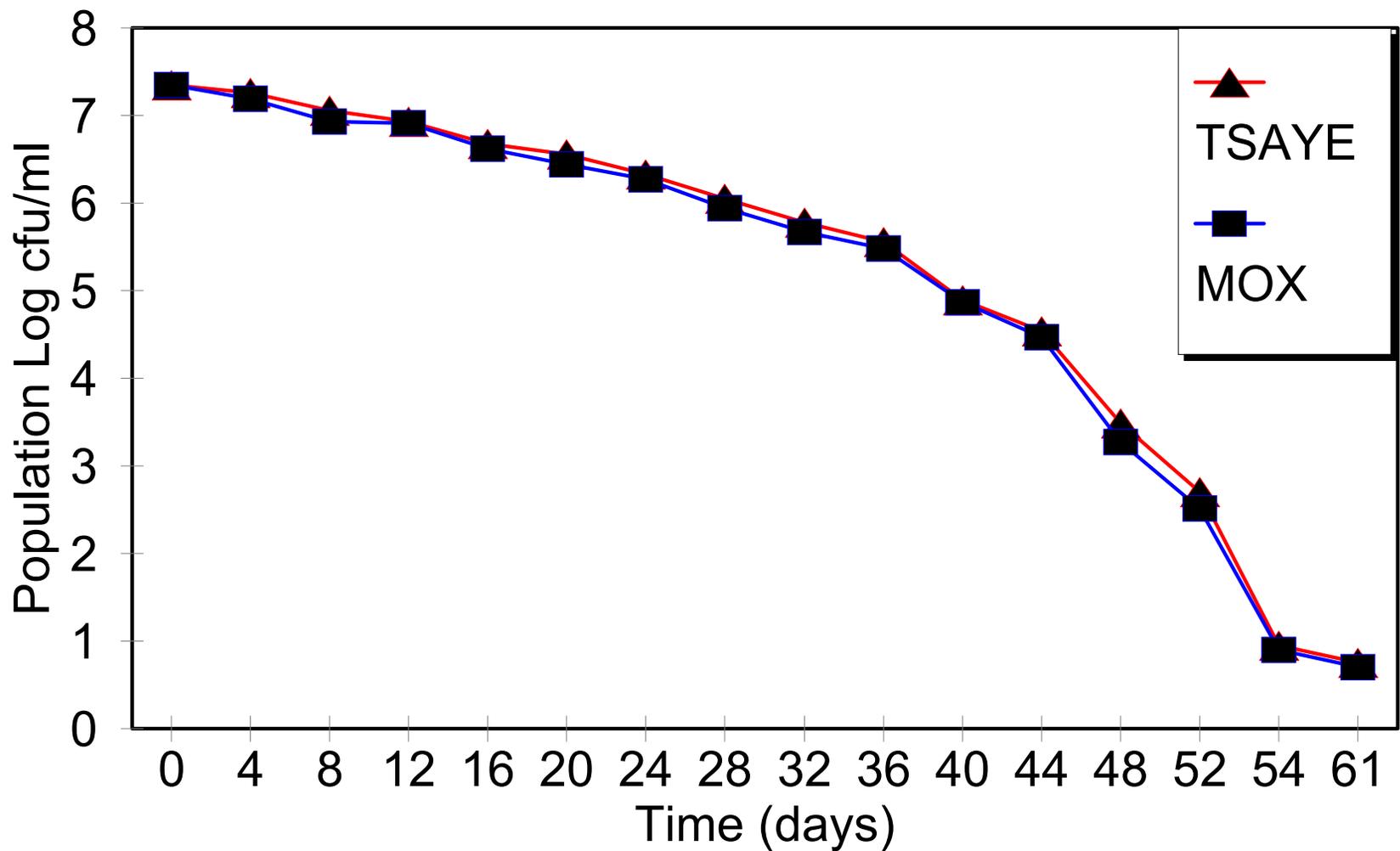


Figure 10: Fate of *Listeria monocytogenes* during refrigerated storage (4°C) in pasteurized orange juice as determined by recovery on Tryptic Soy Agar supplemented with Yeast Extract (TSAYE) and Modified Oxford Agar (MOX). Limit of detection: 1 log cfu/ml. n=3

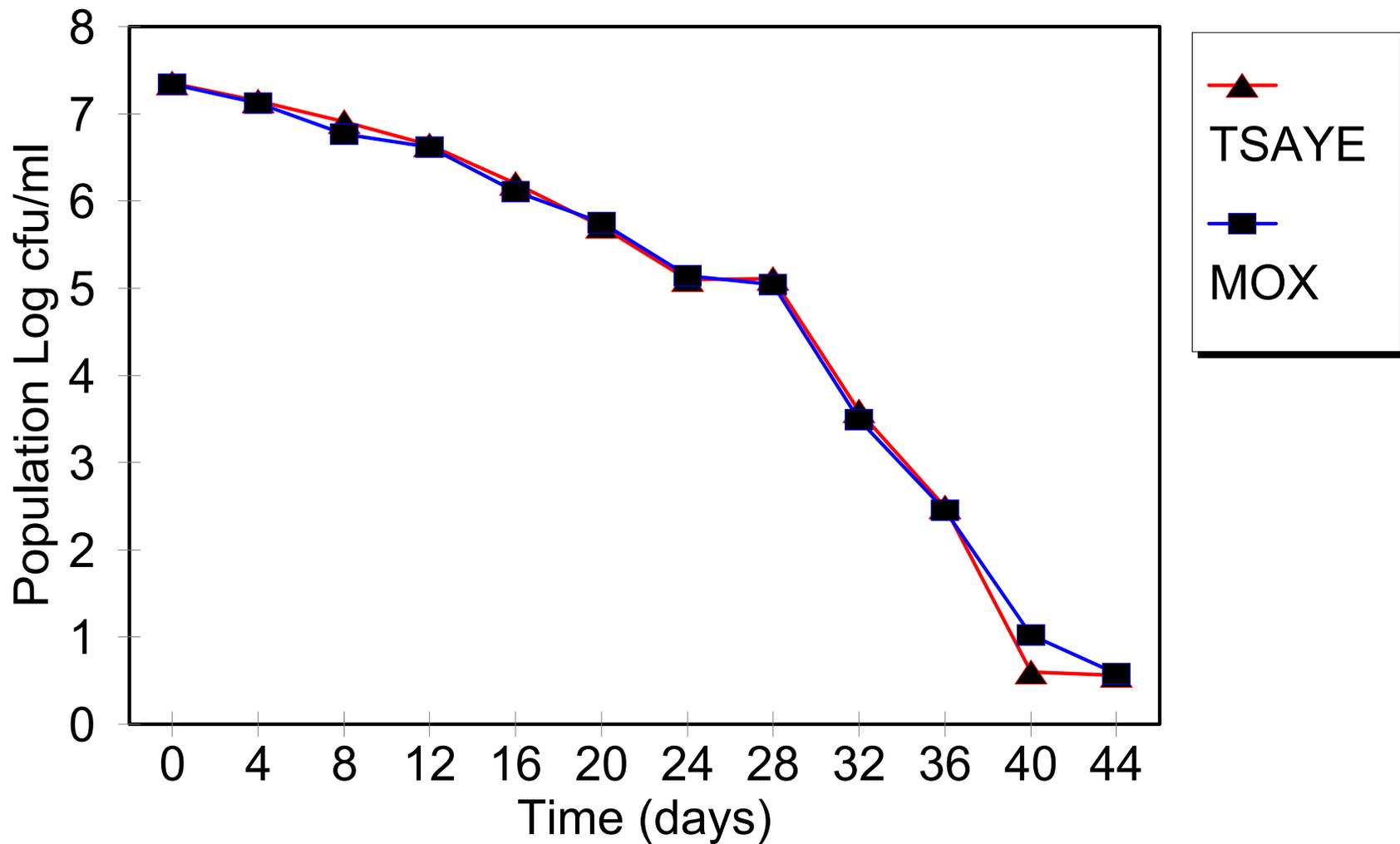


Figure 11: Fate of *Listeria monocytogenes* during abusive-refrigerated storage (10°C) in pasteurized orange juice as determined by recovery on Tryptic Soy Agar supplemented with Yeast Extract (TSAYE) and Modified Oxford Agar (MOX). Limit of detection: 1 log cfu/ml. n=3

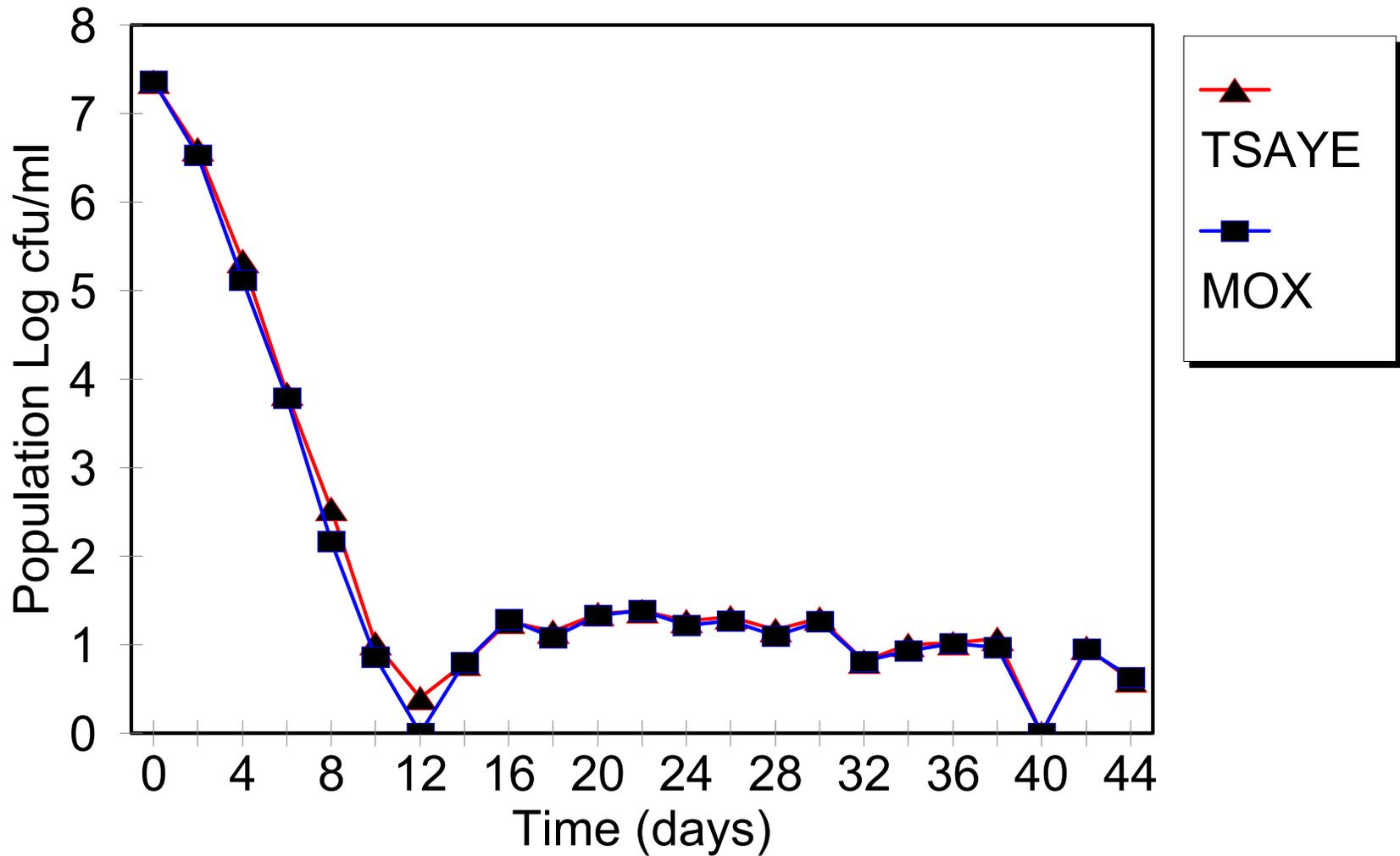


Figure 12: Fate of *Listeria monocytogenes* during ambient storage (24°C) in pasteurized orange juice as determined by recovery on Tryptic Soy Agar supplemented with Yeast Extract (TSAYE) and Modified Oxford Agar (MOX). Limit of detection: 1 log cfu/ml.

n=3

CONCLUSION

The objective of this research was to determine the survival of *Listeria monocytogenes* in juice during storage in refrigeration and abusive temperatures. The results of this study reveal that *L. monocytogenes* is capable of surviving in pasteurized fruit juices for up to 61 days (orange juice). However, survival is influenced by juice type, pH, and storage temperature/time.

Of the juices tested (i.e., apple cider, red grape, white grape and orange juice), the pH of red grape juice appears to have the most inhibitory effect on *L. monocytogenes*. Among the other juices tested, inactivation occurred in the order: white grape juice > apple cider > orange juice, in which *L. monocytogenes* survived at the most, 12, 24 and 61 days, respectively.

Inactivation was generally better at temperatures: 24° C > 10° C > 4° C, except in the case of apple cider which was 24° C > 4° C > 10° C. There was no change in the pH or °Brix throughout the study. There was little or no background microflora present in juice controls, nor was there any *L. monocytogenes* detected in any of the uninoculated juices. Additionally, there was found to be no significant difference in non-neutralized (acidic) versus neutralized plated samples or non-selective (TSAYE) versus selective (MOX) media utilized in this experiment, revealing no statistically significant acid injury.

Numerous foods and beverages are either naturally acidic or are modified to contain organic acids for preservation purposes (O' Driscoll et al., 1996). Many studies have shown that pathogens (i.e., *Escherichia coli* O157:H7, *Salmonella* spp. and *Listeria monocytogenes*) may become resistant to the inherently low “challenge” pH conditions through Acid Tolerance Responses (Datta and Benjamin, 1997). Low pH can allow (mutated) pathogens to adapt and proliferate in high-acid beverages/foods for extended periods of time, possibly causing illness

(Conner et al., 1986). Survival of these enteric, opportunistic pathogens is influenced by the juice's acidity, composition characteristics, processing and storage temperature as well as the growth phase of the contaminating bacterium (Phan-Thanh et al., 2000). It is feasible that sublethal food-processing steps (i.e., thermal, chilling, antimicrobials) may increase the ability of bacteria to survive or grow in such environments, in addition to increased tolerance and virulence effects (O'Driscoll et al., 1996).

There are few published reports on the ability of Listerial survival in beverage environments. This present study demonstrates the need for further research in this area. When considering related studies, to ascertain if results are applicable to beverage manufacturers, processing conditions should be evaluated to determine the effectiveness of their current operations.

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APPENDIX

**Survival of *Listeria monocytogenes* in Fruit Juices
During Refrigerated and Temperature-Abusive Storage**

Micro	Listeria monocytogenes											
Juice	Apple Cider			Orange Juice			White Grape			Red Grape		
Temp	4	10	24	4	10	24	4	10	24	4	10	24
Sampling	0	0	0	0	0	0	0	0	0	0 h	0 h	0 h
(Days)	2	2	2	4	4	2	2	2	1	1 h	1 h	1 h
	4	4	4	8	8	4	4	4	2	2 h	2 h	2 h
Except for	6	6	6	12	12	6	6	6	3	3 h	3 h	3 h
Red Grape	8	8	8	16	16	8	8	8	4	4 h	4 h	4 h
(Hours)	10	10	10	20	20	10	10	10	5	5 h	5 h	5 h
	12	12	12	24	24	12	12	12	6			
	14	14	14	28	28	14	14	14	7			
	16	16	16	32	32	16	16	16	8			
	18	18	18	36	36	18	18	18	9			
	20	20	20	40	40	20	20	20	10			
	22	22	22	44	44	22	22	22	11			
	24	24	24	48	48	24	24	24	12			
	Until	Until	Until	Until	Until	Until	Until	Until	Until	Until	Until	Until
	Death	Death	Death	Death	Death	Death	Death	Death	Death	Death	Death	Death
Media	TSAYE	TSAYE	TSAYE	TSAYE	TSAYE	TSAYE	TSAYE	TSAYE	TSAYE	TSAYE	TSAYE	TSAYE
	MOX	MOX	MOX	MOX	MOX	MOX	MOX	MOX	MOX	MOX	MOX	MOX
Duplicates	x 2	x 2	x 2	x 2	x 2	X 2	x 2	x 2	x 2	x 2	x 2	x 2
Repetitions	3	3	3	3	3	3	3	3	3	3	3	3

VITAE

Christine Lelia was born in Baltimore, Maryland, where she lived the first 15 years of her life. It was during her freshman year at Parkville High School that her initial interest in science took root, stemming from her first biology class with Mr. Faulk. Following that, the next seven years were spent with her parents in Lawrenceville, Georgia, of which she attended the Georgia Institute of Technology and received her Bachelor's degree in Applied Biology in August of 2000. At GT she was advised by Dr. Thomas Tornabene and worked as a lab assistant under Dr. Dwight Hall in the Biology department.

A semester prior to graduation at GT, Christine (Heywood) married Daniel Alan Piotrowski in a ceremony held at the Georgia Renaissance Festival in May of 2000. In August of 1999, Christine's husband was transferred from Atlanta, Georgia to his new position with Norfolk Southern in Virginia; so, following graduation, Christine moved to Roanoke, Virginia to start her own family.

Christine enrolled in the Master's program in Food Science and Technology at Virginia Polytechnic Institute and State University in August of 2001, to follow her microbiology dream with an emphasis in food safety. While at Virginia Tech, she is currently a member of the International Association for Food Protection, the Institute of Food Technologists, and was Treasurer of the IFTSA Virginia Tech chapter of the Food Science Club. Christine plans to continue her education with the FST department at Virginia Tech by beginning her doctoral studies.