Antimicrobial effect of yogurt lactic acid bacteria and muscadine products on Enterobacter sakazakii

Weiien Weng

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ANTIMICROBIAL EFFECT OF YOGURT LACTIC ACID BACTERIA AND MUSCADINE PRODUCTS ON \textit{ENTEROBACTER SAKAZAKII}

By

Wei-Lien Weng

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ANTIMICROBIAL EFFECT OF YOGURT LACTIC ACID BACTERIA AND MUSCADINE PRODUCTS ON *ENTEROBACTER SAKAZAKII*

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Enterobacter sakazakii has been associated with powdered infant formula outbreaks which caused high mortality rate illnesses in infants in recent years. Current research was mainly focused on searching for natural antimicrobial agents which may be incorporated into baby foods to control this emerging pathogen. Yogurt and muscadine products were used in this study.

The antimicrobial effects of yogurt were evaluated on agar plates and in a simulated gastrointestinal model. In the agar spot tests, diluted yogurt sample containing lactic acid bacteria at $10^6$ CFU/mL was antagonistic toward E. sakazakii.
However, the antimicrobial effect of yogurt on *E. sakazakii* in the simulated GI model was not noted. Certain numbers of tested *E. sakazakii* and lactic acid bacteria in yogurt were able to survive the acidic gastric condition and recovered in the intestinal model.

By measuring the viable *E. sakazakii* cells in liquid cultures, the strong antimicrobial activities of muscadine juices and muscadine seed extracts were demonstrated. Within two hours, all inoculated *E. sakazakii* at $10^6$ CFU/mL were decreased to non-detectable level. Juice and seed extract from dark-skinned muscadine demonstrated stronger antimicrobial activities than those from white-skinned muscadine. The characteristics of muscadine juices and seed extracts were also analyzed. The high phenolics and organic acid contents, such as ellagic, gallic, tannic, and tartaric acids in muscadine were correlated to the inhibitory effect observed.

Key words: *Enterobacter sakazakii*, antimicrobial, lactic acid bacteria, muscadine
DEDICATION

To my parents, Chang-Cheng Weng and Mai-Ju Chen.
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CHAPTER I

INTRODUCTION

The outbreaks of foodborne illness associated with Enterobacter sakazakii and their high mortality rates have attracted much attention in recent years (Bowen and Braden 2006; Mullane and others 2007). Enterobacter sakazakii has been reported to grow rapidly in infant formula once it was reconstituted and may remain viable at refrigerated temperature (Bowen and Braden 2006). Although the mechanism of pathogenesis still remains unclear (Drudy and others 2006; Skovgaard 2007), E. sakazakii has been suggested to be sensitive to acidified food product (Kim and Beuchat 2005; Shaker and others 2008).

Considering the potential inhibition effect and the convenience to deliver to infants, two acidic food products, yogurt and muscadine juices, were selected for investigating their antimicrobial effect against E. sakazakii. Yogurt, one of the most popular carriers of lactic acid bacteria, is known to possess antagonistic effect against various pathogens (Shornikova and others 1997; Gagnon and others 2004). High
phenolic contents of muscadines and their health benefits, such as antioxidant activities, anticancer properties, and anti-inflammatory, have been reported (Pastrana-Bonilla and others 2003; Greenspan and others 2005; Mertens-Talcott and others 2006). However, few studies have been conducted to evaluate the antimicrobial effect of muscadines. This research may be the first study to investigate the antimicrobial activity of muscadine juices.

The extracts from muscadine seeds, which are considered a by-product in juice and winery processing, were also used in this study. Grape seeds have been reported to have the antimicrobial effect against several human pathogens (Jayaprakasha and others 2003), and have been used as natural preservatives in ready-to-eat products (Theivendran and others 2006). However, no studies have been reported on the inhibitory effect of muscadine seed on E. sakazakii.

The objectives of this study were to:

1. Evaluate the antagonistic effect of yogurt against E. sakazakii in the agar spot test.
2. Determine the viability of lactic acid bacteria in yogurt and E. sakazakii in a simulated gastrointestinal (GI) system.
3. Evaluate the antagonistic effect of yogurt lactic acid bacteria toward E. sakazakii in
the simulated GI system.

4. Evaluate the antimicrobial effect of muscadine juices subjected to different treatments on inhibition of *E. sakazakii*.

5. Evaluate the inhibitory effect of hot and cold muscadine seed extracts against *E. sakazakii*.

6. Correlate the antimicrobial effect of muscadine seed extracts with the concentration of major phenolics and organic compounds in the seed extracts.
CHAPTER II

LITERATURE REVIEW

Enterobacter sakazakii

*Enterobacter sakazakii* is a member of the family Enterobacteriaceae, and genus *Cronobacter*. The *Enterobacter* spp., a group of motile, peritrichous, non-sporeforming, facultatively anaerobic, Gram-negative straight rods, is able to ferment glucose with production of acids and gases, and gives a positive Voges-Proskauer reaction. It is biochemically similar to *E. cloacae*, and forms yellow colonies on nutrient agar or tryptic soy agar; therefore, *E. sakazakii* was previously called ‘yellow-pigmented *E. cloacae*’ (Richard 1984). Based on the differences between the two species in DNA relatedness, biochemical reactions, pigment production, and antibiotic susceptibility, *E. sakazakii* was formally listed as a new species and named in honor of the Japanese microbiologist Riichi Sakazaki (Farmer and others 1980).
Figure 2.1 shows the remarkably increasing number of publications in regards to *E. sakazakii*, from 1989 to 2007. *E. sakazakii* has been receiving attention in recent years as an emerging, opportunistic pathogen associated with life threatening meningitis, necrotizing enterocolitis and sepsis in infants (Iversen and Forsythe 2003). These *E. sakazakii* related diseases are rare, but high mortality rates of meningitis (40-80%) and enterocolitis (10-55%) have been reported (Nazarowec-White and Farber 1997a; Lai 2001). Among 111 reported cases of *E. sakazakii* infection worldwide from 1958 to 2005, at least 26 deaths are reported in infants and children (Mullane and others 2007). Additionally, cases involving *E. sakazakii* related bacteremia and osteomyelitis in postsurgical adults and immunocompromised elders have also been reported (Lai 2001; Corti and others 2007; Ray and others 2007; See and others 2007). Although *E. sakazakii* affects all age groups, premature and low birth weight (under 2,500 g) neonates (less than 28 days) are found at the highest risk (Mullane and others 2007). Furthermore, irreversible neurological sequela and retarded neural development have been found in meningitis survivors (Muyltjens and others 1983; Willis and Robinson 1988).
In 1980, Farmer and others reported that antibiotic resistance was uncommon in *E. sakazakii*, and only one strain out of over one hundred tested strains was multiple resistance. *Enterobacter sakazakii* was naturally sensitive or intermediate to some antibiotics, including tetracyclines, aminoglycosides, numerous β-lactams (acylureidopeicillins, ticarcillin, and ampicillin), quinolones, antifolates, chloramphenicol, and nitrofurantoin. It had natural resistance to benzylpenicillin, oxacillin, lincosamides, streptogramins, glycopeptidesm, rifampicin, and fusidic acid (Stock and Wiedemann 2002). Ampicillin in combination with gentamicin or chloramphenicol was traditionally used to treat *E. sakazakii* infections (Willis and Robinson 1988; Lai 2001). However, Lai (2001) reported that all *E. sakazakii* isolates were resistant to ampicillin, cefazolin and penicillin, and proposed the use of carbapenems, the third-generation cephalosporins with an aminoglycoside or trimethoprim-sulfamethoxazole. Owing to the acquisition of transposable elements and the production of β-lactamases, *Enterobacter* species are able to inactivate several β-lactam containing antibiotics (Girlich and others 2001; Iversen and Forsythe 2003). Plasmid-mediated quinolone resistance gene (Qnr-A) in *E. sakazakii* was also reported to cause nosocomial spread (Poirel and others 2007).
**Enterobacter sakazakii in food and powdered infant formula**

*Enterobacter sakazakii* is distributed widely in the environment, including water, soil, households, food factories producing powdered milk, chocolate, cereals, potato flour and pasta (Kandhai and others 2004; Friedemann 2007). It has also been isolated from a variety of food products, such as cheese, dried fish products, meat, vegetables, grains, herbs and spices (Kim and Beuchat 2005; Richards and others 2005; Chaves-López and others 2006; Kim and others 2008a). In addition, some researchers believed that rats and flies may be the possible vectors of this bacterium (Gakuya and others 2001; Mramba and others 2007).

Powdered infant formula is designed as a breast milk substitute with formulated nutritional profile. However, many foodborne outbreaks in children have been epidemiologically associated with the ingestion of contaminated powdered infant formula (Biering and others 1989; CDC 2002). During 2005 to 2007, there were 14 out of 49 recalls of infant formula due to *E. sakazakii* contamination worldwide (ICDC 2008). Environmental contamination of infant formula during processing is likely to be the source of *E. sakazakii* (Kandhai and others 2004). In the first survey of the incidence of *E. sakazakii* in 141 infant formulae from 35 countries, *E. sakazakii* was
found at level ranging from 0.36 to 66 CFU/100g in 20 infant formulae from 13 countries (Muutjens and others 1988). Nazarowec-White and Farber (1997) reported that the levels of *E. sakazakii* were 0.36 CFU/100g in 3 positive samples out of 120 cans of infant formulae from five Canadian companies. Generally, *E. sakazakii* is found at low concentration in most positive powdered infant formula samples. However, this bacterium can multiply rapidly in reconstituted powdered infant formula. It takes only 45 min for *E. sakazakii* to double in reconstituted formula at room temperature (Forsythe 2005). Thus, prolonged storage of reconstituted infant formula at room temperature or in a bottle warmer might increase the possibility of *E. sakazakii* infection of infants (Mullane and others 2006).

Powdered infant formulae have a low water activity (*a*<sub>ω</sub>) of about 0.2. Breeuwer and others (2003) reported that *E. sakazakii* in stationary phases were relatively more resistant to dry stress in comparison to *Escherichia coli*, *Salmonella*, and other members of the Enterobacteriaceae family, which are common contaminates found in powdered milk. Following incubation at 25°C, exponential phase *E. sakazakii* decreased by seven log unit in ten days, while stationary phase cells decreased by 1 to 1.5 log units in 46 days. They added that the dry resistance is likely linked to the
trehalose accumulated in the cells. Gurtler and Beuchat (2007) and Lin and Beuchat (2007) studied the parameters affecting *E. sakazakii* survival. They found that *E. sakazakii* strains could survive in powdered infant formula and infant cereal (a$_w$ range from 0.25 to 0.86) for up to 12 months at all tested temperatures (4 to 30°C), even with lower initial inoculation (less than 1 log CFU/g). Although increases in the a$_w$ and temperature cause an increase in the rate of death, the survival of *E. sakazakii* was not influenced by the composition of powdered infant formula or infant cereal (Gurtler and Beuchat 2007; Lin and Beuchat 2007). Some strains of *E. sakazakii* were reported to have the ability to survive in dehydrated powdered infant formula for two years (Edelson-Mammel and others 2005; Caubilla-Barron and Forsythe 2007).

*Enterobacter sakazakii* is one of the most thermal resistant microorganisms among Enterobacteriaceae members found in dairy products (Nazarowec-White and Farber 1997c). Iversen and others (2004) reported the D value of *E. sakazakii* at 58°C was 2.4 min, compared with 4.2 min reported by Nazarowec-White and Farber (1997). The overall z value (5.7-5.8°C) was similar in both studies. Thus, the predicted D value of *E. sakazakii* in infant formula at 71.2°C was 0.7 s. These data would indicate that the high-temperature short-time pasteurization process (71.7°C for
15 s) is effective for the inactivation of *E. sakazakii* in milk (Iversen and others 2004). However, diversity in thermal tolerance among the strains of *E. sakazakii* was found. The D values of 12 strains of *E. sakazakii* at 58°C ranged from 30.5 to 591.9 s (Edelson-Mammel and Buchanan 2004). Thus, some high-temperature tolerant strains of *E. sakazakii* may exist.

Several newer processing technologies, such as irradiation, ultra-high pressure and pulsed electric fields, have been tested for the reduction of *E. sakazakii* in infant formula. When exposed to Gamma irradiation, *E. sakazakii* D_{10}-values ranged from 0.21 to 0.29 kGy and 0.76 to 1.71 kGy in broth and dehydrated powdered infant formula, respectively (Lee and others 2006; Osaili and others 2007). When exposed to high-pressure processing (600 MPa for 1 min), *E. sakazakii* was reduced by 3 to 6.84 log in inoculated reconstituted powder infant formula (González and others 2006). After exposure to pulsed electric fields for 360μs at 40 kV/cm, *E. sakazakii* in reconstituted powder infant formula was reduced 1.2 log CFU/mL (Pina Pérez and others 2007).

Since *E. sakazakii* is possibly introduced to product after pasteurization process by adding other heat sensitive ingredients and/or contamination from nonsterile
equipment, the growth parameters of *E. sakazakii* in reconstituted infant formula were also investigated. The estimated optimal growth temperature was 39.4°C, while *E. sakazakii* was found able to grow in reconstituted infant formula at temperatures between 5.5 to 47°C. The average generation times were 40 min at 23°C and 4.98 h at 10°C (Nazarowec-White and Farber 1997b; Kandhai and others 2006). Therefore, Gurtler and Beuchat (2007) suggested that portions of reconstituted infant formula should be stored at or below 4°C, and no longer than four hours.

Acidification has been found to lower the concentration of *E. sakazakii* (Joosten and Lardeau 2004; Friedemann 2007; Lin and Beuchat 2007). Josten and Lardeau (2004) proposed that acidification, either through fermentation or by direct addition of lactic acid, could prevent the rapid microbial growth in infant formula. Infant rice cereal reconstituted with apple juice at pH 4.29 did not support the growth of inoculated *E. sakazakii* up to 9.3 CFU/mL, while this bacteria grew rapidly in infant rice cereal reconstituted with water, milk, or infant formula (Richards and others 2005).
Pathogenicity and virulence factors

The mechanism of pathogenesis of *E. sakazakii* still remains unknown (Drudy and others 2006; Skovgaard 2007). Generally, the gastrointestinal tract is a suggested portal of entry among patients with *Enterobacter* spp. bacteremia, and meningitis is believed to occur by translocation of invasive bacteria (Iversen and Forsythe 2003; Pagotto and others 2003). The adherence and invasion capacity of *E. sakazakii* to intestinal epithelial cells and the brain microvascular endothelial cells was demonstrated (Mange and others 2006; Mohan Nair and Venkitanarayanan 2007; Townsend and others 2007b; Kim and others 2008a). The more permeable intestinal tracts of premature infants and immunocompromised individuals might make them highly susceptible to *E. sakazakii* (Lenati and others 2008). Furthermore, the less acidic stomach of newborns and the buffering capacity provided by ingested milk also contribute to survival and infection of *E. sakazakii* in infants (Forsythe 2005; Lenati and others 2008).

Contaminated feeding utensils, such as blenders, spoons, and bottles, used for the preparation of infant feed have been linked to *E. sakazakii* infection (Bar-Oz and others 2001). Recent studies found that some *E. sakazakii* strains have the ability to
form biofilms, enabling the organism to attach to common food preparation surfaces, including silicon, latex, polycarbonate, stainless steel, glass, and polyvinyl chloride (Iversen and others 2004; Lehner and others 2005; Kim and others 2006; Oh and others 2007). Inversen and others (2004) reported that higher populations of *E. sakazakii* attached to silicon, latex and polycarbonate than to stainless steel. Kim and others (2006) found the attachment strength of *E. sakazakii* was stronger when growing at 25°C than at 12°C. Biofilms may also provide protection for bacteria from environmental stresses, such as UV light, osmotic stress, heat, starvation, acids, detergents, antibiotics, and bacteriophages (Iversen and others 2004; Lehner and others 2005).

Up until now, there was no evidence for a minimal infectious dose of *E. sakazakii* in humans. It was estimated at 1,000 CFU/ml based on the infectious dose of *E. coli* O157 and *Listeria monocytogenes* 4b (Iversen and Forsythe 2003). In vitro studies in suckling mice showed that the lethal level of tested strains was 1 X10^8 CFU (Pagotto and others 2003). They also proposed that the presence of enterotoxin might be the pathogenesis factor of *E. sakazakii*. Besides, recent research indicated that lipopolysaccharides, a heat stable bacterial endotoxin, may be present in powdered
infant formula. Upon ingestion, the endotoxin may contribute to failure of neonatal intestine integrity, and therefore increase in the bacterial translocation from the gut (Townsend and others 2007a). However, these toxins have not been identified yet.

Health benefits of yogurt and lactic acid bacteria

Yogurt is a semisolid fermented dairy product derived by the activity of a symbiotic blend of lactic acid bacteria (LAB). Although milk of various mammals is used for making yogurt in the world, cow’s milk is the most common one used in yogurt manufacturing. *Streptococcus salavarius* subsp. *thermophilus* and *Lactobacillus delburechii* subsp. *bulgaricus* are the most common starter cultures. These yogurt starter cultures increase the acidity through production of organic acids, mainly lactic acid, during yogurt fermentation, and thus form a coagulum as well as achieve preservation, the original function of fermented foods (Chandan and Shahani 1993).

In addition to yogurt starter cultures, probiotics are often added to yogurts nowadays. Probiotics have been defined by the Food and Agriculture Organization of the United Nations (FAO/WHO) and the World Health Organization (WHO) as ‘live microorganisms which when administered in adequate amounts confer a health benefit
on the host’ (FAO/WHO 2002). Among probiotic cultures, *Lactobacillus acidophilus*, *L. casei* and *Bifidobacterium* spp. are often added to yogurt products (Shah 2001).

In the early twentieth century, the Noble Prize laureate biologist, Élie Metchnikoff first suggested that the long healthy life of Bulgarians may be attributed to the consumption of fermented milk products, in which fermenting bacilli may inhibit toxic microbial activities in the gastrointestinal tract (Holzapfel and others 1998; Hove and others 1999). Many studies since then have discussed more beneficial effects of fermented milk products and lactic acid bacteria, including enhancing the bioavailability of nutrients, enhancing the immune system, reducing symptoms of lactose intolerance and reducing risk of certain cancers, attributed to LAB and their fermented products (Parvez and others 2006).

**Calcium and bone health**

Health benefits of yogurt are mainly attributed to its high nutrition value and to the presence of LAB. The nutrient composition of yogurt is based on the milk it is made from. Milk and its products are rich in protein, calcium, phosphorus, and vitamin B. In the latest food guide pyramid- MyPyramid, two cups (eight fluid ounces) of milk, yogurt and/or other milk products were suggested to meet the daily
calcium requirement, which is vital for bone health (USDA 2005). Calcium in milk and yogurt have been considered as valuable sources due to the high content and its bioavailable form, which is available for absorption and used by human body (McKinley 2005). Calcium must be ionized and in solution to be absorbed. In milk, calcium complexes to lactate, phosphopeptides, and caseins, which are the forms easily to be digested and uptake by human gastrointestinal tract (Bronner and Pansu 1999; Naidu and others 1999). Bronner and Pansu (1999) also reported that the low pH of yogurt may ionize calcium and thus facilitate calcium absorption. A study in 1987 showed greater bone mineralization on yogurt-fed rats compared to the ones fed with calcium carbonate (Adolfsson and others 2004). Particularly for lacto-vegetarians and lactose intolerance individuals, yogurt are great sources of calcium (Smith and others 1985; Weaver and Plawecki 1994).

Alleviation of lactose intolerance symptoms

Lactose, the main carbohydrate in milk, is a disaccharide composed of glucose and galactose. It can be digested by lactase (β-galactosidase) and subsequently absorbed in the small intestine. However, approximately two thirds of the world adult population loses the intestinal lactase activity gradually after weaning (Vesa and others 2004).
The intestinal lactase deficiency leads to the inability to digest lactose, described as lactose maldigestion, malabsorption or lactose intolerance. When the undigested lactose enters the large intestine, it can be fermented by the colonic microflora, causing various degrees of discomfort symptoms, such as flatulence, diarrhea and abdominal cramping (Vesa and others 2000; Adam and others 2004). Many lactase-deficient individuals were reported better able to tolerate fermented dairy products than fresh milk (Savaiano and others 1984; Hertzler and Clancy 2003). Probiotic cultures, such as *L. acidophilus* and *B. longum*, were also reported to aid lactose digestion in humans when incorporated into unfermented milk (Kim and Gilliland 1983; Jiang and others 1996). Both the reduction of lactose during fermentation and the presence of microbial β-galactosidase released by LAB in the gastrointestinal transit are responsible for the improved ability to tolerate lactose (McDonough and others 1987; Hughes and Hoover 1991; Rolfe 2000).

**Immune system modulation**

The immune system is a complex protection barrier in the body. There are two immune mechanisms in healthy humans. The specific immunity is an antigen-specific immune response and is enhanced by repeated exposure. The
nonspecific immunity consists of physical barriers, e.g. mucous membranes and phagocytic cells (Meydani and Ha 2000). Stimulation of both specific and nonspecific immunity by yogurt and probiotics was reported in the literature. The leukocyte phagocytic activity of human blood cells was enhanced by the consumption of a fermented milk product supplemented with *L. acidophilus* La1 and *B. bifidum* Bb12 for 3 wk (Schiffrin and others 1995). Intake of baby formula containing viable *B. lactis* was reported to increase IgA (Immunoglobulin A) level in healthy children (Fukushima and others 1998). It has been indicated that the enhanced immunity by consumption of yogurt and LAB may contribute to the increased resistance to infection and to the decreased incidence of gastrointestinal disorders, cancer and allergic symptoms (Meydani and Ha 2000; Isolauri and others 2002).

**Antimicrobial activity**

The most notable benefit of yogurt and LAB is suppression of pathogens, by which restoring normal microbial composition and functions in the intestinal tract. Certain LAB such as *L. acidophilus, L. bulgaricus* and *B. longum* have been known for their preventative and therapeutic effects against antibiotic-associated diarrhea (Marteau and others 2002). It has also been reported a shortening of rotavirus
diarrhea in children by treatment with *L. rhamnosus* GG, *L. reuteri, B. bifidum* and *S. thermophilus* (Saavedra and others 1994; Shornikova and others 1997; Guandalini and others 2000).

In several in vitro studies, LAB cultures were inhibitory toward various harmful bacteria, such as *Escherichia coli* O157:H7, *Helicobacter pylori, Salmonella* spp. and *Staphylococcus aureus* (Kim and others 2003; Gagnon and others 2004; Millette and others 2004). This beneficial effect has been associated to antimicrobial compounds, such as organic acids and bacteriocins, by which conditions less favorable for pathogenic bacteria and spoilage microorganisms were created (Lourens-Hattingh and Viljoen 2001). Organic acids, mainly lactic and acetic acids are metabolites of LAB. Among all LAB produced organic acids, acetic acid showed strongest antimicrobial activity, inhibiting a wild range of yeasts, molds and bacteria (Vandenbergh 1993; Ouwehand and Vesterlund 2004). Bacteriocins are proteins or peptides secreted by bacteria that inhibit the growth of other bacterial strains or species. Lactic acid bacteria produce various bacteriocins against other Gram- positive bacteria in genera *Bacillus, Clostridium, Enterococcus, Staphylococcus* and *Listeria* (Klaenhammer 1993). Nisin, a well-known example of LAB produced bacteriocin, has been used in
food systems as a preservative (Hansen 1994; Sobrino-López and Martín-Belloso 2008).

In addition to producing antimicrobial compounds and enhancing immunity activities, competitive inhibition by LAB has been proposed as another mechanism found in LAB. Probiotics may prevent harmful bacterial colonization in the intestinal tract by competing essential nutrients for bacterial growth and for adhesion receptors in the intestinal tract (Lourens-Hattingh and Viljoen 2001). However, the adhesion ability is strain and dose dependent. Only a few probiotic strains, such as *L. acidophilus*, *L. casei*, *B. infantis* and *B. breve* showed the adhesive properties to enterocyte cell-line Caco-2 (Bernet and others 1993, 1994).

**Anti-colorectal cancer effect**

Colorectal cancer is cancer that occurs in the colon or rectum. Colorectal cancer is the third most common cancer after prostate and lung cancers and after breast and lung cancers for men and women, respectively. In the United States, colorectal cancer is the second leading cause of cancer-related deaths (CDC 2007). Some studies have shown that LAB and their metabolites may have anti-colorectal cancer effect (Wollowski and others 2001; Rafter 2002). Several postulated mechanisms
that reported to reduce the risk of colorectal cancer are: (i) Mutagen binding and
degradation (Rowland and Grasso 1975; Zhang and Ohta 1991; Orrhage and others
1994); (ii) Inhibition of carcinogen-producing enzymes, such as β-glucuronidase and
nitroreductase (McConnell and Tannock 1993; Ling and others 1994); and (iii)
Stimulation of immunity to better defend against cancer cells (Matsuzaki and others
2004).

Reduction in serum cholesterol

Cholesterol plays essential roles in human and animal body. It acts as a
component of cell membranes and precursor to several vitamins and steroid hormones.
However, high levels of total blood cholesterol or low-density lipoproteins are
considered risk factors for developing coronary heart disease. In animal trials,
feeding of *L. acidophilus* significantly reduced cholesterol levels in the serum of pigs
even fed with a high cholesterol diet (Gilliland and others 1985). Probiotics and
fermented milk products have also been reported to reduce serum cholesterol in
humans although the effects may be culture and strain dependent (Buck and Gilliland
1994; Taylor and Williams 1998; Sanders and Klaenhammer 2001). Two mechanisms
responsible for lowering serum cholesterol have been postulated. One theory is the
ability of some LAB to assimilate cholesterol within bacterial cells and thus reduce the amounts absorbed in blood. Another possibility is that certain intestinal lactobacilli can increase excretion of bile acids through hydrolyzation. Thus, the synthesis of new bile acids from cholesterol in liver can indirectly lower cholesterol level in serum (Gilliland and Speck 1977; Sanders 1999; Ray 2001).

**Muscadine grapes**

Muscadine grapes (*Vitis rotundifolia*) are the predominant grape species in the southeastern United States and have been cultivated for more than 400 years. Indigenous muscadines are now naturalized ‘from Delaware south along the Atlantic coast to central Florida, west along the Gulf of Mexico to eastern Texas, north along the Mississippi River to Missouri, and east to Delaware, with the exclusion of the Appalachian Mountain areas’ (Olien 2001). More than 95% of muscadine production is in the southeastern coastal states. Among 15 muscadine-producing states, Georgia (900 acres), North Carolina (660 acres), and Mississippi (600 acres) ranked top three in muscadine production in 1988 (Olien 1990). During the following years, the production in both Georgia and North Carolina increased to more than 1300 acres.
However, muscadine production in Mississippi decreased to 300 to 400 acres in 2005 (Cline and Fisk 2006; Coblentz 2007).

Compared to the European (*Vitis vinifera*) and American (*Vitis labrusca*) grapes, muscadines are well adapted to the areas of hot and humid climate, having better ability to resist Pierce’s bacterial disease (Chen and others 2001). Unlike other bunch grapes, muscadine fruits are developed individually within a loose cluster that ripens over an extended period. They are harvested from mid-August to mid-September in Mississippi (Chen and others 2001; Coblentz 2007).

Muscadines have unique aroma and flavor, and are currently used for fresh table grapes, wines, juices, jams and jellies, U-pick operations, and some functional byproducts. There are more than 100 different muscadine cultivars available for home planting and commercial production (Mortensen 2001). Usually, they are divided into purple and bronze muscadines, based on the skin color. Purple to black skinned muscadines, such as ‘Noble’ and ‘Ison’, are also called bullace, bull grape, bullet grape etc. ‘Scuppernong’ is the traditional generic name for all bronze muscadine, ‘Carlos’ and ‘Magnolia’ for examples. Actually, ‘Scuppernong’ is
probably the first bronze muscadine cultivar, named after the area in which it was discovered (Olien 1990; Olien 2001).

Muscadines have been reported to contain nutritional and functional components including vitamins, minerals, fiber, and polyphenolics (Ector 2001; Threlfall and others 2007). In recent years, interest in the health benefits of muscadines has increased due to their high phenolics contents, which has been proven to have excellent antioxidant capacity (Pastrana-Bonilla and others 2003).

**Phenolics in muscadines**

The term “phenolic” or “polyphenolic” refers to general structures of molecules which have aromatic rings bearing one or more hydroxyl groups, including functional derivatives such as esters and glycosides. The chemical structures of phenolic compounds range from quite simple to highly complex. The simple phenolic acids include benzoic acid derivatives such as gallic acid, and cinnamic acid derivatives such as caffeic acid. Flavonoids are a group of phenolics having the skeleton of diphenylpropanes with different oxidation level. Kaempferol, quercetin and catechin are examples of flavonoids. Stilbenes (such as resveratrol), lignans, and hydrolysable and condensed tannins are other phenolics with highly polymerized structures.
Phenolic compounds are secondary metabolites unique in higher plants, and serve as a defense system against pathogens and environmental stress (Shahidi and Naczk 1995). For example, resveratrol produced by *Vitis* species has been reported to resist fungal infection and in response to ultraviolet irradiation and mechanical injury (Magee and others 2002).

Most phenolics in grapes and muscadines are located in the seeds and skin (Pastrana-Bonilla and others 2003). Gallic acid, catechin and epicatechin are the main phenolics found in muscadine seeds, while ellagic acid, myricetin, quercetin, kaempferol and resveratrol are the major phenolics in the skin (Pastrana-Bonilla and others 2003). Overall, the major phenolics reported in muscadines are ellagic acid (6.4-6.8mg/100g), kaempferol, myricetin and quercetin. Anthocyanins are the most abundant flavonoids present in whole muscadines (Talcott and Lee 2002). Total anthocyanins range from 2 to 104 mg/L in muscadine juice, depending on cultivar skin color, with red ones having the highest concentration (Threlfall and others 2007).

The presence of ellagic acid and anthocyanins in muscadines is unusual among grape species (Lee and Talcott 2004). However, anthocyanins and ellagic acid and their derivatives are common phenolic compounds in berries, such as blueberries,
raspberries and strawberries, well-known as good antioxidant sources (Puupponen-Pimiä and others 2005c). Anthocyanins, primarily as 3, 5-diglucosides in muscadine, appear as red or purple color substances in the skin. Ellagic acid is a dimeric derivative of gallic acid, and is believed to be formed by hydrolysis from its conjugated forms like ellagic acid glycosides and ellagitannins in muscadines (Lee and Talcott 2002).

**Antioxidant capacity and other protective effects of phenolics**

Positive correlation exists between total phenolics in fruits and vegetables and their antioxidative activities (Velioglu and others 1998). Total phenolics or individual phenolic compounds either in berries or in grapes have been demonstrated to have excellent antioxidant capacity (Kähkönen and others 2001; Pastrana-Bonilla and others 2003; Yilmaz and Toledo 2004). They also have been correlated to the induction of apoptosis, which inhibits growth of cancer cells (Mertens-Talcott and others 2006; Seeram and others 2006), anti-inflammatory property (Greenspan and others 2005; Bralley and others 2007), and antimicrobial activity (Puupponen-Pimiä and others 2001; Nohynek and others 2006).
Velioglu and others (1998) believe that many protective effects of fruits and vegetables are derived from their antioxidant functions. Plant-derived antioxidants function as hydrogen donators, oxygen quenchers, free radical scavengers, peroxide decomposers, metal chelators, enzyme inhibitors, and synergists (Wang and Lin 2000; Puupponen-Pimiä and others 2001). For example, grape seed extract containing catechin and gallic acid, has been shown to prevent oxidative damage in tissues by reducing lipid oxidation and/or blocking the production of free radicals (Yilmaz and Toledo 2004). The inhibitory effect of metalloproteinase of water extracts of raspberries, blackberries and muscadines could contribute to their anticarcinogenesis effect (Tate and others 2004). The anticancer activity of muscadines may be partially based on the ability to quench reactive oxygen species and the protection of critical cellular components such as DNA, proteins, and lipids from oxidative damage (Mertens-Talcott and others 2006). In addition, combinations of phenolics are presented in fruits and vegetables, and may be involved in synergistic interactions. Ellagic acid and quercetin were reported to interact synergistically with resveratrol as anticarcinogens (Mertens-Talcott and Percival 2005).
Antimicrobial effect of phenolics

Berries and their phenolic compounds have also been shown to selectively control several pathogens as natural antimicrobials, which maybe a resolution of antibiotic resistance (Puupponen-Pimiä and others 2005b; Puupponen-Pimiä and others 2005c). Some pure berry phenolics and berry extracts show inhibitory effect against tested gastrointestinal Gram-negative pathogens, like *Helicobacter pylori*, *Escherichia coli*, and *Salmonella enterica*. However, the inhibition activity on Gram-positive bacteria remains ambiguous in the literature. Puupponen-Pimiä and others (2005a) reported berry extracts might possess a selective inhibition effect on Gram-positive bacteria. Berry phenolic compounds have antimicrobial effect on *Staphylococcus aureus*, but not on all lactic acid bacteria (Puupponen-Pimiä and others 2001). It was reported that cranberry (at 10mg extract per mL) is the only berry extract proven to affect *Listeria* strains (Puupponen-Pimiä and others 2005c). Overall, cloudberry, raspberry and strawberry, which are rich in ellagitannins share the strongest inhibitory ability among common Nordic berries (Puupponen-Pimiä and others 2005c; Nohynek and others 2006). So far, only one paper has been published to assess potential antimicrobial effects of muscadines (Kim and others 2008b). They showed that water soluble
muscadine seed extracts with high concentration of tartaric acid has strong antimicrobial activity on \textit{E. coli O157:H7}.

It has been concluded that the complex mixture of phenolic compounds in berries may affect different bacterial species by different mechanisms (Puupponen-Pimiä and others 2005c; Heinonen 2007). First, the different cell surface structures between Gram-positive and Gram-negative bacteria might limit the accessibility to berry phenolics. Some phenolic compounds have been reported to disintegrate the lipopolysaccharide layer of the outer membrane and increase the permeability of Gram-negative bacteria. Cloudberry and raspberry extracts may disintegrate the bacterial outer membrane and cause permeabilisation of \textit{Salmonella} strains (Nohynek and others 2006). Secondly, antiadhesion could block the adherence of bacteria to epithelial cells, which is generally believed as an important virulence character in bacterial infections. Ellagitannins in cranberry have demonstrated possible bacterial antiadhesion activity, and gives cranberry juice the ability to inhibit \textit{E. coli} adherence to the uroepithelium (Puupponen-Pimiä and others 2005b). Thirdly, Puupponen-Pimiä and others (2005b) suggested that the degree of hydroxylation of pure flavonols and flavones might affect their antibacterial activity. For example,
lactic acid bacteria are only inhibited by myricetin but not other more lipophilic flavones (Puupponen-Pimiä and others 2005b).

**Antimicrobial property of organic acids**

Organic acids are known to have antimicrobial effect and are used as food preservatives or additives. Their effectiveness is dependent on the type and concentration of acid, dissociation level, pH reduction, and the type of microorganisms affected. Among these factors, pH is considered a primary factor of antimicrobial activity (Ricke 2003). At low pH (below pKa value of the acid), the acids exist in their undissociated forms and may interact with or pass through the cell membrane, which usually in microorganisms is negatively charged. Therefore, a low pH could alter cell membrane permeability, and further lower the enzyme functions in microorganisms and change their physiological status (Puupponen-Pimiä and others 2005b). However, incorporation with other antimicrobial agents, phenolic acids for example, yields better antibacterial activity than organic acids alone (Eswaranandam and others 2004; Waite and Daeschel 2007).
Effect of phenolic and organic acids on food quality

Astringency and bitterness of food products depends on their phenolic as well as organic acid content. Both of them change as grape ripens. Generally, phenolic content increases as fruit ripens (Lee and Talcott 2004). Tartaric and malic acids, the most prominent organic acids in muscadine grapes, decrease during ripening (Lamikanra and others 1995).

The 3, 5- anthocyanin diglucosides are unstable to oxidation and heat. The anthocyanin degradation may be responsible for the color loss during juice or wine storage (Doremus-Coign 1989; Talcott and Lee 2002). Also, ellagic acid has been identified as the predominant component of the insoluble sediment, considered as a quality defect in muscadine juice and wine (Lee and Talcott 2002).

Phenolics analysis by HPLC

Reversed-phase high performance liquid chromatography (RP-HPLC) with ultraviolet detection has been utilized to analyze many food components, including organic acids and phenolic compounds, with high sensitivity (Lee 2000; Talcott 2003). The mobile phase, a relatively polar buffer, such as water, methanol, or acetonitrile,
changes to a continuous gradient during detection. The stationary phase is relatively nonpolar, such as silica-based C18 column.

For phenolic compound detection, either acid or alkaline hydrolysis is necessary to separate the free phenolic acids from their conjugated form in the samples during the preparation steps. The separation of the phenolic components is based on their relative hydrophobicity. The elution and separation of phenolic compounds by RP-HPLC is in a decreasing polarity order (Harborne 1998; Lee 2000). Their identification and quantification involve comparing the retention time and peak area of interest to those of pure standards. The results are often presented in milligrams per milliliter or gram of food sample (Talcott 2003).
Figure 2.1  Number of publications per year on *Enterobacter sakazakii* in databases PubMed and AGRICOLA.
CHAPTER III

ANTAGONISTIC EFFECT OF YOGURT LACTIC ACID BACTERIA AGAINST *ENTEROBACTER SAKAZAKII*

Abstract

Yogurt, one the most popular carriers of lactic acid bacteria (LAB), is known to exhibit antagonistic activity against human pathogens and also maintain healthiness of the gastrointestinal system. The objective of this study was to evaluate the antagonistic effect of yogurt LAB against *Enterobacter sakazakii*. In agar spot tests, yogurt sample containing LAB at $10^6$ CFU/g were antagonistic toward *E. sakazakii* strains Fec39 and MSDH. The clear zones, indicating inhibitory effect, were significantly bigger with decreased concentration of inoculated *E. sakazakii*. In the simulated gastric model, the D value of *E. sakazakii* (90.5 min) was lower than that of LAB (about 148 min) when pH dropped below 3.0. The recovery of viable *E. sakazakii* was significantly higher than that of LAB after being transferred to the simulated small intestinal compartment. Yogurt LAB were able to survive through
the simulated GI model; however, the expected inhibitory effect of yogurt toward *E. sakazakii* was not clear in this model. A lower initial concentration of *E. sakazakii*, as well as competitive adhesion ability to intestinal epithelium may be considered for further study.

**Introduction**

*Enterobacter sakazakii* is a member of the Enterobacteriaceae family, genus *Cronobacter*. It is a gram-negative, motile, non-spore forming, facultative anaerobic bacillus. It has been considered an emerging opportunistic pathogen causing rare but life threatening meningitis, necrotizing enterocolitis, and sepsis in infants (Iversen and Forsythe 2003). Among 111 reported cases of *E. sakazakii* infection worldwide from 1958 to 2005, at least 26 deaths were reported in infants and children (Mullane and others 2007). Irreversible neurological consequence and retarded neural development have been commonly found in meningitis survivors (Lai 2001). Outbreaks of *E. sakazakii* infections have been associated with the ingestion of contaminated powdered infant formula (Iversen and Forsythe 2003). Although the pathogenesis mechanism of *E. sakazakii* is still unclear, the gastrointestinal (GI) tract is believed a portal of entry of this bacterium. The adherence and invasion capacity of *E. sakazakii* to
intestinal epithelial cells have also been reported (Mange and others 2006; Mohan Nair and Venkitanarayanan 2007).

Lactic acid bacteria cultures were inhibitory toward various harmful bacteria, such as *Bacillus*, *Clostridium*, *Enterococcus*, *Escherichia coli* O157:H7, *Helicobacter pylori*, *Listeria*, *Salmonella*, and *Staphylococcus aureus* (Klaenhammer 1993; Kim and others 2003; Gagnon and others 2004; Millette and others 2004). The antimicrobial effect of LAB, especially those classified as probiotics, may due to the production of organic acids and other antimicrobial compounds, enhancing immunity activities, and competitive exclusion of pathogens (Bernet and others 1993; Klaenhammer 1993; Vandenberg 1993; Collado and others 2006). Probiotics have been clinically demonstrated to be effective therapeutic agents by reducing the incidence of diarrhea in humans (Saavedra and others 1994; Shornikova and others 1997).

Survival of the gastrointestinal (GI) transit is one of the preconditions for bacteria to develop beneficial effects or to cause diseases. After consumption, bacteria should be able to survive the low stomach pH and tolerate the bile salts in the duodenum (Lick and others 2001). Several simulated GI models have been established with simulated gastric and intestinal juice as alternative to the human GI tract, which is relatively
inaccessible and a difficult area to investigate (Minekus and others 1995; Marteau and others 1997; Koo and others 2001).

This study was designed to evaluate the effectiveness of yogurt, the most popular carrier of LAB, on inhibiting *E. sakazakii* in the agar spot test and in a simulated GI model. The ability of yogurt lactic acid bacteria and *E. sakazakii* to resist GI transit *in vitro* by simulation means was also determined.

**Materials and Methods**

**Chemicals and media**

All chemicals used in this study were purchased from Sigma-Aldrich Inc. (St. Louis, MO) and Fisher Scientific Inc. (Pittsburgh, PA), unless otherwise noted. All culture media and ingredients used for bacteria maintaining, dilution, and enumeration were obtained from Difco Laboratories (Sparks, MD).

**Bacterial strains and yogurt sample**

*Enterobacter sakazakii* strains Fec39 and MSDH, *Lactobacillus bulgaricus* LL18, and *L. helveticus* CNRZ32 used in this study were provided by Dr. Yosen Chen (Mississippi State University). Cultures of *E. sakazakii* were grown in Bacto tryptic
soy broth (TSB) at 37°C for 12 h, and cultures of *Lactobacillus* in Bacto Lactobacilli MRS broth (MRS) at 37°C in a BD GasPack EZ anaerobe container system (Becton, Dickinson and Company, Sparks, MD) for 12 h. All cultures were held at 4°C as stock cultures, and transferred to fresh broth to maintain bacterial viability.

Commercial nonfat plain yogurt (Great Value; marketed by Wal-Mart Stores, Inc., Bentonville, AR) labeled to contain *Lactobacillus acidophilus*, *L. bulgaricus*, *Streptococcus thermophilus*, and *Bifidobacterium logum* was purchased from local stores (Starkville, MS). The yogurt sample was stored at 7°C and used for studies before the sell by date.

**Agar spot test**

A modified agar spot test (Gagnon and others 2004) was used to examine the antagonistic effect between LAB and *E. sakazakii*. Yogurt was diluted at 1:100 (w/v) with 0.1% peptone solution. Pure cultures of *L. bulgaricus* LL18 and *L. helveticus* CNRZ32 were grown in MRS broth at 1% (v/v) inoculation and incubated anaerobically at 37°C for 12 h. A 2 μL portion of each LAB sample was first spotted on 1.5% MRS agar plates. Plates were dried at room temperature for 30 min, incubated anaerobically at 37 °C for 18 h, and overlaid with 10 mL soft agar
containing *E. sakazakii*. The overlaid inoculum was prepared by adding full growth *E. sakazakii* cultures (37°C in TSB for 12 h) into TSB containing 0.85% agar at 0.01, 0.1, and 1% ratio (v/v) to obtain final concentrations of 10^4~10^6 CFU/mL. After incubation at 37 °C for 18 h, the zones of clear agar around the LAB spots on the plates were measured.

**Simulated gastrointestinal (GI) model**

To determine the antagonistic activity of yogurt LAB against *E. sakazakii*, a simulated GI model (Marteau and others 1997; Koo and others 2001) with some modifications was also used in this study. As shown in Figure 3.1, two stainless steel containers with closure lids (Royal House Hold Products Inc., Cerritos, CA) were used to represent the gastric and small intestine compartments, respectively. A sterile 1,000 mL Pyrex beaker with a sterile magnetic stirring bar was placed inside of each compartment. The two containers were then placed in an acrylic open bath (UDY Co.; Fort Collins, CO) with a heating immersion circulator (Julabo E-BASIS; Julabo Labortechnik GmbH, Seelbach, Germany) to maintain the temperature of the water bath at 40°C. Two magnetic stirrers (Fisher aluminum-top stirring hotplate; Fisher Scientific Inc.) were set under the open bath for mixing the GI contents at the lowest
speed. For gastric emptying, the half-time was 60 min (Ghoos and others 1993) up to 3 h (Table 3.1). As shown in Table 3.1, the pH curve in the gastric compartment was controlled to reproduce the values found in humans after consumption: pH 5.0 at initiation and pH 4.1, 3.0, 2.1, and 1.8 at 20, 40, 60, 80 min, respectively (Conway and others 1987). In the small intestinal compartment, the pH value was kept around 6.5. The pH of GI the contents was measured with a Corning Pinnacle 530 pH meter (Corning Inc., Corning, NY). An unscented tea light candle (Id Williamsburgh Candle Corp., NY) was placed next to the beaker and burned each time before closing the container to create an oxygen limited environment.

To prepare the *E. sakazakii* sample, 1 mL full growth broth of *E. sakazakii* Fec39 was centrifuged (Eppendorf Centrifuge 5415C; Hamburg, Germany) at 13,000 rpm for 1 min. After discarding the supernatant, 1 mL sterile milk (8.5 g Similac infant formula (Abbott Laboratories. Abbott Park, IL) / 60 mL water) was added to mix with the pellet and then mixed with 9 mL sterile milk. Ten grams of yogurt and/or *E. sakazakii* sample were diluted (1:1, w/v or v/v) with sterile electrolyte solution (6.2 g of NaCl, 2.2 g of KCl, 0.22 g of CaCl₂, and 1.2 g of NaHCO₃ per liter of distilled water) to simulate the *in vivo* dilution by saliva. This mixture was introduced into the gastric
compartment followed by the addition of simulated gastric fluid which containing 0.1 g of pepsin, 3.5 g of mucin, and 8.5 g of NaCl per liter of sterile distilled water, adjusted to pH 1.6 to 1.7 with 1N HCl.

A 9.9 mL portion of simulated small intestinal juice containing 0.1 g of trypsin and 3.5 g of pancreatin per liter of sterile distilled water was added into the simulated intestinal compartment at the transition time 0 h. The pH was maintained around 6.5 with 0.1M NaHCO₃ initially, and with 0.3M NaHCO₃ for the rest of the experiment. In addition, a 12.5 mL of 4% bile extract solution was added at the beginning, and the bile concentration was maintained at 1.55% with 2% bile extract solution for the remaining time.

It was considered as transition time zero when yogurt and/or *E. sakazakii* samples were first added to the simulated gastric or being transferred to the small intestinal compartment. To study the viability of bacterial cells in the gastric compartment, gastric contents were aseptically sampled and analyzed at gastric transition times 0, 20, 40, 60, 80, 120, and 180 min. To exam the viable bacterial cells in the small intestinal compartment, the content was sampled and analyzed at intestinal transition time in 30-min periods for the first hour and in 60-min periods for the remaining 8 h.
Contents from gastric and small intestinal compartments at each defined time point were serially diluted with 0.1% peptone solution. Pour plating with Bacto M17 agar with 0.5% β-lactose and Bacto Lactobacilli MRS agar was used for lactic streptococci and lactobacilli enumeration, respectively. The viable LAB were then enumerated after 3-day anaerobic incubation at 37°C. Viable *E. sakazakii* Fec39 were enumerated on tryptic soy agar after 18~24 h incubation at 37°C. The viable bacterial cell counts were divided by a dilution factor to take the added volumes of simulated fluids into consideration. The dilution factor of each sampling transition time equaled the contents remaining (mL) in each compartment divided by the combined volume (mL) of the remaining contents and all added simulated fluids (NaHCO₃ and bile solution in simulated small intestine).

**Statistical analysis**

A 2 (*E. sakazakii* strains) * 3 (*E. sakazakii* concentrations)* 3 (LAB samples) factorial arrangement in a completely randomized design with three replications was used to analyze the inhibition zone in the agar spot test. Also, a two-way factorial arrangement (samples * transition times) in a split-plot design was used for analyzing the antagonistic effect in the simulated GI model. Data obtained from triplicate
samples were analyzed using PROC GLM procedure, means were separated by Fisher’s protected LSD ($P < 0.05$), and the slopes of viable \textit{E. sakazakii} which used to further determine decimal reduction time (D value) in the simulated gastric model were obtained by linear regression analysis (PROC REG) with SAS program version 9.1 (SAS Institute, Cary, NC).

**Results**

**Antagonistic effect between lactic acid bacteria and \textit{Enterobacter sakazakii}**

The clear zones around the spots of yogurt and LAB indicated the antagonistic activity against \textit{E. sakazakii}. All \textit{Lactobacillus} spp. and yogurt sample were antagonistic toward \textit{E. sakazakii} to varying degrees (Table 3.2). As the concentration of \textit{E. sakazakii} decreased, the diameters of clear inhibition zones were significantly larger. The average diameters of inhibition zones were 12.8, 10.9, and 8.8 mm when 0.01, 0.1, and 1% of \textit{E. sakazakii} ($10^4$–$10^6$CFU/mL) were inoculated, respectively. Generally, there were no significant differences between the two tested \textit{E. sakazakii} strains. Two lactic acid cultures, \textit{L. helveticus} CNRZ32 and \textit{L. bulgaricus} LL18, at about $10^8$ CFU/mL showed significantly stronger antagonistic effect against \textit{E.}
sakazakii than 1:100 diluted yogurt (about $10^6$ CFU/mL) did. The average diameters of clear zones produced by *L. helveticus* CNRZ32, *L. bulgaricus* LL18, and diluted yogurt were 11.6, 11.8, and 10.3 mm, respectively.

**Viabilities of lactic acid bacteria in yogurt and *Enterobacter sakazakii* in the simulated GI model**

The bars in Figures 3.2 - 3.9 were the mean values of the viable cells of yogurt LAB and *E. sakazakii* Fec39 in the simulated GI model expressed in decimal logarithms. Both *Lactobacillus* and *Streptococcus* in yogurt sample as well as *E. sakazakii* showed similar patterns of decrease in the simulated gastric model (Figures 3.2, 3.4, and 3.6). After 180 min in the simulated gastric model, there were 5.5-5.9 log CFU/mL reductions of viable cells of *Lactobacillus* and *E. sakazakii*, regardless of the form of sample introduced to the simulated GI model. The survival rate of bacteria in the simulated gastric model (Table 3.3) equaled the viable cell numbers at the endpoint (transition time: 180 min) divided by the initial (transition time: 0 min) cell numbers. There were no significant differences of the survival rates among all bacterial cells detected in the simulated gastric model, including the survival rate of *E. sakazakii* in the sample combined yogurt with lower initial *E. sakazakii*. 
With the severe decrease in pH, all viable cells decreased rapidly in 40-80 min in the gastric transit (Figures 3.2, 3.4, 3.6 and 3.8). The slope of the decreased pH value was -0.060 (log CFU/ min) during 40-80 min in the gastric model, while it was -0.018 (log CFU/ min) during the whole 180 min in the gastric model. The D values obtained during 40-80 min were significantly lower than that obtained during 0-180 min in the simulated model (Table 3.4). Lactobacillus spp. and Streptococcus in yogurt showed significantly higher D values than E. sakazakii did.

All the gastric contents were transferred to the simulated small intestinal compartment during the gastric transition time: 60-180 min, which was overlapped with the intestinal transition time: 0-2 h. The viable cells of E. sakazakii and yogurt LAB showed an increased trend during 3-9 h in the simulated small intestinal model (Figures 3.3, 3.5, 3.7 and 3.9). The survival rate of bacteria in the small intestine equaled to the viable cell number at the endpoint (intestinal transition time: 9 h) divided by the initial (gastric transition time: 0 min) cell number. Enterobacter sakazakii demonstrated significant higher survival rate in the simulated intestinal model than yogurt LAB did. An approximate 90% recovery of E. sakazakii was noted after passing through the simulated GI model, while less than 70% of yogurt
LAB were recovered. Although the survival rates of *E. sakazakii* at the final endpoint between samples (with or without yogurt) did not differ, the D value of *E. sakazakii* in the samples combined with yogurt was significantly higher than that of *E. sakazakii* when introduced to the GI model alone.

**Discussion and Conclusion**

There is no clear evidence of a minimal infectious dose of *E. sakazakii* in humans. An estimated 1,000 CFU/mL proposed by Iversen and Forsythe (2003) was based on the infectious dose of *E. coli* O157:H7 and *Listeria monocytogenes*. The results of the agar spot tests showed that yogurt and LAB were able to inhibit up to $10^6$ CFU/mL of *E. sakazakii*, although the inhibitory effectiveness of LAB may depend on the concentration of pathogens and LAB. It has been suggested that the antagonistic effect of LAB may be due to the production of antimicrobial substances such as organic acids (Gagnon and others 2004). Organic acids may lower the pH and create conditions less desirable for harmful bacteria. Competition for nutrients necessary for bacterial growth in the environment has also been reported as another possible antimicrobial mechanism of LAB (Lourens-Hattingh and Viljoen 2001). These
factors may explain the antagonistic results demonstrated by the agar spot tests in this experiment.

An approximate 35% of the ingested LAB in yogurt samples remained viable after 180 min in the gastric compartment in this study. However, Marteau and others (1997) reported that *L. bulgaricus* and *S. thermophilus* fell below 1% of the ingested numbers within 70 and 110 min in a simulated stomach. Some probiotics were believed to have stronger antimicrobial effect and better survivability in gastric (acid) condition than other LAB (Meydani and Ha 2000; Millette and others 2004). Among *Lactobacillus* spp., *L. acidophilus* is more resistant to gastric juice than the yogurt starter culture, *L. bulgaricus*, and is more resistant than *S. thermophilus* (Meydani and Ha 2000). *Lactobacillus acidophilus* and *Bifidobacterium longum*, two well known probiotics, claimed by the commercial yogurt used in this test may be responsible for the observed results. To achieve the therapeutic benefits, the minimum probiotic consumption number of $10^8$ live cells per day was suggested (Tamime and others 1995). Consumption of more than 100 g yogurt containing $10^6$ viable cells per day would provide these number (Rybka and Kailasapathy 1995; Tamime and others 1995; Lourens-Hattingh and Viljoen 2001).
The viability of LAB and *E. sakazakii* varies with gastric pH. Viable counts of both *Lactobacillus* and *Streptococcus* in the yogurt rapidly decreased after transition time 40 min in the gastric compartment, when the pH was below 3. Conway and others (1987) showed similar results of the test of bacterial strains in phosphate-buffered saline solution at various pH. The lower D value in the gastric model indicated that *E. sakazakii* was more sensitive to acid than were yogurt LAB. Shaker and others (2008) reported that the populations of inoculated *E. sakazakii* decreased significantly during the last few hours of yogurt fermentation and during cooling and storage of yogurt (pH 4.2-4.7). Edelson-Mannel and others (2006) studied the acid resistance among 12 strains of *E. sakazakii* in TSB with various adjusted pH for 5 h. At pH 3.0, the decline of viable *E. sakazakii* ranged from 4.9 to >6.3 log CFU/mL, while most strains showed only 1 log CFU/mL reduction at pH 3.5. However, it has been reported that the less acidic stomach of newborns, especially that of prematures, and the buffering capacity provided by ingested milk may contribute to survival and infection of *E. sakazakii* in infants (Forsythe 2005; Lenati and others 2008). Therefore, acidification was suggested to lower the concentration of *E. sakazakii* in baby foods (Joosten and Lardeau 2004; Lin and Beuchat 2007).
The lethality of bile salt on LAB and *E. sakazakii* may be lower than the effect of gastric acid in the simulated GI model. All bacterial cells detected showed increasing trend in the small intestinal transit after serious decline under the acid gastric condition. However, *E. sakazakii* had better recovery than that of LAB in the intestinal model, where pH was maintained around 6.5. There was no significant antagonistic effect of yogurt against *E. sakazakii* at concentrations 10^5 and 10^6 CFU/mL in the simulated GI model. However, it was reported that yogurt consumption may increase the numbers of stool anaerobes, predominantly bifidobacteria to suppress coliform, one of the genera of the family *Enterobacteriaceae* (Chen and others 1999). This contradiction may happen due to the higher initial inoculum amount (higher than the estimated minimal infectious dose 1,000 CFU/mL) of *E. sakazakii*. Also, it is probably due to the dilution of the organic acids and antimicrobial compounds, hypothesized earlier, by the fluid in the GI tract. Besides, the ability of LAB to adhere to the intestinal epithelium is another important parameter influencing the antimicrobial effect toward pathogens (Bernet and others 1993, 1994; Parvez and others 2006). Therefore, the adhesion ability and the competitive exclusion effect of yogurt LAB against *E. sakazakii* should be further determined.
Figure 3.1  Simulated gastrointestinal model: (1) stainless steel container, (2) Pyrex beaker with magnetic stirring bar, (3) water bath, (4) heating immersion circulator, (5) magnetic stirrer, and (6) candle.
<table>
<thead>
<tr>
<th></th>
<th>Transition time (min)</th>
<th>0</th>
<th>20</th>
<th>40</th>
<th>60</th>
<th>80</th>
<th>120</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH control</td>
<td></td>
<td>5.0</td>
<td>4.1</td>
<td>3.0</td>
<td>2.1</td>
<td>1.8</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Contents transferred to “Intestinal model”</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>*</td>
<td>*</td>
<td>**</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Transition time (h)</th>
<th>0</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH control</td>
<td></td>
<td>6.5</td>
<td>6.5</td>
<td>6.5</td>
<td>6.5</td>
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<td>6.5</td>
<td>6.5</td>
<td>6.5</td>
<td>6.5</td>
<td>6.5</td>
</tr>
<tr>
<td>Contents transferred from “Gastric model”</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>*</td>
<td>*</td>
<td>**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* At this transition time point, half of the gastric contents were transferred from the gastric compartment to the intestinal compartment.

** At this transition time point, all of the gastric contents were transferred from the gastric compartment to the intestinal compartment.
Table 3.2  Antagonistic effect of yogurt and Lactobacillus spp. on Enterobacter sakazakii as demonstrated by the agar spot test

<table>
<thead>
<tr>
<th></th>
<th>Inhibition zone diameter (mm)</th>
<th>E. sakazakii Fec 39</th>
<th>E. sakazakii MSDH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1%</td>
<td>0.1%</td>
<td>0.01%</td>
</tr>
<tr>
<td>Diluted yogurt (1:100)</td>
<td>8.2 ± 0.8 A, c*</td>
<td>10.3 ± 1.5 A, b</td>
<td>12.8 ± 1.8 A, a</td>
</tr>
<tr>
<td>L. helveticus CNRZ32</td>
<td>9.0 ± 1.4 A, b</td>
<td>11.3 ± 2.8 A, ab</td>
<td>13.9 ± 2.7 A, a</td>
</tr>
<tr>
<td>L. bulgaricus LL18</td>
<td>9.8 ± 1.8 A, a</td>
<td>11.2 ± 2.7 A, a</td>
<td>13.0 ± 2.7 A, a</td>
</tr>
</tbody>
</table>

* Values within columns having the same capital letters are not significantly different (P<0.05).
  Values within rows having the same small letters are not significantly different (P<0.05).
Figure 3.2  Viable yogurt lactic acid bacteria and pH in simulated gastric model. Means sharing the same letters are not significantly different (P<0.05).
Figure 3.3  Viable yogurt lactic acid bacteria and pH in simulated intestinal model.
Figure 3.4  Viable *Enterobacter sakazakii* Fec39 and pH in simulated gastric model. Means sharing the same letters are not significantly different (P<0.05).
Figure 3.5 Viable *Enterobacter sakazakii* Fec39 and pH in simulated intestinal model.
Figure 3.6 Viable yogurt *Lactobacillus* and *Enterobacter sakazakii* Fec39 (10⁸ CFU/mL initial inoculation) and pH in simulated gastric model. Means sharing the same letters are not significantly different (P<0.05).
Figure 3.7  Viable yogurt *Lactobacillus* and *Enterobacter sakazakii* Fec39 (10⁸ CFU/mL initial inoculation) and pH in simulated intestinal model.
Figure 3.8  Viable yogurt *Lactobacillus* and *Enterobacter sakazakii* Fec39 (10^5 CFU/mL initial inoculation) and pH in simulated gastric model. Means sharing the same letters are not significantly different (P<0.05).
Figure 3.9 Viable yogurt *Lactobacillus* and *Enterobacter sakazakii* Fec39 (10^5 CFU/mL initial inoculation) and pH in simulated intestinal model.
### Table 3.3 Viability and survival rate of *Enterobacter sakazakii* and yogurt LAB in the simulated gastrointestinal model

<table>
<thead>
<tr>
<th>Samples introduced to GI model</th>
<th>Viable cells (log CFU/mL)</th>
<th>Survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Endpoint in G†</td>
</tr>
<tr>
<td><em>E. sakazakii</em> alone</td>
<td>8.1</td>
<td>2.3</td>
</tr>
<tr>
<td>Yogurt with high levels of <em>E. sakazakii</em></td>
<td>8.1</td>
<td>3.1</td>
</tr>
<tr>
<td>Yogurt with low levels of <em>E. sakazakii</em></td>
<td>5.4</td>
<td>2.1</td>
</tr>
<tr>
<td>Yogurt alone</td>
<td>9.3</td>
<td>3.3</td>
</tr>
<tr>
<td><em>Lactobacillus</em> Yogurt with high levels of <em>E. sakazakii</em></td>
<td>8.6</td>
<td>3.1</td>
</tr>
<tr>
<td>Yogurt with low levels of <em>E. sakazakii</em></td>
<td>9.3</td>
<td>3.4</td>
</tr>
<tr>
<td><em>Streptococcus</em> Yogurt alone</td>
<td>9.2</td>
<td>3.3</td>
</tr>
</tbody>
</table>

* Values within columns having the same letters are not significantly different (P<0.05).
† G stands for the simulated gastric model, and I stands for the simulated intestinal model.
Table 3.4  Decimal reduction times (D values) of *Enterobacter sakazakii* and yogurt LAB in the simulated gastric model

<table>
<thead>
<tr>
<th>Samples introduced to GI model</th>
<th>D values (min)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-180 min</td>
<td>40-80 min</td>
<td></td>
</tr>
<tr>
<td><em>E. sakazakii</em> alone</td>
<td>177.3</td>
<td>90.5</td>
<td></td>
</tr>
<tr>
<td><em>E. sakazakii</em> Yogurt with high levels of <em>E. sakazakii</em></td>
<td>208.4</td>
<td>103.9</td>
<td></td>
</tr>
<tr>
<td><em>E. sakazakii</em> Yogurt with low levels of <em>E. sakazakii</em></td>
<td>426.3</td>
<td>102.0</td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus</em> Yogurt alone</td>
<td>234.8</td>
<td>149.6</td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus</em> Yogurt alone</td>
<td>236.2</td>
<td>146.2</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER IV

ANTIMICROBIAL EFFECT OF MUSCADINE JUICES
ON ENTEROBACTER SAKAZAKII

Abstract

Muscadine grapes (Vitis rotundifolia Michx.) have been reported to have high polyphenol content and great antioxidant capacity, but little research has been conducted to assess potential antimicrobial effects of muscadines. In this study, red and white muscadine juices and commercial baby juices were investigated for inhibition of Enterobacter sakazakii. Major phenolic compounds and organic acids in juices were also identified and quantified by HPLC. There were only 0.2 to 1.4-log reductions of E. sakazakii in commercial baby juices, while 6-log reductions of E. sakazakii cells were achieved by muscadine juices within 2-hour incubation at 37°C. Overall, among all tested juices, red muscadine juices which had lower pH values and were higher in tartaric acid, total phenolics and major phenolic acids contents demonstrated the strongest antimicrobial effect. These results suggest that muscadine
juices may be utilized as natural antimicrobial agents in baby foods to control the growth of *E. sakakazii*.

**Introduction**

*Enterobacter sakazakii* is considered as an emerging opportunistic pathogen, causing rare but life threatening meningitis, bacteremia, and necrotizing enterocolitis in infants (Nazarowec-White and Farber 1997a; Lai 2001). Ingestion of contaminated powdered infant formula has been implicated in several clinical outbreaks in neonates and infants (Iversen and Forsythe 2003). The gastrointestinal tract has been suggested as the portal of entry among patients with *Enterobacter* spp. bacteremia, and meningitis is believed to occur by translocation of invasive bacteria (Pagotto and others 2003; Mange and others 2006). The less acidic stomach and the more permeable intestinal tracts of infants may make them most susceptible to *E. sakakazii* (Lenati and others 2008).

Muscadine grapes (*Vitis rotundifolia*) have the ability to tolerate hot, humid climates, and resistant to Pierce’s disease, and they are the predominant grape species grown in the southeastern United States (Chen and others 2001). Recently, interest in the health benefits of muscadines has increased due to their high phenolics’ content
(Pastrana-Bonilla and others 2003; Yilmaz and Toledo 2004), excellent antioxidant capacity (Pastrana-Bonilla and others 2003; Lee and Talcott 2004), anti-cancer properties (Yi and others 2005; Mertens-Talcott and others 2006), and anti-inflammatory activity (Greenspan and others 2005; Bralley and others 2007). The presence of ellagic acid in muscadines is unique among grape species. But, ellagic acid and its derivatives are common phenolic compounds in berries, such as blueberries, raspberries and strawberries, which have been demonstrated to possess strong antimicrobial activity (Puupponen-Pimiä and others 2001; Nohynek and others 2006). Other than phenolics, muscadine grapes also contain nutritional and functional components such as vitamins, minerals, fiber, and organic acids (Ector 2001; Threlfall and others 2007). Organic acids, like tartaric and malic acids, have been reported to inhibit foodborne pathogens (Eswaranandam and others 2004; Waite and Daeschel 2007). An earlier study demonstrated the antimicrobial effects of tartaric acid against *Escherichia coli* O157:H7 (Kim and others 2008b).

The antimicrobial effects against *Salmonella typhimurium, Staphylococcus aureus, Escherichia coli, Pseudomonas, Bacillus, Micrococcus*, and *Proteus* spp. of orange juice, yogurt, and the combination of orange juice with yogurt have been demonstrated.
(Mothershaw and Jaffer 2004). Low pH was then suggested to contribute to the inhibitory activity through creating conditions less desirable for these harmful bacteria. Since *E. sakazakii* were not acid resistance bacteria (Edelson-Mammel and others 2006), lower pH may also decrease its survivability. Considering the potential inhibition effect and the convenience to deliver to infants, muscadine juices were investigated for their antimicrobial effect against *E. sakazakii*. The antimicrobial effect via the combination of muscadine juices and yogurt, a common lactic acid bacteria carrier, was also evaluated.

**Materials and Methods**

**Chemicals and reagents**

Folin & Ciocalteu’s phenol reagent, sodium carbonate and pure standards of gallic acid (90% purity), (+)-catechin (95% purity), (-)-epicatechin (90% purity), ellagic acid (95% purity), myricetin (85% purity), quercetin (98%) purity, trans-resveratrol (95% purity), L-malic acid, and tannic acid were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Tartaric acid, acetic acid, hydrochloric acid, sulfuric acid, acetonitrile,
methanol, and HPLC grade water were purchased from Fisher Scientific Inc. (Pittsburgh, PA).

**Juice preparation**

Two varieties of fresh muscadine juices were donated by the Mississippi Agricultural and Forestry Experiment Station (MAFES), Mississippi State University. Red muscadine juice (hot-pressed) was processed from purple grape cultivar ‘Noble’, while white juice (cold-pressed) was obtained from a mixture of bronze grape cultivars ‘Carlos’ and ‘Magnolia’. Fresh juices were divided into portions in 50mL sterile centrifuge tubes (Fisher Scientific Inc.) and except the juices for pasteurization, and were stored at -20°C until use or further treatments.

To prepare the pasteurized juice samples, frozen muscadine juices were thawed at room temperature until no floating ice was observed, and then heated with constantly stirring in an open steam kettle in the Ammerman-Hearnsberger pilot plant, Department of Food Science, Nutrition and Health Promotion, Mississippi State University. The juices were held at 90°C for one minute and then filled into pre-sterile Pyrex media bottles (Corning Inc., Corning, NY). After cooling in a refrigerator overnight, the pasteurized muscadine juices were stored at -20°C until use.
Both frozen (fresh and pasteurized) muscadine juices were thawed in a refrigerator overnight, and used within 3 days.

After thawing in a refrigerator overnight, the hot sterile juice samples were prepared by autoclaving (121°C, 103.4 kPa) the juices in a screw-capped vial (National Scientific Co., Rockwood, TN) for 15 min. In contrast, the cold sterile muscadine juices were obtained by filtration with a 0.20 µm syringe filter (Millipore, Bedford, MA). Both hot and cold sterile juice samples were stored in a 7°C refrigerator until use.

Three types of commercial baby juices (Gerber Products Co., Fremont, MI) were purchased from local stores (Starkville, MS) and used as reference juice samples in this study. The white grape and apple grape juices were made from juice concentrate, and the mixed berry juice was made from juice and puree concentrates (Table 4.1). Once the bottles were opened, all commercial baby juices were transferred from room temperature (about 25°C) to a refrigerator at 7°C, and the opened baby juice samples were no longer used and discarded after 3-days storage in the refrigerator.
**Total phenolics**

Juice samples were diluted appropriately with deionized water and analyzed for total phenolics according to the Folin-Ciocalteu procedure (Waterhouse 2001). Absorption at 765nm was measured in a Spectronic genesys 5 UV-vis spectrophotometer (Fisher Scientific Inc.). The total phenolics were expressed as milligrams of gallic acid equivalents per milliliter of juice.

**Soluble solids concentration and pH**

The soluble solids concentration was measured using Bausch & Lomb Refractometer 33.46.10 (Bauch & Lomb Inc., Rochester, NY). The temperature was maintained at 20°C with cold water circulating through the refractometer during the measurement. After calibration with deionized water before each reading, one drop (about 300 μL) of juice samples was placed on the glass prism. The readings were recorded and expressed as °Brix.

The pH values of juices were measured at room temperature (about 25°C) with a Corning Pinnacle 530 pH meter (Corning Inc.).
Major phenolic and organic compounds

Reversed-phase high-performance liquid chromatography (HPLC) was used to separate and quantify major phenolics and organic acids in juices. Juice samples were mixed with 6 N HCl at 1:10 ratio (v/v) and placed in a water bath for one hour to separate the free phenolic acids from their conjugated forms (Lee 2000). After cooling to room temperature (about 15 min), each supernatant of acid hydrolyzed samples was filtered through a 0.45 µm syringe filter (Millipore, Bedford, MA) and injected into a Gemini C18, 250 x 4.6 mm column (Phenomenex Inc., Torrance, CA) in an Agilent HPLC 1100 series (Agilent Technologies Inc., Santa Clara, CA) equipped with a diode array detector. The injected volumes of samples were 25 µL. The two mobile phases were solvent A: methanol/acetic acid/water (10:2:88, v/v/v), and solvent B: acetonitrile. A linear gradient for phenolics separation was used as follows: at 0 min, 95% solvent A, 5% solvent B; at 1 min, 90% solvent A, 10% solvent B; at 30 min, 30% solvent A, 70% solvent B; at 31 min, 90% solvent A, 10% solvent B; at 32 min, 95% solvent A, 5% solvent B with 5 min post run. The flow rate was 1 mL/min. Individual phenolics were detected at 260 nm.
To separate organic acids, muscadine juices were centrifuged at 12,000 rpm (1,700 g) for 5 min, and each supernatant was filtered and injected into a HPLC system as described above. The mobile phase was 0.01 N H2SO4 with a flow rate of 1 mL/min. Individual organic acids were detected at 215 nm.

Peaks for phenolic compounds and organic acids were integrated and analyzed using the ChemStation software (Agilent Technologies Inc.). Individual compounds were quantified based on the retention times and peak area of the standards.

**Antimicrobial activity**

*Enterobacter sakazakii* Fec39 and *E. sakazakii* MSDH (provided by Dr. Yosheen Chen, Mississippi State University) were used in this study. After incubation at 37°C in tryptic soy broth (Difco Laboratories, Sparks, MD) for seven hours, the bacterial cultures were added to the juice samples at a 1:1000 ratio (v/v) giving an initial 10^6 cells per milliliter inoculum. The inoculum was incubated at 37°C, and the viable cells were recorded every 30 min, for up to 120 min. Viable *E. sakazakii* were enumerated on tryptic soy agar plates (Difco Laboratories) after incubation at 37°C for 18 to 24 h.
In addition, commercial yogurt as described in chapter III was purchased from local stores (Starkville, MS), and was also incorporated into muscadine juices to examine the combined antimicrobial effect. After mixing yogurt sample with pasteurized muscadine juices, *E. sakazakii* were then inoculated to the mixtures. The viable cells of *E. sakazakii* in the mixtures were enumerated as described above.

**Statistical analysis**

Data of pH, total phenolics, phenolic compounds, and organic acids of the muscadine juices were analyzed using a 2 (muscadine cultivars) * 4 (juice treatments) factorial in a randomized complete block design, while a completely randomized design was used to compare muscadine juices with commercial baby juice samples. A two-way factorial design (treatments x time) was used for measurement of antimicrobial activity. Also, a two-way factorial design (juices x bacteria) and linear regression analysis (PROC REG) was used for decimal reduction time (D value) calculation. Data obtained from at least three replications (except soluble solids) were analyzed by PROC GLM, and means of the variables were separated by Fisher’s protected LSD (*P* < 0.05) using SAS 9.1 (SAS Institute, Cary, NC).
Results

Soluble solids, pH, total phenolics, organic acids, and phenolic acids in juices

The soluble solids expressed as °Brix, pH, and organic acids in muscadine and commercial baby juices were presented in Table 4.2. Overall, both muscadine juices were lower \((P<0.05)\) in malic acid content and pH, and were higher in tartaric and tannic acid than commercial baby juices. Red muscadine juices (RMJ) showed lower pH and °Brix \((P<0.05)\) and higher contents of tartaric and tannic acids than white muscadine juices (WMJ). RMJ and commercial mixed berry (MB) juice demonstrated the highest concentrations of total phenolics, followed by commercial apple grape (AP), white grape (WP), and then WMJ. Among the major phenolic compounds detected, the ellagic acid concentration was 7-11 times higher in RMJ than in WMJ and commercial baby juices (Table 4.3). Also, RMJ were higher \((P<0.05)\) in gallic acid and epicatechin contents than WMJ. There were no consistent trends between treatments of muscadine juices on all characteristics detected.
Antimicrobial activity on *Enterobacter sakazakii*

The antimicrobial activity of muscadine juices against *E. sakazakii* was measured in liquid cultures by the plate count method, and experimental results are shown in Figures 4.1 and 4.2. Within two hours, both strains of *E. sakazakii* were reduced to non-detectable levels in all muscadine juices. There were about 6-log reductions in cell number regardless of the muscadine juice type or treatments. During the first 0.5 h in muscadine juices, the decrease in the viable *E. sakazakii* cells showed a lag trend. Thus, the D values were calculated based on the viable *E. sakazakii* changes from 0.5 h to the time no viable cells were detectable (Table 4.4). Overall, the lower D values of RMJ indicated that RMJ had stronger antimicrobial effect against *E. sakazakii* than WMJ did (*P*<0.05). Pasteurized muscadine juices were less effective against *E. sakazakii* than both cold and hot sterile juices.

In contrast, there were only 0.2 to 1.4-log reductions of *E. sakazakii* in inoculated commercial baby juices within four hours (Figure 4.3). Among three baby juices, mixed berry juice demonstrated better antimicrobial effect than white grape and apple grape juices. However, there were no differences between the susceptibility between *E. sakazakii* Fec39 and MSDH to either muscadine or commercial baby juices.
Commercial nonfat plain yogurt was added into pasteurized muscadine juices at 1:10 ratio (w/v) giving an initial $10^8$ CFU of lactic acid bacteria per milliliter inoculation. In this mixture of yogurt and pasteurized muscadine juice, there were ca. 2-log and 6-log reductions of *E. sakazakii* in two and four hours, respectively (Figure 4.4). No significant differences in *E. sakazakii* log reduction were observed between red and white muscadine juices when combined with yogurt. Within four hours, lactic acid bacteria were reduced about 2-log in viable cell numbers either in muscadine juices and/or in the mixture of juices and yogurt (Figure 4.5). The differences of the viable yogurt lactic acid bacteria among six various samples were not significant.

**Discussion and Conclusion**

Hot pressing, heating prior to pressing, is commonly used in juice processing with dark grapes, including dark-skinned muscadines. This technique has been reported to increase juice yield, color, acids, color pigments and other nutraceutical components (Auw and others 1996; Threlfall and others 2005; Leblanc and others 2008). For white/bronze muscadines, hot pressing procedures were often linked to browning reactions affecting juice quality (Lee and Talcott 2004). Comparing to those in WMJ,
the observed higher contents of total phenolics, epicatechin, and tartaric, tannic, ellagic, and gallic acids in red muscadine ‘Noble’ juice may be derived from the differences in the muscadine cultivar and/or juice pressing method, and may be possible reasons for the stronger antimicrobial activity.

Pasteurization is commonly used in the food industry to extend the shelf life of acidic foods by inactivating enzymes and microorganisms. However, the effects of heat treatment on phenolic contents were contradictory in the literature. Del Pozo-Insfran and others (2006) reported that thermal pasteurization decreased anthocyanins, soluble phenolics, and antioxidant capacity in muscadine juices, whereas other researchers reported that heat treatment may increase the concentration of certain phenolic compounds in juices due to the hydrolysis of higher molecular weight compounds (Dawes and Keene 1999; Musingo and others 2001). Stojanovic (2008) found that anthocyanins, phenolics, and antioxidant activity of blueberry juices were not affected by hot fill pasteurization. In this study, pasteurization treatment increased the contents of tannic and tartaric acids in RMJ and WMJ, respectively. The other changes between treatments were not consistent in the contents of organic acids and phenolic compounds. Comparing the antimicrobial effect with both cold
and hot sterile muscadine juices, pasteurized juices had diminished antimicrobial effect against *E. sakazakii*. However, a burned brown color was observed in hot sterile (autoclaved) juices due to extensive high temperature exposure, and it should not be used for juice processing. Cold sterile juices obtained by filter clarification were reported to caused the loss of compounds linked to the proteins, and therefore the lowered concentrations of anthocyanins and pigment destruction were reported (Bakker and others 1992; Wrolstad and others 1994). Some alternative processing techniques other than thermal processing, like high hydrostatic pressure, pulsed electric field, and dense phase CO₂ processing (Talcott and others 2003; Del Pozo-Insfran and others 2006; Del Pozo-Insfran and others 2007; Stojanovic 2008) have been proposed and may be used to examine their effect on juice quality and antimicrobial effect.

It has been suggested that *E. sakazakii* were sensitive to acidic or acidified food products. Kim and Beucahat (2005) reported that *E. sakazakii* grew in tomato juice (pH 4.4) and watermelon juice (pH 5.1), but did not grow in apple juice (pH 4.0) or strawberry juice (pH 3.5) stored at 25°C. Richards and others (2005) also showed that infant rice cereal reconstituted with apple juice (pH 4.32) did not support the growth of *E. sakazakii*. *Enterobacter sakazakii* was also found to grow at the early
stage but declined at the end of the yogurt fermentation process probably due to increasing concentration in lactic acid (Shaker and others 2008). In the current study, a trend was observed that the juices with lower pH values have the better antimicrobial effects. The RMJ (pH 3.18) followed by WMJ (pH 3.37) showed better inhibitory ability than mixed berry juice (pH 3.52) and other baby juices (pH around 3.6). Generally, at low pH, the organic acids exist in their undissociated forms and may interact with or pass through the bacterial cell membrane. Through altering the cell membrane permeability, decreasing enzyme functions, and other physiological changes in bacteria may cause cell death (Puupponen-Pimiä and others 2005b). In addition, there are differences between commercial baby juices and muscadine juices in organic acid contents. Tartaric and tannic acids, the stronger bacterial inhibitors, were the major organic acid in both RMJ and WMJ, while malic acid was the dominant one in commercial baby juices.

Since the pH values of commercial baby juices (3.52-3.67) were lower than those effective pH (3.5-4.32) described in the literature, some other factors may be interfere in their antimicrobial activity. All the commercial baby juices tested in this study were made from concentrates, which are commonly used in the commercial fruit juices.
(Bengoechea and others 1997). The processing procedures of juice concentration, reconstitution, and pasteurization for final products may contribute to the relatively lower antimicrobial activity of commercial baby juices. It has been suggested that although products may be high in phenolic compounds and antioxidant activity, the antiproliferation and other bioactivity activities of blueberry juices may be diminished by harsh processing methods, including spray-dried and juice concentration (Schmidt and others 2005).

Although only one study has been conducted on the antimicrobial effect of muscadines, various berries with similar phenolics profiles to muscadines have been proven to possess antimicrobial effects against several pathogens, e.g. *Helicobacter pylori*, *E. coli*, *Salmonella enterica*, and *Staphylococcus aureus* (Puupponen-Pimiä and others 2005a; Kim and others 2008b). In the current study, total phenolics contents were high in RMJ and mixed berry baby juice. Other than the differences in pH and organic acids, the concentrations of total phenolics, gallic and ellagic acids, and epicatechin were higher in RMJ than in WMJ. Among the three commercial baby juices, mixed berry juice, which demonstrated better antimicrobial effects, had higher total phenolics contents than white grape and apple grape baby juices. Thus, total
phenolics and some phenolic acids may also contribute to the inhibition effect against *E. sakazakii*. Nohynek and others (2006) suggested that disintegrating the bacterial outer membrane and increasing the permeability of bacterial cells observed in cloudberry and raspberry extracts may be one possible mechanism causing bacterial death. Antiadhesions, blocking the adherence of bacteria to epithelial cells, has also been demonstrated by Puupponen-Pimiä and others (2005b) as another possible antimicrobial mechanism of phenolics.

Berry phenolic compounds have selective inhibitory effect on human pathogens, but Bifidobacterium and several strains of *Lactobacillus* spp. were not affected (Puupponen-Pimiä and others 2001; Puupponen-Pimiä and others 2005c). In this study, yogurt lactic acid bacteria (2-log reduction in four hours) were less affected by muscadine juice than *E. sakazakii* (6-log reduction in two hours). However, the mixture of yogurt and muscadine juice was less effective against *E. sakazakii* than muscadine juice alone. The milk-based proteins and sugars in yogurt may provide buffering capacity (the pH value rose from 3.18 to 3.28 in muscadine juices to 3.5 of for mixture), and contributed to the prolonged eradication time of *E. sakazakii*. Since some strains of probiotics have been proven to inhibit and displace preadhered *E.*
*sakazakii*, it was suggested that the inclusion of probiotics in infant formula is a possible way to improve the resistance to these gastrointestinal pathogens when breastfeeding is not possible (Collado and others 2006; Collado and others 2008). In addition, in order to prevent rapid microbial growth in infant formula, acidification, either through fermentation with lactic acid bacteria or by direct addition of lactic acid, has been proposed by Josten and Lardeau (2004).

In conclusion, muscadine juice, a rich source of phenolics and organic acids, demonstrated strong antimicrobial effect against *E. sakazakii*. All inoculated *E. sakazakii* were eradicated within 2 and 4 h in muscadine juices and muscadine juice combined with yogurt, respectively. Comparing juices from two muscadine cultivars, red muscadine ‘Noble’ juices were lower in pH, had higher concentrations of total phenolics, tartaric, tannic, gallic, and ellagic acids, and epicatechin, and showed better antimicrobial effect. Pasteurized juices showed lower D values than cold and hot sterile juices. The commercial baby juices had higher pH and lower tartaric and tannic acids contents, and the effects on *E. sakazakii* were not clear. These results suggest that muscadine juices or in forms incorporated with lactic acid bacteria may have the potential to be used in baby food as a natural *E. sakazakii* inhibitor.
Table 4.1  Ingredients labeled in the commercial baby juices used in this study

<table>
<thead>
<tr>
<th>Baby juices</th>
<th>Ingredients</th>
<th>Other information labeled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apples grape juice</td>
<td>Grape juice concentrate</td>
<td>No added sweeteners</td>
</tr>
<tr>
<td></td>
<td>Apple juice concentrate</td>
<td>No preservatives</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>No artificial flavors or colors</td>
</tr>
<tr>
<td></td>
<td>Ascorbic acid (vitamin C)</td>
<td></td>
</tr>
<tr>
<td>White grape juice</td>
<td>White grape juice concentrate</td>
<td>No added sweeteners</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>No preservatives</td>
</tr>
<tr>
<td></td>
<td>Ascorbic acid (vitamin C)</td>
<td>No artificial flavors or colors</td>
</tr>
<tr>
<td></td>
<td>Citric acid</td>
<td></td>
</tr>
<tr>
<td>Nature Sips mixed berry (just for toddlers)</td>
<td>Apple puree concentrate</td>
<td>Real fruit smoothies</td>
</tr>
<tr>
<td></td>
<td>Blackberry puree</td>
<td>Naturally flavored with natural flavors</td>
</tr>
<tr>
<td></td>
<td>Blueberry puree</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Grape juice concentrate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>White grape juice concentrate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blueberry juice concentrate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blackberry juice concentrate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ascorbic acid (vitamin C)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alpha-tocopheryl acetate (vitamin E)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Natural flavor</td>
<td></td>
</tr>
<tr>
<td>Juice</td>
<td>Treatment/Flavor</td>
<td>Soluble Solids (°Brix)</td>
</tr>
<tr>
<td>------------------------</td>
<td>------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>RMJ *</td>
<td>Fresh</td>
<td>16.3</td>
</tr>
<tr>
<td></td>
<td>Cold Sterile</td>
<td>16.2</td>
</tr>
<tr>
<td></td>
<td>Hot Sterile</td>
<td>15.4</td>
</tr>
<tr>
<td></td>
<td>Pasteurization</td>
<td>16.0</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>16.0</td>
</tr>
<tr>
<td>WMJ *</td>
<td>Fresh</td>
<td>20.1</td>
</tr>
<tr>
<td></td>
<td>Cold Sterile</td>
<td>20.2</td>
</tr>
<tr>
<td></td>
<td>Hot Sterile</td>
<td>19.8</td>
</tr>
<tr>
<td></td>
<td>Pasteurization</td>
<td>20.4</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>20.1</td>
</tr>
<tr>
<td>Commercial Baby Juices</td>
<td>AG *</td>
<td>13.4</td>
</tr>
<tr>
<td></td>
<td>MB *</td>
<td>15.8</td>
</tr>
<tr>
<td></td>
<td>WG *</td>
<td>17.4</td>
</tr>
</tbody>
</table>

† Means of muscadine juices followed by a different small letter differ (P<0.05).
Means of average muscadine juices and commercial baby juices followed by a different capital letter differ (P<0.05).
* RMJ (red muscadine juice), WMJ (white muscadine juice), AG (apple grape juice), MB (mixed berry juice), WG (white grape juice), and ND (not detected).
Table 4.3  Major phenolic compounds in red muscadine, white muscadine, and commercial juices

<table>
<thead>
<tr>
<th>Juice</th>
<th>Treatment/Flavor</th>
<th>Gallic acid (mg/100mL)</th>
<th>Catechin (mg/100mL)</th>
<th>Epicatechin (mg/100mL)</th>
<th>Ellagic acid (mg/100mL)</th>
<th>Myricetin (mg/100mL)</th>
<th>Resveratrol (mg/100mL)</th>
<th>Quercetin (mg/100mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh</td>
<td>21.6 a †</td>
<td>54.0 a</td>
<td>53.7 a</td>
<td>24.4 a</td>
<td>1.1 a</td>
<td>3.8 a</td>
<td>0.9 b</td>
</tr>
<tr>
<td>RMJ*</td>
<td>Cold sterile</td>
<td>21.1 a</td>
<td>53.4 a</td>
<td>50.7 a</td>
<td>29.1 a</td>
<td>0.5 b</td>
<td>4.6 a</td>
<td>1.3 ab</td>
</tr>
<tr>
<td></td>
<td>Hot sterile</td>
<td>20.2 a</td>
<td>43.0 ab</td>
<td>47.6 a</td>
<td>25.5 a</td>
<td>1.4 a</td>
<td>4.4 a</td>
<td>0.6 b</td>
</tr>
<tr>
<td></td>
<td>Pasteurization</td>
<td>18.0 ab</td>
<td>43.2 ab</td>
<td>43.9 a</td>
<td>24.0 a</td>
<td>0.9 ab</td>
<td>4.4 a</td>
<td>1.7 a</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>20.3±2.9 A</td>
<td>48.4±15.4 A</td>
<td>49.1±11.2 A</td>
<td>25.9±7.5 A</td>
<td>0.9±0.6 A</td>
<td>4.3±1.3 AB</td>
<td>1.1±0.7 A</td>
</tr>
<tr>
<td>WMJ*</td>
<td>Fresh</td>
<td>8.6 d</td>
<td>21.1 b</td>
<td>17.4 b</td>
<td>3.1 b</td>
<td>ND *</td>
<td>3.1 a</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Cold sterile</td>
<td>13.9 bc</td>
<td>48.1 ab</td>
<td>22.9 b</td>
<td>4.9 b</td>
<td>0.4 b</td>
<td>3.4 a</td>
<td>1.2 ab</td>
</tr>
<tr>
<td></td>
<td>Hot sterile</td>
<td>13.9 bc</td>
<td>45.7 ab</td>
<td>24.6 b</td>
<td>3.0 b</td>
<td>0.5 b</td>
<td>3.3 a</td>
<td>0.2 c</td>
</tr>
<tr>
<td></td>
<td>Pasteurization</td>
<td>12.4 cd</td>
<td>27.9 ab</td>
<td>16.8 b</td>
<td>3.1 b</td>
<td>0.6 ab</td>
<td>2.4 a</td>
<td>1.2 ab</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>12.4±4.1 B</td>
<td>36.4±21.4 A</td>
<td>20.8±4.3 B</td>
<td>3.4±1.8 B</td>
<td>0.5±0.2 A</td>
<td>3.1±1.5 B</td>
<td>0.9±0.6 A</td>
</tr>
<tr>
<td>Commercial Baby Juice</td>
<td>Apple Grape</td>
<td>17.7 AB</td>
<td>32.5 A</td>
<td>46.5 A</td>
<td>2.60 B</td>
<td>0.56 A</td>
<td>5.05 AB</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Mixed Berry</td>
<td>19.7 A</td>
<td>29.7 A</td>
<td>46.2 A</td>
<td>3.17 B</td>
<td>0.70 A</td>
<td>2.10 B</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>White Grape</td>
<td>22.8 A</td>
<td>26.7 A</td>
<td>14.5 C</td>
<td>2.20 B</td>
<td>0.63 A</td>
<td>6.93 A</td>
<td>0.4 A</td>
</tr>
</tbody>
</table>

† Means of muscadine juices followed by a different small letter differ (P<0.05).

Means of average muscadine juices and commercial baby juices followed by a different capital letter differ (P<0.05).

* RMJ (red muscadine juice), WMJ (white muscadine juice), ND (not detected).
Figure 4.1  Viable cells of (A) *Enterobacter sakazakii* Fec39 and (B) *E. sakazakii* MSDH in red muscadine juices. Different treatments are showed as: (●) pasteurization, (▲) hot sterile, (■) cold sterile. (*) shows control growth curve of *E. sakazakii* in tryptic soy broth.

(A) [Graph showing viable cells of *Enterobacter sakazakii* Fec39]

(B) [Graph showing viable cells of *E. sakazakii* MSDH]
Figure 4.2 Viable cells of (A) Enterobacter sakazakii Fec39 and (B) E. sakazakii MSDH in white muscadine juices. Different treatments are showed as: (●) pasteurization, (▲) hot sterile, (■) cold sterile. (*) shows control growth curve of E. sakazakii in tryptic soy broth.
Table 4.4  Decimal reduction times (D values) for *Enterobacter sakazakii* in muscadine juices

<table>
<thead>
<tr>
<th>Juices</th>
<th>Treatments</th>
<th><em>E. sakazakii Fec39</em></th>
<th><em>E. sakazakii MSDH</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Red muscadine juices</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cold sterile</td>
<td>53.5</td>
<td>71.9</td>
</tr>
<tr>
<td></td>
<td>Hot sterile</td>
<td>69.7</td>
<td>74.8</td>
</tr>
<tr>
<td></td>
<td>Pasteurization</td>
<td>74.5</td>
<td>76.9</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>65.9</td>
<td>74.5</td>
</tr>
<tr>
<td><strong>White muscadine juices</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cold sterile</td>
<td>72.7</td>
<td>69.5</td>
</tr>
<tr>
<td></td>
<td>Hot sterile</td>
<td>53.3</td>
<td>52.9</td>
</tr>
<tr>
<td></td>
<td>Pasteurization</td>
<td>106.7</td>
<td>106.6</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>77.6</td>
<td>76.3</td>
</tr>
</tbody>
</table>
Figure 4.3  Viable cells of (A) *Enterobacter sakazakii* Fec39 and (B) *E. sakazakii* MSDH in commercial baby juices: apple grape juice (Δ), white grape juice (○), and mixed berry juice (□). (*) shows control growth curve of *E. sakazakii* in tryptic soy broth.
Figure 4.4  Viable cells of (A) *Enterobacter sakazakii* Fec39 and (B) *E. sakazakii* MSDH in pasteurized red muscadine juice (Δ), yogurt mixed with pasteurized muscadine juice (▲), pasteurized white muscadine juice (□), yogurt mixed with white pasteurized muscadine juice (■). (*) shows control growth curve of *E. sakazakii* in tryptic soy broth.
Figure 4.5  Viable yogurt lactic acid bacteria in various muscadine juices with yogurt added and with/without *E. sakazakii* inoculated. (*) shows viable lactic acid bacteria in 0.1% peptone solution. RMJ stands for red pasteurized muscadine juice, and WMJ is white pasteurized muscadine juice.
CHAPTER V

ANTIMICROBIAL EFFECT OF MUSCADINE SEED EXTRACTS

ON ENTEROBACTER SAKAZAKII

Abstract

Muscadine grapes (*Vitis rotundifolia*) are recognized for their high polyphenol contents and their antioxidant capacity, but little research has been conducted to assess their potential antimicrobial properties. Seed extracts from two muscadine cultivars (bronze and purple) along with two extraction methods were investigated for their inhibition of *Enterobacter sakazakii*. The relationships between the composition of muscadine seed extracts and their antimicrobial effects were also evaluated. Within two hours, viable *E. sakazakii* in all seed extracts were reduced to non-detectable levels, except in the cold ‘Carlos’ (bronze) seed extract. In general, ‘Ison’ (purple) seed extracts demonstrated stronger inhibition ability to *E. sakazakii*, had lower pH, and were higher in organic acids, phenolic contents, and total phenolics. Ellagic, tannic, and gallic acids, which increased under hot extraction, may play a key role in
the antimicrobial effect on \textit{E. sakazakii}. Results suggested that muscadine seed extracts might be a great natural antimicrobial agent for baby foods and other products.

\section*{Introduction}

\textit{Enterobacter sakazakii} is a Gram-negative, motile bacterium that is considered an emerging opportunistic pathogen. It causes meningitis and necrotizing enterocolitis in infants with high mortality rates, up to 40-80\% and 10-55\%, respectively. The meningitis survivors often develop irreversible neurological sequelae (Lai 2001; Kim and others 2008a). Outbreaks of \textit{E. sakazakii} infections have been associated with the ingestion of infant formula (Iversen and Forsythe 2003). Although \textit{E. sakazakii} exists in powdered infant formula at less than 1 cell per 100g in most positive samples (Muytjens and others 1988; Nazarowec-White and Farber 1997b), this bacterium has been found to grow rapidly in reconstituted infant formula at temperatures between 5.5 to 47°C (Nazarowec-White and Farber 1997b; Forsythe 2005; Kandhai and others 2006).

Muscadine grape (\textit{Vitis rotundifolia}) is native to the southeastern United States. In recent years, interest in the health benefits of muscadines has increased due to their high phenolic contents (Pastrana-Bonilla and others 2003; Yilmaz and Toledo 2004).
The major phenolics in muscadines are ellagic acid and its derivatives, which are unique among grape species. These compounds are common in berries, such as blueberries, raspberries, and strawberries (Pastrana-Bonilla and others 2003; Lee and Talcott 2004). Muscadines and its phenolic compounds have demonstrated excellent antioxidant capacity (Pastrana-Bonilla and others 2003; Lee and Talcott 2004), anti-cancer properties (Yi and others 2005; Mertens-Talcott and others 2006), anti-inflammatory activity (Greenspan and others 2005; Bralley and others 2007), and antimicrobial activity on *E. coli* O157:H7 (Kim and others 2008b).

The muscadine seed, which accounts for 1.5 to 9.2% of whole fruit, is considered a by-product in juice and winery processing (Pastrana-Bonilla and others 2003). Pastrana-Bonilla and others (2003) reported most phenolics in grapes and muscadines are located in the skin and seeds. It has also been reported that dried muscadine seeds have the highest contents of total phenolics and oxygen radical absorbance substances compared to dried skins, whole grapes, and the juice (Striegler and others 2005). Grapes (*Vitis vinifera*) and their by-products have been reported to possess the antimicrobial effect on *Listeria monocytogenes*, *E. coli* O157:H7, *Staphylococcus aureus*, and several *Bacillus* spp. (Jayaprakasha and others 2003; Özkan and others
Several reports have revealed that grape seed extracts combined with nisin can be used as natural preservatives to control *Listeria monocytogenes* on ready-to-eat products (Theivendran and others 2006; Sivarooban and others 2007). However, no studies have reported on their effect on *E. sakazakii*. Therefore, this research is designed to investigate (i) the organic acid and phenolics composition of muscadine seed extracts from two cultivars and the composition differences as affected by two extraction methods, (ii) the inhibition effects of muscadine seed extracts against *E. sakazakii*, and (iii) to correlate the composition profile of muscadine seed extracts with the antimicrobial effect.

**Materials and Methods**

**Chemicals and reagents**

Folin & Ciocalteu’s phenol reagent, sodium carbonate and pure standards of gallic acid (90% purity), (+)-catechin (95% purity), (-)-epicatechin (90% purity), ellagic acid (95% purity), malic acid and tannic acid were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Tartaric acid, acetic acid, hydrochloric acid, sulfuric acid, acetonitrile,
methanol, and water (ACS reagent grade) were purchased from Fisher Scientific Inc. (Pittsburgh, PA).

**Seed extract preparation**

Ground ‘Carlos’ (bronze-skinned) and ‘Ison’ (black-skinned) muscadine seed powders were provided by a local fruit grower (Starkville, MS). Both seed powders were kept at -20°C in the dark until further analysis. To prepare the hot seed extract, one gram of seed powder was first mixed with 5 mL deionized water in a screw-capped vial (National Scientific Co., TN). The mixture was autoclaved (121°C, 103.4 kPa) for 15 min, mixed in a rotary mixer (Dynal Inc., NY) at 40 rpm for one hour at room temperature, and centrifuged at 17,000 x g for 5 min (Eppendorf centrifuge 5415C; Brinkmann Instruments, NY). The supernatant was then filtered through a 0.20 μm syringe filter (Millipore, Bedford, MA). The cold seed extract was prepared as the hot extraction method without autoclaving.

**Total phenolics and pH**

The pH of muscadine seed extracts were measured using a pH meter (Corning Pinnacle 530; Corning Inc., Corning, NY) at room temperature. Total phenolics were
determined according to the Folin-Ciocalteu procedure (Waterhouse 2001). Appropriately diluted samples were mixed with Folin & Ciocalteu’s phenol reagent and sodium carbonate and allowed to stand for two hours. Absorbance at 765nm was measured in a Spectronic Genesys 5 UV-vis spectrophotometer (Fisher Scientific Inc.). Results were expressed as milligrams of gallic acid equivalents per milliliter of seed extract.

**Organic and phenolic acids detection by high-performance liquid chromatographic (HPLC) analysis**

Reversed-phase HPLC was used to separate and quantify major phenolic compounds and organic acids in muscadine seed extracts. Extract samples were mixed with 6 N HCl at 1:10 ratio (v/v) and placed in a water bath for one hour to separate the free phenolic acids from their conjugated forms (Lee 2000). After cooling down to room temperature (about 15 min), each supernatant of acid hydrolyzed samples was filtered through a 0.45 µm syringe filter (Millipore, Bedford, MA) and injected into a Gemini C18, 250 x 4.6 mm column (Phenomenex Inc., Torrance, CA) in an Agilent HPLC 1100 (Agilent Technologies Inc., Santa Clara, CA) equipped with a diode array detector. The injected volume of samples was 25 µL.
The two mobile phases were solvent A: methanol/acetic acid/water (10:2:88, v/v/v), and solvent B: acetonitrile. A linear gradient for phenolics separation was used as follows: at 0 min, 95% solvent A, 5% solvent B; at 1 min, 90% solvent A, 10% solvent B; at 30 min, 30% solvent A, 70% solvent B; at 31 min, 90% solvent A, 10% solvent B; at 32 min, 95% solvent A, 5% solvent B with 5 min post run. The flow rate was 1 mL/min. Individual phenolic compounds were detected at 260 nm.

To separate tartaric, malic and tannic acids, muscadine seed extracts were centrifuged at 12,000 rpm (1,700 g) for 5 min, and the supernatants were filtered and injected into the HPLC system as described above. The mobile phase was 0.01 N H₂SO₄ with a flow rate of 1 mL/min. Individual organic acids were detected at 215 nm.

Peaks for phenolic compounds and organic acids were integrated and analyzed using the ChemStation software (Agilent Technologies Inc.). Individual compounds were quantified based on the retention times and peak area of the standards.

**Antimicrobial activity**

*Enterobacter sakazakii* Fec39 and *E. sakazakii* MSDH (provided by Dr. Yoshen Chen, Mississippi State University) were used in this study. After incubation at 37°C
in tryptic soy broth (Difco Laboratories, Sparks, MD) for seven hours, the bacterial cultures were added into muscadine seed extracts at a 1:1000 ratio (v/v) giving an initial 10^6 CFU per milliliter inoculation. The inoculum was incubated at 37°C, and the viable cells were recorded every 30 min for up to 120 min. Viable *E. sakazakii* were enumerated on tryptic soy agar plates (Difco Laboratories) after incubation at 37°C for 18 to 24 h.

**Statistical design and analysis**

A 2 (muscadine cultivars) * 2 (extraction methods) factorial in a randomized complete block design with three replications was used for detecting pH, total phenolics, organic acids, and phenolic compounds. To measure the antimicrobial activity, a two-way factorial design (treatments x times) was used. Fisher’s protected LSD (*P* < 0.05) used to separate means, Linear regression analysis used for D value determine, Pearson correlations used to analyze the relationship between *E. sakazakii* log reduction and components in muscadine seed extract, and analysis of variance were carried out with SAS 9.1 (SAS Institute, Cary, NC).
**Results**

**pH and total phenolics, organic acid and phenolic acid contents in seed extracts**

Tartaric acid was the major organic acid, while gallic acid had the highest concentration among the detected phenolic acids in muscadine seed extracts (Table 5.1). ‘Ison’ seed extracts had lower pH, and was higher in organic acids than ‘Carlos’ muscadine seed extract. Overall, total phenolics, gallic and ellagic acids were generally higher in ‘Ison’ seed extracts than those in ‘Carlos’ seed extracts. Hot extracts had lower pH and higher concentrations of total phenolics, malic, tannic, ellagic acids, and epicatechin than cold extracts regardless of the cultivar.

**Antimicrobial activity of extracts on Enterobacter sakazakii**

Within two hours incubation, both strains of *E. sakazakii* were reduced to non-detectable levels, about a 6-log reduction, in most muscadine seed extracts (Figure 5.1). Overall, ‘Ison’ seed extracts and hot extractions demonstrated stronger antimicrobial effect than ‘Carlos’ seed extracts and cold extractions, respectively. The hot ‘Ison’ seed extract had the stronger antimicrobial effect on *E. sakazakii* while the cold ‘Carlos’ seed extract had the weakest activity. However, the inhibition
effects of seed extracts between the two tested bacterial strains were similar.

The decimal reduction times (D values) were calculated based on the slope of the linear trend line of viable *E. sakazakii* log reduction. Ison seed extracts showed lower D values, which means better antimicrobial effect than Carlos seed extracts (Table 5.2). The D values of seed extracts prepared by hot extraction method were lower than those prepared by cold extraction.

**Pearson correlation**

Pearson correlations were used to examine the relationships between muscadine seed extract components and viable *E. sakazakii* cell reduction. The determination coefficient ($R^2$) and probability ($p$) were presented in Table 5.3. The log reduction showed a negative correlation with pH ($R^2 = -0.855$), whereas positive determination coefficient were showed with other components of muscadine seed extract. Ellagic, tannic, gallic, malic, and tartaric acids, and pH showed low probability ($p< 0.01$) with high determination coefficient ($R^2$).
Discussion and Conclusion

The minimal infective dose of *E. sakazakii* was estimated to be 1,000 CFU/mL based on the infectious dose of *E. coli* O157:H7 and *Listeria monocytogenes* (Iversen and Forsythe 2003; Lehner and Stephan 2004). In the present study, *E. sakazakii* cells at $10^6$ CFU/mL were inactivated to non-detectable levels by most muscadine seed extracts within 90 min. Also, heat treatment increased the concentrations of organic acids and phenolics, and enhanced their antimicrobial activity of muscadine seed extracts. Heat treatment has been reported to increase several low molecular weight phenolic compounds and the antioxidant activity of citrus peel extracts (Jeong and others 2004). Thus, heat treated muscadine seed extracts may potentially serve as antimicrobials to control *E. sakazakii*.

The characteristics and the antimicrobial effects of muscadine juices observed in the previous chapter were compared with the results of muscadine seed extracts. The hot seed extract was higher in tartaric and tannic acids contents than muscadine juice, but the pH values remained similar. Among all detected phenolic compounds, the seed extracts were only higher in gallic acid contents. The amounts of other phenolics compounds were similar or lower. Especially, the total phenolics
concentrations were much lower in the seed extracts. Although the inoculated *E. sakazakii* at 6-log CFU were inhibited to non-detectable level with 2 h in most muscadine juices and seed extracts, hot ‘Ison’ seed extracts has been demonstrated the fastest antimicrobial activity on *E. sakazakii*.

Several researchers have suggested that acidification may lower the concentration of *E. sakazakii* in food products. Comparing infant rice cereal reconstituted with water and milk, cereal reconstituted with apple juice at pH 4.29 did not support the growth of inoculated *E. sakazakii* (Richards and others 2005). Kim and Beuchat (2005) reported *E. sakazakii* grew in cantaloupe, watermelon, carrot, cucumber, and lettuce juices, but not in apple and strawberry juices. Also, they suggested that low initial pH values of apple and strawberry juices may be the major factor in preventing growth of *E. sakazakii* (Kim and Beuchat 2005). Kim and others (2008b) reported that 5.6-log reduction of *E. coli* O157:H7 was achieved by 10.7 mg/mL tartaric acid alone, the major organic acid in muscadine seed extracts. However, the authors also suggested that other compounds may be involved in the antimicrobial activity since tartaric acid alone did not inactivate *E. coli* as much as the whole seed extracts did.
Since the poor solubility of many phenolic compounds has been suggested to limit the determination of the antimicrobial effect and the minimum inhibitory concentration of individual phenolic compounds on pathogens (Puupponen-Pimiä and others 2005b), the correlation analysis was performed to predict the relationship between the detected components in muscadine seed extracts and the E. sakazakii log reduction. The results showed factors, other than tartaric acid, may be highly correlated to the E. sakazakii log reduction. The antimicrobial activity on E. sakazakii by the muscadine seed extracts correlated well with the increase of ellagic, tannic, gallic, malic, and tartaric acids and with the decrease of pH.

Ellagic acid is a dimeric derivative of gallic acid, and is believed to be formed by hydrolysis from its conjugated forms like ellagic acid glycosides and ellagitannins in muscadines (Lee and Talcott 2002). Ellagitannins and gallotannins (esters of gallic acid and glucose) are two major groups of hydrolysable tannins, commonly referred as to tannic acid (Akiyama and others 2001). Tannins have been reported to inhibit a wide range of fungi, yeasts, viruses and foodborne bacteria, including Enterobacter aerogenes, E. coli, Listeria monocytogenes, Salmonella enteritidis, S. paratyphi, Shigella flexnir and Staphylococcus aureus etc. (Chung and others 1998b). The
inhibitory effect of tannins on microorganisms may be owed to their strong binding capacity to iron, which is critical for the survival of bacteria (Chung and others 1998a). Cloudberry and raspberry extracts, which are rich in ellagitannins, may disintegrate the bacterial outer membrane and cause permeabilisation of *Salmonella* strains (Nohynek and others 2006). Also, ellagitannins in cranberry have demonstrated possible bacterial antiadhesion activities, and give cranberry juice the ability to inhibit *E. coli* 157:H7 adherence to the uroepithelium (Puupponen-Pimiä and others 2005b).

In conclusion, muscadine seed extracts have low pH and are rich sources of phenolic compounds and organic acids, which together contribute to great antimicrobial effects on *E. sakazakii*. Tannic acid and its’ derivatives, ellagitannins and gallotannins, might play a key role in the observed antimicrobial effect. These results revealed that muscadine seed extracts may act as potential natural antimicrobial agents. Before being added to baby foods or used in pharmaceutical industry, further studies are needed to determine the minimum inhibitory concentration of muscadine seed extracts, and to evaluate their sensory characteristics and safety.
Table 5.1  pH, total phenolics, organic acids, and major phenolics concentration of muscadine seed extracts

<table>
<thead>
<tr>
<th>Muscadine cultivars</th>
<th>Extraction methods</th>
<th>Extraction methods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ison</td>
<td>Carlos</td>
</tr>
<tr>
<td></td>
<td>Cold</td>
<td>Hot</td>
</tr>
<tr>
<td>pH</td>
<td>3.37c *</td>
<td>3.30 c</td>
</tr>
<tr>
<td>Total phenolics (mg/mL)</td>
<td>2.11 c</td>
<td>3.45 a</td>
</tr>
<tr>
<td>Individual compounds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tartaric acid (mg/mL)</td>
<td>7.23 a</td>
<td>7.55 a</td>
</tr>
<tr>
<td>Malic acid (mg/mL)</td>
<td>1.43 b</td>
<td>1.89 a</td>
</tr>
<tr>
<td>Tannic acid (mg/mL)</td>
<td>5.25 b</td>
<td>8.58 a</td>
</tr>
<tr>
<td>Gallic acid (mg/100 mL)</td>
<td>26 ab</td>
<td>35 a</td>
</tr>
<tr>
<td>Catechin (mg/100 mL)</td>
<td>11 a</td>
<td>18 a</td>
</tr>
<tr>
<td>Epicatechin (mg/100 mL)</td>
<td>5 c</td>
<td>10 a</td>
</tr>
<tr>
<td>Ellagic acid (mg/100 mL)</td>
<td>9 b</td>
<td>17 a</td>
</tr>
</tbody>
</table>

* Values within rows having the same letters are not different (P<0.05)
Figure 5.1  Viable cells of (A) Enterobacter sakazakii Fec39 and (B) E. sakazakii MSDH in cold Ison seed extract (□), hot Ison seed extract (■), cold Carlos seed extract (Δ) and hot Carlos seed extract (▲). Control (*) shows the viable E. sakazakii in tryptic soy broth.
Table 5.2  Decimal reduction times (D values) for *Enterobacter sakazakii* in muscadine seed extracts

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>Extraction methods</th>
<th>D values (min) for <em>E. sakazakii</em></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ison</td>
<td>Cold</td>
<td>45.3</td>
<td>50.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hot</td>
<td>25.0</td>
<td>41.8</td>
<td></td>
</tr>
<tr>
<td>Carlos</td>
<td>Cold</td>
<td>93.4</td>
<td>146.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hot</td>
<td>55.9</td>
<td>70.3</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.3  Pearson correlation analysis performed between the components in the muscadine seed extracts and *Enterobacter sakazakii* log reduction

<table>
<thead>
<tr>
<th>Components</th>
<th><em>E. sakazakii</em> log deduction</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R^2$</td>
<td>$p$</td>
<td></td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>0.878</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Tannic acid</td>
<td>0.876</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Gallic acid</td>
<td>0.867</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Malic acid</td>
<td>0.861</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>-0.855</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Tartaric acid</td>
<td>0.838</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Catechin</td>
<td>0.804</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Total phenolics</td>
<td>0.699</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Epicatechin</td>
<td>0.634</td>
<td>&gt;0.05</td>
<td></td>
</tr>
</tbody>
</table>
In the agar spot tests, the diluted commercial yogurt containing approximately $10^6$ CFU/mL lactic acid bacteria was antagonistic toward *Enterobacter sakazakii*. The average diameters of clear zones produced by pure cultures of *Lactobacillus helveticus* CNRZ32, *L. bulgaricus* LL18, and diluted yogurt were 11.6, 11.8, and 10.3 mm, respectively. As the concentration of *E. sakazakii* decreased, the increase in yogurt antagonistic effect was observed. The average diameters of inhibition zones were 12.8, 10.9, and 8.8 mm when 0.01, 0.1, and 1% of *E. sakazakii* were applied, respectively.

After 180 min incubation in the simulated gastric model, about 35% of *Lactobacillus*, *Streptococcus*, and *E. sakazakii* in yogurt and/or milk remained viable (5.5-5.9 log CFU/mL reduced). The viable cell numbers decreased rapidly when the pH in the gastric compartment dropped below 3.0. All bacterial cells were recovered after being transferred to the intestinal compartment at various levels. Overall,
comparing to yogurt LAB, *E. sakazakii* was more sensitive to gastric acid with observed lower D value and the lower survival rate in the simulated gastric compartment, *E. sakazakii*, however, recovered better in the intestinal model. There was no significant antagonistic effect of yogurt against *E. sakazakii* at concentration $10^5$ and $10^8$ CFU/mL in the simulated GI model. The adhesion ability and the competitive exclusion effect, two important factors of LAB influencing the antimicrobial effect other than producing antimicrobial compounds, need to be further determined.

Muscadine juices demonstrated inhibitory effect against *E. sakazakii*. Within two hours, the initial inoculated $10^6$ CFU/mL of *E. sakazakii* cells were reduced to non-detectable level in all muscadine juice samples. Overall, RMJ had lower pH values, higher contents of tartaric acid, total phenolics and major phenolic acids, and demonstrated the strongest antimicrobial effect among all tested juice samples. The pasteurized muscadine juices were less effective against *E. sakazakii* than cold sterile muscadine juices. When commercial yogurt was added into muscadine juices, 6-log reduction in viable *E. sakazakii* was observed within four hours, while there was only 2-log decreasing in viable *Lactobacillus* for the same duration.
The characteristics and the antimicrobial effect of the muscadine seed extracts (seed power: distilled water at 1:5) were also evaluated. Between the two muscadine cultivars, seed extracts from ‘Ison’, the dark-skinned muscadine, had lower pH and higher contents of organic acids, total phenolics, and some major phenolics than the seed extracts from the light-skinned ‘Carlos’ muscadine. ‘Ison’ seed extracts also demonstrated stronger inhibitory effects against *E. sakazakii*. Hot extraction method increased the concentrations of organic acids and phenolics in the seed extracts and enhanced their antimicrobial activity. The correlation analysis was performed to predict the relationship between the concentrations of detected components and the antimicrobial effect of muscadine seed extracts. The results indicated that the reduction of *E. sakazakii* caused by muscadine seed extracts was correlated well to pH and the concentrations of ellagic, tannic, gallic, malic, and tartaric acids in the seed extracts.

Results suggested that muscadine juices and seed extracts were strong antimicrobial agents against *E. sakazakii*. Further studies will be necessary for the application of muscadine products in baby food to help controlling the problem of *E. sakazakii*. Although the antimicrobial effect of commercial yogurt was not shown in
the simulated GI model, the antimicrobial effects observed in the agar spot test and in
the mixture incorporated with muscadine juices were demonstrated. Further works
need to be done in combination with muscadine products or with other probiotic
strains.
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Theivendran S, Hettiarachchy NS, Johnson MG. 2006. Inhibition of Listeria monocytogenes by nisin combined with grape seed extract or green tea extract in soy protein film coated on turkey frankfurters. J Food Sci 71:m39-44.


APPENDIX
Figure A.1  Growth curve of (A) Enterobacter sakazakii FEC 39 and (B) E. sakazakii MSDH grow in tryptic soy broth at 37°C.
Figure A.2  Standard curve used in Folin-Ciocalteu procedure for total phenolics determination. The total phenolics were expressed as milligrams of gallic acid equivalents per milliliter.
Table A.1  Linear gradient program used for the separation of phenolic compounds in HPLC analysis

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solvent A (%)</th>
<th>Solvent B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methanol/acetic acid/water</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>0</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>30</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>31</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>32</td>
<td>95</td>
<td>5</td>
</tr>
</tbody>
</table>

Table A.2  Retention times of organic acids and phenolic compounds observed in HPLC analysis

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Retention times (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phenolic compounds at 260 nm</strong></td>
<td></td>
</tr>
<tr>
<td>Gallic acid</td>
<td>5.3</td>
</tr>
<tr>
<td>Catechin</td>
<td>7.9</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>9.2</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>12.9</td>
</tr>
<tr>
<td>Myricetin</td>
<td>15.2</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>15.8</td>
</tr>
<tr>
<td>Quercetin</td>
<td>18.1</td>
</tr>
<tr>
<td><strong>Organic acids at 215 nm</strong></td>
<td></td>
</tr>
<tr>
<td>Tartaric acid</td>
<td>4.3</td>
</tr>
<tr>
<td>Malic acid</td>
<td>5.2</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>23.1</td>
</tr>
</tbody>
</table>
Tartaric Acid

\[ y = 1932.3x + 2.2464 \]

\[ R^2 = 0.9999 \]

Concentration (mg/mL) vs. Peak Area (mAU*s)

Figure A.3  Standard curve used to quantify tartaric acid in HPLC analysis.

Malic Acid

\[ y = 953.44x - 110.93 \]

\[ R^2 = 0.9742 \]

Concentration (mg/mL) vs. Peak Area (mAU*s)

Figure A.4  Standard curve used to quantify malic acid in HPLC analysis.
Figure A.5  Standard curve used to quantify tannic acid in HPLC analysis.

Figure A.6  Standard curve used to quantify gallic acid in HPLC analysis.
Figure A.7  Standard curve used to quantify catechin in HPLC analysis.

Figure A.8  Standard curve used to quantify epicatechin in HPLC analysis.
Figure A.9  Standard curve used to quantify ellagic acid in HPLC analysis.

Figure A.10  Standard curve used to quantify myricetin in HPLC analysis.
Figure A.11  Standard curve used to quantify resveratrol in HPLC analysis.

Figure A.12  Standard curve used to quantify quercetin in HPLC analysis.