

Survival of *Listeria monocytogenes* on Blueberries (*Vaccinium corymbosum*) and Shelf Life Determination under Controlled Atmosphere Storage

Anibal Concha-Meyer

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Food Science and Technology

Joseph D. Eifert, Chair
Robert C. Williams
Joseph E. Marcy
Gregory E. Welbaum

July 26th, 2013
Blacksburg, VA

Keywords: *Listeria monocytogenes*, acid tolerance resistance, blueberries, controlled atmosphere storage, gaseous ozone.

Survival of *Listeria monocytogenes* on Blueberries (*Vaccinium corymbosum*) and Shelf Life Determination under Controlled Atmosphere Storage

Anibal Concha-Meyer

ABSTRACT

Listeria monocytogenes represents a high risk for consumers, because it causes severe illness. This work studied *in-vitro* growth and survival of *L. monocytogenes* in media acidified with malic acid, lactic acid or blueberry extract. The growth of *L. monocytogenes* and shelf life extension of fresh blueberries were evaluated after storage at 4°C or 12°C under different controlled atmosphere conditions, including air (control); 5% O₂ and 15% CO₂, 80% N₂ (CAS); or ozone gas (O₃) 4ppm at 4°C or 2.5ppm at 12°C, at high relative humidity (90-95%) for a total of 10 days.

L. monocytogenes growth in tryptic soy broth with yeast extract (TSB+YE) mixed with different acid solutions (malic acid, lactic acid and blueberry extract) and incubated at 25°C for 24h, was calculated measuring optical density. Complete inhibition occurred in the presence of treatments including malic acid pH 2.0 and pH 3.0; lactic acid pH 2.0, pH 3.0 and pH 4.0; and with blueberry extract pH 2.0 in the mixture. After 6h, there were significant differences among growing treatments. At 18h, there were no significant differences in turbidity among media mixed with blueberry extract at pH 3.0, 4.0 and 5.0, and their optical density values were higher than treatments including media mixed with malic acid 5.0 or lactic acid 5.0. Blueberry extract was not an effective acidifying media and acid adapted *L. monocytogenes* grew in acidified media.

Fresh blueberries inoculated with *L. monocytogenes* were stored at 4°C or 12°C under different controlled atmosphere conditions (Air, CAS or O₃ 4ppm at 4°C or 2.5ppm at 12°C) and sampled on day 0, 1, 4, 7 and 10 for bacterial growth, weight loss, firmness and yeast and molds counts. CAS did not delay or inhibit *Listeria monocytogenes*, yeast, or molds by day 10. Storage at 4°C showed lower weight loss values compared to 12°C. Ozone controlled weight loss and firmness loss. Moreover, gaseous ozone achieved 3 and 2 log reductions when compared with air at 4°C and 12°C, respectively.

Dedication

This is dedicated to my family and especially to my wife Consuelo Oyarzún for her company, comprehension, support, and love throughout this entire process.

Acknowledgements

I would like to recognize everyone in the Department of Food Science and Technology at Virginia Tech. It has not only a great collegial atmosphere, but also a warm and family climate. I would especially like to thank my major professor Dr. Joseph Eifert for always being available to answer my questions and guiding me from the very beginning to accomplish this research project, becoming my mentor and role model.

I am thankful to my committee members, Dr. Williams who gave me lots of useful advice as both teacher and committee member and had a lot of input in my research experience from the initial to final stages of my program. Dr. Welbaum provided me invaluable insight both during classes and meetings concerning my research. He provided blueberries on the experimental farm, and was very instrumental guiding me to the right facts necessary for this project. Dr. Marcy provided me with excellent resources, technical advice, his expertise and support throughout this endeavor.

Dr. Renee Boyer was instrumental providing me with her food microbiology knowledge and managing to obtain the 2011 cantaloupe outbreak *Listeria monocytogenes* from FDA and Dr. Hengjian Wang was supportive with his scientific and statistics knowledge.

I would also like to thank Kim Waterman, Karleigh Huff, Raul Saucedo and Matt Schroeder for their help not only in this project, but also during the classes I took and taught. To the guys at the Graduate Students office, the current and the ones who left, I thank them for keeping the good vibes, collegiality, support and great environment throughout all these years. Lastly, I would like to thank the Food Science and Technology Department of Virginia Tech, responsible for funding this study.

TABLE OF CONTENTS

ABSTRACT.....	ii
DEDICATION.....	iv
ACKNOWLEDGEMENTS.....	v
TABLE OF CONTENTS.....	vi
LIST OF FIGURES.....	ix
LIST OF TABLES.....	x
CHAPTER 1: INTRODUCTION.....	1
REFERENCES	5
CHAPTER 2: REVIEW OF LITERATURE.....	8
PROMOTION OF FRUIT AND VEGETABLE CONSUMPTION.....	8
Foodborne Illness Associated with Fruits and Vegetables.....	9
Blueberry Production.....	10
Health Benefits of Blueberry Consumption.....	11
Processing and Contamination.....	12
<i>LISTERIA MONOCYTOGENES</i>	13
Listeriosis.....	15
Illness Outbreaks Linked to <i>Listeria monocytogenes</i>	16
Illness Outbreak Related with Acid Products.....	17
ACID TOLERANCE RESPONSE (ATR).....	19
ATR Mechanism.....	20
ATR in Foodborne Pathogenic Bacteria.....	21
Organic vs. Inorganic Acid Stress.....	23
<i>In vitro</i> Inducible ATR.....	24
Growth and Survival of <i>Listeria monocytogenes</i> in Low pH Fruits.....	25
HORTICULTURE PRODUCTION AND TECHNICAL INFORMATION OF BLUEBERRIES.....	27
Postharvest Procedures Affecting Quality and Shelf Life.....	30
CONTROLLED ATMOSPHERE OF FRESH PRODUCE FOR SHELF LIFE EXTENSION AND SAFETY.....	31

Modified Atmosphere Packaging.....	34
High Oxygen Packaging Atmosphere.....	35
Use of Chlorine Dioxide Gas in CAS.....	36
OZONE APPLICATION FOR SHELF LIFE EXTENSION AND SAFETY.....	38
Production and Stability of Ozone.....	39
Safety Concerns of Gaseous Ozone.....	39
Measurement of Ozone Concentration.....	40
Ozone Effects on Produce Quality.....	40
Pathogenic and Spoilage Inhibition Studies on Food.....	42
REFERENCES.....	45
CHAPTER 3: <i>LISTERIA MONOCYTOGENES</i> SURVIVAL IN THE PRESENCE OF MALIC ACID, LACTIC ACID OR BLUEBERRY EXTRACT.....	61
ABSTRACT.....	62
INTRODUCTION.....	63
MATERIALS AND METHODS.....	65
RESULTS AND DISCUSSION.....	68
CONCLUSIONS.....	72
REFERENCES.....	74
CHAPTER 4: SHELF LIFE DETERMINATION OF FRESH BLUEBERRIES (<i>VACCINIUM CORYMBOSUM</i>) UNDER CONTROLLED ATMOSPHERE STORAGE.....	81
ABSTRACT.....	82
INTRODUCTION.....	83
MATERIALS AND METHODS.....	85
RESULTS AND DISCUSSION.....	89
CONCLUSIONS.....	97
REFERENCES.....	98
CHAPTER 5: SURVIVAL OF <i>LISTERIA MONOCYTOGENES</i> ON FRESH BLUEBERRIES (<i>VACCINIUM CORYMBOSUM</i>) UNDER CONTROLLED ATMOSPHERE STORAGE.....	110
ABSTRACT.....	111

INTRODUCTION.....	112
MATERIALS AND METHODS.....	114
RESULTS AND DISCUSSION.....	118
REFERENCES.....	122
CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS.....	128
APPENDIX A. EVALUATION OF <i>LISTERIA MONOCYTOGENES</i> GROWTH ON INOCULATED BLUEBERRIES AND STORAGE AT ROOM TEMPERATURE.....	131
MATERIALS AND METHODS.....	131
RESULTS AND DISCUSSION.....	132
APPENDIX B. DETERMINATION OF <i>LISTERIA MONOCYTOGENES</i> INOCULUM VOLUME AND BLUEBERRIES NUMBER TO BE INOCULATED.....	137

LIST OF FIGURES

CHAPTER 3:

Figure 1. <i>Listeria monocytogenes</i> Scott A optical density turbidity growth curve measured using tryptic soy broth with yeast extract (TSB+YE) under different treatments (B, C, or D) with different acids (malic acid, lactic acid and blueberry extract) incubated at 25°C for 24h.....	78
---	----

CHAPTER 4:

Figure 1. Weight loss percentage of highbush blueberries stored at 4°C or 12°C under controlled atmosphere storage conditions (CAS), Ozone (O ₃) or regular atmosphere (Air) during 10 days.....	106
--	-----

CHAPTER 5:

Figure 1. Schematics of controlled atmosphere incubation equipments.....	125
Figure 2. <i>Listeria monocytogenes</i> survival during 10 days storage time under different atmosphere conditions.....	126

APPENDIX A:

Figure A1. Example of an undamaged blueberry.....	135
Figure A2. Example of a damaged blueberry.....	135

LIST OF TABLES

CHAPTER 3:

Table 1. Treatments solutions pH before inoculation with 10 µl of <i>Listeria monocytogenes</i> in 0.1% peptone water.....	79
--	----

Table 2. Mean of optical density turbidity growth curve values and standard deviation (n=12) for <i>Listeria monocytogenes</i> Scott A in TSB+YE under acid treatments incubated at 25°C for 24 h.....	80
--	----

CHAPTER 4:

Table 1. Yeast counts (Log CFU/g) and increase over 10 days (Δ) of highbush blueberries stored at 4°C or 12°C under controlled atmosphere storage conditions (CAS), Ozone (O ₃) or regular atmosphere (Air).....	107
---	-----

Table 2. Mold counts (Log CFU/g) and increase over 10 days (Δ) of highbush blueberries stored at 4°C or 12°C under controlled atmosphere storage conditions (CAS), Ozone (O ₃) or regular atmosphere (Air).....	108
--	-----

Table 3. Firmness penetration peak force (g) of highbush blueberries stored at 4°C or 12°C under controlled atmosphere storage conditions (CAS), Ozone (O ₃) or regular atmosphere (Air) during 10 days.....	109
--	-----

CHAPTER 5:

Table 1. <i>Listeria monocytogenes</i> counts (Log CFU/mL) on fresh highbush blueberries stored at 4°C or 12°C under controlled atmosphere storage conditions (CAS), Ozone (O ₃) or regular atmosphere (Air) during 10 days.....	127
--	-----

APPENDIX A:

Table A1. Recovery counts of different strains of <i>Listeria monocytogenes</i> (Log CFU/20 berries) from inoculated undamaged and damaged blueberries stored at 25°C for 6 days.....	136
---	-----

APPENDIX B:

Table B1. Population recovery of <i>Listeria monocytogenes</i> from blueberries inoculated with different volume of inoculum (15µL and 100 µL) and different quantity of inoculated blueberries per sample (5, 10 and 20 berries inoculated from a total of 50 berries) incubated at 12°C for 48h.....	137
--	-----

CHAPTER 1

INTRODUCTION

Infections caused by microorganisms that contaminate the food supply are evidence of the complicity of the worldwide food web that link humans with animals, plants, and microorganisms. Approximately 46 million foodborne illnesses occur each year just in the United States, in addition to 250,000 hospitalizations and 3,000 deaths (Scallan et al., 2011). More than 9 million of these illnesses are estimated to be caused by major pathogens (Painter et al., 2013). Furthermore the leading causes of death related to foodborne infections are nontyphoidal *Salmonella* spp. (28%), *T. gondii* (24%), *Listeria monocytogenes* (19%), and norovirus (11%) (Scallan et al., 2011).

Total fresh produce consumption in the United States has increased significantly in the last thirty years (U.S. General Accounting Office, 2002). Additionally, the number of outbreaks reported to the Centers for Disease Control and Prevention (CDC) caused by foodborne pathogens that were associated with fresh vegetables and fruit consumption has considerably increased. The latter demonstrates a change from traditional outbreak associated source like food of animal origin, such as eggs meat and dairy products, to fresh produce (Hedberg et al., 1994). Using data from outbreak-associated illnesses from 1998 to 2008, Painter et al. (2013) concluded that produce commodities (fruits, nuts and 5 vegetables) accounted for 46% of total illnesses. Fresh fruit and vegetables eaten uncooked are not necessary safe, despite the fact that they are considered an essential part of a healthy diet (Sivapalasingam et al., 2004).

Listeria monocytogenes is a gram positive bacterial pathogen that can cause life threatening foodborne illness in humans. Consumption of contaminated food can lead to the development of listeriosis, especially in pregnant women, newborns, and adults with weakened

immune systems (McLauchlin, 1996). Manifestations include septicemia, meningitis (or meningoencephalitis), encephalitis, and intrauterine or cervical infections in pregnant women, which may result in spontaneous abortions (2nd/3rd trimester) or stillbirth (FDA, 2010).

This organism is commonly associated with plant matter and soil and it has been suggested that it may be more common in those fruits and vegetables grown in close association with the soil (Brackett, 1999). *Listeria monocytogenes* has been isolated from a variety of raw vegetables and there have been several reported foodborne outbreaks associated with fruit or fruit products (Caggia et al., 2009). An investigation of a multistate outbreak linked with cantaloupe began in September of 2011 (Cosgrove et al., 2011), even though melon consumption had never been implicated in any listeriosis outbreak in United States before. Packing facility conditions that contributed to the introduction, dispersion and growth of *L. monocytogenes* in this situation included insufficient cleaning and sanitization of the processing equipment, failure of a pre-cooling step before cold storage, and poor packing facility design (FDA, 2012).

Moreover, there is association of this pathogen with acidic fruits and fruit juice products, including unpasteurized apple juice (pH 3.78) and apple/raspberry juice (pH 3.75) (Sado et al., 1998), cut and whole strawberries (Johannessen et al., 2002; Flessa et al., 2005), peeled Hamlin oranges (Pao et al., 1998) and orange juice and orange slices (Caggia et al., 2009).

Berries are not usually linked to bacteria outbreaks, but associated with the protozoan parasite Cyclospora, or Hepatitis A virus and Norovirus (Kniel & Shearer, 2009). Despite this fact, five bacterial outbreaks have been attributed to berries in the United States since 1973 (Sivapalasingam et al., 2004). Most of these cases are related with strawberries contaminated with *Staphylococcus aureus* in 1985, *Salmonella* Group B in 2003 and enterohemorrhagic *E. coli* O26 in 2006, this latter including blueberries as well. Blueberries were related to a possible

outbreak of listeriosis in Connecticut in 1984 (Ryser, 1999). Also, 14 years later a producer was forced to recall an unknown volume of frozen blueberries in California, Illinois and Australia due to contamination with *L. monocytogenes* (FDA, 1998). Moreover, other outbreaks have been attributed to salads containing berries, where the fruit was not identified as the original source of the pathogen (CDC, 2006). According to Schlech, (1996) blueberries are considered a food implicated in published reports of foodborne listeriosis. Berries are reported to have antibacterial properties because of their acidity and phenolic compounds (Puupponen-Piämä et al., 2005), however based on extensive research with artificially contaminated berries these properties should not be relied upon to ensure food safety (Kniel & Shearer, 2009).

The use of controlled atmosphere in produce transportation is widely applied by producers, to assure the quality of the product and avoid spoilage. In the case of blueberries that are imported to the U.S. from countries like Chile, that harvest the fruit from October to April, the use of this technology (and refrigeration) during transportation predominates (Baños, 2006). When these products are shipped from Chile to the U.S., they can take 20 days to arrive to their final destination in U.S. ports (Beaudry et al., 1998). Respiration rate and deterioration decreases for some fruits when under CO₂ levels of about 10% to 20%. Some results indicate that O₂ concentrations between 8% and 10% and CO₂ concentrations of 10% to 13% have managed to maintain the quality of the blueberries between 5 and 8 weeks at 0-1°C and 3 days under temperature conditions of 18-20°C (Bounous et al., 1997). At the same time, a CO₂ concentration of 15% would reduce the incidence of fruit decay. Blueberries imported from Chile are usually transported under 16% CO₂ and 5% O₂ controlled atmosphere conditions. Improper control of the gas compositions may lead to undesirable results such as anaerobic respiration, accelerated physiological decay, and shortened shelf life. High relative humidity

inside packages can lead to microbial growth and moisture condensation (Dennis, 1985; Kader et al., 1989), while low relative humidity causes shriveling and moisture loss.

Ozone is a very effective bactericidal substance and is generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (FDA) for broad food applications (Graham, 1997). This gas is widely used in its aqueous state as a water disinfectant in processing facilities and can also be applied in a gaseous form. Compared to other chemical oxidants its advantages include precursor's (O₂) abundance, economical production and no residual chemicals post-contact. Ozone was approved in 2001 for raw commodities treatment by the FDA (Federal Register, 2001). Research on apples found reductions in both weight loss and spoilage when exposed to 0.06 ppm of ozone gas for 4 hours each day (Bazarova, 1982). Moreover, when blackberries were exposed to 0.1 to 0.3 ppm ozone gas for 12 days, a spoilage reduction of 80% by *Botrytis cinerea* was observed (Barth et al., 1995).

The present study evaluated the behavior of *L. monocytogenes* in acidic conditions and also in acidic food product fresh blueberries packaged under controlled atmosphere conditions and gaseous ozone, to estimate if bacterial occurrence, survival and growth in these environments may represent a potential increase in health hazard for consumers. The objectives of this research are 1) to determine the adaptive acid tolerance response (ATR) of *Listeria monocytogenes* under *in vitro* conditions and different pH conditions; 2) evaluate the growth of *L. monocytogenes* in fresh blueberries under different controlled atmosphere conditions (Air; 5% O₂ and 15% CO₂; and Ozone) and temperature storage (4°C and 12°C) for 10 days; and 3) determine the fruit shelf life and quality preservation measuring yeast and mold counts, weight loss and blueberries firmness during storage under different controlled atmosphere storage conditions.

References

- Bañados, M. P. (2006). Blueberry production in South America. *Acta Hort.*, 715, 165-172.
- Barth, M. M., Zhou, C., Mercier, M., & Payne F.A. (1995). Ozone storage effects on anthocyanin content and fungal growth in blackberries. *J. Food Sci.*, 60, 1286-1287.
- Bazarova, V. I. (1982). Use of ozone in storage of apples. *Food Sci. Tech. Abstr.*, 14(11), J1653.
- Beaudry, R. M., Moggia, C. E., Retamales, J. B., & Hancock, J. F. (1998). Quality of 'Ivanhoe' and 'Bluecrop' blueberry fruit transported by air and sea from Chile to North America. *HortScience*, 33, 313-317.
- Bounous, G., Giacalone, G., Guarinoni, A., & Peano, C. (1997). Modified Atmosphere Storage of Highbush Blueberry. *Acta Hort.*, 446, 197-204.
- Brackett, R. E. (1999). Incidence and behavior of *Listeria monocytogenes* in products of plant origin. In: E.T. Ryser & E.H. Marth (Eds.), *Listeria, Listeriosis, and Food Safety, second ed.* (pp. 631-655). New York, NY: Marcel Dekker, Inc.
- Caggia, C., Scifo, G. O., Restuccia, C., & Randazzo, C. L. (2009). Growth of acid-adapted *Listeria monocytogenes* in orange juice and in minimally processed orange slices. *Food Control*, 20, 59-66.
- Center for Disease Control and Prevention. CDC. (2006). *Salmonella* Oranienburg infections associated with fruit salad served in health-care facilities - Northeastern United States and Canada. *MMWR Morb. Mortal. Wkly. Rep.*, (56), 1025-1028. Available at: <http://www.cdc.gov/MMWR/preview/mmwrhtml/mm5639a3.htm>. Accessed 29 May 2013.
- Cosgrove, S., Cronquist, A., Wright, G., Ghosh, T., & Vogt, R. (2011). Multistate outbreak of listeriosis associated with Jensen Farms cantaloupe—United States, August-September 2011. *MMWR Morb. Mortal. Wkly. Rep.*, 60, 1357-1358.
- Dennis, C. (1985). Post-harvest spoilage of fruits and vegetables. In: P.G. Ayres & L. Boddy (Eds.), *Water Fungi and Plants* (p. 343). Cambridge, London: Cambridge University Press.
- FDA. (1998). Frozen blueberries recalled due to *Listeria monocytogenes* contamination. *FDA Enforcement Rep.*, 98-52.
- FDA. (2010). "*Listeria monocytogenes*" Bad Bug Book. Available at <http://www.fda.gov/Food/FoodborneIllnessContaminants/CausesOfIllnessBadBugBook/ucm070064.htm>. Accessed 29 May 2013.
- FDA. (2012). "Information on the Recalled Jensen Farms Whole Cantaloupes" Available at <http://www.fda.gov/Food/RecallsOutbreaksEmergencies/Outbreaks/ucm272372.htm>. Accessed April 30 2013.

- Federal Register. (2001). Secondary direct food additives permitted in food for human consumption, final rule. *Fed. Reg.*, 66, 33829-33830.
- Flessa, S., Lusk, D. M., & Harris, L. J. (2005). Survival of *Listeria monocytogenes* on fresh and frozen strawberries. *Int. J. Food Microbiol.*, 101, 255-262.
- Graham, D.M. (1997). Use of ozone for food processing. *Food Technol.*, 51, 72-75.
- Hedberg, C. W., MacDonald, K. L., & Osterholm, M. T. (1994). Changing epidemiology of food-borne disease: a Minnesota perspective. *Clin. Infect. Dis.*, 18, 671-680.
- Johannessen, G.S., Loncarevic, S., & Kruse, H. (2002). Bacteriological analysis of fresh produce in Norway. *Int. J. Food Microbiol.*, 77, 199-204.
- Kader, A. A., Zagory, D., & Kerbel, E. L. (1989). Modified atmosphere packaging of fruits and vegetables. *CRC Cr. Rev. Food Sci.*, 28(1), 1-30.
- Kniel, K.E., & Shearer, A. E. (2009). Berry Contamination: Outbreaks and Contamination Issues. In: G. Sapers, E. Solomon & K. R. Matthews (Eds.), *The produce contamination problem: causes and solutions* (pp. 271-297). Waltham, MA: Academic Press.
- McLauchlin, J. (1996). The relationship between *Listeria* and listeriosis. *Food Control*, 7, 187-193.
- Painter, J. A., Hoekstra, R. M., Ayers, T., Tauxe, R. V., Braden, C. R., Angulo, F. J., & Griffin, P. M. (2013). Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998–2008. *Emerg. Infect. Dis.*, 19(3), 507-515.
- Pao, S., Brown, E., & Schneider, K. R. (1998). Challenge studies with selected pathogenic bacteria on freshly peeled Hamlin orange. *J. Food Sci.*, 63, 359-362.
- Puupponen-Piämä, R., Nohynel, L., Alakomi, H. L., & Oksman-Caldentey, K. M. (2005). Bioactive berry compounds – Novel tools against human pathogens. *Appl. Microbiol. Biotech.*, 67, 8-18.
- Ryser, E. T. (1999). Foodborne listeriosis. In: E. T. Ryser & E. H. Marth (ed.), *Listeria, Listeriosis and Food Safety*, 2nd ed. (p. 341). New York, NY: Marcel Dekker, Inc.
- Sado, P.N., Jinneman, K. C., Husby, G. J., Sorg, S. M., Omiecinski, C. J. (1998). Identification of *Listeria monocytogenes* from unpasteurized apple juice using rapid test kits. *Food Protect.*, 61, 1199-1202.

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., Jones, J. L., & Griffin, P. M. (2011). Foodborne illness acquired in the United States—major pathogens. *Emerg. Infect. Dis.*, 17(1), 7–15.

Schlech, W.F. (1996). Overview of listeriosis. *Food Control*, 7(4–5), 183-186.

Sivapalasingam, S., Friedman, C. R., Cohen, L., & Tauxe, R. V. (2004). Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. *Food Protect.*, 67, 2342– 2353.

U.S. General Accounting Office. (2002). Fruits and vegetables: Enhanced federal efforts to increase consumption could yield health benefits for Americans. Report GAO-02-657. Available at: <http://www.gao.gov/products/GAO-02-657>.

CHAPTER 2

REVIEW OF LITERATURE

PROMOTION OF FRUIT AND VEGETABLE CONSUMPTION

Government health agencies from many countries recommend consumption of fresh fruit and vegetables, because of their importance as key components of a healthy and balanced diet, as well as the protection they provide against a range of illnesses such as cancers and cardiovascular diseases. The World Health Organization (WHO, 1990) encouraged people to eat daily at least five portions (approximately 400 g) of fruits and vegetables to avoid cancer and other chronic diseases. Furthermore, in 1988 the California Department of Health and Human Services started the social program “5-A-Day” to encourage fruit and vegetable consumption of at least 5 daily servings. In 1991 this initiative turned into a national campaign as a public-private partnership between the US by the National Cancer Institute (NCI) and the Produce for Better Health Foundation (PBH) (Pivonka et al., 2011). Besides the consumption promotion, the program started a major investigation to isolate and identify the compounds responsible for the benefits (Havas et al., 1994). In 2005 this program was changed to the campaign Fruits & Veggies-More Matters™ and fruits and vegetables were categorized as “food groups to encourage” and as “foods to increase” respectively by the Dietary Guidelines for Americans (USDA/HHS, 2010). Recommendations for fruits and vegetables intake by MyPyramid depend on age, sex, and physical activity level (USDA, 2005) and range from 2 to 6.5 cups per day and corresponds to 4 to 13 servings approximately (Guenther et al., 2006). These efforts look to increase consumption, which is critical in kids, since poor intake of fruit and vegetables during childhood may be related to a low intake in later adulthood (Maynard et al., 2006). Therefore, governments have implemented programs that provide free or subsidized fruits and vegetables to

children to increase availability, complementing this with nutrition education in the curriculum, enabling healthy choices in the school environment and communicating with parents to reinforce family guidance regarding this matter (Evans et al., 2012).

Foodborne Illness Associated with Fruits and Vegetables

Foodborne pathogenic microorganisms are the cause of most serious food safety problems in the United States, causing many illnesses. CDC estimates that each year 1 in 6 persons (48 million people) gets sick, 128,000 are hospitalized and 3,000 die of foodborne illnesses in the US (CDC, 2011a). Improper holding temperature and poor personal hygiene of food handlers contributed to most disease incidence (Collins, 1997). Pathogens that cause foodborne illness include viruses, parasites, and bacteria, this last being the major cause.

Fresh produce and especially raw consumed leafy greens are considered important vehicles for transmission of human pathogens that were traditionally associated with animal origin foods. Currently there is need for a deeper understanding on where and how the pathogenic contamination and survival takes place in the fresh produce supply chain (Berger et al., 2010). Produce have an increased high risk for pathogenic transmission, because they are mostly consumed raw with minimal previous processing or no heat treatment. Contamination of fresh produce can be caused in the field by spoilage or pathogenic organisms during pre-harvest and by post-harvest procedures in the field or packing house (Guo et al., 2001; Brenner et al., 2000; Coburn et al., 2007). Moreover, raw fruits and vegetables have been related to bacterial foodborne outbreaks (Burnett & Beuchat, 2001). Pathogens that have been isolated from raw produce include: *Aeromonas*, *Bacillus cereus*, *Campylobacter*, *Clostridium botulinum*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella*, *Staphylococcus spp.* *Shigella*,

Staphylococcus aureus, *Vibrio cholerae* and *Yersinia enterocolitica*, among others (Beuchat, 1998). Recent pathogenic outbreaks in the US involved *Escherichia coli* O157:H7 infections with bagged baby spinach (CDC, 2006), *Salmonella* Saintpaul in hot peppers and possibly tomatoes (CDC, 2008) and *Salmonella* Poona with imported cantaloupes (CDC, 2002), emphasizing the challenges related to fresh produce. Additionally, cantaloupes were implicated in cases of listeriosis in a multistate outbreak of *Listeria monocytogenes* in 2011 (CDC, 2012).

Blueberry Production

The fruit crop blueberries (*Vaccinium* spp.) are native to North America and are the second most important berry in the US exceeded only by strawberries (Saftner et al., 2008). Since 2000 the blueberries market has increased at a rate of 10–20% annually, motivated by the increased desire for a healthy and nutritious diet (Plattner et al., 2012; USDA, 2013). According to the 2007 US Census of Agriculture 25,017 farms grew some type of berry and there were 261,733 acres in production. The majority of berry acreage was devoted to strawberry and blueberry production. During 2007 the production total value of fruits, tree nuts and berries was \$18.6 billion, increasing by \$4.9 billion since 2002, and representing 6.3 percent of all agricultural products sold in the United States. Therefore berries and especially blueberries have become an important crop not just in the US, but also worldwide. Domestic blueberries represent 29% of the total distribution of berry acreage in the US, followed by strawberries with a 22%, while wild blueberries represent 18% (USDA, 2007). The US per capita food availability of blueberries in 1994 was 0.766 lbs, detailed in availability on fresh blueberries of 0.265 and frozen of 0.501 lbs. However, in 2010 this increased to 1.708 lbs, while the value for fresh blueberries was 1.115 lbs and for frozen 0.593 lbs (USDA/Economic Research Service, 2012),

because of a change in consumption by the consumers. Although the US is the world biggest blueberry producer, the American market is so large that it requires importing the fruit from other countries. In 2012 Canada provided more than 25%, while Chile provided more than 50% of the imported blueberries sold in the country (USDA, 2012). The nation imports increased 12 percent from previous year, achieving in 2012 nearly \$419.8 million in blueberries imports (USDA, 2012). Chile is projected to export in the 2012/13 season around 85,000 tons of fresh blueberries, which represents 21 percent more compared to last season. From this projected volume, the fruit destined for the US market is more than 75 percent, providing US retailers an all time record supply during the winter months (Plattner et al., 2012).

Health Benefits of Blueberry Consumption

Important health benefits have been related to diets rich in fruits and vegetables including not only obesity avoidance (World Cancer Research Fund/American Institute for Cancer Research 2007), but also reductions in cardiovascular disease (Ness, 1997; Dauchet, 2006), and many cancers (International Agency for Research on Cancer, 2003; Boffetta et al., 2010). Moreover, many of these diseases are major causes of death in developed countries (WHO, 2003). However, there are some exceptions where researchers have found that there is no association of produce intake with cancer prevention. Koushik et al., 2012 examined a pooled cancer risk analysis of 14 prospective studies from North America, Europe, and Australia (study periods between 1980 and 2005). They found that their results did not provide evidence that fruit and vegetable consumption during adulthood is associated with an overall decreased risk of pancreatic cancer.

Blueberries are recognized for their health promoting compounds (Lila, 2004; Wang et al., 2005), hence they have been widely accepted by consumers. The reasons for high blueberries availability and consumption increase are the widely recognized health benefits of both wild and domestic blueberries that can generate positive effects in reduction of coronary heart disease, treatment of urinary tract disorders, and anticarcinogenic activity (Kalt & Dufour 1997). Other studies show delay in brain aging influenced by blueberry polyphenolics (Joseph et al., 1999; Youdim et al., 2000). Blueberries are ranked high in their antioxidant activity among fruits and vegetables tested to date (Prior et al., 1998). Additionally, blueberries are a significant source of bioactive substances including antioxidants and antimicrobial compounds against human pathogens (Puupponen-Piämä et al., 2001, 2005), such as flavonoids (Hakkinen & Torronen, 2000), anthocyanins and others (Prior et al., 1998).

Processing and Contamination

Minimally processed vegetables or fruits are defined as fresh vegetables or fruits that have been processed to increase their functionality without modifying their natural properties (Salunkhe et al., 1991). The kind of process will vary depending on the fruit or vegetable and some examples include washing, cutting, mixing and packaging. Fresh blueberries are considered minimally processed food since they are only harvested, packed, transported, and sold. Frequently this fruit is not washed or dipped into chlorinated cleaning solutions since this may remove the natural whitish waxy “bloom” on the surface, which is a positive aesthetic quality attribute (Gomez-Rodas et al., 2010). Furthermore, the humidity and fruit moisture gained after a washing step can lead to considerable mold growth during the storage and transportation (Sapers et al., 2001). Good Agricultural and Management Practices are essential to

control food safety hazards in fresh blueberries and need to be implemented by growers, packers and shippers. As well as for other fresh produce, blueberries can suffer contamination at any point within the farm to table food chain where human or animal feces are the principal source of microbial contamination (Gomez-Rodas et al., 2010). Preventive measurements to avoid contamination include strict personal hygiene of food handlers and use of potable water suitable for food processing and fruit irrigation. Fruits and vegetables should not be irrigated with sewage or slurry water at least ten months before harvest (Newell et al., 2010). Fresh produce will in some part be consumed raw by consumers, hence the importance of their awareness of eating raw food and the possible routes of getting foodborne infections. In 1984 consumption of unwashed strawberries, blueberries and/or nectarines was linked to listeriosis in an unpublished report in Connecticut (Ryser, 1999). In the period 2003-2004, Mukherjee et al. (2006) performed a survey of bacterial contamination sampling strawberries, raspberries and blueberries from the Upper Midwest region in the US. Coliform counts averaged approximately 1 to 2 log MPN/g on berries and 1% tested positive for *E. coli* O157:H7.

LISTERIA MONOCYTOGENES

The early reports of *Listeria monocytogenes* mention a 1926 study isolating a gram-positive bacterium as the cause of lethal disease in rabbits and guinea pigs at Cambridge in England (Murray et al., 1926). Animals developed an elevation of white cells monocytes in their blood, thus the researchers named the organism *Bacterium monocytogenes*. Moreover, another study from South Africa identified a gram-positive bacterium as the cause of the deadly “Tiger River” disease (Pirie et al., 1927). Animals affected by this bacterium died from a necrotizing hepatic infection. The organism was named *Listerella hepatolytica* after British surgeon Joseph

Lister, who is considered the father of antiseptics (Tan & Tasaki, 2007). Ultimately, investigators found both bacteria to be the same and named it *Listeria monocytogenes* (Pamer, 2004). The five other species in the genus *Listeria* are *L. grayi*, *L. innocua*, *L. ivanovii*, *L. seeligeri* and *L. welshimeri*. Just *L. ivanovii* is also considered pathogenic, but mainly in mice and other animals, rather than in humans (FDA, 2011).

Listeria monocytogenes is a rod-shaped, Gram-positive bacterium, a facultative anaerobe (meaning that can grow with and without oxygen), and is also motile through its flagella (FDA, 2012). Behraves et al. (2012) included it in a list of foodborne agents that have emerged since 1970 that also includes different bacteria, viruses, parasites, biotoxins, and a prion. This pathogen is considered among the leading sources of death from foodborne illness since it causes listeriosis, a disease with serious outcome for immunocompromised persons, such as pregnant women, newborns, and elderly people (Allerberger & Wagner, 2010). It is ubiquitous in the environment and can be found in moist conditions, soil, and decaying vegetation (FDA, 2012). *L. monocytogenes* has 13 serotypes, including 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7, from which the serotypes linked with the greatest number of foodborne infections are 1/2a, 1/2b, and 4b (FDA, 2012; Bracegirdle et al., 1994).

L. monocytogenes is recognized as a rough and robust pathogen since unlike other pathogens it is highly salt-tolerant (Gandhi & Chikindas, 2007) and not only can survive in temperatures below 1°C, but it also grows in these environments (FDA, 2012; Rocourt & Cossart, 1997). Furthermore, it is distinguished for its resiliency in food manufacturing environments, including the mild methods of fermentation-based preservation used in a vast number of low acid foods, such as meat, cheese, orange juice, salad dressing, and yogurt (Gahan et al., 1996). When this bacterium is exposed to these adverse circumstances, such as acidic food,

gastric secretions, or following phagocytosis, it can develop an acid-tolerance response (ATR) to persist in more severe acidic conditions which cross-protects it against other forms of stress (Ferreira et al., 2003).

The survival of this pathogen on produce or other food surfaces is generally improved when storage temperatures are abused to 10°C or above. In addition to storage temperature and time, growth and survival of *L. monocytogenes* is influenced by pH and type of acid. Adequate processing sanitation, quality control, and HACCP practices are necessary to prevent contamination of minimally processed produce during processing.

Listeriosis

The infection caused by the bacterium *Listeria monocytogenes* develops into the disease called listeriosis, which is diagnosed more often in humans in urban areas in developed countries and has a high death rate due to brain inflammation and blood infection (Anonymous, 2006). It causes a wide variety of clinical syndromes such as meningitis, osteomyelitis, meningoencephalitis, endocarditis, brain abscess, hepatitis, rhombencephalitis, and liver abscess among others (Schelch, 1996). Case fatality rates can range from 15% to 30% when a serious form of the infection is present. If listerial meningitis occurs, overall case-fatality ratio could be up to 70%; and when newborns are infected it can raise to more than 80% (FDA, 2012). Moreover, pregnant women are more susceptible to infection since the placenta provides a secure place for bacterial growth (Bakardjiev et al., 2005), with spontaneous abortions as result, as well as stillbirth, neonatal infection, severe necrotizing hepatitis, placental necrosis, and increased risk of post implantation loss (Abram et al., 2003). Consequences of dormant listeriosis infections in

pregnant woman include habitual abortions, intrauterine deaths and fetal malformations (Winkhaus-Schindl et al., 1966; Romana et al., 1989).

Although the most common infection mechanism of this disease is through ingestion, the pathogen contagion can also happen due to inhalation or direct contact, as well as venereal transmission. In animals it is normally transferred during consumption of contaminated silage or other feed, while for humans the source of contaminated food involves raw meat and unpasteurized dairy and animal derivative products from the originally contaminated animals (Acha & Szyfres 2003). This pathogen can also spoil uncooked vegetables or processed foods that have become contaminated after processing, particularly soft cheeses, deli cold cuts, sliced or grated cheese, and ice cream (FDA, 2012). A variety of RTE foods including soft cheese, cooked meat, pâté's and vegetables have all been implicated in outbreaks of foodborne listeriosis (McLauchlin, 1996).

Since the infective dose of *L. monocytogenes* is unknown, it is conceivable that the dose-response relationship will depend on the strain and susceptibility of the host, in addition to the food matrix involved. For instance it is probable that fewer than 1,000 cells may cause illness in sensitive persons when raw or inadequately pasteurized milk is consumed, but as previously stated the infective dose may differ widely depending on various factors (FDA, 2012).

Illness Outbreaks Linked to *Listeria monocytogenes*

The success of *L. monocytogenes* as a foodborne pathogen is due to its ability to grow in a wide range of adverse environmental conditions, such as low temperature, low pH and high salt concentration. Moreover, the ability of *L. monocytogenes* to react and survive to harsh conditions

plays an important role for its virulence, because this microorganism faces these adverse environments during its way through the human stomach.

The first epidemiologically confirmed outbreak of listeriosis transmitted by food was linked to the consumption of coleslaw in Canada in 1981 (Schlech et al., 1983), while in the US was in 1985 and associated with Mexican style cheese in California (Linnan et al., 1988; James et al., 1985). Hotdogs and cold cuts were involved in a *Listeria monocytogenes* outbreak in 1998 (CDC, 1999). According to Painter et al. (2013) of the 278 deaths attributed to poultry consumption from the outbreaks that occurred during 1998-2008 period, most were attributed to *Listeria monocytogenes* (63%) or *Salmonella* spp. (26%). Furthermore, three massive outbreaks were linked to turkey delicatessen meat contaminated in the post-cooking step from 1998 through 2002 (Gottlieb et al., 2006; Mead et al., 2006; Olsen et al., 2005) and FDA ranked delicatessen sliced meat as the highest risk ready-to-eat food (FDA, 2003).

The Centers for Disease Control and Prevention (CDC) in Atlanta, GA, reported an escalating number of listeriosis illnesses related to cantaloupe consumption in September 2011 (CDC, 2011b). Furthermore, the serotypes associated were 1/2a and 1/2b which were rarely related to wide outbreaks (CDC, 2011b; Cheng et al., 2008). CDC reported a total of 147 cases from 28 US states, causing 33 deaths and 1 miscarriage (CDC, 2011b). After further inspection, the FDA was able to isolate the pathogen from washed cantaloupes and different environmental surfaces in the packing facility (CDC, 2011b).

Illness Outbreak Related with Acid Products

Cases of *E. coli* O157:H7 illness were with unpasteurized apple juice in Seattle WA in 1996 (39 cases), mayonnaise in Oregon in 1993 (63 cases), yoghurt in North Western England in

1991 (49 cases), and apple cider in CT and NY 1996 (66 cases and 1 dead) among others. Due to these outbreaks attention has been drawn to the acid tolerance properties of this and other bacteria. Furthermore, the outbreak of *E. coli* O157:H7 infection associated with drinking unpasteurized apple cider happen in October 1996, occurred at the same time as an outbreak of cider-related *Cryptosporidium parvum* infections in the US northeast. CDC (1997) identified eight case-patients with ages ranging from 2 to 73 years with a mean of 25 years, all residents of six towns within New Haven County. Six of these patients were female and the illness manifestations included bloody diarrhea and abdominal pain in eight patients, vomiting in five, and fever in four. The estimated duration of the illness was 3 to 11 days with a median of 7 days and five patients were hospitalized. Apple cider is a traditional beverage produced and consumed in the fall, which is often is manufactured locally at small cider mills where apples are crushed in presses. Frequently, no pasteurization is performed on the product before sale. This report suggests that in this case practices for producing apple cider may not be adequate to prevent microbial contamination.

After this case the agency recommendations focused on preventing the introduction of animal manure into orchards, avoiding use of apples that have fallen to the ground, washing and brushing apples before pressing, using a preservative such as sodium benzoate and routine product pasteurization. Even though some of these recommended production practices were followed, in a following outbreak in Connecticut apples were washed and brushed before pressing, however, drop apples were used. Moreover, an outbreak in New York the manufacturer did not use drop apples and apples were washed and brushed before pressing, however cattle were present near the farm and the apples were washed with water from a source later determined to contain *E. coli*, an indicator of contamination with animal or human feces.

According to Parish (1997) approximately 2% of all juices sold in the United States are unpasteurized.

ACID TOLERANCE RESPONSE (ATR)

Bacteria in nature are often exposed to dramatic fluctuations in external pH which represents a threat to their viability, thus they develop a resistance that allows them to grow in a moderately acid environment triggering the synthesis of proteins that protect the cell from more extreme acid conditions. The main concept of Acid Tolerance Response (ATR) is that the growth of microorganism in a moderately acidic environment will trigger the synthesis of proteins that will protect the cell from more extreme acid conditions. Survival of microorganisms, therefore, depends on the presence of adaptive mechanisms that sense an acidifying environment and coordinate an appropriate molecular response (Bang et al., 2000). Other known tolerance responses of bacteria are to refrigeration temperatures, heat, salt, and alkali environment conditions. Acid tolerance is perceived to be an important property of probiotic LAB, enabling the cells to survive gastric acidity and volatile fatty acids produced as a result of fermentation in the intestine (Giannella et al., 1972).

Inducible tolerance to acidic and alkaline environments is recognized as an important survival strategy for many prokaryotic and eukaryotic microorganisms. Recent developments in understanding this phenomenon include the identification of regulatory genes, specific tolerance mechanisms and genes associated with tolerance. There is significant evidence linking pH responses with virulence of pathogens (Foster, 1999), since 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 (Phan-Thanh et al., 2000).

ATR Mechanism

Different systems are engaged in the ATR mechanism and they depend on the growth phase, medium, and type of acid stress (i.e. organic and/or inorganic acid). Two major low pH inducible systems have been identified and they are classified based on the growth phase at which each becomes induced. These two are the stationary-phase ATR and log-phase ATR systems. Most studies have focused on the log-phase ATR system induced when exponentially growing cells suddenly undergo a rapid transition to low pH. ATR causes pH effects on gene expression, characterization of systems that protect the cell against acid or alkaline stress, and influence virulence. Over 50 acid shock proteins (ASPs) are produced during this response. Complementary technologies such as DNA microarrays and proteomics have been developed to study this response in different pathogens and environments at a genomic level (Nascimento et al., 2008).

The acquisition of acid tolerance properties in gram positive bacteria can occur by one of three apparently distinct mechanisms (Cotter and Hill, 2003):

- i) ATPase and/or glutamate decarboxylase, which is implicated in intracellular pH homeostasis.
- ii) Metabolic modifications.
- iii) Protein synthesis to protect and/or repair macromolecules.

Lemos et al. (2008) mention different stress tolerance mechanisms involved in ATR including stringent response, proton extrusion F_1F_0 -ATPase, alteration of catabolic pathways, DNA repair, cytoplasm alkalinization, ATP generation, alteration of cell envelope, and protein protection, repair or degradation. The Fur protein, also required for log-phase ATR, is not involved in the stationary-phase ATR indicating that the two acid-inducible acid tolerance

systems function differently. Consistent with this idea, 10 stationary-phase ASPs have been found that are not log-phase ASPs. The membrane-bound F-ATPase (H^+ -translocating ATPase) is considered the primary agent of acid tolerance of *Streptococcus mutans*, because it allows the organism to maintain a cytoplasmic pH that is more alkaline than the extracellular environment (Lemos et al., 2008).

Research by Sheng and Marquis (2006) demonstrated that in starved cells, a sudden drop in pH results in a rapid increase in ATP, followed by a fast decline that enhances protection against acid killing. By using specific inhibitors of F-ATPase, the authors were able to demonstrate that this increase in ATP comes from the enzyme acting as an ATP synthase. Thus, the F-ATPase may play a dual role in acid tolerance, extruding protons from the cells and, under certain conditions, generating ATP for growth and persistence.

ATR in Foodborne Pathogenic Bacteria

The phenomenon of acid tolerance in bacteria has been studied for many years. However, most of the research has been carried out on *E. coli* and *Salmonella*, while studies on *L. monocytogenes* and other gram-positive bacteria are less extensive (Caggia et al., 2009). *Salmonella* works under both log and stationary phase ATR systems that preserve cells at pH as low as 3 for several hours (Bearson et al., 1997). An earlier exposure to mild acid pH (adaptive pH) leads to protection against extreme acid pH, since exponential phase cells that are adapted to pH 5.8 in minimal media, maintain internal pH at levels compatible with survival while exposed to lethal acid stress (pH 3) using pH homeostasis system. Moreover, lowering adaptive pH of *Salmonella* further to 4.5 triggers the presence of 50 ASPs that contribute to acid tolerance response (Foster, 1999). ASPs respond to internal or external pH signals that can lead to acid

tolerance induction, which also provides cross protection to stresses such as oxidative damage, high temperature and high osmolarity (Moat et al., 2003).

In the case of *E. coli*, the most striking difference from other bacteria is found in stationary phase cells, where it becomes dramatically more acid resistant than *Salmonella* when challenged by low pH in complex media. While *Salmonella* survival at pH levels below 3 is very poor, *E. coli* can survive for hours at pH 2 (Tosun et al., 2005). Also *E. coli* possesses log phase and stationary phase ATR mechanisms, but there are some dramatic differences in acid stress responses of these two closely related species (Bearson et al., 1997). This difference is only observed in complex media, because both organisms exhibit roughly equivalent acid resistance when tested in minimal media.

Although the ability of *L. monocytogenes* to grow and survive under acidic conditions has been investigated (Cole et al., 1990; Conner et al., 1990; McClure et al., 1989; Young & Foegeding, 1993), there is still need for research on the influence of environmental agents on the capability of this pathogen to survive under low pH (Davis et al., 1996). ATR in *L. monocytogenes* can increase its resistance against heat shock (52°C), osmotic shock (25–30% NaCl), and alcohol stress (15%) improving its survival in cottage cheese, yogurt, orange juice, salad dressing and even in food system models containing nisin (Cotter & Hill, 2003). Other studies have shown that ATR can result in increased thermal tolerance in apple, orange and white grape juice. Likewise, it can alter the membrane fluidity inducing nisin resistance. Gahan et al. (1996) concluded that prior adaptation to mildly acidic conditions, *L. monocytogenes* can significantly enhance its survival in low pH foods.

Organic vs. Inorganic Acid Stress

To prevent pathogens and spoilage organisms from growing in foods many producers add weak organic acids to their produce (Tompkin, 2002). For example, weak acids such as acetic acid, sorbic acid, and benzoic acid are widely used in ready-to-eat (RTE) products including mayonnaise, salad dressings, pickled foods, and soft cheeses, dairy products, processed fruit and vegetable products, and certain meat products. Organic acids can affect cell growth in at least two ways, by lowering pH_i and by increasing turgor through anion accumulation. Research with *S. typhi* indicates that organic and inorganic acid tolerances are different, because different repair systems are involved. Studies indicate that organic acids at low concentrations (below minimal inhibitory concentrations) do not induce resistance to higher concentrations of these acids at pH 4.4 (Bearson et al., 1997). Questions have persisted whether acid stress caused by organic acids (e.g. acetate) is different from the stress caused by inorganic acids (e.g. HCl). A weak organic acid not only acidifies pH_i of the cell (the protonated form permeates the cytoplasmic membrane and deprotonates based on the pH_i and the pK of the acid) but also accumulates as an intracellular anion. Organic acids have a greater antimicrobial effect on *Listeria* due to the undissociated form of the acids at acidic pH in foods (Eklund, 1989). The antimicrobial mode of action is primarily attributed to the undissociated acid concentration, rather than hydrogen ions, and when this is present in a non-charged state it has a greater capacity to penetrate bacterial cells than dissociated products. Undissociated acids permeate the cellular membrane via porins or permeases. Inorganic acids, such as hydrochloric acid, are nearly completely dissociated in aqueous solutions. The superior antimicrobial effect of organic acids has been probed on *Listeria* in foods at acidic pH (Banwart, 1979; Lueck, 1980). Furthermore, these studies determined that

the effectiveness of the ATR depended on strain type and acidification medium utilized, where organic acids yielded a more destructive response (lowered pH_i) versus inorganic acids.

***In vitro* Inducible ATR**

In vitro inducible ATR is achieved by treating cells at an intermediary non-lethal pH (4.4–5.8) for 2 to 3 hours (Phan-Thanh et al., 2000). Growth media can vary depending on the bacteria, but usually BHI or TSB (+YE) are used and the pH is adjusted with organic (lactic acid) or inorganic (HCl) acids. Davis et al. (1996) studied the ATR of *Listeria monocytogenes* strain Scott A under *in vitro* conditions, achieving maximal acid resistance when bacteria were previously exposed to pH 5.0 for 1h using HCl. The research concluded that *L. monocytogenes* has a growth phase dependent on an acid tolerance system which is independent of pH. The decrease of the growth solution pH during the growth phase contributed to a high level of acid resistance observed in the stationary phase. In Caggia et al. (2009), a procedure for *in vitro* inducible ATR in logarithmic phase cells was used. In this study, overnight cultures were inoculated into fresh TSB–YE and grown at 37°C until the optical density reached approximately 0.15 representing an early exponential growth phase. Duplicated samples were centrifuged (15 min at 1500g at 4°C) and the pellets were re-suspended in an equal volume of TSB–YE with pH adjusted to 5.7 for ATR induction or to 7.0 for control samples and incubated for 3 h at 37°C. To determine the ATR, cells were harvested by centrifugation and the pellet was re-suspended into TSB–YE with pH adjusted to 3.5 for 3 h to challenge the growth. Media acidification was carried out by adding lactic acid and the surviving samples were serially diluted in phosphate buffered saline (PBS, pH 7.2) and surface plated on TSA–YE at interval times of 0, 30, 60, 90 and 120 min. Plates were incubated at 37°C for 36 h before counting colonies. According to Caggia et al.

(2009), *L. monocytogenes* survival rate in acidic conditions was higher at lower temperatures when the strain was inoculated in higher levels, which corresponds the results retrieved by Phan-Thanh et al. (2000) who stated that the acquired acid tolerance persisted if the adapted bacteria were preserved at 4°C.

Growth and Survival of *Listeria monocytogenes* in Low pH Fruits

Caggia et al. (2009) evaluated the behavior of *L. monocytogenes* in the acidic food products orange juice and minimally processed orange slices to estimate if bacterial occurrence and growth in these environments may represent a potential health hazard. The colony count obtained after 3 h incubation at 37°C, showed a rise with the highest peak after 6 h incubation. The strain growth increased by approximately 3.8 log CFU/ml. This discovery highlighted the ability of the acid-adapted strain to grow in orange juice even at pH 2.6. Both growth curves at 4°C and 25°C were rather similar and their peak values were reached after 6 days of storage. At 4°C, an increase of 1.95 log was recorded, while at 25°C such increase reached 2.42 log. Logarithmic growth was clearly reached during storage time at refrigeration temperatures. The results obtained in this study conclude that the cells adapted to acidic environments can grow in lethal pH conditions. In the past, the growth of human pathogens in citrus products was assumed to be avoided because of the acidity of the juice and the heat treatment applied to commercial citrus juices. Moreover, these results showed the adequacy of minimally processed orange fruits as substrate for acid-adapted *L. monocytogenes* growth. Therefore, from an industrial point of view, the human health consequences of the survival or acid adaptation of *Listeria spp.* in acidic conditions, such as orange processing environments, should be better evaluated.

Flessa et al. (2005) studied survival of *L. monocytogenes* on fresh and frozen cut and whole strawberries that were previously surface spot inoculated using a five strain nalidixic acid resistant cocktail. Inoculation was done applying 15µl of pathogen cocktail per strawberry, which were then dried for 1 h at 24°C and stored in containers at 4°C or 24°C. High initial inoculum level was approximately 7.5 log CFU/mL per sample, while low level was 5.6 log CFU/mL on both cut and whole strawberries. On whole strawberries, a decrease of 0.6 and 1.2 log was observed after the 1h drying period. After 48h storage at 24°C, a reduction per sample of 1.4 and 3.3 log was reported for high and low inoculum, respectively. At 4°C and over 7 day storage period, this reduction was approximately 3 log per whole strawberry sample for both inoculums. Moreover, sliced strawberries were inoculated (6.7 log CFU/25 g sample) with or without 20% sucrose and then frozen at -20±2°C. *L. monocytogenes* counts after 28 days of frozen storage showed a decline by 0 to 1.2 log cycles, with and without 20% sucrose, respectively. In conclusion, the results of this study show that although *L. monocytogenes* is not able to grow on the surface of fresh intact or cut strawberries, it is capable of surviving throughout the expected shelf life of the fresh fruit and even in frozen conditions for at least 4 weeks.

Pathogenic growth has been limited to intact surfaces and ambient storage temperatures in fruits. For instance Pao et al. (1998) found that *L. monocytogenes* was capable of growing within 24 h on the surface of peeled Hamlin oranges stored at 24°C, while Beuchat and Brackett (1991) found that on the surface of whole tomatoes stored at 21°C this pathogen grew within 48 h. However, in uncut strawberries stored at 24°C, *L. monocytogenes* experienced a reduction of 2.4 to 3.2 log CFU/berry within 48 h, perhaps explained by the inability of the bacteria to access adequate moisture and nutrients (Flessa et al., 2005). Strawberries used by Flessa et al. (2005)

had a pH of approximately 3.6 to 3.8. Moreover, factors such as storage temperature and time, pH and type of acid can influence the growth and survival of *L. monocytogenes* (Sorrells et al., 1989).

HORTICULTURE PRODUCTION AND TECHNICAL INFORMATION OF BLUEBERRIES

Vaccinium corymbosum L., normally known by the name highbush blueberries, are endemic to North America woody perennial shrubs that grow long-lived, one quarter to three quarter inch diameter berry fruits (Bratsch & Pattison, 2009). This perennial crop is part of the Ericaceae family, which is distinguished by the capability to grow naturally in acid soil (Gough, 1994). Other *Vaccinium* crops with commercial importance are *Vaccinium angustifolium* and *V. ashei* better known as lowbush and rabbiteye blueberries, respectively. Highbush blueberries are widely planted in different producing areas in the US and around the world, and some of the most marketed cultivars are ‘Bluecrop’, ‘Blueray’, ‘Jersey’, and ‘Duke’ (Retamales, 2012). The nomenclature has been brought up to date in accordance with the International Code for the Nomenclature of Cultivated Plants, which recommends the use of the term cultivar (cv.), and not the old horticultural term variety (Trehane et al., 1995).

Northern and southern highbush cultivars can set fruit in monoculture plantings, since their flowers are self fertile. However, larger fruit production, earlier ripening and higher yields can be achieved by using cross-pollination provided by wild bees such as bumblebees to improve pollination. Also during the pollination period, honeybee hives can help promote blueberry cross-pollination. Insects such as fruit-worm species, aphids, mites and blueberry maggots can become an important problem for production (Prodorutti et al., 2007). For instance in New

Jersey the number one insect problem, especially for organic growers, is the blueberry maggot fly (Marucci, 1996). In addition, different fruit-rotting fungi cause significant damage in highbush blueberries. For example, in Michigan, the most serious fruit diseases are caused by *Colletotrichum acutatum* (anthracnose fruit rot or ripe rot), *Alternaria* sp. (Alternaria rot), and *Botrytis cinerea* (Ramsdell, 1989). Proper pruning not only can achieve much higher yields and larger fruit sizes, but also supports fruit rot control. Fruit-rotting pathogens are capable of surviving harsh winter conditions by infecting twigs, and they just require high relative humidity for infection and sporulation (Caruso & Ramsdell, 1995). Another widely used rot control are fungicides applied to the crop as a spray with fixed wing aircraft and various other equipment. Captan (N-Trichloromethylthio-4-cyclohexene-1,2-dicarboximide) is the most used fungicide in Michigan. It is a non-systemic protectant that needs to be applied uniformly as a wettable powder throughout the canopy for better results. The fungicide efficacy can be affected by application equipment characteristics and operation through altering the deposition patterns (Hanson et al., 2000).

The soil for growing purposes can be nutritionally poor organic sand, however it needs to be well drained (Fernández & Hoefft, 2009) and acidity should range from pH 4.5 to 5.2 (Williamson et al., 2006). This crop is considered long-term, since it can be in the field for more than twenty years. However, it requires several years to establish and within the third and fourth season the harvest can be initiated, achieving full production after six to eight years (Bratsch & Pattison, 2009). Best planting sites include gentle slopes that provide good cold air and surface water drainage. Moreover, sloped locations provide several degrees of frost protection compared to lower areas, allowing cold air to flow away from the planting as it settles to low areas (Thiele, 1999). In poorly drained locations the cultivation rows should be marked and if required, raised

in the form of beds. Row alignment with the slope is encouraged, because perpendicular arrangement of these beds across inclined hills may end up in water pooling (Bratsch & Pattison, 2009).

Advantages of mulching soil include better moisture holding, uniformity and improved weed control. Furthermore, mulched soils reduce negative effects of frost heaving, since they remain warmer during the winter and cooler during the summer (Moore, 1990). Larger plants and greater yields over time are advantages of mulching with irrigation.

The water use in blueberries crops is significant and is estimated to range from four to six gallons per day per plant. Another estimate per acre indicates one inch per week for a mature planting and two inches when plants have a full crop load (Bratsch & Pattison, 2009).

Considering its increased efficiency and delivery, drip irrigation is preferred to overhead irrigation. Furthermore, drip irrigation allows simultaneous field work operations and diminishes foliar disease prevalence. However, overhead irrigation should be installed to provide frost protection and additional moisture when the planting site is likely to suffer from frosts (Gomez-Rodas et al., 2010). Water used for sprinkle irrigation, as well as water used for processing and mixing pesticides is a potential source of biological contamination, since it will come in contact with blueberries. Safety measures, such as the use of potable water, can minimize the potential for microbial contamination of fresh or frozen blueberries. Moreover, all along the food chain including production, harvesting, sorting, packing, and transport it is crucial that workers hygiene and sanitation practices reduce the potential risk for infecting blueberries (Gomez-Rodas et al., 2010). The use of animal manure or biosolid waste material for fertilization purposes should be carefully managed to avoid potential pathogenic contamination of fresh fruit. Factors that could influence the fertilization requirements include soil pH and nitrogen source, age of the

plants, use of and the condition of mulch, soil type and percentage of organic matter, and the water applied (Bratsch & Pattison, 2009).

Postharvest Procedures Affecting Quality and Shelf Life

Blueberries can be mechanically or hand harvested (Forney, 2008), however hand harvesting is preferred for fresh fruit markets since mechanical harvesting reduces fruit firmness due to physical abuse (NeSmith et al., 2002) and considerably reduces market life (Mainland et al., 1975). This fruit holds very well on the plant through varying stages of maturity and after the first ripe berry develops. Picking should be done every five to seven days to attain the best fruit quality (Cline, 2005). Although blueberries targeted to processing and industrial market are previously sanitized, hand harvested and field packed fresh berries do not include any previous washing step to maintain product shelf life, relegating this task to the consumer before consumption (Harris et al., 2003). For high value fresh fruit, it is recommended to harvest them immediately into retail containers to avoid bruising, with no previous washing step to avoid moisture increase and mold spoilage (Bower, 2007). Many research studies have found that washing strawberries can achieve 1 to 2 log reductions depending if *Salmonella*, *E. coli* O157:H7 or *L. monocytogenes* were inoculated and the type of sanitizer used (Schlitt-Dittrich et al., 2004; Lukasik et al., 2003; Yu et al., 2001). However, fresh blueberries are manually picked and not washed, since the light waxy covering on them should not be removed, as this will decrease storage life (Bratsch & Pattison, 2009).

Furthermore, berries should be rapidly cooled to 2°C after harvest and are ideally shipped at 1°C to maximize shelf life (Mitcham & Mitchell, 2002). Normally fresh highbush blueberries have a shelf life of 1 to 8 weeks depending on factors such as stage of fruit ripeness, method of

harvest, presence of fruit disease, and storage conditions, which include temperature, relative humidity, and atmosphere conditions (Hancock et al., 2008). Blueberries capacity of quality retention will depend on: the *Vaccinium* species (Makus & Morris, 1993; Silva et al., 2005) and cultivar (Miller et al., 1993; Silva et al., 2005), harvest date (Beaudry et al., 1998), handling directly after harvest (Miller & Smittle, 1987; Jackson et al., 1999), storage conditions (Smittle & Miller, 1988; Beaudry et al., 1998; Rosenfeld et al., 1999) and packaging (Miller et al., 1984).

Everyone involved at any level of production such as farm, packing facility, distribution center, and transport operation, is accountable in a successful food safety program. Workers should be trained and be qualified to monitor and document all the information to track the fruit back through the distribution pathway from the farmer to ensure a successful production.

CONTROLLED ATMOSPHERE OF FRESH PRODUCE FOR SHELF LIFE

EXTENSION AND SAFETY

Although there are differences in storability between cultivars, cold storage, which implies low temperature and high relative humidity conditions, is considered as the most effective method for short term storage, since it allows storage for nearly one month without significant loss of quality. Cold storage controls decay, which shortens the shelf life of blueberries. *Botrytis cinerea*, *Alternaria* and *Colletotrichum* rot are the limiting factors for cold storage of highbush blueberries (Ceponis & Cappellini, 1977). Other technologies have been implemented to complement cold storage and reduce fruit decay, such as controlled atmosphere storage using CO₂ enriched atmospheres (Ceponis & Cappellini, 1983). The use of controlled atmosphere storage with high CO₂ concentration is widely recognized by different researchers to prolong the storage period (Borecka & Pliszka, 1985). Depending on the different cultivars, there

will be various responses to high CO₂. For instance, highbush blueberries ‘Bluecrop’ and ‘Dixie’ under 20% CO₂ and 2% O₂ maintained fruit quality for only 21 days, however the extension of storage period to 56 days resulted in poor quality (Roelofs, 1993). Moreover, ‘Climax’ and ‘Woodard’ cultivars of rabbiteye blueberry (*V. ashei*) stored for 42 days at 5°C in 20% CO₂ and 5% O₂ resulted in a better quality of fruit than those in normal air (Smittle & Miller, 1988) and ‘Burlington’ showed a good quality for up to six weeks at 0°C with 10% CO₂ and 15% O₂ (Forney et al., 2003). Although cultivar ‘Duke’ maintained fruit firmness and acidity content under 12% CO₂ without O₂ reduction, a CO₂ level higher than 12% has a negative impact on flavor, acidity content, and firmness of the fruit (Harb & Streif, 2004).

Mahajan and Goswami (2004) evaluated the postharvest quality of lychee (*Litchi chinensis* Sonn. cv. Bombay) after storage under CAS at 3.5% O₂ and 3.5% CO₂, at 2°C and high relative humidity (92-95%). Weight loss after 56 days of storage achieved a lower value under CAS (4.9%) when compared to regular atmosphere (RA) at 2°C (11.0%) under same humidity conditions, while normal ambient controls achieved 31.1%. Furthermore, fruit firmness and pericarp puncture strength increased 2.2 times in CAS and 3.9 times in RA from the initial level.

Control of decay and also shelf life prolongation can be obtained by using high CO₂ levels in storage and shipping conditions in cherries exported from Chile to Japan (Retamales, 2003). Moreover, gray mold rot incidence in strawberries can be significantly reduced by applying 15% CO₂ and using hot water dips (Garcia et al., 1995), treatments of hot air (42 or 48°C) for 3h in an air oven (Civello et al., 1997). Heat treatments lead to an increase of fruit respiration rates, however once they are finished the fruit recovers to normal levels (Garcia, 1995). Increase of respiration rate after harvest makes fresh produce more susceptible to disease

organisms and limits the fruit shelf life under ambient conditions. Fungal development of blueberries was minimized using CAS when compared to air control (Schotsmans et al., 2007). Duarte et al. (2009) evaluated quality changes of blueberries (*Vaccinium corymbosum* L. cv Brigitta) after storage under CAS conditions at 0°C. Atmospheric air was used as control for three gas concentrations treatments which included CO₂ at 5%, 10%, and 15%, each one combined with 5% O₂. Weight loss was not influenced by treatment and averaged a 0.9% reduction from the initial weight after 48 days. However, better fruit quality was observed under CAS after 24h compared to the control, by day 48 this difference wasn't significant and a high number of unmarketable fruit was observed in all treatments. By day 24, CAS fruit were firmer, had better color, higher anthocyanin and acidity levels, while soluble solids content was not measured (Duarte et al., 2009). High differences in storability depend on the type of cultivar used. According to Eccer et al. (2005) rabbiteye cultivars gave better quality storability results compared to highbush, when stored in five different CAs (2% O₂ and 10% CO₂; 4% O₂ and 10% CO₂; 2% O₂ and 14% CO₂; 4% O₂ and 14% CO₂ using air as control) at 0°C for up to 5 months. In this study, high CO₂ concentrations significantly reduced fruit decay. Schotsmans et al. (2007) found that shrivel and weight loss were not affected on rabbiteye cultivars stored up to 6 weeks at 1.5°C in either controlled atmosphere (2.5kPa O₂ and 15kPa CO₂) or air control confirming that temperature is crucial for minimizing weight loss. Furthermore, maintaining high relative humidity (95% or greater) reduces weight loss and shriveling of blueberries (Forney, 2008), however condensation should be avoided since it can result in increased fruit decay (Sargent et al., 2006). Maximum weight loss of blueberries before becoming non-salable is approximately 5% to 8% (Sanford et al., 1991). Jackson et al. (1999) reported a minimal weight loss of about 2% in blueberries after 14 days of storage at 0°C.

Modified Atmosphere Packaging

Modified atmosphere packaging for products like cut salads has become a popular and satisfactory product in the market. One of the main examples is the use of low oxygen modified atmosphere in iceberg lettuce retarding the browning rate (Cameron, 2003). Most of these types of products contain a modified atmosphere that is less than 1 kPa O₂ and they should be stored at 5 to 10°C for significant browning control (Smyth et al., 1998). Prevention of fungal growth and fungal spore germination can be achieved by using limited O₂ and elevated CO₂ atmospheres (Day et al., 1990; Beaudry, 1999), meanwhile fruit firmness and sensory quality are also maintained.

While controlled atmosphere storage (CAS) involves maintaining a fixed concentration of gases surrounding the product by careful monitoring and continuous addition of gases, the gaseous composition of modified atmosphere packaging (MAP) is constantly changing due to chemical reactions and microbial activity (Mullan & McDowell, 2003). While modified atmosphere packaging technology is largely used for minimally processed, fresh and “ready-to-use” fruits and vegetables convenience products, controlled atmosphere storage (CAS) is used for bulk produce storage (Sandhya, 2010). According to Hintlian and Hotchkiss (1986) the definition of MAP is ‘the packaging of a perishable product in an atmosphere that has been modified so that its composition is other than that of air. Although benefits such as increased shelf life, high quality product, consumer convenience and the absence of chemical preservatives can be achieved with this technology, added costs, temperature control requirement, product safety assurance, increased packaging volume leading to increased retail display space and transport costs are some of the disadvantages (Farber, 1991; Parry, 1993; Davies, 1995; Phillips, 1996).

High Oxygen Packaging Atmosphere

Elevated O₂ levels have been studied as a preservative gas for packaging respiring products with an inhibiting effect on bacterial and yeast growth, reduction in mold development, and prevention of undesired anoxic fermentation (Day, 2001). However, researchers found detrimental effects in organoleptic properties of strawberries under a 100% O₂ controlled atmosphere conditions produced by the increase of fermentative metabolites (Wszelaki & Mitcham, 2000). In the case of blueberries, high O₂ conditions of 40% result in the best treatment with a high gas permeable film in the package stored at 4°C and 12°C (Rosenfield et al., 1999). However acidic taste and blueberry flavor were described after storage at low temperatures, while bitter taste and storage flavor predominated when stored at high temperature. Jacxsens et al. (2003) evaluated high oxygen atmosphere treatment (95% O₂) in raspberry storage that resulted in mold development and yeast growth inhibition, nonetheless there were negative organoleptic effects such as off flavors, which implied a shortening of shelf life. In the study conducted by Stewart (2003), one of the conclusions was that there are benefits in using high oxygen packaging atmospheres, especially at low temperatures, since the levels of cell wall hydrolytic enzymes either reduce or, in the case of cellulase, do not increase during storage thus inducing a better firmness of the packaged fruit. Although high O₂ atmospheres were found to inhibit the growth of several groups of bacteria, yeasts, and molds (Day, 2002), as well as specific food pathogenic and spoilage microorganisms, the CCFRA's recommended optimal gas levels (80-95% O₂ / 5-20% N₂) has become a big safety issue due to the high flammability of pure O₂. This issue must be addressed before such a treatment can become commercially viable (Lu & Toivonen, 2000).

Use of Chlorine Dioxide Gas in CAS

Other gases have been studied that not only increase shelf life in controlled atmosphere storage, but also to inhibit pathogenic bacteria. Many researchers suggest that chlorine dioxide (ClO_2) gas treatment is an effective decontamination technique for improving the safety of strawberries while extending shelf life. For instance, Han et al. (2004) evaluated the efficacy of different concentrations and application systems of ClO_2 gas to control *Escherichia coli* O157:H7 and *Listeria monocytogenes* on strawberries by using spot inoculation and storing fruit for 1 day at 4°C. High ClO_2 gas concentrations increased pathogenic log reductions for both batch and continuous application systems. A reduction higher than 5 log was obtained for each bacteria with application rates of 4 mg/liter ClO_2 for 30 min (batch) and 3 mg/liter ClO_2 for 10 min (continuous). Additionally, no significant change was observed on surface color of strawberries after 1 week and 3.0 mg/liter ClO_2 for 10 min exposure. However, after treatments were applied to the fruit, ClO_2 and chlorite (Cl_2) residues were 0.19 ± 0.33 mg per kg and 1.17 ± 2.02 mg per kg, respectively. After 1 week of storage, these values decreased to 0.07 ± 0.12 mg Cl_2 per kg and a non detectable level in ClO_2 . Sy et al. (2005) studied not only the control of the foodborne pathogen *Salmonella enterica*, but also yeast and molds on blueberries, strawberries and red raspberries using ClO_2 gas. Blueberries were stem scar, calyx tissue or skin inoculated and dried for 2 h at 22°C, then incubated at 4°C for 20 h and 22°C for 2 h under high relative humidity (75-90%) before treatment. ClO_2 concentrations of 4.1; 6.2; and 8.0 mg/liter were applied using chemical reactants sachets, while treatment times were 30, 60 and 120 min, respectively, at 23°C. On blueberries, a significant reduction of the pathogen by 2.4 to 3.7 log CFU/g using 8.0 mg/liter of ClO_2 , was observed, whereas significant reductions of 3.8 to 4.4 log CFU/g on strawberries and 1.5 log CFU/g on raspberries were also noticed. When blueberries

were inoculated on skin a higher lethality was achieved compared with the stem scar tissue inoculation. Yeast and molds populations under 4.1 and 8.0 mg/liter ClO₂ treatments were reduced by 1.4 to 2.5 log CFU/g in blueberries, 1.4 to 4.2 log CFU/g in strawberries and 2.6 to 3.0 log CFU/g in raspberries. Sensory quality was not affected on fruits treated with 4.1 mg/liter of ClO₂ after 10 days of storage at 8°C. However, the authors reported a loss in sensory quality in control blueberries, which may be caused by a reduction in relative humidity during fruit storage (Sy et al., 2005). Popa et al. (2007) evaluated a dry chemical sachet that produced ClO₂ for *Listeria monocytogenes*, *Salmonella* spp. and *E. coli* O157:H7 inactivation in blueberries, as well as yeast and molds popular for fruit spoilage. Samples were exposed to 4 mg/liter ClO₂ gas for 12 h at 22°C with 99% relative humidity. Reductions observed were 3.94 log CFU/g for *L. monocytogenes*, 3.62 log CFU/g for *Salmonella*, 4.25 log CFU/g for *E. coli* O157:H7, 3.10 log CFU/g for yeasts, and 3.17 log CFU/g for molds. Another experimental treatment was to expose stacked pallets of blueberries to 18 mg/liter of ClO₂ for 12 h. This treatment achieved significant reductions of 2.33, 1.47, 0.52, 1.63, and 0.48 log CFU/g for mesophilic aerobic bacteria, coliforms, *E. coli*, yeasts, and molds, respectively, after gassing and compared to controls (Popa et al., 2007).

Wu and Kim (2007) not only studied the control of foodborne pathogens but also yeast and molds on blueberries using aqueous ClO₂. Different foodborne pathogens were spot inoculated on blueberries skins and samples were treated with different concentrations (1, 3, 5, 10, and 15 ppm) of ClO₂ and various contact times (10 s; 1, 5, 10, 20, 30 min; and 1 h and 2 h). *L. monocytogenes* was the most effectively reduced pathogen compared to the other bacteria, achieving a 4.88 log CFU/g reduction. For inhibition of *Salmonella* Typhimurium short

treatment times were more effective than longer ones for most gas concentrations. Yeast and molds were reduced by 2.83 log CFU/g under 15 ppm ClO₂ after 1 h.

Although chlorine based disinfectants are economical bactericides that inactivate all types of vegetative cells, these sanitizers have several disadvantages including being toxic to humans, and irritating in high concentrations, likely to form carcinogenic compounds and being harmful to the environment (Guzel-Seydim et al., 2004). For many years chlorine products have been preferred by industries to sanitize and disinfect food and water, since chlorination can efficiently decrease infectious foodborne diseases from spreading. However, when this compound is combined with many organic substances it can form toxic by-products, that when released to the environment can not only affect this but also human public health. Trihalomethanes (THM) and haloacetic acids (HAA5) are some of the mutagenic and carcinogenic compounds that can cause these problems (Bellar et al., 1974; Trussell & Umphres, 1978).

OZONE APPLICATION FOR SHELF LIFE EXTENSION AND SAFETY

Ozone was discovered in 1839 by C. F. Schonbein and its name is derived from the Greek word “Ozein,” which means “to smell” since this gas has a characteristic pungent odor (Zeynep, 2003). At normal temperatures and pressure this gas is relatively unstable and is partially soluble in water (Muthukumarappan et al., 2000). In 1982 FDA granted ozone the status of generally recognized as safe (GRAS) for use in bottled water (FDA, 1982), while USDA permitted its use for reconditioning recycled poultry chilling water (USDA, 1984). Currently ozone is formally approved by the FDA for use as a disinfectant or sanitizer in the gas or liquid phase on food (21 CFR, Part 173) and its use in direct contact as an antimicrobial agent for

treatment, storage and processing of different foods including raw and minimally processed fruits and vegetables.

Production and Stability of Ozone

High energy sources such as high voltage electrical discharge or ultraviolet radiation are required to convert oxygen molecules to ozone from air or other gases containing oxygen. Because of ozone instability and rapid decomposition to oxygen, it must be manufactured on site for immediate use. One of the most commonly used methods to produce ozone in large amounts is the corona discharge, which produces ozone when a high voltage alternating current is applied across a discharge gap where oxygen is ionized (Kim et al., 1999; Kim et al., 2003).

The time required to reduce ozone concentration to half its value (half-life time) in gaseous states is approximately 12 h at room temperature, while in pure water (pH 7-8) it is 20 to 30 min (Khadre et al., 2001; Graham, 1997). Factors that affect half-life time of ozone include temperature (Strait 1998), since at lower temperatures gas molecules do not move quickly and therefore collide less frequently, while at higher temperatures ozone molecules move faster and collide more frequently. Also when relative humidity increases, half-life time of ozone decreases (McClurking & Maier, 2010; Strait, 1998).

Safety Concerns of Gaseous Ozone

Ozone is highly corrosive and can be lethal to humans at high concentrations and long time exposure. The current permissible exposure level-time weighted average (PEL-TWA) for ozone exposure in the work place environment is 0.1 ppm, as recommended by the American Conference of Governmental Industrial Hygienists (ACGIH, 1986) and adopted by the United

States Occupational Safety and Health Administration (OSHA) (Kim et al., 2003). According to CFR (1997) susceptible individuals can be exposed continually to this ozone concentration during a normal 8 h day/40 h working week without adverse effects. When exposed to 0.3ppm the short term limit time is less than 15 min and four times per day.

Measurement of Ozone Concentration

The most widely used and popular technique for monitoring atmospheric ozone is the UV absorbance method, which is also the method used almost exclusively by the US EPA and environmental agencies internationally. However numerous methods and devices for ozone concentration quantification have been developed in the past based on a variety of chemical luminescent, electrochemical, semiconductor, and photometry techniques (Andersen et al., 2010). Heated metal oxide semi-conductor (HMOS) sensors have been broadly adapted for industrial ozone gas monitoring, since it gives short time response averaging less than a minute. These sensors work by heating a small and thin film substrate (metal oxides such as indium oxide for instance) reaching high temperatures of 200°C approximately. At this temperature, the substrate shows sensitivity to ozone and exhibits a change in resistance that is proportional to the ozone concentration. The electrical circuit of the devices interprets the resistance change and displays the corresponding ozone concentration level as ppm or ppb (Kanan et al., 2009; Korotcenkov et al., 2007).

Ozone Effects on Produce Quality

As previously mentioned, ozone is effective in improving the microbial quality and safety of minimally processed agricultural commodities, due to its high oxidation potential compared to

other oxidants when applied in advanced oxidation processes (AOPs) (Crowe et al., 2007). Kim et al. (2010) evaluated the shelf life extension of blueberries packed in plastic clamshell boxes and stored in a Shelf Life Extender (SLXR) Modified Atmosphere Storage (MAS) container with 15.5% O₃ gas, 193 mg/L sulfur dioxide (SO₂), 18.8% carbon dioxide and normal air as control for 48 days at 20°C. Fruit decay percentage and moisture percentage were not influenced by any gas treatment up to day 34, while by day 48 berries stored under SO₂ and O₃ presented less weight loss compared to other treatments. Fan et al. (2001) studied shelf life extension of highbush blueberries by pretreating them using ozone in CAS at concentrations of 0, 200, or 700 ppb for 1, 2, or 4 days and later storage at 10°C for 7 days in air or 0°C for 4 weeks in CAS (10 kPa CO₂/15 kPa O₂). Fruit marketability was 4 to 7% higher with ozone for 2 or 4 days and then at CAS when compared to controls. Although respiration was stimulated after pretreatment with 200 ppb ozone for 1 or 2 days, no differences were observed between respiration quotient and ethylene production. Moreover, antioxidant capacity (e.g. anthocyanins and phenolic compounds content) was not affected by ozone treatments.

The oxidation potential of chlorine dioxide is 1.50, while ozone is 2.07 (Manley & Niegowski, 1967), this latter having a greater effect against certain microorganisms plus decomposing rapidly into oxygen without leaving residues (White, 1992). Moreover, Wu and Kim (2007) experienced a significant decrease of ClO₂ concentrations over time and even further degradation when exposed to blueberries. The disadvantages of using ozone compared to the use of chlorine are a higher corrosiveness and initial capital cost for generation (Smilanick et al., 1999).

Pathogenic and Spoilage Inhibition Studies on Food

Crowe et al. (2007) studied the inhibition of common spoilage bacteria *Enterobacter agglomerans* and *Pseudomonas fluorescens* isolated from blueberries using 1% hydrogen peroxide (H_2O_2), 100 ppm chlorine and 1 ppm aqueous ozone as well as AOPs including combination of 1% H_2O_2 /UV, 100 ppm chlorine/UV and 1 ppm ozone/1% H_2O_2 /UV. All H_2O_2 and ozone treatments showed significant population decreases (2.5 log CFU/g reductions) when compared to chlorine treatments and the unwashed control; however, treatments were not significantly different from each other. It was concluded that a single treatment of ozone or H_2O_2 are as effective as AOPs and can be considered valid alternatives for disinfection of blueberries with chlorine.

Mold control using ozone and temperature was investigated by Liew and Prange (1994). *Botrytis cinerea* and *Sclerotinia sclerotiorum* were inoculated in whole carrots, which were stored in chambers and flushed with 0.5 l/min air and ozone concentrations of 0, 7.5, 15, 30, or 60 ml/l for 8 h daily for 28 days at 2, 8 and 16°C. Carrot respiration rate increased and less intense and lighter color were observed with higher ozone concentrations, however both fungi daily growth rates were reduced in half when compared to controls, demonstrating the fungistatic properties of this gas.

Pathogenic bacteria resistance to ozone is not associated with vegetative bacteria and especially not with *Listeria monocytogenes*, however it can be observed in bacterial spores from *Bacillus stearothermophilus*, which are an indicator of ozone resistance and commonly used for testing disinfection effectiveness (Khadre et al., 2001). Yet many researchers have studied the antimicrobial efficacy of aqueous and gaseous ozone against different pathogens. For example Bialka and Demirci (2007) used pulsed UV-light, and gaseous and aqueous ozone to estimate

blueberries decontamination effectiveness against *Escherichia coli* O157:H7 or *Salmonella*. The berries were exposed to continuous and pressurized ozone, achieving maximum decrease of both pathogens after 64 min. When ozone was applied using continuous flow after 64 min, *E. coli* O157:H7 showed a 2 log reduction while *Salmonella* just 1 log. Furthermore, when pressurized ozone was used after 64 min, *Salmonella* was reduced 3 logs and *E. coli* O157:H7 by 1.4 logs. No sensory characteristics differences were observed between treated and untreated blueberries.

According to Kim and Hung (2012), a combination of 1 min ozone exposure followed by 2 min of UV can achieve higher *E. coli* O157:H7 reductions on blueberries when compared to both single treatments. Treatment with an ozone concentration of 4000 mg/l reduced this pathogen by 0.66 and 0.72 log CFU/g on calyx and skin, respectively, while UV light at 20mW/cm² for 10 min achieved 2.14 and 4.05 log reductions on calyx and skin of blueberries, respectively.

An in-package ozonation device, which produced ozone inside sealed film bags, was developed by Fan et al. (2012) and tested to preserve fruit quality and inhibition of *Listeria innocua*, *Salmonella* Typhimurium and *E. coli* O157:H7 on tomatoes. Pathogenic stem scar inoculation of the three strains was used on the surface of tomatoes, which were then packed in the bags with an ozone concentration of 1,000 ppm within 1 min of activation. A 4 log CFU reduction of *Listeria innocua* was observed per fruit leading to non detectable levels. *E. coli* and *Salmonella* were reduced by 2 to 3 log CFU/fruit after 2 to 3 min treatments demonstrating that bacteria can respond differently to ozonation. Finally, after a 22 days post treatment storage period there were not any negative effects on fruit texture and color.

Daş et al. (2006) studied growth and survival of spot inoculated or stem injected *Salmonella* Enteritidis in cherry tomatoes stored under passive MAP, CAS, or air at 7 and 22°C.

Gas composition for MAP were 6% O₂ and 4% CO₂, while in CAS CO₂ was maintained at 5%. Factors such as inoculum location on the fruit, suspension cell density, inoculum attachment time and storage temperature were identified to influence on pathogenic growth and survival. Stem scars showed the most protective environments for the pathogen. A low inoculum of 3.0 log CFU/tomato showed undetectable levels by day 4 under MAP and day 6 under CAS and air storage, showing a faster death rate on MAP when compared to CAS and air. An approximate decrease of 4.0 to 5.0 log CFU/tomato during MAP, CAS, and air treatments was observed when a high inoculum (7.0 log CFU/tomato) level on tomato surfaces was studied. The authors observed that the lower the temperature, the faster the pathogen died, since non-detectable levels were reached on day 6 at 7°C, and on day 8 while at 22°C. The effect of an ozone treatment (5-30 mg/l ozone gas for 0-20 min) was also evaluated in this study. For both low and high inoculum of *Salmonella* Enteritidis, 10 mg/l ozone with intervals of 5 and 15 min, respectively, were the most effective treatments to inhibit the pathogen.

References

- Abram, M., Schluter, D., Vuckovic, D., Wraber, B., Doric, M., & Deckert, M. (2003). Murine model of pregnancy-associated *Listeria monocytogenes* infection. *FEMS Immunol. Med. Microbiol.*, 35, 177–182.
- ACGIH. (1986). Threshold limit values for chemical substances in the work environment. American Conference of Governmental Industrial Hygienists, Cincinnati, OH.
- Acha, P. N., & Szyfres, B. (2003). Listeriosis. In: *Zoonoses and communicable diseases common to man and animals. Volume 1. Bacterioses and mycoses. 3rd ed.* (p. 168-179). Washington, DC: Pan American Health Organization [PAHO].
- Allerberger, F., & Wagner, M. (2010). Listeriosis: a resurgent foodborne infection. *Clin Microbiol Infect.*, 16:16–23.
- Andersen, P. C., Williford, C. J., & Birks, J. W. (2010). Miniature Personal Ozone Monitor Based on UV Absorbance. *Analytical Chem.*, 82(19), 7924-7928.
- Anonymous. (2006). Listeriosis. Center for Food Security and Public Health. College of Veterinary Medicine. Ames, IA: Iowa State University. Available at http://www.cfsph.iastate.edu/FastFacts/pdfs/listeriosis_F.PDF. Accessed 13 May 2013.
- Bakardjiev, A. I., Stacy, B. A., & Portnoy, D. A. (2005). Growth of *Listeria monocytogenes* in the guinea pig placenta and role of cell-to-cell spread in fetal infection. *J. Infect. Dis.*, 191, 1889–1897.
- Bang, I. S., Kim, B. H., Foster, J. W., & Park, Y. K. (2000). OmpR regulates the stationary-phase acid tolerance response of *Salmonella enterica* serovar Typhimurium. *J. Bacteriol.*, 182(8), 2245-2252.
- Banwart, G. J. (1979). Basic Food Microbiology. AVI Publishing Co., Inc., Westport, CT.
- Bearson, S., Bearson, B., & Foster, J. W. (1997). Acid stress responses in enterobacteria. *FEMS Microbial Lett.*, 147, 173-180.
- Beaudry, R. M., Moggia, C. E., Retamales, J. B., & Hancock, J. F. (1998). Quality of 'Ivanhoe' and 'Bluecrop' blueberry fruit transported by air and sea from Chile to North America. *HortScience*, 33, 313–317.
- Beaudry, R. M. (1999). Effect of O₂ and CO₂ partial pressure on selected phenomena affecting fruit and vegetable quality. *Postharvest Biol. Technol.*, 15, 293–303.
- Behravesh, C. B., Williams, I. T., & Tauxe, R. V. (2012). Emerging Foodborne Pathogens and Problems: Expanding Prevention Efforts before Slaughter or Harvest. In: *Improving Food Safety*

Through a One Health Approach: Workshop Summary (p. A14). Washington, DC: National Academies Press.

Bellar, T. A., Lichtenberg, J. J., & Kroner, R. C. (1974). The occurrence of organohalides in chlorinated drinking water. *J. Am. Water Works Assoc.*, 66, 703–706.

Berger, C.N., Sodha, S. V., Shaw, R. K., Griffin, P. M., Pink, D., Hand, P., & Frankel, G. (2010). Fresh fruit and vegetables as vehicles for the transmission of human pathogens. *Environ. Microbiol.*, 12(9), 2385-2397.

Beuchat, L.A., and Brackett, R.E. (1991). Behavior of *Listeria monocytogenes* inoculated into raw tomatoes and processed tomato products. *Appl. Environ. Microbiol.*, 57, 1367–1371.

Beuchat, L. R. (1998). Surface decontamination of fruits and vegetables eaten raw: a review. Food Safety Unit, WHO, Report WHO/FSF/FOS/98.2, pp. 1–49.

Bialka, K. L., & Demirci, A. (2007). Decontamination of *Escherichia coli* O157: H7 and *Salmonella* enterica on Blueberries Using Ozone and Pulsed UV-Light. *J. Food Sci.*, 72(9), M391-M396.

Boffetta, P., Couto, E., Wichmann, J., Ferrari, P., Trichopoulos, D., Buenode Mesquita, H. B., van Duijnhoven, F. J., Buchner, F. L., Key, T., Boeing, H. et al. (2010). Fruit and vegetable intake and overall cancer risk in the European Prospective Investigation into Cancer and Nutrition (EPIC). *J Natl Cancer Inst.*, 102, 529–37.

Borecka, H.W., & Pliszka, K. (1985). Quality of blueberry fruit (*Vaccinium corymbosum*) stored under LPS, CA, and normal air storage. *Acta Hort.*, 165, 241–249.

Bower, C. (2007). Postharvest handling, storage, and treatment of fresh market berries. In: Y. Zhao (Eds.), *Berry fruit: value-added products for health promotion* (pp. 262-284). New York, NY: CRC press.

Bracegirdle, P., West, A. A., Lever, M. S., Fitzgeorge, R. B., & Baskerville, A. (1994). A comparison of aerosol and intragastric routes of infection with *Listeria spp.* *Epidemiol Infect.*, 112, 69–79.

Bratsch, A., & Pattison, J. (2009). Specialty crop profile: Blueberries. Publication 438-103. Virginia Cooperative Extension.

Brenner, F. W., Villar, R. G., Angulo, F. J., Tauxe, R., & Swaminathan, B. (2000). *Salmonella* Nomenclature. *J. Clinical Micro.*, 38, 2465-2467.

Burnett, S. L., & Beuchat, L. R. (2001). Human pathogens associated with raw produce and unpasteurized juices, and some reasons related to difficulties in decontamination. *J. Ind. Microbiol. Biotechnol.*, 25, 281-287.

Caggia, C., Scifo, G. O., Restuccia, C., & Randazzo, C. L. (2009). Growth of acid-adapted *Listeria monocytogenes* in orange juice and in minimally processed orange slices. *Food Control*, 20, 59–66.

Cameron, A.C. (2003). Modified-atmosphere packaging of perishable horticultural commodities can be risky business. *Acta Hort.*, 600, 305-310.

Caruso, F.L., & Ramsdell, D.C. (1995). Compendium of blueberry and cranberry diseases. *APS Press, Amer. Phytopathol. Soc.*, St. Paul, Minn.

Center for Disease Control and Prevention. (1997). Outbreaks of *Escherichia coli* O157:H7 Infection and Cryptosporidiosis Associated with Drinking Unpasteurized Apple Cider -- Connecticut and New York. Available in: <http://www.cdc.gov/mmwr/preview/mmwrhtml/00045558.htm>. Accessed 29 May 2013.

Centers for Disease Control and Prevention. (1999). Update: multistate outbreak of listeriosis—United States, 1998–1999. *MMWR Morb. Mortal. Wkly. Rep.*, 47, 1117–8.

Centers for Disease Control and Prevention. (2002). Multistate outbreaks of *Salmonella* serotype Poona infections associated with eating cantaloupe from Mexico – United States and Canada, 2000–2002. *MMWR Morb. Mortal. Wkly. Rep.*, 51, 1044–1047.

Centers for Disease Control and Prevention. (2006). Ongoing multistate outbreak of *Escherichia coli* serotype O157:H7 infections associated with consumption of fresh spinach – United States, September 2006. *MMWR Morb. Mortal. Wkly. Rep.*, 55, 1045–1046.

Centers for Disease Control and Prevention. (2008). Outbreak of *Salmonella* serotype Saintpaul infections associated with multiple raw produce items – United States, 2008. *MMWR Morb. Mortal. Wkly. Rep.*, 57, 929–934.

Center for Disease Control and Prevention. (2011a). Estimates of Foodborne Illness in the United States. Revised on May 8, 2013. Available on <http://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html>. Accessed 29 May 2013.

Centers for Disease Control and Prevention. (2011b). Multistate outbreak of listeriosis associated with Jensen Farms cantaloupe—United States, August–September 2011. *MMWR Morb. Mortal. Wkly. Rep.*, 60, 1357–8.

Centers for Disease Control and Prevention. (2012). Multistate Outbreak of Listeriosis Linked to Whole Cantaloupes from Jensen Farms, Colorado – United States. Available at <http://www.cdc.gov/listeria/outbreaks/cantaloupes-jensen-farms/082712/index.html>. Accessed 22 May 2013.

Ceponis, M. J., & Capellini, R. A. (1977). Vulnerability of stem-end scars of blueberry fruits to postharvest decays. *Phytopathology*, 67, 118–119.

Ceponis, M. J., & Capellini, R. A. (1983). Control of postharvest decays of blueberries by carbon dioxide-enriched atmospheres. *Plant Dis.*, 67, 169–171.

CFR. (1997). "Air Contaminants" Title 29, Vol. 6, Part 1910. Washington, DC: Office Federal Register.

Cheng, Y., Siletzky, R., & Kathariou, S. (2008). Genomic divisions/lineages, epidemic clones, and population structure. In: D. Liu (ed.), *Handbook of Listeria monocytogenes* (pp. 337–57). Boca Raton, FL: CRC Press.

Civello, P. M., Martínez, G. A., Chaves, A. R., & Añón, M. C. (1997). Heat treatments delay ripening and postharvest decay of strawberry fruit. *J. Agric. Food Chem.*, 45, 4589-4594.

Cline, B. (2005). Growing Blueberries for Local Markets. In: *Proceedings of the 4th National Small Farm Conference* (p. 206).

Coburn, B., Grassl, G. A., & Finlay, B. B. (2007). *Salmonella*, the host and disease: a brief review. *Immunol. Cell. Biol.*, 85, 112-8.

Cole, M. B., Jones, M. V., & Holyoak, C. (1990). The effect of pH, salt concentration and temperature on the survival and growth of *Listeria monocytogenes*. *J. Appl. Bacteriol.*, 69, 63–72.

Collins, J.E. (1997). Impact of changing consumer lifestyles on the emergence/reemergence of foodborne pathogens. *Emerg. Infect. Dis.*, 3(4), 471-479.

Conner, D. E, Scott, V. N., & Bernard, D. T. (1990). Growth, inhibition and survival of *Listeria monocytogenes* as affected by acidic conditions. *J. Food Prot.*, 53, 652-655.

Cotter, P. D., & Hill, C. (2003). Surviving the acid test: responses of gram-positive bacteria to low pH. *Microbiol. Mol. Biol. Rev.*, 67(3), 429-453.

Crowe, K. M., Bushway, A. A., Bushway, R. J., Davis-Dentici, K., & Hazen, R. A. (2007). A comparison of single oxidants versus advanced oxidation processes as chlorine-alternatives for wild blueberry processing (*Vaccinium angustifolium*). *Int. J Food Microbiol.*, 116(1), 25-31.

Daş, E., Gürakan, G. C., & Bayındırlı, A. (2006). Effect of controlled atmosphere storage, modified atmosphere packaging and gaseous ozone treatment on the survival of *Salmonella Enteritidis* on cherry tomatoes. *Food Microbiol.*, 23(5), 430-438.

Dauchet, L., Amouyel, P., Hercberg, S., & Dallongeville, J. (2006). Fruit and vegetable consumption and risk of coronary heart disease: a meta-analysis of cohort studies. *J. Nutr.*, 136, 2588–93.

Davies, A.R. (1995). Advances in modified-atmosphere packaging. In: G. W. Gould (ed.), *New Methods of Food Preservation* (pp. 304–320). Glasgow, UK: Blackie.

Davis, M. J., Coote, P. J., & O'Byrne, C. P. (1996). Acid tolerance in *Listeria monocytogenes*: The adaptive acid tolerance response (ATR) and growth-phase-dependent acid resistance. *Microbiology*, 142, 2975–2982.

Day, N. B., Skura, B. J. & Powrie, W. D. (1990). Modified atmosphere packaging of blueberries: Microbiological changes. *Can. Inst. Food Sci. Technol. J.*, 23, 59–65.

Day, B. P. F. (2001). Fresh prepared produce: GMP for high oxygen MAP and nonsulphite dipping. Guideline No. 31. Chipping Campden, UK: CCFRA.

Day, B. P. F. (2002). Industry guidelines for high oxygen MAP of fresh prepared produce. In: *International Conference: Postharvest Unlimited 599* (pp. 81-90).

Duarte, C., Guerra, M., Daniel, P., Camelo, A. L., & Yommi, A. (2009). Quality changes of highbush blueberries fruit stored in CA with different CO₂ levels. *J. Food Sci.*, 74(4), S154-S159.

Eccher, T., Carotti, E., & Gibin, M. (2005). Controlled-Atmosphere Storage of Highbush Blueberries (*Vaccinium corymbosum*) and Rabbiteye Blueberries (*V. ashei*). In: *IX International Controlled Atmosphere Research Conference 857* (pp. 373-380).

Eklund, T. (1989). Organic acids and esters. In: G. W. Gould (ed.), *Mechanisms of action of food preservation procedures* (pp. 160–200). London, UK: Elsevier Science Publishers Ltd.

Evans, C. E., Christian, M. S., Cleghorn, C. L., Greenwood, D. C., & Cade, J. E. (2012). Systematic review and meta-analysis of school-based interventions to improve daily fruit and vegetable intake in children aged 5 to 12 y. *Amer. J. Clin. Nutr.*, 96(4), 889-901.

Fan, L., Forney, C. F., Jordan, M. A., Hildebrand, P. D., Kalt, W., Ryan, D. A. J., & Song, J. (2001). Effect of ozone treatment and controlled atmosphere storage on quality and phytochemicals in highbush blueberries (pp. 417-423). In *VIII International Controlled Atmosphere Research Conference 600*.

Fan, X., Sokorai, K. J., Engemann, J., Gurtler, J. B. & Liu, Y. (2012). Inactivation of *Listeria innocua*, *Salmonella* Typhimurium, and *Escherichia coli* O157: H7 on surface and stem scar areas of tomatoes using in-package ozonation. *J. Food Prot.*, 75(9), 1611-1618.

Farber, J.M. (1991). Microbiological aspects of modified-atmosphere packaging – a review. *J Food Prot.*, 54, 58–70.

FDA. (1982). GRAS status of ozone. *Fed. Reg.* 47, 50209–50210.

FDA. (2003). Quantitative assessment of relative risk to public health from foodborne *Listeria monocytogenes* among selected categories of ready-to-eat foods. Available at

<http://www.fda.gov/downloads/food/scienceresearch/researchareas/riskassessmentsafetyassessment/ucm197330.pdf>. Accessed 20 February 2013.

FDA. (2011). Bacteriological Analytical Manual. Arlington, VA: AOAC International.

FDA. (2012). Bad Bug Book. Food-borne pathogenic microorganisms and natural toxins handbook. Second Edition. Available at <http://www.fda.gov/downloads/Food/FoodborneIllnessContaminants/UCM297627.pdf>. Accessed 29 May 2013.

Fernández, F. G., & Hoeft, R. G. (2009). Managing soil pH and crop nutrients. Illinois Agronomy Handbook, 24th edn. (pp. 91-112). Urbana, IL: University of Illinois.

Ferreira, A., Sue, D., O' Byrne, C., & Boor, K. J. (2003). Role of *Listeria monocytogenes* σ B in survival of lethal acidic conditions and in the acquired acid tolerance response. *Appl. Environ. Microbiol.*, 69, 2692–2698.

Flessa, S., Lusk, D. M., & Harris, L. J. (2005). Survival of *Listeria monocytogenes* on fresh and frozen strawberries. *Int. J. Food Microbiol.*, 101, 255–262.

Forney, C.F., Jordan, M. A., & Nicholas, K. V. K. G. (2003). Effect of CO₂ on physical, chemical, and quality changes in 'Burlington' blueberries. *Acta Hort.*, 600, 587–593.

Forney, C. F. (2008). Postharvest issues in blueberry and cranberry and methods to improve market-life. In: *IX International Vaccinium Symposium 810* (pp. 785-798).

Foster, J.W. (1999). When protons attack microbial strategies of acid adaptation. *Curr. Opin. Microbiol.*, 2, 170–174.

Gahan, C. G., O'Driscoll, B., & Hill, C. (1996). Acid adaptation of *Listeria monocytogenes* can enhance survival in acidic foods and during milk fermentation. *Appl. Environ. Microbiol.*, 62, 3128–3132.

Gandhi, M., & Chikindas, M. L. (2007). *Listeria*: a foodborne pathogen that knows how to survive. *Int. J. Food Microbiol.*, 113, 1-15.

Garcia, J.M., Aguilera, C., & Albi, M. A. (1995). Postharvest heat treatment on Spanish strawberry (*Fragaria anassa* cv. Tudla). *J. Agric. Food Chem.*, 43, 1489-1492.

Giannella, R. A., Broitman, S. A., & Zamcheck, N. (1972). Gastric acid barrier to ingested microorganism in man: studies *in vivo* and *in vitro*. *Gut.*, 13, 251–256.

Gomez-Rodas, A., Bourquin, L., García-Salazar, C., Varela-Gómez, A., & Wise, J. C. (2010). Good Agricultural Practices for Food Safety in Blueberry Production: Basic Principles. Ottawa, MI: Michigan State University Extension Central Region.

- Gottlieb, S. L., Newbern, E. C., Griffin, P. M., Graves, L. M., Hoekstra, R. M., Baker, N. L., Hunter, S. B. et al. (2006). Multistate outbreak of listeriosis linked to turkey deli meat and subsequent changes in US regulatory policy. *Clin. Infect. Dis.*, 42, 29–36.
- Gough, R. E. (1994). The highbush blueberry and its management. Boca Raton, FL: CRC Press.
- Graham, D. M. (1997). Use of ozone for food processing. *Food Technol.*, 51, 72-75.
- Guenther, P. M., Dodd, K. W., Reedy, J., & Krebs-Smith, S. M. (2006). Most Americans eat much less than recommended amounts of fruits and vegetables. *J. Am. Diet. Assoc.*, 106, 1371-1379.
- Guo, X., Chen, J., Brackett, R. E., & Beuchat, L. R. (2001). Survival of *Salmonellae* on and in tomato plants from the time of inoculation at flowering and early stages of fruit development through fruit ripening. *Appl. Environ. Microbiol.*, 67, 4760-4764.
- Guzel-Seydim, Z. B., Greene, A. K., & Seydim, A. C. (2004). Use of ozone in the food industry. *Lebensm-Wiss Technol.*, 37(4), 453-460.
- Hakkinen, S. H., & Torronen, A. R. (2000). Content of flavonols and selected phenolic acids in strawberries and *Vaccinium* species: influence of cultivar, cultivation site and technique. *Food Res. Int.*, 517–524.
- Han, Y., Selby, T. L., Schultze, K. K., Nelson, P. E., & Linton, R. H. (2004). Decontamination of strawberries using batch and continuous chlorine dioxide gas treatments. *J. Food Protect.*, 67(11), 2450-2455.
- Hancock, J., Callow, P., Serce, S., Hanson, E., & Beaudry, R. (2008). Effect of cultivar, controlled atmosphere storage, and fruit ripeness on the long-term storage of highbush blueberries. *Hort. Technol.*, 18, 199–205.
- Hanson, E., Hancock, J., Ramsdell, D. C., Schilder, A., VanEe, G., & Ledebuhr, R. (2000). Sprayer type and pruning affect the incidence of blueberry fruit rots. *HortSci.*, 35(2), 235-238.
- Harb, J. Y., & Streif, J. (2004). Controlled atmosphere storage of highbush blueberries cv. ‘Duke’. *Eur. J. Hort. Sci.*, 69, 66–72.
- Harris, L. J., Farber, J. N., Beuchat, L. R., Parish, M. E., Suslow, T. V., Garrett, E. H., & Busta, F. (2003). Outbreaks associated with fresh produce: Incidence, growth, and survival of pathogens in fresh and fresh-cut produce. *Compr. Rev. Food Sci. F.*, 2(s1), 78-141.
- Havas, S., Heimendinger, J., Reynolds, K., Baranowski, T., Nicklas, T. A., Bishop, D., Buller, D., Sorensen, G., Beresford, S., & Cowan, A. (1994). 5 a day for better health: a new research initiative. *J. Am. Diet. Assoc.*, 94(1), 32–36.

Hintlian, C.B., & Hotchkiss, J. H. (1986). The safety of modified atmosphere packaging: a review. *Food Technol.*, 40(12), 70–76.

International Agency for Research on Cancer. (2003). IARC Handbooks of Cancer Prevention. Vol 8. Fruit and Vegetables. Lyon, France: International Agency for Research on Cancer.

Jackson, E.D., K. A. Sanford, R. A. Lawrence, K. B. McRae, and R. Stark. (1999). Lowbush blueberry quality changes in response to prepacking delays and holding temperatures. *Postharvest Biol Technol.*, 15:117–126.

Jacxsens, L., Devlieghere, F., Van Der Steen, C., Siro, I., & Debevere, J. (2003). Application of ethylene adsorbers in combination with high oxygen atmospheres for the storage of strawberries and raspberries. *Acta Hort.*, 600, 311-318.

James, S. M., Fanin, S. L., Agee, B. A., Hal, B., Parker, E., Vogt, J., Run, G., Williams, J., Lieb, L., Salminen, C., Prendergast, T., Wrner, S. B., & Chin, J. (1985). Listeriosis outbreak associated with Mexican style cheese California. *MMWR Morb. Mortal. Wkly. Rep.*, 34, 357.

Joseph, J. A., Shykitt-Hale, B., Denisova, N. A., Bielinski, D., Martin, A., & McEwen, J. J. (1999). Reversal of age related declines in neuronal signal transduction, cognitive, and motor behavioral defects with blueberry, spinach, or strawberry dietary supplementation. *J. Neurosci.*, 19, 8114–8121.

Kalt, W., & Dufour, D. (1997). Health functionality of blueberries. *Hort Tech.*, 7, 216–221.

Kanan, S. M., El-Kadri, O. M., Abu-Yousef, I. A., & Kanan, M. C. (2009). Semiconducting metal oxide based sensors for selective gas pollutant detection. *Sensors*, 9(10), 8158-8196.

Khadre, M. A., Yousef, A. E., & Kim, J. (2001). Microbiological aspects of ozone applications in food: a review. *J. Food Sci.*, 6, 1242–52.

Kim, C., & Hung, Y. C. (2012). Inactivation of *E. coli* O157: H7 on blueberries by electrolyzed water, ultraviolet light, and ozone. *J. Food Sci.*, 77(4), M206-M211.

Kim, J. G., Yousef, A. E., & Dave, S. (1999). Application of ozone for enhancing the microbiological safety and quality of foods: a review. *J. Food Protect.*, 62, 1071-1087.

Kim, J. G., Yousef, A. E., & Khadre, M. A. (2003). Ozone and its current and future application in the food industry. *Adv. Food Nutr. Res.*, 45, 167-218.

Kim, T. J., Silva, J. L., Tokitkla, A., & Matta, F. B. (2010). Modified atmosphere storage influences quality parameters and shelf life of 'tifblue' blueberries. *J. Mississippi Acad. Sci.*, 4(55), 143-148.

Korotcenkov, G., Blinov, I., Brinzari, V., & Stetter, J. R. (2007). Effect of air humidity on gas response of SnO₂ thin film ozone sensors. *Sensor. Actuat. B-Chem.*, 122(2), 519-526.

Koushik, A., Spiegelman, D., Albanes, D., Anderson, K. E., Bernstein, L., van den Brandt, P. A., & Smith-Warner, S. A. (2012). Intake of fruits and vegetables and risk of pancreatic cancer in a pooled analysis of 14 cohort studies. *Amer. J. Epid.*, 176(5), 373-386.

Lemos, J. A., & Burne, R. A. (2008). A model of efficiency: stress tolerance by *Streptococcus mutans*. *Microbiol.*, 154, 3247–3255.

Liew, C. L., & Prange, R. K. (1994). Effect of ozone and storage temperature on postharvest diseases and physiology of carrots (*Daucus carota* L.). *J. Amer. Soc. Hort. Sci.*, 119(3), 563-567.

Lila, M.A. (2004). Antocyanidins and human health: an *in vitro* investigative approach. *J. Biomed. Biotechnol.*, 5, 306–313.

Linnan, M. J., Mascola, L., Lou, X. D., Goulet, V., May, S., Salminen, C., Hird, D. W., Yonekura, M. L., Hayes, P., Weaver, R., Audurier, A., Pukaytis, B. D., Fannin, S. L., Kleks, A., & Broome, C. V. (1988). Epidemic listeriosis associated with Mexican style cheese. *N. Engl. J. Med.*, 319, 823-828.

Lu, C., & Toivonen, P. (2000). Effect of 1 and 100 kPa O₂ atmospheric pretreatments of whole ‘Spartan’ apples on subsequent quality and shelf life of slices stored in modified atmosphere packages. *Postharvest Biol. Technol.*, 18(2), 99-107.

Lueck, E. (1980). Antimicrobial Food Additives, Characteristics, Uses, Effects. New York, NY: Springer-Verlag.

Lukasik, J., Bradley, M. L., Scott, T. M., Dea, M., Koo, A., Hsu, W. Y., Bartz, J. A., & Farrah, S. R. (2003). Reduction of poliovirus 1, bacteriophages, *Salmonella* Montevideo and *Escherichia coli* O157:H7 on strawberries by physical and disinfectant washes. *J. Food Protect.*, 66, 188–193.

Mahajan, P. V., & Goswami, T. K. (2004). Extended storage life of litchi fruit using controlled atmosphere and low temperature. *J. Food Proc. Preserv.*, 28(5), 388-403.

Mainland, C.M., Kushman, L. J., & Ballinger, W. E. (1975). The effect of mechanical harvesting on yield, quality of fruit and bush damage of highbush blueberry. *J. Amer. Soc. Hort. Sci.*, 100, 129-134.

Makus, D.J., & Morris, J. R. (1993). A comparison of fruit of highbush and rabbiteye blueberry cultivars. *J. Food Qual.*, 16, 417–428.

Manley, T. C., & Niegowski, S. J. (1967). Ozone. In: Encyclopedia of Chemical Technology. Vol. 14, 2nd ed. (pp. 410–432). New York, NY: Wiley.

Marucci, P.E. (1996). Insects and their control. In: P. Eck & N.F. Childers (eds.), *Blueberry culture* (pp.199–235). New Brunswick, NJ: Rutgers University Press.

- Maynard, M., Gunnell, D., Ness, A. R., Abraham, L., Bates, C. J., & Blane, D. (2006). What influences diet in early old age? Prospective and cross-sectional analyses of the Boyd Orr cohort. *Eur. J. Publ. Hlth.*, 16, 316–24.
- McClure, P. J., Roberts, T. A., & Oguru, P. O. (1989). Comparison of the effects of sodium chloride, pH, and temperature on the growth of *Listeria monocytogenes* on gradient plates and in liquid medium. *Lett. Appl. Microbiol.*, 9, 95-99.
- McClurkin, J. D., & Maier, D. E. (2010). Half-life time of ozone as a function of air conditions and movement. *Julius-Kühn-Archiv.*, 425, S-381.
- McLauchlin, J. (1996). The relationship between *Listeria* and listeriosis. *Food Cont.*, 7:187–193.
- Mead, P. S., Dunne, F., Graves, L., Wiedmann, M., Patrick, M., Hunter, S. et al. (2006). Nationwide outbreak of listeriosis due to contaminated meat. *Epidemiol. Infect.*, 134, 744–51.
- Miller, W. R., & Smittle, D. A. (1987). Storage quality of hand harvested and machine harvested rabbiteye blueberries. *J. Am. Soc. Hort. Sci.*, 112, 487–490.
- Miller, W.R., McDonald, R. E., Melvin, C. F., & Munroe, K. A. (1984). Effect of package type and storage time-temperature on weight loss, firmness, and spoilage of rabbiteye blueberries. *HortSci.*, 19, 638–640.
- Miller, W.R., McDonald, R. E., & Crocker, T. E. (1993). Quality of two Florida blueberry cultivars after packaging and storage. *HortSci.*, 28, 144–147.
- Mitcham, E.J., & Mitchell, F. G. (2002). Postharvest handling systems: small fruits. II. Strawberries and cane berries. In: A. Kader (Ed.), *Postharvest Technology of Horticultural Crops, third ed. Publication, vol. 3311* (pp. 364– 368). Oakland, CA: University of California, Division of Agriculture and Natural Resources.
- Moat, A. G., Foster, J. W., & Spector, M. P. (2003). *Microbial Physiology*. New York, NY: Wiley.
- Moore, J.N. (1990). Mulching blueberries: Fact vs. fiction. (pp. 59-63). In: Small Fruit Conf. Proc, MO.
- Mukherjee, A., Speh, D., Jones, A. T., Buesing, K. M., & Diez-Gonzalez, F. (2006). Longitudinal microbiological survey of fresh produce grown by farmers in the upper Midwest. *J Food Protect.*, 69(8), 1928-1936.
- Mullan, M., & McDowell, D. (2003). 10 Modified atmosphere packaging. *Am. Soc. Test. Mater.*, 303.

- Murray, E. G. D., Webb, R. A., & Swann, M. B. R. (1926). A disease of rabbits characterized by a large mononuclear monocyctosis, caused by a hitherto undescribed bacillus *Bacterium monocytogenes*. *J Pathol Bacteriol.*, 29, 407–439.
- Muthukumarappan, K., Halaweish, F., & Naidu, A. S. (2000). Ozone. In: A.S. Naidu (Eds.), *Natural Food Anti-Microbial Systems* (pp. 783–800). Boca Raton, FL: CRC Press.
- Nascimento, M. M., Lemos, J. A., Abranches, J., Lin, V. K., & Burne, R. A. (2008). Role of RelA of *Streptococcus mutans* in global control of gene expression. *J. Bacteriol.*, 190, 28–36.
- NeSmith, D. S., Prussia, S., Tetteh, M., & Krewer, G. (2002). Firmness losses of rabbiteye blueberries (*Vaccinium ashei* Reade) during harvesting and handling. *Acta Hort.*, 574, 287-293.
- Ness, A. R., & Powles, J. W. (1997). Fruit and vegetables, and cardiovascular disease: a review. *Int. J. Epidemiol.*, 26(1), 1–13.
- Newell, D. G., Koopmans, M., Verhoef, L., Duizer, E., Aidara-Kane, A., Sprong, H., Opsteegh, M., Langelaar, M., Threfall, J., Scheutz, F., van der Giessen, J., & Kruse, H. (2010). Food-borne diseases-the challenges of 20 years ago still persist while new ones continue to emerge. *Int. J. Food Microbiol.*, 139(1), S3-S15.
- Olsen, S. J., Patrick, M., Hunter, S. B., Reddy, V., Kornstein, L., MacKenzie, W. R., et al. (2005). Multistate outbreak of *Listeria monocytogenes* infection linked to delicatessen turkey meat. *Clin. Infect. Dis.*, 40, 962–7.
- Painter, J. A., Hoekstra, R. M., Ayers, T., Tauxe, R. V., Braden, C. R., Angulo, F. J., & Griffin, P. (2013). Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998–2008. *Emerg. Infect. Dis.*, 19(3), 507-515.
- Pamer, E. G. (2004). Immune responses to *Listeria monocytogenes*. *Nature Rev. Immunol.*, 4(10), 812-823.
- Pao, S., Brown, E., & Schneider, K. R. (1998). Challenge studies with selected pathogenic bacteria on freshly peeled Hamlin orange. *J. Food Sci.*, 63, 359– 362.
- Parish, M. (1997). Public Health and Nonpasteurized Fruit Juice. *Crit. Rev. Microbiol.*, 23(2), 109-119.
- Parry, R.T. (1993). Principles and Applications of Modified Atmosphere Packaging of Food. Glasgow, UK: Blackie.
- Phan-Thanh, L., Mahouin, F., & Alige, S. (2000). Acid responses of *Listeria monocytogenes*. *Int. J. Food Microbiol.*, 55, 121-126.
- Phillips, C. A. (1996). Review: modified atmosphere packaging and its effects on the microbiological quality and safety of produce. *Int. J. Food Sci. Technol.*, 31(6), 463-479.

- Pirie, J. H. H. (1927). A new disease of veld rodents. 'Tiger River Disease'. *Publ. S. Afr. Inst. Med. Res.*, 3, 163–186.
- Pivonka, E., Seymour, J., McKenna, J., Baxter, S. D., & Williams, S. (2011). Development of the Behaviorally Focused Fruits & Veggies—More Matters Public Health Initiative. *J. Amer. Diet. Assoc.*, 111(10), 1570-1577.
- Plattner, K., Perez, A., & Warm, D. W. R. U. (2012). Fruit and Tree Nuts Outlook. USDA/ERS Report. FTS-354.
- Popa, I., Hanson, E. J., Todd, E. C., Schilder, A. C., & Ryser, E. T. (2007). Efficacy of chlorine dioxide gas sachets for enhancing the microbiological quality and safety of blueberries. *J. Food Protect.*, 70(9), 2084-2088.
- Prior, R. L., Cao, G., Martin, A., Sofic, E., McEwen, J., O'Brien, C., et al. (1998). Antioxidant capacity as influenced by total phenolics and anthocyanins content, maturity, and variety of vaccinium species. *J. Agric. Food Chem.*, 46, 2686–2693.
- Prodorutti, D., Pertot, I., Giongo, L., & Gessler, C. (2007). Highbush blueberry: cultivation, protection, breeding and biotechnology. *Eur. J. Plant Sci. Biotech.*, 1, 44-56.
- Puupponen-Pimia, R., Nohynek, L., Meier, C., Kahkonen, M., Heinonen, M., Hopia, A., & Oksman-Caldentey, K. M. (2001). Antimicrobial properties of phenolic compounds from berries. *J. Appl. Microbiol.*, 90, 494–507.
- Puupponen-Piamia, R., Nohynel, L., Alakomi, H. L., & Oksman-Caldentey, K. M. (2005). Bioactive berry compounds – Novel tools against human pathogens. *Appl. Microbiol. Biotech.*, 67, 8-18.
- Ramsdell, D.C. (1989). Blueberry diseases in Michigan. Ext. Bul. E-1731. East Lansing, MI: Michigan State Univ.
- Retamales, J., Manríquez, D., Castillo, P., & Defilippi, B. (2003). Controlled atmosphere in bing cherries from Chile and problems caused by quarantine treatments for export to Japan. *Acta Hort.*, 600, 149-153.
- Retamales, J. B., & Hancock, J. F. (2012). Blueberries (Vol. 21). In: Crop production science in horticulture. Wallingford, UK: CAB International.
- Rocourt, J., & Cossart, P. (1997). *Listeria monocytogenes*. In: M.P. Doyle, L.R. Buechat & T.J. Montville (Eds.), *Food Microbiology — Fundamentals and Frontiers* (pp. 337–352). Washington DC: American Society for Microbiology (ASM) press.
- Roelofs, F. (1993). Research results of storage trials with blueberries 1992: One berry crop is not the same as another. *Fruittelt.*, 83, 19–24.

Romana, C., Salleras, L., & Sage, M. (1989). Latent listeriosis may cause habitual abortion intrauterine deaths, fetal malformations. When diagnosed and treated adequately normal children will be born. *Acta Microbiol Hung.*, 36, 171–172.

Rosenfeld, H.J., Meberg, K.R., Haffner, K., & Sundell, H.A. (1999). MAP of high bush blueberries; sensory quality in relation to storage temperature, film type and initial high oxygen atmosphere. *Postharvest Biol. Tech.*, 16, 27-36.

Ryser, E. T. (1999). Foodborne listeriosis. In: E. T. Ryser & E. H. Marth (eds.), *Listeria, listeriosis and food safety*, 2nd ed. (p. 341). New York, NY: Marcel Dekker, Inc.

Saftner, R., Polashock, J., Ehlenfeldt, M., & Vinyard, B. (2008). Instrumental and sensory quality characteristics of blueberry fruit from twelve cultivars. *Postharvest Biol. Tech.*, 49(1), 19-26.

Salunkhe, D. K., Bolin, H. R., & Reddy, N. R. (1991). Minimal processing. In: D. K. Salunkhe, H. R. Bolin & N. R. Reddy (Eds.), *Storage, processing, and nutritional quality of fruit and vegetables: volume 2: processed fruit and vegetables*. Boca Raton, FL: CRC Press

Sandhya, S. (2010). Modified atmosphere packaging of fresh produce: current status and future needs. *Lebensm-Wiss Technol.*, 43(3), 381-392.

Sanford, K.A., Lidster, P. D., McRae, K.B., Jackson, E. D., Lawrence, R. A., Stark, R., & Prange, R. K. (1991). Lowbush blueberry quality changes in response to mechanical damage and storage temperature. *J. Am. Soc. Hort. Sci.*, 116, 47–51.

Sapers, G. M. (2001). Efficacy of washing and sanitizing methods for disinfection of fresh fruit and vegetable products. *Food Tech. Biotech.*, 39(4), 305-312.

Sargent, S.A., Brecht, K. K., & Forney, C. F. (2006). Blueberry harvest and postharvest operations: quality maintenance and food safety. In: N. F. Childers & P. M. Lyrene (eds.), *Blueberries for Growers, Gardeners, Promoters* (pp.139-151). DeLeon Springs, FL: E.O. Painter Printing Co.

Schlech, W.F. (1996). Overview of listeriosis. *Food Control*, 7(4–5), 183-186.

Schlech, W. F., Lavigne, P. M., Bortolussi, R. A., Allen, A. C., Haldane, E. V., Wort, A. J., Hightower, A. W., Johnson, S. E., King, S. H., Nicholls, E. S., & Broome, C. V. (1983). Epidemic listeriosis evidence for transmission by food. *N. Engl. J. Med.*, 308(4), 203-206.

Schlitt-Dittrich, F., Beuchat, L. R., & Harris, L. J. (2004). Comparison of inoculation method and drying time for their effects on survival and recovery of *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* inoculated onto apples and strawberries (p. 238). 2004 Annual Meeting, International Association of Food Protection, Phoenix, AZ.

- Schotsmans, W., Molan, A., & MacKay, B. (2007). Controlled atmosphere storage of rabbiteye blueberries enhances postharvest quality aspects. *Postharvest Biol. Tech.*, 44(3), 277-285.
- Sheng, J., & Marquis, R. E. (2006). Enhanced acid resistance of oral streptococci at lethal pH values associated with acid-tolerant catabolism and with ATP synthase activity. *FEMS Microbiol Lett.*, 262, 93-98.
- Silva, J.L., Marroquin, E., Matta, F. B., Garner, J. O. J., & Stojanovic, J. (2005). Physicochemical carbohydrate and sensory characteristics of highbush and rabbiteye blueberry cultivars. *J. Sci. Food Agric.*, 85, 1815-1821.
- Smilanick, J. L., Crisosto, C., & Mlikota, F. (1999). Postharvest use of ozone on fresh fruit. *Perishables Handling Quarterly Issue*. 99(10e): 14.
- Smittle, D.A., & Miller, W. R. (1988). Rabbiteye blueberry storage life and fruit quality in controlled atmospheres and air storage. *J. Am. Soc. Hort. Sci.*, 113, 723-728.
- Smyth, A.B., Song, J., & Cameron, A. C. (1998). Modified atmosphere packaged cut Iceberg lettuce: Effect of temperature and O₂ partial pressure on respiration and quality. *J. Agric. Food Chem.*, 46, 4556-4562.
- Sorrells, K. M., Enigl, D. C., & Hatfield, J. R. (1989). Effect of pH, acidulant, time, and temperature on the growth and survival of *Listeria monocytogenes*. *J. Food Protect.*, 52, 571-573.
- Stewart, D. (2003). Effect of high O₂ and N₂ atmospheres on strawberry quality. *Acta Hort.*, 600, 567-570.
- Strait, C.A. (1998). Efficacy of ozone to control insects and fungi in stored grain. M.S. thesis, Purdue University, West Lafayette, IN.
- Sy, K. V., McWatters, K. H., & Beuchat, L. R. (2005). Efficacy of gaseous chlorine dioxide as a sanitizer for killing *Salmonella*, yeasts, and molds on blueberries, strawberries, and raspberries. *J. Food Protect.*, 68(6), 1165-1175.
- Tan, S. Y., & Tasaki, A. (2007). Joseph Lister (1827-1912): father of antisepsis. *Singap. Med. J.*, 48(7), 605.
- Thiele, G. (1999). Berry fruit. In: D. Jackson, N. Looney, M. Morley-Bunker & G. Thiele (Eds.), *Temperate and Subtropical Fruit Production 2nd Edn*, 209 (pp. 209-228). Wallingford, UK: CAB International.
- Tompkin, R. B. (2002). Control of *Listeria monocytogenes* in the food-processing environment. *J. Food Protect.*, 65(4), 709-25.

- Tosun, H., & Gonul, S. A. (2005). The effect of acid adaptation conditions on acid tolerance response of *Escherichia coli* O157:H7. *Turk J. Biol.*, 29, 197-202.
- Trehane, P., Brickell, C. D., Baum, B. R., Hetterscheid, W. L. A., Leslie, A. C., McNeill, J., Vrugtman, F. et al. (1995). International code of nomenclature for cultivated plants (No. Ed. 6). Wimbourne, UK: Quarterjack Publishing.
- Trussell, R.R., & Umphres, M. D. (1978). The formation of trihalomethanes. *J. Amer. Water Works Assoc.*, 70, 604–612.
- US Department of Agriculture. (1984). Poultry products; chiller water reuse. *Fed. Reg.*, 49, 9409–9411.
- US Department of Agriculture. (2005). MyPyramid Plan. Available at www.mypyramid.gov. Accessed May 13, 2011.
- US Department of Agriculture. (2007). “Fruit, Berries and Tree Nuts” 2007 Census of Agriculture. Available at http://www.agcensus.usda.gov/Publications/2007/Online_Highlights/Fact_Sheets/Production/fbn.pdf. Accessed 27 May 2013.
- US Department of Agriculture. (2012). Non-citrus fruits and nuts 2011 summary. Washington, DC: National Agricultural Statistics Service.
- US Department of Agriculture. (2013). Non-citrus fruit and nuts 2012 preliminary summary. Washington, DC: National Agricultural Statistics Service.
- US Department of Agriculture/Economic Research Service. (2012). U.S. per capita food availability: Individual fruit. Available in http://www.ers.usda.gov/data-products/food-availability-%28per-capita%29-data-system.aspx#.UaqWVtj3X_M. Accessed 20 May 2013.
- US Department of Agriculture and US Department of Health and Human Services. (2010). Dietary Guidelines for Americans, 2010. 7th edition, Washington, DC: US Government Printing Office.
- Wang, Y., Chang, C. F., Chou, J., Chen, H. L., Deng, X., Harvey, B. K., Cadet, J. L., & Bickford, P. C. (2005). Dietary supplementation with blueberries, spinach, or spirulina reduces ischemic brain damage. *Exp. Neurol.*, 193(1), 75-84.
- White, G. C. (1992). Ozone. In: *Handbook of chlorination and alternative disinfectants* (3rd ed.). (pp. 1046-1110). New York, NY: Van Nostrand Reinhold.
- WHO. (1990). Report of a WHO Study Group. Diet, Nutrition, and the Prevention of Chronic Diseases. Geneva, Switzerland: World Health Organization.

WHO. (2003). Diet, nutrition and the prevention of chronic diseases. In: FAO. Geneva, Switzerland: World Health Organization.

Williamson, J., Krewer, G., Pavlis, G., & Mainland, C. M. (2006). Blueberry soil management, nutrition and irrigation. In: N. F. Childers & P. M. Lyrene (Eds.), *Blueberries for growers, gardeners, promoters* (pp. 60-74). De Leon Springs, FL: EO Painter Printing Company.

Winkhaus-Schindl, I., Seeliger, H. P., & Andries, L. (1966). Listeriosis as a confirmed cause in several patients with habitual abortions. *Geburtshilfe Frauenheilkd.*, 26, 1377–1379.

World Cancer Research Fund/American Institute for Cancer Research. (2007). Food, Nutrition, Physical Activity, and the Prevention of Cancer: A Global Perspective. Washington, DC: American Institute for Cancer Research.

Wszelaki, A.L., & Mitcham, E.J. (2000). Effects of superatmospheric oxygen on strawberry fruit quality and decay. *Postharvest Biol. Tech.*, 20, 125-133.

Wu, V. C., & Kim, B. (2007). Effect of a simple chlorine dioxide method for controlling five foodborne pathogens, yeasts and molds on blueberries. *Food Microbiol.*, 24(7), 794-800.

Youdim, K. A., Shukitt-Hale, B., Martin, A., Wang, H., Denisova, N., Bickford, P. C. et al. (2000). Short-term dietary supplementation of blueberry polyphenolics: beneficial effects on aging brain performance and peripheral tissue function. *Nutr. Neurosci.*, 3, 383–397.

Young, K. M., & Foegeding, P. M. (1993). Acetic, lactic and citric acids and pH inhibition of *Listeria monocytogenes* Scott A and the effect of intracellular pH. *J. Appl. Bacteriol.*, 74, 515-520.

Yu, K., Newman, C., Archbold, D. D., & Hamilton-Kemp, T. R. (2001). Survival of *Escherichia coli* O157:H7 on strawberry fruit and reduction of the pathogen population by chemical agents. *J. Food Protect.*, 64, 1334–1340.

Zeynep, G.S. (2003). Use of ozone in the food industry. *Food Microbiol.*, 21(4), 475-79.

CHAPTER 3

Listeria monocytogenes survival in the presence of malic acid, lactic acid or blueberry extract

ABSTRACT

Listeria monocytogenes represents a high risk for consumers, since it can grow under refrigeration conditions and acidic environments by developing acid tolerance resistance.

The aim of this work was to study the growth and survival of ATR *L. monocytogenes* strain Scott A in media acidified with malic acid, lactic acid, or blueberry extract. Blueberry extract (pH 3.06) was obtained from previously blended and centrifuged fresh fruit. Bacterial growth was evaluated using tryptic soy broth with yeast extract (TSB+YE) combined with different acid (malic, lactic acid, or blueberry extract) and incubated at 25°C for 24h. An optical density system (BioScreen C Microbiology Reader) measured growth over time.

Complete inhibition of *L. monocytogenes* occurred in the presence of treatments including malic acid pH 2.0 and 3.0; lactic acid 2.0, 3.0, and 4.0; and with blueberry extract 2.0 in the mixture. No pathogenic growth was observed in treatments under pH 4.5. Turbidity values of growth media mixed with blueberry extract at pH 3.0, 4.0, and 5.0 showed no statistical difference at 18h and solutions had pH 6.13, 6.53, and 6.78, respectively. Lactic acid was more effective inhibiting bacterial growth when compared to malic acid when acid solution pH was the same. Blueberry extract was not effective acidifying the final pH of the solutions when mixed with TSB+YE. *L. monocytogenes* survived and grew in media acidified with low pH blueberry extract, increasing its ability to contaminate acidic foods such as blueberry juices.

Keywords: *Listeria monocytogenes*; acid tolerance resistance; blueberries; lactic acid; malic acid.

1. Introduction

The capability of foodborne pathogenic bacteria to survive under environmental stress, not only inside the host, but also outside, will determine the success to cause disease in humans. Pathogenic bacteria, including *Listeria monocytogenes*, which has a high fatality rate of approximately 17%, become a threat in products that are not protected from, or properly treated to reduce contamination. The U.S. Food and Drug Administration and U.S. Department of Agriculture Food Safety and Inspection Service have established a zero tolerance for *L. monocytogenes* meaning no detectable level is permitted in ready to eat foods including minimally processed fresh fruits and vegetables (Leverentz et al., 2003; FDA, 2003). This pathogen infectious dose is assumed to be high for most healthy adults, however in immunocompromised, elderly, neonate and pregnant persons the minimum dose is unknown and those infected are likely to die (Todd & Notermans, 2011).

L. monocytogenes is common in fruits and vegetables grown near soil (Weis & Seeliger, 1975). Moreover, there is an association of this pathogen with acidic fruits and fruit juice products, including unpasteurized apple juice (pH 3.78) and apple/raspberry juice (pH 3.75), cut and whole strawberries, tomatoes, orange juice and orange slices (Sado et al., 1998; Kniel & Shearer, 2009; Caggia et al., 2009). In 2011, human listeriosis cases, attributed to consumption of contaminated cantaloupe, resulted in 33 deaths (CDC, 2012).

Since 1973 in the US, five bacterial outbreaks have been attributed to berries (Sivapalasingam et al., 2004; Kniel & Shearer, 2009) and most of them were related with strawberries infected with *Staphylococcus aureus* in 1985, *Salmonella* Group B in 2003, and enterohemorrhagic *E. coli* O26 in 2006, in this latter case blueberries were also associated. Although blueberries are considered low risk for foodborne contamination because of their low

pH, they were related to a possible outbreak of listeriosis in Connecticut in 1984 (Ryser, 1999). Furthermore, low pH and bacterial acid tolerance are linked to an increased virulence of pathogens (Foster, 1999; Werbrouck et al., 2009).

Over the past several years consumer interest in minimally processed fruits and vegetables has increased because of their freshness, convenience, and healthy attributes. The U.S. per capita food availability of blueberries increased from 0.26 lbs in 2000 to 1.3 lbs in 2011. Annual average per capita availability of fresh blueberries surpassed frozen, reaching 0.96 lbs compared to 0.54 lbs, respectively, in 2009 (Economic Research Service US Department of Agriculture, 2012). Fresh blueberries are minimally processed after harvest, refrigerated, and stored for several days, without washing or treatment to inhibit microbes.

L. monocytogenes represents a high risk for consumers since it can adapt and grow not only under refrigeration conditions (2-4°C), but also in high acidity foods, high salinity foods and within the host immune system (Gandhi and Chikindas, 2007; Rocourt & Cossart, 1997). Moreover, due to hand harvesting, field packing, and non-composted manure fertilization use, blueberries are vulnerable to bacterial contamination in the field (Wu & Kim, 2007). Blueberries have a pH of approximately 3.7 when fresh and 3.1- 3.35 when frozen (McGlynn, 2000). The measured surface pH of fresh blueberries in this study was 3.83 (data not shown). There are no previous studies on the incidence of acid tolerant resistant *Listeria monocytogenes* on blueberries or blueberry extract.

Turbidimetry is an established predictive microbiology method used to study bacterial growth based on Optical Density (O.D.) measurements, which makes it possible to follow bacterial population growth in real time (Begot et al., 1995). Some authors have attempted to derive growth parameters from O.D. measurements and found a linear correlation with viable

Listeria monocytogenes plate counts, especially in the exponential growth phase (McClure et al., 1993; Dalgaard et al., 1994, Begot et al., 1995; Hudson 1994). Although O.D. is not directly equivalent to bacterial counts, it can be translated but requires many calibration curves to be accurate.

The present study evaluated the growth of *L. monocytogenes* cultured in the presence of organic acids and blueberry extracts. Bacterial occurrence, survival and growth in acid environments with blueberry extracts were monitored to assess the potential health risk for consumers.

2. Materials and Methods

2.1 Bacterial Strain, Induction to Acid Tolerance Response, Media and Growth Conditions

Listeria monocytogenes strain Scott A (Food Science and Technology Department Culture Collection, Virginia Tech) was propagated in trypticase soy broth (BBL™, BD Diagnostics, Sparks, MD) with 0.6% yeast extract (Acros Organics, Fair Lawn, NJ) (TSB+YE) and stored for overnight propagation at 37°C in Precision Scientific Model 805 Incubator (Precision Scientific Corp, Chicago, IL). Afterwards the culture was centrifuged at 2000xg for 10 min at 4°C and the pellets were re-suspended in an equal volume of TSB–YE with pH adjusted to 5.7 to induce the acid tolerance and incubated for 18 h at 37°C. The media acidification was carried out using lactic acid (Caggia et al., 2009). Cultures were stored at -18°C in TSB with 1% glycerol as cryoprotectant.

After incubation at 37°C, 10mL of each culture was centrifuged (Sorvall Legend RT+ centrifuge, Thermo Scientific, Braunschweig, Germany) at 2000xg for 10 min at 4°C. After centrifugation, the supernatant was discarded and replaced with 10mL of 0.1% peptone water.

Solutions were vortexed (Fisher Mini-Shaker Model 58, Fisher Scientific, Pittsburgh, PA) and centrifuged again at 2000xg for 10 min at 4°C. Finally, supernatant was discarded and the remaining pellet was resuspended in 10mL 0.1% peptone water.

2.2 Preparation of Acids and Blueberries Solution

Highbush blueberries [*Vaccinium corymbosum*] fruit, cvs. ‘O’Neil’, ‘Nelson’ and ‘Sunrise’, were hand harvested from irrigated plots at the Virginia Tech Kentland Research Farm (Blacksburg, VA) then stored in a cooler (4-8°C) and transported to the Food Science and Technology Building at Virginia Tech. Blueberries were sorted and only fully developed fruit free of visible defects were selected on a visual basis and stored. Blueberries were minced in a blender (Hamilton Beach, Glen Allen, VA) for 30s, then the sample was passed through a sifter and finally centrifuged at 9820xg for 15min retaining just the liquid phase. The pH of the blueberry supernatants (initial pH 3.06) were adjusted using malic acid 95% (Sigma Chemicals, St Louis, MO) and 3M NaOH; and this mixture was stored at 0°C to avoid enzyme degradation. Malic acid was chosen because of its presence in blueberries. The pH of lactic acid 95% (Fisher Chemicals, Fair Lawn, NJ) (pKa 3.85) and malic acid (pKa₁ 3.40 and pKa₂ 5.20) solutions (both initial pH 1.2) were fixed using the same acid and/or 3M NaOH. A pH meter (Model 220, Denver Instrument, Bohemia, NJ) measured solution pH. All samples were sterilized using a 0.45 µm syringe filter Whatman (PTFE Puradisc, Whatman Inc., Piscataway, NJ). All three different acid treatments were fixed to pH 2.0, 3.0, 4.0, or 5.0.

2.3 Automated Growth Curve Analysis

The effects of lactic acid, malic acid and the blueberry solution on the growth of *L. monocytogenes* strain Scott A, were tested by a Bioscreen C Microbiology Reader (Growth

Curves, Piscataway, NJ), equipped with an incubator and automated turbidimeter to determine O.D. between 420-540nm. Each well of a sterilized 100 well honeycomb microwell plate (Growth Curves, Piscataway, NJ) was filled with 125 µl of TSB+YE pH 7.0 as growth liquid medium, 15 µl of pH fixed prepared acid or extract, and 10 µl of culture (achieving a bacterial concentration of approx. 10^7 cfu/ml). For negative acid controls (uninoculated), wells were filled with 135 µl of growth medium and 15 µl of pH fixed prepared acid or berry solution. For *L. monocytogenes* controls, wells were filled with 140 µl of growth medium and 10 µl of culture. Negative *L. monocytogenes* control wells were filled with 150 µl of growth medium. The mean O.D. of the uninoculated control wells were subtracted from the O.D. of the pathogen inoculated wells for each measurement (Begot et al., 1995), discarding initial turbidity of the sample to obtain only turbidity values due to pathogenic growth. Different samples were labeled A, B, C and D according to 2.0, 3.0, 4.0 and 5.0 pH solution used in the mixture, respectively (Table 1). Microwell plates were incubated at 25°C for 48 h, and O.D. was measured every 15 min with 10 sec of shaking before each reading. However, for data analysis only the first 24 h data was utilized, since after that time no major change in optical densities were observed. Data was generated using EZExperiment software (Growth Curves, Piscataway, NJ) and exported as a spreadsheet (Microsoft Excel, Seattle, WA).

2.4 Experimental Design and Statistical Analysis

Optical density data were analyzed using the Generalized Linear Model (GLM) procedure of SAS (V. 9.13, Statistical Analysis Systems Institute, Inc. 2006). The randomized complete block factorial design with three replications was utilized to test the treatments and their interactions on microbial growth (BioScreen turbidity). All the tests used four samples for

each treatment (malic acid, lactic acid and blueberry extract) and different mixtures (A, B, C or D). If the interactions between treatments were not significant ($P>0.05$), the main effects of the treatments were separated by the Least Significant Difference test using the interaction as the error term.

3. Results and Discussion

According to Hudson (1994), to avoid the error in measuring the time to reach a detectable turbidity, the initial inoculum must be high enough to measure the turbidity, since in case turbidity is not measurable, the calculated lag time becomes the period taken for the culture to reach detectable turbidity. In this study, the initial bacterial inoculum concentration was approx. 10^7 CFU/ml, while the initial O.D. measured in uninoculated medium was 0.125 for malic acid, 0.133 for lactic acid, 0.277 for blueberry extract and 0.143 for the positive control. Optical density measurement technique is a quicker, more convenient and precise than other methods to measure growth changes over time.

Growth curves of *L. monocytogenes* in TSB+YE varied among acid treatments (Figure 1). Malic A, Malic B, Lactic A, Lactic B, Lactic C and Berries A treatments were not included in this graph since they completely inhibited pathogen growth. Pathogen survival and growth in acid treatments Malic D, Lactic D, Berries B, Berries C and Berries D was considerable and included distinct lag, exponential and stationary phases, but were inferior when compared to *L. monocytogenes* positive control (Control).

Despite published reports indicating that minimum pH for growth in laboratory conditions is pH 4.4 to 4.5 (Parish & Higgins, 1989; Sorrells et al., 1989), and from 5.0 to 5.5 when pH of the media is adjusted with lactic acid (Farber et al., 1998), numerous studies have

determined *L. monocytogenes* ability to respond and to survive at even lower pH values (O'Driscoll et al., 1997, Davis et al., 1996; Kroll & Patchett, 1992). Acid tolerance response (ATR) in *L. monocytogenes* can be induced by exposing the organism to mild acidic conditions, pH of 5.5 (1 M lactic acid). Such treatments make the cells resistant to severe acidic conditions (O'Driscoll et al., 1996). The acid response upon exposure of cells to an acidic pH involves several changes in the cell such as protein induction, pH homeostasis and glutamate decarboxylase system (Gandhi & Chikindas, 2007). Proteins such as GroEl, ATP synthase, and various transcriptional regulators showed increased synthesis under acid stress growth (Phan-Thanh & Mahouin, 1999). F₀F₁-ATPase is a multisubunit enzyme involved in the acid adaptation of *L. monocytogenes*, serving as an ATP driven proton channel across the cell membrane, helping maintain cellular pH homeostasis (Cotter et al., 2000). An inadequately maintained intracellular homeostasis can be the cause of loss of cell viability (Chitarra et al., 2000). A study of the role of the glutamate decarboxylase in the acid resistance of *L. monocytogenes* during gastric transit using synthetic human and porcine gastric fluid concluded that addition of glutamate increased the survival of the wild type strain in gastric fluid, creating a concern about contamination in foods containing glutamate (Cotter et al., 2001). Gahan et al. (1996) compared acid-adapted and non-adapted *L. monocytogenes* survival in a variety of acidic food products using a constitutive acid tolerant mutant isolated by prolonged exposure to pH 3.5 (3 M lactic acid). The acid-adapted strain and the acid tolerant mutant demonstrated better survival in commercial natural yogurt and cottage cheese made in a laboratory. Throughout the ripening of hard cheeses, the acid tolerant mutant showed enhanced resistance and the authors were able to recover pathogen cells after the 70-day ripening period. Moreover, during milk fermentation by *Streptococcus thermophilus*, the acid-adapted strain

demonstrated enhanced survival compared to the non-adapted culture. The *L. monocytogenes* acid tolerant mutant also survived similar to the acid-adapted cells during the first 4 h of fermentation. In the present study no bacteria survived at a pH lower than 4.54.

After 6h, turbidity measurements were significantly different among the majority of the survival treatments and the *L. monocytogenes* control during the exponential growth phase. The positive Control showed the highest O.D. value (0.18) after 6h of incubation, while Malic D was the lowest (0.02) (Table 2). Berries C and D were not significantly different at this time, a trend also observed at 12 and 18 h. Between 12 and 18h, nearly all *L. monocytogenes* solutions ceased to grow as the stationary phase was reached. The only exception to the latter was Malic C, which showed a unique growth pattern without a specific lag and exponential growth phase.

Acid dissociation constants (pKa) are linked to antimicrobial activity of organic acids against *L. monocytogenes* as well as with greater permeability of weak acids in their undissociated form through the cell membrane (Vasseur et al., 1999). When the solution pH is lower than the pKa of an acid, the majority of acid in the solution will be undissociated and will be able to pass through the lipid membranes, unlike the dissociated (ionized) form which cannot. In this study, lactic acid solutions were more efficient reducing pathogenic growth when compared to malic acid solutions at a same pH. At same pH lactic acid has a pKa of 3.85, while malic acid has a pKa₁ of 3.40 and both are in the acidic range. Since lactic acid has the higher pKa, therefore it would have higher amounts of undissociated molecules, which accumulation in the cells will lead to lower intracellular pH values and metabolic activity deregulation of the cell, causing a higher inhibitory activity. Lactic acid ability to diffuse through the cell membrane results in a higher inhibitory effect, whereas malic acid weaker inhibitory capacity is because the dissociation form cannot passively penetrate the cell membrane (Vasseur et al., 1999). According

to Phan-Thanh et al. (2000) in aerobic bacteria, the active transport of protons is associated with the electron transport process in respiratory chains with three major constituents, dehydrogenases, quinines and oxydoreductases. In anaerobic bacteria, proton transport occurs through a specific H^+ channel in the F_0F_1 -ATPase molecule using energy from ATP hydrolysis. As a facultative bacterium, *Listeria* may use both processes to control its intracellular pH homeostasis (Phan-Thanh et al., 2000).

Eswaranandam et al. (2004) studied the effectiveness of partial replacement of glycerol with citric, lactic, malic, and tartaric acids on the antimicrobial activities of nisin (205 IU/g protein)-incorporated soy protein film against *L. monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella* Gaminara. In that study, organic acids such as malic and lactic acids had greater antimicrobial activity than citric acid. Moreover, Phan-Thanh et al. (2000) also noted that organic acids were more lethal at low pH than inorganic acids. This appeared to be due to their ability to alter the internal pH of the cell (pH_i) of *L. monocytogenes* to a lower level than that observed with inorganic acids. Miller et al. (2009) indicated that weak organic acids are more efficient against *L. innocua* than a stronger acid, such as HCl, at the same pH. The effectiveness of malic and lactic acid against *L. monocytogenes* has been previously demonstrated (Sorrells et al., 1989) and reinforced in this study.

Except for Berries A, *L. monocytogenes* grew in all blueberry extract treatments. Turbidity on Berries C and D solutions showed no statistical significance until 24h. At 24 h, the O.D. growth between the Malic D and Berries B treatments were not significantly different ($P>0.05$). Malic acid concentration in fresh highbush blueberries can average 0.18% fresh weight (Ehlenfeldt et al., 1994). Although there is no lactic acid content in blueberries, this acid was used in this experiment because of its well known antimicrobial properties.

Wang et al. (2008) reported a total phenolic content in blueberries of 319.3 and 190.3 mg/100 g of fwt (fresh weight) and total anthocyanins content of 131.0 and 82.36 mg/100 g of fwt from organic and conventional cultures, respectively. The antimicrobial activity of berries phenolic compounds and anthocyanins has been intensively studied to control human pathogens (Cavanagh et al., 2003). However several studies show that the growth of *Listeria* species are not inhibited by berry phenolics (Nohynek et al., 2006; Puupponen-Pimiä et al., 2001; Puupponen-Pimiä et al., 2005; Rauha et al., 2000). In this study blueberry extract showed antimicrobial activity over *L. monocytogenes* only when the pH was low, such as in Berries A (pH 3.85), while pathogenic growth was observed in treatments Berries B, C and D with pH 6.13; 6.53 and 6.78, respectively (Table 2). The acidic solution containing blueberries extract showed a lower buffer capacity when compared to the organic acids, thus not achieving lower pH in the final growth solutions. Moreover, considering that blueberry extract contains acids, nutrients, minerals and water, it is understandably why the same effect were not seen when compared with malic and lactic acid, which were concentrated acids.

4. Conclusions

Since consumers these days demand more natural food products, the use of natural food ingredients such as acidic juices appears as an option to control and prevent foodborne illnesses and outbreaks. However, in this study blueberry extract showed a lower buffering capacity when compared to malic and lactic acid and was not effective for the reduction of pH in growth media, and hence the inhibition of *Listeria monocytogenes*. This research confirms that *Listeria monocytogenes* can adapt to acid-stress conditions and survive at pH 4.5. However, according to

the literature it was expected to survive at even lower pH (Davis et al., 1996; Kroll and Patchett, 1992).

Future research should examine growth and survival of inoculated acid-adapted *L. monocytogenes* on fresh blueberries stored under commercial transport conditions, such as controlled atmosphere refrigeration. Additionally, more research is required in the use of blueberry extracts as natural antimicrobial for food.

Acknowledgements

The authors wish to thank Dr. Hengjian Wang for his scientific and statistics support and Dr. Sean O’Keefe for his food chemistry scientific guidance. This publication is the result of a research sponsored by the Virginia Tech Department of Food Science and Technology.

References

- Begot, C., Desnier, I., Daudin, J.D., Labadie, J.C., & Lebert, A. (1995). Recommendations for calculating growth parameters by optical density measurements. *J. Microbiol. Meth.*, 25, 225-232.
- Caggia, C., Scifo, G.O., Restuccia, C., & Randazzo, CL. (2009). Growth of acid-adapted *Listeria monocytogenes* in orange juice and in minimally processed orange slices. *Food Control*, 20, 59–66.
- Cavanagh, H. M. A., Hipwell, M., & Wilkinson, J. M. (2003). Antibacterial activity of berry fruits used for culinary purposes. *J. Med. Food*, 6, 57–61.
- Centers for Disease Control and Prevention. (2012). Multistate outbreak of listeriosis linked to whole cantaloupes from Jensen Farms, Colorado – United States. Available at <http://www.cdc.gov/listeria/outbreaks/cantaloupes-jensen-farms/082712/index.html> Accessed 25.01.12
- Chitarra, L.G., Breeuwer, P., Van den Bulk, R.W., & Abee, T. (2000). Rapid fluorescence assessment of intracellular pH as a viability indicator of *Clavibacter michiganensis* subsp *michiganensis*. *J. Appl. Microbiol.*, 88 (5), 809–816.
- Cotter, P. D., Gahan, C. G. M., & Hill, C. (2000). Analysis of the role of the *Listeria monocytogenes* F0F1-ATPase operon in the acid tolerance response. *Int. J. Food Microbiol*, 60, 137-146.
- Cotter, P. D., Gahan, C. G. M., & Hill, C. (2001). A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid. *Mol. Microbiol*, 40, 465–475.
- Dalgaard, P., Ross, T., Kamperman, L., Neumeyer, K., & McMeekin, T.A. (1994). Estimation of bacterial growth rates from turbidimetric and viable count data. *Int. J. Food Microbiol*, 23(3), 391-404.
- Davis, M., Coote, P., & O’Byrne, C. (1996). Acid tolerance in *Listeria monocytogenes*: The adaptive acid tolerance response (ATR) and growth-phase-dependent acid resistance. *Microbiology*, 142, 2975-2982.
- Economic Research Service, US Department of Agriculture. (2012). Loss-adjusted food availability: fruit per capita availability adjusted for loss. Available from: <http://www.ers.usda.gov/Data/FoodConsumption/FoodGuideIndex.htm#fruit> (accessed in 19 August 2012).
- Ehlenfeldt, M.K., Meredith, F.I., & Ballington, J.R. (1994). Unique organic acid profile of rabbiteye vs. highbush blueberries. *Hortscience*, 29(4), 321-323.

- Eswaranandam, S., Hettiarachchy, N.S. & Johnson, M.G. (2004). Antimicrobial activity of citric, lactic, malic, or tartaric acids and nisin-incorporated soy protein film against *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella* Gaminara. *J. Food Sci.*, 69(3), 79-84.
- Farber, J. M., Wang, S. L., Cai, Y., & Zhang, S. (1998). Changes in population of *Listeria monocytogenes* inoculated on packaged fresh-cut vegetables. *J. Food Prot.*, 61, 192-195.
- FDA. (2003). Quantitative assessment of relative risk to public health from foodborne *Listeria monocytogenes* among selected categories of ready-to-eat foods. College Park, MD: US Food and Drug Administration Center for Food Safety and Applied Nutrition.
- Foster, J. W. (1999). When protons attack: microbial strategies of acid adaptation. *Curr. Opin. Microbiol.*, 2, 170-174.
- Gahan, C. G., O'Driscoll, B., & Hill C. (1996). Acid adaptation of *Listeria monocytogenes* can enhance survival in acidic foods and during milk fermentation. *Appl. Environ. Microbiol.*, 62(9), 3128.
- Gandhi, M., & Chikindas, M.L. (2007). *Listeria*: a foodborne pathogen that knows how to survive. *Int. J. Food Microbiol.*, 113, 1-15.
- Hudson, J.A. (1994). Comparison of response surface models for *Listeria monocytogenes* strains under aerobic conditions. *Food Res. Int.*, 27, 53-59.
- Kniel, K.E. & Shearer, A.E. (2009). Berry Contamination: Outbreaks and Contamination Issues. In: G. Sapers, E. Solomon & K. R. Matthews (Eds.), *The Produce Contamination Problem: Causes and Solutions* (pp. 271-297). San Diego, CA: Academic Press.
- Kroll, R. G., & Patchett, R. A. (1992). Induced acid tolerance in *Listeria monocytogenes*. *Lett. Appl. Microbiol.*, 14, 224-227.
- Leverentz, B., Conway, W. S., Camp, M. J., Janisiewicz, W. J., Abuladze, T., Yang, M., Saftner, R. & Sulakvelidze, A. (2003). Biocontrol of *Listeria monocytogenes* on fresh-cut produce by treatment with lytic bacteriophages and a bacteriocin. *Appl. Environ. Microb.* 69(8): 4519-4526.
- McClure, P.J., Cole, M.B., Davies, K.W., & Anderson, W.A. (1993). The use of automated turbidimetric data for the construction of kinetic models. *J. Industr. Microbiol.*, 12, 277-285.
- McGlynn, W. (2000). The Importance of Food pH in Commercial Canning Operations. Division of Agricultural Sciences and Natural Resources. Oklahoma State University Cooperative Extension Service. Accessed on 19 December 2012
<http://pods.dasnr.okstate.edu/docushare/dsweb/Get/Document-603/FAPC-118pod.pdf>.

- Miller, F. A., Ramos, B., Gil, M. M, Brandão, T. R., Teixeira, P., & Silva, C. L. (2009). Influence of pH, type of acid and recovery media on the thermal inactivation of *Listeria innocua*. *Int. J. Food Microbiol.*, 133(1-2), 121-8.
- Nohynek, L. J., Alakomi, H. -L., Kähkönen, M. P., & Heinonen, M. (2006). Berry phenolics - Antimicrobial properties and mechanisms of action against severe human pathogens. *Nutr. Cancer*. 54:18 – 32.
- O'Driscoll, B., Gahan, C.G., & Hill, C. (1996). Adaptive acid tolerance response in *Listeria monocytogenes*: isolation of an acid-tolerant mutant which demonstrates increased virulence. *Appl. Environ. Microbiol.*, 62 (5), 1693–1698.
- O'Driscoll, B., Gahan, C., & Hill, C. (1997). Two-dimensional polyacrylamide gel electrophoresis analysis of the acid tolerance response in *Listeria monocytogenes* LO28. *Appl. Environ. Microb.*, 63 (7), 2679-2685.
- Parish, M.E. & Higgins, D.P. (1989). Survival of *Listeria monocytogenes* in low pH model broth systems. *J. Food Protect.*, 52, 144-147.
- Phan-Thanh, L., Mahouin, F., & Alige, S. (2000). Acid responses of *Listeria monocytogenes*. *Int. J. Food Microbiol.*, 55, 121-126.
- Phan-Thanh, L., & Mahouin, F. (1999). A proteomic approach to study the acid response in *Listeria monocytogenes*. *Electrophoresis*, 20 (11), 2214-24.
- Puupponen-Pimiä, R., Nohynek, L., Alakomi, H. -L., & Oksman-Caldentey, K. -M. (2005). Bioactive berry compounds -Novel tools against human pathogens. *Appl. Microbiol. Biot.*, 67, 8-18.
- Puupponen-Pimiä, R., Nohynek, L., Meier, C., Kähkönen, M., Heinonen, M., Hopia, A., & Oksman-Caldentey, K. M. (2001). Antimicrobial properties of phenolic compounds from berries. *J. Appl. Microbiol.*, 90(4), 494-507.
- Rauha, J. -P., Remes, S., Heinonen, M., & Hopia, A. (2000). Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *Int. J. Food Microbiol.*, 56, 3 – 12.
- Rocourt, J., & Cossart, P. (1997). *Listeria monocytogenes*. In: M. P. Doyle, L. R. Buechat & T. J. Montville (Eds.), *Food Microbiology — Fundamentals and Frontiers* (pp. 337–352). Washington, DC: American Society for Microbiology (ASM) press.
- Ryser, E. T., & Marth, E. H. (1999). Foodborne listeriosis. In: *Listeria, listeriosis and food safety.*, (Ed. 2) (pp. 299-358). New York, NY: Marcel Dekker, Inc.,

- Sado, P. N., Jinneman, K. C., Husby, G. J., Sorg, S. M., & Omiecinski, C. J. (1998). Identification of *Listeria monocytogenes* from unpasteurized apple juice using rapid test kits. *J. Food Protect.*, 61(9), 1199-1202.
- Sivapalasingam, S., Friedman, C. R., Cohen, L. & Tauxe, R. V. (2004). Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. *J. Food Protect.*, 67, 2342– 2353.
- Sorrells, K. M., Enigl, D. C. & Hatfield, J. R. (1989). Effect of pH, acidulant, time, and temperature on the growth and survival of *Listeria monocytogenes*. *J. Food Protect.*, 52, 571-573.
- Todd, E. C. D., & Notermans, S. (2011). Surveillance of listeriosis and its causative pathogen, *Listeria monocytogenes*. *Food Control*, 22, 1484–1490.
- Vasseur, C., Baverel, L., Hébraud, M., & Labadie, J. (1999). Effect of osmotic, alkaline, acid or thermal stresses on the growth and inhibition of *Listeria monocytogenes*. *J. Appl. Microbiol.*, 86, 469–476.
- Wang, S. Y., Chen, C. T., Sciarappa, W., Wang, C. Y., & Camp, M. J. (2008). Fruit quality, antioxidant capacity, and flavonoid content of organically and conventionally grown blueberries. *J. Agr. Food Chem.*, 56(14), 5788-5794.
- Weis, J. & Seeliger, H. P. R. (1975). Incidence of *Listeria monocytogenes* in nature. *Appl. Microbiol.*, 30: 29–32.
- Werbrouck, H., Vermeulen, A., Van Coillie, E., Messens, W., Herman, L., Devlieghere, F., & Uyttendaele, M. (2009). Influence of acid stress on survival, expression of virulence genes and invasion capacity into Caco-2 cells of *Listeria monocytogenes* strains of different origins. *Int. J. Food Microbiol.*, 134, 140–146.
- Wu, V. C., & Kim, B. (2007). Effect of a simple chlorine dioxide method for controlling five foodborne pathogens, yeasts and molds on blueberries. *Food Microbiol.*, 24(7), 794-800.

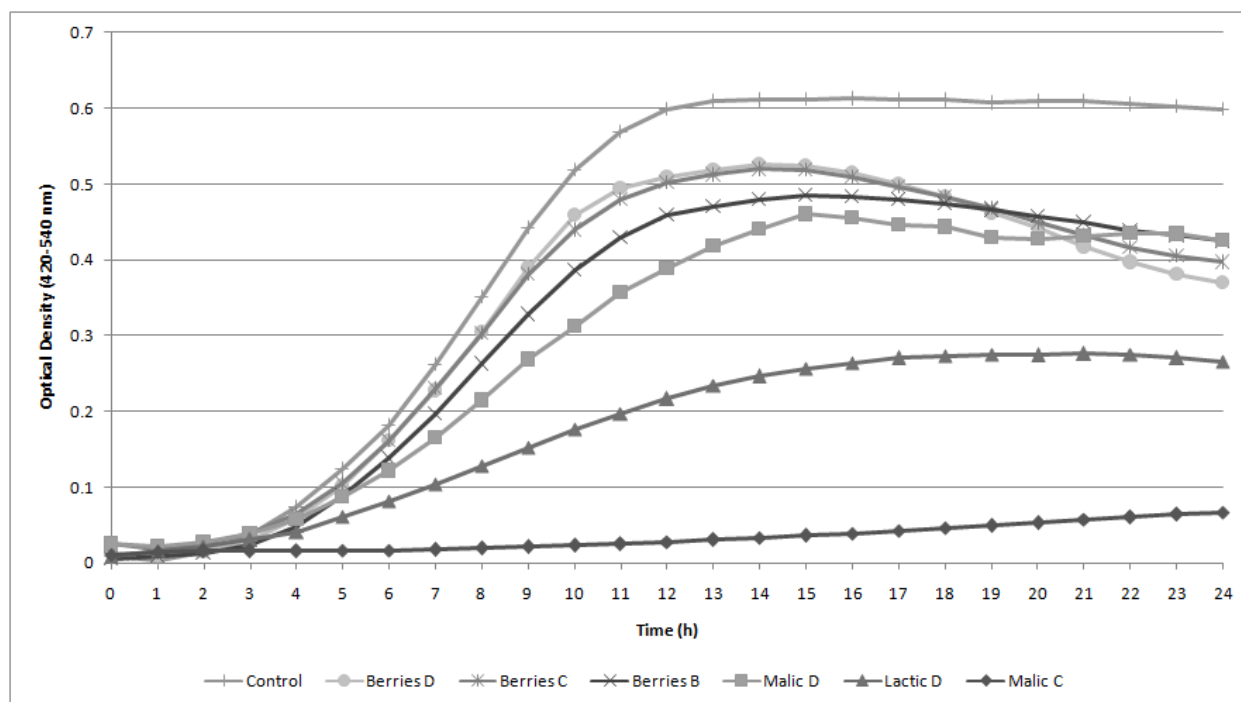


Figure 1. *Listeria monocytogenes* Scott A optical density turbidity growth curve measured using tryptic soy broth with yeast extract (TSB+YE) under different treatments (B, C, or D) with different acids (malic acid, lactic acid and blueberry extract) incubated at 25°C for 24h.

Table 1. Treatments solutions pH before inoculation with 10 µl of *Listeria monocytogenes* in 0.1% peptone water.

Treatment	Acid Solution	Growth Media (pH 7.0)	Treatment pH
Malic A	15µl of Malic acid pH 2.0	125 µl of TSB+YE	3.04±0.02
Malic B	15µl of Malic acid pH 3.0	125 µl of TSB+YE	3.67±0.04
Malic C	15µl of Malic acid pH 4.0	125 µl of TSB+YE	4.54±0.04
Malic D	15µl of Malic acid pH 5.0	125 µl of TSB+YE	5.76±0.10
Lactic A	15µl of Lactic acid pH 2.0	125 µl of TSB+YE	3.02±0.02
Lactic B	15µl of Lactic acid pH 3.0	125 µl of TSB+YE	3.53±0.07
Lactic C	15µl of Lactic acid pH 4.0	125 µl of TSB+YE	4.30±0.08
Lactic D	15µl of Lactic acid pH 5.0	125 µl of TSB+YE	6.04±0.11
Berries A	15µl of Blueberry extract pH 2.0	125 µl of TSB+YE	3.85±0.03
Berries B	15µl of Blueberry extract pH 3.0	125 µl of TSB+YE	6.13±0.05
Berries C	15µl of Blueberry extract pH 4.0	125 µl of TSB+YE	6.53±0.02
Berries D	15µl of Blueberry extract pH 5.0	125 µl of TSB+YE	6.78±0.01

Table 2. Mean of optical density turbidity growth curve values and standard deviation (n=12) for *Listeria monocytogenes* Scott A in TSB+YE under acid treatments incubated at 25°C for 24 h.

Treatment	O.D. 420-540nm			
	6 hours	12 hours	18 hours	24 hours
Control	0.18±0.05 ^A	0.60±0.05 ^A	0.61±0.03 ^A	0.60±0.03 ^A
Berries D	0.16±0.06 ^B	0.51±0.02 ^B	0.48±0.04 ^B	0.37±0.02 ^D
Berries C	0.16±0.05 ^B	0.50±0.04 ^B	0.48±0.04 ^B	0.40±0.02 ^C
Berries B	0.14±0.05 ^C	0.46±0.05 ^C	0.47±0.04 ^B	0.42±0.04 ^B
Malic D	0.12±0.07 ^D	0.39±0.10 ^D	0.44±0.09 ^C	0.43±0.08 ^B
Malic C	0.02±0.01 ^F	0.03±0.02 ^F	0.05±0.03 ^E	0.07±0.04 ^F
Lactic D	0.08±0.07 ^E	0.22±0.14 ^E	0.27±0.14 ^D	0.27±0.12 ^E

Means within each column followed by the same letter are not significantly different (P>0.05) from each other.

Abbreviations: Positive *Listeria monocytogenes* Scott A Control (Control), Blueberry extract (Berries), Malic acid (Malic) and Lactic acid (Lactic).

CHAPTER 4

Shelf life determination of fresh blueberries (*Vaccinium corymbosum*) under controlled atmosphere storage

ABSTRACT

Fresh blueberries are commonly transported refrigerated in controlled atmospheres to protect shelf life for long periods of storage. Ozone is an antimicrobial gas that can extend shelf life and protect fruit from microbial contamination. The aim of this study was to determine the shelf life of fresh highbush blueberries stored at 4°C or 12°C under different controlled atmosphere conditions, including air (control); 5% O₂ and 15% CO₂, 80% N₂ (CAS); and ozone gas (O₃) 4ppm at 4°C or 2.5ppm at 12°C, at high relative humidity (90-95%) for a total of 10 days. Product shelf life was evaluated by determining yeast and mold counts, weight loss and firmness of blueberries during storage.

Fresh blueberries inside a plastic clamshell were stored at 4°C or 12°C in isolated cabinets under air, CAS, and O₃. Samples were evaluated on day 0, 1, 4, 7, and 10 for yeast and molds using 3M Petrifilm (Yeast and Mold Count Plates), weight loss and for firmness using a texture analyzer. Controlled atmosphere storage did not delay or inhibit yeast and molds growth in fresh blueberries after 10 days. Fruit stored at 4°C showed lower weight loss values compared with 12°C. By day 10, berries treated with ozone showed reduced weight loss at 12°C, and firmness loss when compared to the other treatments. Low concentrations of ozone gas together with proper refrigeration temperature can help protect product quality and also ensure safety during transportation. Gaseous ozone is a strong antimicrobial that safely decomposes to oxygen and water without leaving residues and is an alternative to prevent bacterial growth during long-term transport and storage.

Keywords: Blueberries; controlled atmosphere storage; gaseous ozone, shelf life.

1. Introduction

Blueberries are recognized for their contribution to a healthy diet with different beneficial bioactive compounds such as flavonoids, anthocyanins and others (Lila, 2004; Wang et al., 2005; Hakkinen & Torronen, 2000; Prior et al., 1998), that helps avoiding important diseases including different cancers (World Cancer Research Fund/American Institute for Cancer Research, 2007; Ness, 1997; Dauchet et al., 2006; International Agency for Research on Cancer, 2003; Boffetta, 2010). Highbush blueberries (*Vaccinium corymbosum*) together with the other commercial blueberry species are ranked as the second most important berry after strawberries in the U.S., accounting for nearly 850.9 million in 2012 (USDA, 2013). Although the U.S. is the world biggest blueberry producer, the American market is so large that importing the fruit from other countries such as Chile, is required. In 2011, Chile provided more than 50% of the imported blueberries sold in the U.S. (USDA, 2012). Products shipped from Chile can take 20 days to arrive to the U.S. (Beaudry et al., 1998).

Proper storage temperature conditions for blueberries are 0°C, while relative humidity ranges from 90 to 95% for a storage life of 10-18 days (Bachmann & Earles, 2000). The use of controlled atmosphere in fresh produce transportation is widely applied by producers, to assure the quality of the product and avoid spoilage. Respiration rate and deterioration decreases for some fruits when under CO₂ levels of about 10% to 20%. Some results indicate that O₂ concentrations between 8% and 10% and CO₂ concentrations of 10% to 13% have managed to maintain the quality of the blueberries between 5 and 8 weeks at 0-1°C and 3 days under temperature conditions of 18-20°C (Bounous et al., 1997). High levels of CO₂, while fruit was stored and shipped, helped control decay and extend shelf life of cherries transported from Chile to Japan (Retamales, 2003). Moreover, reduction of *Botrytis cinerea* (grey mold rot) in

strawberries can be achieved by applying 15% CO₂ (Garcia et al., 1995). According to Mitcham & Mitchell (2002) grey mold rot and other decay organisms can be minimized by using gas concentrations of 15 to 20% carbon dioxide and 5 to 10% oxygen, that will also decrease respiration and softening rates of shipped blueberries, raspberries and blackberries thus prolonging postharvest life.

Ozone (O₃) is a strong antimicrobial agent with variety of applications in the food industry (Kim et al., 1999). Ozone was designated as generally recognized as safe (GRAS) in 1982 by the FDA for use as a disinfectant or sanitizer in the gas or liquid phase on food (21 CFR, Part 173) and for direct contact use as an antimicrobial for treatment, storage and processing on diverse foods including raw and minimally processed fruits and vegetables (FDA, 1982). Ozone decomposes rapidly into oxygen without leaving residues (White, 1992) and its application at a postharvest level has increased (Parish et al., 2003). Moreover, its use in cold rooms helps reduce the ethylene (C₂H₄) level in air, extending the storage life of fruits and vegetables such as apples and oranges (Skog & Chu, 2000). Other studies have proven inhibition of spoilage microorganisms and shelf life extension using O₃ in bananas (Gane, 1936), potatoes, onions and beetroot (Baranovskaya et al., 1979), tomatoes and mandarins (Jin et al., 1989), blackberries (Barth et al., 1995), lettuce and carrot (Singh et al., 2002) and strawberries (Pérez et al., 1999).

However, other studies have found ozone to be ineffective to control spoilage on apples, blueberries, green beans, muskmelons, peaches, and strawberries (Liew & Prange, 1994). Furthermore, apples, cantaloupes, cranberries and corn kernels, showed increased decay and spoilage contamination when ozone was used on them (Barger et al., 1948; Brooks & Csallany, 1978; Norton, 1968; Schomer & McCulloch, 1948; Spalding, 1968).

According to Palou et al. (2002) 0.3 $\mu\text{L/L}$ O_3 on peaches and table grapes did not delay decay, however grey mold was inhibited on grapes. Pérez et al. (1999) found 0.35 $\mu\text{L/L}$ O_3 inefficient to prevent fungal decay in addition to negative effects on the sensory properties of strawberries. Although, high O_3 doses may be needed for an effective elimination of microorganisms, this may alter negatively the sensory attributes of fresh fruit (Mallison and Spalding, 1966; Ogawa et al., 1990; Achen, 2000).

The present study will evaluate shelf life extension and quality preservation of fresh blueberries packaged under different controlled atmosphere conditions (Air; 5% O_2 ; 15% CO_2 ; 80% N_2 ; and Ozone) and temperature storage (4°C and 12°C) for 10 days, measuring yeast and mold counts, weight loss and blueberries firmness during storage.

2. Materials and Methods

2.1 Fruit Samples

The trial was carried out during the summer of 2012 using highbush blueberries (*Vaccinium corymbosum* L., cv Ozark Blue) that were hand harvested from the commercial planting Three Bird's Berry Farm located in Blacksburg, VA. Two replications were conducted each consisting of fruit harvested at two different dates. Only fully colored fruits were used.

2.2 Fruit Selection and Storage

After harvest collection, samples were stored in a cooler (4-8°C) and transported to the Food Science and Technology Building at Virginia Tech. Blueberries were sorted and only fully developed fruit free of visible defects were selected on a visual basis and stored in a 4.4 oz PET retail clamshell box (Highland Corporation, Mulberry, FL). Fifty blueberries per clamshell were

used as one sample and each were placed in the controlled atmosphere chamber and incubated at different temperatures (4°C and 12°C), and one of three atmospheres (Air as a control (21% O₂ and 0.03% CO₂); 5% O₂: 15% CO₂: 80% N₂; Ozone). Berries were sampled on day 0, 1, 4, 7 and 10.

2.3 Controlled Atmosphere and Ozone Treatment

Clear acrylic desiccator cabinets with exterior dimensions of 12"W x 12"H x 12"D with gas ports including hygrometer (Cole-Parmer, Lansing, MI, USA) were used to store fruit in clamshells, inside an incubator (Precision Incubator, Thermo Scientific, Waltham, MA) and held at 4°C or 12°C with 90-95% relative humidity (RH). Temperature and RH were monitored by a sensor (Traceable Jumbo Thermo-Humidity Meter, Fisher Scientific, Pittsburgh, PA).

For controlled atmosphere storage a certified standard-spec gas tank was used (MID-Saint Louis SGL (SAP)-MO). Compressed gas was composed by 5% O₂, 15% CO₂ and balanced nitrogen (80%). Gas concentrations were monitored two times a day (morning and late afternoon) using an O₂/CO₂ gas analyzer (PBI Dansensor O₂/CO₂ Check Point, PBI-Dansensor, Ringsted, Denmark). When gas concentrations experienced a $\geq 3\%$ reduction, cabinets were refushed.

A corona discharge ozone generator (FreshFridge 2.0 Refrigerator Air Purifier Model GH 2138, IonCare G&H Industrial Ltd., China) and a small air circulating fan were installed in the chambers. Ozone concentration in chambers was monitored, controlled and recorded continuously using an ozone analyzer (Model ES-600, Ozone Solutions Inc., IA, USA) and a Dell Latitude D830 computer as data logger (Dell, Round Rock, Texas). Ozone concentration mean and standard deviation values were 4.0 ± 1.8 ppm for 4°C and 2.5 ± 1.5 ppm at 12°C. A

third chamber without an ozone generator served as the control (Air). Blueberries were exposed to controlled atmosphere and ozone in these chambers for a total of 10 days.

2.4 Weight Loss

Each clamshell with fifty blueberries was weighted after harvest and the re-weighted after 1, 4, 7 and 10 days of storage. Weight was recorded using a scale with an accuracy of 0.01g (Mettler, Madrid, Spain). Weight loss was estimated by subtracting sample data weight from the initial weight and expressed as accumulated weight loss percentage per unit time (Al-qurashi, 2002).

2.5 Yeast and Molds

Yeast and Molds Petrifilm (3M Microbiology Products, St. Paul, MN) pouches were stored unopened at $<8^{\circ}\text{C}$. After opening, pouches were resealed by folding and taping the open end and stored at $<8^{\circ}\text{C}$ in a dry place. To aseptically prepare 1:10 dilutions, 10g of blueberries were weight and then added to a stomacher bag with 90mL of sterile 0.1% peptone water. Samples were blended and stomached (AES Laboratoire Easy Mix, Microbiology International, Combourg, France) for 120 seconds. Samples were plated on Yeast and Mold Petrifilm placing plate in a flat surface, lifting the top film, holding pipette perpendicular to plate and carefully inoculating 1mL of previously diluted suspension onto center of film base. After this top lift was placed down onto inoculum and a lift plastic spreader with a circular handle and concave side was used to distribute suspension evenly using gentle downward pressure on center of spreader. Duplicate plates were made at each dilution. Plates were incubated in horizontal position, clear side up, in stacks not exceeding 20 units for 3 days at room temperature 20-25°C.

Yeasts appeared as blue-green or off-white in color and form small defined edge colonies. Mold colonies are usually blue but may also assume their natural pigmentation (e.g., black, yellow, green). They tend to be larger and with more diffuse edges than yeast colonies, usually with a focus in a center of colony. Yeast and molds were enumerated using the AOAC official method 997.02 (AOAC, 1998).

2.6 Firmness Texture Analysis

Textural measurements were carried out at harvest and after 1, 4, 7 and 10 days during postharvest storage. Berry samples for analysis were randomly selected and sample size was fifteen berries each testing, according with Doving et al. (2005). Each blueberry fruit was measured individually using a TA.XT*Plus* Texture Analyzer and a TA-52 2 mm diameter stainless steel puncture probe (Stable Microsystems, Godalming, Surrey, UK). The probe height was calibrated to 13 mm above the TA-90 base platform so that the blueberry could be aligned directly under the probe and a 50 Kg load transducer was used. The probes were cleaned between each test replicate to remove any adhered material. Fruit penetration was set to reach 3 mm. The following test settings were used: Measure Force in Compression; Return to Start; pre-test speed of 2mm/s and test speeds of 1 mm/s with an automatic trigger set to 5 grams of force; test distance of 3 mm into the blueberries; and a post-test withdrawal speed of 2 mm/s. Each time a set of fruits was measured, the equipment was force and height calibrated.

2.7 Experimental Design and Statistical Analysis

Data were analyzed using the Standard Least Square procedure of JMP Pro 10 (SAS Institute Inc, Cary, NC). The randomized complete block factorial design with two replications was utilized to test the treatments and their interactions on weight loss, yeast and mold growth

and texture firmness. All the tests used two samples for each treatment (Air, CAS and O₃). If the interactions between treatments were not significant ($P>0.05$), the main effects of the treatments were separated by the Student's t Significant Difference test using the interaction as the error term.

3. Results and Discussion

3.1. Weight Loss

Although weight loss was minimal in this study and ranged from 0.18–2.64 % within 10 days of storage, significant differences in treatments were observed mostly at 12°C storage conditions. Weight loss of fruit stored at 4°C showed no significant difference among treatments or time of storage, and achieved lower than 1% values (Figure 1). To control weight loss of blueberries it is crucial to keep low temperatures (0-1°C) (Harb & Streif, 2004; Sanford et al., 1991) and high relative humidity during storage. Forney (2008) found that maintaining a high relative humidity (95% or greater) helped minimize weight loss and shrivel of blueberries. However, this can increase decay development if condensation is not properly controlled (Sargent et al., 2006). Schotsmans et al. (2007) concluded that CA storage (2.5 kPa O₂ +15 kPa CO₂) presents a clear benefit for longer term blueberry storage up to 42 days, but weight loss and shrivel of rabbiteye blueberries were not decreased by it followed by an exposure shelf life period at 20°C. Furthermore, a significant weight loss (9-14%) was not observed until day 6 of exposure and the antioxidant activities and total phenolic content of blueberries were not affected adversely (Schotsmans et al. 2007).

Despite that in the present study the same ozonator device was used at 4°C and 12°C, ozone gas concentration averaged 4ppm and 2.5ppm, respectively, in agreement with Liew and

Prange (1994) who observed that residual ozone concentration is less at higher storage temperatures. When storage temperature was 12°C, O₃ was the treatment that showed the lowest weight loss along time. O₃ differences were significant from Air and CAS at day 1, 4 and day 10 (Figure 1). Nevertheless, weight loss in blueberries treated with O₃ was always lower when compared to Air and CAS during the 10 days at 12°C. Sanford et al. (1991) reported that maximum weight loss before blueberries become non-saleable is approximately 5% to 8%, implying that in this study there was not a loss of quality since values were far from this range. The results in this study are equivalent to other research that showed around 2% loss from initial weight after 10 days of storage at 5°C and 10°C (Nunes et al., 2004) and minimal weight loss of 2% after 14 days at 0°C (Jackson et al., 1999).

O₃ kept weight loss low and helped control decay at 12°C, while other authors found reciprocal results on stored highbush blueberries at 10°C for 7 days using 700 ppb and no phytotoxicity compounds were observed (Fan et al., 2001). According to Kim et al. (2010) SO₂ and O₃ reduce weight loss and fruit decay in blueberries increasing fruit quality and storage life. In the present study by day 10, CAS and Air showed no significant difference in weight loss at 12°C, implying that CAS is not as effective in a long time storage period. Although Bounous et al. (1997) observed that three cultivars of highbush blueberries lost more weight when stored in Air and O₃ when compared to CAS, at the end of a period of 6 week storage CAS weight loss was higher than air and O₃.

Weight loss in fruit is directly related to respiration rate (Jackson et al., 1999). A low O₂ and rich CO₂ atmosphere can potentially reduce not only the respiration rate but also ethylene sensitivity and production, oxidation and fruit decay (Fonseca et al., 2002; Gorris & Tauscher, 1999; Kader et al., 1989; Saltveit, 1997). Moreover, O₃ inhibition of enzymatic reaction can

cause a decrease in fruit respiration leading to less weight loss (Kim et al., 2010). Other gases can produce a decrease of enzymatic activity in fruits such as SO₂ (Kim et al., 2010), Na₂S₂O₅ sodium metabisulfite (Petri et al., 2008) and CO₂ (Bounous et al., 1997). According to Aguayo et al. (2006) O₃ stimulated the respiration rate in both whole and cut tomatoes only during the first 2 days of storage, decreasing after that period. Other authors have confirmed that O₃ decreases respiration rate in whole tomatoes (Maguire & Solverg, 1980; Jin et al., 1989), bananas (Gane, 1936) and peaches (Palou et al., 2002), however Liew and Prange (1994) observed an increase on carrots under O₃ treatments, depending on doses and storage time. The ratio of CO₂ produced to O₂ consumed, known as the respiratory quotient (RQ), is normally assumed to be equal to 1.0 if the metabolic substrates are carbohydrates (Fonseca et al., 2002). Beaudry et al. (1992) explained an observed RQ of 1.3 for blueberries by their high content of citric acid and sugars. The RQ is much greater than 1.0 when anaerobic respiration takes place. Oxygen concentration should low but no zero, to avoid anaerobic respiration on fruit stored in controlled atmosphere conditions. In this study oxygen concentrations under CAS were 5%. O₃ was effective to control weight loss of fresh blueberries stored at 12°C with high relative humidity, when compared to air and CAS.

3.2. Yeast and Molds

Yeast counts increased from Day 0 to Day 10 along all treatments and temperatures (Table 1). Initial counts in CAS and O₃ were higher than in Air. At 12°C yeast increase was higher in O₃ and CAS with 2.59 and 2.37 log CFU/g, respectively, when compared 1.87 and 1.86 at 4°C. Air did not show much of a difference in increase along temperature. Variability in the results can be observed, since fruits were collected from the field and were not surface-sterilized

before testing, so naturally occurring yeast and molds was measured. Initial yeast and mold counts and high variability in them is influenced by time of the year, weather and harvest conditions, as well as the fruit wetness when picked (Cline, 1996). Moreover, if natural protective wax bloom of blueberries is absent due to weather or picking practices, it is more likely to grow yeast and molds. For a more confident number maybe an initial inoculation with a known concentration is required for future research.

Increase overtime in molds counts were similar for blueberries stored in air (control) at 4°C (2.74 log CFU/g) and 12°C (2.75 log CFU/g) (Table 2). The increase overtime of mold for CAS blueberries at 4°C (2.10 CFU/g) was higher compared to 12°C storage (0.20 CFU/g). Beuchat and Brackett (1990) observed slower growth of molds at 10°C when compared to 5°C on lettuce. Lower counts at warmer temperatures were observed by day 10 on O₃ and CAS treatments. The increase in mold counts for the O₃ treatment at 4°C was calculated using the recovery counts of day 7, since at day 10 at 4°C no plate growth occurred at a detection limit of 10⁻⁴. Growth of visible mold on samples was only observed in air treatment at 12°C in Day 10 (1/50 berries).

Ozone inhibition of bacteria is more noticeable than in yeast and molds (Aguayo et al., 2006; Kim et al., 1999). Moreover, Palou et al. (2001) indicated that O₃ delays but does not reduce fungi incidence after one week and cannot control molds in wounded fruits. Ozone was fungistatic against grey mold (*Botrytis cinerea*) and not fungicidal (Liew & Prange, 1994). Ozone treatment was ineffective in preventing fungal decay in strawberries after 4 days at 20°C (Perez et al., 1999). According Schotsman et al. (2007) CA effectiveness to decrease fungal and blemish development is achieved after 28 days of storage at refrigeration conditions.

3.3. Firmness

Table 3 shows that firmness penetration force values were not significantly different ($p < 0.05$) between treatments after 10 day of storage at 4°C. However at 12°C, penetration force for the control berries (219.77g) was significantly higher compared to berries stored under O₃ (169.19g) and CAS (182.67g). According to Schotsman et al. (2007) a high touch firmness value means the blueberry fruit is perceived as being softer. The enhanced resistance of the fruit to the probe penetration can be interpreted as excessive elasticity or gumminess, due to a strong loss of internal water turgor pressure (Giongo et al., 2013). This characteristic is unfavorable since it can mislead fruit grading and texture determination (Marshall et al., 2008). Turgidity is the most critical texture component in blueberry (Giongo et al., 2013).

At 4°C texture within O₃ treatments did not have significant differences over time, while Air only showed differences at day 1 when compared to the rest of the days. This latter could be because heat shock from harvest temperature to the refrigeration temperatures. Aguayo et al. (2006) observed that the firmness of tomato slices did not change after cyclical O₃ enriched airflow exposure throughout 5°C cold storage time when compared to control. However, in whole tomatoes, the O₃ treatment reduced softness. Mushrooms treated with O₃ (0.03 mg s⁻¹) for 15 or 30 min showed no significant difference in firmness change during storage (Escriche et al., 2001).

At 12°C CAS blueberries showed a significant increase in texture after day 0 (196.0 g) when compared to day 1 (150.1 g) and day 4 (138.5 g). In literature can be found many reports where strawberry firmness increased during low temperature storage (Larsen & Watkins, 1995a; Watkins et al., 1999) and high CO₂ levels (Ueda & Bai, 1993; Larsen & Watkins, 1995b; Goto et al., 1996). CO₂ firming effect and its magnitude are possibly cultivar dependent (Smith & Skog,

1992; Watkins et al., 1999). Moreover, CO₂ indirect effect on the apoplastic pH with the following precipitation of soluble pectins and the enhancement of cell-to-cell bonding (Harker et al., 2000) are likely responsible for the firming response (Pelayo et al., 2003). Higher firmness of Bluecrop blueberries during storage could also be related to the presence of stone cells in the fruit (Allan-Wojtas et al., 2001).

The firmness increase in CAS at 12°C when comparing day 0 (196.0 g) to 7 (146.3 g) is equivalent to the results of the study by Mahajan and Goswami (2004) where litchi fruit firmness increased, achieving acceptable puncture strength within the storage period, perhaps because of the moisture loss from litchi fruit during storage, which may be explained by fruit drying and hardening characteristics. In the present study blueberries stored under CAS at 12°C obtained high weight loss values when compared to ozone. Pelayo et al. (2003) found beneficial effect of CO₂ during storage increasing firmness in two cultivars with no detectable effects on external color.

Texture is affected by cellular organelles, biochemical constituents, water content or turgor and cell wall composition (Jackman and Stanley, 1995). High humidity allows degradation of the middle lamella and disintegration of the primary cell wall, that are important factors determining fruit softening (Deng et al., 2005). During blueberry ripening, the total water soluble pectin decreases and the degradation of the cell wall and middle lamella is responsible for the loss of firmness (Deng et al., 2005). Furthermore changes in texture occur due to changes in the chemistry of the primary cell wall components cellulose, pectins and hemicelluloses that occur during growth and development can also affect texture (Chiabrando et al., 2009). This variation may be attributed to cultivar differences and/or their interaction with postharvest

storage conditions (Forney et al., 1998). In the present study the only cultivar used was Ozark Blue under high humidity environments (90-95%).

Absence of significant changes in firmness among treatments at 4°C in the present study associated with the results obtained by Chiabrando et al. (2009) who found that during storage, firmness of blueberries was not considered a critical quality factor since it remained constant during the postharvest storage period storage at 0°C, indicating that low-temperature conditions may delay berry softening by inhibiting enzymatic activities and ethylene production.

The high variability (Standard Deviation) of average blueberries firmness observed can be due to the different berry sizes within the samples, since smaller blueberry tended to be slightly firmer than larger ones (Smagula et al., 1996; Khazaei & Mann, 2004) confirming the negative relation between size and firmness of blueberries from the same cultivar (Donahue et al. 2000; Schotsman et al., 2007). Moreover, Doving and Mage (2002) and Chiabrando et al. (2009) also observed a significant amount of fruit-to-fruit variability in firmness values. Texture is not easy to define particularly in small fruits such as blueberries, since a common standardized method does not exist. Many instruments and techniques have been studied widely (Barrit & Torre, 1980; Bourne, 1980; Bernstein & Lustig, 1981; Mencarelli et al., 1994; Duprat et al., 1997; Barreiro and Ruiz-Altisent, 2000; Khanizadeh et al., 2000; Doving & Mage, 2002) and the majority of these methods record a measurement of the force needed to puncture, penetrate or deform the fruit (Chiabrando et al., 2009). In this study the measurement measured was the penetration firmness like other authors with blueberries (Silva et al., 2005; Swift, 2010)

At 12°C by day 10 the penetration peak force for blueberries stored in air was significantly higher on day 10 (219.8 g), which was the highest value obtained in the entire experiment, compared to day 0 (178.0 g), 1 (167.6 g) and 7 (179.5 g). This increase in firmness

may be attributed to moisture loss during storage, which corresponds to fruit drying and hardening characteristics (Mahajan & Goswami, 2004; Chiabrando et al., 2009). This behavior is due to the fact that a less turgid berry generally presents an extended tissue deformation before the probe breaks the superficial tension and enters into the plasticity phase, where irreversible rupture occurs (Giongo et al. 2013). A soft and more deformable and elastic berry structure are probably caused due to water leak as reported for other products (Kader, 1993).

Moreover at day 10 all treatments showed significant differences when compared to each other and CAS obtained 182.7 g, while O₃ was the lowest value with 169.2 g thus with a better texture. In whole tomatoes O₃ treatment reduced softness after 15 days of storage, providing a better retention of the texture of fruit compared to control (Aguayo et al., 2006). Daş et al. (2006) observed that ozone gas concentration of 30 mg/l did not change or soften texture of cherry tomatoes. Fan et al. (2012) developed an in-package ozonation device, which produced ozone inside sealed film bags, and showed no negative effects on cherry tomatoes texture, preserving fruit quality after 22 days post treatment storage.

At 12°C fruit firmness increased initially in all treatments when comparing Day 0 and Day 1, while with the advancement of storage period it declined, in agreement with results reported by Allan-Wojtas et al. (2001), Chiabrando et al. (2009) and Basiouny and Chen (1988) in blueberries, and Pelayo et al. (2003) in strawberries. Shriveling of blueberries over time was observed regardless of storage temperature in agreement with other authors (Nunes et al., 2004; Chiabrando et al., 2009).

Firmness testing of fruits is used to describe mechanical properties of the fruit tissue (Gunness et al., 2009) and mostly used for scientific purposes and provides information on the storability and resistance to injury of the product during handling (Doving & Mage, 2002;

Harker et al., 1997). Instrumental measurements of texture are preferred rather than sensory evaluation since instruments may reduce variation among measurements due to human factors and are in general more precise (Abbot et al., 1997). The present study results show that the variations in texture between individual fruit are large, in agreement with previous reports (Doving & Mage 2002; Doving et al., 2005).

4. Conclusions

Ozone was more effective controlling weight loss at 12°C, although weight loss in fruit was never more than 3%. A high weight loss in fruit is significant commercially, because it can produce decay. Naturally occurring yeast and molds on blueberries were not affected by O₃ and CAS treatments, since they were able to grow overtime at both temperatures. During storage time, firmness was better maintained with O₃ and CAS treatment when compared to Air. These treatments can be used during cold storage, meaning an advantage to producers who may wish to delay or extend marketing of fruits (Ceponis & Capellini, 1985). The O₃ did not cause any external damage. The O₃ treatment reported here can be used in fresh highbush blueberries to maintain quality and extend shelf life. Future research could study the impact of O₃ and CAS on flavor using a sensory panel.

Acknowledgements

The author wishes to thank Mr. Bill Sembello from Three Birds Berry Farm for his help and support providing fresh blueberries. Also to acknowledge Mr. Matthew Schroeder for his scientific and laboratory support. This publication is the result of research sponsored by the Virginia Tech Department of Food Science and Technology.

References

- Abbot, J.A., Lu, R., Upchurch, B.L., & Stroshine, R.L. (1997). Technologies for nondestructive quality evaluation of fruits and vegetables. *Hortic. Rev.*, 20, 1–120.
- Achen, M. (2000). Efficacy of ozone in inactivating *Escherichia coli* O157:H7 in pure cell suspensions and on apples. M.S. thesis. Columbus, OH: The Ohio State University.
- Aguayo, E., Escalona, V.H., & Artés, F. (2006). Effect of cyclic exposure to ozone gas on physicochemical, sensorial and microbial quality of whole and sliced tomatoes. *Postharvest Biol. Tec.*, 39(2), 169-177.
- Al-qurashi, A.D. (2002). Establishment and early growth of blueberry cultivars in northern Mississippi, and postharvest studies. Ph.D. Dissertation. Starkville, MI: Mississippi State University.
- Allan-Wojtas, P.M., Forney, C.F., Carbyn, S.E., & Nicholas, K.U. (2001). Microstructural indicators of quality-related characteristics of blueberries: an integrated approach. *Lebensm. Wiss. Technol.*, 20: 23–32.
- AOAC. (1998). Official Method 997.02. Yeast and molds counts in foods. Dry rehydratable film method. Petrifilm method. Gaithersburg, MD: AOAC International.
- Bachmann, J., & Earles, R. (2000). Postharvest handling of fruits and vegetables. ATTRA.
- Baranovskaya, V.A., Zapolskii, O.B., Ovrutskaya, I.Y., Obodovskaya, N.N., Pschenichnaya, E.E., & Yushkevich, O.I. (1979). Use of ozone gas sterilization during storage of potatoes and vegetables. *Konservn. Ovoshchesus. Promst.*, 4, 10–12.
- Barger, W.R., J.S. Wiant, W.T. Pentzer, A.L. Ryall, & D.H. Dewey. (1948). A comparison of fungicidal treatments for the control of decay in California cantaloupes. *Phytopathology*, 38, 1019–1024.
- Barreiro, P., & Ruiz-Altisent, M. (2000). Instrumentación de la calidad en frutas y hortalizas frescas. *Horticultura*, 8, 14–20.
- Barrit, B.H., & Torre, L.C. (1980). Fruit firmness measurements in red raspberry. *HortScience*, 15, 38–39.
- Barth, M.M., Zhou, C., Mercier, M., & Payne, F.A. (1995). Ozone storage effects on anthocyanin content and fungal growth in blackberries. *J. Food Sci*, 60, 1286–1287.
- Basiouny, F.M., & Chen, Y. (1988). Effects of harvest date, maturity and storage interval on postharvest quality of rabbiteye blueberry (*Vaccinium ashei* Reade). *Proc. Fla. State. Hortic. Soc.*, 101, 281–284.

- Beaudry, R. M., Cameron, A.C., Shirazi, A., & Dostal-Lange, D.L. (1992). Modified atmosphere packaging of blueberry fruit: effect of temperature on package O₂ and CO₂. *J. Am. Soc. Hortic. Sci.*, 117, 436–441.
- Beaudry, R. M., Moggia, C. E., Retamales, J. B., & Hancock, J. F. (1998). Quality of 'Ivanhoe' and 'Bluecrop' blueberry fruit transported by air and sea from Chile to North America. *HortScience*, 33, 313–317.
- Bernstein, Z., & Lustig, I. (1981). A new method of firmness measurement of grape berries and other juicy fruits. *Vitis*, 20, 15–21.
- Beuchat, L. R., & Brackett, R. E. (1990). Survival and growth of *Listeria monocytogenes* on lettuce as influenced by shredding, chlorine treatment, modified atmosphere packaging and temperature. *J. Food Sci.*, 55(3), 755–758.
- Boffetta, P., E. Couto, J. Wichmann, P. Ferrari, D. Trichopoulos, H. B. Buenode Mesquita, F. J. van Duijnhoven, F. L. Buchner, T. Key, H. Boeing, et al. (2010). Fruit and vegetable intake and overall cancer risk in the European Prospective Investigation into Cancer and Nutrition (EPIC). *J. Natl. Cancer Inst.*, 102, 529–37.
- Bounous, G., Giacalone, G., Guarinoni, A., & Peano, C. (1997). Modified atmosphere storage of high bush blueberry. *Acta Hortic.*, 446, 197–203.
- Bourne, M.C. (1980). Textural evaluation of horticultural crops. *HortScience*, 15, 51–56.
- Brooks, R.I., & Csallany, A.S. (1978). Effects of air, ozone, and nitrogen dioxide exposure on the oxidation of corn and soybean lipids. *J. Agr. Food Chem*, 26, 1203–1209.
- Ceponis, M.J., & Cappellini, R.A. (1985). Reducing decay in fresh blueberries with controlled atmospheres. *HortScience*, 20, 228–229.
- Chiabrando, V., Giacalone, G., & Rolle, L. (2009). Mechanical behaviour and quality traits of highbush blueberry during postharvest storage. *J. Sci. Food Agr.*, 89(6), 989–992.
- Cline, W.O. (1996). Postharvest infection of highbush blueberries following contact with infested surfaces. *HortScience*, 31(6), 981–983.
- Daş, E., Gürakan, G. C., & Bayındırlı, A. (2006). Effect of controlled atmosphere storage, modified atmosphere packaging and gaseous ozone treatment on the survival of *Salmonella Enteritidis* on cherry tomatoes. *Food Microbiol.*, 23(5), 430–438.
- Dauchet, L., Amouyel, P., Hercberg, S., & Dallongeville, J. (2006). Fruit and vegetable consumption and risk of coronary heart disease: a meta-analysis of cohort studies. *J. Nutr.*, 136, 2588–93.

- Deng, Y., Wu, Y., & Li, Y. (2005). Effect of high O₂ levels on postharvest quality and shelf life of table grapes during long-term storage. *Eur. Food Res. Technol.*, 221, 392–397.
- Donahue, D.W., Benoit, P.W., Lagasse, B.J., & Buss, W.R. (2000). Consumer and instrumental evaluation of Maine wild blueberries for the fresh pack market. *Postharvest Biol. Technol.*, 19, 221–228.
- Doving, A., Mage, F., & Vestrheim, S. (2005). Methods for testing strawberry fruit firmness: a review. *Small Fruit Rev.*, 4:11–34.
- Doving, A., & Mage, F. (2002). Methods of testing strawberry fruit firmness. *Acta Agr. Scand. B-S P.*, 52, 43–51.
- Duprat, F., Grotte, M.G., Pietri, E., & Loonis, D. (1997). The acoustic impulse response method for measuring the overall firmness of fruit. *J. Agri. Eng. Res.*, 66, 251–259.
- Escriche, I., Serra, J. A., Gómez, M., & Galotto, M. J. (2001). Effect of ozone treatment and storage temperature on physicochemical properties of mushrooms (*Agaricus bisporus*). *Food Sci. Tech. Int.*, 7(3), 251–258.
- Fan, L., Forney, C. F., Jordan, M. A., Hildebrand, P. D., Kalt, W., Ryan, D. A. J., & Song, J. (2001). Effect of ozone treatment and controlled atmosphere storage on quality and phytochemicals in highbush blueberries (pp. 417-423). In *VIII International Controlled Atmosphere Research Conference 600*.
- Fan, X., Sokorai, K. J., Engemann, J., Gurtler, J. B. & Liu, Y. (2012). Inactivation of *Listeria innocua*, *Salmonella* Typhimurium, and *Escherichia coli* O157: H7 on surface and stem scar areas of tomatoes using in-package ozonation. *J. Food Prot.*, 75(9), 1611-1618.
- FDA. (1982). GRAS status of ozone. *Fed. Reg.*, 47, 50209–50210.
- Fonseca, S.C., Oliveira, F.A., & Brecht, J.K. (2002). Modelling respiration rate of fresh fruits and vegetables for modified atmosphere packages: a review. *J. Food Eng.*, 52(2), 99-119.
- Forney, C.F. (2008). Postharvest issues in blueberry and cranberry and methods to improve market-life (pp. 785-798). In: *IX International Vaccinium Symposium 810*.
- Forney, C.F., Kumudini, U.K.G.N., & Jordan, M.A. (1998). Effect of postharvest storage conditions on firmness of ‘Burlington’ blueberry fruit (pp. 227–232). In: *Proceedings of the 8th North American Blueberry Research and Extension Workers Conference*.
- Gane, R. (1936). The respiration of bananas in presence of ethylene. *New Phytol*, 36, 170–178.
- Garcia, J.M., Aguilera, C. & Albi, M. A. (1995). Postharvest heat treatment on Spanish strawberry (*Fragaria anassa* cv. Tudla). *J. Agric. Food Chem.*, 43, 1489-1492.

- Giongo, L., Poncetta, P., Loretto, P., & Costa, F. (2013). Texture profiling of blueberries (*Vaccinium* spp.) during fruit development, ripening and storage. *Postharvest Biol. Tec.*, 76, 34-39.
- Gorris, L., & Tauscher, B. (1999). Quality and safety aspects of novel minimal processing technology. In F. A. R. Oliveira, & J. C. Oliveira (Eds.), *Processing of foods: Quality optimization and process assessment* (pp. 325–339). Boca Raton, FL: CRC Press.
- Goto, T., Goto, M., Chachin, K., & Iwata, T. (1996). The mechanism of the increase of firmness in strawberry fruit treated with 100% CO₂. *Nippon Shokuhin Kagaku Kaishi*, 43, 1158-1162.
- Gunness, P., Kravchuk, O., Nottingham, S. M., D'Arcy, B. R., & Gidley, M. J. (2009). Sensory analysis of individual strawberry fruit and comparison with instrumental analysis. *Postharvest Biol. Tec.*, 52(2), 164-172.
- Hakkinen, S. H., & Torronen, A. R. (2000). Content of flavonols and selected phenolic acids in strawberries and *Vaccinium* species: influence of cultivar, cultivation site and technique. *Food Res. Int.*, 33(6), 517–524.
- Harb, J.Y., & Streif, J. (2004). Controlled atmosphere storage of highbush blueberries cv. 'Duke'. *Eur. J. Hort. Sci.*, 69, 66–72.
- Harker, F.R., Elgar, H.J., Watkins, C.B., Jackson, P.J., & Hallett, I.C. (2000). Physical and mechanical changes in strawberry fruit after high carbon dioxide treatments. *Postharvest Biol. Tec.*, 19, 139-146.
- Harker, F.R., Stec, M.G.H., Hallet, I.C., & Bennett, C.L. (1997). Texture of parenchymatous plant tissue: a comparison between tensile and other instrumental and sensory measurements of tissue strength and juiciness. *Postharvest Biol. Tec.*, 11, 63–72.
- International Agency for Research on Cancer. (2003). IARC Handbooks of Cancer Prevention. Vol 8. Fruit and Vegetables. Lyon, France: International Agency for Research on Cancer.
- Jackman, R.L., & Stanley, D.W. (1995). Perspectives in the textural evaluation on plant foods. *Food Sci. Technol.*, 6, 187–194.
- Jackson, E.D., Sanford, K.A., Lawrence, R.A., McRae, K.B., & Stark, R. (1999). Lowbush blueberry quality changes in response to prepacking, delays and holding temperatures. *Postharvest Biol. Tec.*, 15, 117-126.
- Jin, L., Xiaoyu, W., Honglin, Y., Zonggan, Y., Jiaxun, W., & Yaguang, L. (1989). Influence of discharge products on post-harvest physiology of fruit (pp. 1-4). In: *Proc. 6th International Symp. High Voltage Eng., New Orleans, LA, August*.
- Kader, A. A., Zagory, D., & Kerbel, E. L. (1989). Modified atmosphere packaging of fruits and vegetables. *Crit. Rev. Food Sci.*, 28, 1–30.

- Kader, A.A. (1993). Postharvest handling In: J. E. Preece & P. E. Read (Eds.), *The Biology of Horticulture: An Introductory Textbook* (pp. 353–377). New York, NY: Wiley.
- Khanizadeh, S., DeEll, J., & Cousineau, J. (2000). Comparison of three methods to evaluate fruit firmness in advanced strawberry selections. *Acta Hort.*, 517, 463–465.
- Khazaei J., & Mann, D.D. (2004). Effects of temperature and loading characteristics on mechanical and stress–relaxation properties of sea buckthorn berries. Part 1. Compression tests. *Agricultural Engineering International: CIGR Journal of Scientific Research and Development*. Liège, Belgium: International Commission of Agricultural Engineering.
- Kim, J. G., Yousef, A. E. & Dave, S. (1999). Application of ozone for enhancing the microbiological safety and quality of foods: a review. *J Food Protect.*, 62, 1071–1087.
- Kim, T. J., Silva, J. L., Tokitkla, A., & Matta F.B. (2010). Modified Atmosphere Storage Influences Quality Parameters and Shelf Life of 'Tifblue' Blueberries. *J Mississippi Academy of Sciences*, 4(55), 143–148.
- Larsen, M., & Watkins, C.B. (1995a). Firmness and concentration of acetaldehyde, ethyl acetate and ethanol in strawberries stored in controlled and modified atmospheres. *Postharvest. Biol. Tec.*, 5, 39–50.
- Larsen, M., & Watkins, C.B. (1995b). Firmness and aroma composition of strawberries following short-term high carbon dioxide treatments. *HortScience*, 30, 303–305.
- Liew, C. L., & Prange, R. K. (1994). Effect of ozone and storage temperature on postharvest diseases and physiology of carrots (*Daucus carota* L.). *J. Am. Soc. Hortic. Sci.*, 119(3), 563–567.
- Lila, M.A. (2004). Antocyanins and human health: an in vitro investigative approach. *J. Biomed. Biotechnol.*, 5, 306–313.
- Maguire, Y.P., & Solverg, M. (1980). Influence of atmospheric oxygen and ozone on ripening indices of normal (*Rin*) and ripening inhibited (*rin*) tomato cultivars. *J. Food Biochem.*, 4, 99–110.
- Mahajan, P.B., & Goswami, T.K. (2004). Extended storage life of litchi fruit using controlled atmosphere and low temperature. *J. Food Process. Preserv.*, 28, 388–403.
- Mallison, E.D., & Spalding, D.H. (1966). Use of ozone in tomato ripening rooms (p. 10). USDA ARS 52-17.
- Marshall, D.A., Spiers, J.M., & Stringer, S.J. (2008). Blueberry splitting tendencies as predicted by fruit firmness. *HortScience*, 43, 567–570.

Mencarelli, F., Massantini, R., Lanzarotta, L., & Rotondi, R. (1994). Accurate detection of firmness and color changes in the packing of table grapes with paper dividers. *J. Hortic Sci*, 69, 299–304.

Mitcham, E.J., & Mitchell, F. G. (2002). Postharvest handling systems: small fruits. II. Strawberries and cane berries. In: A. Kader (Ed.), *Postharvest Technology of Horticultural Crops, third ed. Publication, vol. 3311* (pp. 364– 368). Oakland, CA: University of California, Division of Agriculture and Natural Resources.

Ness, A. R., & Powles, J. W. (1997). Fruit and vegetables, and cardiovascular disease: a review. *Int. J. Epidemiol*, 26(1), 1–13.

Norton, J.S., Charig, A.J., & Demoranville, I.E. 1968. The effect of ozone on storage of cranberries. *Proc. Amer. Soc. Hort. Sci*, 93, 792–796.

Nunes, M.C.N., Emond, J.P., & Brecht, J.K. (2004). Quality curves for highbush blueberries as a function of the storage temperature. *Small Fruits Rev.*, 3(3-4), 423-440.

Ogawa, J.M., Feliciano, A.J., & Manji, B.T. (1990). Evaluation of ozone as a disinfectant in postharvest dump tank treatments for tomato. *Phytopathology*, 80, 1020.

Palou, L., Crisosto, C.H., Smilanick, J.L., Adaskaveg, J.E., & Zoffoli, J.P. (2002). Effects of continuous 0.3 ppm ozone exposure on decay development and physiological responses of peaches and tables grapes in cold storage. *Postharvest Biol. Tec.*, 24, 39–48.

Palou, L., Smilanick, J. L., Crisosto, C. H., & Mansour, M. (2001). Effect of gaseous ozone exposure on the development of green and blue molds on cold stored citrus fruit. *Plant dis.*, 85(6), 632-638.

Parish, M.E., Beuchat, L.R., Suslow, T.W., Harris, L.J., Garret, E.H., Farber, J.N., & Busta, F.F. (2003). Methods to reduce eliminate pathogens from fresh and fresh-cut produce. *Compr. Rev. Food Sci. F.*, 2(s1), 161–173.

Pelayo, C., Ebeler, S. E., & Kader, A. A. (2003). Postharvest life and flavor quality of three strawberry cultivars kept at 5° C in air or air+ 20 kPa CO₂. *Postharvest Biol. Tec.*, 27(2), 171-183.

Pérez, A.G., Sanz, C., Ríos, J.J., Olías, R., & Olías, J.M. (1999). Effects of ozone treatment on postharvest strawberry quality. *J. Agric. Food Chem.*, 47, 1652–1656.

Petri, E., Arroqui, C., Anogós, I., & Vírveda, P. (2008). Effect of Preservative Agents on the Respiration Rate of Minimally Processed Potato (*Solanum tuberosum* cv. Monalisa). *J. Food Sci.*, 73, C122-26.

- Prior, R. L., Cao, G., Martin, A., Sofic, E., McEwen, J., O'Brien, C., et al. (1998). Antioxidant capacity as influenced by total phenolics and anthocyanins content, maturity, and variety of vaccinium species. *J. Agric. Food Chem.*, 46, 2686–2693.
- Retamales, J., Manríquez, D., Castillo, P., & Defilippi, B. (2003). Controlled atmosphere in bing cherries from Chile and problems caused by quarantine treatments for export to Japan. *Acta Hort.*, 600, 149-153.
- Saltveit, M. E., Jr. (1997). A summary of CA and MA requirements and recommendations for harvested vegetables. In: M. E. Saltveit (Ed.), *Proceedings of the 7th international controlled atmosphere research conference: Vol. 4* (pp. 98–117). Davis, CA: Postharvest Horticulture Service.
- Sanford, K.A., Lidster, P.D., McRae, K.B., Jackson, E.D., Lawrence, R.A., Stark, R., & Prange, R.K. (1991). Lowbush blueberry quality changes in response to mechanical damage and storage temperature. *J. Am. Soc. Hort. Sci.*, 116, 47–51.
- Sargent, S.A., Brecht, J.K., & Forney, C.F. (2006). Blueberry harvest and postharvest operations: quality maintenance and food safety. In: N.F. Childers & P.M. Lyrene (eds.), *Blueberries for Growers, Gardeners, Promoters* (pp. 139-151). DeLeon Springs, FL: E.O. Painter Printing Co.
- Schomer, H.A., & McCulloch, L.P. (1948). Ozone in relation to storage of apples. *U.S. Dept. of Agr. Circ.*, 765.
- Schotsmans, W., Molan, A., & MacKay, B. (2007). Controlled atmosphere storage of rabbiteye blueberries enhances postharvest quality aspects. *Postharvest Biol Tec.*, 44(3), 277-285.
- Silva, J.L., Marroquin, E., Matta, F.B., Garner, J.O.J., & Stojanovic, J. (2005). Physicochemical carbohydrate and sensory characteristics of highbush and rabbiteye blueberry cultivars. *J. Sci. Food Agric.*, 85, 1815–1821.
- Singh, N., Singh, R.K., Bhunia, A.K., & Stroshine, R.L. (2002). Efficacy of chlorine dioxide, ozone, and thyme essential oil or a sequential washing in killing *Escherichia coli* O157:H7 on lettuce and baby carrots. *Lebensm. Wiss. Technol.*, 35, 720–729.
- Skog, L.J., & Chu, C.L. (2000). Ozone technology for shelf life extension of fruits and vegetables. In: R. Ben-Aire & S. Philosoph-Hadas (Eds.), *Proceedings of the Fourth Conference On Postharvest*, vol. II, *Acta Hort.*, 553, ISHS, 285–291.
- Smagula, J.M., Litten, W., Chen, Y., & Dunham, S. (1996). Variation of fruit set and fruit characteristics of wild lowbush blueberries (*Vaccinium angustifolium* Ait.) in a managed field. *Acta Hort.*, 446, 109–115.
- Smith, R.B., & Skog, L.J. (1992). Postharvest carbon dioxide treatment enhances firmness of several cultivars of strawberry. *HortScience*, 27, 420-421.

Spalding, D.H. (1968). Effects of ozone atmospheres on spoilage of fruits and vegetables after harvest. *U.S. Dept. of Agr. Mktg. Res. Rpt*, 801.

Swift, J. E. (2010). Effects of Frozen Storage and Harvest Time on the Textural and Sensory Properties of Rabbiteye Blueberries (*Vaccinium virgatum* Aiton). MSc. Thesis. Raleigh, NC: NCSU.

Ueda, Y., & Bai, J. (1993). Effect of short term exposure of elevated CO₂ on flesh firmness and ester production of strawberries. *J. Jpn. Soc. Hortic. Sci.*, 62, 457-464.

US Department of Agriculture. (2013). Non-citrus fruit and nuts 2012 preliminary summary. Washington, DC: National Agricultural Statistics Service.

US Department of Agriculture. (2012). Non-citrus fruits and nuts 2011 summary. Washington, DC: National Agricultural Statistics Service.

Wang, Y., Chang, C.F., Chou, J. Chen, H.L., Deng, X., Harvey, B.K., Cadet, J.L. & Bickford, P.C. (2005). Dietary supplementation with blueberries, spinach, or spirulina reduces ischemic brain damage. *Exp. Neurol*, 193(1), 75-84.

Watkins, C.B., Manzano-Mendez, J.E., Nock, J.F., Zhang, J., & Maloney, K.E. (1999). Cultivar variation in response of strawberry fruit to high carbon dioxide treatments. *J. Sci. Food Agric.*, 79, 886-890.

White, G.C. (1992). Ozone. In: *Handbook of chlorination and alternative disinfectants (3rd ed.)* (pp. 1046 - 1110). New York, NY: Van Nostrand Reinhold.

World Cancer Research Fund/American Institute for Cancer Research. (2007). Food, Nutrition, Physical Activity, and the Prevention of Cancer: A Global Perspective. Washington, DC: American Institute for Cancer Research.

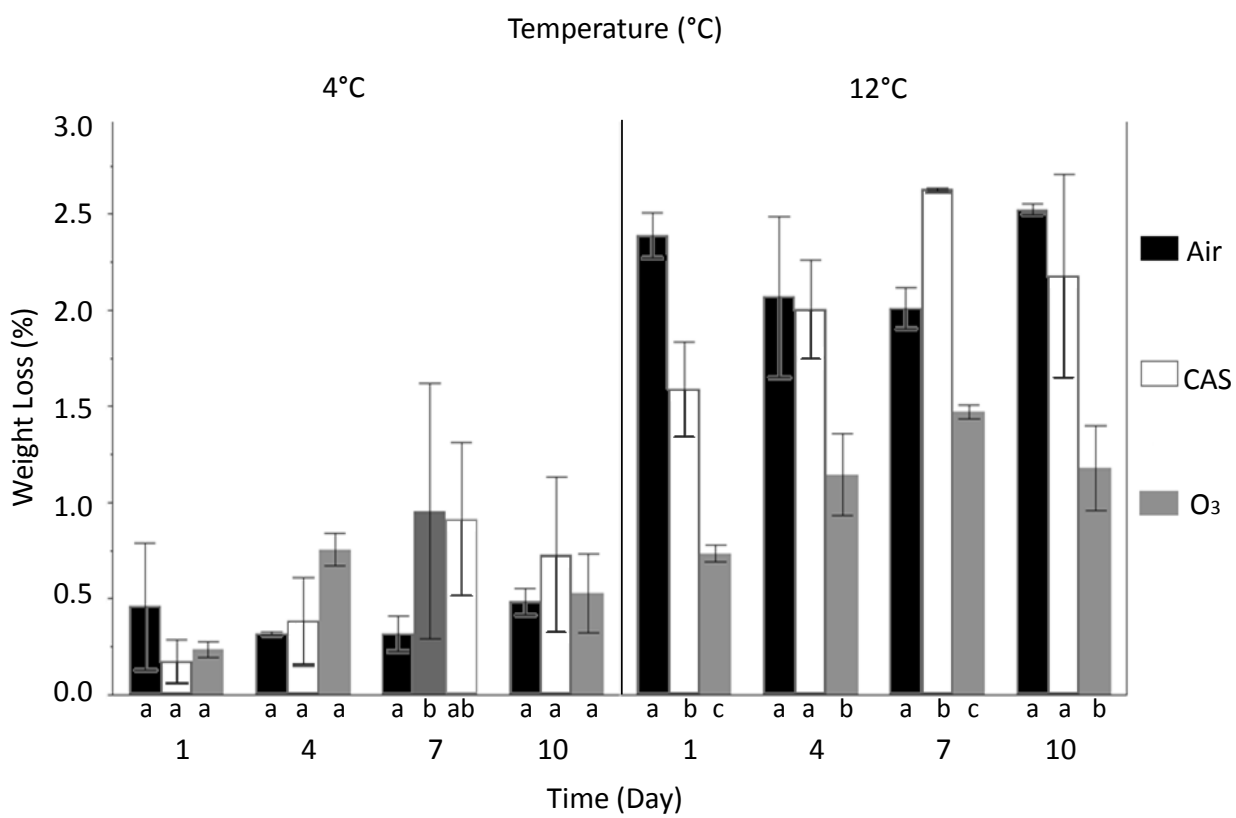


Figure 1. Weight loss percentage of highbush blueberries stored at 4°C or 12°C under controlled atmosphere storage conditions (CAS), Ozone (O₃) or regular atmosphere (Air) during 10 days.

Columns within each day at same temperature followed by the same lower case letter are not significantly different ($P>0.05$) from each other.

Table 1. Yeast counts (Log CFU/g) and increase over 10 days (Δ) of highbush blueberries stored at 4°C or 12°C under controlled atmosphere storage conditions (CAS), Ozone (O₃) or regular atmosphere (Air).

Treatment	4°C			12°C		
	Day 0	Day 10	Δ	Day 0	Day 10	Δ
Air	3.01±0.10	3.95±0.27	0.94	3.01±0.10	3.93±0.15	0.92
CAS	5.43±0.05	7.30±0.10	1.87	3.83±0.30	6.42±0.03	2.59
O ₃	5.43±0.05	7.29±0.14	1.86	3.77±0.36	6.14±0.06	2.37

Table 2. Mold counts (Log CFU/g) and increase over 10 days (Δ) of highbush blueberries stored at 4 °C or 12°C under controlled atmosphere storage conditions (CAS), Ozone (O₃) or regular atmosphere (Air).

Treatment	4°C			12°C		
	Day 0	Day 10	Δ	Day 0	Day 10	Δ
Air	<1.00*	3.74	2.74	<1.00*	3.75	2.75
CAS	2.85	4.95	2.10	2.24	2.60	0.20
O ₃	2.85	4.74**	1.89	2.24	3.39	1.15

* No growth at limit of detection.

** Mold counts at Day 7 used, because of no growth at Day 10 due to high limit of detection.

Table 3. Firmness penetration peak force (g) of highbush blueberries stored at 4°C or 12°C under controlled atmosphere storage conditions (CAS), Ozone (O₃) or regular atmosphere (Air) during 10 days.

Firmness Penetration Peak Force (g)					
Temperature 4°C					
Treatment	Day 0	Day 1	Day 4	Day 7	Day 10
Air	178.0±42.5 ^{Aa}	138.8±23.7 ^{Ba}	180.8±17.5 ^{Aa}	195.3±46.3 ^{Aa}	185.6±34.3 ^{Aa}
CAS	159.2±24.7 ^{CDa}	183.0±26.0 ^{ABb}	140.5±20.9 ^{Db}	167.8±36.2 ^{BCa}	194.4±44.2 ^{Aa}
O ₃	159.2±24.7 ^{Aa}	169.0±26.6 ^{Ab}	170.1±33.8 ^{Aa}	176.1±39.1 ^{Aa}	173.4±51.7 ^{Aa}
Temperature 12°C					
Treatment	Day 0	Day 1	Day 4	Day 7	Day 10
Air	178.0±42.5 ^{Aa}	167.6±23.3 ^{Aa}	192.0±17.5 ^{Aa}	179.5±23.4 ^{Aa}	219.8±57.3 ^{Ba}
CAS	196.0±28.9 ^{Aab}	150.1±26.8 ^{Bb}	138.5±21.9 ^{Bb}	146.3±18.4 ^{Bb}	182.7±35.5 ^{Ab}
O ₃	202.8±24.8 ^{Ab}	146.1±15.1 ^{Bb}	153.1±15.6 ^{Bc}	143.5±16.1 ^{Bb}	169.2±26.6 ^{Cb}

Means within each row followed by the same capital letter are not significantly different (P>0.05) from each other. Means within each column followed by the same lower case letter are not significantly different (P>0.05) from each other.

CHAPTER 5

Survival of *Listeria monocytogenes* on fresh blueberries
(*Vaccinium corymbosum*) under controlled atmosphere storage

ABSTRACT

Listeria monocytogenes is a foodborne pathogen that represents a high risk for consumers, since it can grow under refrigeration conditions and can also develop acid tolerance. Fresh blueberries are hand-picked, packed and transported under refrigeration without receiving a microbial inactivation treatment.

The aim of this work was to study the survival of *L. monocytogenes* in fresh highbush blueberries stored at 4°C or 12°C under different controlled atmosphere conditions, including air (control); 5% O₂ and 15% CO₂, 80% N₂ (CAS); or ozone gas (O₃) 4ppm at 4°C or 2.5ppm at 12°C, at high relative humidity (90-95%) for a total of 10 days.

Fresh blueberries inside a plastic clamshell were spot inoculated with the bacteria and were stored at 4°C or 12°C in isolated cabinets under air, CAS, and O₃ atmospheric conditions. Samples were evaluated on day 0, 1, 4, 7, and 10 for microbial growth using modified oxford agar.

Controlled atmosphere storage did not delay or inhibit *Listeria monocytogenes* growth in fresh blueberries after 10 days. Gaseous ozone achieved 3 log and 2 log reductions when compared with air treatment at 4°C and 12°C, respectively. Low concentrations of ozone gas together with proper refrigeration temperature can ensure product safety throughout transportation. Ozone is a strong antimicrobial that safely decomposes to oxygen and water without leaving residues and is an alternative to prevent bacterial growth during a long transport period.

Keywords: *Listeria monocytogenes*, spot inoculation, blueberries, controlled atmosphere, ozone

1. Introduction

Over the past several years there has been growing consumer interest in minimally processed fruits and vegetables for their freshness, convenience, and healthy attributes. The US per capita food availability of blueberries increased from 0.766 lbs (fresh 0.265 lbs and frozen 0.501 lbs) in 1994 to 1.708 lbs (fresh 1.115 lbs and frozen 0.593 lbs) in 2010 (USDA/Economic Research Service, 2012). Although the US is the world biggest blueberry producer, the American market is so large that it requires importing the fruit from other countries. The nation's imports of blueberries increased 12 percent from the previous year to nearly \$419.8 million in 2012 (USDA, 2012). In 2012 Chile provided more than 50% of the imported blueberries sold in the country (USDA, 2012). Chile is projected to export in the 2012/13 season around 85,000 tons of fresh blueberries, which represents 21 percent more compared to last season (Plattner et al., 2012). Transportation of blueberries from Valparaíso, Chile to Wilmington, DE can take 20 days in sea freight shipping containers (Beaudry et al., 1998). Controlled atmosphere is widely used for fresh produce and also during transportation of blueberries (Bañados, 2006). CO₂ gas in a controlled atmosphere may have significant and direct antimicrobial activity due to alteration of cell membrane function (Farber, 1991). However researchers have found that limiting O₂ concentrations and including high CO₂ concentrations in controlled atmosphere storage conditions do not assure foodborne bacteria inhibition (Daş et al., 2006).

Pathogenic bacteria, including *Listeria monocytogenes*, become a threat in products that are not protected from, or properly treated to reduce, contamination. *L. monocytogenes* is commonly associated with plant matter and soil and it may be more common in those fruits and vegetables grown in close association with the soil (Brackett, 1999). It is a facultative anaerobe that can grow with or without oxygen as well as grow under refrigeration conditions and can

develop an acid tolerance response (Davis et al., 1996). Fresh blueberries are minimally processed after harvest and stored refrigerated for several days, without any previous microbial inhibition treatment like washing. Although *L. monocytogenes* has been mainly linked to dairy, meat and seafood products contamination, currently it has been associated with fresh produce outbreaks (Cosgrove et al., 2011) and therefore, there is concern about its behavior under controlled atmosphere conditions.

In 2001 the U.S. Food and Drug Administration approved ozone for the treatment of raw agricultural commodities such as fresh fruits and vegetables (Federal Register, 2001). Gaseous ozone (O₃) can be used during controlled atmosphere storage to protect fruit from foodborne bacteria, since studies have demonstrated that it is highly effective reducing different bacteria including *Listeria monocytogenes* in orange juice (Patil et al., 2010) *Listeria innocua* in tomatoes (Fan et al., 2012), *Salmonella* spp. and *Escherichia coli* O157:H7 in blueberries (Bialka & Demirci, 2007) and *Salmonella* Enteritidis in tomatoes (Daş et al., 2006). The oxidation potential of chlorine dioxide is 1.50, while ozone is 2.07 (Manley & Niegowski, 1967), the latter having a greater effect against certain microorganisms while decomposing rapidly into oxygen without leaving residues (White, 1992).

There are no previous reports on the growth or survival of *L. monocytogenes* inoculated onto blueberries stored under controlled atmosphere conditions and gaseous ozone. The present study will evaluate the behavior of *L. monocytogenes* in an acidic food product, such as fresh blueberries packaged under refrigerated controlled atmosphere conditions, to estimate if bacterial occurrence, survival and growth in these environments may represent a potential increase in health hazard for consumers. Also, the effectiveness of ozone as an antimicrobial to protect fruit during transportation will be evaluated.

2. Materials and Methods

2.1 Inocula Preparation

The US Food and Drug Administration (FDA-CFSAN), provided the bacterial strains of *Listeria monocytogenes* from the 2011 cantaloupe outbreak (Jensen Farms, CO). Strains were adapted to grow in the presence of nalidixic acid (50 µg/mL). Cultures were stored at -18°C in trypticase soy broth (TSB) with 1% glycerol as cryoprotectant (Virginia Tech Food Science and Technology Culture Collection).

Bacterial propagation occurred at 37°C for 24 h in trypticase soy broth (BBL™, BD Diagnostics, Sparks, MD) with 0.6% yeast extract (Acros Organics, Fair Lawn, NJ) (TSB+YE) supplemented with nalidixic acid (50 µg/mL). After propagation, the culture was centrifuged (Sorvall Legend RT+ centrifuge, Thermo Scientific, Braunschweig, Germany) at 2000xg for 10 min. After centrifugation, the supernatant was discarded and replaced with 10mL of 0.1% peptone water. Solutions were vortexed (Fisher Mini-Shaker Model 58, Fisher Scientific, Pittsburgh, PA) and centrifuged again at 2000xg for 10 min. Finally, the supernatant was discarded and the remaining pellet was dissolved in 10mL 0.1% peptone water to a final concentration of 10⁹ CFU/mL. Samples of the cocktail were plated onto Modified Oxford Agar supplemented with nalidixic acid (50 µg/mL) (MOXN, Difco, Becton Dickinson, Sparks, MD) to verify initial inocula levels.

2.2 Fruit Samples

Highbush blueberries (*Vaccinium corymbosum* L.) were purchased from a local retailer distributor in Blacksburg, VA. Two replications were conducted each consisting of fruit obtained

different dates. Once received only fully colored and non damaged fruit was used for the experiment.

2.3 Fruit Inoculation Procedure

Blueberries were sorted and only fully developed fruit free of visible defects were selected on a visual basis and stored in a 4.4 oz PET retail clamshell box (Highland Corporation, Mulberry, FL). Blueberries for inoculation were placed in single layers and each layer had 8 berries facing their stem scar up. Each blueberry was individually spot inoculated with 15 μ l of the prepared *L. monocytogenes* cocktail on the stem scar surface of the fruit using a multichannel micropipette. Blueberries were then held at room temperature for 1 h under a biological laminar flow safety cabinet (Nu Aire, Inc., Minneapolis, MN) with the fan running to facilitate drying.

2.4 Fruit Incubation

After drying, five inoculated blueberries were placed in clamshells containing 45 uninoculated berries for total of 50 blueberries per sample clamshell. This protocol was adopted after performing a preliminary inoculation study. Since the amount of inoculum per sample was 75 μ l, the estimated initial inoculum per sample was approximately 10^7 CFU/mL. Clamshells were placed in the controlled atmosphere chamber and incubated at different temperatures (4°C or 12°C), and one of three atmospheres (air as a control (21% O₂ and 0.03% CO₂); 5% O₂: 15% CO₂:80% N₂; or O₃). Berries were sampled on day 0, 1, 4, 7, and 10.

2.5 Controlled Atmosphere and Ozone Treatment

Clear acrylic desiccator cabinets (12"W x 12"H x 12"D exterior) with gas ports including hygrometer (Cole-Parmer, Lansing, MI, USA) were used to store fruit in clamshells, inside an incubator (Precision Incubator, Thermo Scientific, Waltham, MA). The incubator was held at 4°C or 12°C and relative humidity (RH) inside cabinets was 90-95%. Temperature and RH were monitored by a sensor (Traceable Jumbo Thermo-Humidity Meter, Fisher Scientific, Pittsburgh, PA).

For controlled atmosphere storage a certified standard-spec gas tank was used (MID-Saint Louis SGL (SAP)-MO). Compressed gas was composed by 5% O₂, 15% CO₂ and balanced nitrogen (80%). Gas concentrations were monitored two times a day (morning and late afternoon) using an O₂/CO₂ gas analyzer (PBI Dansensor O₂/CO₂ Check Point, PBI-Dansensor, Ringsted, Denmark). When gas concentrations experienced a $\geq 3\%$ reduction, cabinets were refushed.

A corona discharge ozone generator (FreshFridge 2.0 Refrigerator Air Purifier Model GH 2138, IonCare G&H Industrial Ltd., China) and a small air circulating fan were installed in the chambers. Ozone concentration in chambers was monitored, controlled and recorded continuously using an ozone analyzer (Model ES-600, Ozone Solutions Inc., IA) and a Dell Latitude D830 computer as data logger (Dell, Round Rock, TX). Ozone concentration mean and standard deviation values were 4.0 ± 1.8 ppm for 4°C and 2.5 ± 1.5 ppm at 12°C. A third chamber without an ozone generator served as the control (Air). A schematic of the experiment equipment connections can be observed in Figure 1. Blueberries were exposed to controlled atmosphere and ozone in these chambers for a total of 10 days.

2.6 *L. monocytogenes* Recovery from Blueberries

Blueberries were transferred to a sterile 8 oz holding cup (Snap-Seal™ Container, Corning Inc., Corning, NY) and rinsed in 100 mL of 0.1% peptone water. Cups were agitated in an orbital shaker (New Brunswick Scientific, Controlled Environment Incubator Shaker, PsycroTherm, Edison, NJ) for 150 RPM for 2 min and then sonicated at 72 Joules (15 Watts) (Ultrasonic Processor Model CV18, Cole Parmer, Vernon Hills, IL) for a total of 10 seconds, in 3 intervals of 5 seconds with a pause in between (1:1:1). Subsequent dilutions of the rinsing solution were made using 0.1% peptone water blanks and the samples were plated onto MOXN using a SpiralPlate Biotech Autoplate 4000 (Spiral Biotech Inc., Bethesda, MD, USA). Presumptive *L. monocytogenes* counts were manually determined after incubation (37°C for 48 h) using a colony counter (Darkfield Quebec Colony Counter, American Optical Company, Buffalo, NY) and following the Autoplate 4000 User Guide procedure.

2.7 Experimental Design and Statistical Analysis

Data were analyzed using the Standard Least Square procedure of JMP Pro 10 (SAS Institute Inc, Cary, NC). The randomized complete block factorial design with two replications was utilized to test the treatments, temperatures, time and their interactions on *Listeria monocytogenes* counts (CFU/mL). All the tests used two samples for each treatment (Air, CAS and O₃) at temperature (4°C and 12°C). If the interactions between treatments were not significant ($P > 0.05$), the main effects of the treatments were separated by the Tukey Honestly Significant Difference test using the interaction as the error term.

3. Results and Discussion

Figure 2 shows the growth curves of *L. monocytogenes* in fresh blueberries under different temperatures and atmosphere conditions. Spot inoculation was used since it permits the application of a known amount of bacteria onto blueberry surfaces regardless of blueberry size and also allows more accurate calculation of the reductions in the number of viable cells caused by sanitation treatment (Han et al., 2004; Sy et al., 2005). Nalidixic acid was used to suppress the growth of the background microbial populations found on uninoculated blueberries (Knudsen et al., 2001; Flessa et al. 2005).

Bacteria were able to survive during the 10 days of storage demonstrating its resiliency to refrigeration temperatures and even to low pH conditions. Caggia et al. (2009) observed that acid-adapted *L. monocytogenes* was able to grow into minimally processed orange slices within 6 days of storage at 4 and 25°C with initial inoculation rates of 5–7 log (CFU/mL). Moreover, an approximate initial inoculum of 7.7 log (CFU/sample) of *L. monocytogenes* cocktail survived on the surface of whole strawberry samples experiencing a reduction of 3 log (CFU/sample) of viable organisms after 7 days of storage at 4°C (Flessa et al., 2005).

The initial counts on Day 0 showed no significant difference among treatments at 4°C nor at 12°C, indicating a homogenous inoculation procedure for all fruit (Table 1). After day 0, storage in air resulted in higher enumerated populations when compared per sampling day to CAS and O₃. However, CAS and air showed no significant difference ($P>0.05$) at 4°C on day 0, 1 and 4. CAS showed significant difference achieving 5.36 log (CFU/mL) when compared to air treatment 4.83 log (CFU/mL) by day 10 at 4°C, however no difference was observed at 12°C.

CAS was less effective inhibiting *L. monocytogenes* at 12°C when compared to O₃, this could be because this pathogen as a facultative anaerobe pathogen can survive under low oxygen

conditions. Berrang et al. (1989) agrees that although CAS extends the quality and shelf life of vegetables for a longer period of time, it does not affect *L. monocytogenes* growth. They investigated fresh asparagus, broccoli and cauliflower that were inoculated with the foodborne bacteria and stored under CAS and air at 4°C or 15°C for a 21 and 10 day period, respectively. When incubated at 15°C all vegetables showed an increase of the bacteria of 3 log (CFU/g) in asparagus (after 6 days), broccoli (after 10 days) and cauliflower (after 8 days).

L. monocytogenes inoculated on the surface of the blueberries was very sensitive to ozone treatments. Bacterial recovery in O₃ by day 10 at 4°C (2.27 log CFU/mL) showed a significant difference ($P < 0.05$) with air (5.36 log CFU/mL) achieving 3 log reduction when compared. Furthermore, by day 10 *L. monocytogenes* counts from O₃ at 12°C (3.14 log CFU/mL) was also significantly different than air (5.15 log CFU/mL), but achieving a 2 log reduction. Although the same ozone generator equipment was used for the trials at 4°C and 12°C, the mean gas concentrations were 4ppm and 2.5ppm, respectively. This is in agreement with Liew and Prange (1994) who observed that residual ozone concentration is less at higher storage temperatures. The higher concentration of ozone at 4°C could explain the lower counts *L. monocytogenes* (2.27 log CFU/mL), when compared to 3.14 CFU/mL that was observed at 12°C (Table 1), however there are no significant differences ($P > 0.05$) between these two values.

Fan et al. (2012) inoculated tomatoes on their stem scar with *Listeria innocua* and achieved a 4 log reduction CFU/fruit by packing the fruit in a bag with an ozone concentration of 1,000 ppm for 1 min. Moreover, they observed that after a 22 days post treatment storage period there were not any negative effects on fruit texture and color. Bialka and Demirci (2007) accomplished a 3 log reduction CFU/g of *Salmonella enterica* on highbush blueberries using a pressurized ozone concentration of 5% wt/wt (equivalent to 50,000 ppm) for 64 min, however

this treatment affected the sensory properties resulting in darker berries. No changes in color and appearance of blueberries were observed in the present study.

Panglioli and Hung, (2013) developed an innovative washing technology using ozonated water to reduce *E.coli* O157:H7 on surface spot inoculated blueberries. Water with a concentration of 1.5 ppm ozone inhibited *E. coli* O157:H7 by 3.5 log CFU/g after 5 min which was significantly higher than reduction from using tap water. In the present study a washing step was discarded since the recommendation for high value fresh fruit is to harvest them immediately into retail containers to avoid bruising, with no previous washing step to avoid moisture increase and mold spoilage (Bower, 2007). Moreover, a washing step will remove the natural light waxy “bloom” covering the blueberries surface, not only causing a negative aesthetic quality attribute (Gomez-Rodas et al., 2010), but also decreasing storage life since fruit will more likely to grow yeast and molds (Bratsch & Pattison, 2009). Further, the time required to reduce ozone concentration to half its value (half-life time) in gaseous states is longer than when dissolved in pure water (Khadre et al., 2001; Graham, 1997).

The use of chlorinated water sprays at permitted concentrations in blueberry processing has been studied, resulting in a mean population reduction of <1.25 log with an average residence time of 60 s (Crowe et al., 2005). Although chlorine is a disinfectant that has been historically preferred in the produce industry, regulatory agencies have questioned its use because of findings on toxic by-products production, such as trihalomethanes and haloacetic acids production, which are acknowledged mutagenic and carcinogenic compounds (Bellar et al., 1974; Trussell & Umphres, 1978), while ozone does not leave residues.

Blueberries have a pH of approximately 3.7 when fresh and 3.1- 3.35 when frozen (McGlynn, 2000), while the calculated surface pH of fresh blueberries in this study was 3.83

(data not shown). Although published reports indicate that minimum pH for growth in laboratory conditions is pH 4.4 to 4.5 (Parish & Higgins, 1989; Sorrells et al., 1989), and from 5.0 to 5.5 when pH of the media is adjusted with lactic acid (Farber et al., 1998), numerous studies have determined *L. monocytogenes* ability to respond and to survive at even lower pH values (O'Driscoll et al., 1997, Davis et al., 1996; Kroll & Patchett, 1992).

The present study provides information for commercial applications of gas ozone in controlled atmosphere conditions to achieve desirable reductions of microorganisms. Blueberries that are stored and transported under refrigeration conditions can include ozone ranging in concentration from 2.5 - 4 ppm that can reduce the pathogenic bacteria *Listeria monocytogenes* by 2-3 log reductions while not affecting the visible quality of the blueberries.

This research confirms that *Listeria monocytogenes* can survive in low oxygen atmospheres, acid-stress and refrigeration conditions in fresh blueberries. Ozone gas can be used in low concentrations to prevent microbial contamination. However, fresh blueberries should be considered a low risk for listeriosis, because of their inability to support the growth of *L. monocytogenes*.

Acknowledgements

The author wishes to thank Dr. Renee Boyer at Virginia Tech for her help to get the bacterial strains of *Listeria monocytogenes* from the 2011 cantaloupe outbreak (Jensen Farms, CO). This publication is the result of research sponsored by the Virginia Tech Department of Food Science and Technology.

References

- Bañados, M. P. (2006). Blueberry production in South America. *Acta Hort.*, 715, 165-172.
- Beaudry, R. M., Moggia, C. E., Retamales, J. B., & Hancock, J. F. (1998). Quality of 'Ivanhoe' and 'Bluecrop' blueberry fruit transported by air and sea from Chile to North America. *HortScience*, 33, 313–317.
- Bellar, T. A., Lichtenberg, J. J., & Kroner, R. C. (1974). The occurrence of organohalides in chlorinated drinking water. *J. Am. Water Works Assoc.*, 66, 703–706.
- Berrang, M. E., Brackett, R. E., & Beuchat, L. R. (1989). Growth of *Listeria monocytogenes* on fresh vegetables stored under controlled atmosphere. *J. Food Protect.*, 52(10), 702-705.
- Bialka, K. L., & Demirci, A. (2007). Decontamination of *Escherichia coli* O157:H7 and *Salmonella enterica* on blueberries using ozone and pulsed UV-light. *J. Food Sci.*, 72, M391–M396.
- Bower, C. (2007). Postharvest handling, storage, and treatment of fresh market berries. In: Y. Zhao (Eds.), *Berry fruit: value-added products for health promotion* (pp. 262-284). New York, NY: CRC press.
- Brackett, R. E. (1999). Incidence and behavior of *Listeria monocytogenes* in products of plant origin. In: E.T. Ryser & E.H. Marth (Eds.), *Listeria, Listeriosis, and Food Safety, second ed.* (pp. 631–655). New York, NY: Marcel Dekker, Inc.
- Bratsch, A., & Pattison, J. (2009). Specialty crop profile: Blueberries. Publication 438-103. Virginia Cooperative Extension.
- Caggia, C., Scifo, G. O., Restuccia, C., & Randazzo, C. L. (2009). Growth of acid-adapted *Listeria monocytogenes* in orange juice and in minimally processed orange slices. *Food Control*, 20, 59-66.
- Cosgrove, S., Cronquist, A., Wright, G., Ghosh, T., & Vogt, R. (2011). Multistate outbreak of listeriosis associated with Jensen Farms cantaloupe—United States, August–September 2011. *MMWR Morb. Mortal. Wkly. Rep.*, 60, 1357-1358.
- Crowe, K. M., Bushway, A. A., & Bushway, R. J. (2005). Effects of alternative postharvest treatments on the microbiological quality of lowbush blueberries. *Small Fruits Rev.*, 4(3), 29-39.
- Daş, E., Gürakan, G. C., & Bayındırlı, A. (2006). Effect of controlled atmosphere storage, modified atmosphere packaging and gaseous ozone treatment on the survival of *Salmonella Enteritidis* on cherry tomatoes. *Food Microbiol.*, 23(5), 430-438.

Davis, M. J., Coote, P. J., & O'Byrne, C. P. (1996). Acid tolerance in *Listeria monocytogenes*: The adaptive acid tolerance response (ATR) and growth-phase-dependent acid resistance. *Microbiology.*, 142, 2975–2982.

Fan, X., Sokorai, K. J., Engemann, J., Gurtler, J. B., & Liu, Y. (2012). Inactivation of *Listeria innocua*, *Salmonella Typhimurium*, and *Escherichia coli* O157: H7 on surface and stem scar areas of tomatoes using in-package ozonation. *J. Food Protect.*, 75(9), 1611-1618.

Farber, J.M. (1991). Microbiological aspects of modified-atmosphere packaging – a review. *J Food Prot.*, 54, 58–70.

Farber, J. M., Wang, S. L., Cai, Y., & Zhang, S. (1998). Changes in population of *Listeria monocytogenes* inoculated on packaged fresh-cut vegetables. *J. Food Prot*, 61, 192-195.

Federal Register. (2001). Secondary direct food additives permitted in food for human consumption, final rule. *Fed. Reg.*, 66, 33829-33830.

Flessa, S., Lusk, D. M., & Harris, L. J. (2005). Survival of *Listeria monocytogenes* on fresh and frozen strawberries. *Int. J. Food Microbiol.*, 101, 255-262.

Gomez-Rodas, A., Bourquin, L., García-Salazar, C., Varela-Gómez, A., & Wise, J. C. (2010). Good Agricultural Practices for Food Safety in Blueberry Production: Basic Principles. Ottawa, MI: Michigan State University Extension Central Region.

Graham, D.M. (1997). Use of ozone for food processing. *Food Technol.*, 51, 72-75.

Han, Y., Selby, T. L., Schultze, K. K., Nelson, P. E., & Linton, R. H. (2004). Decontamination of strawberries using batch and continuous chlorine dioxide gas treatments. *J. Food Protect.*, 67(11), 2450-2455.

Khadre, M. A., Yousef, A. E., & Kim, J. (2001). Microbiological aspects of ozone applications in food: a review. *J. Food Sci.*, 6, 1242–52.

Knudsen, D. M., Yamamoto, S. A. & Harris, L. J. (2001). Survival of *Salmonella* spp. and *E. coli* O157:H7 on fresh and frozen strawberries. *J. Food Protect.*, 64, 1483–1488.

Kroll, R. G., & Patchett, R. A. (1992). Induced acid tolerance in *Listeria monocytogenes*. *Lett. Appl. Microbiol.*, 14, 224-227.

Liew, C. L., & Prange, R. K. (1994). Effect of ozone and storage temperature on postharvest diseases and physiology of carrots (*Daucus carota* L.). *J. Amer. Soc. Hort. Sci.*, 119(3), 563-567.

Manley, T. C., & Niegowski, S. J. (1967). Ozone. In: Encyclopedia of chemical technology. Vol. 14, 2nd ed. (pp. 410–432). New York, NY: Wiley.

McGlynn, W. (2000). The Importance of Food pH in Commercial Canning Operations. Division of Agricultural Sciences and Natural Resources. Oklahoma State University Cooperative Extension Service. Accessed on 19 December 2012

<http://pods.dasnr.okstate.edu/docushare/dsweb/Get/Document-603/FAPC-118pod.pdf>.

O'Driscoll, B., Gahan, C., & Hill, C. (1997). Two-dimensional polyacrylamide gel electrophoresis analysis of the acid tolerance response in *Listeria monocytogenes* LO28. *Appl. Environ. Microbiol.*, 63 (7), 2679-2685.

Pangloli, P., & Hung, Y. C. (2013). Reducing microbiological safety risk on blueberries through innovative washing technologies. *Food Control*, 32, 621-625.

Parish, M.E. & Higgins, D.P. (1989). Survival of *Listeria monocytogenes* in low pH model broth systems. *J. Food Protect.*, 52, 144-147.

Patil, S., Valdramidis, V. P., Cullen, P. J., Frias, J. M., and Bourke, P. (2010). Ozone inactivation of acid stressed *Listeria monocytogenes* and *Listeria innocua* in orange juice using a bubble column. *Food Control*, 21, 1723–1730.

Plattner, K., Perez, A., & Warm, D. W. R. U. (2012). Fruit and Tree Nuts Outlook. USDA/ERS Report. FTS-354.

Sorrells, K. M., Enigl, D. C., & Hatfield, J. R. (1989). Effect of pH, acidulant, time, and temperature on the growth and survival of *Listeria monocytogenes*. *J. Food Protect.*, 52, 571-573.

Sy, K. V., McWatters, K. H., & Beuchat, L. R. (2005). Efficacy of gaseous chlorine dioxide as a sanitizer for killing *Salmonella*, yeasts, and molds on blueberries, strawberries, and raspberries. *J. Food Protect.*, 68(6), 1165-1175.

Trussell, R.R., & Umphres, M. D. (1978). The formation of trihalomethanes. *J. Amer. Water Works Assoc.*, 70, 604–612.

US Department of Agriculture. (2012). Non-citrus fruits and nuts 2011 summary. Washington, DC: National Agricultural Statistics Service.

US Department of Agriculture/Economic Research Service. (2012). U.S. per capita food availability: Individual fruit. Available in http://www.ers.usda.gov/data-products/food-availability-%28per-capita%29-data-system.aspx#.UaqWVtj3X_M. Accessed 20 May 2013.

White, G. C. (1992). Ozone. In: *Handbook of chlorination and alternative disinfectants* (3rd ed.). (pp. 1046-1110). New York, NY: Van Nostrand Reinhold.

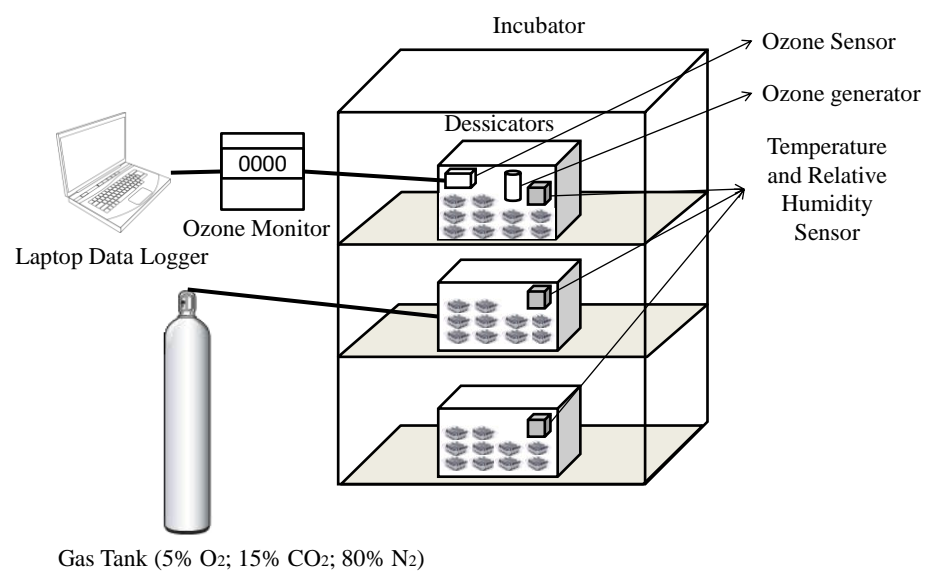


Figure 1. Schematics of controlled atmosphere incubation equipments.

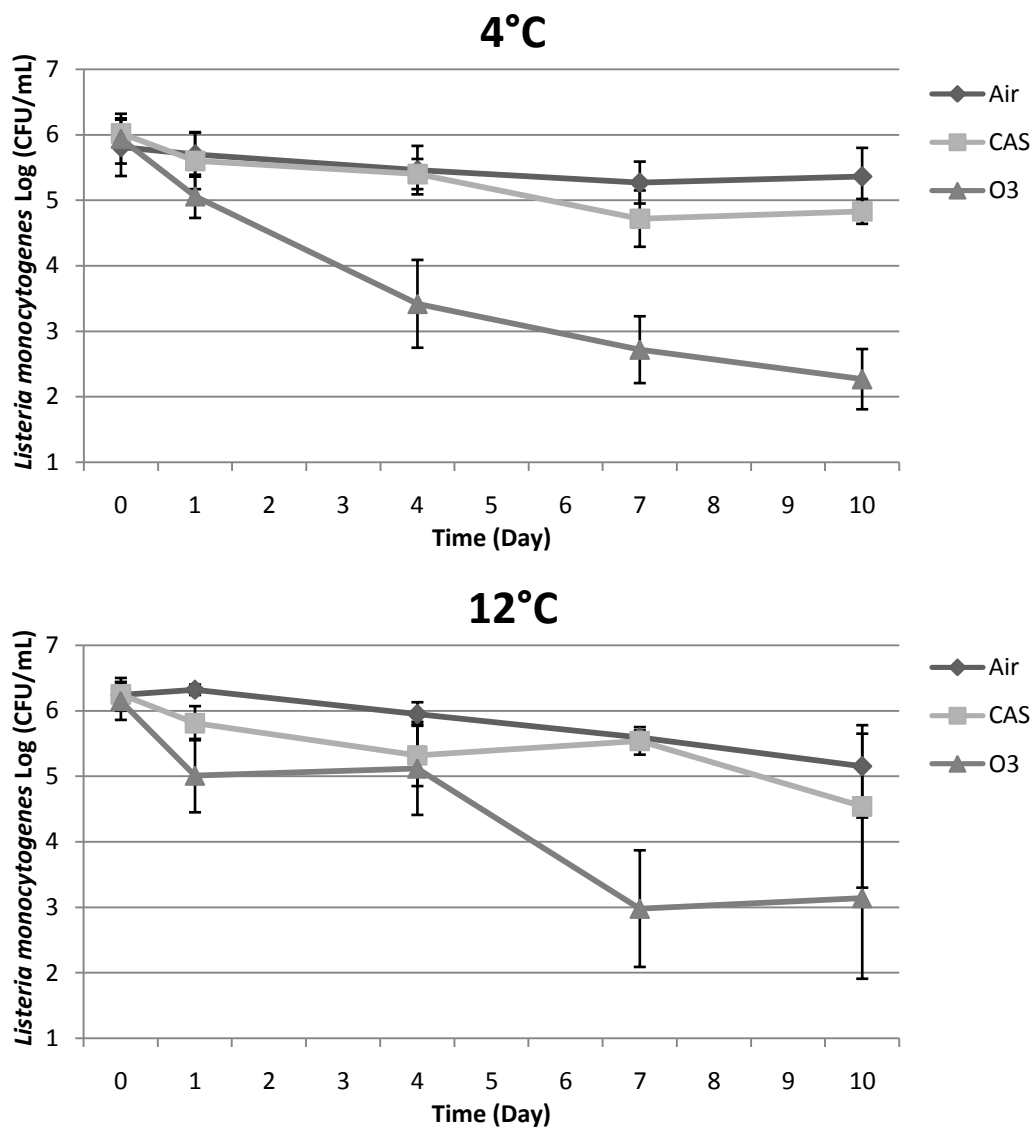


Figure 2. *Listeria monocytogenes* survival during 10 days storage time under different atmosphere conditions, which were maintained and monitored continually over time.

Table 1. *Listeria monocytogenes* counts (Log CFU/mL) on fresh highbush blueberries stored at 4°C or 12°C under controlled atmosphere storage conditions (CAS), Ozone (O₃) or regular atmosphere (Air) during 10 days.

<i>Listeria monocytogenes</i> counts Log (CFU/mL)					
Temperature 4°C					
Treatment	Day 0	Day 1	Day 4	Day 7	Day 10
Air	5.81±0.44 ^{Aa}	5.70±0.34 ^{ABa}	5.46±0.37 ^{ABa}	5.27±0.32 ^{Ba}	5.36±0.44 ^{Ba}
CAS	6.02±0.21 ^{Aa}	5.60±0.43 ^{Ba}	5.40±0.23 ^{Ba}	4.72±0.43 ^{Cb}	4.83±0.19 ^{Cb}
O ₃	5.94±0.38 ^{Aa}	5.06±0.33 ^{Bb}	3.42±0.67 ^{Cb}	2.72±0.51 ^{Dc}	2.27±0.46 ^{Dc}
Temperature 12°C					
Treatment	Day 0	Day 1	Day 4	Day 7	Day 10
Air	6.24±0.19 ^{ABa}	6.32±0.08 ^{Aa}	5.95±0.18 ^{Ba}	5.59±0.11 ^{Ca}	5.15±0.50 ^{Da}
CAS	6.25±0.25 ^{Aa}	5.81±0.26 ^{ABb}	5.32±0.47 ^{Bb}	5.54±0.21 ^{ABa}	4.54±1.24 ^{Ca}
O ₃	6.15±0.29 ^{Aa}	5.01±0.56 ^{Bc}	5.12±0.71 ^{Bb}	2.98±0.89 ^{Cb}	3.14±1.23 ^{Cb}

Means within each row followed by the same capital letter are not significantly different (P>0.05) from each other. Means within each column followed by the same lower case letter are not significantly different (P>0.05) from each other.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

The purpose of this research was to create knowledge on microbial food safety of the high value crop highbush blueberries. Consumption of this fruit in the United States is very high and increases every year. Other fresh produce has been related to different multistate foodborne illness outbreaks revealing that they are not impervious to pathogenic bacteria. Blueberries are well known to have antioxidants and an acidic pH that could lead to antimicrobial activity, however research has demonstrated that this does not assure them to be protected from pathogenic bacteria. The present study provides information on the interactions of the pathogenic bacteria *Listeria monocytogenes* with, blueberry extract and fresh blueberries, survival and, growth of this pathogen, and potential antimicrobial properties of this fruit.

The results of this research demonstrate that blueberry extract showed a lower buffering capacity when compared to lactic and malic acid; and it was not effective acidifying the growth media therefore not inhibiting *L. monocytogenes*. Although, according to literature the acid adapted pathogen was expected to survive at a pH lower than 4.6, in this study it was not able to survive under pH 4.5 on acidified media. Since consumers these days demand more natural food products, the use of natural food ingredients such as fruit acidic juices appear as an option to control and prevent foodborne illnesses and outbreaks. Even though acid solutions had the same pH, when mixed with TSB+YE media, different final pH values were achieved and blueberry extract at pH 3.0 and 4.0 were not capable of lowering final pH under 4.6. Consumers and producers should take into account the buffering capacity of an extract when trying to protect their food products from bacterial contamination.

Low storage temperature was the main factor to achieve low weight loss on stored fresh blueberries. When used in controlled atmosphere storage on fresh blueberries, ozone gas was more effective controlling weight loss at 12°C than air and CAS (5% O₂: 15% CO₂: 80% N₂), although weight loss in fruit never attained more than 3%. Weight loss is a significant commercial parameter that needs to be controlled to avoid fruit decay, and cargo rejection at port arrival. O₃ and CAS treatments did not affect naturally occurring yeast and molds counts on blueberries and they were able to grow at both temperatures overtime. During storage time blueberries firmness was better maintained with O₃ and CAS treatment than air, becoming an advantage when used during cold storage to extend texture positive characteristics of the fruits. O₃ treatment did not cause any fruit external and visible damage and can be used in fresh highbush blueberries to maintain quality and extend shelf life, however it does not assure the inhibition of naturally occurring yeast and molds.

Although the surface of fresh highbush blueberries does not support the growth of *Listeria monocytogenes*, this pathogen can survive at 4°C and 12°C for a period of 10 days under high relative humidity storage conditions. This research confirms that *Listeria monocytogenes* can survive in low oxygen atmospheres (CAS), acid-stress and refrigeration conditions in fresh blueberries. Ozone gas can be used at 4°C (4 ppm) and 12°C (2.5 ppm) to achieve 3 log and 2 log reductions when compared with air treatment, respectively. Minimally processed fresh blueberries are not commonly associated with foodborne contamination and there is not much scientific information regarding *L. monocytogenes* incidence on them. Moreover, this study provides relevant results that cover concerns mentioned previously. However, is important to highlight that fresh blueberries should be considered a low risk for listeriosis, because of their inability to support the growth of *L. monocytogenes*.

Regardless of the use of the same ozonator device in both shelf life determination and *L. monocytogenes* survival studies at 4°C and 12°C, the O₃ gas concentration averaged 4ppm and 2.5ppm, respectively. According to literature residual ozone concentration is less at higher storage temperatures, therefore for upcoming studies a system that assures same O₃ gas concentrations at different temperatures should be developed.

Future research should examine the effectiveness of blueberry extracts when used as a natural antimicrobial for food and how effective are blueberry phenolics compounds and anthocyanins inhibiting pathogenic bacteria in neutral pH environments. While optical density measurement is a convenient, fast and simple method to observe growth changes over time, nonetheless for future work quantitative bacterial counts should be included. More research should be conducted on the sensory impact of O₃ and CAS storage on fresh blueberries using a sensory panel. In the future, higher temperatures could be used in the same storage conditions to observe significant changes in weight loss. High variability in the yeast and molds results could be avoided by including a fruit surface-sterilization and then an inoculation with a known concentration of yeast and molds for a more confident number to evaluate the fungicidal properties of CAS and O₃.

In conclusion, this study recommends the inclusion of gaseous ozone for storage of blueberries, since this reduces *L. monocytogenes* after 10 days. Although, this research did not prove fungicidal properties of ozone and CAS, and consequently shelf life extension of the fruit, it demonstrated that they retained fruit firmness better than air by day 10 of storage. Moreover, no visible mold in blueberries was observed after 10 days of storage under CAS and O₃ treatments. Finally, when the fruit was stored under an abusive storage temperature (12°C), weight loss of fruit was significantly lower when ozone was included.

Appendix A.

Evaluation of *Listeria monocytogenes* growth on inoculated blueberries and storage at room temperature.

To estimate survival of *Listeria monocytogenes* strains Scott A, V7, D43 and LCDC in inoculated blueberries, select the most resilient strain and to obtain a recovery range for future evaluations, this study was developed. Also the pH of the fruit was evaluated on the surface and fruit blend.

Materials and Methods

Different cultivars of highbush blueberries (*Vaccinium corymbosum* L.) were used in this study. Fruit samples were harvested and collected from the plot located in Virginia Tech Kentland farm (5250 Whitethorne Road, Blacksburg, VA). Only mature fruits were harvested on the basis of the development of a uniform size and fully blue coloration along the surface. Moreover, undamaged and damaged blueberries were selected and collected. Approximately more than 2000g of fruit were harvested and transported in a cooler to the FST building in Blacksburg, VA. Samples were separated by damaged and undamaged and kept separated stored at 4°C.

Inoculation of Blueberries

In this study the inoculation was realized using 37°C overnight incubated *Listeria monocytogenes* strains (Scott A, V7, D43 and LCDC). Tryptic soy broth (TSB) and Tryptic soy broth with yeast extract (TSB+YE) were used as propagation media. TSA was used for pathogenic cultures used in the damaged berries and TSB+YE for the undamaged berries samples. Eighty berries were inoculated in a solution of 1 mL of overnight cultured on media with 99ml of Buffered Peptone Water (BPW) using a 18 oz.- 532 ml capacity and 4-1/2" Wide x

9" Longitude (11.5 x 23 cm) sterile polyethylene bags (Whirl-Pak, Fisher Scientific, Pittsburgh, Pa.). These inoculated blueberries were suspended in this solution for 30min and then were removed, separated in 20 berries samples and placed on sterile empty Petri plates for drying under laminar flow of the cabinet for 2h. After this plates were incubated at room temperature 25-27°C.

Microbial Enumeration

Initial counts of inoculation solution of each strain and day 0 of blueberries inoculated were calculated after completion of inoculation and drying. Inoculated blueberries samples were mixed with 100mL 0.1% peptone water and shaking for 1min after that 1ml was serial diluted using 9mL 0.1% peptone water blank tubes for microbial enumeration. MOX plates were used as plating media and were incubated at 37°C for 48h. Serial dilution was used to get representative recounts.

pH Measurements

pH measurements of blueberries were done by using a pH/conductivity meter (Accumet ® Excel XL20) and a flat surface electrode (ThermoScientific). Surface pH measurements were made in fresh blueberries samples. 50g of undamaged (39 days) and damaged (9 days) blueberries were blended for 30s using a blender (Osterizer Corp., Milwaukee, WI).

Results and Discussion

Table 1 shows the recovery counts of different strains of *Listeria monocytogenes* (Log CFU/20 berries) from inoculated undamaged (Figure A2) and damaged (Figure A3) blueberries stored at 25°C for 6 days. Inoculated damaged and undamaged blueberries showed a decrease of 1.34 to 2.75 log by day 0 when compared to the initial inocula. In the case of undamaged V7 and

Scott A both showed growth between 6.5 and 7 log (CFU/20berries). Maybe the use of less dilution in the plating should be required and the use of sonication of inoculated samples to remove more bacteria to recover. Counts on day 1 for undamaged samples were not accurate, since not enough dilutions were performed. Although in some cases undamaged berries obtained higher counts than damaged, there was not a considerable difference. Also not considerable differences were observed among the different strains of the pathogen. *Listeria* showed a great capacity to survive and even grow at 25°C.

Figure A1 shows the pH of surface and a blend of blueberries. Although there was no considerable difference in pH between blended damaged and undamaged blueberries (approximately 3.0), surface pH mean was 3.82.

By day 4 visual yeast and molds were observed on undamaged blueberries stored at 25°C, while for damaged blueberries this happened by day 3. Although *Listeria monocytogenes* strains did not have major problem to growth in TSB, for future experiment TSB+YE will be used following FDA's BAM recommendations. Also the fruit inoculation should be done by spot inoculation, since this provides a known concentration of the pathogen and does not add moisture as much as to rinse the product in the inoculum. Furthermore, the use of sonication could improve bacteria recovery counts from the fruit. Sterile polyethylene bags should be replaced for sterile hard plastic container to facilitate sample orbital shaking. Although BBL *Listeria* ChromAgar was tried in some of the bacteria plating, MOX agar was more accurate and suitable for the experiment purpose of identifying *Listeria monocytogenes*. Inoculated blueberries samples should be incubated in holed plastic containers such as clamshells instead of the use of Petri dishes.

After this experiment we can conclude that under room temperatures, *Listeria monocytogenes* is able to grow in fresh blueberries. Surface pH of blueberries is higher than blended blueberries. Future research needs to study the survival of the pathogen on surface inoculated blueberries stored under refrigerated and controlled atmosphere conditions.



Figure A1. Example of an undamaged blueberry.



Figure A2. Example of a damaged blueberry.

Table A1. Recovery counts of different strains of *Listeria monocytogenes* (Log CFU/20 berries) from inoculated undamaged and damaged blueberries stored at 25°C for 6 days.

Population (Log CFU/20 berries) of <i>Listeria monocytogenes</i>								
Time (Day)	Undamaged Berries				Damaged Berries			
	Scott A	V7	D43	LCDC	Scott A	V7	D43	LCDC
Inoculum	8.32	8.15	7.38	7.46	8.38	8.00	7.89	8.75
0	6.78	6.48	< 6.00	< 6.00	< 6.00	< 6.00	< 6.00	< 6.00
1	>3.00	>3.00	>3.00	>3.00	8.15	8.04	8.20	8.00
5	8.80	8.72	8.43	8.49	7.51	7.60	7.49	8.11
6	8.08	7.43	8.08	8.23	7.86	7.83	7.53	8.00

Appendix B.

Determination of *Listeria monocytogenes* inoculum volume and blueberries number to be inoculated.

Table B1. Population recovery of *Listeria monocytogenes* from blueberries inoculated with different volumes of inoculum (15µL and 100 µL) and different quantity of inoculated blueberries per sample (5, 10 and 20 berries inoculated from a total of 50 berries) incubated at 12°C for 48h.

Population (Log CFU/mL) of <i>Listeria monocytogenes</i>						
	15 µL			100 µL		
Time (h)	5/50	10/50	20/50	5/50	10/50	20/50
0	6.80	6.13	5.38	4.20	6.02	5.98
48	6.75	6.80	5.30	7.02	7.31	6.35