2006

Antimicrobial action of selected plant-derived compounds against Listeria monocytogenes

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Antimicrobial action of selected plant-derived compounds against *Listeria monocytogenes*

by

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A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Food Science and Technology

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2006

UMI Number: 3244381

UMI Microform 3244381

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300 North Zeeb Road
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Ann Arbor, MI 48106-1346

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ACKNOWLEDGMENTS

I would like to acknowledge all those people who directly or indirectly contributed to this thesis. Thank you to all my committee members Dr. Pometto, Dr. Dickson, Dr. Sebranek, and Dr. DiSpirito for their time and efforts through my studies at Iowa State University. I would specially like to thank my major professor, mentor, and friend, Dr. Mendonca. Thank you for giving me the chance to join the "Microbial Food Safety Laboratory" crew back in 2002. I will forever treasure the experiences we shared both in and outside the lab. I would like to express my gratitude to my lab mates through the past four years for their willingness to help me whenever necessary.

To my friends, thank you for EVERYTHING. You are in my heart and in my mind no matter where we go. Jose, thank you for loving me and helping me throughout this past year; you have giving me more than I could have ever asked for.

To my family, words cannot say how much you mean to me. Your love and support have carried me through the highs and lows of my every experience. Thank you for not giving up on me so that I would not give up on myself.

I thank God everyday for all my blessings.

ABSTRACT

Listeria monocytogenes causes listeriosis, a disease that can be fatal to immunocompromised individuals. Due to the number of outbreaks, recalls, and deaths linked to the consumption of contaminated ready-to-eat (RTE) foods, the United States government issued a directive for the control of *L. monocytogenes* in the production of RTE meats including the use of a post-lethality treatment and/or the addition of a growth inhibitor. Several methods to inhibit pathogenic bacteria in RTE foods are currently utilized. One method actively being studied involves the use of natural plant products as food antimicrobials. The present study evaluated the effectiveness of plant-derived compounds in controlling the growth of *L. monocytogenes*. Four antimicrobials (cranberry-CB, grape seed-GS, oregano-OR, and green tea-GT) were evaluated in culture media and two (CB and GS in combination with sodium lauryl sulfate-SLS) as dips for frankfurters (formulated with or without sodium lactate-SL). In culture media, GS had the highest inhibitory activity at both storage temperatures (4 and 10°C) and at all concentrations tested. In frankfurters without SL, 4 and 6 log reductions in the initial numbers of the pathogen were obtained with CB (+SLS) and GS (+SLS), respectively, and growth of survivors was inhibited up to day 14. In frankfurters with SL, a similar bacteriocidal effect was obtained with both CB (+SLS) and GS (+SLS) but growth of survivors was prevented for 90 days. The survival of acid adapted *L. monocytogenes* in culture media containing CB and GS was also assessed. Acid adapted cells were less affected by CB than the non-acid adapted at weeks 1 and 2; no differences in sensitivity to GS was observed between acid adapted and non-acid adapted cells. Lastly, preliminary

evidence on the mode of action of GS on *L. monocytogenes* cells using transmission electron microscopy indicated possible damage to the cytoplasmic membrane.

CHAPTER 1. GENERAL INTRODUCTION

The Centers for Disease Control and Prevention (CDC) has estimated that *Listeria monocytogenes* causes up to 2,500 cases of foodborne illness per year resulting in approximately 500 deaths, a hospitalization rate of 91%, and a case fatality rate of 20% (*115*). In addition, cases of listeriosis account for approximately $200 million in monetary loss in the United States annually (*31*). The ubiquitous nature of *L. monocytogenes* and its ability to proliferate at refrigerated temperatures make this organism a threat to the safety of a number of food products. Special attention has been given to *L. monocytogenes* and ready-to-eat (RTE) products due to several deadly foodborne outbreaks. The lack of a heating or cooking step prior to the consumption of these food items presents a risk, especially to susceptible individuals. The presence of *L. monocytogenes* in the processing environment and the potential for cross-contamination of the pathogen into RTE foods has been troublesome for the food industry, regulatory agencies, and the consumer.

Although *L. monocytogenes* should be considered potentially present in all raw foods and ingredients, the pathogen has been primarily linked to the consumption of contaminated foods including raw milk, cheeses (particularly soft-ripened), ice cream, raw vegetables, raw meats, sliceable turkey deli meat, hot dogs, and raw and smoked fish (*29, 30, 63*). Listeriosis is of particular concern for the elderly, pregnant women, neonates, and immunocompromised individuals. The disease can cause septicemia, meningitis, encephalitis, and death; in pregnant women, listeriosis may result in miscarriages or stillbirths. The overall mortality of listeric meningitis may be as high as

70%, for septicemia 50%, and from perinatal/neonatal infections greater than 80% (*63*).

Several large outbreaks that occurred in the early 1980s prompted regulatory agencies to issue a "zero tolerance" policy for *L. monocytogenes* in RTE foods (*76*). Despite efforts to eliminate this pathogen from RTE foods, contamination still occurs. Results from monitoring activities and surveillance by the United States Department of Agriculture (USDA) and the Food and Drug Administration (FDA) indicate that approximately 5% of RTE foods contain detectable levels of *L. monocytogenes* (*76*). In 2003 and 2004, respectively, 45,251 and 502,845 pounds RTE refrigerated meats (including chicken, beef, and turkey) were recalled due to *L. monocytogenes* contamination (*66*). Since the mid-1980s the government and the food industry have been actively seeking and implementing strategies to control *L. monocytogenes*. Research efforts continue to focus on new ways to further protect the consumer from foodborne listeriosis.

Among the many conventional methods (e.g. pasteurization) utilized to preserve foods by inhibiting (or slowing down) the growth of unwanted microorganisms is the use of chemical agents. Some compounds added to foods are industrially synthesized, while others are naturally occurring. These natural substances may display antimicrobial properties in the foods where they are normally found, or the compounds may be extracted from those foods and commercially added to others (*163*). Much of the research on the area on naturally occurring antimicrobials are conducted because consumers' desire for more "healthy" and "natural" foods. The food industry has been working on lowering the use synthetic agents while increasing the use of compounds from natural sources.

The history of plant derived compounds such as herbs and spices dates back as far as 6000 B.C. These substances were utilized as flavor agents, food preservatives, and for medicinal purposes (*37*). For a great number of these compounds, the uses are the same today. Efforts to maximize the utilization of these natural compounds as antimicrobials in foods continue to drive the food industry and the scientific community forward.

DISSERTATION ORGANIZATION

This dissertation contains a literature review (chapter 2), four journal articles (chapters 3-6), and a comprehensive conclusion (chapter 7). Each journal article is made up of the following sections: abstract, introduction, materials and methods, results and discussion, and references. The papers and all references (chapter 1-6) are formatted following the guidelines for submission to the Journal of Food Protection.

CHAPTER 2. LITERATURE REVIEW

1. *Listeria* - Historical Background

In early 1891, *Listeria* may have been recognized in tissue samples from German patients; it was isolated from the liver of rabbits in 1911 in Sweden. The symptoms from the disease were recognized in sheep in Germany in 1925 *(79, 112)*.

In 1926, Murray et al. *(122)* first described *L. monocytogenes* following an investigation on the causative agent of an epizootic disease in rabbits and guinea pigs. He isolated the bacterium from the blood of infected animals that demonstrated symptoms of mononucleosis; hence he named the organism *Bacterium monocytogenes*. In 1927, Pirie named the same organism *Listerella hepatolytica* based on the liver marks of infected rodents in South Africa and in honor of the surgeon Lord Lister *(151)*. Once those researchers discovered that they had isolated the same organism, the name was changed to *Listerella monocytogenes*. Finally in 1940, Pirie proposed the name *Listeria (136)*.

Until the late 1940s, *L. monocytogenes* was the only species in the genus *Listeria*. In 1948 *L. denitrificans* was included in the genus *(165)*; this species was later excluded from the genus following 16s rRNA studies that revealed differences with other species within the genus *Listeria* and similarities with the coryneform bacteria *(143)*. In 1966, *L. grayi* was added to the list *(97)*, followed by *L. murrayi* in 1971 (later to be added to *L. grayi*) *(167, 178)*, *L. innocua* in 1981 *(152)*, *L. ivanovii* in 1985 *(154)*, and *L. welshimeri* and *L. seeligeri* in 1983 *(141)*; although *L. monocytogenes* is considered the only pathogenic species, there were instances when *L. ivanovii* (three cases) *(26, 142)* and *L. seeligeri* (one case) *(85)* were reported to cause human infection.

2. Characteristics of *Listeria* species

Listeria bacteria are small gram-positive rods measuring approximately 0.4-0.5 µm in diameter by 0.5-2.0 µm in length *(153)*. Members of this species are nonsporeforming, aerobic, microaerophilic, facultatively anaerobic, with both psychrotrophic and mesophilic features. They are motile by means of peritrichous flagella when grown at 20-25ºC, but show little to no movement when cultured at 37ºC *(71, 140)*. They are catalase-positive (most strains) and oxidase negative; they can catabolize glucose both aerobically and anaerobically by the Embden-Meyerhoff pathway *(140, 153)*.

3. Characteristics of *Listeria monocytogenes*

3.1. Strains

L. monocytogenes strains can be differentiated by serological typing. Strains differ in the antigenic determinants expressed on their cell surface; more than 14 serotypes of *L. monocytogenes* have been designated (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4bX, 4c, 4d, 5, 6a, 6b) *(78)*. Although *L. monocytogenes* is widely found in nature, only three serotypes (1/2a, 1/2b, and 4b) account for approximately 96% of the human infections documented in the United States *(170)*.

3.2. Temperature

L. monocytogenes grows in temperatures ranging from -0.4 to 50ºC *(53)* with an optimum temperature between 30 and 37ºC *(133)*. *L. monocytogenes* was reported by Hudson et al. *(87)* to grow at -1.5ºC in vacuum-packaged sliced roast beef, the lowest growth temperature reported for this organism. The pathogen was reported to survive freezing and frozen storage at -18ºC in ground turkey *(131)*.

3.3. pH and Water Activity

Some strains of *L. monocytogenes* can survive a pH as low as 4.1 and as high as 9.6 *(90)*, although the optimum pH for the organism ranges between 6 and 8. A minimum water activity of 0.90 was found to support growth of *L. monocytogenes* in certain foods *(105)*.

3.4. Salt Tolerance

L. monocytogenes can grow, although slowly, in nutrient broth containing up to 10% NaCl *(153)*. The survival of this organism in salt solutions can be significantly increased by lowering the incubation temperature *(87)*.

4. *Listeria monocytogenes* as a Foodborne Pathogen

Several factors have contributed to the development of *L. monocytogenes* as a foodborne pathogen. One important factor is the increased use of refrigeration to preserve foods for extended periods of time. Although most organisms may be inhibited by cold temperatures, this type of storage provides conditions adequate for the growth and survival of *L. monocytogenes*. Another factor includes changes in the population, such as an increase in the number of elderly people and immunocompromised individuals; all of whom are highly susceptible to acquiring listeriosis. Also, the consumption of RTE products, which do not require an additional cooking step, is increasing. Studies have shown that societal changes, for example single headed households, have caused people to have less time for food preparation. In 2003, frozen and RTE foods accounted for more than one-quarter of dinner entrees while the number one trend in the food industry that year was the ready-to-eat, heat-and-eat, and packaged food products for on-the-go consumption *(160, 161)*.

4.1. Listeriosis in Humans

It has been estimated that approximately 5-10% of healthy humans harbor *L. monocytogenes* in their intestinal tract *(47, 115)*. Carriers of the pathogen as indicated by examination of fecal samples ranges from 0.5% (9 of 1,732) (low) to 69.2% (36 of 52) or 91.7% (11 of 12 female laboratory technicians) (high) *(53)*. Although humans might shed the bacteria in their feces, they may never show signs of the illness *(74)*.

Immunocompromised individuals are typically more susceptible to listeriosis; for example, patients with cancer or AIDS, pregnant woman or neonates, patients undergoing treatment with steroids or cytotoxic drugs, alcoholic patients, and those with diabetes *(48, 162)*. Another predisposing factor to listeriosis is age; a case-fatality rate of 11% was documented in people under 40 years of age, while a 63% was noted for those over the age of 60 *(74)*. Reasons for this predisposition may include a poor immune system, the presence of immunesuppressive disorders, and the use of immunesuppressive drugs *(48)*.

The risk of acquiring listeriosis for a pregnant woman is 20 times higher than that of a healthy adult. The illness usually occurs during the third trimester of the pregnancy and the mother may experience a mild flu-like illness with fever, headache, and gastrointestinal symptoms *(148)*. Transplacental transmission of *L. monocytogenes* to the fetus may occur following maternal bacteremia, although some infections occur from vaginal colonization. A severely ill mother may enter labor prematurely and a preterm labor may result in spontaneous abortion, stillbirth, or early-onset neonatal infection *(162)*.

Two forms of listeriosis can occur in adults, invasive and noninvasive. The invasive form is characterized by symptoms such as conjunctivitis septicemia, meningitis,

meningoencephalitis, and endocarditis. The incubation period for this form of the illness is approximately 30 days *(48)*. The noninvasive form of listeriosis has been proposed to occur in people that consume foods contaminated with an infectious dose of *L. monocytogenes*. Gastrointestinal and flu-like symptoms (fatigue, fever, nausea, cramps, vomiting, and diarrhea) have been observed in approximately one-third of the documented cases of listeriosis *(48)*. The incubation period for this form of the illness is much shorter; symptoms typically appear within 18 to 20 hours. Febrile gastroenteritis has been noted in several outbreaks *(40, 145)*, although the frequency of gastroenteritis as a result of *L. monocytogenes* infection is still unknown. The infectious dose that causes the gastroenteritis has also not been determined, as are host characteristics associated with the syndrome *(48)*.

4.2. Pathogenesis of *Listeria monocytogenes*

It has been demonstrated that when *L. monocytogenes* is contracted orally, the pathogen colonizes the intestinal tract and then invades other tissues, including the placenta in pregnant women. The organism can enter the blood stream, from where it can reach other susceptible body cells *(90)*.

Macrophages actively ingest *L. monocytogenes* cells. The expression of a pore forming cytotoxin protein hemolysin, LLO (listeriolysin O), allows the pathogen to survive inside the macrophages by escaping from the phagolysosomal membrane into the cytoplasm *(90)*. Once inside the cytoplasm, the protein ActA aids in the development of actin tails that move the pathogen toward the cytoplasmic membrane. Once at the membrane, a combination of LLO and two phospholipases free the bacteria from the macrophage and the process is repeated again once the pathogen enters adjacent host cells *(90)*. Entry of *L.*

monocytogenes into nonphagocytic cells require the presence of In1A and In1B expressed on the surface of the bacterium *(102)*.

4.3. Epidemic Listeriosis

In the 1980s, several foodborne outbreaks were positively linked to the consumption of cheese and raw vegetables contaminated with *L. monocytogenes*. Although these outbreaks were the first documented evidence of foodborne transmission of listeriosis, the history of this illness can be traced back to when *L. monocytogenes* was first isolated. For example, an outbreak in Germany from 1949 to 1957 was suspected to have been caused by *L. monocytogenes* contaminated raw milk, but the difficulties in isolating the organism from food and environmental sources impeded the conclusive identification of the pathogen in the milk *(144)*.

The first documented outbreak of listeriosis occurred in Canada in 1981 *(179)*. The implicated food in the outbreak was locally produced coleslaw made with inadequately disinfected cabbage. In this outbreak, forty-one people became ill and eighteen died. The largest outbreak of listeriosis in the United States occurred in 1985 in California, where Mexican-style fresh cheese was made with a mixture of pasteurized and unpasteurized milk. One hundred and forty two cases were detected over an 8-month period, with an approximate 30% case fatality rate *(103)*.

Several large listeriosis outbreaks have also occurred in Europe (Table 1). In France, 1992, pork tongue in jelly was the implicated food in an outbreak where two hundred and seventy nine people became ill and eighty-five died. In Italy, 1997, over fifteen hundred people became ill after consuming contaminated corn salad.

A multistate outbreak occurred in the United States in 1998 linked to contaminated

Table 1. Foodborne outbreaks of listeriosis.

Year	Place	No. of Cases (deaths)	Vehicle	Serotype	Reference
1979	MA, United States	20 (5)	Raw vegetables	4b	44
1981	Canada	41 (18)	Coleslaw	4b	45
1983	MA, United States	49 (14)	Pasteurized milk	4b	46
1985	CA, United States	142 (48)	Mexican-style cheese	4b	47
1983-1987	Switzerland	122 (34)	Soft cheese	4b	48
1986-1987	PA, United States	36 (16)	Ice cream, salami, brie cheese	4b, 1/2a, 1/2b	49
1988-1989	United Kingdom	366 (94)	Pâté	4b	50
1989	CT, United States	9 (1)	Shrimp	4b	51
1992	France	279 (85)	Pork tongue in jelly	4b	52
1993	Italy	18 (0)	Rice salad	1/2b	39
1994	IL, United States	48 (0)	Pasteurized chocolate milk	1/2b	38
1997	Italy	1566 (0)	Corn salad	4b	53
1998	24 states, United States	108 (18)	Hot dogs	4b	54
1999	France	26 (7)	Pork tongue in jelly	4b	55
2000	10 states, United States	29 (4)	Deli turkey meat	4b	6
2000	NC, United States	12 (5)	Mexican-style cheese	4b	56
2001	Canada	5 (0)	Flat whipping cream	1/2a	57
2002	Canada	47 (1)	Cheese	4b	57
2002	Canada	86 (0)	Cheese (water)	1/2a	57
2002	Canada	17 (0)	Heat-treated soft and firm cheese	1/2a	57
2002	8 states, United States	46 (10)	Sliceable turkey deli meat	4b	2
2003	United Kingdom	5 (0)	Pre-packaged sandwiches	1/2a	58

hot dogs. The outbreak caused illness in one hundred and eight people residing in 24 states and caused fourteen deaths and four stillbirths *(77)*. In Canada, 2002, two cases of *L. monocytogenes* meningitis lead to the recall of potentially contaminated cheese; a total of forty-seven cases of human listeriosis were identified including two cases of bacteremia in pregnancy associated with a miscarriage *(130)*.

In 2000 and 2002, two multistate outbreaks occurred in the United States linked to contaminated deli turkey meat. The first outbreak involved ten states with twenty-nine cases and four deaths and three miscarriages or stillbirths; the second outbreak involved eight states with forty-six cases and seven deaths and three miscarriages or stillbirths *(30, 31)*. In the United Kingdom, 2003, a listeriosis outbreak was linked to the consumption of pre-packaged sandwiches purchased from a hospital retail shop. Samples of sandwiches taken from the local sandwich supplier tested positive for *L. monocytogenes*, as did samples taken form the premises (chopping boards, sink, and cleaning sponge) *(43)*.

4.4. Efforts to Control Listeriosis

The outbreaks of listeriosis that occurred during the 1980s and the 1990s prompted U. S. federal regulatory agencies and the food industry to develop ways to control *L. monocytogenes* in foods. Strong efforts to control this human pathogen and to prevent further outbreaks have continued over the years.

In response to the high number of outbreaks linked to dairy products and meats, the FDA developed the Dairy Safety Initiatives Program in April of 1986 *(38)* and the USDA developed the monitoring/verification program for *L. monocytogenes* in meat products in September of 1987 *(62)*. In November of 1998, The FDA's Compliance Program for Domestic and Imported Cheese and Cheese Products issued guidelines, such as

conducting inspections and examining samples of imported and domestic cheese, after recognizing that *L. monocytogenes* contaminated cheese could cause human illness *(62)*.

In the United States, many of the outbreaks and recalls of contaminated cooked and RTE products have been attributed to post-processing contamination. The implementation of good manufacturing practices (GMPs), standard operating procedures (SOPs), and Hazard Analysis and Critical Control Points (HACCP) programs have significantly contributed to the reduction in recalls of these products *(48)*. In the late 1980s, the Food Safety and Inspection Service (FSIS) established a "zero tolerance" policy for *L. monocytogenes* in RTE products. The policy is currently defined as detection of *L. monocytogenes* in a 25-gram sample of food, which renders the product adulterated in agreement with the Food, Drug, and Cosmetic Act *(59)*.

FSIS defines an RTE food as a meat or poultry product (includes frozen) in the form that it is edible without additional preparation to achieve food safety *(176)*. These products are not required to show safe-handling instructions or other labels that direct the consumer to cook or treat the product for safety *(174)*. RTE meats such as deli-type products which are sliced at the establishment or may be sliced at the retail store are considered high-risk foods *(175)*. Government agencies have published recommendations advising certain portions of the population (e.g. pregnant woman) to minimize or restrict their consumption of high-risk foods.

As a result of these efforts, in the United States between 1989 and 1993, the rates of invasive listeriosis decreased by 49% while the number of deaths decreased by 48% *(170)*. During this period, the annual incidence rate of listeriosis declined from 7.9 to 4.2 cases per million persons; to the year 2000, the number has remained stable at 5.0 cases

per million persons *(28)*.

In 2003, the FSIS introduced an interim final rule regarding the "control of *Listeria monocytogenes* in RTE meat and poultry products". Under this rule, the food industry was required to implement a post-lethality treatment and/or the use of an antimicrobial (growth inhibitor) for *L. monocytogenes* or to utilize a sanitation procedure in the processing environment to control the pathogen *(64)*.

5. Methods to Detect and Isolate *Listeria monocytogenes*

5.1. Conventional Methods

L. monocytogenes can be easily cultured on bacteriological media such as Tryptose Agar and Nutrient Agar. However, isolation of the pathogen from inoculated or naturally contaminated foods and clinical specimens using nonselective media can be more difficult *(46)*. Complex samples containing low numbers of *L. monocytogenes* and/or large amounts of background flora requires selective enrichment of the organism before it can be detected. Combinations of direct plating, cold enrichment, and selective enrichment can be used to detect *L. monocytogenes* in clinical, environmental, and food samples.

The cold enrichment method for recovering *L. monocytogenes* was adopted as the "standard procedure" in the late 1940s and first reported by Gray et al. in 1948 *(80)*. This method requires incubation of the sample in non-selective broth for several weeks at 4ºC *(46)*; only a few weeks are usually needed for recovering *Listeria*, but some samples can require months of refrigerated storage before detection is possible. Therefore, although this procedure can greatly enhance the likelihood of isolating *L. monocytogenes* from a variety of specimens, the time constraint has led to the use less time-consuming methods.

The selective enrichment method at higher temperatures (30-37ºC) involves the addition of inhibitory agents that prevent the growth of background flora while allowing growth of *Listeria (46)*. Selective enrichment requires the use of inhibitory agents such as lithium chloride, nalidixic acid, acriflavine, polymyxin B, moxalactam, colistin, ceftazidime. Although this procedure relies on the resistance of *L. monocytogenes* to various selective agents and antibiotics, the process is not always clear-cut; many such inhibitory agents can partially inhibit growth of the pathogen, especially when cells maybe sublethally injured *(46)*.

Following selective enrichment, samples are plated on isolation media. Most solid media will include an indicator substrate, such as chromogens or blood to distinguish *Listeria* from other bacteria present in the sample or *L. monocytogenes* from other *Listeria (180)*. The two most common types of solid media used to isolate *Listeria* include Modified Oxford Agar (MOX) and PALCAM Agar. Both contain esculin and ferric ammonium citrate to produce black *Listeria* colonies from esculin hydrolysis *(46)*.

5.2. Rapid Methods

Most of the techniques developed in the 1980s for the isolation and enumeration of *L. monocytogenes* in foods require long periods of incubation. These methods may not be rapid enough to ensure the safety of perishable foods before consumption. Regulation passed by the United States government, such as the “zero tolerance” rule for *L. monocytogenes* in RTE products, prompted the food industry to develop ways to rapidly detect the pathogen in such foods.

Immunoassays (monoclonal or polyclonal antibodies), nucleic acid probes, and polymerase chain reaction (PCR) have been used to detect both *L. monocytogenes* and

Listeria spp. in foods. The choice of which method to use depends primarily in factors such as simplicity, cost, speed, and sensitivity *(11)*. Today, commercial test systems exist that combine one or a few of the above methods in order to more rapidly and easily detect *L. monocytogenes.*

Some newly developed rapid methods can detect *Listeria* within a 24 hour to a 3 day time period. The API *Listeria* system (BioMérieux, La Balme-les-Grottes, France) consists of reaction strips that rely on 10 different tests (presence or absence of arylamidase, esculin hydrolysis, α-mannosidase, etc.) to differentiate *Listeria* isolates within 18 to 24 h *(20)*. The BAX® system by Dupont utilizes PCR to rapidly amplify millions of copies of a specific DNA fragment of *L. monocytogenes* for reliable detection *(84)*. A tablet, included in the BAX® kit, contains all of the PCR ingredients necessary for the reaction. *L. monocytogenes* cells are lysed and the lysate hydrates the tablet; an automatic unit takes less than 4 hours to process a rack of 96 tests. Results for *Listeria* spp. may be obtained within 24 to 28 h following enrichment. Other rapid tests available in the market include the Oxoid Biochemical Identification System – Mono (O.B.I.S.), VIP® by Biocontrol and Singlepath® by Merck.

6. Distribution of *Listeria*

6.1. *Listeria monocytogenes* in the Environment

L. monocytogenes is ubiquitous in the environment and it can be found in water, soil, and decaying plant material. Along with water and soil, the bacteria have the ability to survive for long periods of time in fecal material and animal feed. This capacity to survive extended periods of time may explain why the natural environment can act as a reservoir of *L. monocytogenes* with the high risk of contaminating animals and plant food

products *(55)*.

Agricultural soil and silage are often contaminated with *L. monocytogenes*. Silage which has not been properly baled can create a good environment for mold growth which may cause the pH to rise, therefore, making it possible for *L. monocytogenes* to replicate to high numbers *(54)*; in turn, animals fed diets of silage commonly shed the organism in their feces *(56)*. The organism has also been found in water and sewage, with some studies showing transmission to foods via those routes. For example, high rates of contamination by *L. monocytogenes* were found in mussels and oysters from areas in the ocean polluted with sewage *(23)*. Although the organism may be found in soil, most studies have shown that the widespread presence of the pathogen in soil most likely results from contamination by fecal material and decaying vegetation *(55)*.

6.2. *Listeria* in the Processing Environment and in Foods

L. monocytogenes can be readily isolated from humans, pets, homes, and the food processing environment; it can be found in meats, poultry, vegetables, dairy products, and fishery products *(172)*. The organism is highly pervasive and able to persist in the environment for long periods of time and over a wide range of challenging conditions. These characteristics make *L. monocytogenes* extremely difficult to eradicate, especially in the food processing environment where many reservoirs have been found to harbor the pathogen. Floors, drains, condensate on pipes, and cleaning tools (sponges/brushes) frequently contain high numbers of *Listeria* spp.; other sites of contamination include conveyor belts, packaging equipment, slicers/dicers/blenders, and hand tools/gloves/aprons *(172)*. Table 2 provides common sites of contamination and reservoirs of *L. monocytogenes* in the processing plant.

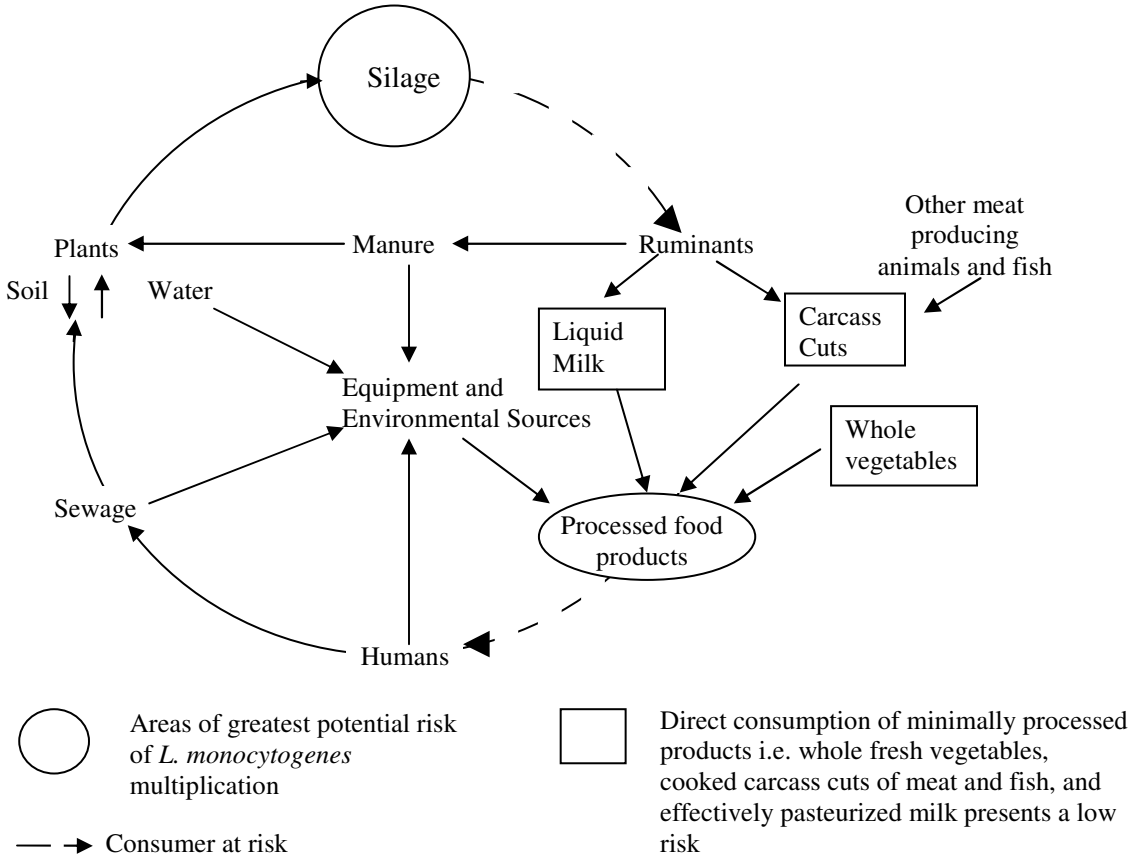
One of the most important tasks for the food industry and the focus of much research and regulation is the control of *L. monocytogenes* in the food processing environment to prevent contamination of finished products. Of these, special attention has been given to RTE foods because of the lack of a heating or cooking step required before their consumption and the severity of the illness to susceptible individuals. Studies have shown that contamination of the final product, in some cases, occurs during processing because the strains found in the raw materials are different from those found in the final products *(6, 106)*. Other studies have demonstrated that the source of contamination of final products is indeed the incoming raw material because the *L. monocytogenes* strains in the two are identical *(75, 99)*. Figure 1 illustrates a flow diagram of *L. monocytogenes* contamination from the natural environment through the food chain.

The prevalence of *Listeria* spp. in fresh meats can be as high as 68% with even higher percentages in comminuted meats *(91)*. In a study by Loura et al. *(106)*, samples of chicken breast, livers, surfaces of saws and tables, hands and gloves from an industrial poultry processing plant, were examined for contamination with *Listeria* spp. Forty percent of the chicken breast samples analyzed were contaminated with *L. monocytogenes*. Thirty percent of livers contained *Listeria* spp., while 80% of samples from tables and 30% from saws were positive for *L. monocytogenes*. In another study, Autio et al. *(7)* collected 373 samples from ten pig slaughterhouses. Six establishments and 9% of all samples were positive for *L. monocytogenes*. Another area of concern with the presence of *L. monocytogenes* in the processing environment is the ability of the organism to attach to stainless steel, glass, and rubber to produce biofilms *(21)*.

Table 2. Common sites of *Listeria monocytogenes* contamination (top) and reservoirs of the pathogen (bottom) in the processing plant.

Collators used for assembling/arranging product for packaging
Containers such as bins, tubs, or baskets used for holding food while it is waiting to be further processed or packaged
Conveyors
Filling or packaging equipment
Hand tools, gloves, aprons, etc. that contact exposed finished product
Slicers, dicers, shredders, blenders, etc. used after heating or decontamination and before packaging
Solutions used in chilling food
Spiral freezers/blast freezers
Racks for transporting finished product
Ceilings, overhead structures, catwalks
Cleaning tools such as sponges, brushes, floor scrubbers
Condensate
Drains
Equipment framework and other equipment in the area
Floors
Insulation in walls or around pipes and cooling units that has become wet
Maintenance tools
Trolleys, forklifts, walk-alongs
Walls, especially if there are cracks that retain moisture

Adapted from Tompkin, 1999



Adapted from Fenlon, 1999

Figure 1. *Listeria monocytogenes* from the natural environment through the food chain.

Some biofilms have been shown to resist the action of chlorine, iodine, anionic acid, and quaternary ammonium sanitizers *(69). L. monocytogenes* is capable of surviving and growing in such biofilms; improper cleaning and disinfection of equipment could lead to the reintroduction of the pathogen into new processed foods.

A report by Levine et al. *(100)* showed the results of a 10-year cumulative study on the prevalence of *L. monocytogenes* in RTE meat and poultry products. The data was obtained by the FSIS from 1,800 federally inspected establishments (production facilities only) from 1990 to 1999. The results indicated prevalence of *L. monocytogenes* in those products as follows: jerky, 0.52%; cooked, uncured poultry products, 2.12%; large-diameter cooked sausages, 1.31%; small-diameter cooked sausages, 3.56%; cooked beef, roast beef, and cooked corned beef, 3.09%; salads, spreads, and pâtés, 3.03%; and sliced ham and luncheon meat, 5.16%. A 3-year cumulative study for dry and semidry fermented sausages showed *L. monocytogenes* at 3.25%. In 2003, 0.75% of random samples of RTE meats collected by the FSIS over a 9-month period tested positive for *L. monocytogenes (65).* While in the same year, Gombas et al. *(76)* reported the results of a survey of RTE foods collected over a 2-year period from retail stores in Maryland and northern California. The product categories included luncheon meats, deli salads, fresh soft “Hispanic-style” cheeses, bagged salads, blue-veined and soft mold-ripened cheeses, smoked seafood, and seafood salads; the prevalence of *L. monocytogenes* ranged from 0.17 to 4.7% depending on the food sample.

7. Stress Adaptation

Organisms deal with environmental stress, such as lack of food or water, on a daily basis. When it comes to bacteria, stress can be defined as any variation from optimum

growth conditions leading to reduced growth rate or sublethal damage of cellular components *(166)*. Over time, bacteria have evolved adaptive mechanisms to deal with the constant changes of the environment and to survive the challenges of stress. In some organisms (e.g. *Bacillus subtilis*) the strategy to survive stress involves differentiation to spores; while other organisms (e.g. *Escherichia coli*) enter what is known as stationary phase. In this non-differentiated state, cells may undergo physiological changes that allow for their survival under several environmental stresses; some of those challenges include: starvation, high energy wavelengths, high or low temperatures, low water activity, acids, alkali, heavy metals, osmolarity, and oxidative challenge *(166)*. Studies have shown that a number of proteins synthesized under normal conditions are repressed when the cells undergo some environmental stress, while other proteins (such as stress-induced) are up-regulated or novel *(134)*. Regardless of the type of stress, the adaptive response involves a number of genetic changes that control the metabolic modifications taking place within the cells *(1)*.

7.1. General Stress Response

Studies have shown that the modification of sigma (σ) factors, whose main role is to bind core RNA polymerases to confer promoter specificity, is a common regulatory mechanism in bacteria undergoing environmental stress. The protein sigma B (σ^B), coded by *sigB*, regulates the expression of ten genes coding for general stress response proteins in *L. monocytogenes (166)*. Becker et al. *(15)* described an increase in the transcription of *sigB* in *L. monocytogenes*, implying an increase in σ^B activity, when exponentially growing cells had been exposed to acid, ethanol, osmotic, heat, and oxidative stresses. Ferreira et al. *(57)* demonstrated that a *sigB* null mutant strain of *L. monocytogenes* lost

viability more rapidly than the parent strain when exposed to acid, oxidative stress, and carbon starvation.

7.2. Acid Response

Acid stress is defined as the biological effect of H^+ ions in combination with weak acid concentrations on the bacterial cell. Extreme low pH outside the cell (pH_o) causes H^+ ions to leak across the membrane resulting in a decreased intracellular pH (pH_i). This drop in pH_i can be detrimental to the cell, causing disruptions of macromolecular structures and biochemical reactions *(166)*. Maintaining pH homeostasis is a very important aspect of bacterial cell physiology, and a process that is tightly regulated.

Cell membranes have very low permeability for H^+ ions. Protons move in and out of the cell by mechanisms that control H^+ transport, including F_oF_1 ATPase, Na^+/H^+ antiporters, and respiratory chains *(95, 135)*. Many studies have shown that weak organic acids are more detrimental to bacteria than strong inorganic acids such as HCl. Organic acids, particularly short chain lipophilic acids, diffuse across cell membranes in their protonated forms, then completely dissociate into H^+ and anions in the cytoplasm. Such acids will enter the cell through porins or permeases and once inside of the cell, they cannot diffuse out. Inside the cell, these acids dissociate into protons (H^+) and anions. The presence of the H^+ ions lowers the pH_i causing severe damage to the cell if it cannot actively pump the ions out.

Acid stress management systems have been described for many bacteria. These allow organisms to cope with the changes in pH in the environment. Some of those mechanisms include the acid tolerance response (ATR), acid shock response (ASR), and acid adaptation or habituation. For example, Phan-Thanh et al. *(135)* showed that *L.*

monocytogenes grown to mid-exponential phase in media containing HCl (pH 3.5-4), displayed growth arrest (cell density constant over a period of time). When the pH was dropped below those values, the bacteria began to die. However, prior exposure of cells to non-lethal pH (~5) for a few hours drastically increased their ability to resist subsequent acid stress at pH < 4.0 (induced ATR).

Acid adaptation in *E. coli* was demonstrated by Buchanan et al. *(24)*. Several strains grown overnight in tryptic soy broth with or without 1% glucose (final pHs of cultures at ~5 and 7, respectively) were used to inoculate Brain Heart Infusion broth adjusted to pH 3. Based on numbers of viable cells that survived over a 7 h period, all *E. coli* strains tested were acid resistant.

A great number of proteins, including some known as acid shock proteins, confer protection from acid stress encountered by bacteria. A study by Phan-Thanh and Gormon *(134)* demonstrated that when *L. monocytogenes* experienced acid shock, approximately 65% of the proteins expressed by the bacteria during normal conditions were repressed, while 22 proteins were up-regulated; 10 of those being novel proteins. Foster *(68)* defined adaptive acid tolerance in *Salmonella* as a two-stage process that required the pre-shock induction of the ATR-specific pH homeostasis system and the post-shock synthesis of acid shock survival proteins.

7.3. Cross-Protection

One of the main concerns of stress adaptation by bacteria, to any environmental challenge, is the threat of cross-protection. Studies have shown that adaptation to one specific stress (e.g. acid) provides organisms a general cross-protection against other stresses. Phan-Thanh et al. *(135)* showed that acid adapted *L. monocytogenes* had an

increased resistance to osmotic shock, heat shock, and alcohol stress. Lou and Yousef *(104)* demonstrated that heat shock significantly increased the resistance of *L. monocytogenes* to ethanol and NaCl. O'Driscoll et al. *(127)* also showed that induction of an ATR in *L. monocytogenes* provided the pathogen cross-protection against ethanol, osmotic and thermal stress, and crystal violet (surface-active agent). This study also demonstrated the increased lethality of the stress adapted pathogen in mice. More recently, Mendonca et al. *(117)* demonstrated that starvation in *L. monocytogenes* increased the pathogen's resistance to ionizing radiation.

Several studies have shown that *L. monocytogenes* not only responds to environmental challenges by regulating the synthesis of a great number of stress proteins, but also by regulating the synthesis of specific or essential virulence factors *(113, 123)*. The presence of these factors, along with stress proteins, allows the pathogen to infect and cause disease by surviving through the gastrointestinal tract of the host and in the phagosomes of attacking macrophages. Begley et al. *(17)* demonstrated that stationary-phase cultures of *L. monocytogenes* were capable of tolerating concentrations of bovine, porcine, and human bile in excess amounts of those encountered in vivo.

Another area of concern for the food industry involving the issue of cross-protection is the use of organic acid as sanitizers in the processing environment. Organic acids are used alone or in combination to sanitize processing equipment; these compounds have been shown to have good antimicrobial activity against a broad spectrum of vegetative cells *(137)*. But the potential for certain organisms, such as *L. monocytogenes*, to develop acid resistance when exposed continuously to an acidic environment may create a problem. Not only would the sanitizer's effectiveness decline, but these acid adapted cells

could develop resistance to other stresses (e.g. heat) or form difficult to remove biofilms on the equipment *(128)*. All of these factors could contribute to the re-contamination of foods with *L. monocytogenes*.

7.4. Bacteria Stress Hardening and Food Safety

Organisms are normally stressed during food processing. Food preservation and the safety of food products heavily rely on these stresses which are considered hurdles for organisms to overcome, especially foodborne pathogens. For example, in the manufacture of cheese, organisms must survive the hydrogen peroxide that may be added to the raw milk, the acid developed during fermentation, the salt added to the curd, and the heat from the thermal treatment. During sausage fermentation, organisms are stressed by salt, the acid developed during fermentation, and the heat treatment *(104)*.

The phenomenon of stress hardening refers to the increased resistance of an organism to certain lethal factors after adaptation to environmental stress *(104)*. This occurrence may lessen the effectiveness of the food preservation hurdles applied to or inherently present in foods, with the potential of compromising the safety of such products. Gahan et al. *(70)* examined the survival of acid adapted and non-acid adapted *L. monocytogenes* in low pH foods and acidified dairy products. Acid adaptation greatly improved the survival of the pathogen in orange juice, salad dressing. cottage cheese, yogurt, and whole-fat cheddar cheese.

Buchanan et al. *(25)* demonstrated that acid adapted *E. coli* cells in apple juice displayed radiation D-values (doses needed to decrease the population by 90%) higher than non-adapted cells. Mazzotta *(110)* found that heat resistance of *E. coli*, *Salmonella*, and *L. monocytogenes* in orange, apple, and white grape juices increased significantly

after acid adaptation. The ability of *L. monocytogenes,* and other pathogenic organisms, to survive harsh environments is of great interest to the food industry as hurdle technology is regularly used by food processors to prevent bacterial growth in foods.

8. Methods to Inhibit the Growth of *Listeria monocytogenes*

In the past few years, ways to inhibit growth of *L. monocytogenes* and to control post-processing contamination of foods by the pathogen have been extensively researched. Although some methods have not yet been approved by the U. S. government for use in certain food products (e.g. irradiation of RTE meats), other methods are being utilized by the food industry to lower the microbial load in raw products and/or to prevent post-processing product adulteration. Some of those methods include the use chemical rinses for carcasses and dips for surface treatment of RTE products and/or the incorporation of additives to processed meats.

Ionizing radiation has been approved for use in a number of food products, including spices, some vegetables, poultry, and fresh and frozen red meats *(32*). Two different processes, gamma rays (cobalt-60 or cesium-137) or electron beams are now used to irradiate such foods. The use of gamma and electron beam irradiation to kill *L. monocytogenes* in RTE meats has been studied by several investigators. Studies have shown that these processes are effective in reducing *L. monocytogenes* in RTE meats *(67, 121)*; it has also been shown that irradiation causes minor quality changes in such products *(184).*

Decontamination technologies on raw meat and poultry have been used extensively evaluated in the United States to reduce microbial load by *L. monocytogenes* and other pathogenic bacteria. The development and commercial application of decontamination

procedures continues to increase. Technologies under evaluation include pH enhancement (9.6) with ammonia gas on boneless lean beef trimmings *(124)*, live animal cleaning/washing, chemical dehairing, removing physical contaminants on carcasses with a knife, spot-cleaning carcasses with a steam vacuum, spray washing/rinsing of carcasses with water (low or high pressures and temperatures) or with chemical solutions (organic acids), and pressurized steam to the sides of the carcass *(51, 88)*. Studies have shown that if applied correctly and under appropriate conditions, these technologies may reduce microbiological counts by approximately 1-3 log CFU/cm^2. Some of these have already been approved and are employed in commercial applications (steam vacuum; carcass spray/washing with water, chlorine, organic acid, or trisodium phosphate; hot water spraying/rinsing; pressurized steam) *(164)*.

8.1. Inhibition of *Listeria* in Ready-to-Eat Foods

Due to the severe outbreaks involving contaminated RTE meats and the zero tolerance policy currently in place, several methods have been implemented, and new methods investigated, in an effort to prevent post-processing contamination of such products. Some methods involved the use of antimicrobials (e.g. acetic acid, sodium acetate) as post-processing dips for frankfurters and/or sausages *(147)*; organic acids are probably most effective as surface treatments since contamination of these products is usually concentrated on the surface following peeling and before packaging *(107)*. Other methods of inhibition involve the addition of antimicrobials to the formulation of such RTE products (e.g. sodium lactate). Lactic acid salts have been studied for their antimicrobial effects in foods although their primary role in RTE products has been as humectants, flavor enhancers, and to increase water holding capacity and cooking yields *(111, 158)*.

Samelis et al. *(146)* demonstrated that the addition of 1.8% sodium lactate (SL) to the formulation of frankfurters significantly inhibited the growth of *L. monocytogenes* for 5 weeks of storage at 4°C. Samelis et al. *(147)* conducted a study to test the effectiveness of organic acids (lactic or acetic) and their salts (sodium acetate, sodium diacetate, sodium lactate, potassium sorbate, or potassium benzoate) as dipping solutions for sliced pork bologna inoculated with *L. monocytogenes*. The authors found that no significant increase in *L. monocytogenes* occurred from day 0 to 120 in bologna dipped for 1 min in 2.5 or 5% acetic acid or 5% sodium diacetate or 5% potassium benzoate. In a similar study, Nuñez et al. *(126)* demonstrated that dipping frankfurters in a 3.4% solution of lactic acid reduced initial numbers of *L. monocytogenes* by approximately 4 logs.

In order to enhance the antimicrobial effect of some dipping solutions, surfactants can be added. Surface-active agents, such as sodium lauryl sulfate (SLS), decrease the surface or interfacial energies between the antimicrobial and in this case, bacterial cells (increasing the contact between the two). Greater inhibition can be achieved by maximizing this interaction *(157)*. Although SLS is used as an emulsifier, wetting agent, and whipping agent in food products such as egg whites and edible fats and oils *(61)*, it possesses some antimicrobial activity or enhances the efficacy of antibacterial compounds. A study by Tamblyn and Conner *(169)* demonstrated that the addition of SLS to 0.5% lactic acid reduced counts of *Salmonella typhimurium* on breast chicken skin under simulated chill. As a post-process dip, the addition of SLS to tartaric or citric acids also increased their antimicrobial activity.

The techniques of using dipping solutions or incorporating antimicrobials into product formulation can be combined in order to obtain an additive or synergistic effect to

eliminate *L. monocytogenes*. The antimicrobial effect of additives used alone or in combination with other antimicrobials against the pathogen, depend largely on the processing method (the final pH, water activity, moisture, fat, nitrite, and salt content of the product) and the storage conditions (temperature and packaging atmosphere) *(16)*.

Some studies have also explored the use of antimicrobial packaging to protect RTE meats from contamination with *L. monocytogenes*. Edible films such as whey protein, corn zein, soy protein, casein, collagen, and chitosan can be used as carriers for antimicrobial compounds. A study by Eswaranandam et al. *(52)* demonstrated that soy protein edible films containing organic acids and nisin reduced *L. monocytogenes*, *S. gaminara*, and *E. coli* O157:H7 by 2.8, 6, and 2.1 log CFU/ml, respectively. Cagri et al. *(27)* examined the use of whey protein isolate containing p-aminobenzoic acid (PABA) to inhibit *L. monocytogenes*, mesophilic aerobic bacteria, lactic acid bacteria, and yeast/molds in frankfurters stored at 4°C. After 42 days, *Listeria* populations remained unchanged in PABA coated frankfurters while numbers of the pathogen in natural casings increased by 2.5 logs. The authors also obtained 1-3 log reductions with the PABA films on the populations of the other organisms tested.

9. Additional Methods to Inhibit *Listeria*

The development of new and innovative techniques for the control of pathogenic organisms in foods is ongoing. Some methods being explored or currently used by the food industry include mild heating, modified atmosphere, vacuum packaging, high hydrostatic pressure, and the use of natural antimicrobials *(1)*. Consumers are largely responsible for the continuous development of new technology, procedures, and food products. The food industry has been trying to keep up with consumers' demands for

more convenient foods (such as RTE products), containing fewer preservatives (e.g. salt, nitrites), and of higher quality (both from a sensory and a microbial safety perspective).

The increasing interest in more "natural" foods and ingredients has prompted the food industry to search for alternative antimicrobial agents to be used in foods. Concerns about the use of conventional antimicrobial agents have been debated for several decades due to potential unfavorable effects on food production processes *(94, 132)*. The combination of these factors, have driven the food industry and the research community to focus their efforts on the use of naturally occurring compounds as antibacterial agents.

Natural antimicrobials can come from barks, stems, leaves, flowers, seeds, fruits, various animal tissues, and microorganisms. Publicized sources of natural antimicrobials include herbs (leaves), spices (roots, flowers, seeds, and barks), fruits, milk, eggs, and lactic acid bacteria used in fermentation *(163)*.

9.1. History of Plant Products as Antimicrobial Agents

Throughout history, records show that herbs and spices have been used for medicinal purposes and to flavor foods. Historical passages tell us that as early as 6000 B.C. in China, botanicals were used for flavor and medication *(49)*; around 1550 B.C., the ancient Egyptians not only utilized spices to preserve foods, but also to embalm their dead *(42)*. Much scientific research in the 19th century on plant products has been focused on the antimicrobial properties of herbs, spices, and their constituents; the interest in the properties of these compounds continues to grow *(183)*.

Many of the studies on herbs and spices have been conducted on their essential oils, major components of the oils, and their antimicrobial effect on various organisms. One of the earliest reports on the antimicrobial activity of plant extracts was published by

Chamberland in 1887 *(33)* who tested over 100 essential oils against spores of *Bacillus anthracis* and found that the vapor of the cinnamon oil was lethal to the spores. In 1911, Hoffman and Evans *(86)* reported that cinnamon, mustard, and clove were good preservatives for apple sauce. They also found that allspice and nutmeg had some antimicrobial activity, while black pepper, cayenne pepper, and ginger did not. In the past decade, a majority of the research on plant extracts have been on screening studies to determine which extracts are effective, under which conditions, and against what organisms. The activity of such extracts in microbiological media may not be the same as their activity in food systems although antimicrobial efficacy of a few extracts has been shown in both.

9.2. Plants and Their Components

It is estimated that plants on Earth range from 250,000 to 500,000 species, of which approximately 1 to 10% are used as foods by humans and animals; possible more are used for medicinal purposes *(22, 120)*. Plants have the ability to synthesize aromatic substances such as phenols and their oxygen-substituted derivatives *(73)*. At least 12,000 of these metabolites have been isolated, although that number is believed to be less than 10% of the total *(149)*. Many of these secondary metabolites serve as plant defense mechanisms against predators (microbes, insects, etc.). Others give plants their odors (e.g. terpenoids), pigments (e.g. quinones and tannins), and flavors (e.g. terpenoid capsaicin from chili peppers) *(37)*.

Some of the metabolites produced by plants have been shown to possess antimicrobial properties. Several of these compounds have been placed in categories; these include phenolics and polyphenols, flavones, flavonoids, flavonols, quinones, tannins, coumarins,

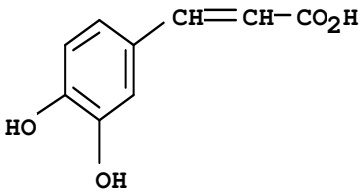
terpenoids, alkaloids, lectins, and polypeptides *(37)*. The antimicrobial activity of some of the compounds in the above categories involve disruption of bacterial cell walls and cell membranes, complex formation with extracellular proteins, surface-exposed adhesins, and cell wall polypeptides, and inactivation of transport proteins *(37)*. Figure 2 illustrates the structures of a few of these antimicrobials and their respective categories.

9.3. Plant Antimicrobial Agents Today

A list of some plant products shown to possess antimicrobial activity is given in Table 3. As already mentioned, thousands of plants on Earth may harbor compounds exhibiting antimicrobial activities. The use of new and improved techniques to isolate compounds and to develop applications for plant-derived antimicrobials, keeps the list growing. The following nine compounds have been shown by many research studies to be highly antimicrobial: basil *(8, 50)*, black cumin *(44, 156)*, cinnamon *(9, 44, 168)*, clove *(4, 9, 44)*, garlic *(4, 44)*, mustard *(116, 168)*, oregano *(9, 10, 14, 50, 129)*, rosemary *(9, 10)*, and thyme *(8, 129, 168)*. A large number of these studies are carried out using essential oils or extracts of the plant products. The methods used to isolate these substances may be as simple as crushing spices with a pestle and mortar then mixing with water *(4)* or more elaborate such as using alcohol extractions *(44)* or distillation *(129)*. A small portion of the research is actually conducted using fresh plants. A few reasons that restrict the use of fresh products include: high pigmentation of plant parts may hinder the use of certain equipment (e.g. spectrophotometers) to further research these products, strong odors and flavors (sensory), higher concentrations needed to achieve effective microbial inhibition, and inability to precisely determine the active inhibitory substance. Although barriers exist, research continues in hopes of overcoming the obstacles and maximizing the use of

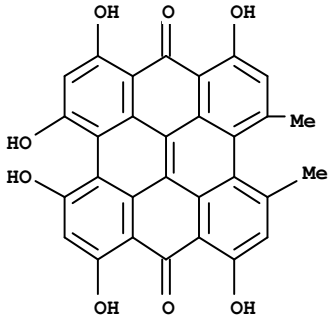
Phenolics and Polyphenols

Phenolic acids



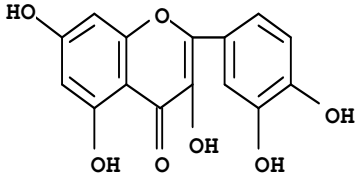
caffeic acid (thyme)
Other example: cinnamic

Quinones



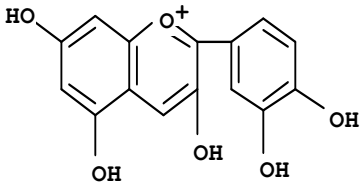
hypericin (St. John's wort)
Other example: quinone

Flavonols



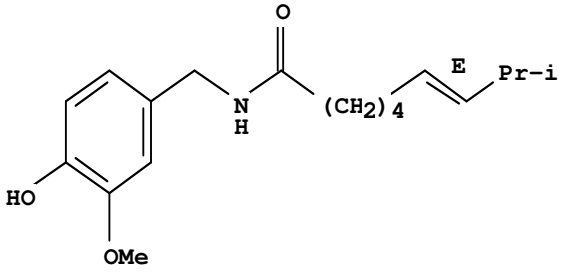
quercetin (cranberry)
Other example: myricetin

Anthocyanidins



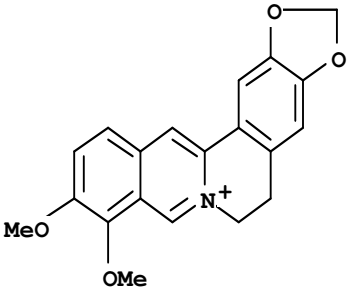
cyanidin (raspberry)
Other example: delphinidin

Terpenoids



capsaicin (chili peppers)
Other example: menthol

Alkaloids



berberine (Oregon grape)
Other examples: harmane

Figure 2. Structures of common plant-derived antimicrobials under their specific categories, names in parenthesis are familiar sources of the compounds.

Table 3. Plant-derived compounds having antimicrobial activity.

Common Name	Scientific Name	Antimicrobial Category
Allspice	*Pimenta dioica*	Essential oil
Basil	*Ocimum basilicum*	Terpenoids
Bay	*Laurus nobilis*	Terpenoids
Black cumin	Bunium persicum	Terpenoids
Black pepper	*Piper nigrum*	Alkaloid
Caraway	*Carum carvi*	Coumarins
Chili pepper	*Capsicum annuum*	Terpenoid
Clove	*Syzygium aromaticum*	Terpenoid
Coriander	*Coriandrum sativum*	Terpenoid
Cranberry	*Vaccinium* spp.	Polyphenols
Dill	*Anethum graveolens*	Terpenoid
Garlic	*Allium sativum*	Sulfoxide
Grapefruit (peel)	*Citrus paradisa*	Terpenoid
Green tea	*Camellia sinensis*	Flavonoid
Horseradish	*Armoracia rusticana*	Terpenoids
Licorice	*Glycyrrhiza glabra*	Phenolic alcohol
Onion	*Allium cepa*	Sulfoxide
Orange (peel)	*Citrus sinensis*	Terpenoid
Peppermint	*Mentha piperita*	Terpenoid
Rosemary	*Rosmarinus officinalis*	Terpenoid
Thyme	*Thymus vulgaris*	Terpenoid
		Polyphenols
		Flavones
Turmeric	*Curcuma longa*	Terpenoids
Wintergreen	*Gaultheria procumbens*	Polyphenols

Adapted from Cowan, 1999 and Draughon, 2004

plant-derived antimicrobials.

9.4. Selected Plant-Derived Antimicrobials

Cranberry

Although many in the scientific community would not find the evidence reliable, traditionally, cranberry has been used to treat and prevent urinary tract infections *(108)*. Cranberries were used by Native Americans for bladder and kidney diseases. During the 17th century therapeutic uses of the fruit included the relief of blood disorders, stomach and liver disease, vomiting, scurvy, and cancer *(159)*. Today, many continue to use the fruit for specific medicinal purposes, others simply for its health benefits (antioxidants); in addition, a number of research studies have demonstrated that cranberries possess antimicrobial activity.

The American cranberry (*Vaccinium macrocarpon*) is about 88% water and contains high amounts of ascorbic acid. Other biologically active substances present in cranberries include phenols, polyphenols, and phenolic acids. Chen et al. *(34)* found that freshly squeezed cranberry juice contained flavonoids (56%) and other phenolic compounds (44%). In this study, the major flavonols (class of flavonoids) found were quercetin and myricetin; the major phenolic compound was benzoic acid followed by the phenolic acids *p*-coumaric and sinapic *(34, 185)*. Benzoic and phenolic acids were found to occur mainly in the bound form, with only approximately 10% occurring as free acid *(185)*. Cranberry juice also contains quinic, malic, and citric organic acids in lower concentrations *(36)*. Another group of polymeric phenolic substances found in cranberry are the proanthocyanidins (condensed tannins). These compounds are believed to prevent a number of illnesses and are commonly found in beverages such as green teas and red

wines *(155)*.

Benzoic acid is one of the oldest chemical preservatives used by the drug, cosmetic, and food industries *(35)*. Cranberries contain both phenolic compounds and phenolic acids that have antimicrobial activities. Phenolic compounds have been used for their antimicrobial and antiseptic properties for over 100 years *(41)*. In this regard, cranberry, and its extracts and derivatives should be ideal for the control of pathogenic and spoilage organisms in foods. Puupponen-Pimiä et al. *(138)* obtained a 3 log reduction in *S. typhimurium* when cells were incubated for 2 h in culture media containing 1mg/ml of cranberry extract. In another study, the antimicrobial activity of oregano extract increased with the addition of cranberry extract. This mixture was bacteriocidal to *L. monocytogenes* in beef and fish slices *(101)*. Nogueira et al. *(125)* demonstrated bacterial inactivation (5 log) of *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* in cranberry concentrate at or above temperatures commonly used to transport or store such products.

Grape Seed

The grape (*Vitis vinifera*), is one of the fruit crops most extensively grown in the world. Numerous investigations have demonstrated that grapes contain large amounts of phenolic compounds. Many of these compounds have been shown to have a positive effect on human health including lowering low-density lipoprotein *(119)* and reducing coronary heart disease *(177)*. Extracts from grape seeds and grape pomace (skin and seed), by-products of the wine and juice industries, have been used as natural antioxidants and are known to also contain a large number of phenolic and polyphenolic compounds *(13)*. Grape seeds are particularly rich in tannins. Grape seed extracts made to contain > 90% proanthocyanidins have already appeared in the market for various

applications including their addition into cereals, bars, certain beverages *(93)*.

The antibacterial effects of grape seed extracts have been demonstrated both *in vitro* and *in vivo* in a number of studies. Baydar et al. *(12)* was able to show the antimicrobial activity of grape seed extracts against food spoilage and pathogenic organisms such as *Bacillus subtilis*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Bacillus cereus*, and *L. monocytogenes* using the paper disc diffusion method. In another study, these authors tested grape seed extract against fifteen bacteria using the agar well diffusion method. They showed that all tested bacteria were inhibited by grape seed extract *(13)*. Ahn et al. *(3)* were able to inhibit *E. coli* O157:H7, *S. typhimurium*, and *L. monocytogenes* in raw ground beef using 1% grape seed extract.

9.5. Plant-Derived Antimicrobials - Application in Foods

The activity of many plant products against pathogenic organisms has been extensively studied in microbiological media. However, only a small number of those antimicrobials have actually been tested in foods. The presence of lipids, surface-active agents, and proteins in food systems have been shown to reduce the antibacterial activity of some plant extracts *(39, 45)*. If high amounts or high concentrations of plant extracts have to be used in order to counteract the action of those substances, detrimental changes in the flavor and other attributes (sensory) of the food products may occur *(2)*.

Research to demonstrate the effectiveness and possible application of plant extracts in foods continues to increase. Larson et al. *(98)* demonstrated that hop extracts could be used to inhibit *L. monocytogenes* in culture media and in certain foods; they concluded that these extracts would work best in minimally processed foods with low fat content. Cinnamon powder was examined for activity against *L. monocytogenes* in meat and

cheese. When the foods were held at 30°C, 6% cinnamon reduced the organism by 1-2 logs in comparison to the untreated controls *(118)*. Yin and Cheng *(182)* demonstrated that two garlic-derived organosulfur compounds significantly reduced total aerobes and inhibited the growth of *S. typhimurium*, *E. coli* O157:H7, *L. monocytogenes*, *Staphylococcus aureus* and *Campylobacter jejuni* in ground beef. In another study, populations of *E. coli* O157:H7, *S. typhimurium*, and *L. monocytogenes* were reduced by 1.08, 1.24, and 1.33 log CFU/g, respectively, in raw ground beef treated with either 1% Pycnogenol (pine bark extract), 1% ActiVin (grape seed extract), or 1% oleoresin rosemary after 9 days of refrigerated storage *(3)*.

Clove (eugenol) extract reduced *L. monocytogenes* and *Aeromonas hydrophila* by 1.4 and 2.5 log, respectively, in refrigerated skinless chicken breast meat after 4 days at 5°C *(81)*. Pimento extract reduced *L. monocytogenes* and *A. hydrophila* by 1.3 and 1.2, respectively, in refrigerated cooked beef slices after 4 days *(82)*. Tsigarida et al. *(173)* demonstrated that 0.8% oregano essential oil reduced the initial bacterial population, including lactic acid bacteria and *L. monocytogenes*, by 2 to 3 log CFU/g on naturally contaminated beef fillets stored at 5°C, regardless of the gaseous environment used to package the meat.

Although some essential oils have been shown to possess good antimicrobial activity, the high concentrations needed to obtain inhibition in certain foods *(8)* could reduce their sensory acceptability. In order overcome this obstacle, some researchers have encapsulated the compounds in surfactant micelles. The increased solubility of an essential oil due to the surfactant, could improve the interaction between the active compound and the microorganism. A study by Gaysinsky et al. *(72)* showed that very low

concentrations of eugenol and carvacrol (0.15% and 0.025% , respectively) encapsulated with 1% surfactant were sufficient to inhibit growth of four strains of *E. coli* and three strains of *L. monocytogenes* in an aqueous system. Therefore, the use of a surfactant such as SLS could improve the antimicrobial activity of certain plant extracts in foods.

The potential use of edible films in combination with natural antimicrobial plant extracts has also been studied. Theivendran et al. *(171)* evaluated the inhibitory effect against *L. monocytogenes* of soy protein film containing 1% green tea (GT) or 1% grape seed (GS) extract and nisin (10,000 IU) coated on turkey frankfurters. The authors obtained greater than 2 log reductions in samples coated with nisin combined with either GT or GS after 28 d at 4 and 10°C.

9.6. Safety and Regulation

Most herbs, spices and other natural seasonings and flavorings including essential oils, oleoresins, and natural extracts (solvent-free), fall under the category of GRAS (generally recognized as safe by qualified experts under the conditions of intended use) because they are used at low levels to enhance flavors in foods *(60)*. For preservation purposes, higher levels than those normally used for flavor (intended use) may be needed; if so, the submission of a GRAS notice to the FDA would be required.

Although most herbs and spices are considered GRAS and have been utilized safely by humans for centuries part of the population is sensitive to some herbal compounds and strong aromatic ingredients. Some of the reported sensitivities include contact dermatitis (cinnamon, clove, ginger, allspice) *(92)* and hypersensitivity reaction (basil, oregano, thyme) *(18)*. Some of these sensitivities should be taken into account when using plants extracts in foods.

9.7. The Future of Natural Antimicrobials

Naturally occurring antimicrobials have been found to be effective against a number of spoilage and pathogenic organisms when used individually and in combination with other methods of food preservation. In addition, these antimicrobials are found in large quantities in the environment and in light of consumer demands for more "natural" food ingredients, the interest in these substances continues to rise.

Although much has been accomplished thus far, to expand the use of natural antimicrobials, there is a need for more research to look further into: their efficacy and functionality in foods, the standardization of isolation/extraction methods, toxicology and safety in food formulations, interactions with food components, mechanisms of action against organisms, influences on quality (nutritional and sensory), commercial use, and economical production *(163).*

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CHAPTER 3. INHIBITION OF *LISTERIA MONOCYTOGENES* BY PLANT-DERIVED ANTIMICROBIALS IN BRAIN HEART INFUSION BROTH AT 4 AND 10ºC

A paper to be submitted to the Journal of Food Protection

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ABSTRACT

The inhibition of five strains of *Listeria monocytogenes* by selected plant-derived compounds in Brain Heart Infusion (BHI) broth was evaluated. *L. monocytogenes* cells were grown at 35ºC for 18-20 h in BHI broth, harvested by centrifugation, and washed once in 0.85% saline. The antimicrobials were added separately to 100 ml of BHI broth in Erlenmeyer flasks to obtain the following concentrations: 0, 1, 3, 5, and 10% for cranberry; 0, 1, 5, and 10% for grape seed; 0, 1, 2, and 3% for oregano; 0, 3, 5, and 10% for green tea. Broth samples were inoculated with the washed *L. monocytogenes* cells to give a final bacterial concentration of 10^5 CFU/ml and incubated at 4 and 10ºC. On days 0, 1, 7, 14, 28, and 42, samples of inoculated broth were plated in duplicate on BHI agar and bacterial colonies counted after incubation at 35ºC for 24 h. Cranberry concentrate (3 or 5%) was bacteriostatic while 10% was bacteriocidal (at 4 and 10ºC). Grape seed extract (1%) was bacteriostatic while 5 and 10% were bacteriocidal at both temperatures. At 4ºC oregano extract (1%) was bacteriostatic whereas 2 and 3% were bacteriocidal. At

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10ºC 3% oregano was also bactericidal. Green tea extract was bacteriocidal at all concentrations tested and at both storage temperatures. The results of these experiments indicate that cranberry concentrate, grape seed extract, oregano extract, and green tea extract are good antimicrobials that may be potentially utilized by the food industry to inhibit the growth of *Listeria monocytogenes*. Further studies are necessary to determine the effectiveness of these plant-derived compounds for controlling human enteric pathogens in actual food systems.

INTRODUCTION

The post-processing contamination of ready-to-eat meats with *Listeria monocytogenes* has become a threat not only to consumers but to food manufacturers as well. The consumption of food products such as hot dogs, deli turkey meat, and corn salad has been attributed to recent outbreaks of listeriosis *(19)*. *L. monocytogenes* has been isolated from poultry, meat, milk and milk products, seafood, and vegetables *(10)*. Food processors, food safety researchers, and regulatory agencies have directed much of their attention to the possible contamination of RTE food products by *Listeria* during processing and to ways of controlling the pathogen following packaging. This is primarily due to the hardy nature of the organism and the absence of a cooking step prior to the consumption of such foods.

Many characteristics make this organism difficult to control, including its ability to survive under adverse environmental conditions. *L. monocytogenes* is a non-spore forming, psychrotrophic, gram-positive foodborne pathogen that can persist on processing equipment for long periods of time *(19)*. *L. monocytogenes* is an opportunistic

pathogen and the disease that it causes is manifested in children, the elderly, and immunocompromised individuals as meningoencephalitis, and/or septicemia *(14, 20)*. Severe consequences due to infection have been observed in pregnant women; perinatal infections may occur transplacentally leading to abortion and stillbirth. Meningitis, gastroenteritis, endocarditis, and disseminated granulomatous lesions can occur in adults *(20)*. Outbreaks of listeriosis involving deli turkey meat have been identified in 10 states since May 2000. These cases included four deaths and three miscarriages or stillbirths *(17)*.

Natural plant-derived compounds have been generally used as herbs and spices *(2*). Since prehistoric times, spices have been used as condiments to enhance the flavor of foods. History also tells us that many spices and their oils have been found to possess some antimicrobial activity. The use of spices as preservatives dates back to 1550 B. C., when the ancient Egyptians used these substances to preserve food and to embalm their dead *(6)*.

Although early studies cast doubt on the effectiveness of spices as food preservatives *(13*), it is now widely accepted that spices have germicidal and medicinal properties *(3)*. For example, compounds extracted from clove, garlic, mustard, onion, and cinnamon have been found to be bacteriostatic; however, there is a significant variation in resistance of different organisms to the same spice and of the same organisms to different spices *(3)*. Some of these differences can be attributed to changes in test methods, bacterial strains, and sources of antimicrobial samples used *(9)*. Natural extracts actively used today against microorganisms include cloves, rosemary, sage, garlic, hops, thyme, and coriander *(2)*. It has been reported that more than 1,340 plants are known to produce

potential antimicrobial compounds *(24)*. The compounds present in some of these plants may be lethal to microbial cells or they may inhibit the production of a metabolite *(5)*.

The interest by the food industry in the use of plant-derived compounds has increased in recent years due to consumers demand for more "natural" foods with less chemical preservatives. Many research efforts have been focused on the use of such plant-derived substances as natural antimicrobials to inhibit the growth of pathogenic organisms in foods.

The objective of this study was to evaluate the inhibitory effect of cranberry concentrate, grape seed extract, oregano extract, and green tea extract on *L. monocytogenes* in Brain Heart Infusion broth at 4 and 10ºC over a 42-day period.

MATERIALS AND METHODS

Microorganisms and culture conditions. Five strains of *Listeria monocytogenes* were used in this study. Strains H7962-4b, H7762-4b, H7596-non4b, H7969-4b, and NADC-2045 were obtained from the culture collection of the Microbial Food Safety Laboratory at Iowa State University. The stock cultures were kept in Brain Heart Infusion (BHI) broth (Difco Laboratories, Detroit, Michigan) supplemented with 10% glycerol at -70ºC. Each culture was transferred at least twice, successively, in 10 ml of BHI broth and incubated at 35ºC overnight (18 h) prior to each experiment.

Preparation of cell suspension. *L. monocytogenes* strains were each grown separately in Erlenmeyer flasks containing 100 ml of BHI broth. After 18 h the cells were harvested by centrifugation (Sorvall® Super T21, Sorvall Product, L.P., Newtown, Connecticut) at 10,000 x g for 10 min at 4ºC, washed once in saline (0.85% NaCl) (Fisher Scientific

Company L.L.C., Pittsburgh, Pennsylvania) to remove left over growth medium, then suspended in fresh saline (4ºC). The cell suspensions (cocktail of the five strains) were adjusted to give approximately 10^7 CFU/ml.

Plant-derived compounds. The antimicrobials and the concentrations used in this study were selected based on preliminary data and existing scientific literature suggesting these substances would be inhibitory against *L. monocytogenes* in vitro. The compounds included cranberry concentrate (CB) (Tree of Life, St. Augustine, Florida) in liquid form, grape seed extract (GS) (Gravinol®, Kikkoman Corporation, Tokyo, Japan) in powder form, oregano extract (OR) (Origanox, Barrington Nutritionals, Harrison, New York) in powder form, and green tea extract (GT) (Rexall Sundown, Boca Raton, Florida) in capsule form. The capsules were opened by hand and the powder collected in a sterile flask; to minimize contamination this was performed under a laminar flow hood. CB, OR, and GT easily dissolved in BHI, although some insoluble debris was found in the GT. GS was the least soluble of the four substances.

Total phenolic content. The concentration of total phenolics of each antimicrobial was determined by the Price and Butler method *(27)*. The assay is based on the oxidation of phenolate ion in which ferric ions are reduced to the ferrous state, detected by the formation of the Prussian blue complex $[Fe_4[Fe(CN)_6]_3]$ with a potassium ferricyanide reagent *(16)*. A 1% solution of each antimicrobial in 10% EtOh and deionized water were prepared. The absorbance of each sample was measured at 720 nm and the phenolic contents expressed as catechins equivalents (% concentration in 1% sample).

Treatments and microbiological analysis. The antimicrobials were added separately to 100 ml of BHI broth in Erlenmeyer flaks to obtain the following concentrations: 0, 1, 3,

5, and 10% (v/v) for cranberry; 0, 1, 5, and 10% (w/v) for grape seed; 0, 1, 2, and 3% (w/v) for oregano; 0, 3, 5, and 10% (w/v) for green tea. Flasks were inoculated with 0.1 ml of the five strain cocktail of *L. monocytogenes* (to give a final concentration of approximately 10^5 CFU/ml). Inoculated broth samples were incubated at 4 and 10ºC and tested on days 0, 1, 7, 14, 28, and 42. Appropriate dilutions in 0.1% peptone water were surface-plated, in duplicate, onto plates of BHI agar. All inoculated plates were incubated at 35ºC and bacterial colonies counted after 24 h.

Measurement of pH. The pH of each antimicrobial was measured at day 0 (prior to inoculation) and at test day 42 (both storage temperatures) using an Orion Model 525 pH meter (Orion Research, Inc., Boston, Massachusetts) fitted with a glass electrode.

Statistical analysis. Data from three independent replications were subjected to statistical analysis. Data were analyzed using SAS 9.1 (SAS Institute, Cary, North Carolina). Differences between samples were determined using Tukey's honestly significant difference pairwise test ($p<0.05$).

RESULTS AND DISCUSSION

A number of natural compounds found in dietary plants, such as extracts of herbs and fruits, have been shown to possess antimicrobial activities against *L. monocytogenes (2)*. Although plant extracts differ significantly in their ability to inhibit growth of pathogens, in the present study, the four compounds, cranberry concentrate, grape seed extract, oregano extract, and green tea extract were effective at inhibiting growth of *L. monocytogenes* in Brain Heart Infusion broth.

Figure 1 shows the effect of cranberry concentrate (CB) on the growth of *L.*

monocytogenes at 4ºC and at 10ºC based on enumeration of bacterial colonies on BHI agar. Results indicate that CB was bacteriostatic at concentrations of 3 and 5% at both storage temperatures over the storage period (significantly different from the control, $p<0.05$). CB was bacteriocidal at a 10% concentration at both temperatures, with approximately a 3 log reduction at 4ºC and 5 log reduction at 10ºC both after 7 days. There were no significant differences among CB concentrations between the two storage temperatures.

Cranberries are known to contain benzoic acid, one of the oldest and most commonly used food preservatives. This compound also occurs naturally in raspberries, plums, prunes, cinnamon, and cloves *(4)*. Studies have shown that benzoic acid and other low-molecular-weight components in cranberry juice are antifungal at pH 2.8 (the natural pH of cranberries) *(4)*. At this pH the undissociated form of the acid molecule diffuses through the microbial cell membrane, where it ionizes, causing the acidification of the interior of the cell *(4)*. Studies have shown that benzoic acid inhibits microbial growth by interfering with substrate transport, oxidative phosphorylation, and by preventing the uptake of certain amino acids *(8)*. Such interference with the microorganism's metabolic processes is likely to result in bacteriostasis as observed with 3 and 5% CB in the present study. Therefore, it is likely that the pH lowering effect of 3, 5, and 10% CB contributed to inhibition of *L. monocytogenes*.

The antimicrobial properties of cranberries have also been attributed to the presence of phenolic and polyphenolic compounds such as flavonols (flavonoids) and proanthocyanidins (condensed tannins) *(7)*. Antimicrobial activity of phenolics has been shown against several bacteria including *Escherichia coli*, *Staphylococcus aureus*, and

Clostridium perfringens (24). Most studies have shown that gram-positive bacteria are generally more sensitive to these types of compounds. The phenolic compounds in the cranberry concentrate may exert antimicrobial activity by damaging the cytoplasmic membrane to cause leakage of cellular contents *(24).* The total phenolic concentration in 1% CB was found to be 0.01%. It is likely that the phenolic compounds in 10% CB may have contributed to the death of *L. monocytogenes* via disruption of the cytoplasmic membrane.

Figure 2 shows the effect of grape seed extract (GS) on the growth of *L. monocytogenes* at 4ºC and at 10ºC based on enumeration of bacterial colonies on BHI agar. Results indicate that GS was bacteriostatic at a 1% concentration at both storage temperatures (significantly different from the control). The extract was bactericidal at concentrations of 5 and 10% at both temperatures, with approximately a 3 log reduction at 4ºC and a 5 log reduction at 10ºC after 7 days at both concentrations (significantly different from each respective control). There were no significant differences among concentrations between the two storage temperatures.

Like cranberries, grape seeds are abundant in phenolic compounds. Grape seeds have been shown to contain high amounts of catechins (flavonoids) and tannins *(21).* Proanthocyanidin-rich grape seed extracts have been produced and many made commercially available *(25).* Many of these compounds possess a wide range of pharmacological activities (including antiulcer properties) *(21)*; they have been investigated for their antioxidative, antimutagenic, and anticarcinogenic activities *(2).* However, there is little information with regard to the inhibitory effects of the extracts on foodborne pathogens. The total phenolic concentration in 1% Gravinol® was found to be

0.95%. Based on the high phenolic content of GS we can assume that, as with CB, those compounds are responsible for the inhibitory effect of the extract on *L. monocytogenes*.

The presence of fatty acids may also contribute to the inhibitory effect of GS. Fatty acid content in grape seeds (% whole seed) include: 2.4% of C18:0 (stearic acid), 3.4% of C18:1 (oleic acid), and 14.2% C18:2 (linoleic acid). Generally, fatty acids are most effective against gram positive bacteria. With regard to antibacterial effects, the most effective chain length for saturated fatty acids is 12 carbons, the most effective monounsaturated is pamitoleic, and the most active polyunsaturated is linoleic *(15)*. Various mechanisms for the antimicrobial activity of fatty acids have been postulated. Some studies have suggested that antimicrobial activity is due to inhibition of membrane transport; more specifically, that unsaturated fatty acids undergo oxidation and the free radicals formed are able to inhibit bacteria by attaching to critical sites of the cell membrane *(18)*.

Figure 3 shows the effect of oregano extract (OR) on the growth of *L. monocytogenes* at 4ºC and at 10ºC, respectively. Compared to the control, all concentrations of OR significantly inhibited the growth of *L. monocytogenes* at 4°C ($p<0.05$). Although OR was bacteriostatic at a 1% concentration at 4ºC, no inhibition was found at the same concentration at 10ºC. The extract was bactericidal at concentrations of 2 and 3% at 4ºC and 3% at 10ºC, with approximately a 2 log reduction at 4ºC (both concentrations) and 4 log reduction at 10ºC after 7 days.

Origanox is a natural extract from the edible herb species belonging to the Labitae family (such as *Origanum vulgare* and *Salvia officinalis*). As in cranberry and grape seed, phenolic compounds in oregano have been shown to have antimicrobial activity. The two

major phenolic compounds found in oregano include thymol and carvacrol. The reported antimicrobial properties of thymol and carvacrol are 20.0 and 1.5 times that of phenol, respectively *(1)*. The total phenolic concentration in 1% OR was found to be 0.18%.

A number of studies have shown the antimicrobial activity of oregano extracts against certain foodborne pathogens. For example, Seaberg et al. *(22)* demonstrated that oregano extract effectively inhibited the growth of *L. monocytogenes* in a broth system after 24 h and in a meat system after 48 h.

Figure 4 shows the effect of green tea extract (GT) on the growth of *L. monocytogenes* at 4ºC and at 10ºC. Results indicate that all concentrations of GT significantly inhibited the growth of *L. monocytogenes* irrespective of storage temperature ($p<0.05$). GT was bacteriocidal at all concentrations with approximately a 2 log reduction at 4ºC and 4 log reduction at 10ºC after 7 days. There were no significant differences among treatments between the two storage temperatures.

The antimicrobial properties of tea have also been attributed to the presence of polyphenolic compounds, which account for up to 15% of the dry weight of green tea leaves *(11)*. The active components responsible for such biological activities are catechins, such as epicatechin, epicatechin gallate, and epigallocatechin gallate *(26)*. In all tea catechins, the epigallocatechin gallate and epicatechin gallate forms accounts for about 36 and 24%, respectively *(23)*. The total phenolic concentration in 1% GT was found to be 0.18%. Tea extracts have shown strong antimicrobial activity against a wide variety of microorganisms, including methicillin resistant *S. aureus*, enteropathogenic bacteria, and fungi *(26)*. A study of the mechanism of antibacterial activity of tea catechins showed that the primary target site is the bacterial membrane *(12)*.

Table 1 shows the pH values for cranberry extract, grape seed extract, oregano, and green tea in BHI at all concentrations and at both 4ºC and at 10ºC at days 0 and 42. *L. monocytogenes* can grow at pH values from 4.4 to 9.4. The results indicated that the pH of the BHI containing either antimicrobial fell within the range of growth of *L. monocytogenes*. Therefore, we did not expect pH to have a strong inhibitory effect on the pathogen under these growth conditions.

Consumers' desire for so-called "natural" foods (less or no synthetic preservatives added) has also been increasing *(24)*. Therefore, there is a growing trend towards more widespread use of natural antimicrobials. Isolation, purification, and incorporation into foods of some natural plant extracts, or their constituents, are being researched by many scientists. The challenge is that the addition of the extracts should not unfavorably affect sensory, nutritional, and safety characteristics of the food.

The results of the present study indicate that cranberry concentrate, grape seed extract, oregano extract, and green tea extract are effective antimicrobials that may be potentially utilized by the food industry to inhibit the growth of *L. monocytogenes*. Further studies are necessary to determine the effect of these natural antimicrobials in appropriate food systems.

ACKNOWLEDGMENTS

The authors thank Kikkoman Corporation (2-1-1, Nishi-Shinbashi, Minato-ku, Tokyo, Japan, zip code: 105-8428) for providing the grape seed extract for all of the experiments. Dr. White at Iowa State University for giving us permission to use the materials and equipment in her laboratory for the phenolic content experiment, Jose

Gerde for his assistance with that experiment, and the statistical analysis and expertise provided by Man-Yu Yum (Department of Statistics, Iowa State University).

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Table 1. The pH values of cranberry concentrate, grape seed extract, oregano, and green tea in BHI at all concentrations and at days 0 (before inoculation) and at test day 42 (at 4 and 10°C). Values are an average of three independent replications.

TREATMENT	CONCENTRATION	Day 0	Day 42	
			4ºC	10ºC
Cranberry Extract (CB)	0%	7.5	5.6	5.5
	1%	6.7	4.8	4.8
	3%	5.4	4.6	4.6
	5%	4.7	4.5	4.6
	10%	4.0	3.9	3.6
Grape Seed Extract (GS)	0%	7.5	5.7	5.6
	1%	7.3	7.1	6.8
	5%	6.9	6.6	6.5
	10%	6.5	6.1	6.0
Oregano (OR)	0%	7.5	5.7	5.6
	1%	7.1	5.1	5.0
	2%	6.8	6.3	5.7
	3%	6.5	6.0	5.6
Green Tea (GT)	0%	7.5	5.8	5.6
	3%	7.0	6.2	6.0
	5%	6.8	5.9	5.8
	10%	6.3	5.5	5.5

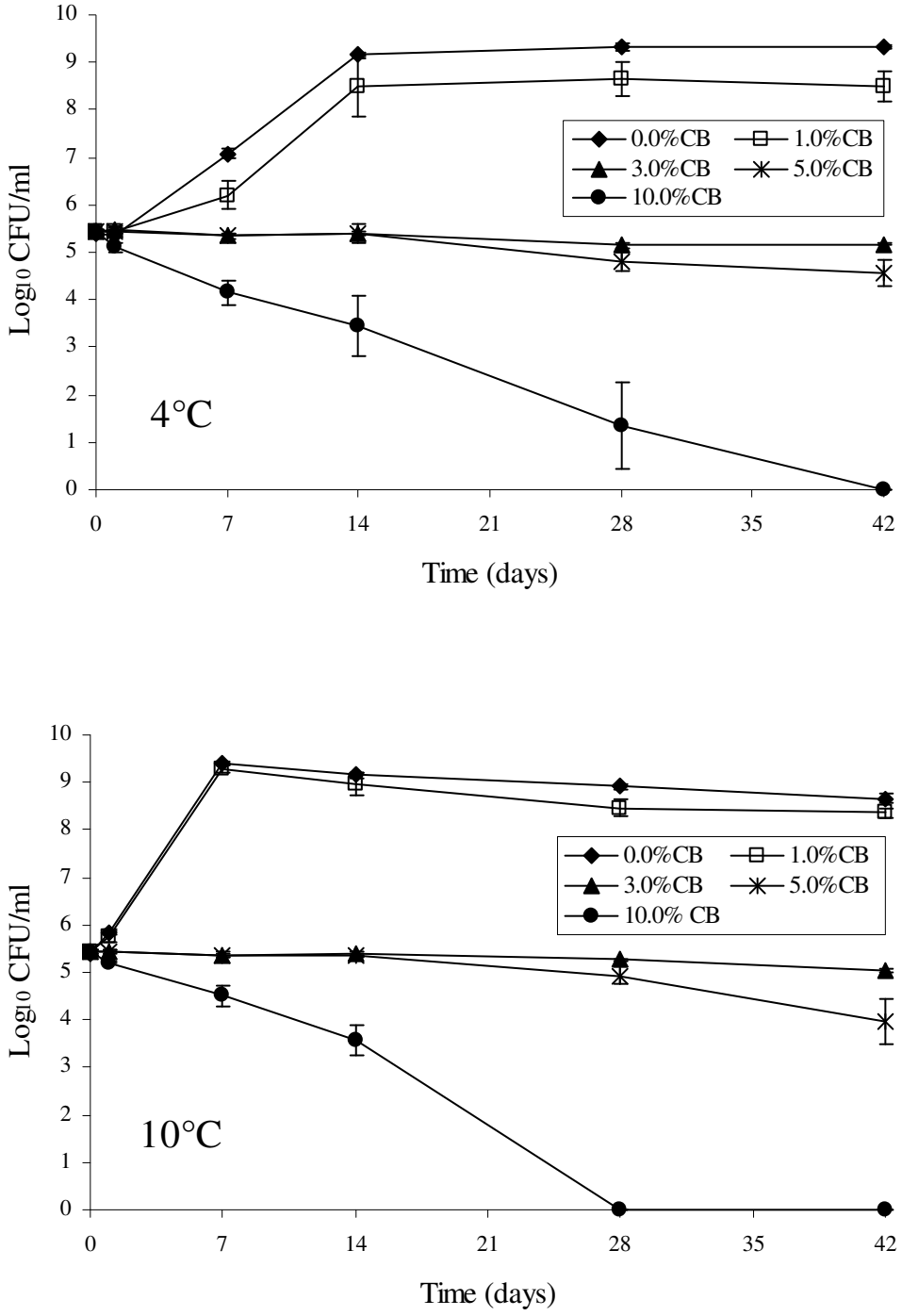

Figure 1. Effect of cranberry concentrate (CB) on the growth of *Listeria monocytogenes* in BHI at 4 and 10°C. Values at each time point are an average of three independent replications. Error bars represent standard error of the mean.

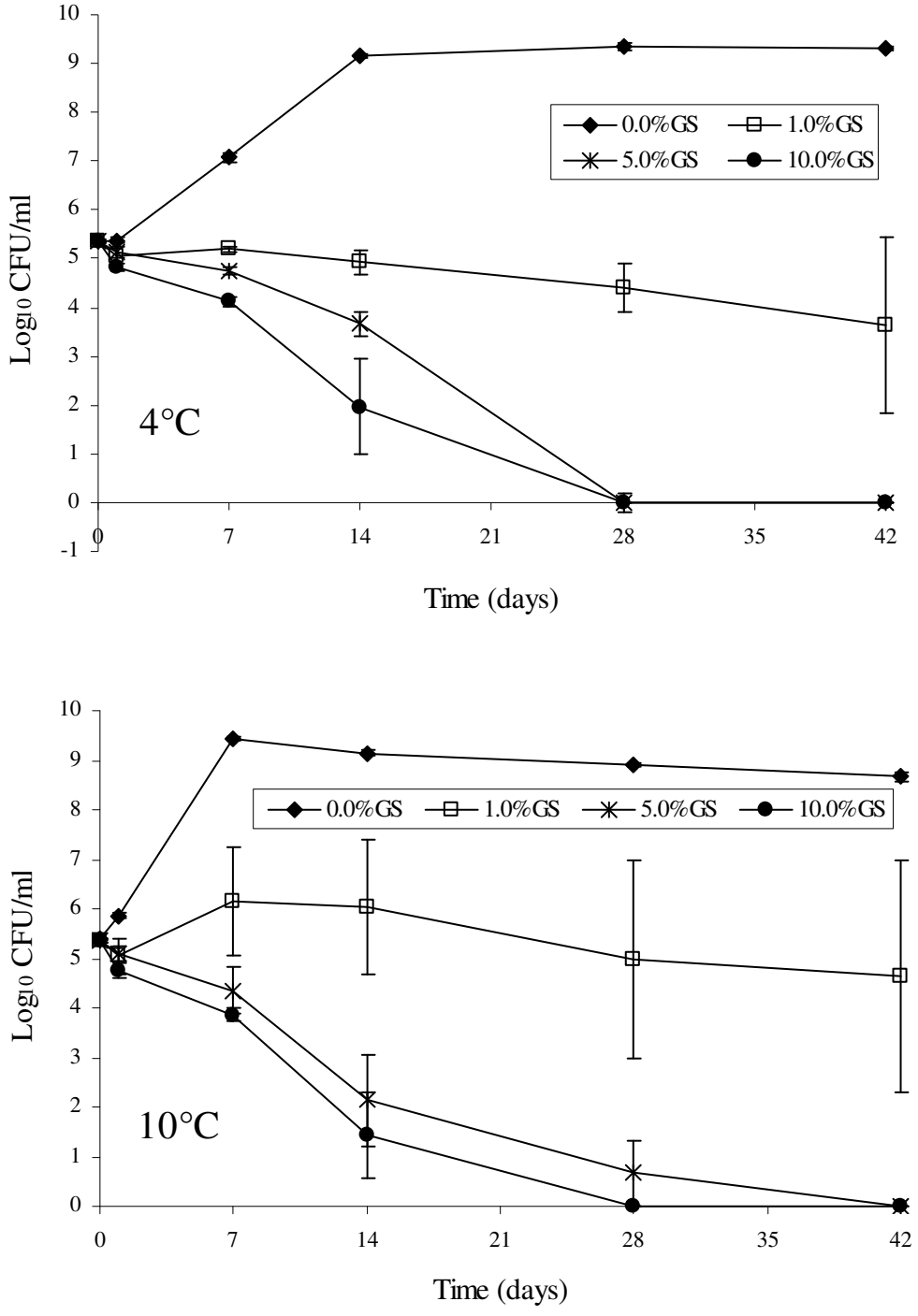

Figure 2. Effect of grape seed extract (GS) on the growth of *Listeria monocytogenes* in BHI at 4 and 10°C. Values at each time point are an average of three independent replications. Error bars represent standard error of the mean.

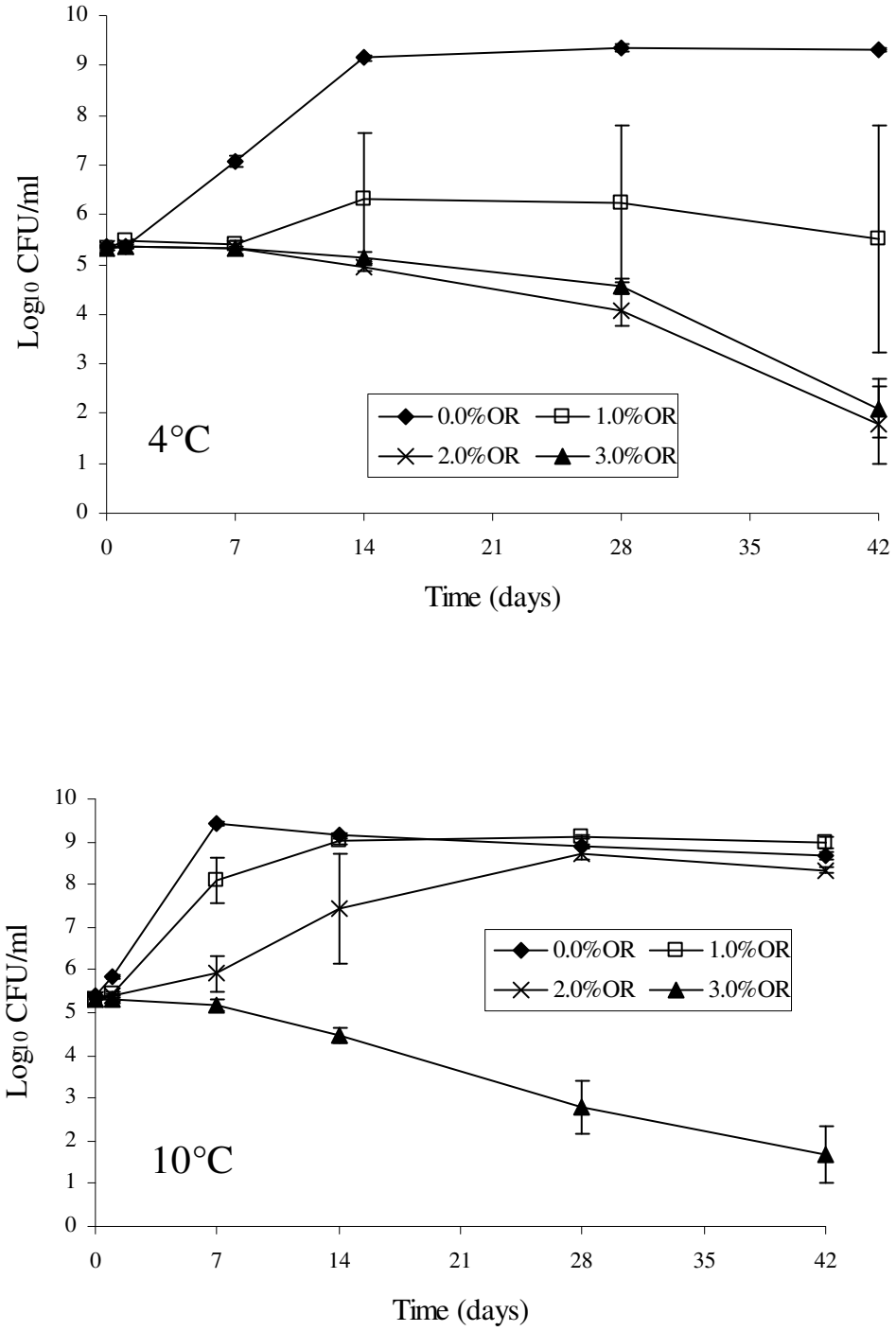

Figure 3. Effect of oregano (OR) on the growth of *Listeria monocytogenes* in BHI at 4 and 10°C. Values at each time point are an average of three independent replications. Error bars represent standard error of the mean.

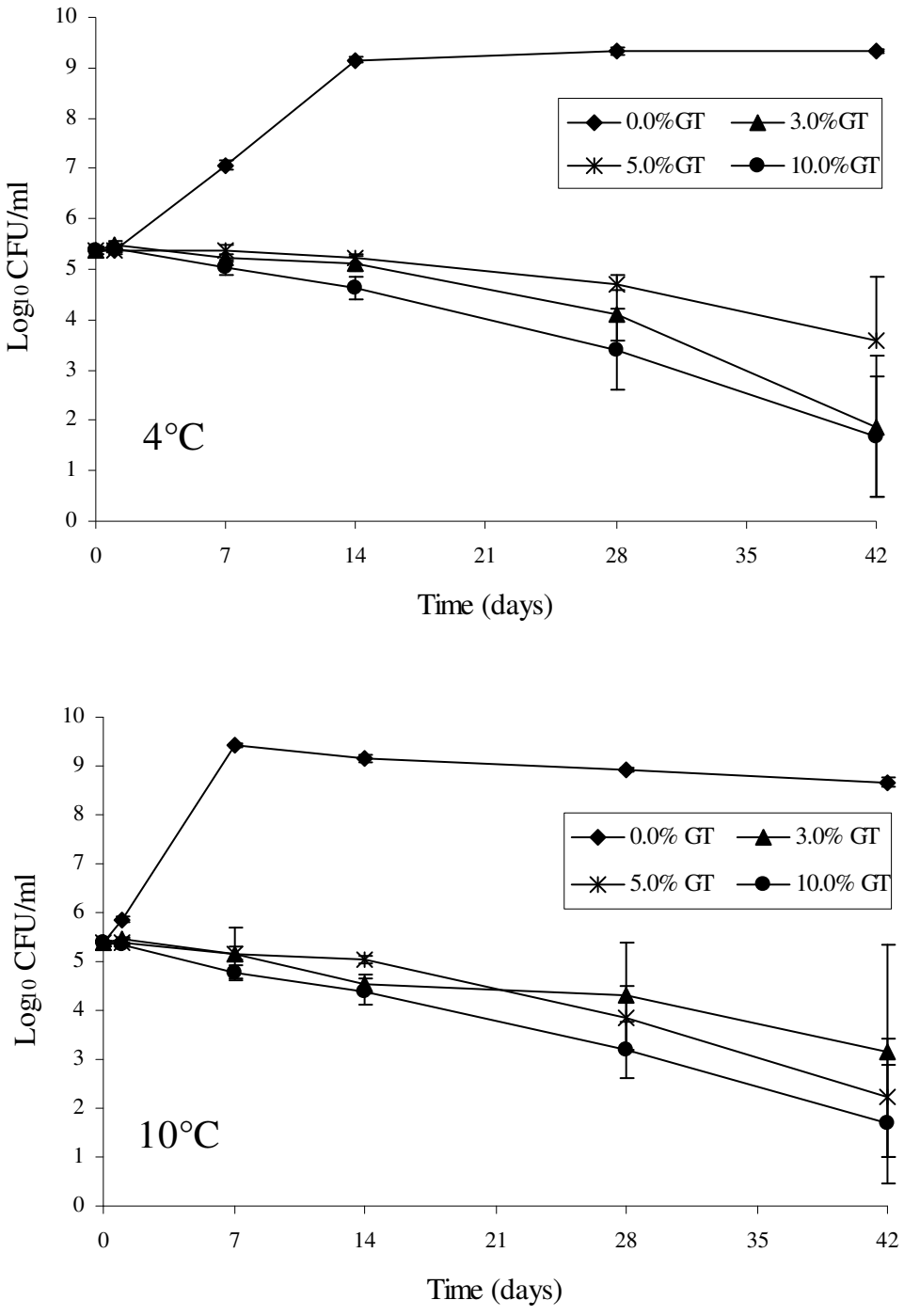

Figure 4. Effect of green tea (GT) on the growth of *Listeria monocytogenes* in BHI at 4 and 10°C. Values at each time point are an average of three independent replications. Error bars represent standard error of the mean.

CHAPTER 4. SURVIVAL OF ACID ADAPTED AND NON-ACID ADAPTED *LISTERIA MONOCYTOGENES* IN BRAIN HEART INFUSION BROTH CONTAINING CRANBERRY CONCENTRATE OR GRAPE SEED EXTRACT AND STORED AT 4 AND 10ºC

A paper to be submitted to the Journal of Food Protection

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ABSTRACT

The survival of a five strain cocktail of acid adapted and non-acid adapted *Listeria monocytogenes* in Brain Heart Infusion (BHI) broth containing cranberry concentrate (CB) or grape seed extract (GS) was evaluated. *L. monocytogenes* cells were grown overnight at 35ºC in BHI supplemented with 1% glucose (BHI+G) or in BHI without glucose (BHI-G). Final pH of the BHI+G and BHI-G cultures was approximately 4.4 and 6.8, respectively. Cells were then harvested by centrifugation and washed once in 0.85% saline. CB and GS were added separately to 100 ml BHI in Erlenmeyer flaks in the following concentrations: 0 and 10% for CB, 0 and 7% for GS. All flasks contained a final *L. monocytogenes* concentration of 10^5 CFU/ml. Flasks were incubated at 4 and 10ºC and tested on days 0, 1, 7, 14, 28, and 42. All samples were plated in duplicate on BHI agar and incubated at 35ºC for 24h. In the presence of CB, non-acid adapted *L. monocytogenes* was reduced by approximately 5 logs by day 14, whereas the acid

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adapted was reduced by approximately 1 log, regardless of storage temperature. In the presence of GS, populations of acid adapted and non-acid adapted *L. monocytogenes* were reduced by approximately 2 logs by day 14, regardless of storage temperature. The results of this experiment indicate that acid adaptation in *L. monocytogenes* may cross-protect the organism against inhibition by CB, but may be ineffective in protecting the pathogen in the presence of GS.

INTRODUCTION

Listeria monocytogenes is a foodborne pathogen that causes serious disease in immunocompromised individuals, children, and the elderly. Diseases range from gastroenteritis to meningitis or encephalitis; in pregnant woman, infection may lead to abortion or stillbirth *(12)*. A mortality rate of approximately 20% has been associated with infection by *L. monocytogenes (21)*. In the past decade, outbreaks of listeriosis have been linked to the consumption of different types of foods including raw milk, cheese, coleslaw, hot dogs, and sliceable turkey meat *(8, 18)*. *L. monocytogenes* has also been found in various environments including: soil, water, agricultural and industrial wastes, and vegetation *(20)*.

In such as harsh environments as those described above and during food processing, pathogens such as *L. monocytogenes* are commonly stressed. For example, in the manufacture of cheese, organisms are affected by the hydrogen peroxide that may be added to the raw milk, the acid developed during fermentation, the salt, and the heat from the thermal treatment; during sausage fermentation, organisms are stressed by salt, acid, and heat *(17)*. These stresses, in combination, are considered hurdles that may be difficult

for foodborne pathogens to overcome. Food safety and food preservation are heavily dependent on the presence of these hurdles to prevent the growth of pathogenic and spoilage organisms *(13)*.

Several studies have demonstrated that exposure of organisms to certain stresses causes them to adapt and to become more resistant to further stress. In one study, adaptation of *L. monocytogenes* to ethanol, starvation, hydrogen peroxide, and acid increased the resistance of the pathogen to heat *(16, 17)*. In other studies, acid adapted *Escherichia coli* was found to be more resistant to weak acids that its non-adapted counterpart *(9, 10)* and acid adapted *Salmonella typhimurium* showed an increase in acid resistance *(5)*. Such adaptations to environmental factors may reduce the efficacy of food preservation and compromise food safety.

An unfavorable environmental condition such as acidity is encountered both in natural habitats (acid rain, manure, silage, fermented foods, and feed) and in infected hosts (gastric secretions) *(20)*. *L. monocytogenes* has the ability to adapt to low pH environments; this organism encounters such environments as it passes through the stomach and while it resides in the macrophage phagosome *(19)*. The ability of *L. monocytogenes,* and other pathogenic organisms, to survive such harsh environments is also of great interest to the food industry as organic acids are regularly used by food processors to prevent bacterial growth in foods *(19)*.

Natural plant extracts have been used since prehistoric times as condiments to enhance the flavor of foods and to preserve its quality. Many spices and their oils are known to possess antimicrobial activity. Compounds isolated from clove, garlic, mustard, onion, and cinnamon have been found to inhibit bacterial growth; however, there is a

significant variation in resistance of different organisms to the same spice *(2)*. Some of these differences can be attributed to changes in test methods, bacterial strains, and sources of antimicrobial samples used *(7)*. Some natural extracts actively used today against microorganisms include: cloves, rosemary, sage, garlic, hops, thyme, and coriander *(1)*. Other natural plant extracts, such as cranberry, grape seed, and oregano, are currently being studied for their antimicrobial properties against *L. monocytogenes* and other pathogenic or spoilage bacteria.

The wide range of environmental factors that microorganisms may encounter should be taken into account when assessing the antimicrobial effectiveness of any natural or synthetic antimicrobial to be used in food systems. The exposure of a pathogen to an acidic environment may induce acid habituation potentially leading to cross-protection of the organism against other inhibitory factors. Accordingly, the objective of this study was to determine the influence of acid adaptation on the survival of *L. monocytogenes* in the presence of cranberry concentrate and grape seed extract.

MATERIALS AND METHODS

Microorganisms and culture conditions. Five strains of *Listeria monocytogenes* were used in this study. Strains H7962-4b, H7762-4b, H7596-non4b, H7969-4b, and NADC-2045 were obtained from the culture collection of the Microbial Food Safety Laboratory at Iowa State University. The stock cultures were kept in Brain Heart Infusion (BHI) broth (Difco Laboratories, Detroit, Michigan) supplemented with 10% glycerol at -70ºC. Each culture was transferred at least twice, successively, in 10 ml of BHI broth and incubated at 35ºC overnight prior to each experiment.

Acid adaptation of *L. monocytogenes*. *L. monocytogenes* cultures were acid adapted by growing them separately for 18 h at 35ºC in 100 ml of BHI broth supplemented with glucose to a concentration of 10g/liter (1%) (BHI+G) (1). Cells grown in BHI without glucose (Difco Laboratories) served as control (BHI-G). The acid adapted and control cultures final pH values were 4.4 and 6.8, respectively.

Preparation of cell suspensions. Overnight *L. monocytogenes* cultures (BHI+G and BHI-G) were harvested by centrifugation (Sorvall® Super T21, Sorvall Product, L.P., Newtown, Connecticut) at 10,000 x g for 10 min at 4ºC, washed once in saline (0.85% NaCl) (Fisher Scientific Company L.L.C., Pittsburgh, Pennsylvania) to remove left over growth medium, then suspended in fresh saline (4ºC). The cell suspensions (cocktail of the five strains) were adjusted to give approximately 10^7 CFU/ml.

Antimicrobials. The antibacterial compounds used during this study included cranberry concentrate (Tree of Life, St. Augustine, Florida) in liquid form and grape seed extract (Gavinol®, Kikkoman Corporation, Tokyo, Japan) in powder form.

Treatments and microbiological analysis. The antimicrobials were added separately to 100 ml of BHI broth in Erlenmeyer flaks in the following concentrations: 0 and 10% (v/v) for cranberry and 0 and 7% (w/v) for grape seed. Flasks were inoculated with 0.1 ml of the five strain cocktail of acid adapted or non-acid adapted *L. monocytogenes* (to give a final concentration of approximately 10^5 CFU/ml). Flasks were incubated at 4 and 10ºC and tested on days 0, 1, 7, 14, 28, and 42. Appropriate dilutions in 0.1% peptone water were surface-plated, in duplicate, onto plates of BHI agar. All inoculated plates were incubated at 35ºC and bacterial colonies counted after 24 h.

Statistical analysis. Data from three independent replications were subjected to

statistical analysis. Data were analyzed using SAS 9.1 (SAS Institute, Cary, North Carolina). Differences between samples were determined using Tukey's honestly significant difference pairwise test ($p<0.05$).

RESULTS AND DISCUSSION

L. monocytogenes has been shown to adapt to low pH environments upon exposure to mild acid conditions; this adaptation, cross-protected the pathogen against other stresses including heat shock and osmotic stress *(15)*. In the food industry, acids are commonly used as forms of preservatives or as sanitizers. It is important that adaptation and cross-protection to stresses be evaluated when testing the efficacy of new or existing antimicrobials such as cranberry concentrate and grape seed extract.

Figure 1 shows the effect of cranberry concentrate on the growth of acid adapted (AA) and non-acid adapted (NAA) *L. monocytogenes* at 4ºC and at 10ºC based on enumeration of bacterial colonies on BHI agar. Results indicate that a 4.8 log reduction was obtained after 14 days of exposure of NAA *L. monocytogenes* to cranberry, regardless of storage temperature. A 1 log reduction was obtained after 14 days of exposure of AA *L. monocytogenes*, regardless of storage temperature. At days 7 and 14 they were significant differences in surviving populations of NAA *L. monocytogenes* and AA *L. monocytogenes* ($p<0.05$).

The results indicate that, in the absence of cranberry, there were no significant differences between the growth patterns between AA and NAA *L. monocytogenes* cells. In the presence of the cranberry extract, inhibition occurred in both AA and NAA types; although significant differences in the death rates between the two were found. Overall,

the NAA population declined at a faster rate than the AA population. By day 21, no viable cell counts were obtained for the NAA *Listeria* indicating the greater sensitivity of the organism to cranberry in the non-acid adapted state. In contrast, viable counts of AA were detected up to day 42.

The increased survival of acid adapted organisms in acidic foods and/or environments has previously been reported *(8, 15)*. In the present study, prior exposure of *L. monocytogenes* to low pH media enhanced its survival in the presence of the antimicrobial. The cranberry concentrate is an acidic mixture (pH 4) that is made up of phenolic compounds and a number of organic acids, including benzoic acid *(3)*. At low pH, the undissociated acid molecules present in the antimicrobial diffuse through the microbial cell membrane, where they ionize, causing the acidification of the interior of the cell *(3)*. Other studies have shown that benzoic acid inhibits microbial growth by interfering with substrate transport, oxidative phosphorylation, and by preventing the uptake of certain amino acids *(6)*.

In acid adapted organisms, the synthesis of certain proteins, including outer membrane and heat shock proteins, is believed to provide a mechanism for sustaining intracellular homeostasis in lethal environments *(8, 11)*. In the present study, acid adapted *L. monocytogenes* may have developed such a defense mechanism while growing in the acidic media. The subsequent survival of AA cells for a longer storage period was most likely enhanced by the synthesis of protective proteins, compared with that of the non-adapted cells *(20)*.

Figure 2 shows the effect of grape seed extract on the growth of AA and NAA *L. monocytogenes* at 4ºC and at 10ºC. Results indicate that approximately a 2 log reduction

was obtained after 14 days of exposure of AA and NAA *L. monocytogenes* to grape seed, regardless of storage temperature ($p>0.05$). The pH of a 7% concentration of grape seed extract was approximately 7.0.

Prior exposure of *L. monocytogenes* to acidic conditions did not enhance the organism's survival in the presence of grape seed extract. As with the cranberry concentrate experiment, no significant difference in growth of NAA and AA were found in the absence of the grape seed extract. In the presence of the antimicrobial, both NAA and AA were equally inhibited. Like cranberries, grape seeds are abundant in phenolic compounds *(22)* but do not contain organic acids. Many phenols and polyphenols are known to cause disruption of the cell membrane; leakage of intracellular compounds has been associated with a loss in membrane permeability *(4)*. Certain microorganisms have developed resistance mechanisms against compounds such as polyphenols; for example, efflux pumps for extrusion of toxic compounds.

The results of the present study indicate that acid adaptation increased the resistance of *L. monocytogenes* to cranberry concentrate but not to grape seed extract. Certain constituents of the extracts may be responsible for the increase resistance or lack of resistance of each physiological state of the pathogen. Acid adaptation has been shown to increase resistance of certain microorganisms to various organic acids and to also increase their survival in some foods *(14, 15)*. Therefore, the increase resistance of acid adapted *Listeria* to the cranberry concentrate should be taken into account when conducting laboratory food challenge studies involving this natural antimicrobial.

ACKNOWLEDGMENTS

The authors thank Kikkoman Corporation (2-1-1, Nishi-Shinbashi, Minato-ku,

Tokyo, Japan, zip code: 105-8428) for providing the grape seed extract for all of the experiments. Also, the statistical analysis and expertise provided by Man-Yu Yum (Department of Statistics, Iowa State University).

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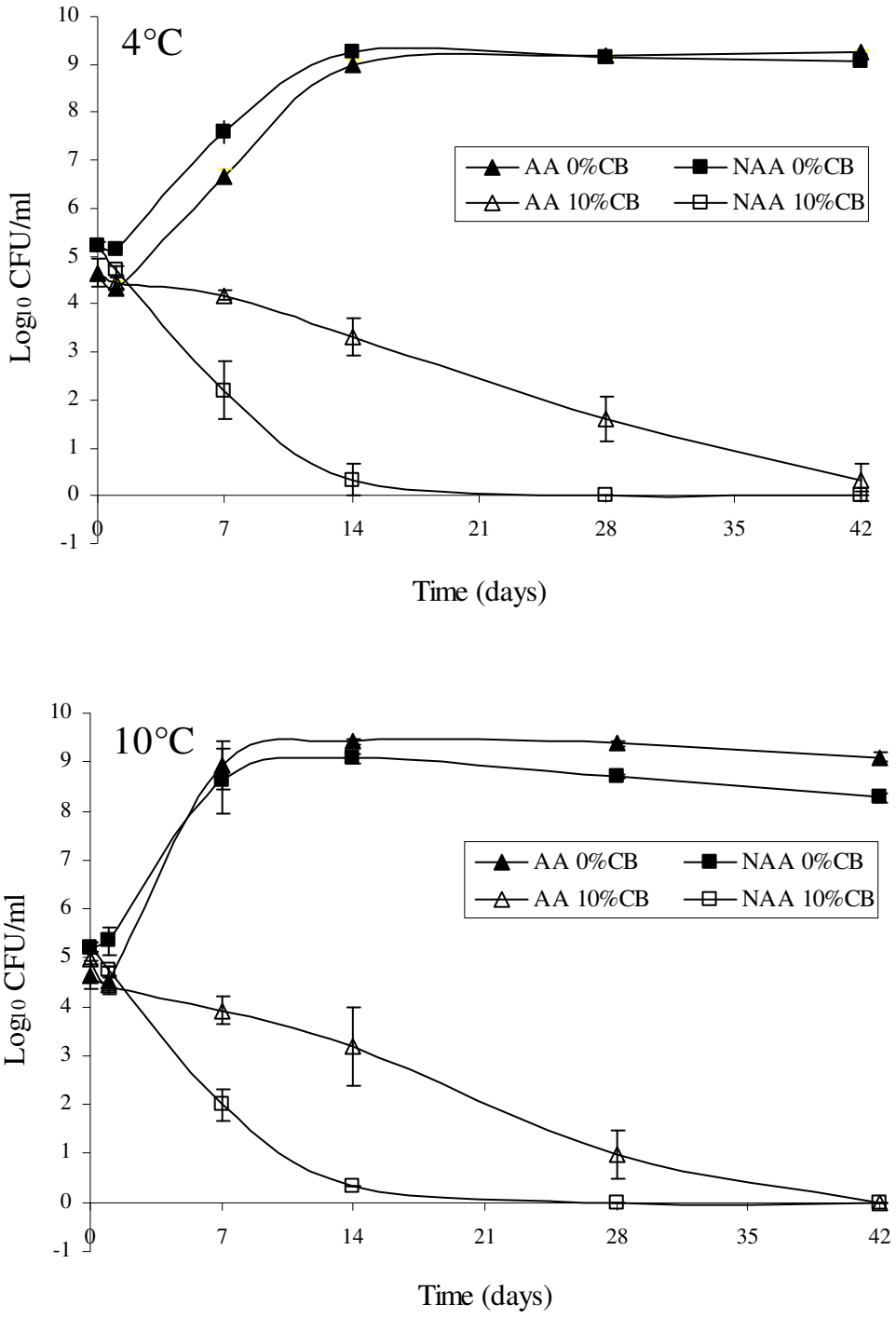

Figure 1. Effect of 10% cranberry concentrate (CB) on the growth of acid adapted and non-acid adapted *Listeria monocytogenes* in BHI at 4 and 10ºC. Values at each time point are an average of three independent replications. Error bars represent standard error of the mean.

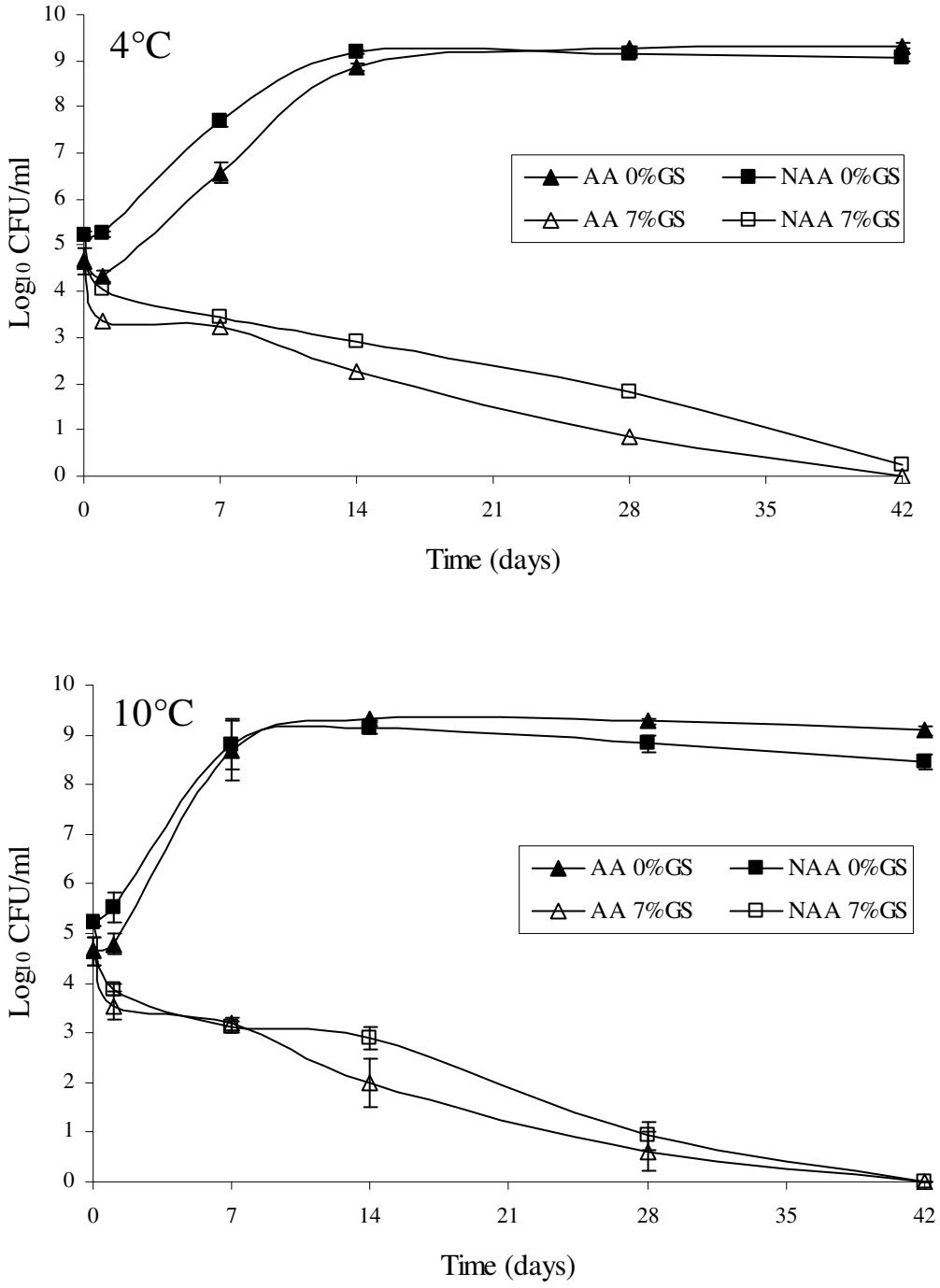

Figure 2. Effect of 7% grape seed extract (GS) on the growth of acid adapted and non-acid adapted *Listeria monocytogenes* in BHI at 4 and 10ºC. Values at each time point are an average of three independent replications. Error bars represent standard error of the mean.

CHAPTER 5. ANTIMICROBIAL EFFICACY OF CRANBERRY CONCENTRATE OR GRAPE SEED EXTRACT ALONE OR COMBINED WITH SODIUM LAURYL SULFATE AGAINST *LISTERIA MONOCYTOGENES* IN VACUUM PACKAGED FRANKFURTERS AT 4ºC

A paper to be submitted to the Journal of Food Protection

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ABSTRACT

A study was conducted to evaluate the antimicrobial efficacy of cranberry concentrate (CB) and grape seed extract (GS) against *Listeria monocytogenes* in refrigerated vacuum packaged frankfurters. Frankfurters formulated with or without 2% (w/w) sodium lactate (SL) were immersed (2.0 min) in CB (2 or 3% v/v) or GS (2 or 3% w/v) alone or combined with 1% (w/v) sodium lauryl sulfate (SLS). Frankfurters dipped in sterile distilled water served as control. After dipping, frankfurters were drained (15 s), placed in vacuum packaging bags, inoculated with a 5-strain mixture of *L. monocytogenes* to give ~5 x 10^7 CFU/frankfurter, vacuumed packaged, and stored at 4ºC for 90 days. At set time intervals *L. monocytogenes* survivors were enumerated by washing the frankfurters with 0.1% peptone, plating on Modified Oxford Medium (MOX), and incubating at 35ºC for 48 h. CB (3%) and GS (3%) reduced initial numbers of *L. monocytogenes* by 0.7 and 4.0 log, respectively, on frankfurters without SL. The addition of 1% SLS enhanced their

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antimicrobial activity. CB (2 or 3%) + 1% SLS and GS (2 or 3%) + 1% SLS decreased initial numbers by ~ 4 and 6 log, respectively. Greater reductions in initial numbers of the pathogen were observed in frankfurters with SL. CB (2 or 3%) + 1% SLS and GS (2 or 3%) + 1% SLS produced reductions of ~ 5.0 and 7.0 log, respectively. While survivors grew in frankfurters without SL, no growth occurred in frankfurters that contained SL. Based on these results the combined application of CB or GS with SLS on the surface of frankfurters (formulated with 2% SL) has good potential for substantially reducing initial numbers of *L. monocytogenes* and inhibiting growth of survivors in these popular ready-to-eat meat products.

INTRODUCTION

In the past decade, *Listeria monocytogenes* has been implicated in a number of foodborne illness outbreaks involving ready-to-eat (RTE) meat or poultry products. The food products accounting for the highest risk for listeriosis included frankfurters and deli meats *(1)*, which are consumed without further cooking or reheating. To healthy adults, listeriosis may cause only flu-like symptoms and minor discomforts, whereas for immunocompromised individuals the disease can be deadly. The associated mortality rate is as high as 20% and those at risk include infants, pregnant woman, and elderly persons *(9)*.

L. monocytogenes is widely distributed in the environment and has the ability to grow at refrigeration temperatures and in the presence or absence of oxygen. It can tolerate salt concentrations up to 12% and a pH range of 4.5 to 9.0 *(3, 8, 9, 13)*. The hardy nature of this pathogen and its presence in the processing environment has

prompted regulatory agencies to establish requirements for the control of *L. monocytogenes* in RTE meat. One of those requirements includes the use of a postlethality treatment (may be an antimicrobial agent) to reduce or eliminate the pathogen and an antimicrobial to limit or suppress its growth *(4)*.

Many processed meats contain sodium chloride and nitrite salts, and although they possess antimicrobial properties, their presence does not inhibit growth of *L. monocytogenes* under refrigerated storage conditions *(7)*. The use of additional substances can serve as antimicrobial hurdles to suppress the growth of the pathogen. This may include the incorporation of lactates or sorbates into the meat formulation and/or the application of an antimicrobial to the meat surface.

The use of plant-derived compounds by the food industry as natural antimicrobials has increased over the last few years due to consumer demand for more natural food ingredients. A number of extracts including cloves, rosemary, sage, garlic, hops, thyme, and coriander are actively used against various foodborne organisms *(12)*. These compounds are generally recognized as safe and may be employed as dipping solutions or in meat formulations to control enteric pathogens.

The objective of this study was to investigate the antimicrobial effect cranberry concentrate and grape seed extract as antimicrobial dips alone or in combination with sodium lauryl sulfate, an anionic surfactant, in frankfurters containing sodium lactate.

MATERIALS AND METHODS

Microorganisms and culture conditions. Five strains of *Listeria monocytogenes* were used in this study. Strains H7962-4b, H7762-4b, H7596-non4b, H7969-4b, and NADC-

2045 were obtained from the culture collection of the Microbial Food Safety Laboratory at Iowa State University. The stock cultures were kept in Brain Heart Infusion (BHI) broth (Difco Laboratories, Detroit, Michigan) supplemented with 10% glycerol at -70ºC. Each culture was transferred at least twice, successively, in 10 ml of BHI broth and incubated at 35ºC overnight prior to each experiment.

Preparation of cell suspension. *L. monocytogenes* strains were each grown separately in Erlenmeyer flasks containing 100 ml of BHI broth. After 18 h the cells were harvested by centrifugation (Sorvall® Super T21, Sorvall Product, L.P., Newtown, Connecticut) at 10,000 x g for 10 min at 4ºC, washed once in saline (0.85% NaCl) (Fisher Scientific Company L.L.C., Pittsburgh, Pennsylvania) to remove left over growth medium, then suspended in fresh saline (4ºC). The cell suspensions (cocktail of the five strains) were adjusted to give approximately 5x 10^7 CFU/ml as determined by plate counts on BHI agar.

Antimicrobials. The antimicrobials used during this study included cranberry concentrate (CB) (Tree of Life, St. Augustine, Florida) and grape seed extract (GS) (Gravinol®, Kikkoman Corporation, Tokyo, Japan). Sodium lauryl sulfate (SLS) (Fisher Scientific, Pittsburgh, Pennsylvania) was added to increase the solubility of the antimicrobials. The dips were made by dissolving 2.0 or 3.0% of CB (v/v) or GS (w/v) alone or combined with 1% SLS (w/v) in sterile distilled water.

Total phenolic content. The concentration of total phenolics of each antimicrobial was determined by the Price and Butler method *(14)*. The assay is based on the oxidation of phenolate ion in which ferric ions are reduced to the ferrous state, detected by the formation of the Prussian blue complex $[Fe_4[Fe(CN)_6]_3]$ with a potassium ferricyanide

reagent *(6)*. A 1% solution of each antimicrobial in 10% EtOh and deionized water were prepared. The absorbance of each sample was measured at 720 nm and the phenolic contents expressed as catechins equivalents (% concentration in 1% sample).

Preparation of frankfurters. The frankfurters were prepared by the Iowa State University Meat Laboratory. The frankfurters were formulated to contain: beef, pork, water, salt, ground mustard, spices, dextrose, sodium phosphates, garlic powder, sodium erythorbate, and sodium nitrite for the control; the sodium lactate (SL) frankfurters contained the same ingredients along with the addition of SL at 2% of the final product weight .

Immersion in the antimicrobial solutions. Each frankfurter was dipped in antimicrobial solution for 2 min, drained for 15 s, and placed in stomacher bags (Stomacher 400, Brinkmann Instruments Inc., Westbury, New York). Frankfurters dipped in sterile distilled water served as control. Each antimicrobial dip and water control was changed after ten frankfurters were individually dipped (the two types of frankfurters, +/-SL, were dipped in separate antimicrobial solutions).

Inoculation of frankfurters. The frankfurters were inoculated with the 5-strain mixture of *L. monocytogenes* to obtain a final concentration of 5×10^7 CFU/frankfurter. The frankfurters were then vacuum packaged (Multivac, Multivac Inc., Kansas City, Kansas) and stored at 4ºC.

Microbiological analysis. Frankfurters were analyzed on days 1, 14, 28, 42, 56, 70, and 90 (two frankfurters were analyzed per treatment). On the corresponding sampling day, bags were aseptically cut opened, 100 ml of 0.1% peptone water added, and frankfurters massaged by hand continuously for 30 s. Appropriate dilutions of the wash solution (in

0.1% peptone water) were surface-plated, in duplicate, onto Modified Oxford Agar (MOX) (BD Diagnostics, Sparks, Maryland) plates. All inoculated plates were incubated at 35ºC and bacterial colonies counted after 48 h.

Sample enrichment. Throughout the study, samples that contained *L. monocytogenes* cells below detectable levels were enriched (2 frankfurters per treatment). The recovery was performed following the USDA's enrichment procedure for the isolation of *L. monocytogenes (11)*. Frankfurters treated with 2 and 3% CB each combined with SLS were analyzed on storage day 90. Frankfurters treated with 2% GS combined with SLS were analyzed on storage day 56 and those treated with 3% GS combined with SLS were analyzed on days 14, 42, and 70.

Statistical analysis. Data from three independent replications were subjected to statistical analysis. Data were analyzed using SAS 9.1 (SAS Institute, Cary, North Carolina). Differences between samples were determined using Tukey's honestly significant difference pairwise test ($p<0.05$).

RESULTS AND DISCUSSION

Figure 1 shows the effect of surface treatment of frankfurters (no added SL) with CB and SLS, alone or in combination, on the growth of *L. monocytogenes* at 4°C. The initial numbers of *L. monocytogenes* increased to approximately 10 log CFU/frankfurter at 28 days and remained relatively unchanged up to 90 days. Dipping frankfurters in 3% CB did not significantly inhibit the growth of *L. monocytogenes* under the storage conditions ($p>0.05$). At day 1, no significant differences were found between the 1% SLS dip and the control ($p>0.05$), however, differences were found at days 14 and 28 (3.4 and

2.5 log difference, respectively) ($p<0.05$). Dipping the frankfurters in a mixture of 3% CB or 2% CB and 1% SLS reduced the initial population of *L. monocytogenes* by approximately 4 logs after 1 day of storage. This 4 log reduction was maintained until day 28. By day 42, there were no significant differences between the treated frankfurters and the untreated control ($p>0.05$).

Similar results, although with greater log reductions, were obtained when using GS as the antimicrobial. Dipping frankfurters in a mixture of 3% GS or 2% GS, each with 1% SLS, reduced the initial population of *L. monocytogenes* by approximately 6 logs (Figure 1). Significant differences in populations of the pathogen between the treated frankfurters and the control persisted for more than 28 days. When the frankfurters were dipped in 3% GS alone the initial population of *L. monocytogenes* was reduced by approximately 3 logs; significant differences between this treatment and the control were observed until day 28 ($p<0.05$).

These results indicate that although immersion of frankfurters (without SL) in a mixture of CB or GS, in combination with SLS, can substantially reduce initial populations of *L. monocytogenes* and inhibit growth during storage, the bacterial counts may eventually reach the high levels similar to those of the control. In contrast, in samples that were formulated with SL, the *L. monocytogenes* counts in the dipped frankfurters, including the control, were lower throughout the storage period.

Figure 2 shows the effect of surface treatment of frankfurters (containing SL) with CB and SLS, alone or in combination, on the growth of *L. monocytogenes* at 4°C. A bacteriostatic effect was obtained when the SL-containing frankfurters were dipped in water (control) and 3% CB. A bacteriocidal effect was obtained when frankfurters were

dipped in 1% SLS and in both 3% CB or 2% CB each with 1% SLS ($p<0.05$). Dipping the frankfurters in the CB/SLS mixture reduced the initial population of *L. monocytogenes* by approximately 5 logs. The numbers of bacterial survivors remained below 2 logs for 90 days.

Dipping SL-containing frankfurters in 3% GS alone reduced initial viable counts by 3.5 logs and exerted a bacteriostatic effect on survivors. In this instance, significant differences in numbers of survivors were found between the treatment and the control throughout storage ($p<0.05$) (Figure 2). As with the results obtained for CB, dipping the frankfurters in 3% GS or 2% GS, each combined with 1% SLS, was bacteriocidal resulting in initial log reductions of approximately 7 and 6 logs, respectively. Numbers of survivors remained below 1 log for both dips, with counts falling below detectable levels at specific days throughout the storage period.

The enrichment procedure was carried out to recover injured *L. monocytogenes* in frankfurters with cell numbers below detectable levels. The only frankfurters enriched were those treated with 2 and 3% CB and GS each combined with SLS. Following primary enrichment, *L. monocytogenes* was recovered from all of the 2% CB and GS (+SLS) samples. Further analysis with secondary enrichment was needed in order to recover *L. monocytogenes* from the 3% CB and GS (+SLS) treated frankfurters.

The antimicrobial properties of cranberry and grape seed extract have been attributed to the presence of phenolic and polyphenolic compounds such as flavonols (flavonoids) and proanthocyanidins (condensed tannins) *(2, 15)*. The total phenolic concentration in 1% CB and 1% GS was found to be 0.01 and 0.95%, respectively. Antimicrobial activity of phenolics has been shown against several bacteria including

Escherichia coli, *Staphylococcus aureus*, and *Clostridium perfringens*. These compounds may exert antimicrobial activity by injuring membranes, which result in leakage of cellular contents *(12)*. SLS is an anionic surfactant commonly used as an emulsifier or a whipping agent in certain foods. This surfactant was used to increase the surface contact on the frankfurter between the plant extract and the pathogen. Studies have shown the activity of SLS as a microbicide with virucidal activity suggesting that SLS may denature membrane proteins of cells and pathogens *(5, 10)*.

Based on the results of the present study, formulation of frankfurters with 2% SL and surface treatment of these RTE products with 2 or 3% CB or GS combined with 1% SLS, has good potential for controlling *L. monocytogenes* during storage at 4°C.

ACKNOWLEDGMENTS

The authors greatly acknowledge The Institute for Food Safety and Security at Iowa State University for their assistance and the Kikkoman Corporation (2-1-1, Nishi-Shinbashi, Minato-ku, Tokyo, Japan ZipCode: 105-8428) for providing the grape seed extract for all of the experiments. Also, Dr. White at Iowa State University for giving us permission to use the materials and equipment in her laboratory for the phenolic content experiment, Jose Gerde for his assistance with that experiment, and the statistical analysis and expertise provided by Man-Yu Yum (Department of Statistics, Iowa State University).

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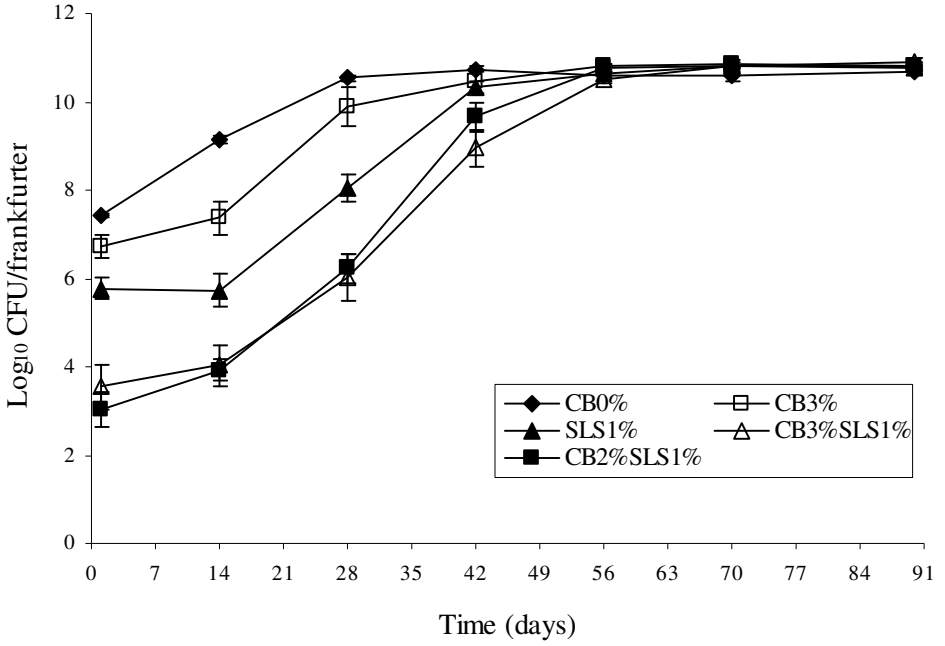


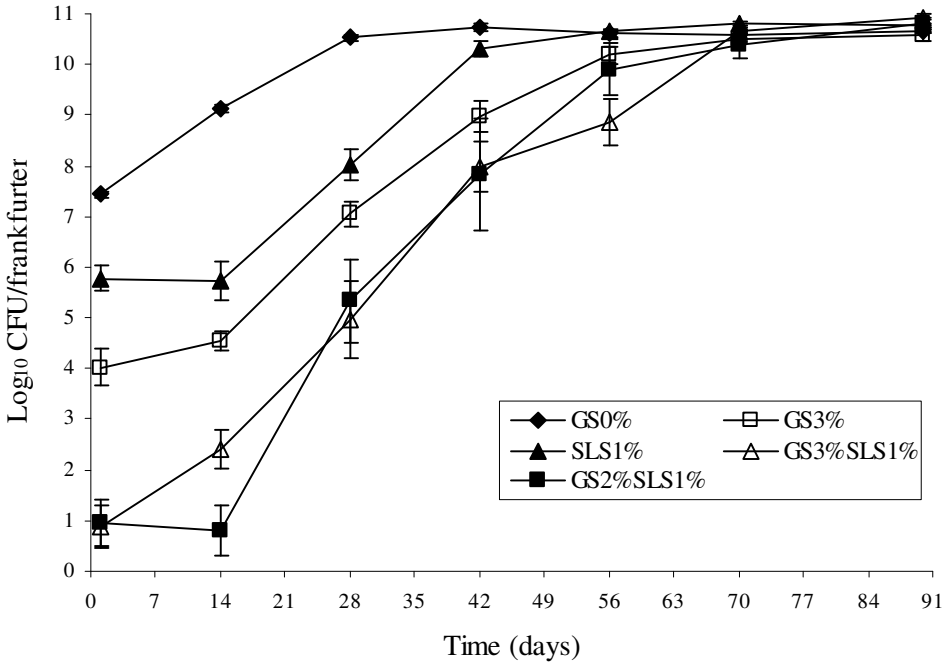
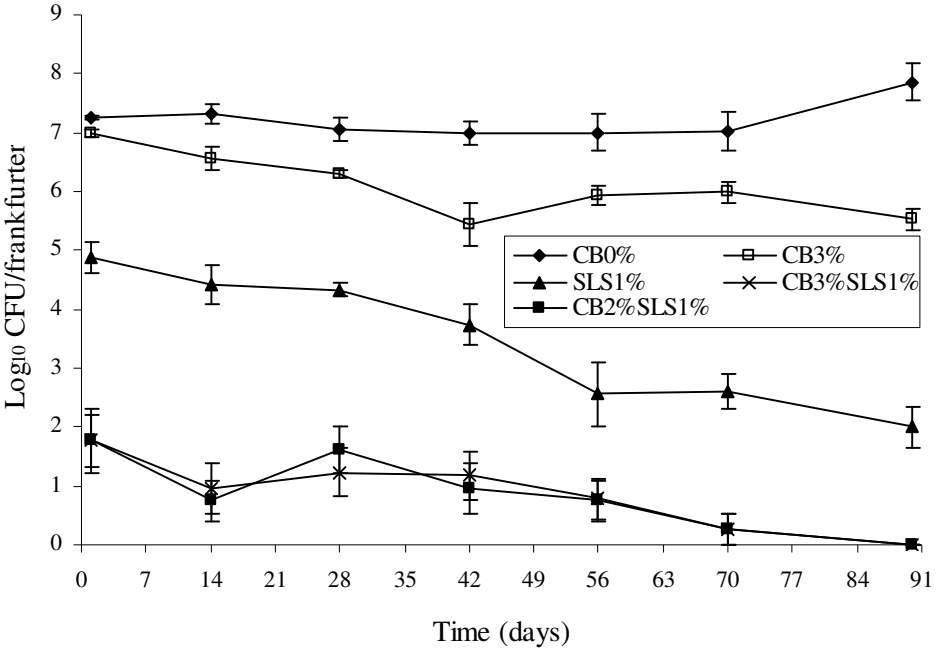


Figure 1. Effect of surface treatment of frankfurters (without sodium lactate) with cranberry concentrate (CB) or grape seed extract (GS), alone or in combination with sodium lauryl sulfate (SLS), on the growth of *Listeria monocytogenes* at 4°C (test days: 1, 14, 28, 42, 56, 70, 90). Values at each time point are an average of three independent replications. Error bars represent standard error of the mean.



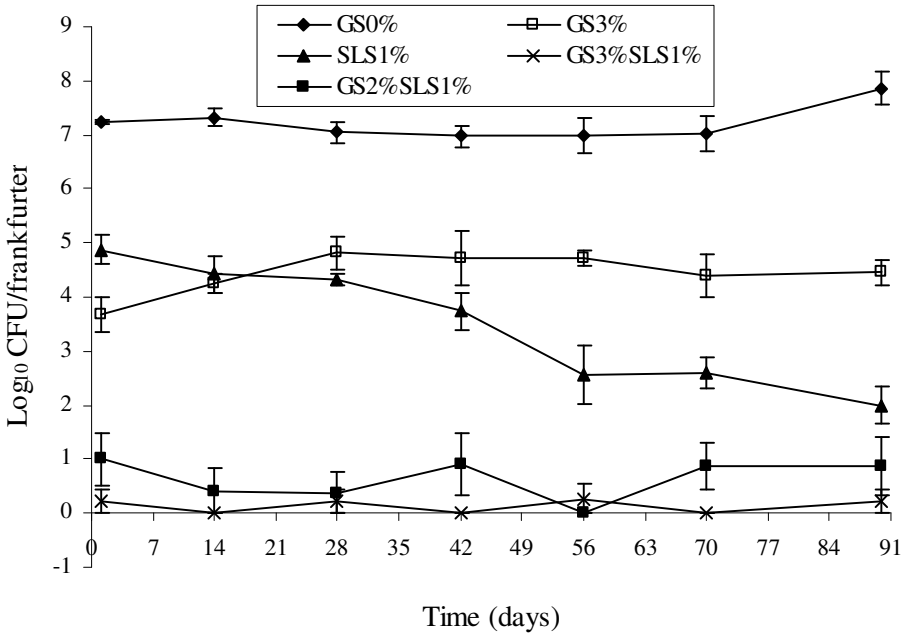


Figure 2. Effect of surface treatment of frankfurters (containing sodium lactate) with cranberry concentrate (CB) or grape seed extract (GS), alone or in combination sodium lauryl sulfate (SLS), on the growth of *Listeria monocytogenes* at 4°C (test days: 1, 14, 28, 42, 56, 70, 90). Values at each time point are an average of three independent replications. Error bars represent standard error of the mean.

CHAPTER 6. ANTIMICROBIAL MODE OF ACTION OF GRAPE SEED EXTRACT AGAINST *LISTERIA MONOCYTOGENES* INVOLVES DAMAGE TO THE CYTOPLASMIC MEMBRANE

A paper to be submitted to the Journal of Food Protection

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ABSTRACT

The objective of the present study was to elucidate the antimicrobial mode of action of grape seed extract (GS) against *Listeria monocytogenes* Scott A. Stationary phase cells, grown overnight (18 h) in Brain Heart Infusion (BHI) broth at 35°C, were harvested by centrifugation and washed once in 0.85% NaCl (saline). The washed cells were used to evaluate the minimum inhibitory concentration (MIC) of GS for *L. monocytogenes* at 10^5 CFU/ml of BHI broth (35°C) using broth dilution susceptibility tests. Washed cells were also added to tubes of saline containing 0 (control), 2.5, 5.0, and 10.0 mg/ml GS to obtain a final concentration of 10^8 CFU/ml. Inoculated tubes were held at 35°C and at pre-determined time intervals viability of the pathogen was evaluated by plating aliquots (0.1-ml) of serially diluted (1:10) samples on BHI agar and counting bacterial colonies after 24 h of incubation (30°C). Transmission electron microscopy (TEM) was used to determine alterations in cell structure. The MIC of GS for *L. monocytogenes* was 1.25

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mg/ml. A rapid loss in viability of *L. monocytogenes* (6 log reduction in 10 min) was observed following exposure of the pathogen to GS at 1.25 (MIC), 2.5, 5.0, or 10.0 mg/ml. When observed by TEM, cells exposed to GS exhibited distorted shapes and evidence of cell rupture. Based on these results it was concluded that the bactericidal effect of GS against *L. monocytogenes* involves damage to the cytoplasmic membrane.

INTRODUCTION

Listeria monocytogenes has been known as a foodborne pathogen only within the past two decades, although it has been recognized as a human pathogen for over 70 years. While listeriosis causes a mild illness in healthy adults, it can be life-threatening to immunocompromised individuals and may involve septicemia, meningitis, and encephalitis *(12, 14)*. The Centers for Disease Control and Prevention estimate the burden of listeriosis at 2,493 cases and 499 deaths per year in the United States *(15)*. Studies have shown that *Listeria* spp. are frequently isolated from raw and processed meats *(13, 18)*, vegetables *(6, 19)*, fish and seafood *(2, 5)*, and certain types of soft cheeses *(11)*. Although ready-to-eat products, such as hot dogs, lunch meats, soft cheeses, and smoked fish, are the foods most commonly associated with foodborne illness by *L. monocytogenes*.

The demand by consumers for more “natural” products with less chemical preservatives has been rapidly growing. Due to this demand, many research efforts in recent years have been focused on the use of natural plant extracts as a means to control microorganisms in foods. The antimicrobial activities of several plant extracts have been demonstrated in vitro and in food model systems. Some of those extracts include

rosemary *(16)*, cranberry *(10)*, oregano *(10)*, and tea *(8)*. Ahn et al. *(1)* showed the inhibitory effect of grape seed extract *in vitro* and in raw ground beef. The compounds in the extracts responsible for their antimicrobial activities are mostly phenolic and polyphenolic components, including phenolic acids, flavonoids, and tannins. Many of the phenolic compounds in plant extracts have been shown to exert antimicrobial activity by injuring lipid-containing membranes, which results in leakage of cellular contents *(20)*.

The grape seed extract (Gravinol®) utilized in this study is a naturally occurring antioxidant containing high amounts proanthocyanidins (condensed tannins) extracted from grape seeds. Preliminary experiments conducted in our laboratory indicate that Gravinol® has the potential to kill *L. monocytogenes in vitro* and in a food system. To our knowledge, there are no published reports on how Gravinol® kills *L. monocytogenes*. In this regard, the objective of the present study was to elucidate the antimicrobial mode of action of GS against *L. monocytogenes* Scott A.

MATERIALS AND METHODS

Microorganism and culture conditions. *Listeria monocytogenes* NADC-2045 (Scott A) was obtained from the culture collection of the Microbial Food Safety Laboratory at Iowa State University. The stock culture was kept in Brain Heart Infusion (BHI) broth (Difco Laboratories, Detroit, Michigan) supplemented with 10% glycerol at -70ºC. The culture was transferred at least twice, successively, in 10 ml of BHI broth and incubated at 35ºC overnight prior to each experiment.

Preparation and inoculation of test samples. The *L. monocytogenes* culture was grown in an Erlenmeyer flask containing 100 ml of BHI broth. After 18 h the cells were

harvested by centrifugation (Sorvall® Super T21, Sorvall Product, L.P., Newtown, Connecticut) at 10,000 x g for 10 min at 4ºC and washed once in saline (0.85% NaCl) (Fisher Scientific Company L.L.C., Pittsburgh, Pennsylvania) to remove left over growth medium. Pelleted cells were suspended in fresh saline to give a final viable cell concentration of approximately 4.0 x 10^9 CFU/ml as determined by plate counts on BHI agar.

Antimicrobial plant extract. The antimicrobial used in this study was grape seed extract (GS) (Kikkoman Corporation, Tokyo, Japan). A stock solution of the GS (100 mg/ml) was prepared in BHI broth containing 10% (v/v) ethanol (to increase solubility of the extract) for the MIC experiment or autoclaved distilled water, also containing 10% ethanol, for the cell viability experiment.

Total phenolic content. The concentration of total phenolics of the antimicrobial was determined by the Price and Butler method *(21)*. The assay is based on the oxidation of phenolate ion in which ferric ions are reduced to the ferrous state, detected by the formation of the Prussian blue complex $[Fe_4[Fe(CN)_6]_3]$ with a potassium ferricyanide reagent *(7)*. A 1% solution of each antimicrobial in 10% EtOh and deionized water were prepared. The absorbance of each sample was measured at 720 nm and the phenolic contents expressed as catechins equivalents (% concentration in 1% sample).

Minimal Inhibitory Concentration (MIC). The MIC was calculated using the Microbiology Reader Bioscreen C (MTX Lab Systems Incorporated, Vienna, Virginia) which measures the development of turbidity (growth) by vertical photometry. The organisms were grown under continuous agitation at 35ºC for 18 h with optical density ($OD_{600\ nm}$) readings measured every 15 min. The MIC of the Gravinol® for *L.*

monocytogenes was considered as the lowest concentration that completely inhibited the growth of the pathogen after 18 h.

The antimicrobial assay was performed in a 96-well microtiter plate (Thermo Electron Corporation, Waltham, Massachusetts) containing GS in BHI at final concentrations ranging from 0.005 to 5.0 mg/ml. These final concentrations were obtained by serially diluting the stock solution of Gravinol® (100 mg/ml) following the Clinical and Laboratory Standards Institute (CLSI) methodology for preparing solutions of antimicrobial agents to be used in broth dilution susceptibility tests *(3)*. Each microtiter well contained 200 µl of total volume with a final concentration of 1 x 10^5 CFU/well of *L. monocytogenes*. All tests were conducted in triplicate and included two control samples with no antimicrobial; one control consisted of BHI alone and the other consisted of BHI + 1.0% (v/v) ethanol.

Cell viability. GS was added to separate tubes in the following concentrations: 0 (with and without 1.0% ethanol (v/v)), 1.25, 2.5, 5.0, and 10 mg/ml. Cell viability was determined by inoculating 10 ml of saline in 50 ml screw-cap cell culture tubes with a suspension of washed *L. monocytogenes* cells to obtain a final concentration of approximately 10^8 CFU/ml. The tubes were incubated in a Gyratory shaker water bath (News Brunswick Scientific, Edison, New Jersey) shaking at 5 rpms set at 35ºC for: 0 (control), 10, 20, 30, 40, 50, 60, 70, 80, 100, and 120 min (one tube per incubation time). Samples from each tube were serially diluted (1:10) in peptone water and surface plated on BHI agar. Plates were incubated at 30ºC and bacterial colonies counted at 24 h. Three independent replications of the experiment were conducted.

Transmission electron microscopy. As with the cell viability experiment, tubes

containing 10 ml of saline were inoculated with the *L. monocytogenes* culture to a final concentration of approximately 10^9 CFU/ml. GS was added at a concentration of 10 mg/ml (0 mg/ml for the control). The tubes were placed in the shaker water bath at 35ºC for 5 and 10 min incubation times. Following the incubation period, cells were harvested by centrifugation (10,000 x g, 10 min, 4ºC) and resuspended in fixative containing 2.0% glutaraldehyde (w/v) and 2.0% paraformaldehyde (w/v) in 0.1 M cacodylate and then held for 48 h at 4ºC. Samples were rinsed 2 times in sterile distilled water and pelleted after each step in a microcentrifuge (Beckman Coulter, Inc., Fullerton, California). This and all subsequent experiments except for polymerization were conducted at room temperature. The cells were post-fixed in 1.0% osmium tetroxide in 0.1 M cacodylate for 1 h, followed by a 5 min wash in distilled water and enbloc staining with 2.0% uranyl acetate for 30 min. The samples were then dehydrated in a graded ethanol series, cleared with ultra-pure acetone, infiltrated, and embedded using a modified EPON epoxy resin (Embed 812, Electron Microscopy Sciences, Ft. Washington, Pennsylvania). Resin blocks were polymerized for 48 h at 70ºC; thick and ultrathin sections were made using a Reichert Ultracut S ultramicrotome (Leeds Precision Instruments, Minneapolis, Minnesota). Ultrathin sections were collected onto copper grids and images were captured using a JEOL 1200EX scanning and transmission electron microscope (Japan Electron Optic Laboratories, Peabody, Massachusetts).

Statistical analysis. For the MIC and viability experiments, data from three independent replications were subjected to statistical analysis. Data were analyzed using SAS 9.1 (SAS Institute, Cary, North Carolina). Differences between samples were determined using Tukey's honestly significant difference pairwise test ($p<0.05$).

RESULTS AND DISCUSSION

A number of natural components, such as polyphenolic compounds in plants, have been shown to possess antimicrobial activities against *L. monocytogenes (10).* A number of studies have demonstrated the inhibitory properties of grape seed extract against pathogenic organisms *(1).* Many of those studies suggest that the phenolic compounds present in the grape seed damage the cytoplasmic membrane causing cell leakage *(22).* However, direct evidence on the mode of action of the grape seed extract used in this study, has not yet been shown.

Antimicrobial activity of grape seed extract in liquid medium. The MIC of Gravinol® was determined to be 1.25 mg/ml. This concentration effectively inhibited growth of *L. monocytogenes* at the initial population of 5.0 log CFU per microtiter plate well. Figure 1 shows the effect of several concentrations of GS on the growth of *L. monocytogenes*; although substantial differences in the starting OD_{600} for the GS containing samples were obtained (data not shown), the Bioscreen compensated for background pigmentation by normalizing the data to give a common starting OD_{600} value. There were no significant differences in the growth of *L. monocytogenes* in both controls. As expected, the level of ethanol did not affect the growth of the bacteria.

Ahn et al. *(1)* described an MIC of 4.0 mg/ml of grape seed extract (ActiVin) on an initial *L. monocytogenes* population of approximately 5.0 log. The difference in the MIC obtained in the present study and that reported by Ahn et al. may be attributed to the difference in the brands of the grape seed extract used in the studies. In many cases, the antimicrobial effect of spices can vary significantly because of varietal or brand differences *(22).* Many of the plants from where these extracts are derived are grown in

several countries around the world where diseases, climate, and geographical factors may affect yield of crops and quality of the final product *(17)*. The extraction method for the isolation of active ingredients and the quantities of each may also be different depending on the manufacture of the plant extract.

The effect of Gravinol® on the viability of *L. monocytogenes* was determined using 1.25, 2.5, 5.0, and 10.0 mg/ml of the antimicrobial. A 6 log reduction in counts was obtained after 10 min of exposure with all four concentrations of the GS compared with the controls (Figure 2). For the remaining exposure times, the viability of *L. monocytogenes* remained between approximately 2.0 and 3.0 log depending on the concentration of GS. The rapid loss in cell viability indicated that GS was bacteriocidal toward *L. monocytogenes* at these concentrations.

Effect of grape seed extract on cell morphology. The cell morphology of treated bacterial cultures was examined through TEM pictures to aid understanding of the antimicrobial action of Gravinol®. A 10 mg/ml concentration of GS was chosen in order to ensure the effect of the extract would be clearly captured by the TEM images. The 5 and 10 min exposure times were chosen based on the results from the viability experiment. The rapid death rate of *L. monocytogenes* cells, within the first 10 min of exposure to Gravinol®, was an indication of severe damage to the cells, possibly membrane damage. A control, containing ethanol, was not included in the TEM experiment because the results from the MIC and viability experiments indicated that ethanol did not have an inhibitory effect on *L. monocytogenes*.

TEM images of *L. monocytogenes* cells without treatment with GS, independent of exposure time, showed a homogeneous staining of bacterial cells, well-defined cell

membranes and cell walls, and no cytoplasmic contents leaking from the cells (Figure 1, panel A). In contrast, the *L. monocytogenes* treated with 10 mg/ml Gravinol® for 5 min revealed loss of homogeneity and aggregation of cytoplasmic materials within the cells (Figure 1, panel B). These characteristics were more pronounced in those cells treated for 10 min (Figure 1, panel C). In some cells, the cytoplasmic membrane appeared to bulge against the cell wall. Some cells that exhibited signs of lysis (broken cell walls and membranes) were also observed (Figure 1, inserted boxes).

The presence of phenolic compounds in the grape seed extract may be responsible for the membrane damage of the *L. monocytogenes* cells. Gravinol® is predominantly made up of proanthocyanidins (condensed tannins) which have been shown to target microbial cell surface-exposed adhesions, cell wall polypeptides, and membrane-bound enzymes; they also complex with polysaccharide *(4)*. The total phenolic concentration in 1% GS was found to be 0.95%. Previous studies have shown that phenolic compounds may act on the microbial cell wall or membrane, suggesting that inhibition of microbial growth is by change of cell permeability leading to loss of intracellular molecules (proteins, DNA, RNA, etc.) *(9)*.

In order to further demonstrate damage to the cell membrane, an experiment was conducted to detect leakage of cytoplasmic contents at 260 nm. Spectrophotometer readings of treated samples fell outside of the detection range of the equipment. This was possibly due to the increased levels of pigmented compounds in the GS. When diluted samples were utilized, it was very difficult to successfully detect leakage differences between the control and treated cells. A look at the UV/VIS absorbance range of the extract indicated that pigments in GS absorbed in the range of 260-280 nm. Therefore,

absorbance readings under the parameters of the leakage experiment were unattainable.

The antimicrobial activity of grape seed extract is likely due to the presence of high amounts of polyphenolic compounds, such as tannins. The rapid cell death of *L. monocytogenes* treated with the concentrations of the GS used in the present study was indicative of membrane damage. The TEM images of cells treated with 10 mg/ml Gravinol® revealed perturbation of the intracellular matrix, loss of cell membrane integrity, and in some instances, leakage through holes in the cell membranes and cell walls. Based on these results it was concluded that the bactericidal effect of GS against *L. monocytogenes* involves damage to the cytoplasmic membrane.

ACKNOWLEDGMENTS

The authors thank the Kikkoman Corporation for providing the grape seed extract for the experiments. Also, Dr. White at Iowa State University for giving us permission to use the materials and equipment in her laboratory for the phenolic content experiment, Jose Gerde for his assistance with the phenolic and viability experiments, Bledar Bisha for assisting with the MIC work, the Iowa State University Microscopy Facility and Tracey Pepper for preparing the samples for electron microscopy and generating the electron micrographs. The authors also acknowledge the financial and kind support from The Institute of Food Safety and Security at Iowa State University.

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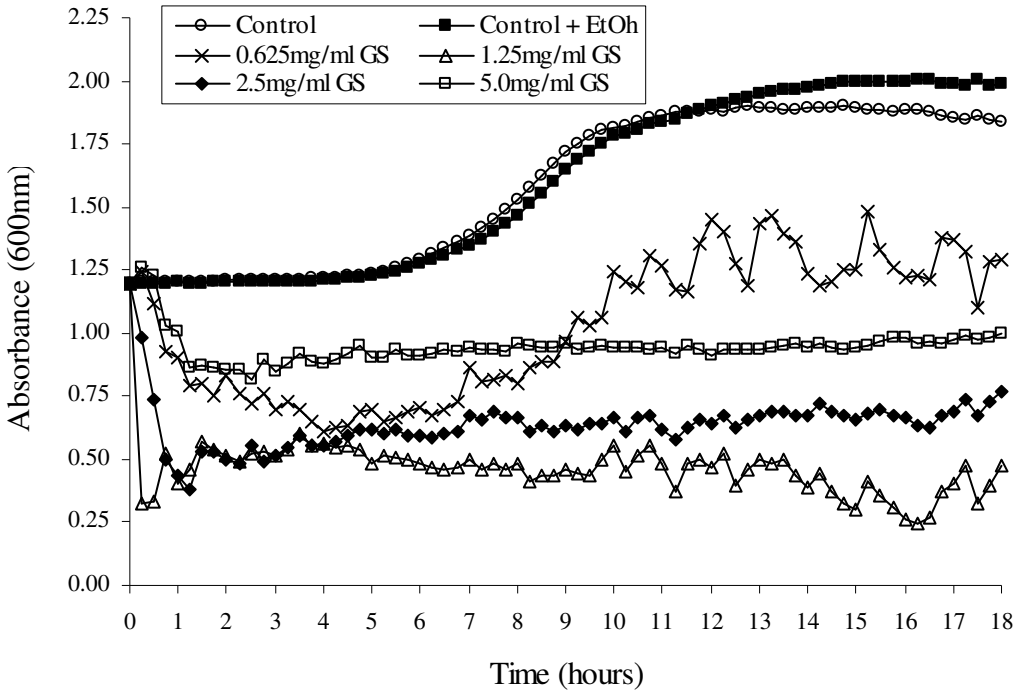

Figure 1. The growth of *Listeria monocytogenes* in the absence (control with and without ethanol) or presence of 0.625, 1.25, 2.5, or 5.0 mg/ml grape seed extract (GS) in BHI broth at 35°C.

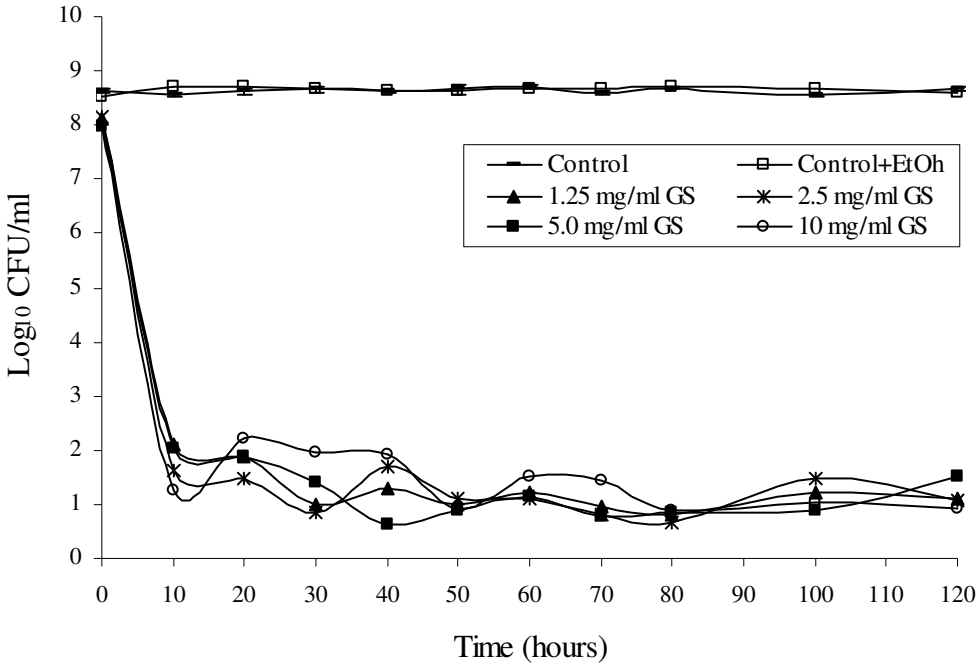

Figure 2. Viability of *L. monocytogenes* in the absence (control with and without 1% ethanol) or the presence of 1.25, 2.5, 5.0, or 10 mg/ml grape seed extract (GS) in saline at 35°C. Colonies on BHI were enumerated after incubation at 30°C for 24h.

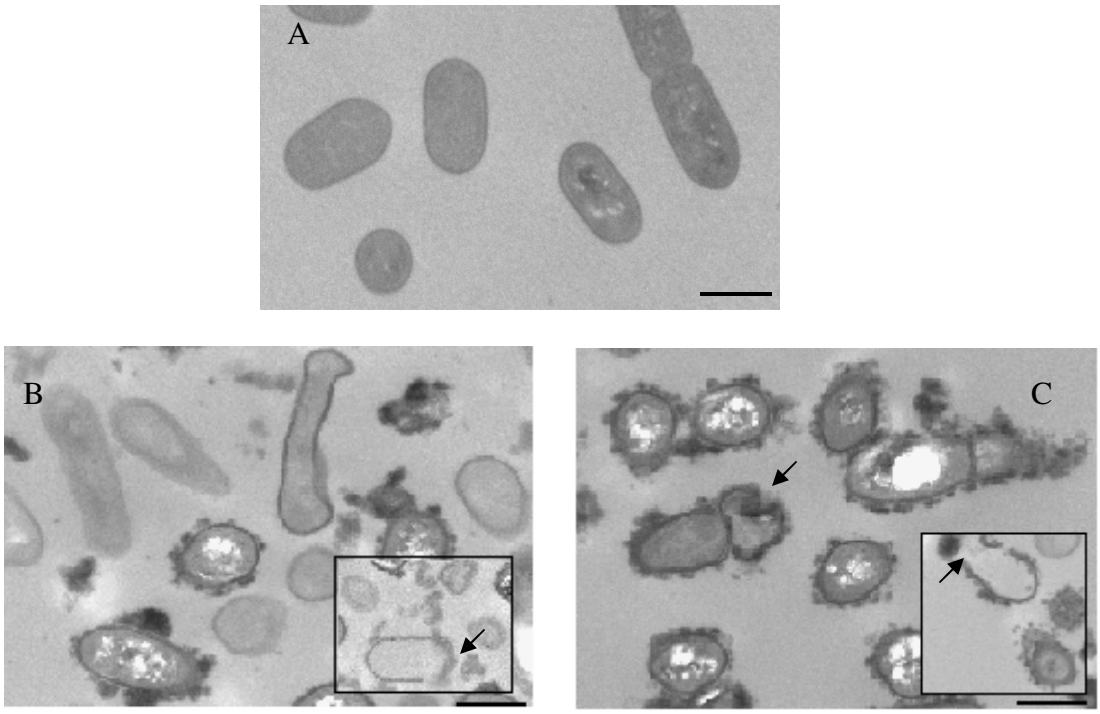

Figure 3. Transmission electron micrographs of *L. monocytogenes* exposed to 10 mg/ml GS in saline for 5 min (panel B) and 10 min (panel C). Inserted boxes display cells with signs of lysis. Panel A is control without GS, bar 0.5 µm.

CHAPTER 7. GENERAL CONCLUSIONS

The efforts of the food industry to maintain the quality and safety of ready-to-eat products will undoubtedly continue. Although the presence of *Listeria monocytogenes* in the processing environment may be unavoidable, processors continue to use control measures and innovating technologies to inhibit the growth of the pathogen in these products. Changes in consumer preferences have also prompted the search for new types of inhibitory substances. In turn, research on the use of natural derived plants extracts as antimicrobials against pathogenic and spoilage organisms in foods have increased.

The evaluation of natural antimicrobials against *L. monocytogenes* was initially conducted in Brain Heart Infusion (BHI) at two storage temperatures (4°C and 10°C). The four compounds tested (cranberry-CB, grape seed-GS, oregano-OR, and green tea-GT), showed activity against the pathogen in BHI at both storage temperatures, but not at all concentrations. The highest inhibitory activity was obtained with GS, based on log reductions and concentration. Although the four antimicrobials were active against *L. monocytogenes* in vitro, only two were tested in a food system. CB and GS were used in combination with a surfactant (sodium lauryl sulfate-SLS) as a surface treatment for frankfurters with or without sodium lactate (SL) in the formulation. Both substances displayed bacteriocidal activity when used in combination with SLS and in frankfurters containing SL. It can be concluded that CB and GS possess antimicrobial activity against *L. monocytogenes* and have good potential for use as part of a multiple hurdle approach in controlling the growth of this pathogens in RTE meats.

To prevent the growth of microorganisms, organic acids are readily used in foods and in the processing environment to sanitize equipment. The ability of *L. monocytogenes* to

adapt to environmental stress (e.g. acid stress) has been shown to increase the pathogen's resistance of other stresses (e.g. antimicrobials). The acid adaptation ability of *L. monocytogenes* was tested in BHI in the presence of CB and GS. Based on results of this study, acid adapted cells exhibit an increased resistance to CB but not to GS. In this regard it can be concluded that prior acid stress in *L. monocytogenes* is ineffective in cross-protecting the pathogen from the antimicrobial action of GS.

Preliminary evidence on the mode of action of GS against *L. monocytogenes* demonstrated possible disruption of the cell membrane based on results from transmission electron microscopy (TEM). The presence of active phenolic compounds such as proanthocyanidins (tannins) in GS may be responsible for membrane damage. Inhibition by these components has been proposed to occur by a change of cell permeability leading to loss of intracellular molecules (proteins, DNA, RNA).

The results compiled in this dissertation show that plant derived compounds can be used as antimicrobials to inhibit the growth of *L. monocytogenes* in foods. The acid resistance of the pathogen should be assessed before utilizing inhibitory substances that may rely on an organic acid for antimicrobial activity. The findings of this study and much of the on-going research on natural antimicrobials offer the industry alternative ways for the control of *L. monocytogenes* in ready-to-eat foods and to prevent further outbreaks.

APPENDIX

Specification sheet for Origanox

BARRINGTON NUTRITIONALS

ORIGANOX
Powerful Natural Antioxidant

Description
Origanox is a natural, completely water soluble, powerful antioxidant, extracted from edible herb species, belonging to the Labiatae family (such as *Origanum vulgare* and *Salvia officinalis*) and considered as GRAS by the FDA. A special feature of Origanox is its stability and carry-through properties under high frying and baking temperatures.

Standard Specification

Appearance	Yellow to light brown powder
Smell	Imparts a mild, tea-like odor
Antioxidant activity	Standardized
Total plate count	≤ 1,000 CFU/g
E. coli	Absent
Salmonella	Absent
Yeast and molds	≤ 100 CFU/g
Lead	≤ 0.5 ppm
Arsenic	≤ 0.5 ppm
Mercury	≤ 0.5 ppm
Total heavy metals	≤ 10.0 ppm
Solubility	Completely soluble in water
Heat stability	Stable and active at 180ºC / 356ºF
Toxicity	Non toxic

Shelf-life
2 years, when stored under appropriate conditions

Labeling
Origanox may be labeled as natural plant extract or natural flavor

Packing
Available in 10 kg aluminum foil bags. Other packing available upon request

Manufacturing conditions
Manufactured under conditions conform to GMP and ISO 9002

Recommended storage conditions
Store in a cool and dry place

Specification sheet for grape seed extract

CERTIFICATE OF ANALYSIS

KIKKOMAN CORPORATION

GRAVINOL
Grape seed extract

PO # LOT # GVS2007

Quantity 10 g Packing size 10 g x 1

CRITERIA	SPECIFICATION	VALUE
Total flavanols (%)	≥ 95	104.7
Proanthocyanidins (%)	≥ 83	92.9
Porter's method value (PVU)	≥ 380	510
Loss on drying	≤ 7	4.0
Lead (ppm)	≤ 2	good
Arsenic (ppm)	≤ 2	good
Aerobic plate cound (CFU/g)	1 x 10^3	good
Coliform	negative	negative

Storage : Dark and cool (preferably desiccated)

The above information is certified to be a true and accurate copy of data obtained for this material.
DATE : April 5, 2004

Edogawa Plant
Biochemicals Division
Kikkoman Corporation