SURVIVAL AND GROWTH OF *ENTEROBACTER SAKAZAKII* ON PRODUCE, CONDITIONS AFFECTING BIOFILM FORMATION, AND ITS SENSITIVITY TO SANITIZERS

by

HOIKYUNG KIM

(Under the direction of LARRY R. BEUCHAT)

ABSTRACT

Survival and growth of *Enterobacter sakazakii* on produce and conditions affecting its attachment and biofilm formation were investigated. Sensitivity of the pathogen to sanitizers and disinfectants was determined. *E. sakazakii* grew on fresh-cut produce and in unpasteurized fruit and vegetable juice stored at 12 and 25°C, and survived at least 5 days but did not grow at 4°C. Populations significantly decreased on whole produce stored at 4, 12, and 25°C. Retention of viability was enhanced at refrigerator temperatures. Treatment of apples, tomatoes, and lettuce with chlorine, chlorine dioxide, and Tsunami 200®, a peroxyacetic acid sanitizer, caused significant reductions in populations of *E. sakazakii*, although the extent of lethality depended on the type of produce, sanitizer concentration, and treatment time. Attachment of *E. sakazakii* to enteral feeding tubes and stainless steel was enhanced at 25°C compared to 12°C. The pathogen formed biofilm on enteral feeding tubes and stainless steel at 25°C when immersed in infant formula broth but not in tryptic soy broth or lettuce juice broth; biofilm was not formed at 12°C. When the surface of stainless steel was spot inoculated with infant formula or water containing *E. sakazakii*, dried, and exposed to a relative humidity of 43% at 4, 25, and 37°C, the pathogen

survived for at least 60 days. Survival was enhanced at 4°C, compared to 25 and 37°C, and when formula rather than water was used as a carrier. The efficacy of sanitizers in killing *E. sakazakii* in dried inoculum and in biofilm on the surface of stainless steel varied, depending on the composition of the carrier used to suspend cells before drying, type of sanitizer, and treatment time. The overall order of resistance of *E. sakazakii* to disinfectants routinely used in hospitals, day-care centers, and food service kitchens was planktonic cells < cells spot inoculated and dried on stainless steel < cells in biofilms on stainless steel. This study provides information useful in assessing the potential of produce to serve as a vehicle for *E. sakazakii* infections, understanding attachment and biofilm formation on abiotic surfaces, and evaluating the effectiveness of sanitizers and disinfectants for its elimination.

INDEX WORDS: *Enterobacter sakazakii*, Fruit, Vegetable, Apple, Cantaloupe, Strawberry, Watermelon, Cabbage, Carrot, Cucumber, Lettuce, Tomato, Juice, Lactic acid bacteria, Molds, Yeasts, Chlorine, Chlorine dioxide, Peroxyacetic acid, Attachment, Biofilm, Stainless steel, Enteral tube, Infant formula, Sanitizer, Disinfectant, Quaternary ammonium compounds, Phenolic compounds, Hydrogen peroxide

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DEDICATION

First, I would like to dedicate this dissertation to my parents, Mr. Yeonsik Kim and Ms. Yeoja Park. Mom and Dad, Thank you for believing me that I made a right decision when I decided pursuing my degrees in U. S. Without your unconditional love and support, I had never even thought of beginning my study. I love you.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Enterobacter sakazakii infections rarely occur, compared to infections caused by several other foodborne pathogens; however, the bacterium can be life-threatening to neonates, infants, and immunocompromised adults. *E. sakazakii* has been categorized by the International Commission for Microbiological Specifications for Foods (ICMSF, 2002) as a "Severe hazard for restricted populations, life-threatening, or substantial chronic sequence or long duration" in a ranking of foodborne pathogens and toxins into hazard groups.

The most common food vehicle associated with *E. sakazakii* infections is powdered infant formula. In surveys done to determine the presence of *E. sakazakii* in powdered infant formula, the organism was detected in 2.4 - 14.2% of the products tested (Muytjens et al., 1988; Iversen and Forsythe, 2004). However, the organism has also been isolated from other foods, including lettuce (Soriano et al., 2001), tomatoes (Jung and Park, 2006), alfalfa sprouts (Cruz et al., 2004), other vegetables, cheese, minced beef, and sausage meat (Leclercq et al., 2002), and in household and food factory environments (Kandhai et al., 2004). The presence of *E. sakazakii* in produce raises possibilities of an increased risk of infection in elderly or immunocompromised people. Foodborne disease outbreaks associated with consumption of fresh produce have been caused by several genera in family Enterobacteriaceae. Therefore, the presence of *E. sakazakii* on produce and its ability to cause infections in elderly, immunocompromised people raises the need to know more about its behavior on produce.

In addition to its potential lethality and apparent ubiquity, *E. sakazakii* has been reported to form biofilms (Iversen et al., 2004; Lehner et al., 2005). *E. sakazakii* has been detected on utensils used for infant formula preparation in hospitals (Simmons et al. 1989; Clark et al., 1990;

Noriega et al., 1990; Bar-Oz et al., 2001). Attachment and biofilm by *E. sakazakii* on food contact surfaces may increase the risk of *E. sakazakii* infections.

Various sanitizers and disinfectants have been effectively used to kill or eliminate foodborne pathogens that may be contaminants in produce processing environments and in food preparation areas in hospitals. However, the efficacy of these sanitizers and disinfectants in killing *E. sakazakii* has not been reported. This information would be useful when developing strategies to reduce the risk of *E. sakazakii* infections.

ENTEROBACTER SAKAZAKII

General characteristics

E. sakazakii was initially referred to as yellow pigmented *Enterobacter cloacae* and was first reported to cause an outbreak of infections in 1958 (Urmenyi and Franklin, 1961). Later, it was proposed that *E. sakazakii* be distinguished from *E. cloacae* as a separate species based on differences in DNA relatedness, pigment production, biotyping, and antibiotic susceptibility (Farmer et al., 1980; Izard et al., 1983). The two bacteria have many similar biochemical reactions. *E. sakazakii*, however, produces a yellow pigment and does not ferment D-sorbitol (Table 1-1).

E. sakazakii is a gram-negative rod, motile, and non-sporulating bacterium known to cause meningitis (Gallagher and Ball, 1991; Burdette and Santos, 2000), sepsis (Simmons et al., 1989), bacteremia (Noriega et al., 1990), and necrotizing enterocolitis (Van Acker et al., 2001) in preterm neonates and immunocompromised adults (Jimenez, et al., 1982; Pribyl et al., 1985; Hawkins, et al., 1991; Emery and Weymouth, 1997; Lai, 2001).

Table 1-1. Differences of biochemical reactions between *E. cloacae* and *E. sakazakii*[a]

(Modified from Farmer and Kelly, 1992; Nazarowec-White and Farber, 1997a)

Biochemical reaction	*E. sakazakii*	*E. cloacae*
Yellow pigmentation	+	−
Fermentation of:		
sucrose	+	+
dulcitol	−	(−)
adonitol	−	(−)
D-sorbitol	−	+
raffinose	+	+
α-methyl-D-glucoside	+	(+)
D-arabitol	−	(−)
Lysine decarboxylase	−	−
Arginine dihydrolase	+	+
Ornithine decarboxylase	+	+
KCN, growth in	+	+

[a] +: 90 - 100% positive; (+): 75 - 89% positive; (−): 10 - 24% positive; −: 0 - 9% positive.

A mortality rate of 40 - 80% has been reported (Urmenyi and Franklin, 1961; Joker et al., 1965; Adamson and Rogers, 1981; Kleiman et al., 1981; Muytjens et al., 1983; Willis and Robinson, 1988; Nazarowec-White and Farber, 1997a). The oral infectious dose has been approximated to range from 10^3 (Iversen and Forsythe, 2003) to $\geq 10^8$ CFU (Pagotto et al., 2003). However, pathogenesis and virulence factors of *E. sakazakii* have not been fully characterized.

The yellow pigmentation of *E. sakazakii* is a unique characteristic distinguished from other *Enterobacter* species. The pigment is produced in greater quantities at 25°C than at 37°C

(Nazarowec-White and Farber, 1997b). Growth characteristics of *E. sakazakii* in infant formula and laboratory media have been determined. *E. sakazakii* is capable of growing at temperatures as low as 5.5°C in brain heart infusion (BHI) broth and as high as 47°C in infant formula milk and BHI broth (Nazarowec-White and Farber, 1997b; Breeuwer et al., 2003; Iversen et al., 2004). When *E. sakazakii* was grown in reconstituted infant formula at 23°C, the lag time and generation time were 2.76 h and 0.67 h, respectively (Nazarowec-White and Farber, 1997b). The optimum growth temperature of six strains of *E. sakazakii* in whitely impedance broth, BHI broth, infant formula milk, and tryptic soy broth ranged from 37 to 43°C (Iversen et al., 2004).

An environmental reservoir of *E. sakazakii* has not been defined. However, several possible reservoirs exist. *E. sakazakii* was isolated from 9 - 44% of test samples of environments in households and food factories (milk powder, chocolate, potato, and pasta) (Kandhai et al., 2004). Other studies showed the presence of *E. sakazakii* in Mexican fruit flies *Anastrepha ludens* (Kuzina et al., 2001) and stable flies *Stomoxys calcitrans* (Hamilton et al., 2003). This indicates that the bacterium is wide spread in the environment. Iversen and Forsythe (2003) demonstrated that soil, water, and vegetables may be the principal sources of *E. sakazakii* contamination because the organism is not a part of the normal animal and human gut biota.

A method for isolating and enumerating *E. sakazakii* in dehydrated powdered infant formula has been developed by the U. S. FDA (2002). The method includes pre-enriching samples in distilled water, enriching in Enterobacteriaceae enrichment (EE) broth, surface plating on violet red bile glucose (VRBG) agar, and confirming the presumptive colonies with API 20E® bioassay kit (Figure 1-1).

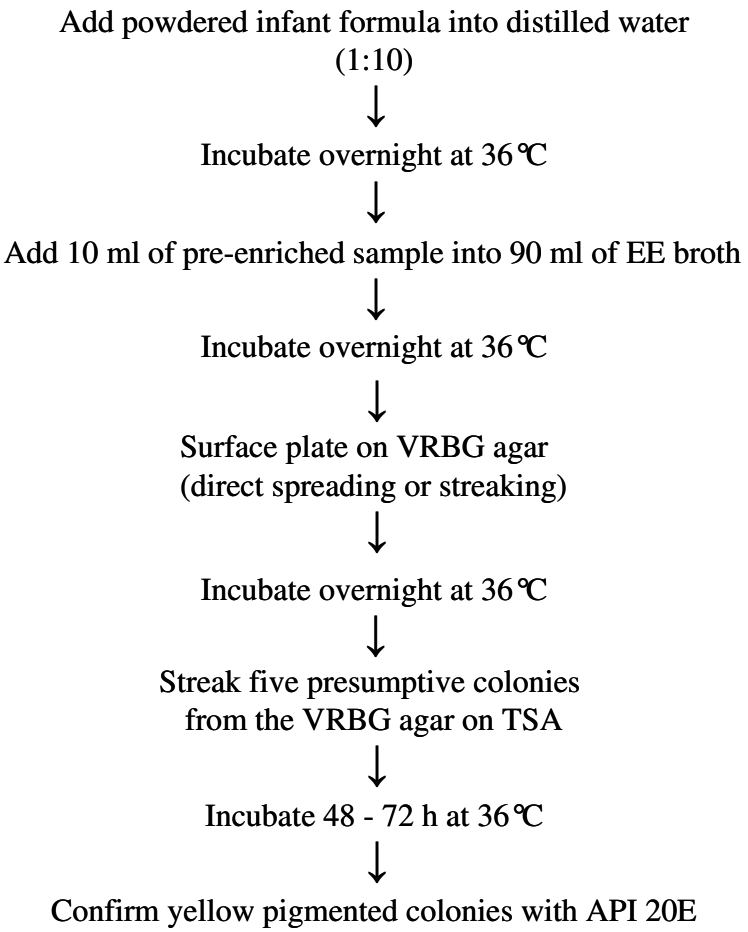


Figure 1-1. Procedure of *E. sakazakii* isolation from powdered infant formula (U. S. FDA, 2002)

Epidemiology

At least 76 cases of *E. sakazakii* infections, resulting in 19 deaths of neonates, infants, and young children have been documented (Iversen and Forsythe, 2003), while at least 9 cases have been reported among adults (Jimenez and Gimenez, 1982; Pribyl et al., 1985; Hawkins et al., 1991; Emery and Weymouth, 1997; Lai, 2001; Dennison and Morris, 2002). Table 1-2 lists outbreaks and cases of *E. sakazakii* infections that have been documented since 1958.

The first two reported cases of *E. sakazakii* infections causing neonatal meningitis occurred in 1958 in England, but the causative bacterium was considered to be *E. cloacae*

Table 1-2. Partial list of outbreaks and cases of *E. sakazakii* infections documented since 1958.

Year	Number of cases	Location	References
1958	2	England	Urmenyi and Franklin (1961)
1958	1	Denmark	Joker et al. (1965)
1958	1	USA (Macon, GA)	Monroe and Tift (1979)
1958	1	USA (Indianapolis, IN)	Kleiman et al. (1981)
1977 - 1981	8	Netherlands	Muytjens et al. (1983)
1984	11	Greece	Arseni et al. (1987)
1984	2	USA (Boston, MA)	Willis and Robinson (1988)
1986 - 1987	3	Iceland	Bierling et al. (1989)
1988	4	USA (Memphis, TN)	Simmons et al. (1989)
1988	1	USA (Baltimore, MD)	Noriega et al. (1990)
1988	1	USA (Cincinnati, OH)	Gallagher and Ball (1991)
1993	1	Israel	Block et al. (2002)
1995	1	Israel	Block et al. (2002)
1995 - 1996	5	USA (Boston, MA)	Lai (2001)
1997	1	Israel	Block et al. (2002)
1998	1	Israel	Block et al. (2002)
1998	12	Belgium	Van Acker et al. (2001)
1999 - 2000	2	Israel	Bar-Oz et al. (2001)
2001	11	USA (Knoxville, TN)	Himelright et al. (2002)

(Urmenyi and Franklin, 1961). In The Netherlands, eight cases of neonatal meningitis caused by *E. sakazakii* were reported between 1977 and 1981 (Muytens et al., 1983). Two of the infected infants had both necrotizing enterocolitis and meningitis and the mortality rate was 75%, although antibiotics were given. Three cases of *E. sakazakii* infections were reported between in 1986 and 1987 in Iceland (Biering et al., 1989). One of the three infants had Down's syndrome.

All three infants were fed reconstituted powdered infant formula. One died and the two recovered, but with severe neurologic sequelae.

In 1998, 12 cases of necrotizing enterocolitis were reported in Belgium (van Acker et al., 2001). Fifty neonates were admitted to neonatal intensive care units in June and July, 1998. All twelve infants with necrotizing colitis had low birth weights (< 2 kg) and had been fed powdered infant formula. Of the 12 neonates, ten were fed with the same formula and six presented blood, anal swab, or stomach aspirate cultures positive for *E. sakazakii*. *E. sakazakii* was isolated not only from infant formulas that were fed to the patients but also from unopened cans of products. Partial strain similarity between powdered infant formula and patient cultures was confirmed with molecular typing (AP-PCR).

In the U. S., outbreaks of *E. sakazakii* infections were reported in Tennessee in 1988 (Simmons et al., 1989). The outbreak involved four infants. One had bloody diarrhea, one had sepsis, and two had both diarrhea and sepsis. In 2001, a premature infant was infected with *E. sakazakii* in Knoxville, TN (CDC, 2002a; Himelright et al., 2002). Forty-nine infants were examined for *E. sakazakii*; stool and urine samples of ten infants were positive for the organism. Powdered infant formula was suspected to be the source of *E. sakazakii* that caused infections.

Resistance to stress

Observations on the occasional presence of *E. sakazakii* in powdered infant formula, which is subjected to heat treatment during processing, have raised interest in determining its heat resistance. Thermal resistance has been reported to vary significantly, depending on the strain and suspending medium. Nazarowec-White and Farber (1997b) determined D values of *E. sakazakii* in reconstituted powdered infant formula. D values at 52, 54, 56, 58, and 60°C were

54.8, 23.7, 10.3, 4.20, and 2.50 min, respectively. Iversen et al. (2004) reported D values of *E. sakazakii* at 54, 56, 58, 60, and 62°C to be 11.7 - 16.4, 3.9 - 5.1, 2.6 - 3.8, 1.1 - 1.8, and 0.2 - 0.3 min, respectively. D values at 58°C of strain ATCC 51329 and a clinical strain in rehydrated powdered infant formula were 0.51 and 9.87 min, respectively (Buchanan and Edelson, 1999). *E. sakazakii* in disodium hydrogen phosphate potassium buffer (pH 7.0) have D values ranging from 0.39 to 0.60 min at 58°C (Breeuwer et al., 2003). D values were definitely lower than those of cells in reconstituted powdered infant formula.

Electromagnetic radiation (2,450 MHz) was tested for effectiveness in killing *E. sakazakii* (Kindle et al., 1996). Five infant formulas were inoculated with *E. sakazakii* at ca. 5 log CFU/ml and heated by microwave treatment until they boiled. *E. sakazakii* survived in one of the four formulas.

Resistance of *E. sakazakii* to low water activity has been studied. Caubilla-Barron et al. (2004) found that *E. sakazakii* can survive in powdered infant formula for at least 12 months. *E. sakazakii* in stationary growth phase was observed to be more resistant to osmotic and desiccation stresses than were *Enterobacter agglomerans*, *Escherichia coli*, *Salmonella* Senftenberg, *S.* Typhimurium, and *S.* Enteritidis (Breeuwer et al., 2003). Stationary phase cells accumulated tremendous amounts of trehalose, compared to the cells in exponential phase, when the organism was dried at 25°C. This may explain why *E. sakazakii* in stationary phase is more resistant to desiccation stress, compared to other Enterobacteriaceae, since trehalose has been determined to stabilize phospholipids in membranes, thereby protecting cells during desiccation (Crowe et al., 1992; Strom and Kaasen, 1993; Potts, 1994; Kempf and Bremer, 1998; Welsh and Herbert, 1999).

The resistance of *E. sakazakii* to acid pH has been studied. Ten of twelve test strains of *E. sakazakii* showed less than a 1-log reduction in tryptic soy broth 5 h after the pH was adjusted to 3.5 with HCl; a 4.9 to > 6.3 log reduction occurred at pH 3.0 (Edelson-Mammel and Buchanan, 2004). *E. sakazakii* inoculated into milk was reported to ferment milk rapidly and reduce the pH from 6.6 to 5.6 at 30°C in < 20 h (Skladal et al., 1993).

Foods from which* E. sakazakii *has been isolated

Powdered infant formula and milk powder have been implicated as sources of *E. sakazakii* in outbreaks of infections (Muytjens et al., 1983; Biering et al., 1989; Simmons et al., 1989; Noriega et al., 1990; Van Acker et al., 2001; Bar-Oz et al., 2001; Block et al., 2002; Himelright et al., 2002). The first outbreak of *E. sakazakii* infection confirmed to be caused by powdered infant formula occurred in 2001 (CDC, 2002a; Himelright et al., 2002; Weir, 2002). The organism was isolated from an unopened can of the formula.

E. sakazakii was detected in 20 of 141 (14.2 %) powdered infant formulas originating from 35 countries (Muytjens et al., 1988) and in 2 of 82 (2.4%) infant formulas manufactured in South Africa, South Korea, Holland, Spain, Switzerland, USA, Belgium, Ireland, Slovenia, and UK (Iversen and Forsythe, 2004). Iversen and Forsythe (2004) surveyed other infant foods and milk-based products, including dried infant foods, milk powders, lactose powders, and cheese products for presence of *E. sakazakii*. They found the bacterium in 5 of 49 (10.2%) dried infant foods, 3 of 72 (4.1%) milk powders, and 2 of 62 cheese products.

Although outbreaks of *E. sakazakii* infections have been linked only to powdered infant formula, the organism has been isolated from various types of ready-to-eat foods, including lettuce (Soriano et al, 2001), four types of vegetables, cheese, minced beef, and sausage meat

(Leclercq et al., 2002), rice seed (Cottyn et al., 2001), beer mugs (Schindler and Metz, 1990), cured meat (Watanabe and Esaki, 1994), tofu (No et al., 2002), sour tea (Tamura et al., 1995), fermented bread (Gassem, 1999), tomatoes (Jung and Park, 2006), and alfalfa sprouts (Cruz et al., 2004).

FOODBORNE DISEASES ASSOCIATED WITH FRESH PRODUCE

In the past two decades, concomitant with an increased per capita consumption of fresh produce in the U. S., the number and frequency of outbreaks of illness associated with fresh produce have increased. Approximately 12% of cases of foodborne illness in the U. S. have been associated with consumption of fresh fruits and vegetables (Tauxe, 1997). Several genera have been involved. Examples of pathogens causing infections include *E. coli* O157:H7 linked to the consumption of lettuce (Hilborn et al., 1999), alfalfa (Breuer et al., 2001), and apple juice (CDC, 1997), *Salmonella* linked to tomatoes (Cummings et al., 2001) and cantaloupe (CDC, 2002b), *Shigella* linked to parsley (CDC, 1999), *Vibrio cholerae* on raw vegetables (CDC, 2004), and *Cyclospora cayetanesis* linked to raspberries (Herwaldt et al., 1997). *Listeria monocytogenes*, *Campylobacter*, *Aeromonas*, and *Clostridium botulinum* have also been concerns of fresh fruits and vegetables (Beuchat, 1996; Harris et al., 2003).

Many factors have contributed to the increase fresh produce-related outbreaks. These include changes in production, handling practices, and consumption patterns (Beuchat and Ryu, 1997). Consequently, various sanitizing methods have been developed and applied by the produce industry to improve safety of fresh produce for consumers (Beuchat, 1998).

The presence of *E. sakazakii* on produce raises concern about safety risks to immunocompromised adults. To date, however, outbreaks of *E. sakazakii* infections have not

been linked to consumption of produce. The efficacy of sanitizers routinely used in the produce industry in killing *E. sakazakii* on produce has not been reported.

BIOFILM

Definitions and mechanisms

The term "biofilm" has been described as a biological matrix of microbial cells and extracellular substances (Bakke et al., 1984). Biofilms have also been defined as sessile communities of bacterial cells attached to a surface or to each other, usually embedded in polymeric substances produced by the bacteria (Marshall, 1992; Costerton et al., 1995).

Attachment of bacterial cells to surfaces is followed by growth, production of exopolysaccharide, and biofilm formation (Kumar and Anand, 1998). Several mechanisms of biofilm formation have been proposed by researchers. Development of biofilms was detailed by Stoodley et al. (2002) as a five-step process: (1) reversible attachment of cell to surface, (2) irreversible attachment, (3) development of biofilm matrix, (4) maturation of biofilm, and (5) dispersal of biofilm. Marshall et al. (1971) viewed the biofilm formation in two- step process consisting of reversible attachment followed by irreversible attachment. Kumar and Anand (1998) proposed that biofilm formation involved a five-stage process: (1) formation of conditioning film, (2) attachment of bacterial cells, (3) development of microcolonies, (4) biofilm formation, and (5) dispersion of biofilms.

Prior to attachment of cells, conditioning films develop by adsorbing organic or inorganic materials to the surface which changes the surface characteristics, e.g., free energy, hydrophobicity, and electrostatic charges (Dickson and Koohmaraie, 1989). In food processing plants, food residues remaining on equipment surfaces may behave as conditioning films. After

the conditioning film forms, reversible attachment of cells occurs followed by irreversible attachment. Van der Waals forces, electrostatic interaction, and hydrophobic interaction play important roles in a reversible attachment (Chmielewski and Frank, 2003). Dipole-dipole interaction, hydrogen bonding, and ionic covalent bonding are involved in irreversible attachment (Bower et al., 1996; Briandet et al., 1999). During the attachment process, cells utilize nutrients from the fluid environment and form microcolonies on surfaces (Kumar and Anand, 1998).

Microorganisms produce extracellular polymeric substances (Characklis and Marshall, 1990), which generally firmly attach cells to surfaces (Eginton et al., 1998). This leads to formation of the biofilm matrix, which is a three-dimensional structure. Once biofilms form, cells continue to grow. As the biofilms mature, however, cells begin to detach and colonize new niches (Kumar and Anand, 1998). The detached cells move to another location and continue the process to form biofilms (Marshall, 1992).

Another process describing biofilm formation was proposed by Busscher and Weerkamp (1987). They proposed a mechanism based on distance between bacterial cells and the supporting surface. It was found that at a distance of > 50 nm, 10 - 20 nm, and < 1.5 nm, Van der Waals forces only, Van der Waals forces and electrostatic interactions, and additional specific forces are involved, respectively.

Factors affecting attachment and biofilm formation

Factors affecting attachment of and biofilm formation by microorganisms include nutrient availability, extracellular polymeric substances, pH of the surrounding medium, and the nature of the cell surfaces (Frank, 2001). There are numerous studies showing that nutrient

availability affects attachment and biofilm formation. Hood and Zottola (1997b) observed that composition of growth and conditioning media influence the attachment of *S.* Typhimurium and *L. monocytogenes* to stainless steel surfaces. Wrangstadh et al. (1986) reported that starvation of *Pseudomonas* sp. S9 cells adversely affect production of exopolysaccharide resulting in enhanced attachment of the cells. Increased nutrient levels enhance biofilm formation by *L. monocytogenes* (Jeong and Frank, 1994). Extracellular polymeric substances can change hydrophobicity of surfaces to enhance or inhibit adhesion of cells. *Serratia marcescens*, for example, has been found to secrete lipopeptide that can make hydrophilic surfaces hydrophobic (Matsuyama et al., 1992). Ryu et al. (2004) observed that nutrient availability and exopolysaccharide produced by cells affect the ability of *E. coli* O157:H7 to form biofilm on stainless steel.

Properties of cells that affect attachment include cell surface structures, hydrophobicity, and surface charges. Fimbriae, the outer membrane, and S layer can play important roles in the attachment of cells (Frank, 2001). Hydrophobic cells have better ability than hydrophilic cells to attach to surfaces (van Loosdrecht et al., 1987). Cells tend to attach to surfaces that have opposite charges from themselves (Frank, 2001). *Staphylococcus aureus*, which is negatively charged, has greater ability to adhere to positively charged surfaces than to negatively charged surfaces (Hogt et al., 1986). However, these interactions are not always a prediction of cell attachment.

Significance of bacterial attachment and biofilms in food processing and preparation areas

Bacterial biofilms are known to be formed on food contact surfaces, which may result in cross-contamination of products (Zottola and Sashara, 1994; Wirtanen et al., 1996; Hood and

Zottola, 1997a; Kumar and Anand, 1998; Frank, 2001). Stainless steel is a commonly used material for food contact surfaces in processing and preparation area. Numerous bacteria, including *E. coli* O157:H7 (Ryu et al., 2004; Ryu and Beuchat, 2005b), *B. cereus* (Ryu and Beuchat, 2005a), *L. monocytogenes* (Hassan et al., 2004; Folsom et al., 2006), *E. sakazakii* (Iversen et al., 2004), and *Pseudomonas putida* (Antoniou and Frank, 2005) have been shown to attach and form biofilms on stainless steel surfaces.

Attachment of microorganisms followed by biofilm formation on biotic or abiotic surfaces is known to enhance the resistance of cells to environmental stresses and provide protection against sanitizers (Kumar and Anand, 1998; Norwood and Gilmour, 2000; Frank et al., 2003; Ryu and Beuchat, 2005b). Several studies have shown that attached microorganisms have higher resistance than planktonic cells to environmental stresses and antimicrobials. For instance, cells of *L. monocytogenes* on glass or stainless steel are more resistant to benzalkonium chloride (BAC), anionic acid sanitizers, and heat (50°C and 70°C), compared to planktonic cells (Frank and Koffi, 1990; Mafu et al., 1991). Delissalde and Amábile-Cuevas (2004) reported that *P. aeruginosa* in biofilm showed higher resistance than non-biofilm formers to several antibiotics. Peracetic acid, mercuric chloride, and formaldehyde have been shown to be ineffective in killing microorganisms in biofilms (Carpentier and Cerf, 1993). Ryu and Beuchat (2005b) reported that the resistance of *E. coli* O157:H7 to chlorine significantly increased as cells formed biofilm on the surface of stainless steel.

Mechanisms of the resistance of cells to environmental stresses have been described. Extracellular polymeric substances produced by microorganisms during biofilm formation behave as protective barriers from stresses (Costerton et al., 1995; Lewis, 2001; Mah and O'Toole, 2001). Oxidizing sanitizers, including hypochlorite and hydrogen peroxide, can be

neutralized and become less effective upon contact with outer layers of biofilms (de Beer et al., 1994; Chen and Stewart, 1996; Xu et al., 1996; Mah and O'Toole, 2001). There have been reports that *P. aeruginosa* in biofilms produce significantly more β-lactamase, an antibiotic-degrading enzyme, than planktonic cells (Tuomanen et al., 1986). The composition of cell wall protein of microorganisms in biofilm can be altered in biofilm (O'Toole et al., 2000).

Biofilm formation by E. sakazakii

E. sakazakii has been found to attach to and form biofilms on silicon, latex, polycarbonate, stainless steel, glass, and polyvinyl chloride (PVC) (Iversen et al., 2004; Lehner et al., 2005). In addition, *E. sakazakii* has been observed to produce extracellular polysaccharide (Scheepe-Leberkühne and Wagner, 1986; Lehner et al., 2005). Attachment and biofilm formation by *E. sakazakii* on equipment surfaces in formula preparation or feeding areas or in produce processing plants may increase the risk of infections. Presence of *E. sakazakii* on a spoon, brush, and blender used for infant formula preparation has been documented in a clinical setting where neonatal infections had been reported (Simmmons et al. 1989; Clark et al., 1990; Noriega et al., 1990; Bar-Oz et al., 2001). Reuse of infant feeding equipment, e.g., infusion tubes and delivery bags, after washing with water is hypothesized to increase the risk of microbial infections (Oie and Kamiya, 2001). Nosocomial infections caused by *E. sakazakii* can occur through contaminated utensils (Martin, 1997).

SANITIZERS AND DISINFECTANTS

Sanitizers used for fresh produce

Various sanitizers have been applied to fresh produce for the purpose of eliminating microorganisms capable of causing diseases. Chlorinated water, chlorine dioxide, and peracetic acid-based sanitizers are among the sanitizers used in the fresh fruit and vegetable industry.

Chlorine

In the U.S., chlorine was first used to treat drinking water in the early 20th century, and then used as a sanitizer in dairy processing plants and other food processing plants (Troller, 1993). Chlorine has several advantages as a sanitizer, including its effectiveness in killing a broad spectrum of microorganisms, low cost, and ease of handling. Disadvantages are that chlorine is corrosive, causes discoloration, is inactivated by organic materials, and can produce off flavors (Troller, 1993). Water containing free chlorine concentrations of 50 - 200 μg/ml is used to sanitize fresh produce (Beuchat et al., 1998; Rogers et al., 2004). Free chlorine is defined as HOCl (hypochlorous acid), OCl^- (hypochlorite ion) or Cl_2 (elementary chlorine) (Weidenkopf, 1953). Of the free forms, HOCl exhibits the most effective bactericidal activity. Consequently, antimicrobial activity of chlorinated water depends on the amount of HOCl present (Beuchat and Ryu, 1997). When Cl_2 is added to water, HOCl forms by the following reaction:

$$Cl_2 + H_2O \rightarrow HOCl + H^+ + Cl^-$$

Then, HOCl dissociates into H+ and OCl^- in water:

$$HOCl \rightarrow H^+ + OCl^-$$

Modes of antimicrobial action that have been proposed include inhibiting glucose oxidation, disrupting protein synthesis, reacting with nucleic acids, purines, and pyrimidines, inhibiting oxygen uptake, forming toxic chloramines, and changing cell permeability (Troller, 1993; Marriott and Gravani, 2006).

Chlorine has been shown to be effective in reducing populations of *E. coli* O157:H7 (Beuchat et al., 1998; Beuchat, 1999; Park and Beuchat, 1999; Fett, 2002; Ryu and Beuchat, 2005b), *Salmonella* (Zhuang et al., 1995; Park and Beuchat, 1999; Weissinger et al., 2000; Beuchat et al., 2001; Fett, 2002), and *L. monocytogenes* (Ukuku and Fett, 2002; Beuchat et al., 2004) on fresh produce. For instance, Beuchat et al. (1998) reported that spraying with 200 µg/ml chlorine and soaking for ≥ 3 min resulted in significant decreases in populations of *E. coli* O157:H7 and *Salmonella* on apples, tomatoes, and lettuce, compared untreated produce. Beuchat et al. (2001) observed a 3.07-log reduction in *Salmonella* on tomatoes sprayed with 200 µg/ml of chlorine. Populations of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* on tomatoes decreased by > 3.04, 4.00, and > 4.83 log/tomato, respectively, following treatment with 200 µg/ml of chlorinated water (Lang et al., 2004). Lettuce treated with chlorine at 200 µg/ml for 5 min showed only 1.45, 1.85, and 1.15 log-reductions in *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes*, respectively (Lang et al., 2004).

Chlorine dioxide

Aqueous chlorine dioxide has been approved as a disinfectant used in bottling plants and food processing plants (storage and handling areas) by the U. S. Environmental Protection Agency (EPA) (1967). The gaseous form of chlorine dioxide was approved as a sterilant for equipment and environmental surfaces in 1988 (U. S. EPA, 2006). Aqueous chlorine dioxide has

been authorized for use as a sanitizer for whole fresh produce (U. S. FDA, 1998). Chlorine dioxide has been increasingly used to enhance the microbiological safety on fruit and vegetable processing plants (Synan, 1979; Costilow et al., 1984; Roberts and Reymond, 1989) due to its bactericidal activity over a wide pH range, rapid bactericidal action (Bernarde et al, 1965; 1967; Rav-Acha, 1984; McGuire and Dishinger, 1984), and limited reaction with organic materials (Richardson et al, 1994; Long et al, 1999). Chlorine dioxide is produced by following reactions (Troller, 1993; Marriott and Gravani, 2006):

$$NaOCl + HCl \rightarrow NaCl + HOCl$$

$$5\ NaClO_2 + 4\ HCl \rightarrow 4\ ClO_2 + 5\ NaCl + 2\ H_2O$$

$$HOCl + 2\ NaClO_2 \rightarrow 2\ ClO_2 + 2\ NaCl + 2\ H_2O$$

Aqueous and gaseous chlorine dioxide have been reported to kill *E. coli* O157:H7 on lettuce and baby carrots (Singh et al., 2002) and *E. coli* O157:H7 and *L. monocytogenes* on green peppers (Han et al., 2001), apples, lettuce, strawberries, and cantaloupes (Rodgers et al., 2004). Du et al. (2003) reported reduction in populations ranged from 4.5 - 7.4 log CFU/site on apple treated with 1.1 - 12 μg/ml gaseous chlorine dioxide. Chlorine dioxide gas (4.1 μg/ml) significantly reduced populations of *Salmonella*, *E. coli* O157: H7, and *L. monocytogenes* on fresh-cut cabbage, carrot, and lettuce (Sy et al., 2005).

Peroxyacetic acid

The U. S. EPA (1987) registered peroxyacetic acid, an oxidizing agent, as a sanitizer for food establishments, medical facilities, and dairy and cheese processing plants. Peroxyacetic acid has been used as a sanitizer for fruit processing operations (Wisniewsky et al., 2000).

Peroxyacetic acid (peracetic acid) is produce by combining of hydrogen peroxide and acetic acid as follows.

$$H_2O_2 + CH_2COOH \rightarrow CH_2COO\text{-}OH + H_2O$$

Peroxyacetic acid has been known to disrupt osmotic function employed by lipoprotein of cytoplasmic membrane (Block, 2001). It is less corrosive than chlorine-based sanitizers and does not produce toxic residues, which makes it more acceptable for use in food processing plants (Dychdala, 1988; Marriott and Gravani, 2006).

Peroxyacetic acid has been used to reduce microbial populations in process water (Hilgren and Salverda, 2000) and on apples (Wisniewsky et al., 2000; Rodgers et al, 2004), cantaloupes (Park and Beuchat, 1999; Rodgers et al., 2004), lettuce (Beuchat et al., 2004; Rodgers et al., 2004), strawberries (Rodgers et al., 2004), honeydew melons, and asparagus (Park and Beuchat, 1999). For instance, treatment with peroxyacetic acid was shown to reduce the percentage of inoculated stainless steel chips positive for *E. coli* O157:H7 by 17 - 42%, compared to treatment with water (Farrell et al., 1998), and has a bactericidal effect to *P. aeruginosa* and *S. aureus* in biofilms (Holah et al., 1990). Wright et al. (2000) reported that the population of *E. coli* O157:H7 on apples was reduced by 2.5 log CFU/cm^2 by upon treatment with 80 µg/ml peroxyacetic acid. Wisniewsky et al. (2000) reported that a 3-log CFU/apple reduction of *E. coli* O157:H7 occurred on whole apples treated with Tsunami 100® (80 µg/ml), a peroxyacetic acid-based sanitizer, for 5 min.

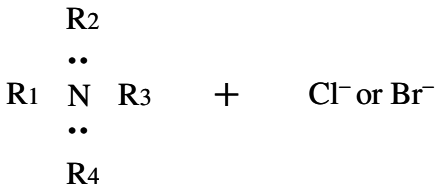
Sanitizers and disinfectants used for food-contact surfaces

Disinfection is described as a process eliminating vegetative cells of potential pathogenic bacteria on inanimate objects (Rutala and Weber, 1999; Exner et al., 2004). Surface disinfection

is routinely done in hospitals by cleaning with a liquid chemical disinfectant since surfaces may contribute to cross-contamination or nosocomial infections. Commercial surface disinfectants have been based largely on quaternary ammonium compounds, phenolic compounds, alcohol, chlorine, and iodophor.

Quaternary ammonium compounds

Quaternary ammonium compounds (quats) are one of the most frequently used chemical disinfectants to control microorganisms in clinical and industrial areas (MacBain et al., 2004). Quats consist of four organic groups linked to nitrogen. The chemical structure of quats is:



The nature of the organic groups may alter biological activity of the quats (Li et al., 2000). Chloride and bromide are most commonly used for commercial quats compounds.

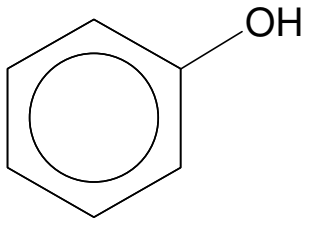
The bactericidal mechanism of quats has not been fully characterized. However, quats have been reported to have different bactericidal activity than chlorine and chlorine dioxide. In addition, bactericidal activity of quats is less affected than chlorine-based sanitizers by organic materials (Marriott and Gravani, 2006). Quats produce a film on surfaces that is bactericidal to vegetative cells and bacteriostatic to spores (Marriott and Gravani, 2006). Quats kill microorganisms by interacting with lipopolysaccharides or lipids of the cell membrane followed by penetration of cells (Russell and Gould, 1988). When *P. aeruginosa* is treated with quats, the fatty acid composition of the cell membrane is altered (Guérin-Méchin et al., 1999). Quats have major advantages in that they are odorless, colorless, non-irritating, less affected by presence of

organic materials than chlorine, non-toxic, and non-corrosive; Disadvantages are that quats may foam excessively and are ineffective against gram-negative bacteria, unstable with soap or anionic detergent, form film on surfaces, and may enhance bacterial resistance (Troller, 1993; Anonymous, 1997; Russell, 2002). The recommended concentration of quats used to sanitize stainless steel is 200 µg/ml (Troller, 1993).

L. monocytogenes cells showed significant reductions in populations when treated with 50 or 100 µg/ml of benzalkonium chloride and cetylpyridinium chloride, major components of quats, compared to treatment with water (Taormina and Beuchat, 2002). Planktonic and attached cells of *B. cereus* were reduced by 5.72 log CFU/ml and 4.22 log CFU/stainless steel chip, respectively, after exposure to quats (dialkyldimethyl ammonium chloride) at 100 µg/ml for 30 sec (Peng et al., 2002). Treatment with 20 µg/ml of quats (benzyldimethyl tetradecylammonium chloride) caused > 90% mortality of planktonic cells and sessile cells of *L. monocytogenes* (Chavant et al., 2004). Antibiotic-susceptible isolates of *E. coli*, *S. aureus*, *S. epidermidis*, and *E. cloacae* were not detected after exposure to quats (9.5% alkyl dimethyl ammonium chlorides) for 10 min (Guimarães et al., 2000).

Phenolic compounds

Another group of compounds commonly used for disinfecting abiotic surfaces are phenols. Phenols are effective in killing bacteria, fungi, and many viruses. The structure of phenol is as follows:



Phenolic compounds are less inactivated by organic soil than is hypochlorite (Tyler and Ayliffe, 1987).

Use of phenolic compounds to disinfect floors has been shown to decrease bacterial populations (Vesley and Michaelsen, 1964). Application of phenolic germicides to hospital floors reduced microbial populations in the environment for 6 months (Kundsin and Walter, 1961). Phenolic disinfectant was less effective than quats in inactivating 70 isolates of gram-negative bacteria from a hospital (Navajas et al., 1992). Phenolic disinfectants retain more activity in the presence of organic material than do iodine and chlorine-containing disinfectants (Bloomfield and Miller). Gram-negative bacteria, including members of the Enterobacteriaceae family isolated from hospitals, have been reported to be resistant to quats and phenolic disinfectants (Russell et al., 1986; Hammond et al., 1987).

Hydrogen peroxide

Hydrogen peroxide (H_2O_2) has been used as an antiseptic. However, it rapidly decomposes to water and oxygen upon contact with organic materials, resulting in no residual bactericidal effects (Lück and Jager, 1997). Hydrogen peroxide is declared to be Generally Recognized as Safe (GRAS) (21 CFR 184.1366) (U. S. FDA, 1986) and allowed to be used for packaging and surface sterilization in food processing plants (21 CFR 178.1010) (U. S. FDA, 1977). Hydrogen peroxide does not behave as an oxidizer but produces powerful oxidants, viz., hydroxyl radical, singlet oxygen, and superoxide radicals (Davidson and Branen, 1993). Of the these oxidants, the hydroxyl radical (HO•) plays an important role in toxicity to bacteria (Imlay and Linn, 1988). It increases lipid peroxidation and ion permeability of the cell membrane (Anzai et al., 1991).

Hydrogen peroxide has been shown to have bactericidal effects on various foodborne pathogens. Populations of planktonic cells of *L. monocytogenes* were reduced by 5.4 and 8.7 log CFU/ml after exposure to a 3.5% hydrogen peroxide solution for 5 and 10 min, respectively (Robbins et al., 2005). These researchers also showed a 4.14-log reduction of *L. monocytogenes* in biofilms on stainless steel chips treated with a 5% solution of hydrogen peroxide. *S.* Stanley freshly attached to the surface of cantaloupe showed ca. 3-log CFU/cm^2 decrease in population following treatment with a 5% solution of hydrogen peroxide (Ukuku and Sapers, 2001).

OBJECTIVES

The objectives of the research reported in this dissertation are as follows:

1. *Study survival characteristics of* E. sakazakii *in fresh-cut produce and unpasteurized juice as affected by storage temperature*

Outbreaks of *E. sakazakii* infections have been associated with infant formulas but documented presence of the organism in a wide range of ready-to-eat foods, including lettuce, tomatoes, bean sprouts, and other raw vegetables raises interest in knowing more about its behavior on produce. Although *E. sakazakii* has been reported to present on vegetables, thereby raising a potential public health concern, to date, outbreaks of infection linked to fresh produce have not been documented. Factors influencing survival and growth of *E. sakazakii* in produce have not been studied. Findings from this study will provide information on the level of potential risk of *E. sakazakii* infections that may be associated with fresh-cut produce and produce juice.

2. *Determine survival characteristics of* E. sakazakii *on the surface of whole produce as affected by storage temperature and its resistance to produce sanitizers, including chlorine, chlorine dioxide, and a peracetic acid-based sanitizer*

E. sakazakii has caused infections in neonates, infants, and elderly immunocompromised adults. Sources of the pathogen have most frequently implicated powdered infant formulas. Observations from this study will provide insights to predicting *E. sakazakii* survival on whole (uncut) produce and the efficacy of sanitizers in killing the bacterium, thereby enabling the development of effective treatments to reduce the risk of *E. sakazakii* infections in immunocompromised individuals.

3. *Investigate influences of temperature and nutrient availability on attachment and biofilm formation by* E. sakazakii *on surfaces of stainless steel and enteral feeding tubes*

E. sakazakii infections in neonates have been associated with consumption of reconstituted infant formula but the pathogen also has been isolated from a wide range of foods, including meat, dairy, cereal, and vegetable products. The pathogen has been reported to form biofilms on stainless steel, latex, polycarbonate, and silicon surfaces, but environmental conditions affecting attachment and biofilm formation on abiotic surfaces such as stainless steel and enteral feeding tubes have not been described. This study will provide insights to the attachment of and biofilm formation by *E. sakazakii* on stainless steel and enteral feeding tubes upon exposure to various nutrients and at various temperatures.

4. *Determine resistance of planktonic cells, spot-inoculated cells, and cells of* E. sakazakii *in biofilms to disinfectants commonly used in formula preparation areas and in food service, hospital, day-care, and home feeding settings*

Presence of *E. sakazakii* on the surface of utensils used for infant formula preparation has been documented to occur in a hospital setting where neonatal infections have been documented. However, the efficacy of sanitizers and disinfectants used in formula and meal preparation areas in hospitals, day-care centers, assisted living facilities, and home feeding environments in killing *E. sakazakii* has not been reported. Results gained from this study will provide information useful in assessing the efficacy of disinfectants used in these environments.

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CHAPTER 2

SURVIVAL AND GROWTH OF *ENTEROBACTER SAKAZAKII* ON FRESH-CUT FRUITS AND VEGETABLES AND IN UNPASTEURIZED JUICES AS AFFECTED BY STORAGE TEMPERATURE[1]

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ABSTRACT

Enterobacter sakazakii is an emerging foodborne pathogen that has caused illnesses and deaths of infants and elderly immunocompromised adults. Outbreaks of *E. sakazakii* infections have been associated with infant formulas but its documented presence in a wide range of ready-to-eat foods, including lettuce and other raw vegetables, raises interest in knowing more about its behavior in these environments. We did a study to determine the survival and growth characteristics of *E. sakazakii* on fresh-cut apple, cantaloupe, strawberry, watermelon, cabbage, carrot, cucumber, lettuce, and tomato and in juice prepared from these fruits and vegetables. Produce and juice were inoculated with *E. sakazakii* at populations of 2 - 3 log CFU/g and 1 - 2 log CFU/ml, respectively, and stored at 4, 12, or 25°C. Populations did not change or gradually decreased in fresh-cut produce and juice stored at 4°C but grew on fresh-cut apple, cantaloupe, watermelon, cucumber, and tomato and in all juices except apple, strawberry, cabbage, and tomato juice at 12°C. All fresh-cut fruits and vegetables except strawberry supported growth of *E. sakazakii* at 25°C. Growth occurred in all juices except apple, strawberry, and cabbage juice, followed by decreases in population to < 1 CFU/ml after 48 - 72 h, which coincided with decreases in pH and an increase in population of lactic acid bacteria. Increases in total counts occurred in all juices except strawberry juice stored at 25°C and apple and strawberry juice stored at 12°C. Total counts increased in cantaloupe, carrot, cucumber, and lettuce juice stored at 4°C. Populations of molds and yeasts increased in apple and tomato juice stored at 25°C but decreased to < 1 CFU/ml in cabbage, lettuce, and cucumber juice. Further characterization of the behavior of *E. sakazakii* on fresh produce and in unpasteurized juice as affected by commercial packaging and handling practices is warranted.

Key words: *Enterobacter sakazakii*, fruit, vegetable, apple, cantaloupe, strawberry, watermelon, cabbage, carrot, cucumber, lettuce, tomato, juice, lactic acid bacteria, molds, yeasts

INTRODUCTION

Enterobacter sakazakii is a gram-negative motile, rod-shaped, non-sporulating bacterium belonging to the family Enterobacteriaceae. The organism has been referred as a yellow-pigmented *Enterobacter cloacae* and was first implicated in a case of neonatal infection and death in 1958 (*51*). *E. sakazakii* was distinguished from *E. cloacae* as a separate species in 1980 (*17*) and has since been documented to cause meningitis (*5, 19, 29, 54*), sepsis (*48*), bacteremia (*13,* 37*, 40*), and necrotizing enterocolitis (*25, 53*) in preterm neonates. The mortality rate is 40 - 80% (*39*) and the oral infectious dose has been approximated to range from 10^3 CFU (*26*) to ≥ 10^8 CFU (*41*). Death of elderly, immunocompromised adults infected with *E. sakazakii* has also been reported (*6, 31*).

Approximately 60 cases of *E. sakazakii* infections have been documented internationally over the past 40 years (*26*). Outbreaks have been primarily associated with reconstituted powdered infant formula and milk powder (*3, 26, 39*). Muytjens et al. (*38*) detected *E. sakazakii* in 20 out of 141 powdered infant formulas originating from 13 countries. The organism has also been isolated from various other foods, food processing plants, and the environment (*26, 27*). Iversen and Forsythe (*26*) stated that soil, water, and vegetables may be principal sources of *E. sakazakii* contamination because the organism is not a part of the normal animal and human gut flora.

In recent years, the incidence of foodborne diseases associated with fresh produce has increased (*4, 9*). During the decade preceding 1999, approximately 12% of foodborne illnesses in the U. S. have been linked to fresh fruits and vegetables (*52*). Bacteria belonging to the family Enterobacteriaceae have caused or been associated with outbreaks of foodborne illnesses implicating unpasteurized juice and fresh fruits and vegetables. Examples of these outbreaks

include *Escherichia coli* O157:H7 infection linked to the consumption of lettuce (*24*) and apple cider (*7*), salmonellosis linked to tomatoes (*14*) and cantaloupe (*10*), and shigellosis linked to parsley (*8*).

Outbreaks of *E. sakazakii* infections associated with fresh produce have not been documented. However, Kandhai et al. (*27*) isolated *E. sakazakii* from 8 out of 9 food factories and from 5 out of 16 households, and the organism has been isolated from lettuce (*49*) and other vegetables (*32*). Because of its presence in the environment, there is a risk of contamination of fresh produce with *E. sakazakii*. Its ability to grow at temperatures as low as 5.5°C (*39*) raises concern about survival and growth on fresh-cut produce and in unpasteurized juice at storage temperatures used at retail and in food service and home environments.

The objective of this study was to determine the survival and growth characteristics of *E. sakazakii* on fresh-cut produce and unpasteurized fruit and vegetable juice stored at 4, 12, and 25°C. Populations of mesophilic aerobic bacteria (total counts), lactic acid bacteria, and molds and yeasts in juice inoculated with *E. sakazakii* were also monitored.

MATERIALS AND METHODS

Bacterial strains and preparation of inocula. Five strains of *E. sakazakii* (4923, A9002, 1625, 8397, and LCDC 674) isolated from clinical specimens were grown in tryptic soy broth (TSB) at 37°C for 24 h. Nalidixic acid-adapted cells of each strain were isolated by surface plating 0.1 ml of culture on violet red bile glucose agar (Becton, Dickinson and Company, Sparks, Md) supplemented with nalidixic acid (50 μg/liter) and pyruvate (0.1%) (VRBGNP) and incubating plates at 37°C for 24 h. Cells from colonies of each strain were confirmed to be *E. sakazakii* using the API 20E assay (bioMérieux Vitek, Inc., Hazelwood, Mo.).

Nalidixic acid-adapted cells were cultured in TSB supplemented with 1% glucose and nalidixic acid (50 μg/liter) (TSBGN) at 37°C for 24 h. After three consecutive transfers of ca. 10 μl of culture into TSBGN at 24-h intervals, 2 ml of culture of each strain were combined to give 10 ml of a five-strain mixture containing approximately equal populations of each strain and centrifuged (1,250 × *g*, 15 min, 21°C). After centrifugation, the supernatant was decanted and cells in the pellet were resuspended in 10 ml of sterile distilled water.

Preparation of fresh-cut produce. Apples (*Malus domestica* Borkh. cv. Delicious), cantaloupes (*Cucumis melo* var.*reticulatis*), strawberries (*Fragaria virginiana* Duschesne), watermelon (*Citrullus lanatus* Mansf.), cabbage (*Brassica oeracea* var. *capitata*), carrots (*Daucus carota* ssp. *sativus* Acreng), cucumbers (*Cucumis sativus* L. var. *sativus*), iceberg lettuce (*Lactuca sativa* L.), and tomatoes (*Lycopersicon esculentum* Mill, Roma cv.) were purchased at a grocery store in Griffin, Ga. 2 days prior to use in experiments. For the purpose of this study, apples, cantaloupe, strawberries, and watermelon are referred to fruits and cabbage, carrots, cucumbers, lettuce, and tomatoes are referred to vegetables. The edible portions of apples, cantaloupes, watermelon, carrots, cucumbers, and tomatoes were cut into pieces measuring ca. 1 × 1 × 1 cm. Strawberries were sliced into pieces approximately 0.5 cm thick, and lettuce and cabbage were cut into pieces ca. 1 × 1 cm. Each sample (25 g) was placed in a stomacher 400 bag (Seward Medical, London, U.K.) and stored at 4°C overnight before inoculating with *E. sakazakii*.

Preparation of produce juice. The same types of produce used in the fresh-cut produce study were used to make juice. Cucumbers and carrots were peeled, stems were removed from strawberries, and only the edible portions of apples, cantaloupe, watermelon, cabbage, lettuce, and tomatoes were used to prepare juice. Each fruit or vegetable at 4°C was homogenized in a

juice blender (ETL Testing Laboratories Inc., Cortland, N.Y.). Juice (300 ml) was placed in sterile 500-ml bottles and inoculated with *E. sakazakii* within 1 h after preparation.

Inoculation and storage conditions. The five-strain *E. sakazakii* cell suspension was serially diluted in sterile distilled water to give suspensions containing 10^4 - 10^5 CFU/ml and 10^3 - 10^4 CFU/ml for use in inoculating fresh-cut produce and produce juice, respectively. Each fresh-cut produce sample (25 g) was inoculated with 0.25 ml of the five-strain mixture of *E. sakazakii* to give 10^2 - 10^3 CFU/g. The inoculated samples were mixed thoroughly by gently shaking and rolling. Fruit and vegetable juice (300 ml) was inoculated with 3 ml of the five-strain mixture to give 10^1 - 10^2 CFU/ml. After inoculation, all samples of fresh-cut produce and juice were stored at 4, 12, or 25°C for up to 7 days.

Measurement of pH and soluble solids content. At various storage times, the pH and soluble solids content (°Brix) of produce juice were measured using a basic pH meter (Denver Instrument Co., Arvada, Colo.) and ABBE-3L refractometer (Spectronic Instruments Inc., Rochester, N.Y.), respectively.

Microbiological analyses of fresh-cut produce and produce juice. Populations of *E. sakazakii* were determined on fresh-cut produce stored at 4, 12, and 25°C for up to 144 h (6 days). Fresh-cut produce (25 g) was combined with 225 ml of Enterobacteriaceae enrichment broth (Becton, Dickinson and Company) supplemented with nalidixic acid (50 μg/liter) and pyruvate (0.1%) (EENP), pummeled at normal speed using a Stomacher 400 (Seward Medical) for 1 min, serially diluted in sterile 0.1% peptone water, and surface plated on VRBGNP. Plates were incubated at 37°C for 24 h before presumptive *E. sakazakii* colonies were counted. When presumptive colonies were not detected on VRBGNP, the mixture of fresh-cut produce and EENP broth incubated at 37°C for 24 h were streaked on VRBGNP. Plates were incubated at

37°C for 24 h before examining for presumptive *E. sakazakii* colonies. The presumptive colonies were confirmed using the API 20E assay.

Produce juice (5 ml) was enriched in EENP broth (45 ml). Populations of *E. sakazakii*, mesophilic aerobic bacteria (total counts), lactic acid bacteria, and molds and yeasts in juice were determined by surface plating undiluted juice and juice serially diluted in sterile 0.1% peptone water on VRBGNP agar, tryptic soy agar (TSA) (Becton, Dickinson and Company), deMan Rogosa Sharpe (MRS) agar (Becton, Dickinson and Company) with an MRS agar overlay applied, and dichloran rose bengal chloramphenicol (DRBC) agar (Becton, Dickinson and Company), respectively. VRBGNP plates were incubated at 37°C for 24 h before presumptive *E. sakazakii* colonies were counted. When presumptive colonies were not detected on VRBGNP, the juice/EENP mixture incubated at 37°C for 24 h was streaked on VRBGNP. Plates were incubated at 37°C for 24 h before examining for presumptive *E. sakazakii* colonies. Presumptive colonies were confirmed with the API 20E assay. TSA, MRS agar, and DRBC agar plates were incubated at 37°C for 1 day, 30°C for 3 days, and 25°C for 5 days, respectively, before colonies were counted.

Statistical analysis. All experiments were performed in triplicate using produce from three different lots. Data were analyzed using the general linear model of the Statistical Analysis Systems procedure (SAS; SAS Institute, Cary, N.C.). Fisher's Least Significant Difference (LSD) test was used to determine if numbers of *E. sakazakii*, total counts, lactic acid bacteria, and molds and yeasts detected in each type of fresh-cut produce and produce juice as well as pH and °Brix values were significantly affected by storage time and temperature. Significant differences are presented at a 95% confidence level ($P \leq 0.05$).

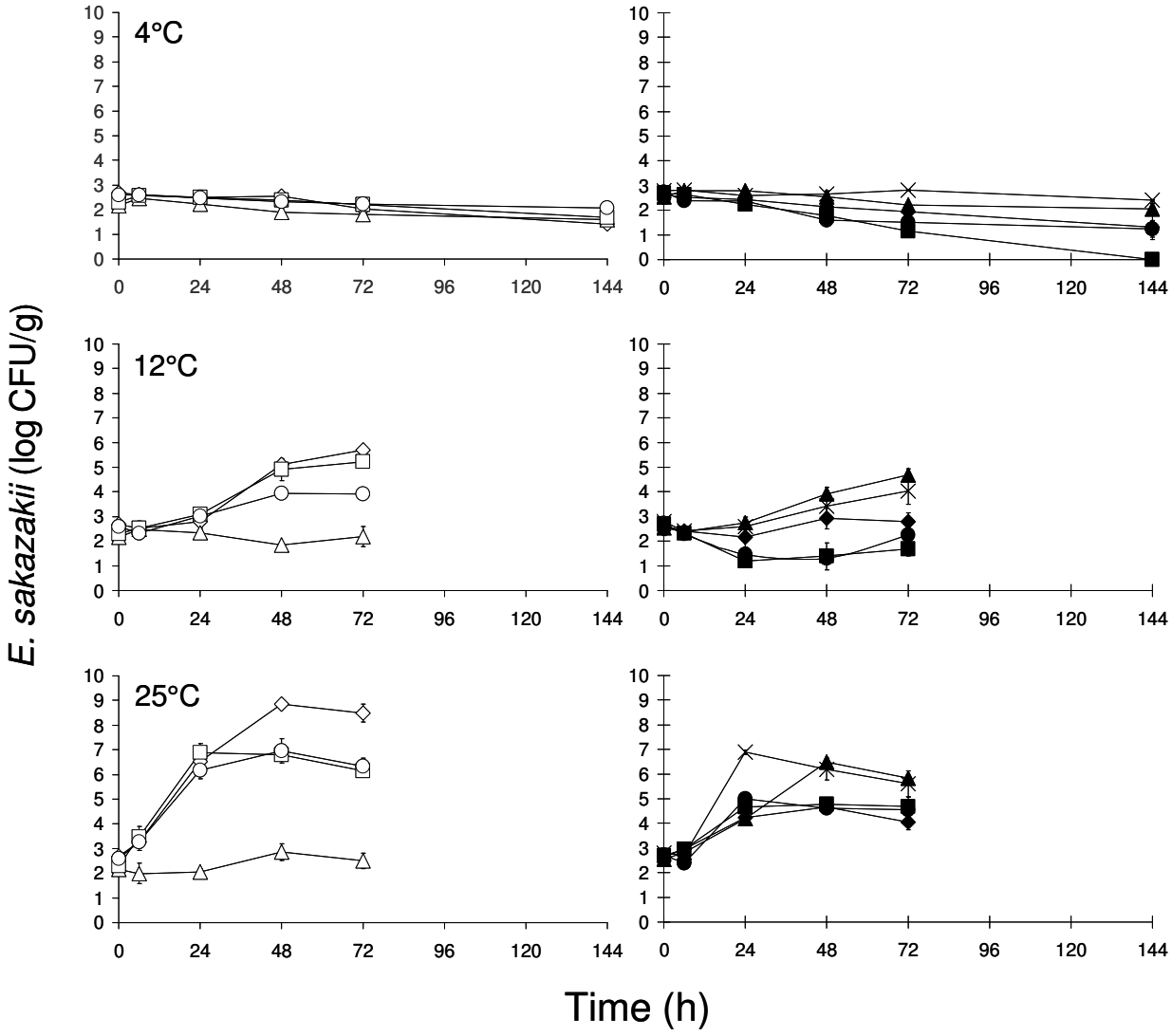
RESULTS

Behavior of *E. sakazakii* on fresh-cut produce. Figure 2-1 shows populations of *E. sakazakii* on inoculated fresh-cut apple, cantaloupe, strawberry, watermelon, cabbage, carrot, cucumber, lettuce, and tomato stored at 4, 12, or 25°C. Survival and growth of the organism was influenced by produce type as well as incubation temperature. At 4°C, populations of *E. sakazakii* on fresh-cut fruits and vegetables did not change or gradually decreased as storage time progressed. Populations of *E. sakazakii* on fresh-cut cabbage, carrot, and lettuce decreased significantly within 48 h. After 144 h at 4°C, *E. sakazakii* was not recovered from fresh-cut carrot but only a slight reduction (ca. 0.5 log CFU/g) in population occurred on apple, cantaloupe, strawberry, watermelon, cucumber, and tomato.

With the exception of fresh-cut strawberry, significant increases in populations of *E. sakazakii* occurred within 24 - 48 h on fruits stored at 12°C (Figure 2-1). Produce stored for more than 72 h at 12°C or 25°C were not analyzed for *E. sakazakii* because produce was visually spoiled and not considered edible. After 72 h at 12°C, populations of *E. sakazakii* on fresh-cut apple, cantaloupe, and watermelon were 3.9, 5.2, and 5.7 log CFU/g, respectively. *E. sakazakii* did not grow on fresh-cut strawberries stored at 4, 12, or 25°C. Populations of *E. sakazakii* on fresh-cut cabbage and carrot stored at 12°C decreased significantly within 6 and 24 h, respectively, but did not change significantly on lettuce stored for 72 h. Populations on cucumber and tomato stored at 12°C increased significantly between 24 and 48 h and reached 4.7 and 4.0 log CFU/g, respectively, at 72 h of storage. The population of *E. sakazakii* on carrot was the lowest (1.7 log CFU/g) among test fruits and vegetables after storage at 12°C for 72 h.

At 25°C, all fresh-cut fruits and vegetables except strawberry supported growth of *E. sakazakii* (Figure 2-1). Lag phases were much shorter compared those on produce stored at

Figure 2-1. Populations of *E. sakazakii* recovered from fresh-cut apples (○), cantaloupe (□), strawberries (△), watermelon (◇), cabbage (●), carrots (■), cucumbers (▲), lettuce (◆), and tomatoes (×) stored at 4, 12 or 25°C for up to 6 days.


4°C
12°C
25°C
E. sakazakii (log CFU/g)
Time (h)

12°C. Populations of *E. sakazakii* on apple, cantaloupe, and watermelon increased significantly within 6 h and reached 6.2, 6.9, and 6.5 log CFU/g, respectively, within 24 h. Populations on fresh-cut apple and cantaloupe remained unchanged during storage for 48 h but continued to increase significantly on watermelon to reach 8.9 log CFU/g at 48 h, the highest population achieved on fresh-cut fruits. The population of *E. sakazakii* on fresh-cut tomato stored at 25°C reached 6.9 log CFU/g within 24 h, whereas populations on other fresh-cut vegetables except cucumber reached a plateau of 4.2 - 5.0 log CFU/g. The population on cucumber reached 6.7 log CFU/g within 48 h at 25°C. The population on fresh-cut tomato decreased significantly from 6.9 log CFU/g at 24 h to 5.6 log CFU/g at 72 h. Overall, among the vegetables stored at 25°C, *E. sakazakii* reached the highest populations on tomato and cucumber.

Changes in pH of fresh-cut produce. The pH values of the cut surfaces of fresh-cut produce stored at 4, 12, or 25°C are listed in Table 2-1. Cantaloupe had the highest initial pH (6.8 - 7.1) and strawberry had the lowest pH (3.8 - 3.9) among test fruits. During storage, changes in pH values of all cut fruit surfaces except strawberry occurred, depending on the incubation temperature. The pH values of fresh-cut cantaloupe and watermelon stored at 4°C remained constant for up to 72 - 144 h but gradually decreased after storage for 24 - 48 h at 12°C and 6 - 24 h at 25°C. The pH of strawberry remained constant at 4, 12, and 25°C during storage for 72 h.

With exception of tomato, the pH values on the surfaces of fresh-cut vegetables were generally higher than those of fruits. This is attributed in part to lower amounts of organic acids and fermentable sugars in vegetables compared to fruits. The pH values of fresh-cut cabbage, carrot, lettuce, and tomato gradually decreased during storage, regardless of temperature. The pH of cucumber stored at 4 or 12°C, however, did not change significantly. Reductions in pH of

Table 2-1. *pH values of fresh-cut produce surfaces as affected by storage time and temperature.*

Produce	Temp (°C)	pH [a]					
		0 h	6 h	24 h	48 h	72 h	144 h
Fruit							
Apple	4	A 5.7 ab	A 6.0 a	A 6.0 a	A 5.9 a	A 5.1 b	5.7 ab
	12	A 5.8 ab	A 5.2 ab	A 5.8 a	A 5.5 ab	A 5.4 b	nd[b]
	25	A 5.6 ab	A 5.4 ab	A 5.6 a	B 4.7 c	A 4.8 bc	nd
Cantaloupe	4	A 6.8 a	A 6.5 ab	A 6.8 a	A 6.7 a	A 6.3 ab	5.4 b
	12	A 7.0 a	A 6.8 a	B 6.1 b	B 5.1 c	B 4.8 c	nd
	25	A 7.1 a	A 7.0 a	C 4.8 b	B 4.4 bc	B 4.3 c	nd
Strawberry	4	A 3.8 a	A 4.0 a	A 4.3 a	A 4.4 a	A 4.2 a	4.7 a
	12	A 3.9 a	A 3.6 a	A 4.4 a	A 3.7 a	A 3.8 a	nd
	25	A 3.8 a	A 3.8 a	A 4.4 a	A 3.7 a	A 4.0 a	nd
Watermelon	4	A 5.4 a	A 5.5 a	AB 5.4 a	A 5.3 a	A 5.6 a	4.4 a
	12	A 5.3 a	A 5.2 a	A 6.1 a	B 4.5 b	B 4.0 b	nd
	25	A 5.5 a	A 5.5 a	B 5.8 b	C 4.6 c	B 4.2 c	nd
Vegetable							
Cabbage	4	A 7.1 a	A 5.7 bc	A 5.4 c	A 5.7 bc	A 6.3 b	5.8 c
	12	A 6.9 a	A 5.4 b	A 5.6 b	A 5.4 b	A 6.6 a	nd
	25	A 7.1 a	A 5.7 b	A 5.9 b	A 5.8 b	A 6.1 b	nd
Carrot	4	A 7.1 a	A 5.7 c	A 5.6 c	A 6.2 bc	A 6.6 ab	5.6 c
	12	A 7.1 a	A 5.6 cd	A 5.2 d	A 5.9 c	A 6.4 b	nd
	25	A 7.1 a	A 5.3 c	A 5.6 c	A 6.1 b	A 6.0 bc	nd
Cucumber	4	A 6.0 a	A 5.9 a	A 6.0 a	A 5.8 a	A 6.1 a	5.9 a
	12	A 5.9 a	A 5.8 a	AB 5.8 a	A 5.7 a	A 5.4 a	nd
	25	A 5.9 a	A 6.0 a	B 4.8 b	B 4.3 c	B 4.1 c	nd
Lettuce	4	A 7.1 a	A 6.1 b	A 5.8 b	A 6.1 b	A 6.3 b	6.2 b
	12	A 7.1 a	A 6.0 b	A 5.8 b	A 5.6 b	A 6.1 b	nd
	25	A 7.1 a	A 5.9 b	A 5.5 b	A 5.6 b	A 6.1 b	nd
Tomato	4	A 4.9 a	A 4.8 a	A 4.8 a	A 4.8 a	A 4.9 a	4.5 b
	12	A 4.9 a	A 4.8 ab	A 4.7 b	A 4.7 b	A 4.5 c	nd
	25	A 4.9 a	A 4.7 a	A 4.5 b	A 4.1 c	A 4.1 c	nd

[a] Within each type of produce and storage time, mean values that are not preceded by the same capital letter are significantly different ($P \leq 0.05$). Within the same row, mean values that are not followed by the same lower case letter are significantly different ($P \leq 0.05$).

[b] Not determined.

the surfaces of all fresh-cut vegetables except cucumber were more rapid than those of fresh-cut fruits stored at 4, 12, or 25°C. At 12 and 25°C, significant reductions in pH of vegetables were detected within 6 - 24 h.

Behavior of *E. sakazakii* in produce juice. Shown in Figure 2-2 are populations of *E. sakazakii* recovered from inoculated juices stored at 4, 12, or 25°C for up to 168 h (7 days). Initial populations (1.2 - 1.6 log CFU/ml) decreased only slightly in cantaloupe, watermelon, carrot, cucumber, lettuce, and tomato juice stored at 4°C for 7 days. *E. sakazakii* was not detected (< 1 CFU/ml) in apple juice stored at 4°C on day 7, and only 0.3 and 0.2 log CFU/ml, were detected in strawberry juice and cabbage juice, respectively.

Populations of *E. sakazakii* in cantaloupe juice and watermelon juice stored at 12°C increased to 3.1 and 4.5 log CFU/ml, respectively, within 72 - 96 h and remained constant for an additional 72 - 96 h. However, the number of *E. sakazakii* recovered from apple, strawberry, cabbage, carrot, cucumber, lettuce, and tomato juice stored at 12°C decreased to an undetectable level (< 1 CFU/ml) by day 7.

All fruit and vegetable juices except apple, strawberry, and cabbage juice stored at 25°C supported an exponential increase in population of *E. sakazakii* within 24 - 48 h, which was followed by decreases to undetectable levels (< 1CFU/ml) by 48 - 72 h. The highest numbers of *E. sakazakii* were detected in watermelon (8.1 log CFU/ml) and carrot (7.3 log CFU/ml) juice. This occurred 30 h after inoculation. The population of *E. sakazakii* increased to 1.7 log CFU/ml of apple juice within 48 h at 25°C and decreased to an undetectable level (< 1 CFU/ml) at 168 h. *E. sakazakii* did not grow in strawberry and cabbage juice at 25°C but survived for up to 36 and 48 h, respectively. With the exception of apple juice, *E. sakazakii* was not detected by enrichment of juices stored at 25°C for 72 h or longer (Table 2-2). The pathogen was detected

Figure 2-2. Populations of *E. sakazakii* recovered from apple (○), cantaloupe (□), strawberry (△), watermelon (◇), cabbage (●), carrot (■), cucumber (▲), lettuce (◆), and tomato (×) juice stored at 4, 12 or 25°C for 7 days.

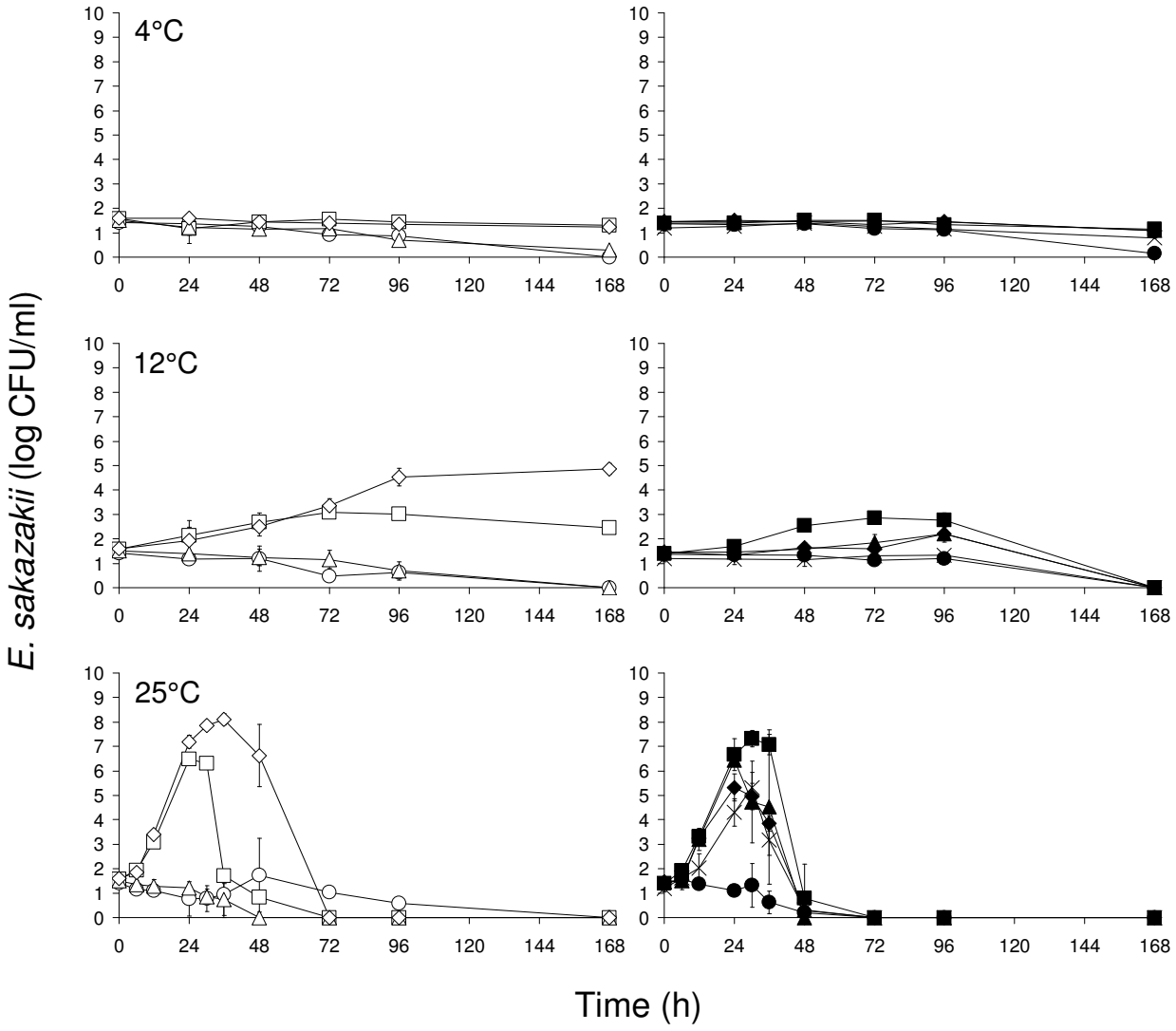

4°C
12°C
25°C
E. sakazakii (log CFU/ml)
Time (h)
0
24
48
72
96
120
144
168
1
2
3
4
5
6
7
8
9
10

Table 2-2. *Populations of* E. sakazakii *recovered from fruit and vegetable juice stored at 25 ℃ for 7 days.*

	Population (log CFU/ml)[a]					
	0 h	24 h	48 h	72 h	96 h	168 h
Fruit						
Apple	1.4 a	0.8 a	1.7 a	1.0 a	0.6 a	1/3[b]
Cantaloupe	1.6 c	6.5 a	2.5 b	0/3	0/3	0/3
Strawberry	1.5 a	1.2 b	1/3	0/3	0/3	0/3
Watermelon	1.6 b	7.2 a	6.6 a	0/3	0/3	0/3
Vegetable						
Cabbage	1.4 a	1.1 a	0.2 b	0/3	0/3	0/3
Carrot	1.4 b	6.7 a	0.8 b	0/3	0/3	0/3
Cucumber	1.5 b	6.4 a	1/3	0/3	0/3	0/3
Lettuce	1.5 b	5.3 a	0.3 c	0/3	0/3	0/3
Tomato	1.2 b	4.3 a	0.3 b	0/3	0/3	0/3

[a] Within the same row, mean values that are not followed by the same letter are significantly different ($P \leq 0.05$).

[b] Samples (5 ml) were enriched with EENP broth (45 ml) and incubated at 37 ℃ for 24 h, followed by streaking on VRBGNP to determine the presence of *E. sakazakii*; numbers indicate the number of samples positive for *E. sakazakii* out of three samples analyzed in three replicate trials.

by enrichment of apple juice stored for 7 days.

Total counts in juice. Total counts for fruit and vegetable juices inoculated with *E. sakazakii* and stored at 4, 12, or 25°C for up to 168 h were monitored. Higher initial counts were detected in vegetable juice (4.3 - 5.5 log CFU/ml) than in fruit juice (2.0 - 3.5 log CFU/ml) (Figure 2-3). Total counts in cantaloupe, carrot, cucumber, and lettuce juice stored at 4°C for 7 days increased significantly to 6.6, 7.7, 7.4, and 7.3 log CFU/ml, respectively. Apple, strawberry, cabbage, and tomato juice stored at 4°C did not support significant increases in total counts throughout the 7-day storage period.

At 12°C, total counts in cantaloupe and watermelon juice increased significantly after 24 - 48 h; counts of 9.0 and 8.8 log CFU/ml, respectively, were observed after 7 days. Total counts gradually increased for 7 days in vegetable juices stored at 12°C. Lettuce juice contained the highest initial (5.4 log CFU/ml) and final (8.7 log CFU/ml) total counts among the vegetable juices tested.

Dramatic increases in total counts were observed in all juices stored at 25°C except strawberry juice. Total counts to 7.2 - 8.7 log CFU/ml were detected within 24 h in all juices except apple, strawberry, and cabbage juice. Total counts in cucumber and lettuce juice decreased between 4 and 7 days to similar levels detected on day 0. Total counts increased to 5.0 log CFU/ml of apple juice within 36 - 48 h at 25°C and reached to 8.4 log CFU/ml after 7 days. Strawberry juice did not support increases in total counts at 25°C.

Lactic acid bacteria in juice. There were no significant changes in populations of lactic acid bacteria in apple, strawberry, cabbage, and tomato juice inoculated with *E. sakazakii* and held at 4°C for 7 days (Figure 2-4). Lactic acid bacteria grew to populations of 5.8 and 5.6 log CFU/ml in cantaloupe and lettuce juice, respectively, but more slowly in watermelon (3.0 log

Figure 2-3. Populations of mesophilic aerobic bacteria (total counts) recovered from apple (○), cantaloupe (□), strawberry (△), watermelon (◇), cabbage (●), carrot (■), cucumber (▲), lettuce (◆), and tomato (×) juice inoculated with *E. sakazakii* and stored at 4, 12 or 25°C for 7 days.

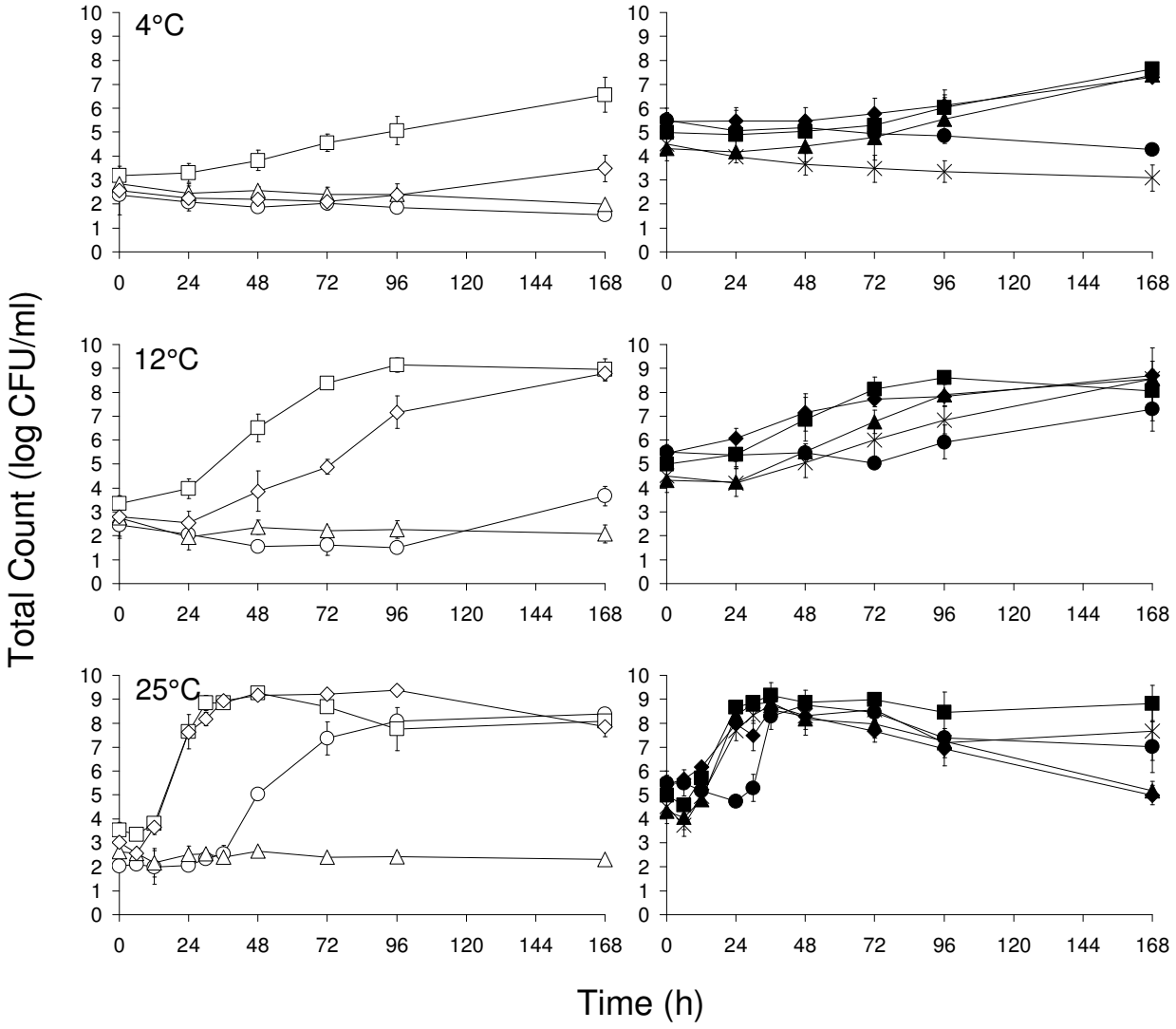
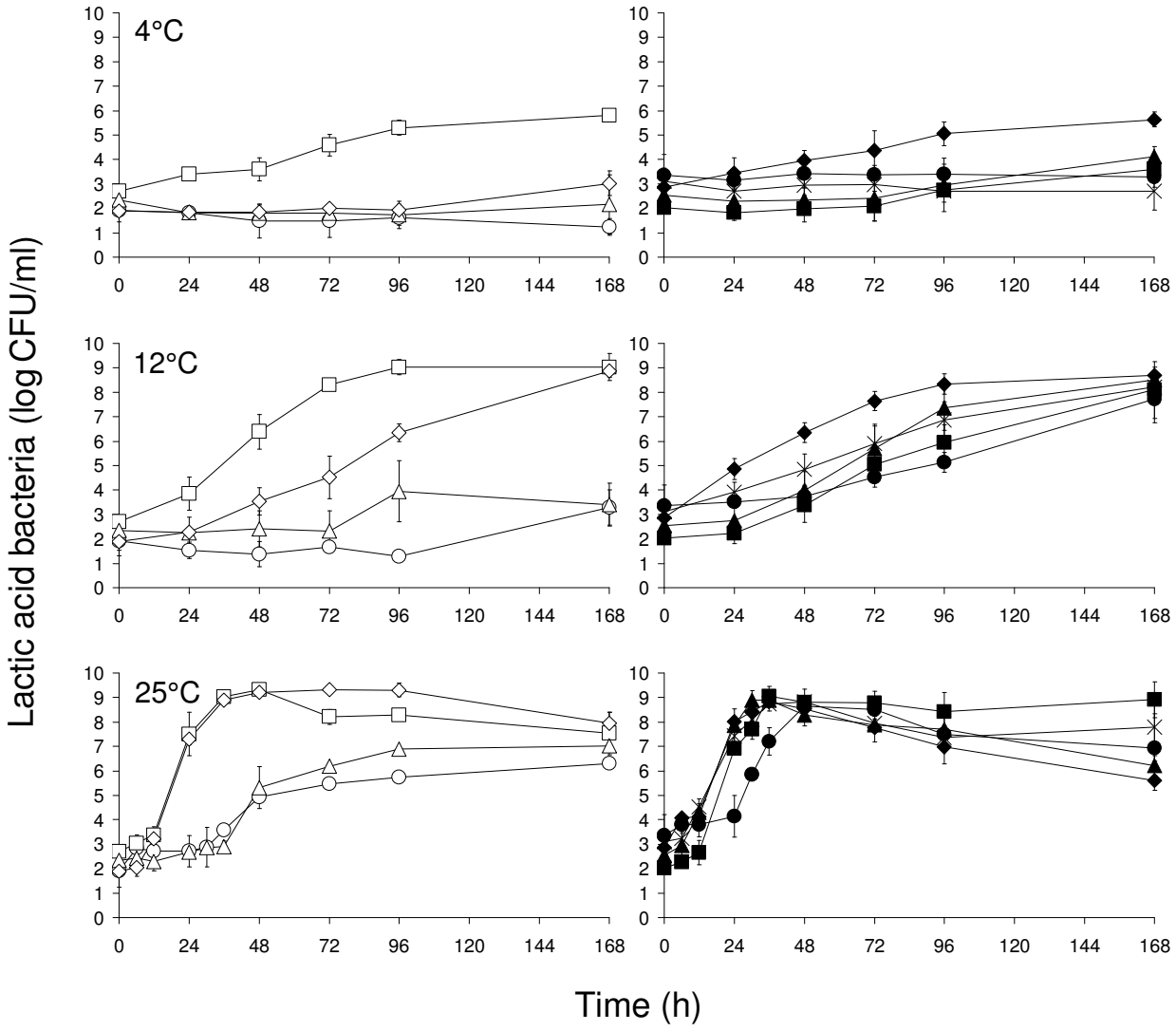

4°C
12°C
25°C
Total Count (log CFU/ml)
Time (h)
0
24
48
72
96
120
144
168
1
2
3
4
5
6
7
8
9
10

Figure 2-4. Populations of lactic acid bacteria recovered from apple (○), cantaloupe (□), strawberry (△), watermelon (◇), cabbage (●), carrot (■), cucumber (▲), lettuce (◆), and tomato (×) juice inoculated with *E. sakazakii* and stored at 4, 12 or 25°C for 7 days.


4°C
12°C
25°C
Lactic acid bacteria (log CFU/ml)
Time (h)
0 24 48 72 96 120 144 168
0 1 2 3 4 5 6 7 8 9 10

CFU/ml), carrot (3.6 log CFU/ml), and cucumber (4.1 log CFU/ml) juice stored at 4°C for 7 days.

All fruit and vegetable juices except apple and strawberry juice supported growth of lactic acid bacteria at 12°C. Populations of 7.7 - 9.1 log CFU/ml of all juices except apple and strawberry juice were reached within 7 days.

All juices supported growth of lactic acid bacteria at 25°C. Similar maximum populations, compared to those in juice stored at 12°C, occurred earlier in the 7-day storage period, i. e., within 24 - 48 h at 25°C compared to 96 - 168 h at 12°C.

Molds and yeasts in juice. Changes in populations of molds and yeasts in juice inoculated with *E. sakazakii* and stored at 4, 12, and 25°C were monitored (Figure 2-5). The initial populations ranged from 2.3 to 5.1 log CFU/ml. Molds and yeasts did not grow but survived in all juices stored for 7 days at 4 °C and in cantaloupe, strawberry, watermelon, carrot, and cucumber juice at 12°C.

Molds and yeasts were not detected (< 1 CFU/ml) in cabbage juice and lettuce juice stored at 12°C for 7 days. Apple and tomato juice stored at 12 and 25 °C supported the growth of molds and yeasts. Populations in apple juice increased to 4.2 and 4.6 log CFU/ml at 12 and 25°C, respectively, by the end of the 7-day storage period. There was a 3.8-log CFU/ml increase of mold and yeast population in tomato juice stored at 25°C. Populations in cantaloupe, watermelon, cabbage, carrot, cucumber, and lettuce juice were suppressed at 25°C. The populations in cabbage, cucumber, and lettuce juice decreased to undetectable levels (< 1 CFU/ml).

Changes in pH and soluble solids content of produce juice. pH values of fruit and vegetable juices inoculated with *E. sakazakii* were measured during the 7-day storage period at 4, 12, and 25°C (Table 2-3). With the exceptions of carrot juice and lettuce juice, there were no

Figure 2-5. Populations of molds or yeasts recovered from apples (○), cantaloupe (□), strawberries (△), watermelon (◇), cabbage (●), carrots (■), cucumbers (▲), lettuce (◆), and tomatoes (×) juice inoculated with *E. sakazakii* and stored at 4, 12 or 25°C for 7 days.

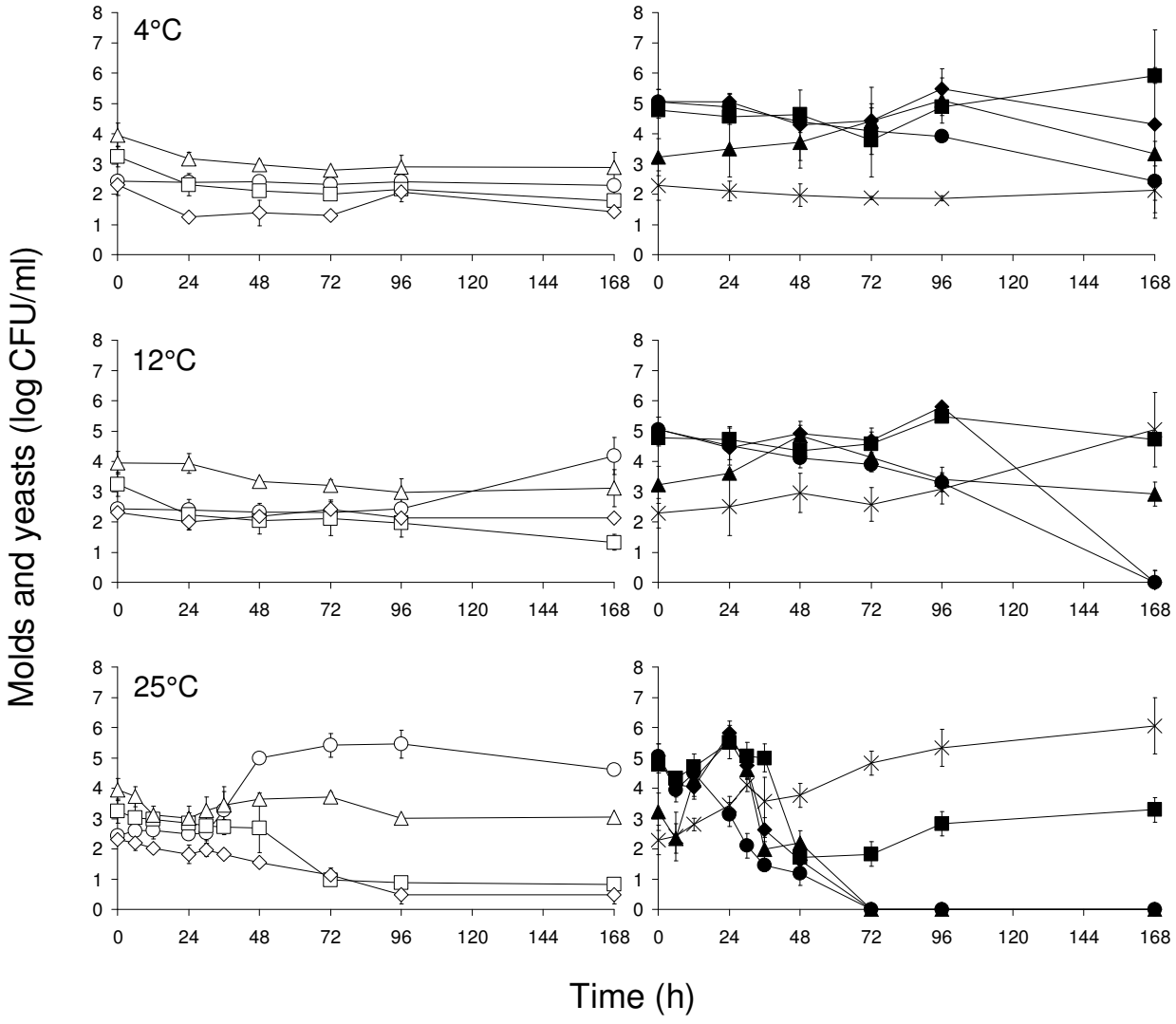

4°C
12°C
25°C
Molds and yeasts (log CFU/ml)
Time (h)
0
24
48
72
96
120
144
168
1
2
3
4
5
6
7
8

Table 2-3. *pH values of fruit and vegetable juice as affected by storage time and temperature.*

Juice	Temp (°C)	pH [a]					
		0 h	24 h	48 h	72 h	96 h	168 h
Fruit							
Apple	4	A 3.9 a	A 3.9 a	A 3.9 a	A 3.9 a	A 3.9 a	A 3.9 a
	12	A 3.9 a	A 3.9 a	A 3.9 a	A 3.9 a	A 3.9 a	A 3.9 a
	25	A 4.0 ab	A 3.9 b	A 3.9 b	A 4.1 ab	A 4.1 a	A 3.9 b
Cantaloupe	4	A 6.8 a	A 6.7 a	A 6.6 a	A 6.6 a	A 6.7 a	A 6.5 a
	12	A 6.8 a	A 6.7 a	A 6.7 a	B 6.1 b	B 4.6 c	B 4.3 c
	25	A 6.8 a	A 6.6 a	B 4.2 b	C 4.2 b	C 4.2 b	B 4.1 b
Strawberry	4	A 3.7 a	A 3.6 a	A 3.7 a	A 3.7 a	A 3.7 a	A 3.6 a
	12	A 3.6 a	A 3.6 a	A 3.6 a	A 3.6 a	A 3.6 a	A 3.5 a
	25	A 3.5 a	A 3.4 a	A 3.4 a	A 3.4 a	A 3.4 a	A 3.5 a
Watermelon	4	A 5.0 a	A 5.3 a	A 5.3 a	A 5.3 a	A 5.3 a	A 5.3 a
	12	A 5.0 a	A 5.3 a	A 5.3 a	A 5.3 a	A 5.2 a	B 4.3 b
	25	A 5.1 a	A 5.3 a	B 4.0 b	B 3.8 b	B 3.7 b	C 3.7 b
Vegetable							
Cabbage	4	A 6.3 a	A 6.3 a	A 6.4 a	A 6.5 a	A 6.5 a	A 6.5 a
	12	A 6.3 ab	A 6.4 a	A 6.5 a	A 6.5 a	A 6.4 a	B 5.6 b
	25	A 6.3 a	A 6.9 a	B 5.0 b	B 3.9 b	B 3.9 b	C 3.9 b
Carrot	4	A 6.4 a	A 6.4 a	A 6.4 a	A 6.4 a	A 6.5 a	A 5.9 b
	12	A 6.4 a	A 6.3 a	A 6.2 a	B 5.9 b	B 5.7 b	B 4.6 c
	25	A 6.4 a	B 5.7 b	B 4.5 c	C 4.3 d	C 4.2 d	B 4.2 d
Cucumber	4	A 5.5 a	A 5.5 a	A 5.5 a	A 5.6 a	A 5.6 a	A 5.7 a
	12	A 5.5 a	A 5.5 a	A 5.7 a	A 5.5 a	A 5.3 a	B 4.2 b
	25	A 5.5 a	A 5.1 a	B 4.0 b	B 3.9 b	B 3.9 b	B 4.0 b
Lettuce	4	A 6.1 a	A 6.0 a	A 6.0 a	A 6.0 a	A 6.0 a	A 5.6 b
	12	A 6.1 a	A 6.0 a	A 5.8 ab	A 5.4 bc	AB 5.3 c	B 4.1 d
	25	A 6.1 a	B 5.2 ab	B 4.4 bc	B 4.4 bc	B 4.4 bc	B 4.0 c
Tomato	4	A 4.4 a	A 4.5 a	A 4.5 a	A 4.6 a	A 4.6 a	A 4.5 a
	12	A 4.4 a	A 4.5 a	A 4.5 a	A 4.6 a	A 4.6 a	AB 4.4 a
	25	A 4.4 a	A 4.5 a	B 3.9 b	B 3.9 b	B 4.0 b	B 3.9 b

[a] Within each juice and storage time, mean values that are not preceded by the same capital letter are significantly different ($P \leq 0.05$). Within the same row, mean values that are not followed by the same lower case letter are significantly different ($P \leq 0.05$).

significant changes in the pH of juices stored at 4°C. A significant decrease in pH of carrot and lettuce juice occurred between 96 and 168 h. At 12°C, the pH of cantaloupe, carrot, and lettuce juice decreased significantly to 6.1, 5.9, and 5.4, respectively, within 72 h; the pH of watermelon, cabbage, and cucumber juice decreased significantly to 4.3, 5.6, and 4.2, respectively, within 168 h. At 25°C, significant decreases in the pH of all juices except apple and strawberry juice occurred within 24 - 48 h. The pH of all juices stored at 25°C for 168 h was 3.5 - 4.2. The decrease of pH coincided with an increase in populations of lactic acid bacteria and decrease in population of *E. sakazakii*.

°Brix values of fruit and vegetable juices inoculated with *E. sakazakii* were monitored for 168 h. No significant changes of soluble solids content of juices were observed at 4 or 12°C (Table 2-4). At 25°C, the soluble solids content of strawberry, carrot, cucumber, lettuce, and tomato juice significantly decreased from initial values of 8.7, 8.8, 3.0, 3.8, and 4.0 to 3.9, 7.1, 2.5, 3.2, and 3.2, respectively, for 7 days. The other juices did not show changes in °Brix at 25°C during the 168-h storage period. Even though there was no significant change in soluble solids content of apple, watermelon, and cabbage juice stored at 25°C, a decreasing trend was evident.

DISCUSSION

Results show that *E. sakazakii* did not grow but survived or died on fresh-cut fruits and vegetables stored at 4°C for up to 6 days. Except for fresh-cut strawberry and tomato, the initial pH of the fresh-cut produce would not be expected to kill or prevent the growth of *E. sakazakii*. Because fresh-cut cantaloupe had the highest initial pH (6.8 - 7.1) among test fruits, it was expected to support the growth of *E. sakazakii*. Del Rosario and Beuchat (*15*) monitored the fate

Table 2-4. *°Brix values of fruit and vegetable juice as affected by storage time and temperature.*

Juice	Temp (°C)	pH [a]					
		0 h	24 h	48 h	72 h	96 h	168 h
Fruit							
Apple	4	A 13.5 a	A 13.8 a	A 14.0 a	A 14.0 a	A 13.7 a	A 13.8 a
	12	A 13.1 a	A 13.1 a	A 13.1 a	A 13.1 a	A 12.7 a	A 13.1 a
	25	A 13.1 a	A 13.2 a	A 13.4 a	A 13.2 a	A 13.0 a	A 12.4 a
Cantaloupe	4	A 8.6 a	A 9.0 a	A 9.2 a	A 9.0 a	A 9.0 a	A 9.3 a
	12	A 8.2 a	A 8.4 a	A 8.5 a	A 8.4 a	A 8.1 a	AB 8.0 a
	25	A 8.2 a	A 9.7 a	A 9.3 a	A 9.0 a	A 9.1 a	B 8.9 a
Strawberry	4	A 9.1 b	A 9.4 ab	A 9.7 a	A 9.7 ab	A 9.6 ab	A 9.7 a
	12	A 8.7 a	A 9.2 a	A 9.6 a	A 9.2 a	A 9.4 a	A 9.0 a
	25	A 8.7 a	A 9.2 a	A 8.6 a	B 5.5 b	B 3.8 c	B 3.9 c
Watermelon	4	A 9.1 a	A 9.6 a	A 9.9 a	A 9.6 a	A 9.2 a	A 9.7 a
	12	A 9.0 a	A 9.1 a	A 9.2 a	A 9.4 a	A 9.1 a	A 8.8 a
	25	A 9.0 a	A 9.4 a	A 9.2 a	A 8.7 a	A 8.3 a	A 8.3 a
Vegetable							
Cabbage	4	A 6.3 a	A 6.3 a	A 6.3 a	A 5.3 a	A 6.5 a	A 6.2 a
	12	A 6.5 a	A 6.0 a	A 6.0 a	A 6.1 a	A 6.1 a	A 6.0 a
	25	A 6.5 a	A 6.0 a	A 5.8 a	A 5.4 a	A 5.5 a	A 5.6 a
Carrot	4	A 7.7 a	A 9.0 a	A 9.1 a	A 9.2 a	A 9.1 a	A 9.1 a
	12	A 8.8 a	A 8.7 a	A 8.7 a	A 8.7 a	AB 8.2 a	A 7.4 a
	25	A 8.8 a	A 8.5 ab	A 7.5 ab	B 7.3 ab	B 7.2 b	A 7.1 b
Cucumber	4	A 3.7 a	A 3.1 a	A 3.0 a	A 3.0 a	A 2.9 a	A 2.9 a
	12	A 3.0 a	A 2.9 a	A 2.9 a	A 3.0 a	A 3.1 a	A 2.7 a
	25	A 3.0 ab	A 3.2 a	A 2.8 bc	A 2.6 bc	A 2.4 c	A 2.5 bc
Lettuce	4	A 4.5 a	A 3.7 a	A 3.5 a	A 3.6 a	A 3.5 a	A 3.6 a
	12	A 3.8 a	A 3.5 a	A 3.5 a	A 3.6 a	A 3.5 a	A 3.0 a
	25	A 3.8 a	A 3.7 ab	A 3.4 ab	A 3.3 ab	A 3.2 ab	A 3.2 b
Tomato	4	A 4.2 a	A 4.4 a	A 4.0 a	A 4.1 a	AB 4.0 a	A 4.2 a
	12	A 4.0 a	A 4.1 a	A 3.9 a	A 4.1 a	A 4.2 a	A 3.9 a
	25	A 4.0 a	A 4.1 a	A 3.6 ab	A 3.5 ab	B 3.5 ab	A 3.2 b

[a] Within each juice and storage time, mean values that are not preceded by the same capital letter are significantly different ($P \leq 0.05$). Within the same row, mean values of each produce juice that are not followed by the same lower case letter are significantly different ($P \leq 0.05$).

of *E. coli* O157:H7 in cantaloupe. They reported that *E. coli* O157:H7, inoculated at ca. 3 log CFU/g, remained viable at 5°C for 34 h and increased in population to ca. 6.5 log CFU/g at 25°C. At 4°C, the population of *Salmonella* Stanley, at an inoculum of 2 log CFU/g, did not change on fresh-cut cantaloupe during storage for 5 days (*50*). *E. sakazakii* behaved similarly to *E. coli* O157:H7 in cantaloupe.

Fresh-cut cucumber had a higher pH (5.9 - 6.0) than fresh-cut, apple, watermelon, or tomato but significantly higher populations of *E. sakazakii* were reached in the latter produce. Of the fresh-cut produce stored at 12 and 25°C, highest populations of *E. sakazakii* were reached on watermelon (pH 5.3 - 5.4). *Escherichia coli* O157:H7, at inoculum level of ca. 3 log CFU/g, also grew significantly better on watermelon than on cantaloupe at 25°C (*15*). This suggests that watermelon may provide a superior quantity or balance of nutrients that more than counteracts the more favorable higher pH of other produce, thus supporting more luxuriant growth of *E. sakazakii.* Watermelon had a high °Brix value, which reflects an elevated level of sugars that would enhance the growth of *E. sakazakii*. The population of *E. sakazakii* decreased minimally on tomatoes (pH 4.9) stored at 4°C. This implies that fresh-cut tomato, compared to the other test vegetables, also provides favorable conditions that may have ameliorated the adverse environment imposed by low pH. Interestingly, the decrease in pH of tomato was lowest (0.8 unit) among fresh-cut produce stored at 25°C for 7 days. *Salmonella* Montevideo has been reported to not grow on tomatoes at 10°C but grew to populations exceeding 3 log and 5 log CFU/cm^2 at 20 and 30°C, respectively (*56*). It has been reported the population of *E. coli* O157:H7 on the surface of uncut tomatoes incubated at 4°C decreased by only 1 log CFU/g during 10 days (*16*). Our observations confirm that fresh-cut produce stored at an appropriate storage temperature can support the growth of *E. sakazakii* as well as other Enterobacteriaceae.

The population of *E. sakazakii* on fresh-cut carrot decreased markedly to < 1 CFU/g within 144 h at 4°C. This indicates that carrot may have antibacterial activity at 4°C. This inhibitory effect was not observed when carrot was stored at 25°C, suggesting that active components are inhibitory or lethal to *E. sakazakii* only at low temperature or that inhibitory compounds are not produced in carrot at 25°C. It has been reported that carrot inhibits or kills *Listeria monocytogenes* (*2*) and *E. coli* O157:H7 (*1*). *Escherichia coli* O157:H7 has been shown to grow on shredded lettuce and sliced cucumber at 12 and 21°C (*1*). In another study, cucumber was reported to produce volatiles such as (*E, Z*)-2, 6-nonadienal and (*E*)-2-nonenal (NDE) that inhibit *E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium (*12*). In our study, *E. sakazakii* grew on fresh-cut cucumber at 25°C, indicating that inhibition by these chemicals did not occur.

Enterobacter sakazakii did not grow in fruit and vegetable juices at 4°C and grew only in cantaloupe, watermelon, and carrot juice at 12°C. Lag times for *E. sakazakii* at 10°C and 23°C have been reported to be 29 - 37 h and 2.8 h, respectively, in infant formula (*39*). Generation times at 10°C and 23°C were 4.2 - 5.1 h and 40 - 41 min, respectively. In our study, the lag times of *E. sakazakii* at 12°C and 25°C in produce juice were longer (approximately 24 - 48 h and 6 h, respectively). The initial population of *E. sakazakii* was 1.2 - 1.6 log CFU/ml of juice. The initial population of *E. sakazakii* in infant formula was higher (ca. 3 log CFU/ml), which may have resulted shorter lag times. A richer nutrient content of infant formula, compared to produce juice, would also enhance the growth of *E. sakazakii*.

Results indicate that *E. sakazakii* did not grow in apple, strawberry, and cabbage juice at 4, 12, and 25°C. Apple and strawberry juice have higher °Brix values than do cantaloupe, cucumber, carrot, lettuce, and tomato juice. Apple and strawberry juice contain 37.8 and 17.7 mg of non-cellulosic neutral sugar per 100 mg of tissue wall, respectively (*22*). These neutral

sugars consists of rhamnose, fructose, arabinose, xylose, mannose, galactose, and glucose, some of which are fermentable by *Enterobacter* spp. and other bacteria found on produce. However, apple and strawberry juice have very low initial pH values (below 4.0) which would retard or inhibit growth. The low pH of apple and strawberry apparently plays the major role in preventing growth and inactivating *E. sakazakii*. The predominant acids of apple and strawberry are citric and malic acids, both known to have bactericidal activity (*34, 36, 46, 55*). Han and Linton (*23*) reported that 1-log and 6-log reductions of *L. monocytogenes* populations occurred in strawberry juice stored for 3 days at 4°C and 37°C, respectively. *Enterobacter agglomerans* and *Pseudomonas* are inactivated by 0.5% citric acid (*42*).

Cabbage juice initially contained a lactic acid bacteria population of more than 3 log CFU/ml. During 150 h of fermentation, lactic acid in a non-sterile mixture of cabbage and carrot juice has been reported to increase from 0.124 to 4.959 g/dm^3 (*28*). Ryu and Beuchat (*47*) showed that lactic acid inactivated *E. coli* O157:H7 at pH below 3.9. In our study, *E. sakazakii* in cabbage juice, after 1 day of incubation at 25°C, may have been inactivated by the reduction in pH caused by production of lactic acid or other organic acids during bacterial fermentation. The pH of cabbage juice decreased to 3.9 and the number of lactic acid bacteria increased by more than 8 log CFU/ml within 3 days. Malic acid, the major organic acid in cabbage, can also have a bactericidal effect. Cabbage contains various sulfur compounds such as allyl isothiocyanate and methyl methanethiosulfinate that have inhibitory activity against bacteria (*11, 30, 33*). These factors may act simultaneously to inactivate *E. sakazakii* in cabbage juice.

Cantaloupe, watermelon, carrot, cucumber, and tomato juice supported greater than 5-log increases in *E. sakazakii* populations within 24 - 30 h, followed by reductions to < 1 CFU/ml by 48 - 72 h at 25°C. Richards et al. (*45*) reported that cantaloupe juice supported growth of

Salmonella Poona to a population exceeding 8 log CFU/g within 48 h. In our study, populations of *E. sakazakii* in watermelon were ca. 1 log higher than in cantaloupe juice after 96 h at 12°C and 24 h at 25°C. Golden et al. (*21*) showed the same trend for *Salmonella* populations in cut watermelon and cantaloupe. They reported ca. 1.0 log higher populations in watermelon than in cantaloupe and honeydew melon stored at 23°C for 24 h. Watermelon and cantaloupe were examined for their ability to support the growth of *Salmonella* and *L. monocytogenes* at 10, 20, and 30°C (*43, 44*). It was observed that the population of *S.* Enteriditis and *L. monocytogenes* on cantaloupe and watermelon increased by 5 - 6 log CFU/g within 12 h and 24 h, respectively, at 30°C but growth was delayed at 10 and 20°C. These studies as well as our observations on *E. sakazakii* show that watermelon and cantaloupe are excellent substrates for growth of Enterobacteriaceae.

Although *E. sakazakii* did not grow and, in some cases, did not survive in produce juice stored at 4°C, total bacterial counts increased in cantaloupe, carrot, cucumber, and lettuce juice. Numbers increased by more than 2 logs during the storage period at 4°C. This indicates that psychrophiles or psychrotrophs grew in these juices. Total counts in watermelon, cabbage, and tomato juice remained constant at 4°C but significantly increased at 12°C for 7 days. Even though *E. sakazakii* was not detected after 3 days at 25°C, largely attributed to low pH, total counts remained constant for an additional 96 h. The growth of acid-tolerant bacteria in these and other juices stored at 12°C and 25°C is evident.

Dramatic growth of lactic acid bacteria caused a reduction in pH of cantaloupe, watermelon, carrot, cabbage, cucumber, lettuce, and tomato juice stored for 24 - 48 h at 25°C. Numbers of lactic acid bacteria in cantaloupe and watermelon juice and in all vegetable juices stored at 25°C reached ca. 8.3 - 9.3 log CFU/ml within 48 h and remained unchanged or

decreased gradually thereafter. The population of lactic acid bacteria increased more slowly in apple and strawberry juices at 25°C; populations of only 4.9 and 5.3 log CFU/ml, respectively, were reached 48 h after inoculation. Vegetable juice inoculated with lactic acid bacteria is known to decrease in pH (*20*). Fleming et al. (*18*) reported that *Leuconostoc mesenteroides* is the predominant lactic acid bacteria during the initial fermentation of many vegetables but its populations decrease as the pH is reduced to 4.0 or below. Another study showed that pH 3.8 inhibited *Leuconostoc* in cucumber juice (*35*). Our study showed that lactic acid bacteria did not grow well in apple and strawberry juice, with initial pH values below 4.0, compared to other juices.

Molds and yeasts grew in some of the juices but did not reach populations achieved by *E. sakazakii*, total counts, and lactic acid bacteria. Growth occurred in only apple and tomato juice at 12 and 25°C. Molds and yeasts were apparently less competitive than bacteria in produce juice inoculated with *E. sakazakii*.

This study clearly demonstrates that *E. sakazakii* can survive and grow in several types of fresh-cut produce and produce juice. Temperature affects survival and growth as does the type of produce. Temperature abuse (≥ 10°C) of produce during post harvest storage occurs at retail and in food service and home settings, which would increase the risk for growth of *E. sakazakii.* *Enterobacter sakazakii* is able to grow at mildly abusive temperatures (e. g., 12°C) and survives at 4°C. More research attention is needed to characterize the potential for *E. sakazakii* infections associated with these products. Further studies focused on determining the fate of *E. sakazakii* on fresh produce upon exposure to conditions and practices simulating those used in commercial processing and handling operations as well as in food service and home settings are warranted.

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CHAPTER 3

SURVIVAL OF *ENTEROBACTER SAKAZAKII* ON FRESH PRODUCE AS AFECTED BY TEMPERATURE, AND EFFECTIVENESS OF SANITIZERS FOR ITS ELIMINATION [1]

[1] Kim, Hoikyung and Larry R. Beuchat. Accepted for publication in *International Journal of Food Microbiology*. Reprinted here with permission of publisher, 2006

Abstract

A study was done to determine the survival characteristics of *Enterobacter sakazakii* on the surface of apples, cantaloupes, strawberries, lettuce, and tomatoes stored at 4, 12, and 25°C for 8 - 28 days. Populations significantly decreased ($p \leq 0.05$) on all test produce at all storage temperatures. The efficacy of chlorine, chlorine dioxide, and a peroxyacetic acid-based sanitizer (Tsunami 200®) treatments (1 and 5 min) in killing the bacterium on apples, tomatoes, and lettuce was determined. Chlorine and chlorine dioxide, at ≥ 50 µg/ml, were equivalent in killing *E. sakazakii* on apples. Populations of *E. sakazakii* on apples treated with 10 µg/ml chlorine dioxide for 1 or 5 min were significantly reduced ($p \leq 0.05$) by 3.38 and 3.77 log CFU/apple, respectively, compared to the number remaining on apples after washing with water. Treatment with Tsunami 200 at 40 µg/ml for 1 min caused reductions of ≥ 4.00 log CFU/apple. Reductions of ≥ 3.70 log CFU/tomato were achieved by treatment with 10 µg/ml chlorine or chlorine dioxide or 40 µg/ml Tsunami 200 for 5 min. Reductions in populations of *E. sakazakii* on lettuce treated with chlorine at 10, 50, and 100 µg/ml for 1 min ranged from 1.61 to 2.50 log CFU/sample (26 ± 4g), compared to populations remaining on lettuce washed with water. Chlorine was less effective in killing *E. sakazakii* on lettuce than on apples or tomatoes. Treatment of lettuce with Tsunami 200 (40 and 80 µg/ml) for 5 min caused a reduction of ≥ 5.31 log CFU/sample. Results provide insights to predicting survival characteristics of *E. sakazakii* on produce and the efficacy of sanitizers in killing the bacterium.

Keywords: Enterobacter sakazakii; fruit; vegetable; apple; cantaloupe; strawberry; lettuce; tomato; chlorine; chlorine dioxide; peroxyacetic acid

1. Introduction

During the past two decades, consumption of fresh fruits and vegetables has increased in the U. S. Concomitant with this trend, the number and frequency of outbreaks of illness associated with fresh produce have increased (Harris et al., 2003). Outbreaks of *Escherichia coli* O157:H7 infections, for example, have been linked to lettuce (Hilborn et al., 1999) and alfalfa sprouts (Breuer et al., 2001) and salmonellosis has been associated with consumption of tomatoes (Cummings et al., 2001) and cantaloupe (Centers for Disease Control and Prevention, 2002). Factors affecting survival and growth characteristics of foodborne pathogens on fresh and fresh-cut produce that may impact the level of risk of causing infections have not been fully defined.

Enterobacter sakazakii is an emerging foodborne pathogen known to cause meningitis (Burdette and Santos, 2000; Gallagher and Ball, 1991), sepsis (Simmons et al., 1980), bacteremia (Noriega et al., 1990), and necrotizing enterocolitis (Van Acker et al., 2001) in preterm neonates and immunocompromised adults (Lai, 2001). This bacterium has been found in several types of foods, food processing plants, and the environment (Iversen and Forsythe, 2003; Kandhai et al., 2004; Gurtler et al., 2005), although outbreaks of infection have been associated primarily with reconstituted, infant formula (Himelright et al., 2002; Muytjens and Kollee, 1990; Van Acker et al., 2001). While *E. sakazakii* has not been reported to cause illnesses linked to the consumption of fresh produce, it has been isolated from lettuce (Soriano et al, 2001) and other vegetables (Leclercq et al., 2002), thereby presenting a potential risk of foodborne infections. We have observed that *E. sakazakii* can grow on several types of fresh-cut produce and in fruit and vegetable juices (Kim and Beuchat, 2005).

Chlorinated water, chlorine dioxide (gaseous and aqueous), and peracetic acid-based sanitizers are among the chemical treatments used to reduce populations of microorganisms on fresh fruits and vegetables. Water containing free chlorine concentrations of 50 - 200 μg/ml can be used to sanitize produce (Beuchat, 1998; Food and Drug Administration, 1998). Chlorine has been shown to be effective in reducing populations of *E. coli* O157:H7 (Beuchat, 1999; Park and Beuchat, 1999; Fett, 2002a; Ryu and Beuchat, 2005), *Salmonella* (Zhuang et al., 1995; Park and Beuchat, 1999; Weissinger et al., 2000; Fett, 2002a), and *Listeria monocytogenes* (Ukuku and Fett, 2002; Beuchat et al., 2004) on fresh produce. Aqueous chlorine dioxide has been reported to kill *E. coli* O157:H7 on lettuce and baby carrots (Singh et al., 2002) and *E. coli* O157:H7 and *L. monocytogenes* on green peppers (Han et al., 2001), apples, lettuce, strawberries, and cantaloupes (Rodgers et al., 2004). Peroxyacetic acid has been used to reduce microbial populations in process water (Hilgren and Salverda, 2000) and on apples (Wisniewsky et al., 2000; Rodgers et al, 2004), cantaloupes (Park and Beuchat, 1999; Rodgers et al., 2004), lettuce (Beuchat et al., 2004; Rodgers et al., 2004), strawberries (Rodgers et al., 2004), honeydew melons, and asparagus (Park and Beuchat, 1999). However, the efficacy of sanitizers in killing *E. sakazakii* on fresh fruits and vegetables has not been reported.

An objective of this study was to determine the survival characteristics of *E. sakazakii* on the surface of apples, cantaloupes, strawberries, lettuce, and tomatoes stored at 4, 12, and 25°C for up to 28 days. A second objective was to determine the effectiveness of chlorine, aqueous chlorine dioxide, and a peroxyacetic acid-based sanitizer in killing *E. sakazakii* inoculated in an organic carrier (horse serum) onto the surface of apples, tomatoes, and lettuce.

2. Materials and methods

2.1. Preparation of inoculum

Five clinical isolates of *E. sakazakii* (strain 4923, A9002, 1625, 8397, and LCDC 674) were grown in tryptic soy broth (TSB; BBL/Difco, Sparks, Maryland) at 37°C for 24 h. Nalidixic acid-adapted cells of each strain were selected by surface plating 0.1 ml of a 24-h culture on tryptic soy agar (TSA; BBL/Difco) supplemented with nalidixic acid (50 μg/liter) and pyruvate (0.1%) (TSANP) and incubating plates at 37°C for 24 h. Nalidixic acid-adapted cells were picked from colonies and cultured in TSB supplemented with 1% glucose and nalidixic acid (50 μg/liter) (TSBGN) at 37°C for 24 h. After three consecutive transfers of ca. 10 μl into TSBGN at 24-h intervals, cells from 30 ml of culture of each strain were collected by centrifugation (1,250 × *g*, 15 min, 21°C) and the supernate was decanted. Cells were resuspended in 15 ml of sterile 5% horse serum (Sigma-Aldrich, St. Louis, Missouri) and appropriate volumes of suspensions of each strain were combined to give approximately equal populations (10 log CFU/ml) of each strain.

2.2. Preparation of fresh produce

Unwaxed organically grown apples (*Malus domestica* Borkh., Red Delicious cv.) and unwaxed organically grown tomatoes (*Lycopersicon esculentum* Mill, Roma cv.) were purchased at a local farmers' market 2 - 3 days prior to use in experiments. Cantaloupes (*Cucumis melo* var. *cantalupensis*), strawberries (*Fragaria virginiana* Duschesne), and iceberg lettuce (*Lactuca sativa* L.) were purchased at a retail grocery store 1 day prior to use in experiments. After purchase, apples, tomatoes, and cantaloupes were stored at room temperature (22 ± 2°C) and lettuce and strawberries were kept at 12°C until inoculated. Two or three of the wrapper leaves

were removed from each head of lettuce and discarded. Samples of lettuce subjected to inoculation and analysis consisted of three 9 × 9 cm pieces cut from the 2 - 3 leaves beneath the wrapper leaves. Two 5 × 5 cm areas marked on the rind surface of each cantaloupe (1,150 ± 70 g) served as inoculation sites from which samples were eventually excised and analyzed. Each sample of apple (186 ± 24 g) and tomato (82 ± 9 g) consisted of a single fruit. A sample of lettuce consisted of three 9 × 9 cm pieces (26 ± 4 g) and a sample of strawberries consisted of three fruits (50 ± 4 g total).

2.3. Storage test

2.3.1. Inoculation and storage of produce

Each produce sample was placed on a wire screen elevated 7 cm above the work surface in a laminar flow biosafety cabinet. Using a micropipette, 100 µl of the five-strain mixture of *E. sakazakii* were deposited on the surface of each produce to give ca. 9 log CFU/produce sample. The inoculated produce was dried for 2 h (35% relative humidity) at 22 ± 2°C in the laminar flow hood. Inoculated apples, cantaloupes, strawberries, and tomatoes were placed in separate plastic trays and lettuce samples were placed in stomacher 400 bags (Seward Medical, Ltd., London, UK). Each type of inoculated produce was placed in a separate plastic tub and stored at 4, 12, or 25°C for up to 28 days before being subjected to microbiological analysis; respective atmospheric relative humidities at the three storage temperatures were 85, 45, and 45%.

2.3.2. Microbiological analysis

Populations of *E. sakazakii* on inoculated produce stored at 4, 12, and 25°C for 0 (2 h after inoculation), 1, 4, 7, 8, 14, 21, and/or 28 days were determined. At each sampling time,

apple, tomato, and strawberry samples were transferred to quart-sized (0.95 liter) Ziploc® bags (S. C. Johnson, Racine, Wisconsin). Fifty milliliters of sterile 0.1% peptone water were added to each bag. Apples and tomatoes were firmly hand rubbed and strawberries were gently shaken for 1 min. Tissues from inoculated areas (5 × 5 cm, 0.5 cm deep) on the rind of inoculated cantaloupes were removed using a sterile stainless steel scalpel and placed in stomacher 400 bags containing a filter septum. Fifty milliliters of 0.1% peptone water were added to each bag containing a cantaloupe or lettuce sample and the mixtures were pummeled in a Stomacher 400 (Seward Medical, Ltd.) at normal speed for 1 min. After samples were hand-rubbed, shaken, or pummeled, the peptone wash solution or homogenate were serially diluted in sterile 0.1% peptone water. Undiluted samples (0.25 ml in quadruplicate and 0.1 ml in duplicate) and serially diluted samples (0.1 ml in duplicate) were surface plated on TSANP. Plates were incubated at 37°C for 24 h and presumptive *E. sakazakii* colonies were counted. Selected colonies were subjected to confirmation using the API 20E bioassay kit (BioMérieux, Hazelwood, Missouri).

2.3.3. Measurement of pH

The pH of the surface of produce was measured on each day samples were analyzed for populations of *E. sakazakii*. A basic pH meter (Denver Instrument Co., Arvada, Colorado) equipped with a surface electrode was used. The pH of internal tissue 0.5 cm below the surface of all produce except lettuce was also measured.

2.4. Sanitizer efficacy test

2.4.1. Inoculation of fresh produce

Apples, tomatoes, and lettuce were selected to determine the efficacy of sanitizers in killing *E. sakazakii*. Produce was purchased, prepared for inoculation, and inoculated with *E. sakazakii* as described above for the storage study. After inoculation, apples and tomatoes were dried in a laminar flow (22 ± 2°C) for 24 h before treating with sanitizers. Inoculated lettuce was dried in a laminar flow (22 ± 2°C) for 2 h, transferred into Stomacher 400 bags, and held at 4°C for 22 h before treating with sanitizers.

2.4.2. Preparation of sanitizers

Solutions containing chlorine, chlorine dioxide, and a peroxyacetic acid-based sanitizer (Tsunami® 200, Ecolab, St. Paul, Minnesota) were evaluated for effectiveness in killing *E. sakazakii* on produce. Chlorinated water (10, 50, and 100 µg/ml) was prepared by combining NaOCl (Aldrich, Milwaukee, Wisconsin) with 0.05 M potassium phosphate buffer (pH 6.8, 22 ± 2°C). Chlorine dioxide solution (10, 50, and 100 µg/ml) at pH 8.56 - 11.02 was prepared from sodium chlorite solution (80%, Sigma-Aldrich) using patent-pending electrochemical technologies (Roselle et al., 2004). Concentrations of free chlorine and chlorine dioxide were determined using a chlorine colorimeter (model Dr/820, Hatch, Loveland, Colorado) immediately before use. Two concentrations (40 and 80 µg/ml) of Tsunami 200® were prepared and the concentrations were measured according to the manufacturer's instructions.

2.4.3. Treatment of produce

Inoculated apples and tomatoes at 22 ± 2°C and lettuce at 4°C were treated with sanitizers. Each apple, tomato, or lettuce sample was placed in a 0.95-liter Ziploc bag. Two hundred milliliters of sterile deionized water (control), chlorinated water (10, 50, or 100 μg/ml), chlorine dioxide (10, 50, or 100 μg/ml), or Tsunami 200 (40 or 80 μg/ml), all at 22 ± 2°C, were added to each bag containing a produce sample. Bags containing produce and sanitizer solution were separately placed in 1-liter beakers, secured on a rotary shaker, and agitated at 150 rpm for 1 or 5 min. After treatment, apples and tomatoes were aseptically transferred to another 0.95-liter Ziploc bag and lettuce was transferred to another Stomacher 400 bag. Fifty milliliters of Dey-Engley (DE) neutralizing broth (BBL/Difco) were immediately added to the bags. Treatment solutions, as well as the DE wash and homogenate, were analyzed for populations of *E. sakazakii*.

2.4.4. Microbiological analysis

Immediately after each treatment, 5 ml of water or sanitizer treatment solution were transferred to a sterile test tube containing 5 ml of double-strength DE broth. The undiluted mixture (0.25 ml in quadruplicate and 0.1 ml in duplicate) and serially diluted mixture (0.1 ml in duplicate) were surface plated on TSANP. Plates were incubated at 37°C for 24 h before colonies were counted. Selected colonies were subjected to API 20E confirmation tests.

Treated apples or tomatoes and DE broth in bags were firmly hand rubbed for 1 min. Bags containing lettuce were pummeled in a Stomacher 400 for 1 min. Undiluted and serially diluted samples were surface plated on TSANP. Enumeration of colonies was done after incubating plates at 37°C for 24 h. Presumptive *E. sakazakii* colonies (2 - 4 for each sample)

were randomly selected for confirmation as described above. Values are reported as log CFU/produce sample.

2.5. Statistical analysis

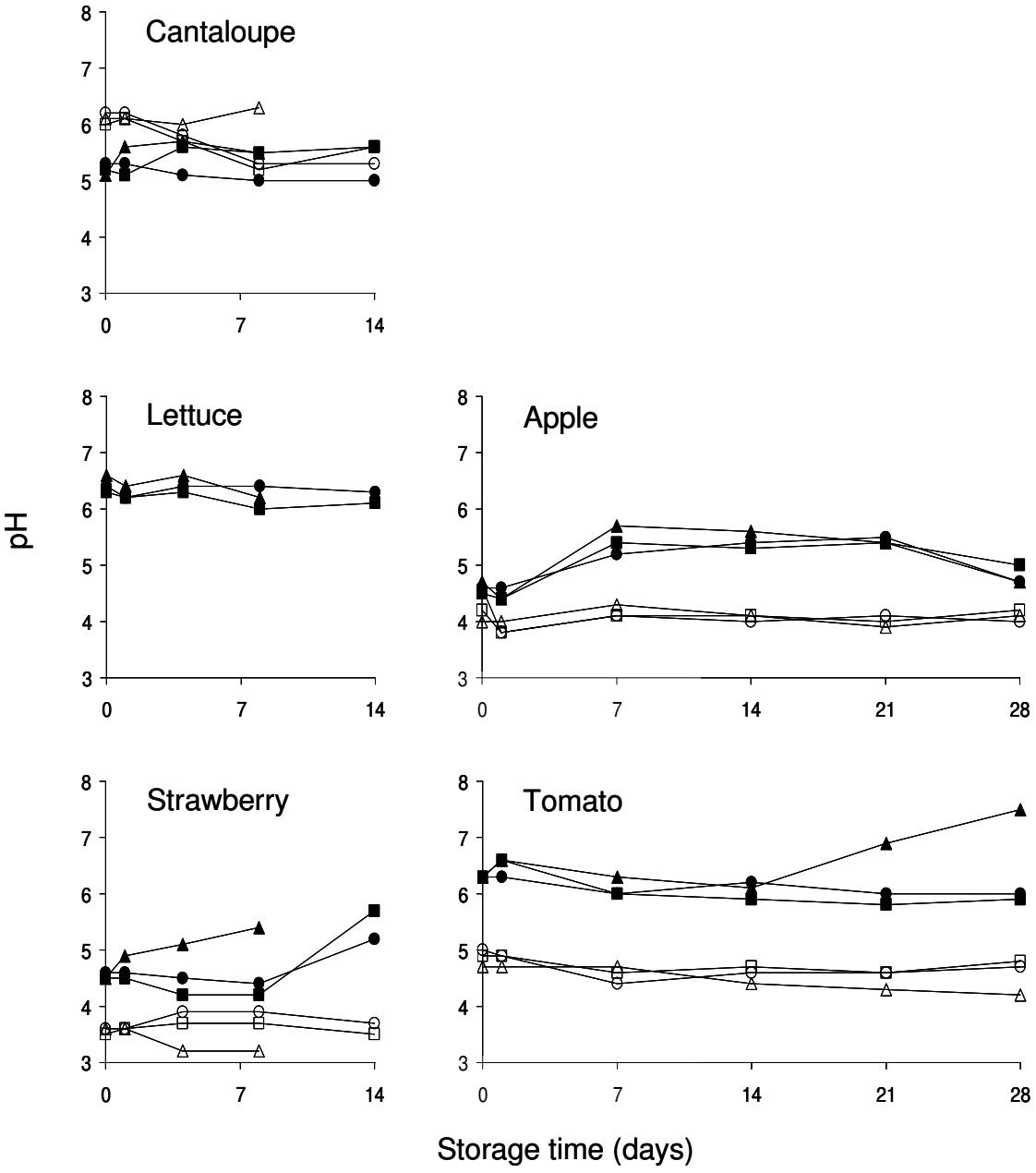
All experiments were performed in triplicate using produce from different lots in each experiment. Two samples representing each combination of test parameters were analyzed at each sampling time; diluted samples were plated in duplicate on TSANP. Data were analyzed using the general linear model of the Statistical Analysis Systems procedure (SAS; SAS Institute, Cary, North Carolina). Fisher's Least Significant Difference (LSD) test was used to determine if populations of *E. sakazakii* significantly decreased or increased stored at various conditions and as a result of treatment with various sanitizers. Significant differences are presented at a 95% confidence level ($p \leq 0.05$).

3. Results and discussion

3.1. pH changes during storage

The pH of the surface and subsurface tissue of inoculated produce stored for up to 28 days at 4, 12, and 25°C under atmospheric relative humidities of approximately 85, 45, and 45%, respectively, was measured. The initial pH (6.0 - 6.2) of internal tissue of cantaloupes was higher than the surface pH (5.1 - 5.3), whereas the reverse was true for strawberry, apple, and tomato (Figure 3-1). The internal tissues of apples and strawberries had the lowest initial pH (3.5 - 4.6) among the test produce. The initial surface pH of apples was 4.5 - 4.7. In a previous study, we attributed the acidic pH of apple juice to inhibiting growth of *E. sakazakii* (Kim and Beuchat, 2005). The pH of internal tissue of strawberries was 3.2 - 3.9 throughout the storage

Figure 3-1. The pH of surface tissue (closed symbols) and internal tissue (open symbols) of apples, cantaloupes, strawberries, lettuce, and tomatoes inoculated with *E. sakazakii* and stored at 4°C (circles), 12°C (squares), and 25°C (triangles) for up to 28 days.


Cantaloupe
Lettuce
Apple
Strawberry
Tomato
pH
Storage time (days)
8
7
6
5
4
3
0
7
14
21
28

period, whereas the initial surface pH (4.5 - 4.6) increased to 5.4 after 8 days at 25°C, 5.7 after 14 days at 12°C, and 5.2 after 14 days at 4°C.

Mold growth was visible on the surface of strawberries within 1 day at 25°C and 8 days at 4 and 12°C. Mold growth on tomatoes stored at 25°C was observed at 14 days. The surfacepH of tomatoes remained constant at 4°C and 12°C, but increased between 14 and 28 days at 25°C. The increase in surface pH may have been caused by molds growing on strawberries and tomatoes. It is known that the pH of internal tissue of tomatoes inoculated with proteolytic molds and stored at 4, 15, or 25°C increases (Wade and Beuchat, 2003a). Wade and Beuchat (2003b) concluded that utilization of acids and secretion alkaline by-products by molds increases the pH of tomato tissues during decay. An increase in pH of acidic tissues of produce would provide a more favorable environment for survival and growth of *E. sakazakii*.

3.2. Fate of E. sakazakii *on stored produce*

Populations of *E. sakazakii* detected on the surface of apples, cantaloupes, strawberries, lettuce, and tomatoes stored at 4, 12, and 25°C for up to 28 days are shown in Table 3-1. Initial populations (8.60 - 8.78 log CFU/produce) significantly ($p \leq 0.05$) decreased on all test produce at all temperatures. However, the rate of death varied, depending on temperature and type of produce. Regardless of the type of produce, however, reductions did not exceed 4.03 log CFU/produce sample (apples stored at 25°C) during the 28-day storage period. In a few instances, storage at 4 or 12°C was protective against death of *E. sakazakii*. Similar phenomena have been observed with other pathogens on produce. Riordan et al. (2000) reported that populations of *E. coli* O157:H7 decreased more slowly on apples stored at 4°C than at 22 ± 2°C.

Table 3-1
Population of *E. sakazakii* recovered from produce stored at 4, 12, and 25°C for up to 28 days

Produce	Temp. (°C)	Number of *E. sakazakii* (log CFU/produce) [a] 0 day	1 day	4 day	7 day	8 day	14 day	21 day	28 day
Apple	4	A 8.78 A	B 8.40 A	-[b]	C 8.12 A	-	D 7.58 B	DE 7.57 A	E 7.32 AB
	12	A 8.78 A	B 8.42 A	-	B 8.27 A	-	B 8.30 A	B 8.32 A	C 8.03 A
	25	A 8.78 A	B 8.37 A	-	AB 7.53 B	-	AB 7.60 B	BC 6.27 A	C 4.75 B
Cantaloupe	4	A 8.45 A	A 8.28 A	B 7.52 A	-	B 7.38 AB	C 7.01 B	-	-
	12	A 8.45 A	B 7.85 B	C 7.47 A	-	C 7.58 A	B 7.81 A	-	-
	25	A 8.45 A	B 7.82 B	C 6.82 B	-	C 7.15 B	-	-	-
Strawberry	4	A 8.78 A	A 8.47 A	A 8.47 A	-	B 7.63 A	B 7.35 A	-	-
	12	A 8.78 A	A 8.43 A	AB 8.30 A	-	B 7.52 A	C 5.42 B	-	-
	25	A 8.78 A	A 8.13 B	B 6.80 B	-	C 4.88 B	-	-	-
Lettuce	4	A 8.85 A	A 8.73 A	B 8.45 A	-	B 8.35 A	C 8.03 B	-	-
	12	A 8.85 A	A 8.83 A	B 8.50 A	-	B 8.42 A	B 8.40 A	-	-
	25	A 8.85 A	A 8.83 A	B 8.53 A	-	B 8.40 A	-	-	-
Tomato	4	A 8.60 A	B 8.30 A	-	C 7.80 B	-	CD 7.75 B	CD 7.68 B	D 7.53 B
	12	A 8.60 A	B 8.20 A	-	B 8.12 A	-	B 8.17 A	AB 8.35 A	B 8.07 A
	25	A 8.60 A	B 8.10 A	-	C 7.65 B	-	BC 7.80 B	BC 7.92 B	D 7.15 B

[a] Within the same row, mean values that are not preceded by the same letter are significantly different ($p \leq 0.05$). Within each type of produce and storage time, mean values that are not followed by the same letter are significantly different ($p \leq 0.05$).
[b] Not determined.

More rapid reductions in *E. coli* O157:H7 populations occurred on apples stored at 21°C than at 2°C (Kenney and Beuchat, 2002). Populations of native mesophilic aerobes and *Listeria monocytogenes* on the rind of cantaloupes were reported to decrease more rapidly at 20°C than at 4°C (Ukuku and Fett, 2002).

Reductions in populations of *E. sakazakii* on cantaloupes, lettuce, and tomatoes were ≤ 1.45 log CFU/produce sample, regardless of storage temperature during storage for up to 8 - 28 days. The behavior of other Enterobacteriaceae on cantaloupes, lettuce, and tomatoes has been described. *E. coli* O157:H7 on the rind surface of cantaloupes grew at 25°C within 4 days but the population significantly decreased at 5°C within 4 days (Del Rosario and Beuchat, 1995). Beuchat (1999) reported decreases in numbers of *E. coli* O157:H7 inoculated at high and low populations on lettuce leaves during storage at 4°C for 8 and 15 days, respectively. Li et al. (2001) reported decreases in the number of *E. coli* O157:H7 on fresh-cut lettuce at 5°C but increases at 15°C. Populations of *E. coli* O157:H7 on the surface of tomatoes decreased by less than 1 log at 4°C and 25°C when inoculated at a high population (6 - 7 log CFU/g) (Eribo and Ashenafi, 2003). Beuchat and Scouten (2004) observed significant decreases in *Salmonella* Poona populations on the intact rind surface of cantaloupes stored at 4°C for 7 days and 21°C for 3 days. Populations of *Salmonella* Baildon on shredded lettuce held at 4°C for 12 days decreased by 96% (Weissinger et al., 2000). The number of *Salmonella* inoculated on the surface of tomatoes stored at 20°C significantly decreased by ca. 4 log CFU/tomato for 14 days (Guo et al., 2002). However, others have shown that *Salmonella* inoculated at a low population (ca. 2 log CFU/g) on tomatoes grew at 15 or 25°C during a 10-day storage period (Wade and Beuchat, 2003a).

Among the largest reductions in *E. sakazakii* populations were those on apples stored at 25°C and strawberries stored at 12 and 25°C. In a previous study (Kim and Beuchat, 2005), we observed that *E. sakazakii* did not grow or was inactivated in apple juice (pH 3.9 - 4.0) and strawberry juice (pH 3.5 - 3.7) stored at 4, 12, or 25°C for 7 days. *Campylobacter jejuni*, inoculated at ca. 6 log CFU/g of strawberry (pH 3.26 - 3.51) and stored at 21°C, was not detected within 2 days (Kärenlampi and Hänninen, 2004). Death was more rapid at 21°C than at 7°C. Approximately 1 - 2 log and 2 log reductions of *Salmonella* and *E. coli* O157:H7, respectively, occurred on the intact surfaces of strawberries stored at 4°C for 7 days (Knudsen et al., 2001). Populations of *E. coli* O157:H7 decreased by 1.30 - 1.77 and 2.08 - 2.25 log CFU/g of strawberries held at 5 and 10°C, respectively, for 3 days, and 0.24 - 0.66 log CFU/g within 1 day at 23°C (Yu et al., 2001). In our study, the number of *E. sakazakii* on strawberries stored at 25°C was reduced to 4.9 log CFU/sample within 8 days, whereas reductions at 4 or 12°C decreased by 1.2 - 1.3 log CFU/sample within the same time period. The high relative humidity (85 %) at 4°C may have contributed to the persistence of *E. sakazakii*. Results, however, show that *E. sakazakii*, like other Enterobacteriaceae capable of causing foodborne illness, can survive on various types of fresh produce for several days. These observations point out the need to assess safety risks for fresh produce stored at refrigerator as well as room temperatures. Like other pathogenic Enterobacteriaceae, *E. sakazakii* survives better on produce at refrigerator temperature.

3.3. Effectiveness of sanitizers

The effectiveness of chlorine (10, 50, and 100 μg/ml), chlorine dioxide (10, 50, and 100 μg/ml), and Tsunami 200 (40 and 80 μg/ml) in killing *E. sakazakii* inoculated onto the surface of

apples, tomatoes, and lettuce was determined. The number of *E. sakazakii* deposited on the surface of each apple, tomato, and lettuce sample was 9.14, 9.20, and 9.20 log CFU, respectively. Populations were reduced to 8.13, 8.15, and 8.39 log CFU/apple, tomato, and lettuce sample, respectively, during drying. Regardless of the type of produce, sanitizer, or treatment time, *E. sakazakii* was not detected in the sanitizer solutions after treating produce (detection limit was 2.60 log CFU/produce).

3.3.1. Studies on apples

Populations of *E. sakazakii* recovered from apples treated with chlorine, chlorine dioxide, and Tsunami 200 for 1 and 5 min are shown in Tables 3-2 and 3-3, respectively. The pathogen was not recovered from treatment solutions, regardless of the type or concentration of sanitizer, indicating that cells removed from apples were killed. Washing apples with water (control) for 1 or 5 min caused 1.94 and 2.18 log CFU/apple reductions, respectively. Treatment with ≥ 50 µg/ml chlorine, ≥ 10 µg/ml chlorine dioxide, and ≥ 40 µg/ml Tsunami 200 for 1 min significantly reduced populations (≥ 2.90 log-reductions), compared to numbers of *E. sakazakii* remaining on apples washed with water. Beuchat et al. (1998) observed that the number of *E. coli* O157:H7 on apples was reduced from an initial population of 1.52 log CFU/cm^2 to 1.14 and 0.87 CFU/cm^2 following 1-min and 5-min treatments, respectively, with 200 µg/ml chlorine.

Treatment of apples for 1 min with 100 µg/ml chlorine dioxide resulted in the highest reduction of *E. sakazakii* populations (≥ 4.49 log CFU/apple) among the treatments evaluated. Treatment for 5 min with ≥ 10 µg/ml chlorine, ≥ 10 µg/ml chlorine dioxide, and ≥ 40 µg/ml Tsunami 200 caused significant reductions of ≥ 2.42, ≥ 3.77, and ≥ 4.25 log CFU/apple, respectively, compared to the numbers of *E. sakazakii* remaining on apples after washing with

Table 3-2
Populations of *E. sakazakii* recovered from apples treated with water or sanitizers for 1min

			Population (log CFU/apple)[a]					
			DE broth[b]			Treatment solution[b]		
Treatment	Concentration (µg/ml)	pH	log CFU/apple		Reduction[c]	log CFU/apple		Reduction
Chlorine	0	6.80	A 6.19	A	-	A 8.08	A	-
	10	6.88	AB 4.48	AB	1.71	B < 2.60	B	≥ 5.48
	50	6.93	BC 3.01	BC	3.18	B < 2.60	B	≥ 5.48
	100	6.92	C 1.96	C	4.23	B < 2.60	B	≥ 5.48
Chlorine dioxide	0	6.93	A 6.19	A	-	A 8.08	A	-
	10	8.56	B 2.81	BC	3.38	B < 2.60	B	≥ 5.48
	50	10.13	B 3.29	BC	2.90	B < 2.60	B	≥ 5.48
	100	11.02	B < 1.70	C	≥ 4.49	B < 2.60	B	≥ 5.48
Tsunami 200	0	6.94	A 6.19	A	-	A 8.08	A	-
	40	3.87	B 2.19	C	4.00	B < 2.60	B	≥ 5.48
	80	3.37	B 2.05	C	4.14	B < 2.60	B	≥ 5.48

[a] Population before treatment was 8.13 log CFU/apple. Within the same sanitizer and solution (DE wash broth or treatment solution), mean values that are not preceded by the same letter are significantly different ($p \leq 0.05$). Mean values in the same column that are not followed by the same letter are significantly different ($p \leq 0.05$).

[b] Populations in DE broth after washing treated apples and in water and sanitizer solutions after treating apples were used to calculate log CFU/apple.

[c] Reduction in population after treatment compared with the population after treatment with water (control).

Table 3-3
Populations of *E. sakazakii* recovered from apples treated with water or sanitizers for 5 min

			Population (log CFU/apple)[a]			
			DE broth[b]		Treatment solution[b]	
Treatment	Concentration (µg/ml)	pH	log CFU/apple	Reduction[c]	log CFU/apple	Reduction
Chlorine	0	6.80	A 5.95 A	-	A 8.06 A	-
	10	6.88	B 3.53 B	2.42	B < 2.60 B	≥ 5.46
	50	6.93	C 1.80 C	4.15	B < 2.60 B	≥ 5.46
	100	6.92	C 2.10 C	3.85	B < 2.60 B	≥ 5.46
Chlorine dioxide	0	6.93	A 5.95 A	-	A 8.06 A	-
	10	8.56	B 2.18 C	3.77	B < 2.60 B	≥ 5.46
	50	10.13	B 2.06 C	3.89	B < 2.60 B	≥ 5.46
	100	11.02	B < 1.70 C	≥ 4.25	B < 2.60 B	≥ 5.46
Tsunami 200	0	6.94	A 5.95 A	-	A 8.06 A	-
	40	3.87	B < 1.70 C	≥ 4.25	B < 2.60 B	≥ 5.46
	80	3.37	B < 1.70 C	≥ 4.25	B < 2.60 B	≥ 5.46

[a] Population before treatment was 8.13 log CFU/apple. Within the same sanitizer and solution (DE wash broth or treatment solution), mean values that are not preceded by the same letter are significantly different ($p \leq 0.05$). Mean values in the same column that are not followed by the same letter are significantly different ($p \leq 0.05$).

[b] Populations in DE broth after washing treated apples and in water and sanitizer solutions after treating apples were used to calculate log CFU/apple.

[c] Reduction in population after treatment compared with the population after treatment with water (control).

water. The highest reduction (≥ 4.25 log CFU/apple) resulted from treatment of apples with 100 µg/ml chlorine dioxide or ≥ 40 µg/ml Tsunami 200 for 5 min. Populations significantly decreased by 3.38 and 3.77 log CFU/apple upon treatment with chlorine dioxide (10 µg/ml) for 1 and 5 min, respectively, compared to the numbers on apples washed with water. At 50 and 100 µg/ml, chlorine and chlorine dioxide were equivalent in killing *E. sakazakii* on apples, regardless of treatment time. Treatment with 100 µg/ml chlorine dioxide for 1 or 5 min decreased the population of *E. sakazakii* to an undetectable level (< 1.70 log CFU/apple). The effectiveness of gaseous chlorine dioxide in killing *E. coli* O157:H7 and *L. monocytogenes* inoculated on apples has been studied (Du et al., 2002; Du et al., 2003). Treating apples with chlorine dioxide gas at 1.1 - 18 µg/liter and 1.0 - 4.0 µg/ml of air caused 2.8 - ≥ 7.0 and 3.2 - 5.5-log reductions of *E. coli* O157:H7 and *L. monocytogenes*, respectively. Treatment with chlorine dioxide (80 µg/ml) for 5 min was reported to cause less than a 4-log reduction of *E. coli* O157:H7 on apples (Wisniewsky et al., 2000).

Treatment with Tsunami 200 at 40 and 80 µg/ml caused ≥ 4.00 log CFU/apple reductions in *E. sakazakii*, compared to washing with water, regardless of treatment time. Others have studied the efficacy of peroxyacetic acid in killing pathogens on apples. Wright et al. (2000) reported that the population of *E. coli* O157:H7 was reduced by 2.5 log CFU/cm^2 of apple surface upon treatment with 80 µg/ml peroxyacetic acid. Rodgers et al. (2004) reported that treatment with 80 µg/ml peracetic acid was less effective than 5 µg/ml aqueous chlorine dioxide in killing *E. coli* O157:H7 and *L. monocytogenes* on apples. A 3-log reduction of *E. coli* O157:H7 occurred on apples upon treatment with Tsunami 100 (80 µg/ml) for 5 min (Wisniewsky et al., 2000). Our study indicates that *E. sakazakii* is more sensitive than *E. coli* O157:H7 to peroxyacetic acid.

Table 3-4
Populations of *E. sakazakii* recovered from tomatoes treated with water or sanitizers for 1min

			Population (log CFU/tomato)[a]					
			DE broth[b]			Treatment solution[b]		
Treatment	Concentration (µg/ml)	pH	log CFU/tomato		Reduction[c]	log CFU/tomato		Reduction
Chlorine	0	6.80	A 5.29	A	-	A 8.10	A	-
	10	6.88	AB 3.65	AB	1.64	B < 2.60	B	≥ 5.50
	50	6.93	AB 3.23	BC	2.06	B < 2.60	B	≥ 5.50
	100	6.92	B 1.80	C	3.49	B < 2.60	B	≥ 5.50
Chlorine dioxide	0	6.93	A 5.29	A	-	A 8.10	A	-
	10	8.56	B 2.99	BC	2.30	B < 2.60	B	≥ 5.50
	50	10.13	B 2.49	BC	2.80	B < 2.60	B	≥ 5.50
	100	11.02	B < 1.70	C	≥ 3.59	B < 2.60	B	≥ 5.50
Tsunami 200	0	6.94	A 5.29	A	-	A 8.10	A	-
	40	3.87	B < 1.70	C	≥ 3.59	B < 2.60	B	≥ 5.50
	80	3.37	B 2.31	BC	2.98	B < 2.60	B	≥ 5.50

[a] Population before treatment was 8.15 log CFU/tomato. Within the same sanitizer and solution (DE wash broth or treatment solution), mean values that are not preceded by the same letter are significantly different ($p \leq 0.05$). Mean values in the same column that are not followed by the same letter are significantly different ($p \leq 0.05$).

[b] Populations in DE broth after washing treated tomatoes and in water and sanitizer solutions after treating tomatoes were used to calculate log CFU/tomato.

[c] Reduction in population after treatment compared with the population after treatment with water (control).

Table 3-5
Populations of *E. sakazakii* recovered from tomatoes treated with water or sanitizers for 5 min

			Population (log CFU/tomato)[a]			
			DE broth[b]		Treatment solution[b]	
Treatment	Concentration (µg/ml)	pH	log CFU/tomato	Reduction[c]	log CFU/tomato	Reduction
Chlorine	0	6.80	A 5.40 A	-	A 7.91 A	-
	10	6.88	B < 1.70 B	≥ 3.70	B < 2.60 B	≥ 5.31
	50	6.93	B 2.19 B	3.21	B < 2.60 B	≥ 5.31
	100	6.92	B < 1.70 B	≥ 3.70	B < 2.60 B	≥ 5.31
Chlorine dioxide	0	6.93	A 5.40 A	-	A 7.91 A	-
	10	8.56	B < 1.70 B	≥ 3.70	B < 2.60 B	≥ 5.31
	50	10.13	B < 1.70 B	≥ 3.70	B < 2.60 B	≥ 5.31
	100	11.02	B < 1.70 B	≥ 3.70	B < 2.60 B	≥ 5.31
Tsunami 200	0	6.94	A 5.40 A	-	A 7.91 A	-
	40	3.87	B < 1.70 B	≥ 3.70	B < 2.60 B	≥ 5.31
	80	3.37	B < 1.70 B	≥ 3.70	B < 2.60 B	≥ 5.31

[a] Population before treatment was 8.15 log CFU/tomato. Within the same sanitizer and solution (DE wash broth or treatment solution), mean values that are not preceded by the same letter are significantly different ($p \leq 0.05$). Mean values in the same column that are not followed by the same letter are significantly different ($p \leq 0.05$).

[b] Populations in DE broth after washing treated tomatoes and in water and sanitizer solutions after treating tomatoes were used to calculate log CFU/tomato.

[c] Reduction in population after treatment compared with the population after treatment with water (control).

3.3.2. Studies on tomatoes

Shown in Tables 3-4 and 3-5 are populations of *E. sakazakii* recovered from tomatoes treated with chlorine, chlorine dioxide, and Tsunami 200 for 1 and 5 min, respectively. As with apples, the pathogen was not recovered from sanitizer solutions after treating tomatoes. Treatment with 100 μg/ml chlorine, ≥ 10 μg/ml chlorine dioxide, or ≥ 40 μg/ml Tsunami 200 caused significant reductions in populations, compared to the number of *E. sakazakii* remaining on tomatoes after treatment with water. Treatment for 5 min was clearly more effective than treatment for 1 min in reducing populations. There was no significant difference in efficacy among all sanitizers at all test concentrations in killing *E. sakazakii* when the treatment time was 5 min (Table 3-5). Beuchat et al. (2001) reported that a 3.07-log CFU/tomato reduction in *Salmonella* was achieved by spray-treating tomatoes with 200 μg/ml of chlorine. Treatment of tomatoes with ≥ 60 μg/ml chlorine caused significant reductions in the number of *Salmonella* Montevideo (Zhuang et al., 1995). Populations of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* on the surface of tomatoes decreased by > 3.04, 4.00, and > 4.83 log/tomato, respectively, as a result of treatment with chlorine at 200 μg/ml (Lang et al., 2004). As with apples, the *E. sakazakii* population was reduced to an undetectable level (< 1.70 log CFU/produce) when tomatoes were treated with 100 μg/ml chlorine dioxide for 1 min. Following a 1-min exposure to 100 μg/ml aqueous chlorine dioxide, slightly higher numbers of *E. sakazakii* were inactivated on tomatoes, compared to treatment with 100 μg/ml chlorine. A significant decrease in *Salmonella* populations has been observed on tomatoes treated with gaseous chlorine dioxide (Sy et al., 2005).

Treatment with Tsunami 200 (40 μg/ml) for 1 min decreased the number of *E. sakazakii* to an undetectable level (< 1.70 log CFU/tomato). In contrast, treatment of alfalfa sprout

irrigation water with Tsunami 100 (40 µg/ml) did not decrease or only slightly decreased the number of mesophilic aerobes and coliforms on sprouts (Fett, 2002b). Treatment of shredded carrots with peroxyacetic acid (80 µg/ml) resulted in a significant decrease in the number of *E. coli* O157:H7 (Gonzalez et al., 2004) and reduced the population of aerobic bacteria by 1.07, 0.84, and 1.54 log CFU/g of celery, cabbage, and potatoes, respectively (Hilgren and Salverda, 2000). Overall, Tsunami 200 (40 - 80 µg/ml) was equivalent to chlorine and chlorine dioxide (10 - 200 µg/ml) in killing *E. sakazakii* on tomatoes.

3.3.3. Studies on lettuce

Tables 3-6 and 3-7 show the number of *E. sakazakii* recovered from lettuce treated with water, chlorine, chlorine dioxide, and Tsunami 200 for 1 and 5 min, respectively. The pathogen was not recovered from sanitizer solutions after treatment of lettuce. The initial population on lettuce (8.39 log CFU/lettuce sample) at the time of treatment was similar to those on apples (8.13 log CFU/apple) and tomatoes (8.15 log CFU/tomato). Lower numbers of *E. sakazakii* were removed from lettuce washed with water for 1 or 5 min, compared to populations removed by washing apples and tomatoes with water. This may indicate that the washing process is less efficient in removing *E. sakazakii* from the surface of lettuce. Reductions caused by treatment of lettuce with 10, 50, and 100 µg/ml chlorine for 1 min were in the range of 1.61 - 2.50, compared to the number remaining on lettuce after washing with water. Populations of *E. coli* O157:H7 inoculated on lettuce leaves and treated with 200 µg/ml chlorine for ≥ 1min have been reported to significantly decrease (Beuchat, 1999). Beuchat et al. (2004) reported a 0.86-log CFU/g reduction in *L. monocytogenes* population on iceberg lettuce treated with 100 µg/ml of chlorine. Treatment of lettuce with chlorine dioxide (100 µg/ml) for 1 min decreased the population of *E.*

Table 3-6
Populations of *E. sakazakii* recovered from lettuce treated with water or sanitizers for 1min

			Population (log CFU/lettuce)[a]					
			DE broth[b]			Treatment solution[b]		
Treatment	Concentration (μg/ml)	pH	log CFU/lettuce		Reduction[c]	log CFU/lettuce		Reduction
Chlorine	0	6.80	A 7.04	A	-	A 7.47	A	-
	10	6.88	B 5.43	BC	1.61	B < 2.60	B	≥ 4.87
	50	6.93	B 4.54	D	2.50	B < 2.60	B	≥ 4.87
	100	6.92	B 4.69	D	2.35	B < 2.60	B	≥ 4.87
Chlorine dioxide	0	6.93	A 7.04	A	-	A 7.47	A	-
	10	8.56	B 5.59	B	1.45	B < 2.60	B	≥ 4.87
	50	10.13	C 4.27	D	2.77	B < 2.60	B	≥ 4.87
	100	11.02	D 2.99	E	4.05	B < 2.60	B	≥ 4.87
Tsunami 200	0	6.94	A 7.04	A	-	A 7.47	A	-
	40	3.87	B 4.78	CD	2.26	B < 2.60	B	≥ 4.87
	80	3.37	B 4.59	D	2.45	B < 2.60	B	≥ 4.87

[a] Population before treatment was 8.39 log CFU/lettuce. Within the same sanitizer and solution (DE wash broth or treatment solution), mean values that are not preceded by the same letter are significantly different ($p \leq 0.05$). Mean values in the same column that are not followed by the same letter are significantly different ($p \leq 0.05$).
[b] Populations in DE broth after washing treated lettuce and in water and sanitizer solutions after treating lettuce were used to calculate log CFU/lettuce.
[c] Reduction in population after treatment compared with the population after treatment with water (control).

Table 3-7
Populations of *E. sakazakii* recovered from lettuce treated with water or sanitizers for 5 min

			Population (log CFU/tomato)[a]			
			DE broth[b]		Treatment solution[b]	
Treatment	Concentration (µg/ml)	pH	log CFU/lettuce	Reduction[c]	log CFU/lettuce	Reduction
Chlorine	0	6.80	A 7.16 A	-	A 7.41 A	-
	10	6.88	B 3.15 B	4.01	B < 2.60 B	≥ 4.81
	50	6.93	B 2.54 B	4.62	B < 2.60 B	≥ 4.81
	100	6.92	B < 1.70 B	≥ 5.46	B < 2.60 B	≥ 4.81
Chlorine dioxide	0	6.93	A 7.16 A	-	A 7.41 A	-
	10	8.56	B 2.88 B	4.28	B < 2.60 B	≥ 4.81
	50	10.13	B < 1.70 B	≥ 5.46	B < 2.60 B	≥ 4.81
	100	11.02	B < 1.70 B	≥ 5.46	B < 2.60 B	≥ 4.81
Tsunami 200	0	6.94	A 7.16 A	-	A 7.41 A	-
	40	3.87	B 1.85 B	5.31	B < 2.60 B	≥ 4.81
	80	3.37	B < 1.70 B	≥ 5.46	B < 2.60 B	≥ 4.81

[a] Population before treatment was 8.39 log CFU/lettuce. Within the same sanitizer and solution (DE wash broth or treatment solution), mean values that are not preceded by the same letter are significantly different ($p \leq 0.05$). Mean values in the same column that are not followed by the same letter are significantly different ($p \leq 0.05$).

[b] Populations in DE broth after washing treated lettuce and in water and sanitizer solutions after treating lettuce were used to calculate log CFU/lettuce.

[c] Reduction in population after treatment compared with the population after treatment with water (control).

sakazakii by 4.05 log CFU/sample, compared to treatment with water. This reduction was significantly higher than the 2.35 log CFU/sample reduction achieved by treating lettuce with 100 µg/ml chlorine for 1 min. At 10 and 50 µg/ml, chlorine and chlorine dioxide were equivalent in lethality to *E. sakazakii* on lettuce, regardless of treatment time. Treatment of lettuce with gaseous chlorine dioxide (4.3 mg/liter) has been reported to cause 3.4-log, 4.3-log, and 5.0-log reductions in *E. coli* O157:H7, *Salmonella* Typhimurium, and *L. monocytogenes*, respectively (Lee et al., 2004).

Treatment of lettuce with Tsunami 200 (80 µg/ml) for 1 min decreased the *E. sakazakii* population by 2.45 log CFU/sample, compared to washing with water. Treatment of lettuce with 100 µg/ml chlorine, 50 or 100 µg/ml chlorine dioxide, or 80 µg/ml Tsunami 200 for 5 min caused ≥ 5.46 log CFU/sample reductions in *E. sakazakii*, compared to treatment with water. Reductions caused by treatment with all test concentrations of chlorine, chlorine dioxide, and Tsunami 200 for 5 min were not significantly different. This is in agreement with observations reported by Beuchat et al. (2004) showing that the reductions of *L. monocytogenes* on pieces of iceberg lettuce, shredded iceberg lettuce, and Romaine lettuce were not significantly different upon treatment with 100 µg/ml chlorine or 80 µg/ml Tsunami 100. Overall, treatment of lettuce with chlorine dioxide caused the higher reductions in populations of *E. sakazakii*, compared to treatment with chlorine or Tsunami 200.

The effectiveness of chlorine, chlorine dioxide, and Tsunami 200 in killing *E. sakazakii* on lettuce was somewhat less than observed for apples and tomatoes. Crevices on the surface of lettuce may provide protection for cells, limiting exposure to sanitizers. In addition, the cut edges of the lettuce released tissue fluids that may neutralize sanitizers, thereby reducing bactericidal activity. Other foodborne pathogens have exhibited higher resistance to sanitizers

on lettuce, compared to other produce. Chlorine dioxide was less effective in killing *E. coli* O157:H7 on lettuce than on baby carrots (Singh et al., 2002). Populations of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* spot-inoculated on lettuce were reduced by less than 2 logs upon treatment with chlorine, whereas treatment of parsley caused a reduction of ≥ 3.30 log CFU/parsley sample (Lang et al., 2004). Sy et al. (2005) reported that lower reductions (< 2 log CFU/g) in *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* occurred on fresh-cut lettuce than on cabbage and carrot treated with gaseous chlorine dioxide. Rodgers et al. (2004) showed that longer treatment times were needed to inactivate *E. coli* O157:H7 and *L. monocytogenes* on lettuce than on apples with peracetic acid (80 μg/ml) or chlorine dioxide (3 and 5 μg/ml). They also showed that peracetic acid and chlorine dioxide were significantly more effective in killing *L. monocytogenes* and *E. coli* O157:H7 on whole lettuce leaves than on shredded lettuce.

4. Summary

In summary, *E. sakazakii* has been shown to survive on produce stored at refrigerator and ambient temperatures. Retention of viability is enhanced at refrigerator temperatures. Chlorine, chlorine dioxide, and Tsunami 200 caused significant reductions of *E. sakazaki*i on apples, tomatoes, and lettuce, although the extent of lethality depended on the type of produce and sanitizer concentration. Further studies to determine the survival, growth, and sanitizer resistance characteristics of *E. sakazakii* attached to surfaces that produce may come in contact with in processing and preparation areas, as well as the presence of *E. sakazakii* in biofilms on these surfaces, are needed. These studies will provide valuable information when developing interventions to control the pathogen and reduce any risk of illness that may exist.

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CHAPTER 4

ATTACHMENT AND BIOFILM FORMATION BY *ENTEROBACTER SAKAZAKII* ON STAINLESS STEEL AND ENTERAL FEEDING TUBES [1]

[1] Kim, Hoikyung and Larry R. Beuchat. Accepted for publication in *Applied and Environmental Microbiology*, 2006. Reprinted here with permission of publisher, 2006

ABSTRACT

Enterobacter sakazakii has been reported to form biofilms but environmental conditions affecting attachment to and biofilm formation on abiotic surfaces have not been described. We did a study to determine the effects of temperature and nutrient availability on attachment and biofilm formation by *E. sakazakii* on stainless steel and enteral feeding tubes. Five strains grown to stationary phase in tryptic soy broth (TSB), infant formula broth (IFB), and lettuce juice broth (LJB) at 12°C and 25°C were examined for the extent to which they attach to these materials. Higher populations attached at 25°C than at 12°C. Stainless steel coupons and polyvinyl chloride enteral feeding tubes were immersed for 24 h at 4°C in phosphate-buffered saline suspensions (7 log CFU/ml) to facilitate attachment of 5.33 - 5.51 and 5.03 - 5.12 log CFU/cm^2, respectively, before immersing in TSB, IFB, or LJB and incubating at 12°C or 25°C for up to 10 days. Biofilms were not produced at 12°C. The number of cells of test strains increased by 1.42 - 1.67 log CFU/cm^2 and 1.16 - 1.31 log CFU/cm^2 in biofilms formed on stainless steel and feeding tubes, respectively, immersed in IFB at 25°C; biofilms were not formed on TSB and LJB at 25°C, indicating that nutrient availability plays a major role in processes leading to the accumulation of biometrices on the surfaces of these inert materials. These observations emphasize the importance of temperature control in reconstituted infant formula preparation and storage areas in preventing attachment and biofilm formation by *E. sakazakii*.

Keywords: *Enterobacter sakazakii*, biofilm, infant formula, lettuce

INTRODUCTION

Enterobacter sakazakii is a foodborne pathogen capable of causing meningitis (4, 12, 35), sepsis (35), bacteremia (37), and necrotizing enterocolitis (52) in preterm neonates and immunocompromised adults (8, 14, 23, 28, 42). Powdered infant formula and milk powder have been implicated as vehicles in outbreaks of *E. sakazakii* infections (3, 17, 34, 35, 37, 48, 52). *E. sakazakii* was detected in 20 of 141 (14.2 %) powdered infant formulas originating from 13 countries (34). The pathogen has been isolated from various clinical sources, food processing plants, the environment (21, 25), lettuce (49), alfalfa sprouts (5), tomatoes (24), and other vegetables, cheese, minced beef, and sausage (29). Its presence in fresh produce raises the possibility of this food group serving as a vehicle of the pathogen for infections in immunocompromised adults, particularly patients in hospitals and elderly adult assisted-care facilities.

Attachment of bacterial cells to surfaces may be followed by growth, production of exopolysaccharide, and biofilm formation (27). Biofilms have been defined as sessile communities of cells attached to a surface or to each other, usually embedded in polymeric substances produced by the bacteria (31). Attachment of microorganisms to biotic or abiotic surfaces followed by biofilm formation is known to enhance the resistance of cells to environmental stresses and provide protection against sanitizers (11, 27, 38, 45). Factors affecting attachment and biofilm formation by microorganisms include nutrient availability, pH of the surrounding medium, and nature of the cell and abiotic surfaces (10).

E. sakazakii has been reported to be able to attach to and form biofilms on silicon, latex, polycarbonate, stainless steel, glass, and polyvinyl chloride (PVC) (22, 30). Foods such as powdered infant formula and fresh produce represent potential vehicles of *E. sakazakii* infections

in infants and immunocompromised adults, respectively. Contact of these and other foods containing the pathogen with abiotic or biotic surfaces could result in attachment and biofilm formation. Attachment or biofilms of *E. sakazakii* on equipment surfaces used in formula preparation and feeding areas or in produce processing plants may increase the risk of infections to infants and immunocompromised adults, respectively. *E. sakazakii* colonization on surfaces of a spoon, brush, and blender used for infant formula preparation has been documented to occur in a clinical setting where neonatal infections were reported (1, 35, 37, 48). Reuse of enteral feeding tubes and delivery bags after washing may increase the risk of microbial infections (39). Biofilm formation by pathogens on fresh fruits and vegetables during harvesting, transporting, processing, and storage has been conjectured to potentially increase the risk of foodborne diseases in individuals consuming these products (2). We have observed that *E. sakazakii* is able to grow on fresh-cut produce and in produce juice (26).

Removal or inactivation of pathogens on inert surfaces in infant formula preparation areas and produce processing environments by washing with water or treating with disinfectants or sanitizers is not always achieved, possibly because cells are enmeshed in biofilms or otherwise protected against exposure to antimicrobials. Attachment and biofilm formation by *E. sakazakii* as affected by temperature and nutrient availability have been given only meager research attention. The objectives of this study were to determine growth characteristics of *E. sakazakii* in a rich microbiological medium, reconstituted infant formula, and lettuce juice as a produce model and characterize subsequent attachment of stationary phase cells and biofilm formation on the surfaces of stainless steel and enteral feeding tubes as affected by temperature and nutrients provided by these media.

MATERIALS AND METHODS

Strains examined. Five clinical strains (2855, 3231, 3234, 3290, and 3295), four strains (2871, 3270, 3437, and 3439) isolated from foods, and one strain (3396) isolated from an environmental source were examined for colony morphology on tryptic soy agar (TSA; BBL/Difco, Sparks, Md.), infant formula agar (IFA), and lettuce juice agar (LJA) (see **Preparation of media**, below). Cultures grown into 10 ml of tryptic soy broth (TSB; BBL/Difco) at 37°C for 24 h were streaked on TSA, IFA, and LJA and incubated at 12, 25, and 37°C for 6, 2, and 1 days, respectively. Three strains (3231, 3234, and 3396) producing colonies distinctly wet and mucoid in appearance and three strains (2855, 3295, and 3439) producing drier non-mucoid colonies were selected for determining growth curves in TSB, infant formula broth (IFB), and lettuce juice broth (LJB). Strains 3231, 3234, 3295, 3439, and 3396 were used for the attachment study; because strain 2855 showed a very different growth pattern compared to the other five strains, it was not included. For the biofilm study, *E. sakazakii* strain 3231, which produces mucoid colonies, and strain 3439, which produces non-mucoid colonies on IFA, were used to determined if there are significantly different patterns of attachment or biofilm formation based, in part, on these characteristics.

Preparation of media. Similac Neosure Advance infant formula (Ross Pediatrics, Abbott Laboratories, Columbus, Ohio) was selected to make infant formula broth (IFB) because preliminary studies showed that the development of mucoid colonies by some of the test strains was more evident on IFA prepared using this formula than on IFA prepared using four other formulas. Milk-based infant formulas such as Similac Neosure Advance are consumed more widely than are soy-based formulas, giving additional incentive for its use in attachment and biofilm studies. IFB (pH 6.6) was made by combining Similac Neosure Advance infant formula

with distilled water at a 1:10 ratio (w/v), dissolving by heating at 50 - 60°C, and autoclaving. To prepare LJB (pH 6.3), iceberg lettuce (*Lactuca sativa* L.) was blended in an automatic juice extractor (The Juiceman Jr., Trillium Health Products, J. M. Marketing, Inc., Seattle, Wash.), filtered through 16 layers of coarsely woven cheese cloth, brought to a boil, and re-filtered through 16 layers of cheese cloth to remove coarse particles. TSB (pH 7.0) and TSA were prepared according to the manufacturer's directions. Powdered infant formula (100 g) and agar (BBL/Difco) (15 g) were combined with 900 ml of distilled water, heated to dissolve, and autoclaved (15 min at 121°C) to make IFA. LJA was made by adding 15 g of agar to 1 liter of LJB, heated to dissolve, and autoclaved. For the growth curve study, 300 ml of each broth in 500-ml capped bottles were used. For attachment and biofilm experiments, 30 ml of each broth in 50-ml centrifuge tubes were used.

Phosphate-buffered saline (PBS) (pH 7.4) used to resuspend *E. sakazakii* cells for use in biofilm experiments was prepared by combining NaCl (8 g), KCl (0.2 g), Na_2HPO_4 (1.44 g), and KH_2PO_4 (0.24 g) with distilled water (1 liter) and autoclaving. Glass beads (425 - 600 microns, Sigma-Aldrich, St. Louis, Mo.) were added to PBS to facilitate removal of adherent *E. sakazakii* cells from the surfaces of stainless steel and the feeding tubes. PBS (30 ml) containing 3 g of acid-washed glass beads in 50-ml centrifuge tubes was sterilized by autoclaving. All media and the PBS were stored at 4°C and used within 2 days.

Determination of growth curves. *E. sakazakii* strains 2855, 3231, 3234, 3295, 3439, and 3396 grown in TSB at 37°C for 24 h were serially diluted in sterile distilled water to make suspensions containing ca. 4 log CFU/ml. Three milliliters of each suspension were added to 300 ml of TSB, IFB, and LJB and the mixture was shaken at 250 rpm using a Controlled Environment Incubator Shaker (New Brunswick Scientific Co. Edison, N.J.) for 1 min. The

inoculated TSB, IFB, and LJB were incubated at 12 or 25°C for up to 10 days. Populations of *E. sakazakii* were determined regular intervals up to 10 days. At each sampling time, 0.1 ml was surface plated on TSA using an Autoplate 4000 Automated Spiral Plater (Spiral Biotech, Norwood, Mass.). Colonies formed on plates incubated at 37°C for 24 h were counted.

Preparation of enteral feeding tubes and stainless steel coupons for attachment and biofilm studies. Enteral feeding tubes (PVC, 0.2 cm external diameter, Fr 7; Vygon Co., Ltd., Norristown, Pa.) were used. The feeding tubes were cut into 5-cm long pieces with a sterile blade and the ends of each piece were sealed by melting with flame followed by applying pressure. The pieces of feeding tubes were immersed in 70% ethanol for 10 min to surface disinfect, followed by drying in a laminar flow biosafety hood. Each piece of tube was placed in a sterile 15-ml test tube. The outer surface of each piece was 3.14 cm^2.

Stainless steel coupons (type 304, 5 cm × 2 cm, surface area = 20 cm^2) with no. 4 finish were also used in attachment and biofilm studies. Coupons were sonicated (model 250D, VWR, Chester, Pa.) in a 15% phosphoric acid solution at 80°C for 20 min, rinsed with distilled water, sonicated in alkali detergent solution (FS Pro-Chlor; Zep, Atlanta, Ga.) at 80°C for 20 min, and rinsed again with distilled water. Washed stainless steel coupons were dried, placed in 50-ml test tubes, and autoclaved.

Preparation of cell suspension for attachment to enteral feeding tubes and stainless steel. *E. sakazakii* strains 3231, 3234, 3295, 3439, and 3396 grown in TSB at 37°C for 24 h were serially diluted in sterile distilled water to give ca. 4 log CFU/ml. TSB, IFB, and LJB (500 ml) were inoculated with each cell suspension (5 ml) to give ca. 2 log CFU/ml and mixed thoroughly. The inoculated TSB, IFB, and LJB were incubated at 25°C for 2 days; TSB was incubated at 12°C for 6 days, and IFB and LJB were incubated at 12°C for 8 days.

Attachment of *E. sakazakii*. Stationary-phase cultures (25 ml) were deposited in sterile 50-ml test tubes, each containing a sterile stainless coupon; 10 ml of each culture were deposited in a sterile 15-ml test tube containing a sterile piece of feeding tube. Stainless steel coupons and feeding tubes immersed in cultures which had been grown at 12 and 25°C were, respectively, incubated at 12 and 25°C for 4 h to facilitate attachment of cells, aseptically removed with a sterile forceps, immersed in 400 ml of sterile distilled water (22 ± 2°C), and gently agitated for 15 sec. Coupons and tubes were rinsed in 200 ml of sterile distilled water with agitation for 5 sec, then deposited in 30 ml of sterile PBS containing 3 g of glass beads in 50 ml-centrifuge tubes. The PBS containing a stainless steel coupon or a feeding tube with 3 g of glass beads was vortexed (model Vortex Genie-2; Scientific Industries, Inc., Bohemia, N.Y.) at maximum speed for 1 min. Immediately after vortexing, suspensions were serially diluted in 0.1% peptone water and surface plated (0.1 ml, in duplicate) on TSA to determine populations of *E. sakazakii* attached to the surfaces of stainless steel coupons and feeding tubes. Simultaneously, media in which cells had grown and attached to surfaces of stainless steel coupons and feeding tubes were serially diluted in 0.1% peptone water and surface plated on TSA to determine the number of planktonic cells in the surrounding media.

Biofilm formation. *E. sakazakii* strains 3231 and 3439 grown to stationary phase in TSB at 37°C for 24 h were centrifuged (4,000 × *g*, 15 min, 4°C) and cells were resuspended in PBS (pH 7.4) to give 7 log CFU/ml. Cell suspensions were kept at 4°C for no longer than 30 min to minimize changes in cell numbers and physiological state before use in experiments. Suspensions (25 ml) of each strain were deposited in sterile 50-ml test tubes containing a sterile stainless coupon; 10 ml of suspension were deposited in a sterile 15-ml test tube containing a sterile piece of feeding tube. After incubating the coupons and tubes at 4°C for 24 h, they were

removed from test tubes with a sterile forceps, immersed in 400 ml of sterile distilled water (22 ± 2°C) for 15 sec, and rinsed in 200 ml of sterile distilled water with gently agitation for 5 sec. The rinsed stainless steel coupons and feeding tubes were deposited, respectively, in 30 ml and 10 ml of TSB, IFB, and LJB in 50-ml and 15-ml centrifuge tubes, respectively, and incubated at 12 or 25°C for up to 10 days. Numbers of *E. sakazakii* in biofilms formed on coupons and tubes were determined on day 0 (24 h after immersing in PBS cell suspension at 4°C), and after 2, 4, 6, 8, and 10 days at 12°C and 1, 2, 4, 6, 8, and 10 days at 25°C. Coupons and tubes were removed from each medium and rinsed in 400 ml of sterile water (22 ± 2°C) with agitation for 15 sec. After rinsing again in sterile distilled water (200 ml) for 5 sec, coupons and tubes were placed in 50-ml centrifuge tubes containing 3 g of glass beads and 30 ml of sterile PBS and vortexed for 1 min. The PBS suspension and each medium in which coupons or tubes had been immersed were serially diluted in 0.1% peptone water, surface plated (0.1 ml, in duplicate) on TSA, and incubated at 37°C. The number of colonies formed on plates within 24 h was counted.

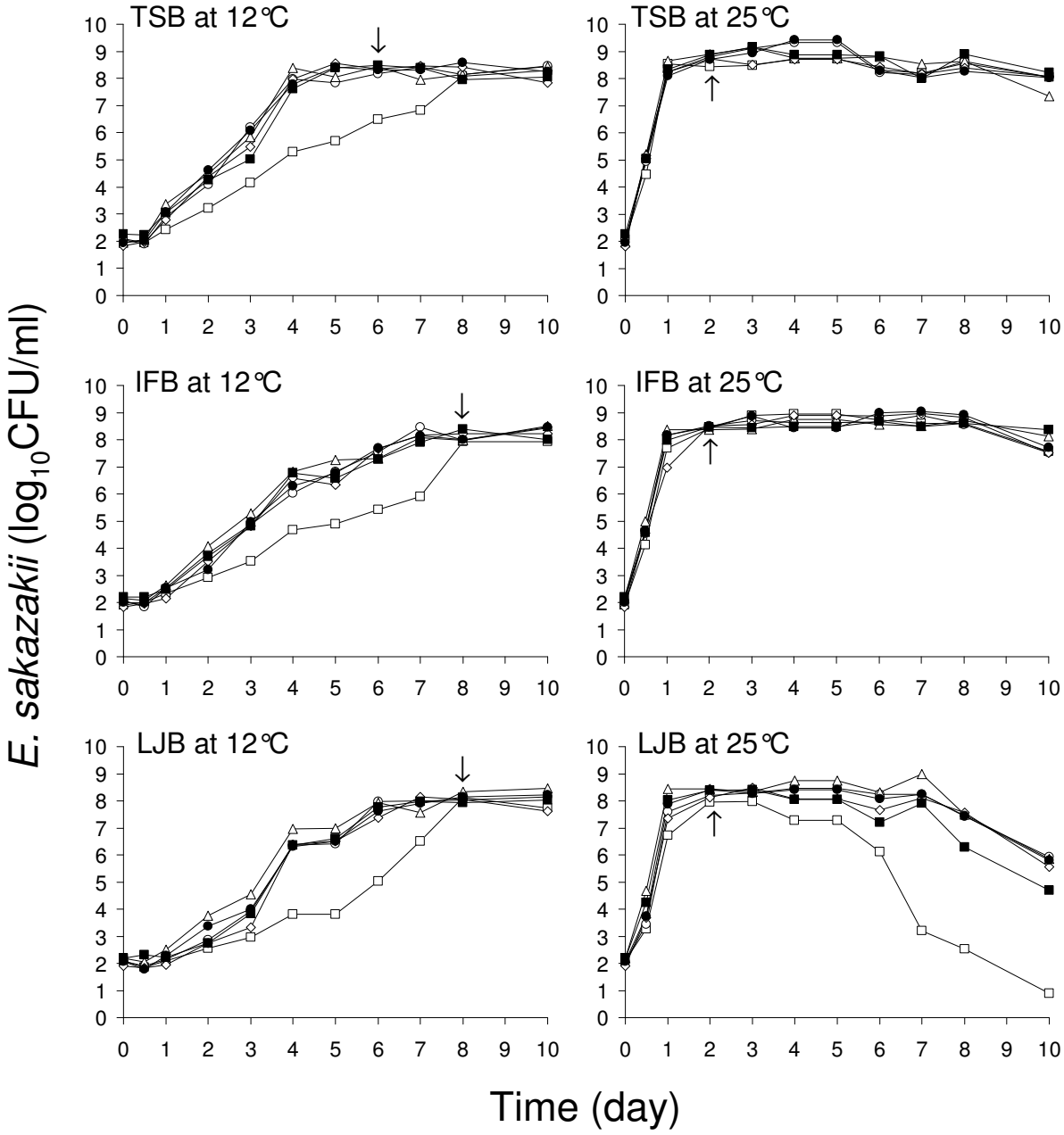
Statistical analysis. All experiments were performed in triplicate and two samples representing each combination of test parameters were analyzed at each sampling time. Data were analyzed using the general linear model of the Statistical Analysis Systems procedure (SAS; SAS Institute, Cary, N.C.). Fisher's Least Significant Difference (LSD) test was used to determine if attachment and biofilm formation of *E. sakazakii* on stainless steel coupons and enteral feeding tubes were significantly affected by temperature and type of media. Significant differences are presented at a 95% confidence level ($P \leq 0.05$).

RESULTS AND DISCUSSION

Growth curves of *E. sakazakii*. Six strains (2855, 3231, 3234, 3295, 3439, and 3396) of *E. sakazakii* were examined for growth characteristics in TSB, IFB, and LJB at 12 and 25°C. Growth characteristics varied, depending on the strain, medium, and incubation temperature. Figure 4-1 shows the growth curves of the six test strains in TSB, IFB, and LJB at 12 and 25°C. All strains except strain 2855 showed very similar growth rates in TSB at 12°C. Strains 3231, 3234, 3295, 3439, and 3396 reached the stationary phase in TSB within 6 days at 12°C, but strain 2855 required about 8 days to reach the stationary phase. Populations of the first five strains increased to 7.63 - 8.38 log CFU/ml within 4 days at 12°C. However, the growth rate of strain 2855 in TSB at 12°C was significantly slower than the other five strains. At 25°C, all six strains showed very similar growth patterns in TSB throughout the first 8 days of incubation. Populations of all strains reached 8.09 - 8.64 log CFU/ml within 1 day at 25°C and stationary phase within 1 - 2 days. Growth rates of *E. sakazakii* in TSB at temperatures ranging from 6 to 49°C were determined by Iversen et al. (22). In their study, the higher the temperature (< 40°C), the faster the rate of growth.

E. sakazakii reached early stationary phase of growth within 7 - 8 days in IFB at 12°C (Figure 4-1). With the exception of strain 2855, populations in IFB incubated at 12°C increased to 7.30 - 7.68 log CFU/ml within 6 days. As in TSB, compared to the other five strains, strain 2855 grew markedly slower in IFB. All strains reached the early stationary phase of growth in IFB within 1 - 2 days at 25°C. Others have studied the growth rate of *E. sakazakii* in infant formulas and cereals. At temperatures between 6 and 30°C, growth rates in TSB were similar to those in infant formula milk (22). In our study, the growth rate of *E. sakazakii* in IFB at 12°C

Figure 4-1. Populations of *E. sakazakii* strains 2855 (□), 3231 (○), 3234 (△), 3295 (◇), 3439 (■), and 3396 (●) grown in TSB, IFB, and LJB at 12°C and 25°C for up to 10 days. Arrows (↓) indicate early stationary phase of growth for strains 3231, 3234, 3295, and 3396 at 12°C and all test strains at 25°C.


TSB at 12℃
TSB at 25℃
IFB at 12℃
IFB at 25℃
LJB at 12℃
LJB at 25℃
E. sakazakii (log10CFU/ml)
Time (day)

was slower than that in TSB. Differences in growth rate observed in the two studies are attributed in part to differences in nutrient content of the infant formula. Richards et al. (44) reported that *E. sakazakii* grew to populations of 7.5 and 4.6 log CFU/ml within 72 h at 21 and 12°C, respectively, in infant rice cereal reconstituted with liquid infant formula initially containing ca. 1.0 log CFU/ml.

The growth curves of *E. sakazakii* in LJB incubated at 12 °C were very similar to those observed in IFB at 12°C (Figure 4-1). All test strains reached early stationary phase after 1 - 2 days and 7 - 8 days of incubation at 12 and 25°C, respectively. *E. coli* O157:H7 grown in LJB reached the stationary phase of growth within 1 - 2 days at 22°C and 4 - 10 days at 12°C (46). In a previous study (26), we observed that *E. sakazakii* reached a maximum population (ca. 5 log CFU/ml) within 24 h in unpasteurized lettuce juice at 25°C, began to decrease at 36 h, and was undetectable (< 1 log CFU/ml) after 72 h. The LJB used in the present study was sterilized, so a reduction in pH caused by fermentation by microorganisms naturally present in unpasteurized lettuce juice that may otherwise have an effect on viability of *E. sakazakii* did not play a role. *E. sakazakii* has been known to survive for > 4 days at 12°C when inoculated into unpasteurized lettuce juice at a population of ca. 1.5 log CFU/ml (26). Even though all six test strains reached the stationary phase within 2 days at 25°C in IFB and LJB, death of strain 2855 and the other five strains in LJB began after 3 and 7 days, respectively. Populations of strain 2855 decreased significantly ($P \leq 0.05$) from 7.98 log CFU/ml at 3 days to 7.29 log CFU/ml after 4 days, 3.20 log CFU/ml after 7 days, and 0.90 log CFU/ml after 10 days in LJB at 25°C. This strain clearly showed growth and death patterns different than those of the other five strains. Lower amounts and different types of nutrients in LJB, compared to TSB and IFB, may have shortened the stationary phase of growth. A lower buffering capacity of LJB, compared to TSB and IFB, may

also have contributed to the rapid initiation of the death phase in LJB. Fermentation of sugars in all test media would result in acid production, with concomitant decreases in pH, particularly if the buffer capacity is minimum as would be the case in LJB. Since, the growth rate of strain 2855 in TSB, IFB, and LJB was slower than that of the five other strains in those media, particularly at 12°C, it was not included in subsequent studies on attachment and biofilm formation. The arrows in Figure 4-1 indicate the incubation times at which cells were harvested for use in an attachment experiment.

Attachment of *E. sakazakii*. Attachment and biofilm formation by *E. sakazakii* on abiotic surfaces was simulated in the laboratory. Stainless steel and enteral feeding tubes were used as models and TSB, IFB, and LJB served as nutrient sources. Cells in early stationary phase of growth were used to minimize the potential influence of different physiological states on attachment and biofilm formation. The effect of temperature on attachment of strains 3231, 3234, 3295, 3439, and 3396 grown in TSB, IFB, and LJB at 12 and 25°C to stainless steel and enteral feeding tubes immersed for 4 h at the same respective temperatures in the same respective broths was investigated. Populations of *E. sakazakii* in early stationary phase that remained in TSB, IFB, and LJB, i.e., planktonic cells, in which tubes were immersed for 4 h at 12 or 25°C, as well as the number of cells which attached to the tubes, are shown in Table 4-1. Strains 3231 and 3396 attached in significantly higher ($P \leq 0.05$) numbers to the feeding tubes immersed in a given broth at 25°C than at 12°C. Strains 3295 and 3439 in TSB and strain 3234 in TSB and IFB attached in significantly higher ($P \leq 0.05$) numbers to the feeding tubes at 25°C than at 12°C. Except for strains 3234, 3295, and 3439 at 12°C, significantly higher ($P \leq 0.05$) numbers of planktonic cells were recovered from TSB and IFB than from LJB incubated at 12 and 25°C. Higher numbers of planktonic cells in media did not always correlate higher numbers

Table 4-1. Populations of *E. sakazakii* in suspension and attached to surfaces of enteral feeding tubes immersed in TSB, IFB, and LJB at 12 and 25°C for 4 h[a].

		12°C		25°C	
		Attached	Planktonic	Attached	Planktonic
Strain	Media	(log CFU/cm^2)	(log CFU/ml)	(log CFU/cm^2)	(log CFU/ml)
3231	TSB	b 5.47 b	b 7.89 a	a 6.09 a	a 8.93 a
	IFB	b 5.86 a	a 8.17 a	a 6.16 a	a 9.10 a
	LJB	b 2.04 c	b 6.09 b	a 4.67 b	a 7.47 b
3234	TSB	b 2.88 a	a 8.05 a	a 4.31 a	a 8.99 a
	IFB	b 2.81 a	b 8.13 a	a 3.55 b	a 8.96 a
	LJB	a 2.34 a	a 7.47 a	a 3.05 c	a 8.11 b
3295	TSB	b 3.14 b	b 7.86 a	a 5.54 a	a 8.92 a
	IFB	a 5.17 a	b 7.82 a	a 5.33 a	a 8.73 a
	LJB	a 3.60 b	a 7.81 a	a 3.76 b	a 7.52 b
3439	TSB	b 3.59 b	b 7.59 a	a 6.03 a	a 8.94 a
	IFB	a 5.28 a	b 7.75 a	a 5.45 b	a 8.91 a
	LJB	a 2.87 b	a 7.20 a	a 3.09 c	a 7.99 b
3396	TSB	b 5.06 b	a 8.05 a	a 6.29 a	a 8.79 a
	IFB	b 5.79 a	b 8.11 a	a 6.28 a	a 9.22 a
	LJB	b 3.35 c	b 6.76 b	a 4.81 b	a 8.12 b

[a] Within the same strain, mean values that are not followed by the same lowercase letter are significantly different ($P \leq 0.05$). Within the same strain, mean values that are not followed by the same capital letter are significantly different ($P \leq 0.05$).

of cells attached to the tubes. This could be due in part differences in the initial number of planktonic cells in the three test broths. Except for strain 3234 at 12°C, cells of test strains attached in significantly higher ($P \leq 0.05$) numbers to enteral feeding tubes immersed in IFB than to tubes in LJB incubated at 12 and 25°C. At 25°C, significantly higher ($P \leq 0.05$) numbers of all strains attached to the tubes in TSB and IFB than in LJB.

Compared to cells grown in LJB, cells grown in TSB and IFB may have more ability to attach to the hydrophobic surfaces of enteral feeding tubes. A major difference among the three broths is that TSB and IFB are protein-rich media, compared to LJB. TSB and IFB used in our study both contain casein and IFB also contains whey protein. Other researchers have determined that nutrients and other components in media affect attachment of microorganisms to surfaces of various materials. Hood and Zottola (19) concluded that composition of growth and conditioning media influence the attachment of *S. enterica* serovar Typhimurium and *L. monocytogenes* to stainless steel surfaces. They observed that *S. enterica* serovar Typhimurium grown in TSB and *L. monocytogenes* grown in 1% reconstituted skim milk supplemented with sucrose attach in the highest numbers to stainless steel chips which were conditioned with TSB and 1% reconstituted skim milk, respectively, when TSB, 1% reconstituted skim milk, and 1% reconstituted skim milk with sucrose were used as growth and conditioning media. Casein has been shown to promote attachment of *E. coli*, *Pseudomonas fluorescens*, and *Aeromonas liquifaciens* to glass surfaces (33) and attachment of milk-associated microorganisms to stainless steel surfaces was enhanced in the presence of whey protein (50). The opposite effects were observed by others. Milk and milk protein were reported to inhibit adhesion of *L. monocytogenes*, *S.* Typhimurium, *S. aureus*, *Serratia marcescens*, and *E. coli* to abiotic surfaces, including stainless steel or Buna-N rubber (15, 18, 19). These observations were attributed to

repulsion between the negative charges of milk protein and bacterial cells (15) and equilibrium between protein in the surrounding medium and proteins which absorb to contact surfaces (32).

Table 4-2 shows populations of *E. sakazakii* attached to the surface of stainless steel coupons and numbers of planktonic cells in the media at the end of the 4-h immersion period. Significantly higher ($P \leq 0.05$) numbers of strains 3234 and 3439 attached to the surfaces of stainless steel coupons immersed in a given broth at 25°C, compared to 12°C. Strains 3231, 3295, and 3396 also showed the same trend, although in some cases the number of cells that attached at 25°C in a particular broth was not statistically different than the number that attached at 12°C. Attachment of *E. sakazakii* to the surface of stainless steel was not correlated with the type of medium in which cells had grown or the number of planktonic cells in the surrounding medium. Except for strain 3439, the number of *E. sakazakii* cells that attached to stainless steel immersed in TSB and IFB was not significantly different than the number that attached to coupons in LJB at 25°C. Under the same attachment conditions, in most cases, higher numbers of *E. sakazakii* adhered to the feeding tubes than to stainless steel. Stainless steel is moderately hydrophilic whereas PVC (enteral feeding tube) is highly hydrophobic. Most bacteria adhere more readily to hydrophobic surfaces (43).

Influences of temperatures on attachment of other foodborne pathogens to biotic and abiotic surfaces have been investigated by others. Our observations are in agreement with study by Gorski et al. (13), who reported that temperature plays a role in attachment of *L. monocytogenes* to radish tissue. In their study, *L. monocytogenes* cells attached in higher numbers to the radish tissues at 20°C than at 10°C after ≥ 1 h of exposure. Higher numbers of *E. coli* O157:H7 attached to lettuce leaf at 22°C than at 4 or 10°C (51). Factors affecting attachment at different temperatures, however, may vary, depending on the nature of surfaces to

Table 4-2. Populations of *E. sakazakii* in suspension and attached to surfaces of stainless steel coupons immersed in TSB, IFB, and LJB at 12 and 25°C for 4 h[a].

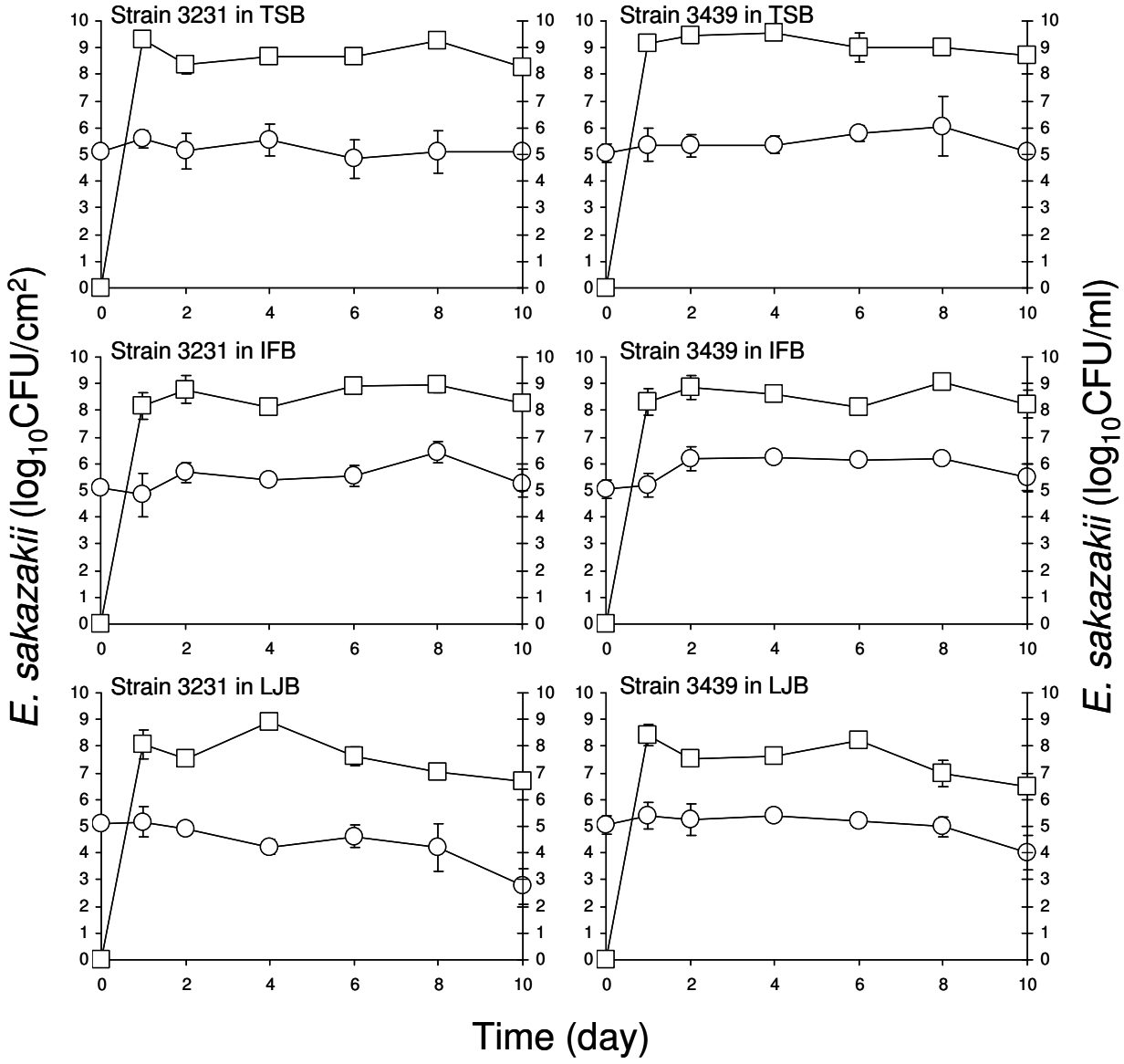
		12°C		25°C	
		Attached	Planktonic	Attached	Planktonic
Strain	Media	(log CFU/cm^2)	(log CFU/ml)	(log CFU/cm^2)	(log CFU/ml)
3231	TSB	b 3.55 b	a 7.98 b	a 4.50 a	a 8.83 a
	IFB	b 3.41 b	a 8.57 a	a 4.28 a	a 9.14 a
	LJB	a 4.19 a	a 7.65 b	a 4.45 a	a 7.69 b
3234	TSB	b 3.46 a	a 8.24 a	a 4.17 a	a 8.85 b
	IFB	b 3.01 b	a 7.96 a	a 4.02 a	a 9.25 a
	LJB	b 2.30 c	a 7.55 a	a 3.71 a	a 7.65 c
3295	TSB	b 2.92 b	a 7.30 a	a 4.53 a	a 8.39 a
	IFB	a 4.06 a	b 7.54 a	a 4.10 a	a 8.87 a
	LJB	b 3.31 b	a 7.41 a	a 4.81 a	a 7.22 b
3439	TSB	b 3.64 a	a 8.01 a	a 4.62 a	a 9.03 a
	IFB	b 3.60 a	a 7.82 a	a 4.31 a	a 8.11 ab
	LJB	b 3.01 b	a 7.36 a	a 3.95 b	a 7.54 b
3396	TSB	b 3.54 a	a 7.49 a	a 5.11 a	a 8.56 a
	IFB	a 4.07 a	b 7.79 a	a 4.78 a	a 8.74 a
	LJB	a 4.02 a	a 7.74 a	a 4.36 a	a 7.64 b

[a] Within the same strain, mean values that are not followed by the same lowercase letter are significantly different ($P \leq 0.05$). Within the same strain, mean values that are not followed by the same capital letter are significantly different ($P \leq 0.05$).

which cells are exposed. Pompermayer and Gaylarde (40), for example, showed that higher numbers of *S. aureus* attached to polypropylene at 12°C than at 30°C after ≥ 4 h of contact time. They also observed that numbers of *E. coli* attached to polypropylene within 4 h of exposure at 12°C and 30°C were not significantly different. Adhesion of *Salmonella enterica* serovar Montevideo to tomatoes and tomatillos was not influenced by temperature alone (12, 22, and 30°C) but also by combinations of temperature and relative humidity (20). *E. sakazakii* grown under different conditions in our study could have had different surface charges and hydrophobicity, which are known to affect the ability of bacterial cells to adhere to surfaces at various temperatures (9, 53).

Biofilm formation by *E. sakazakii*. Stainless steel coupons and enteral feeding tubes were immersed in cell suspensions in PBS (ca. 7 log CFU/ml) at 4°C for 24 h to facilitate attachment, followed by rinsing, transferring into TSB, IFB, or LJB, and incubating at 12 or 25°C for up to 10 days. Figure 4-2 shows the number of *E. sakazakii* cells (strains 3231 and 3439) retrieved from biofilms that formed on feeding tubes. The number of *E. sakazakii* cells initially attached to the surface of the feeding tubes did not change significantly ($P \leq 0.05$) during immersion in TSB at 25°C. Some of the cells would be expected to naturally detach from the surface due to the changes in physiochemical properties (46), starvation (41), and death but, on balance, the number remained the same throughout the 10-day period. Equilibrium was apparently reached between the number of cells that detached from the tube surface, newly-attached cells originating from TSB, and newly formed cells originating from cells attached to or in biofilms on the tube surface. Populations of planktonic cells of strains 3231 and 3439 in TSB increased to ≥ 9.15 log CFU/ml within 1 day, which was followed by slight decreases during following 9 days. *E. sakazakii* initially attached to the feeding tubes grew when the tubes were

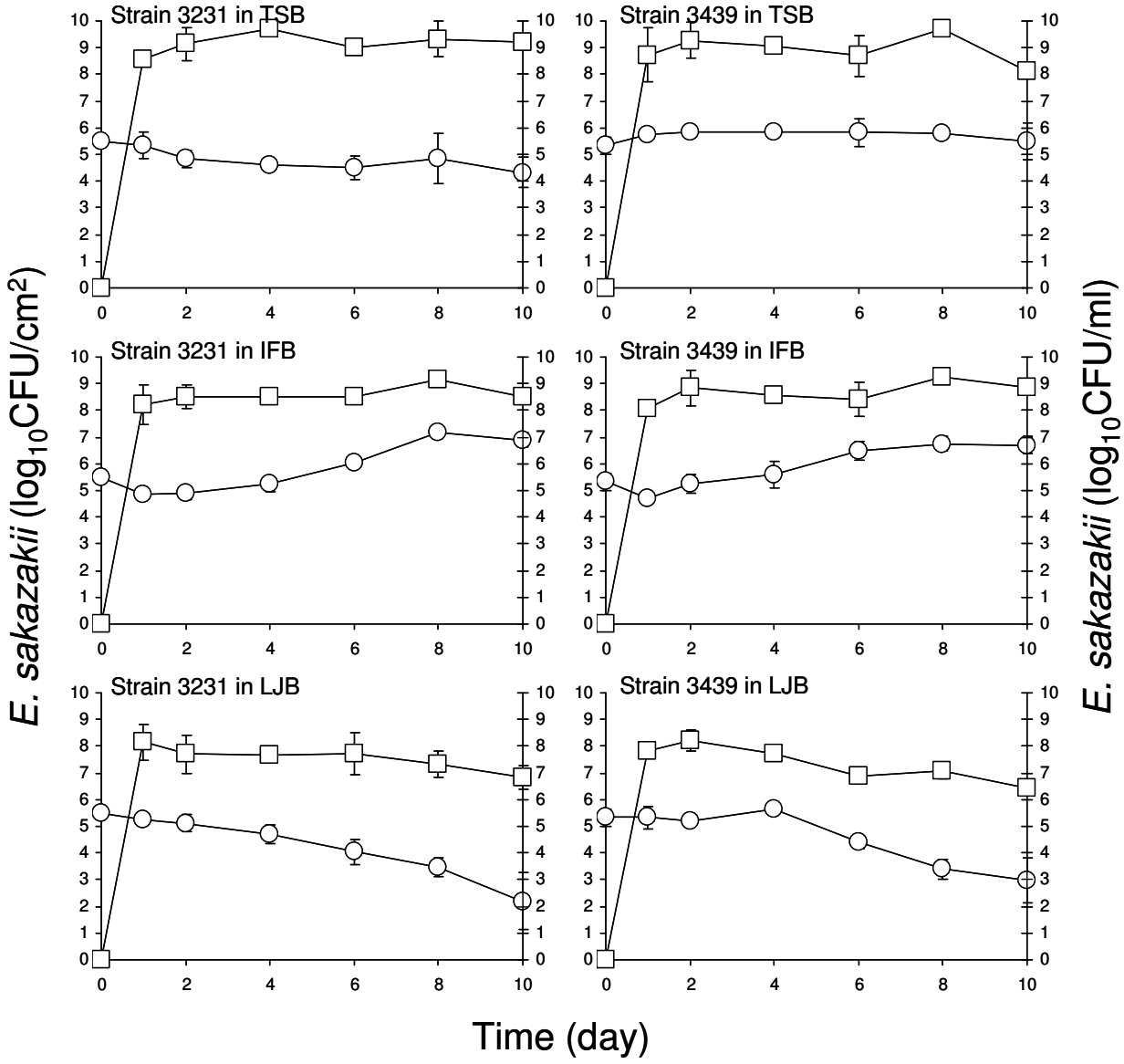
Figure 4-2. Populations of *E. sakazakii* strains 3231 and 3396 attached to the surface or in biofilms formed on the surface of enteral feeding tubes (log CFU/cm^2, ○) immersed in TSB, IFB, and LJB and in broths (log CFU/ml, □) in which tubes were immersed and incubated at 25°C for up to 10 days. Bars indicate standard deviations.


Strain 3231 in TSB
Strain 3439 in TSB
Strain 3231 in IFB
Strain 3439 in IFB
Strain 3231 in LJB
Strain 3439 in LJB
E. sakazakii (log10CFU/cm2)
E. sakazakii (log10CFU/ml)
Time (day)

immersed in IFB at 25°C. Populations of strains 3231 and 3439 increased significantly ($P \leq 0.05$) from initial populations of 5.12 and 5.03 CFU/cm^2, respectively, to 6.43 and 6.20 log CFU/cm^2 on the feeding tube surface at 8 days. Planktonic *E. sakazakii* in IFB surrounding tubes reached the stationary phase of growth within 2 days. Populations on tubes immersed in LJB at 25°C did not change significantly for the first 8 days but decreased between 8 and 10 days. Planktonic *E. sakazakii* in LJB reached the stationary phase of growth within 2 days. The death phase for strains 3231 and 3439 began at 4 and 6 days, respectively.

Populations of *E. sakazakii* strains 3231 and 3439 recovered from the surface of stainless steel coupons immersed in TSB, IFB, and LJB at 25°C are shown in Figure 4-3. The number of *E. sakazakii* strains 3231 and 3439 initially attached to the stainless steel did not change significantly when coupons were immersed in TSB. Initial populations (5.33 - 5.51 log CFU/cm^2) on coupons immersed in IFB increased significantly ($P \leq 0.05$) to 6.69 - 6.89 log CFU/cm^2 within 10 days. The infant formula used to prepare IFB contains non-fat milk, corn syrup, lactose, soy oil, whey protein concentrate, and other nutrients, thus providing protein, fat, carbohydrate, vitamins, and minerals as nutrient sources. As noted above, milk or milk proteins have been shown to either enhance or inhibit bacterial attachment to abiotic surfaces. In our study, *E. sakazakii* attached to surfaces of stainless steel in a PBS suspension, followed by immersion in IFB. The IFB may provide nutrients necessary for *E. sakazakii* cells pre-attached to the stainless steel or feeding tubes to survive and grow. This observation is in agreement with Helke and Wong (16), who reported that *L. monocytogenes* cells pre-attached to stainless steel grew in the presence of milk at 25°C. We observed that as incubation time progressed at 25°C, the texture of IFB became markedly viscous. The high viscosity of IFB may facilitate harborage of *E. sakazakii* cells. Biofilm formation by *E. sakazakii* on latex, silicon, and stainless steel

Figure 4-3. Populations of *E. sakazakii* strains 3231 and 3396 attached to the surface or in biofilms formed on the surface of stainless steel coupons (log CFU/cm^2, ○) immersed in TSB, IFB, and LJB and in broths (log CFU/ml, □) in which coupons were immersed and incubated at 25°C for up to 10 days. Bars indicate standard deviations.

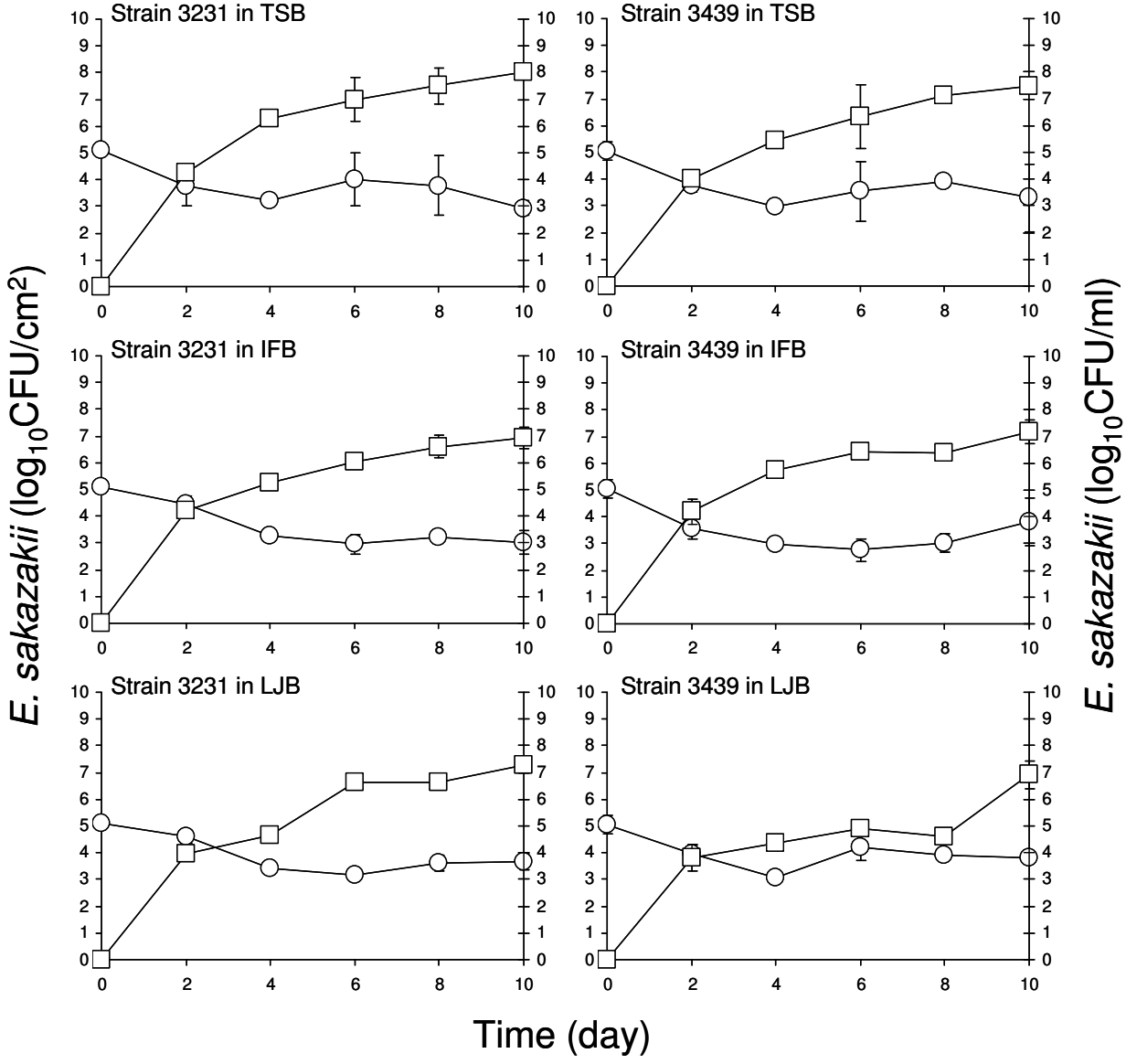

Strain 3231 in TSB
Strain 3439 in TSB
Strain 3231 in IFB
Strain 3439 in IFB
Strain 3231 in LJB
Strain 3439 in LJB
E. sakazakii (log10CFU/cm2)
E. sakazakii (log10CFU/ml)
Time (day)

immersed in infant formula milk at 37°C has been reported (22). Lehner et al. (30) observed that biofilms were formed by 59% of *E. sakazakii* test strains in PVC microtiter wells and extracellular polysaccharide was produced by 42.8 % of the test strains. Extracellular polysaccharide is known to be involved in bacterial adhesion and biofilm formation (6, 7, 10). In the temperature rage of 0 - 30°C, the highest amount of polysaccharide synthesized by *E. sakazakii* was at 27°C (47).

Populations of *E. sakazakii* initially attached to stainless steel remained constant for 2 - 4 days when immersed in LJB, then significantly decreased ($P \leq 0.05$) by ≥ 2.38 log CFU/cm^2 after 10 days (Figure 4-3). Planktonic *E. sakazakii* in the LJB reached 7.82 - 8.15 log CFU/ml within 1 day, followed by slow decreases during the remainder of the 10-day incubation period. A similar phenomenon was reported by Ryu et al. (46), who showed that *E. coli* O157:H7 attached to stainless steel coupons did not form biofilm when immersed in LJB. The number of *E. sakazakii* and *E. coli* O157:H7 cells that detached from the surface of stainless steel is apparently higher than number of newly-attached cells and pre-attached cells that grow. Lack of nutrients in LJB, compared to TSB and IFB, as well as a reduction in pH during incubation may also contribute to significant decreases in surface populations.

Figure 4-4 shows the number of *E. sakazakii* strains 3231 and 3439 detected on the surface of enteral feeding tubes and its growth in TSB, IFB, and LJB in which tubes were immersed at 12°C. None of the strains produced biofilms on tubes immersed in TSB. Unlike the behavior of cells in TSB at 25°C, the number of adherent cells gradually decreased at 12°C. As growth of *E. sakazakii* is very slow at 12°C, biofilm formation in TSB was also restricted. Decreases in populations of the adherent cells were observed within 2 days. Even though *E. sakazakii* reached a stationary growth phase in TSB in which tubes were immersed within 8 - 10

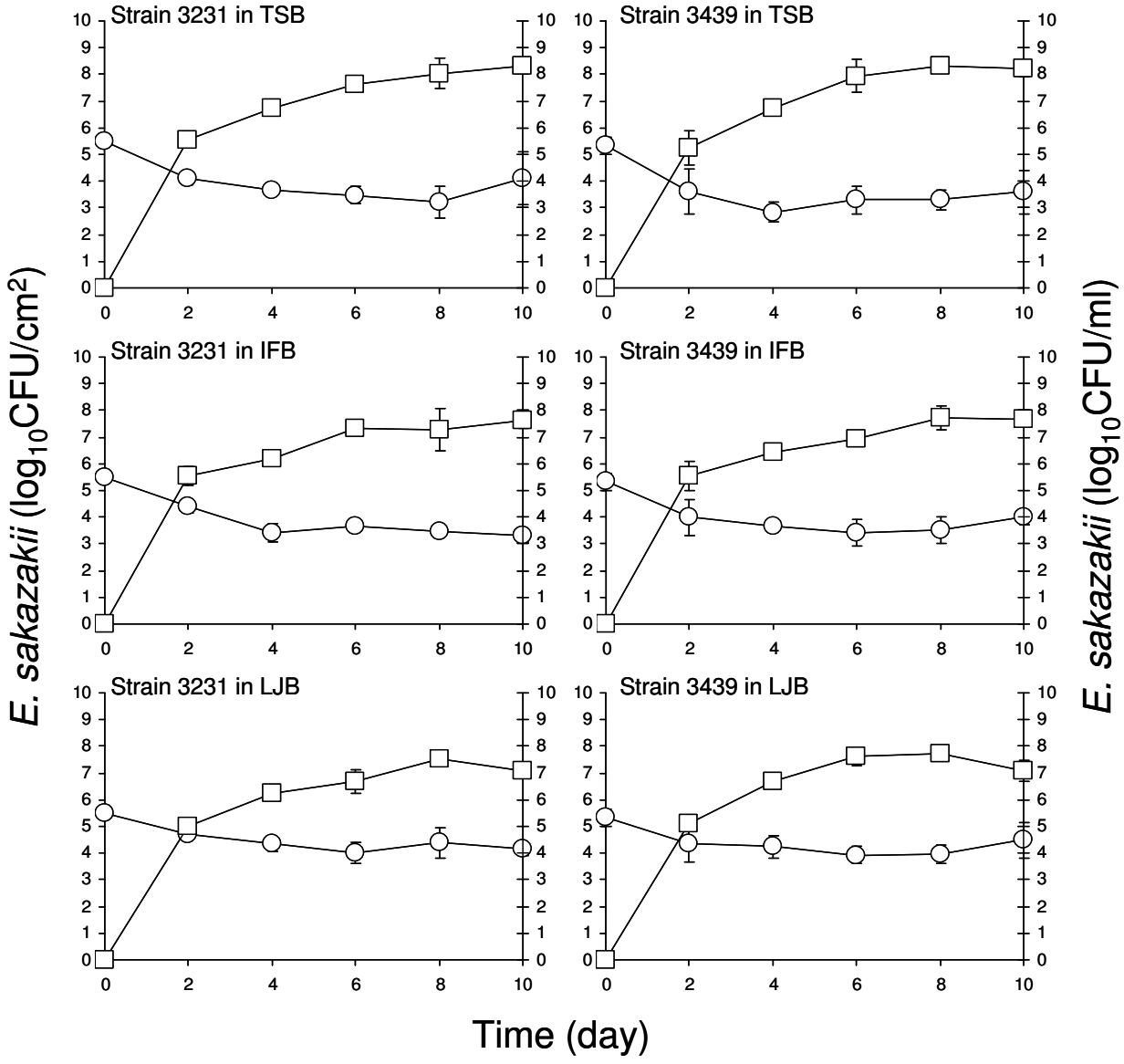
Figure 4-4. Populations of *E. sakazakii* strains 3231 and 3396 attached to the surface or in biofilms formed on the surface of enteral feeding tubes (log CFU/cm^2, ○) immersed in TSB, IFB, and LJB and in broths (log CFU/ml, □) in which tubes were immersed and incubated at 12°C for up to 10 days. Bars indicate standard deviations.


Strain 3231 in TSB
Strain 3439 in TSB
Strain 3231 in IFB
Strain 3439 in IFB
Strain 3231 in LJB
Strain 3439 in LJB
E. sakazakii (log10CFU/cm2)
E. sakazakii (log10CFU/ml)
Time (day)

days, the number of the cells remaining attached to the surfaces of tubes simultaneously decreased. This may be due in part to the presence of fewer planktonic cells at 12°C, compared to 25°C, to a slower rate of growth of cells pre-attached to the surfaces at 12°C, and to different physiological state of cells in media, which has been reported to affect cell surface characteristics of *E. coli* (54). *E. sakazakii*, initially attached at 5.03 - 5.12 log CFU/cm^2 of tube surface, decreased significantly ($P \leq 0.05$) on tubes immersed in IFB at 12°C. This is in contrast to its behavior at 25°C (Figure 4-2) and indicates that temperature plays an important role in biofilm formation by *E. sakazakii* in IFB. *E. sakazakii* reached the stationary phase of growth in IFB surrounding the feeding tubes within 8 - 10 days (Figure 4-4). Reductions in the number of adherent cells on the surface of feeding tubes immersed in LJB at 12°C were 1.23 - 1.47 log CFU/cm^2, which are not substantially different than the 1.05 - 2.37 log CFU/cm^2 reduction at 25°C (Figure 4-2).

Figure 4-5 shows the number of *E. sakazakii* strains 3231 and 3439 on stainless steel coupons immersed in TSB, IFB, and LJB at 12°C and in the immersion broths. As with feeding tubes incubated at 12°C, none of the strains formed biofilms on stainless steel in TSB. Strains 3231 and 3439 attached to stainless steel coupons in PBS cell suspension (ca. 7 log CFU/ml) during incubation at 4°C for 24 h at populations of 5.51 and 5.33 log CFU/cm^2. Immersion of these coupons in TSB resulted in a reduction of 1.4 log CFU/cm^2 for strain 3231 and 1.7 log CFU/cm^2 for strain 3439 during incubation at 12°C for 10 days. *E. sakazakii* reached the stationary phase of growth in TSB at 25°C within 1 - 2 days (Figure 4-3) but required 6 - 8 days when incubated at 12°C (Figure 4-5). Numbers of the cells in stationary phase at 12°C reached ≥ 8.0 log CFU/ml of TSB after 8 days. Unlike the significant growth of *E. sakazakii* observed on stainless steel in IFB at 25°C, initial numbers of adherent cells decreased significantly ($P \leq 0.05$)

Figure 4-5. Populations of *E. sakazakii* strains 3231 and 3396 attached to the surface or in biofilms formed on the surface of stainless steel coupons (log CFU/cm^2, ○) immersed in TSB, IFB, and LJB and in broths (log CFU/ml, □) in which coupons were immersed and incubated at 12°C for up to 10 days. Bars indicate standard deviations.


Strain 3231 in TSB
Strain 3439 in TSB
Strain 3231 in IFB
Strain 3439 in IFB
Strain 3231 in LJB
Strain 3439 in LJB
E. sakazakii (log10CFU/cm2)
E. sakazakii (log10CFU/ml)
Time (day)

by 1.33 - 2.20 log CFU/cm^2 after 10 days at 12°C. *E. sakazakii* did not form biofilm on stainless steel immersed in LJB at 12°C, as was the case at 25°C. However, a slower reduction occurred in the number of cells adhering to stainless steel immersed in LJB at 12°C, compared to 25°C. Populations of strains 3231 and 3439 attached to stainless steel in LJB decreased by 3.32 and 2.38 log CFU/cm^2, respectively, at 25°C (Figure 4-3) but only 1.37 and 0.84 log CFU/cm^2, respectively, at 12°C (Figure 4-5). The larger decreases in populations at 25°C than at 12°C are attributed in part to death of adherent cells on stainless steel immersed in LJB at 25°C. Like its behavior in TSB and IFB at 12°C, *E. sakazakii* also reached the stationary phase of growth in LJB after 8 - 10 days. *E. sakazakii* behaved similarly in TSB, IFB, and LJB at 12°C. Differences in composition of three media did not have an influence on biofilm formation of *E. sakazakii* at 12°C. Results suggest that nutrient depletion did not cause great decreases in the populations of cells attached to feeding tubes or stainless steel coupons at 12°C. It is not the surrounding medium or incubation temperature alone, but rather a combination of medium, temperature, or other factors that influence biofilm formation of *E. sakazakii*.

In summary, *E. sakazakii* attached better to the surfaces of enteral feeding tubes and stainless steel in TSB, IFB, and LJB at 25°C than at 12°C. *E. sakazakii*, pre-attached to stainless steel and enteral feeding tubes and immersed in IFB, formed biofilms at 25°C. The importance of preventing contamination of contact surfaces in areas where powdered infant formulas are reconstituted or fed to infants is reinforced by observations that *E. sakazakii* can attach to and form biofilms on these surfaces. Results emphasize the importance of temperature control in infant formula and produce processing industries to inhibit attachment and biofilm formation by *E. sakazakii*. Further studies will be required to determine the resistance of *E. sakazakii* in

biofilm to various sanitizers and disinfectants used in these industries and in formula reconstitution and feeding areas.

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CHAPTER 5

EFFECTIVENESS OF DISINFECTANTS IN KILLING *ENTERBACTER SAKAZAKII* IN SUSPENSION, DRIED ON THE SURFACE OF STAINLESS STEEL, AND IN BIOFILM [1]

[1] Kim, Hoikyung and Larry R. Beuchat. To be submitted for publication in *Applied and Environmental Microbiology*.

ABSTRACT

Survival of *Enterobacter sakazakii* on the surface of stainless steel coupons as affected by temperature was determined. Resistance of cells in suspension dried on the surface of stainless steel and in biofilms on stainless steel to disinfectants commonly used in hospitals, day-care centers, and food service kitchens was studied. Initial populations of 7.36 - 8.61 log CFU/coupon on the surface of stainless steel held at 4, 25, and 37°C decreased significantly ($p \leq 0.05$) within 3 - 10, 1 - 3, and 1 day(s), respectively, but the pathogen remained viable for up to 60 days. The rate of death was more rapid when cells were suspended in water and dried on stainless steel compared to the rate of death of cells that had been suspended in infant formula before drying. The largest reduction (6.71 - 6.98 log CFU/coupon) during the 60-day monitoring period occurred at 37°C when cells were suspended in water before drying on stainless steel. The smallest reduction (1.07 - 1.21 log CFU/coupon) occurred when cells in infant formula were dried on stainless steel and stored for 60 days at 4°C. Cells of *E. sakazakii* in suspension, dried on the surface of stainless steel, and in biofilm exhibited differences in resistance to quaternary ammonium and phenolic disinfectants, depending on the amount and type of organic matrix surrounding cells and exposure time. The presence of infant formula enhanced the resistance of planktonic cells and cells inoculated and dried on the surface of stainless steel to the disinfectants. The overall order of efficacy of disinfectants in killing *E. sakazakii* was planktonic cells > cells inoculated and dried on stainless steel > cells in biofilms on stainless steel. Findings confirm the exceptional resistance of *E. sakazakii* to desiccation and demonstrate the ineffectiveness of some disinfectants routinely used in hospital, day-care and home settings in killing *E. sakazakii* embedded in organic matrices.

Keywords: *Enterobacter sakazakii*, biofilm, infant formula, disinfectant, sanitizer

INTRODUCTION

Enterobacter sakazakii can cause meningitis (Gallagher and Ball, 1991; Burdette and Santos, 2000), sepsis (Simmons et al., 1989), bacteremia (Noriega et al., 1990), and necrotizing enterocolitis (Van Acker et al., 2001) in preterm neonates. Powdered infant formula has been implicated as a source of *E. sakazakii* in outbreaks of infections (Muytjens et al., 1983; Biering et al., 1989; Simmons et al., 1989; Noriega et al., 1990; Van Acker et al., 2001; Bar-Oz et al., 2001; Block et al., 2002; Himelright et al., 2002). In surveys done to determine the presence of *E. sakazakii* in powdered infant formula, the organism was detected in 2.4 - 14.2% of the products tested (Muytjens et al., 1988; Iversen et al., 2004).

The presence of *E. sakazakii* on the surface of utensils and equipment used for infant formula preparation has been reported to occur in clinical settings where neonatal infections have been documented (Simmons et al. 1989; Clark et al., 1990; Noriega et al., 1990; Bar-Oz et al., 2001). Resistance of the bacterium to desiccation (Breeuwer et al., 2003) and its ability to form biofilms on abiotic surfaces (Iversen et al., 2004; Lehner et al., 2005; Kim et al., 2006) raises the possibility that infections may occur following cross-contamination in formula preparation areas in hospitals, day care centers, and the home.

Foodborne pathogens, e.g., *Escherichia coli* O157:H7 and *Listeria monocytogenes*, and spoilage bacteria such as *Pseudomonas* in biofilms have enhanced resistance to sanitizers (Frank and Koffi, 1990; Delissalde and Amábile-Cuevas, 2004; Ryu and Beuchat, 2005; Folsom and Frank, 2006). We have observed that effectiveness of some of the chemical sanitizers commonly used to decontaminate foods and food-contact surfaces is diminished when *E. sakazakii* in embedded in biofilm (Kim and Beuchat, 2006).

Surface disinfection is routinely carried out in formula preparation areas in hospitals by applying liquid chemical disinfectants to food contact and non-food contact surfaces. Commercial surface cleaners and disinfectants are largely based on quaternary ammonium compounds, phenolic compounds, organic acids, alcohols, chlorine, and iodophors. Various commercial hard-surface cleaners and disinfectants have been evaluated for their efficacy in killing bacteria capable of causing foodborne infections (Vijayakumar and Wolf-hall, 2002; Taormina and Beuchat, 2002; Exner et al., 2004; Sharma and Beuchat, 2004). During formula preparation and infant feeding, reconstituted infant formula containing *E. sakazakii* may contaminate abiotic surfaces. These surfaces may be treated with disinfectants immediately after contamination occurs, after the formula remains on the surface and dries, or after the formation of biofilm. The efficacy of commercial disinfectants used in formula preparation areas in hospitals and day-care centers in killing *E. sakazakii* in dried infant formula and biofilm has not been described.

We undertook studies to determine the effectiveness of disinfectants in killing *E. sakazakii* in suspension, dried on the surface of stainless steel, and embedded in biofilm on stainless steel. Quaternary ammonium and phenolic disinfectants commonly used in infant formula preparation and processing areas, laboratories, and hospital and food service settings were evaluated. The effects of time elapsed after drying cells on stainless steel as well as the age of biofilms on resistance of cells to disinfectants were determined.

MATERIALS AND METHODS

Bacterial strains and preparation of cells for treatment with disinfectants. *E. sakazakii* strains 3231, isolated from cerebral spinal fluid of an infant, and 3439, isolated from a

commercially manufactured powdered infant formula, were grown in tryptic soy broth (TSB; BBL/Difco, Sparks, Md.) at 37°C for 24 h. Cultures were transferred by loop inoculum (ca. 10 µl) three times at 24-h intervals. Each culture was centrifuged at 4,000 × *g* for 15 min at 4°C. Cells resuspended in sterile synthetic hard water (400 µg of $CaCO_3$/ml) or reconstituted infant formula were used in various experiments.

Media in which cells were suspended. Standard synthetic hard water was prepared according to the AOAC International (2000) official methods of analysis. Phosphate buffered saline (PBS) (pH 7.4) containing (per liter of distilled water) NaCl (8 g), KCl (0.2 g), Na_2HPO_4 (1.44 g), and KH_2PO_4 (0.24 g) was used to as a medium to suspend cells for attachment to stainless steel. Reconstituted infant formula was made by combining Similac Neosure Advance powdered infant formula (Ross Pediatrics, Abbott Laboratories, Columbus, Ohio) with distilled water at a ratio of 1:10 (w/v), dissolving by heating at 50 - 60°C, and autoclaving at 121°C for 15 min.

Fate of *E. sakazakii* dried on the surface of stainless steel. *E. sakazakii* strains 3231 and 3439 grown in TSB were collected by centrifugation at 4,000 × *g* for 15 min at 4°C and each pellet was resuspended in sterile distilled water or reconstituted infant formula. Stainless steel coupons were separately inoculated with 100 µl of cell suspension of each strain to give ca. 8 log CFU/coupon and dried in a laminar flow biosafety cabinet (22 ± 2°C) for 2 h. Each coupon on which inoculum had dried was placed in a 50-ml conical centrifuge tube containing 0.8 ml of saturated potassium carbonate solution, which equilibrates a ca. 43% atmospheric relative humidity, and the caps were firmly tightened. The tubes containing inoculated coupons and the salt solution were incubated at 4, 25, and 37°C for up to 60 days.

Populations of *E. sakazakii* surviving on the surface of the stainless steel coupons were determined after holding inoculated coupons for 2 h at 22 ± 2°C (day 0) and 1, 3, 10, 20, 30, 45, and 60 days at 4, 25, and 37°C. At each sampling time, coupons were transferred to 50-ml centrifuge tubes containing 30 ml of sterile 0.1% peptone water and 3 g of glass beads (425 - 600 microns in diameter, Sigma-Aldrich, St. Louis, Mo.) and the tubes were vortexed (model Vortex Genie-2; Scientific Industries, Inc., Bohemia, N.Y.) at maximum speed for 1 min to dislodge and disperse cells. Immediately after vortexing, undiluted samples (0.25 ml in quadruplicate and 0.1 ml in duplicate) and samples (0.1 ml in duplicate) serially diluted in 0.1% peptone water were surface plated on tryptic soy agar (TSA; BBL/Difco) to determine the number of *E. sakazakii* that survived on the surface of stainless steel coupons. Plates were incubated at 37°C for 24 h before colonies were counted. The number of *E. sakazakii* (CFU/coupon) recovered was calculated. The mixture of 30 ml of 0.1% peptone containing cells removed from each coupon, coupons that had been inoculated for up to 60 days, and 3 g of glass beads were transferred to 270 ml of Enterobacteriaceae enrichment (EE) broth (Becton, Dickinson and Company, Sparks, Md.) and incubated at 37°C for 24 h. When *E. sakazakii* colonies were not observed on TSA, EE cultures were streaked on TSA. Plates were incubated at 37°C for 24 h before examining for the presence of *E. sakazakii* colonies.

Preparation of disinfectant solutions. Descriptions of disinfectants evaluated in the study are shown in Table 5-1. All disinfectants were tested at minimum concentrations recommended by the manufacturers. Thirteen products were evaluated for their efficacy in killing planktonic cells of *E. sakazakii*. Disinfectants 1 - 9 were prepared at double the strength of the desired treatment concentrations. Equal volumes of cell suspensions and 2X disinfectant solutions were combined to form the reaction mixture. Disinfectants 10 - 13, intended to be used

Table 5-1. Disinfectants evaluated for lethality to *E. sakazakii*

Disinfectant number	Product name	Type of active ingredients	Active ingedients (listed on label)	Conc. of active ingredient (%)[a]	Applications recommended ny manufacturers
1	ZEP FS Amine Z[b]	Quaternary ammonium compounds	Octyl decyl dimethyl ammonium chloride Didecyl dimethyl ammonium chloride Dioctyl dimethyl ammonium chloride Alkyl (50% C_{14}, 40% C_{12},10% C_{16}) dimethyl benzyl ammonium chloride	3.0 1.5 1.5 4.0	Dairies, restaurants, kitchens, food processing, dairy farms, bakeries, meat packaging plants, sanitary services, bottling plants, etc.
2	ZEP DZ-7[b]	Quaternary ammonium compounds	Octyl decyl dimethyl ammonium chloride Dioctyl dimethyl ammonium chloride Didecyl dimethyl ammonium chloride Alkyl (50% C_{14}, 40% C_{12},10% C_{16}) dimethyl benzyl ammonium chloride	0.814 0.407 0.407 1.085	Major hospital areas including recovery rooms, patient rooms, nursery, maternity, pediatrics, animal research areas and office areas, nursing homes, child day care services, etc.
3	Lemonex[b]	Quaternary ammonium compounds	n-Alkyl dimethyl(50% C_{14}, 30% C_{16},5% C_{12},5% C_{18}) benzyl ammonium chlorides n-Alkyl dimethyl ethylbenzyl ammonium chlorides	0.8 0.8	Bathrooms, kennels, nursing home, hospitals, hotels, child day care services, etc.
4	ZEP Micronex[b]	Quaternary ammonium compounds	Didecyl dimethyl ammonium chloride n-Alkyl (50% C_{14}, 40% C_{12},10% C_{16}) dimethyl benzyl ammonium chloride	10.14 6.76	Major hospital areas including recovery rooms, patient rooms, nursery, maternity, pediatrics, etc.
5	T.B.Q.[c]	Quaternary ammonium compounds	Alkyl (50% C_{14}, 40% C_{12},10% C_{16}) dimethyl benzyl ammonium chloride	8	Veterinary and research institutions, industrial plants, food preparation and service facilities, etc.
6	ZEP FS Formula 386 L[b]	Quaternary ammonium compounds/acid	Octyl decyl dimethyl ammonium chloride Dioctyl dimethyl ammonium chloride Didecyl dimethyl ammonium chloride Alkyl (50% C_{14}, 40% C_{12},10% C_{16}) dimethyl benzyl ammonium chloride	2.295 0.918 1.377 3.060	Dairies, restaurants, kitchens, food processing, dairy farms, bakeries, meat packaging plants, nursing homes, hospitals, etc.
7	Perosan Liquid Sanitizer[b]	Peroxyacetic acid/ hydrogen peroxide	Peroxyacetic acid hydrogen peroxide	5.1 21.7	Eating and drinking establishments, bottling companies, food preparation (manufacturing), etc.
8	LpH se[c]	Phenolic compounds	o-phenylphenol p-tertiary amylphenol	7.7 7.6	Floors, walls, and other equipments in hospitals, nursing homes, clinics, etc.
9	Vesphene IIse[c]	Phenolic compounds	o-phenylphenol p-tertiary amylphenol	9.09 7.66	Hospitals, nursing homes, medical and dental offices, pharmaceutical plants, etc.
10	Coverage Spray HB Plus[c]	Quaternary ammonium compounds	Octyl decyl dimethyl ammonium chloride Dioctyl dimethyl ammonium chloride Didecyl dimethyl ammonium chloride Alkyl (50% C_{14}, 40% C_{12},10% C_{16}) dimethyl benzyl ammonium chloride	0.025 0.010 0.015 0.034	Laboratories and veterinary clinics
11	Coverage Spray TB[c]	Quaternary ammonium compounds	n-Alkyl dimethyl (50% C_{14}, 30% C_{16},5% C_{12},5% C_{18}) benzyl ammonium n-Alkyl dimethyl ethylbenzyl ammonium chlorides	0.105 0.105	Infant care equipment, laboratory equpment and surfaces, medical and dental equipment surfaces, etc.
12	ZEP Kitchen Surface Sanitizer[b]	Quaternary ammonium compounds	Octyl decyl dimethyl ammonium chloride Didecyl dimethyl ammonium chloride Dioctyl dimethyl ammonium chloride Alkyl (50% C_{14}, 40% C_{12},10% C_{16}) dimethyl benzyl ammonium chloride	0.01050 0.00525 0.00525 0.01400	Industiral and institutional kitchens, restaurants delis, cafeterias, butcher shops, bakeries, supermarkets
13	ZEP FS RTU-D2[b]	Alcohol and quaternary ammonium compounds	Isopropyl alcohol Octyl decyl dimethyl ammonium chloride Didecyl dimethyl ammonium chloride Dioctyl dimethyl ammonium chloride	58.6000 0.0075 0.0045 0.0030	Poultry, red meat, and dairy processing, restaurants, beverage plants, food service locations, and other food processing facilities

[a] Undiluted disinfectants
[b] ZEP Manufacturing Co., Altlanta, Ga.
[c] Steris Co., St. Louis, Mo.

without dilution, were tested as received the manufacturers. For experiments involving spot-inoculation of cells on stainless steel and cells in biofilms, disinfectants 2, 5, 6, 7, and 9 were diluted in sterile hard water to obtain the minimum treatment concentrations recommend; undiluted disinfectant 11 was also tested.

Efficacy of disinfectants in killing planktonic cells. Cells in pellets obtained as described above were resuspended in 100 ml of sterile hard water or reconstituted infant formula to give populations of ca. 7 log CFU/ml. Ten milliliters of cell suspension were deposited in a sterile 25- by 150-mm test tube containing 10 ml of 2X disinfectants 1 - 9 or sterile hard water (control) at 22 ± 2°C and thoroughly mixed. For evaluation of disinfectants 10 - 13, cells from pellets were resuspended in 1 ml of sterile hard water or reconstituted infant formula to give populations of ca. 9 log CFU/ml. Cell suspensions (0.1 ml) were added to sterile 25- by 150-mm test tubes containing 20 ml of disinfectants 10 - 13 or sterile hard water (control) and mixed thoroughly.

At 0 time (within 10 sec after combining cell suspension with sterile hard water) and after holding the reaction mixtures (suspension to which water [control] or disinfectants were added) for 1, 5, and 10 min at 22 ± 2°C, 2 ml of the suspension were withdrawn and combined with 2 ml of 2X Dey-Engley (DE) neutralizing broth (BBL/Difco). Undiluted suspensions (0.25 ml in quadruplicate and 0.1 ml in duplicate) and suspensions (0.1 ml in duplicate) serially diluted in 0.1% peptone water were surface plated on TSA. Plates were incubated at 37°C for 48 h before colonies were counted.

Efficacy of disinfectants in killing *E. sakazakii* spot-inoculated and dried on stainless steel. Sterile stainless steel coupons (type 304, 5 cm × 2 cm, no. 4 finish) were placed on a wire screen elevated 7 cm above the work surface in a laminar flow biosafety cabinet. Suspensions

(100 µl) of *E. sakazakii* in sterile hard water or reconstituted infant formula, prepared as described above, were deposited on each coupon to give ca. 8 log CFU/coupon. The inoculum was dried for 20 h (45 ± 7 % relative humidity) at 22 ± 2°C in a laminar flow biosafety cabinet. Inoculated coupons were immersed in sterile 25- by 150-mm test tubes containing 25 ml of disinfectants 2, 5, 6, 7, or 9 prepared at minimum treatment concentrations recommended by manufacturers, 25 ml of undiluted (full-strength) disinfectant 11, or sterile hard water (control) at 22 ± 2°C and thoroughly mixed. After treatment for 0 min (within 10 sec after immersing coupons in sterile water) and after treatment for 1, 5, and 10 min in water or disinfectant solution, each coupon was transferred to a 50-ml centrifuge tube containing 30 ml of DE broth and 3 g of sterile glass beads. The tube containing DE broth, coupon, and glass beads was vortexed at maximum speed for 1 min. Immediately after vortexing, undiluted samples (0.25 ml in quadruplicate and 0.1 ml in duplicate) and samples (0.1 ml in duplicate) serially diluted in 0.1% peptone water were surface plated on TSA and incubated at 37°C for 48 h. Colonies were counted and populations (log CFU/coupon) of *E. sakazakii* remaining on stainless steel coupons before and after treatment with disinfectants were calculated.

Efficacy of disinfectants in killing *E. sakazakii* in biofilm on stainless steel. Each sterile stainless steel coupon was immersed in a sterile 25- by 150-mm test tube containing 25 ml of a suspension of *E. sakazakii* in PBS (ca.7 log CFU/ml) and incubated at 4°C for 24 h to facilitate attachment of cells. The coupons were transferred into 50-ml centrifuge tubes containing 30 ml of sterile reconstituted infant formula prepared as described above and incubated at 25°C for 6 or 12 days. Coupons were removed from the formula and washed in 400 ml of sterile water (22 ± 2°C) with agitation for 15 sec to remove most of the cells not present in or firmly attached to the biofilm matrix. The washed coupons were transferred to sterile 25- by

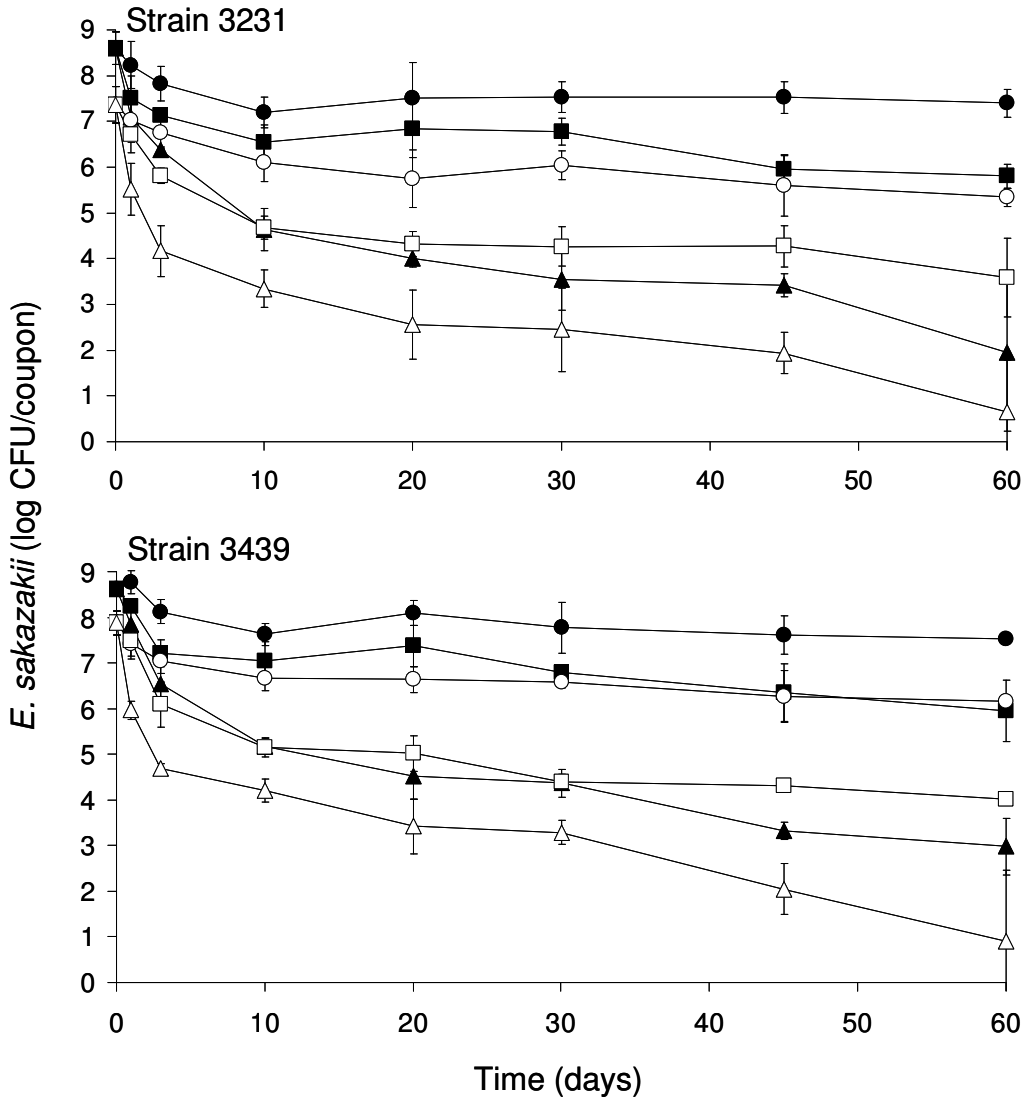
150-mm test tubes containing 25 ml of disinfectants 2, 5, 6, 7, and 9, prepared at minimum treatment concentrations recommended by manufacturers, undiluted disinfectant 11, or sterile hard water (control). After treatment for 0 min (within 10 sec after immersing coupons in sterile water) and after treatment for 1, 5, and 10 min in water or disinfectant solution, each coupon was transferred to a 50-ml centrifuge tube containing 30 ml of DE broth and 3 g of sterile glass beads and vortexed at maximum speed for 1 min to dislodge cells from the biofilms. The undiluted suspensions (0.25 ml in quadruplicate and 0.1 ml in duplicate) and suspensions (0.1 ml in duplicate) serially diluted in 0.1% peptone water were surface plated on TSA. Plates were incubated at 37°C for 48 h before the colonies were counted and the number of cells (log CFU/coupon) surviving treatment was calculated.

Statistical analysis. All experiments were replicated three times. In experiments involving stainless steel coupons, two coupons were examined at each sampling time. Data were analyzed using the general linear model of the Statistical Analysis Systems procedure (SAS; SAS Institute, Cary, N. C.). Statistically significant differences in populations of planktonic cells, spot inoculated, dried cells, and cells of *E. sakazakii* in biofilms caused by treatment with disinfectants were determined. The influence of the presence of infant formula on efficacy of disinfectants in reducing populations of planktonic cells and spot inoculated dried cells, and the efficacy of disinfectants in killing cells in biofilms as affected by the maturation period (age) were determined using Fisher's Least Significant Difference (LSD) test. Significant differences are presented at a 95% confidence level ($p \leq 0.05$).

RESULTS AND DISCUSSION

Fate of *E. sakazakii* dried on the surface of stainless steel. Figure 5-1 shows populations of *E. sakazakii* strains 3231 and 3439 that survived on the surface of stainless steel held at 4, 25, and 37°C for up to 60 days. Populations significantly ($p \leq 0.05$) decreased at all temperatures within 1 - 10 days, depending on type of carrier, storage temperature, and strain. During a 2-h drying period at 22°C, initial populations of strains 3231 and 3439 suspended in water decreased by 0.94 - 1.46 log CFU/coupon, which was less than decreases in the number of cells (0.21 log CFU/coupon) suspended in infant formula and dried on coupons. During subsequent storage, the rate of death, particularly in the first three days, varied depending on storage temperature and composition of the cell carrier. We observed a similar phenomenon in a previous study (Kim and Beuchat, 2006). In that study, populations of *E. sakazakii* on whole (uncut) produce decreased more rapidly at 25°C than at 4 or 12°C during storage for up to 28 days. Regardless of strain or storage temperature, at a given storage time throughout the 60-day storage period, reductions were significantly greater when cells had been suspended in water than in infant formula before drying on the stainless steel surfaces. Infant formula may protect cells against environmental stresses such desiccation and unfavorable temperature during storage. Largest reductions in the populations occurred at 37°C, regardless of type of cell carrier and strain. Populations of cells of both strains suspended in water decreased by 1.84 - 1.91 log CFU/coupon within 1 day at 37°C. This compares to a reduction of 0.76 - 1.52 log CFU/coupon inoculated with cells suspended in infant formula. After incubation at 37°C for 60 days, populations recovered from coupons that had been inoculated with cells suspended in water and infant formula, respectively, decreased by 6.71 - 6.98 and 5.63 - 6.66 log CFU/coupon. Reductions of 1.73 - 2.02 and 1.07 - 1.21 log CFU/coupon occurred when cell suspensions in

Figure 5-1. Populations of *E. sakazakii* strains 3231 and 3439 suspended in sterile distilled water (open symbols) and reconstituted infant formula (closed symbols), inoculated and dried on the surface of stainless steel, and incubated at 4°C (circle), 25°C (square), and 37°C (triangle) for up to 60 days.


Strain 3231
Strain 3439
E. sakazakii (log CFU/coupon)
Time (days)
0
1
2
3
4
5
6
7
8
9
0
10
20
30
40
50
60

water and infant formula, respectively, were dried on coupons and stored at 4°C for 60 days.

Resistance of planktonic cells to disinfectants. Numbers of *E. sakazakii* strain 3231 (Table 5-2) and strain 3439 (Table 5-3) suspended in hard water and reconstituted infant formula not containing disinfectants did not change significantly ($p > 0.05$) within 10 min at 22°C. Treatment of both strains suspended in water containing disinfectants for 1 min resulted in significant reductions ($p \leq 0.05$) in populations, compared to the number of cells recovered from water not containing disinfectants. Considering disinfectants 1 - 9, with the exceptions of planktonic cells in water treated with disinfectants 1, 5, and 6, populations of *E. sakazakii* strain 3231 (Table 5-2) decreased to < 0.30 log CFU/ml within 1 min; with the exceptions of disinfectants 5 and 6, populations of strain 3439 (Table 5-3) were reduced to < 0.30 log CFU/ml within 1 min. This indicates that disinfectants 1, 5, and 6, at the concentrations tested, have the lowest lethality among disinfectants 1 - 9 to *E. sakazakii* suspended in water. Disinfectants 1, 5, and 6 contain alkyl (50% C_{14}, 40% C_{12}, and 10% C_{16}) dimethyl benzyl ammonium chloride as a major active ingredient (3 - 8%). After treatment of cells in water with disinfectants 1 - 9 for 5 min, populations of strains 3231 and 3439 were reduced from initial populations of 7.01 and 7.60 log CFU/ml, respectively, to < 0.30 log CFU/ml, indicating that with sufficient exposure time, disinfectants 1 - 9 are equivalent in lethality to *E. sakazakii*. Populations of strains 3231 and 3439 suspended in infant formula were decreased significantly ($p \leq 0.05$) by treatment with disinfectants 3 and 7 for 1 - 5 min while treatment with the other disinfectants for 10 min did not decrease the populations.

Disinfectants 10 - 13 are spray products which are ready-to-use without dilution or additional preparation. Treatment of *E. sakazakii* strains 3231 (Table 5-2) and 3439 (Table 5-3) in water containing disinfectants 10 - 13 reduced initial populations of 7.01 and 7.60 log CFU/ml,

Table 5-2. Survival of planktonic cells of *E. sakazakii* strain 3231 as affected by treatment with disinfectants

Disinfectant[b]	Carrier for cells	pH[c]	Population (log CFU/ml) after exposure for:[a] 0 min	1 min	5 min	10 min
Water (control)	Water	7.39	A 7.33 A	a A 7.01 A	a A 7.20 A	a A 7.19 A
	Infant formula	7.01	A 7.17 A	a A 7.26 A	ab A 7.33 A	a A 7.27 A
1	Water	7.60	A 7.22 A	c B 1.76 B	b B < 0.30[d] C	b B < 0.30 C
	Infant formula	6.96	A 7.06 AB	b A 6.78 B	a A 7.38 A	a A 7.39 A
2	Water	6.94	A 7.22 A	e B < 0.30 B	b B < 0.30 B	b B < 0.30 B
	Infant formula	6.82	A 7.06 A	a A 7.29 A	ab A 6.99 A	a A 7.04 A
3	Water	10.71	A 7.22 A	e B < 0.30 B	b B < 0.30 B	b B < 0.30 B
	Infant formula	9.69	A 7.06 A	b A 6.70 A	c A 5.26 B	c A 4.01 C
4	Water	8.33	A 7.22 A	e B < 0.30 B	b B < 0.30 B	b B < 0.30 B
	Infant formula	6.90	A 7.06 A	a A 7.21 A	ab A 7.13 A	a A 7.25 A
5	Water	9.30	A 7.31 A	d B 0.91 B	b B < 0.30 B	b B < 0.30 B
	Infant formula	7.61	A 7.19 A	a A 7.16 A	b A 6.79 A	a A 6.67 A
6	Water	2.72	A 7.22 A	b B 3.00 B	b B < 0.30 C	b B < 0.30 C
	Infant formula	5.64	A 7.06 A	a A 7.26 A	ab A 7.15 A	a A 7.18 A
7	Water	5.31	A 7.22 A	e B < 0.30 B	b B < 0.30 B	b B < 0.30 B
	Infant formula	6.02	A 7.06 A	c A 6.12 B	c A 5.76 BC	b A 5.18 C
8	Water	2.76	A 7.31 A	e B < 0.30 B	b B < 0.30 B	b B < 0.30 B
	Infant formula	5.77	A 7.19 A	a A 7.06 A	ab A 7.11 A	a A 7.32 A
9	Water	9.91	A 7.31 A	e B < 0.30 B	b B < 0.30 B	b B < 0.30 B
	Infant formula	8.75	A 7.19 A	a A 7.29 A	ab A 7.10 A	a A 7.11 A
Water (control)	Water	7.39	A 7.33 A	a A 7.01 A	a A 7.20 A	a A 7.19 A
	Infant formula	7.01	A 7.17 A	a A 7.26 A	a A 7.33 A	a A 7.27 A
10	Water	10.31	A 7.31 A	b A < 0.30 B	b A < 0.30 B	b A < 0.30 B
	Infant formula	10.31	A 7.19 A	b A 1.02 B	b A < 0.30 B	b A < 0.30 B
11	Water	12.14	A 7.31 A	b A < 0.30 B	b A < 0.30 B	b A < 0.30 B
	Infant formula	12.17	A 7.19 A	b A < 0.30 B	b A < 0.30 B	b A < 0.30 B
12	Water	8.33	A 7.31 A	b A < 0.30 B	b A < 0.30 B	b A < 0.30 B
	Infant formula	8.57	A 7.19 A	b A < 0.30 B	b A < 0.30 B	b A < 0.30 B
13	Water	9.56	A 7.31 A	b A < 0.30 B	b A < 0.30 B	b A < 0.30 B
	Infant formula	9.03	A 7.19 A	b A < 0.30 B	b A < 0.30 B	b A < 0.30 B

[a] Comparison of the effect of treatment time: mean values in the same row that are not followed by the same letter are significantly different ($p \leq 0.05$). Comparison of the effect of carrier (water versus infant formula); within treatment time and within water (control) or each disinfectant treatment, mean values that are not preceded by the same upper case letter are significantly different ($p \leq 0.05$). Comparison of the effect of disinfectants 1 - 9: within water

Table 5-2, continued

(control) and all disinfectant treatments 1 - 9, type of carrier, and treatment time, mean values that are not preceded by the same lower case letter are significantly different ($p \leq 0.05$). Comparison of the effect of disinfectants 10 - 13: within water (control) and all disinfectant treatments 10 - 13, type of carrier, and treatment time, mean values that are not preceded by the same lower case letter are significantly different ($p \leq 0.05$).

[b] See Table 5-1 for description of disinfectants. Disinfectants 1 - 9 were prepared by diluting with water to minimum concentrations as per manufacturers' recommendations. Disinfectants 10 - 13 were applied without dilution (full-strength) as per manufacturer's recommendations.

[c] pH of treatment mixture (water or infant formula plus disinfectant)

[d] Detection limit was 2 CFU/ml (0.30 log CFU/ml).

Table 5-3. Survival of planktonic cells of *E. sakazakii* strain 3439 as affected by treatment with disinfectants

Disinfectant[b]	Carrier for cells	pH[c]	Population (log CFU/ml) after exposure for:[a] 0 min	1 min	5 min	10 min
Water (control)	Water	7.39	A 7.42 A	a A 7.60 A	a A 7.32 A	a A 7.12 A
	Infant formula	7.01	A 7.44 A	a A 7.41 A	a A 7.26 A	a A 7.25 A
1	Water	7.60	A 7.40 A	d B < 0.30[d] B	b B < 0.30 B	b B < 0.30 B
	Infant formula	6.96	A 6.91 B	a A 7.28 AB	a A 7.32 AB	a A 7.40 A
2	Water	6.94	A 7.40 A	d B < 0.30 B	b B < 0.30 B	b B < 0.30 B
	Infant formula	6.82	A 6.91 A	a A 7.10 A	a A 6.99 A	a A 7.15 A
3	Water	10.71	A 7.40 A	d B < 0.30 B	b B < 0.30 B	b B < 0.30 B
	Infant formula	9.69	A 6.91 A	b A 6.41 A	c A 4.04 B	c A 3.22 B
4	Water	8.33	A 7.40 A	d B < 0.30 B	b B < 0.30 B	b B < 0.30 B
	Infant formula	6.90	A 6.91 A	a A 7.24 A	a A 7.04 A	a A 7.02 A
5	Water	9.30	A 7.37 A	c B 3.15 B	b B < 0.30 C	b B < 0.30 C
	Infant formula	7.61	A 7.46 A	a A 7.17 A	a A 6.85 A	a A 6.81 A
6	Water	2.72	A 7.40 A	b B 3.71 B	b B < 0.30 C	b B < 0.30 C
	Infant formula	5.64	A 6.91 A	a A 7.24 A	a A 7.22 A	a A 7.38 A
7	Water	5.31	A 7.40 A	d B < 0.30 B	b B < 0.30 B	b B < 0.30 B
	Infant formula	6.02	A 6.91 A	b A 6.12 B	b A 5.63 BC	b A 5.16 C
8	Water	2.76	A 7.37 A	d B < 0.30 B	b B < 0.30 B	b B < 0.30 B
	Infant formula	5.77	A 7.46 A	a A 7.49 A	a A 7.15 A	a A 7.37 A
9	Water	9.91	A 7.37 A	d B < 0.30 B	b B < 0.30 B	b B < 0.30 B
	Infant formula	8.75	A 7.46 A	a A 7.50 A	a A 7.33 A	a A 7.31 A
Water (control)	Water	7.39	A 7.42 A	a A 7.60 A	a A 7.32 A	a A 7.12 A
	Infant formula	7.01	A 7.44 A	a A 7.41 A	a A 7.26 A	a A 7.25 A
10	Water	10.31	A 7.37 A	b A 0.30 B	b A < 0.30 B	b A < 0.30 B
	Infant formula	10.31	A 7.46 A	b A 0.56 B	b A < 0.30 B	b A < 0.30 B
11	Water	12.14	A 7.37 A	b A < 0.30 B	b A < 0.30 B	b A < 0.30 B
	Infant formula	12.17	A 7.46 A	b A < 0.30 B	b A < 0.30 B	b A < 0.30 B
12	Water	8.33	A 7.37 A	b A < 0.30 B	b A < 0.30 B	b A < 0.30 B
	Infant formula	8.57	A 7.46 A	b A < 0.30 B	b A < 0.30 B	b A < 0.30 B
13	Water	9.56	A 7.37 A	b A < 0.30 B	b A < 0.30 B	b A < 0.30 B
	Infant formula	9.03	A 7.46 A	b A < 0.30 B	b A < 0.30 B	b A < 0.30 B

[a] Comparison of the effect of treatment time: mean values in the same row that are not followed by the same letter are significantly different ($p \leq 0.05$). Comparison of the effect of carrier (water versus infant formula); within treatment time and within water (control) or each disinfectant treatment, mean values that are not preceded by the same upper case letter are significantly different ($p \leq 0.05$). Comparison of the effect of disinfectants 1 - 9: within water

Table 5-3, continued

(control) and all disinfectant treatments 1 - 9, type of carrier, and treatment time, mean values that are not preceded by the same lower case letter are significantly different ($p \leq 0.05$). Comparison of the effect of disinfectants 10 - 13: within water (control) and all disinfectant treatments 10 - 13, type of carrier, and treatment time, mean values that are not preceded by the same lower case letter are significantly different ($p \leq 0.05$).

[b] See Table 5-1 for description of disinfectants. Disinfectants 1 - 9 were prepared by diluting with water to minimum concentrations as per manufacturers' recommendations. Disinfectants 10 - 13 were applied without dilution (full-strength) as per manufacturer's recommendations.

[c] pH of treatment mixture (water or infant formula plus disinfectant)

[d] Detection limit was 2 CFU/ml (0.30 log CFU/ml).

respectively, to ≤ 0.30 within 1 min. Of the ready-to-use products tested, disinfectant 10 had the lowest initial lethality to *E. sakazakii*, regardless of carrier composition.

Disinfectants 1 - 6 and 10 - 12 are quaternary ammonium-based disinfectants. At the concentrations tested, all contain alkyl dimethyl benzyl ammonium chloride (benzalkonium) at concentrations of 0.006 - 0.105%. The time required to achieve the same level of lethality to *E. sakazakii* in water, however, differed among these disinfectants, indicating that pH and constituents other than benzalkonium chloride contribute to bactericidal activity. Disinfectants 3 and 11, containing alkyl (60% C_{14}, 30% C_{16}, 5% C_{12}, and 5% C_{18}) dimethyl benzyl ammonium chloride, showed the greatest lethality, whereas disinfectants 1, 5, 6, and 10, which had lowest lethality, contain alkyl (50% C_{14}, 40% C_{12}, and 10% C_{16}) dimethyl benzyl ammonium chloride. These differences may in part be responsible for differences in efficacy of these two groups of quaternary ammonium compounds in killing *E. sakazakii* suspended in water. Benzalkonium chloride is known to have different bactericidal effects depending on length of its hydrophobic chain (Jono et al., 1986). The general order of antibacterial activity, which depends on the length of the alkyl chain, has been reported to be $C_{14} > C_{16} > C_{12} \geq C_{18} > C_{10} > C_8$ (Shelton et al., 1946; Jono et al., 1986; Kitahara et al., 2004). Merianos (1991) reported that C_{12}, C_{14}, and C_{16} homologues are most effective in inactivating yeasts and molds, gram-positive bacteria, and gram-negative bacteria, respectively. Observations in our study that quaternary ammonium compounds with highest alkyl C_{14} and C_{16} content cause higher lethality to *E. sakazakii* are in agreement with these findings.

Type of cell carrier, i.e., water or infant formula, in which *E. sakazakii* was suspended significantly ($p \leq 0.05$) affected the efficacy of disinfectants 1 - 9 in killing both test strains (Tables 5-2 and 5-3). The effectiveness of all disinfectants was markedly decreased in the

presence of infant formula. The microbicidal activity of chlorine, quaternary ammonium compounds, peroxyacetic acid, hydrogen peroxide, and phenolic compounds is known to be reduced upon contact with organic materials (Ostrander and Griffith, 1964; Troller, 1993; Beuchat et al., 2004; Kennedy et al., 2006; Kim and Beuchat, 2006; Marriott and Gravani, 2006). In addition, upon combining infant formula (pH 6.6) with disinfectant solutions, the pH shifted toward 7.0. Disinfectants 6 and 8, for example, had the lowest pH (2.72 and 2.76, respectively) when combined with cells suspended in water, while respective pH values were 5.64 and 5.77, respectively, when combined with infant formula. The pH of disinfectants 3, 5, and 9 ranged from 9.30 - 10.71 when combined with water containing cells, but was reduced to 7.61 - 9.69 when combined with infant formula. Exposure of *E. sakazakii* to low pH (< 4.0) is known to cause reductions in populations (Edelson-Mammel and Buchanan, 2004; Kim and Beuchat, 2005). Exposure of *E. sakazakii* to environments at pH 9.30 - 10.71, however, may not cause immediate death. Gurtler and Beuchat (2005) reported that populations of *E. sakazakii* decreased by less than 0.5 log CFU/ml when cells were exposed to an environment at pH 11.25 for 5 min. Considering disinfectants 1 - 9, only in inoculated formulas treated with disinfectants 3 and 7 did significant reductions in populations of both strains occur within 10 min. Disinfectant 3 contains n-alkyl (60% C_{14}, 30% C_{16}, 5% C_{12}, and 5% C_{18},) dimethyl benzyl ammonium chloride and n-alkyl dimethyl ethylbenzyl ammonium chloride, whereas disinfectant 7 contains peroxyacetic acid and hydrogen peroxide as active ingredients.

In contrast to the depressing effect infant formula has on lethality of disinfectants 1 - 9 to *E. sakazakii*, the type of carrier had no affect on lethality of disinfectants 10 - 13. This is attributed to the low concentration of organic material in the reaction mixture, which was 100-

fold less than the amount introduced by the infant formula in experiments involving the evaluation of disinfectants 1 - 9.

Resistance of spot inoculated, dried cells to disinfectants. Table 5-4 shows populations of *E. sakazakii* strains 3231 and 3439 recovered from the surface of stainless steel on which inocula in water and infant formula were dried and treated with disinfectants 2, 5, 6, 7, 9, and 11. These disinfectants were selected for evaluation because they showed different degrees of lethality to *E. sakazakii* in suspension, represent a wide range of recommended applications, and are based on various types of bacterocides, viz., quaternary ammonium compounds, phenolic compounds, and a combination of peroxyacetic acid and hydrogen peroxide. Treatment of coupons that had been inoculated with cells suspended in water with disinfectant 7, containing peroxyacetic acid and hydrogen peroxide, and disinfectant 9, containing phenolic compounds, reduced initial populations of 7.98 and 8.01 log CFU/coupon for strains 3231 and 3439, respectively, to < 1.48 log CFU/coupon within 5 min. Both strains were reduced to populations < 1.48 log CFU/coupon by treating coupons for 1 min with disinfectant 11, a quaternary ammonium compound product. Numerous studies have shown that hydrogen peroxide (Robbins et al., 2005), peroxyacetic acid (Farrell et al., 1998), and phenolic compounds (Vesley and Michaelsen, 1964) are effective in reducing microbial populations on abiotic surfaces. We have observed a peroxyacetic acid-based sanitizer to be effective in killing *E. sakazakii* on produce (Kim and Beuchat, 2006). Cells of strains 3231 and 3439 applied to stainless steel using water as a carrier and initially at populations of 7.98 and 8.01 log CFU/coupon, respectively, survived 10-min treatments with disinfectants 2, 5, and 6, all quaternary ammonium products containing alkyl (50% C_{14}, 40% C_{12}, and 10% C_{16}) dimethyl benzyl ammonium chloride as the major microbicide. Disinfectant 11, the most effective among the six disinfectants tested, contains

Table 5-4. Survival of *E. sakazakii* strains 3231 and 3439 spot inoculated and dried on the surface of stainless steel coupons as affected by the type of carrier (water or infant formula) in which cells were suspended

			Population (log CFU/coupon)							
			Strain 3231				Strain 3439			
			Water		Infant formula		Water		Infant formula	
Disinfectants[a]	pH[b]	Treatment time (min)	Recovered[c]	R[d]	Recovered[c]	R[d]	Recovered[c]	R[d]	Recovered[c]	R[d]
Water	7.20	0	7.98 A		8.74 A		8.01 A		8.65 A	
(control)		1	A 8.01 A		A 8.44 A		A 8.35 A		BC 8.58 A	
		5	A 8.22 A		A 8.51 A		A 8.31 A		A 8.44 A	
		10	A 8.29 A		A 8.45 A		A 8.29 A		AB 8.43 A	
2	6.68	0	7.98 A		8.74 A		8.01 A		8.65 B	
		1	C 6.43 AB	1.58 A	A 8.46 A	+0.02 B	B 7.62 AB	0.73 A	A 9.01 A	+0.43 B
		5	C 5.21 B	3.01 A	A 8.04 B	0.47 B	B 7.14 AB	1.17 A	A 8.38 B	0.06 B
		10	D 1.08 C	7.21 A	D 7.40 C	1.05 B	B 6.78 B	1.51 A	A 8.53 B	+0.1 B
5	8.99	0	7.98 A		8.74 A		8.01 A		8.65 A	
		1	B 7.54 A	0.47 A	A 8.22 A	0.22 A	B 7.87 A	0.48 A	BC 8.63 A	+0.05 B
		5	B 6.86 B	1.36 A	A 8.44 A	0.07 B	B 7.34 AB	0.97 A	A 8.63 A	+0.19 B
		10	C 4.75 C	3.54 A	AB 8.36 A	0.09 B	B 6.42 B	1.87 A	A 8.55 A	+0.12 B
6	2.72	0	7.98 A		8.74 A		8.01 A		8.65 A	
		1	C 6.64 B	1.37 A	A 8.61 AB	+0.17 B	B 7.49 AB	0.86 A	AB 8.75 A	+0.17 A
		5	B 6.35 B	1.87 A	A 8.16 AB	0.35 B	B 7.07 AB	1.24 A	A 8.60 A	+0.16 A
		10	B 6.21 B	2.08 A	BC 8.02 B	0.43 B	B 6.76 B	1.53 A	AB 8.28 A	0.15 A
7	5.24	0	7.98 A		8.74 A		8.01 A		8.65 A	
		1	C 6.40 B	1.61 A	A 8.16 A	0.28 B	B 7.29 A	1.06 A	B 8.70 A	+0.12 B
		5	D < 1.48[e] C	≥ 6.74 A	B 4.96 B	3.55 B	C < 1.48[e] B	≥ 6.83 A	B 6.48 B	1.96 B
		10	D < 1.48 C	≥ 6.81	E < 1.48 C	≥ 6.97	C < 1.48 B	≥ 6.81	C < 1.48 C	≥ 6.95
9	9.98	0	7.98 A		8.74 A		8.01 A		8.65 AB	
		1	D 4.37 B	3.64 A	A 8.67 A	+0.23 B	C 5.79 B	2.56 A	AB 8.87 A	+0.29 B
		5	D < 1.48 C	≥ 6.74 A	A 8.59 A	+0.08 B	C < 1.48 C	≥ 6.83 A	A 8.28 BC	0.16 B
		10	D < 1.48 C	≥ 6.81 A	C 7.95 B	0.50 B	C < 1.48 C	≥ 6.81 A	B 8.04 C	0.39 B
11	12.04	0	7.98 A		8.74 A		8.01 A		8.65 A	
		1	E < 1.48 B	≥ 6.53 A	A 8.35 B	0.10 B	D < 1.48 B	≥ 6.87 A	C 8.36 A	0.07 B
		5	D < 1.48 B	≥ 6.74	C < 1.48 C	≥ 7.03	C < 1.48 B	≥ 6.83	C < 1.48 B	≥ 6.96
		10	D < 1.48 B	≥ 6.81	E < 1.48 C	≥ 6.97	C < 1.48 B	≥ 6.81	C < 1.48 B	≥ 6.95

[a] See Table 5-1 for description of disinfectants

Table 5-4, continued

[b] pH of water or treatment solution (disinfectant)
[c] Comparison of the effect of disinfectants: within the same strain and the same treatment time, mean values in the same column that are not preceded by the same letter are significantly different ($p \leq 0.05$). Comparison of the effect of treatment time: within the same strain and within water or each disinfectant treatment, mean values in the same column that are not followed by the same letter are significantly different ($p \leq 0.05$).
[d] Reduction or increase in population compared to the number of *E. sakazakii* recovered from stainless steel coupons treated with water (control) for the same length of time. Comparison of the effect of carrier (water versus infant formula): within the same strain, mean values for R in the same row that are not followed by the same letter are significantly different ($p \leq 0.05$).
[e] Detection limit was 30 CFU/coupon (1.48 log CFU/coupon).

alkyl (50% C_{14}, 30% C_{16}, 5% C_{12}, and 5% C_{18}) dimethyl benzyl ammonium chloride and n-alkyl dimethyl ethylbenzyl ammonium chloride.

As with planktonic cells treated with disinfectants 1 - 9 (Tables 5-2 and 5-3), the composition of the carrier used to suspend cells significantly ($p \leq 0.05$) affected the efficacy of disinfectants in killing cells of *E. sakazakii* strains 3231 and 3439 dried on the surface of stainless steel (Table 5-4). Cells of both strains of *E. sakazakii* in infant formula dried on stainless steel showed significantly higher resistance to all disinfectants, compared to the resistance of cells applied to stainless steel using water as a carrier (Tables 5-4). With the exceptions of disinfectants 7 and 11, ≥ 7.40 and ≥ 8.04 log CFU/coupon of strains 3231 and 3439, respectively, survived in infant formula initially containing 8.74 and 8.65 log CFU/coupon, respectively, after treatment with disinfectants for 10 min. Treatment with disinfectants 7 and 11 for 10 and 5 min, respectively, decreased the number of *E. sakazakii* in dried infant formula to < 1.48 log CFU/coupon.

The greatest reductions in populations of spot-inoculated, dried cells were achieved by treatment with disinfectant 11, regardless of type of cell carrier and strain (Table 5-4). In addition to its unique alkyl ammonium chloride composition, among the disinfectants tested, disinfectant 11 has the highest pH (12.04). The alkaline pH of disinfectant 11 may in part contribute additively to the reductions in populations. Highly alkaline pH environments can cause disruption of cell membrane of gram negative bacteria, resulting in leakage of cytoplasm and death (Mendonca et al., 1994).

Compared to planktonic cells, a lower percentage of cells dried on stainless steel coupons was exposed to the treatment solutions, which may explain in part the lower effectiveness of disinfectants in killing *E. sakazakii* dried on stainless steel. Although a portion of the dried cells

would be expected to be injured by desiccation, thereby increasing sensitivity to disinfectants, organic material (in the case of the infant formula carrier) and cells at or near the surface of the dried inoculum would provide protective barriers against contact with disinfectants. Some of the cells dried on the surface of stainless steel may have undergone starvation during drying for 20 h, a condition known to increase the resistance bacteria to sanitizers (Berg et al., 1982; Jones and Pickup, 1989; Walters et al., 2003). Mosley et al. (1976) reported that bacteria inoculated on stainless steel strips were more resistant than planktonic cells to sanitizers. Treatment with various sanitizers, including quaternary ammonium compounds and peroxyacetic acid, was reported to be effective in killing *Pseudomonas fluorescens* and *Yersinia enterocolitica* in liquid suspension but relatively ineffective in killing cells attached to surfaces (Mosteller and Bishop, 1993). Our observations on the behavior of *E. sakazakii* are in agreement with these reports.

Efficacy of disinfectants in killing *E. sakazakii* in biofilm. Biofilms formed by *E. sakazakii* on stainless steel immersed in rehydrated infant formula for 6 or 12 days were treated with water and disinfectants 2, 5, 6, 7, 9, and 11 for 1, 5, and 10 min. Populations of strains 3231 and 3439 surviving treatment are shown in Tables 5-5. Treatments with disinfectants 2, 5, and 6 did not significantly ($p > 0.05$) reduce the population of strain 3231 (7.68 log CFU/coupon) in 6-day-old biofilm, compared to treatment with water (control). Treatment with disinfectants 7 and 9 for 10 min significantly ($p \leq 0.05$) reduced populations, but only by 2.45 and 0.81 log CFU/coupon, respectively. Treatment with disinfectant 11 decreased the population of strain 3231 in 6-day-old biofilm by 0.77 log CFU/coupon within 1 min and subsequently caused decreases to an undetectable level (<1.48 log CFU/coupon) at 5 min. As with strain 3231, treatment with disinfectant 11 for 5 min resulted in significant reductions in populations of strain

Table 5-5. Survival of *E. sakazakii* strains 3231 and 3439 in biofilm formed on the surface of stainless steel coupons immersed in infant formula at 25°C for 6 or 12 days as affected by treatment with disinfectants

			Population (log CFU/coupon)							
			Strain 3231				Strain 3439			
			6-day biofilm		12-day biofilm		6-day biofilm		12-day biofilm	
Disinfectants[a]	pH[b]	Treatment time (min)	Recovered[c]	R[d]	Recovered[c]	R[d]	Recovered[c]	R[d]	Recovered[c]	R[d]
Water (control)	7.20	0	7.68 A		7.80 A		7.74 A		8.23 A	
		1	A 7.74 A		A 7.91 A		A 7.67 A		A 8.07 A	
		5	A 7.69 A		A 7.85 A		A 7.82 A		A 7.99 A	
		10	A 7.71 A		A 7.81 A		A 7.55 A		A 8.11 A	
2	6.68	0	7.68 A		7.80 A		7.74 A		8.23 A	
		1	AB 7.45 A	0.29 A	A 7.24 AB	0.67 A	A 7.71 A	+0.04 A	A 7.79 AB	0.28 A
		5	A 7.12 A	0.57 A	AB 6.79 B	1.06 A	B 7.06 B	0.76 A	AB 7.34 BC	0.65 A
		10	A 7.03 A	0.68 A	AB 6.43 B	1.38 A	A 6.67 B	0.88 A	A 6.86 C	1.25 A
5	8.99	0	7.68 A		7.80 A		7.74 A		8.23 A	
		1	A 7.67 A	0.07 A	A 7.35 AB	0.56 A	A 7.80 A	+0.13 A	A 7.87 AB	0.20 A
		5	A 7.08 A	0.61 A	AB 6.84 B	1.01 A	A 7.80 A	0.02 A	A 7.89 AB	0.10 A
		10	A 7.12 A	0.59 A	A 6.95 B	0.86 A	A 7.16 B	0.39 A	A 7.30 B	0.81 A
6	2.72	0	7.68 A		7.80 A		7.74 A		8.23 A	
		1	A 7.69 A	0.05 A	A 7.30 AB	0.61 A	A 7.63 A	0.04 A	A 7.96 AB	0.11 A
		5	A 7.09 A	0.60 A	B 6.45 B	1.40 A	B 7.21 A	0.61 A	A 7.41 BC	0.58 A
		10	A 7.02 A	0.69 A	A 6.98 AB	0.83 A	A 6.35 B	1.20 A	A 6.87 C	1.24 A
7	5.24	0	7.68 A		7.80 A		7.74 A		8.23 A	
		1	AB 7.46 AB	0.28 A	AB 7.01 AB	0.90 A	B 6.71 A	0.96 A	A 7.19 AB	0.88 A
		5	A 6.61 B	1.08 A	B 6.47 BC	1.38 A	C 5.70 A	2.12 A	B 6.73 B	1.26 A
		10	B 5.26 C	2.45 A	B 5.70 C	2.11 A	B 2.72 B	4.83 A	B 5.22 C	2.89 A
9	9.98	0	7.68 A		7.80 A		7.74 A		8.23 A	
		1	A 7.64 A	0.10 A	A 7.84 A	0.07 A	A 7.63 AB	0.04 A	A 7.96 AB	0.11 A
		5	A 7.04 AB	0.65 A	A 7.39 AB	0.46 A	B 7.09 BC	0.73 A	A 7.57 B	0.42 A
		10	A 6.90 B	0.81 A	A 7.19 B	0.62 A	A 6.60 C	0.95 A	A 6.75 C	1.36 A
11	12.04	0	7.68 A		7.80 A		7.74 A		8.23 A	
		1	B 6.97 B	0.77 B	B 6.37 B	1.54 A	C 3.68 B	3.99 A	B 5.01 B	3.06 A
		5	B < 1.48[e] C	≥ 6.21	C < 1.48 C	≥ 6.37	D < 1.48[e] C	≥ 6.34	C < 1.48 C	≥ 6.51
		10	C < 1.48 C	≥ 6.23	C < 1.48 C	≥ 6.33	C < 1.48 C	≥ 6.07	C < 1.48 C	≥ 6.63

[a] See Table 5-1 for description of disinfectants

Table 5-5, continued

[b] pH of water or treatment solution (disinfectant)
[c] Comparison of the effect of disinfectants: within the same strain and the same treatment time, mean values in the same column that are not preceded by the same letter are significantly different ($p \leq 0.05$). Comparison of the effect of treatment time: within the same strain and within water or each disinfectant treatment, mean values in the same column that are not followed by the same letter are significantly different ($p \leq 0.05$).
[d] Reduction or increase in population compared to the number of *E. sakazakii* recovered from stainless steel coupons treated with water (control) for the same length of time. Comparison of the effect of carrier (water versus infant formula): within the same strain, mean values for R in the same row that are not followed by the same letter are significantly different ($p \leq 0.05$).
[e] Detection limit was 30 CFU/coupon (1.48 log CFU/coupon).

3439 in 6-day-old biofilms to an undetectable level (< 1.48 log CFU/coupon). Exposure of biofilms to disinfectants 2, 5, 6, and 9 for 5 - 10 min caused significant reductions in populations of strain 3439 in 6-day-old biofilms, but ≥ 6.35 log CFU/coupon survived after treatment for 10 min.

The behavior of *E. sakazakii* in 12-day-old biofilms was similar to that in 6-day-old biofilms, indicating that the age of the biofilms did not have a major effect on the resistance of cells to disinfectants. Significant ($p \leq 0.05$) reductions in numbers of both strains of *E. sakazakii* in 12-day-old biofilms to < 1.48 log CFU/coupon occurred only upon treatment with disinfectant 11 for 5 min. Treatment with all other disinfectants significantly reduced populations of strains 3231 and 3439 in 12-day-old biofilms; however, reductions in populations were ≤ 2.89 log CFU/coupon upon treatment for 10 min. Regardless of strain or age of biofilms, disinfectant 11 had the greatest lethality to *E. sakazakii*; disinfectant 7 had the second greatest lethality.

At the concentrations tested, the lethality of all test disinfectants to *E. sakazakii* in biofilms was lower than that observed for planktonic cells or spot inoculated, dried cells on stainless steel. The overall order of efficacy of all disinfectants in killing *E. sakazakii* was planktonic cells > spot inoculated, dried cells > cells in biofilms. *E. sakazakii* has been observed to form biofilms on the surfaces of stainless steel, silicon, latex, polycarbonate, glass, and polyvinyl chloride (Iversen et al., 2004; Lehner et al., 2005; Kim et al., 2006); however, inactivation of the bacterium in biofilms by the disinfectants examined in our study was not reported.

Mechanisms that enhance the resistance of bacteria in biofilms to environmental stresses have been proposed. Extracellular polymeric substances produced by microorganisms during biofilm formation behave as protective barriers against exposure to environmental stresses

(Costerton et al., 1995; Mah and O'Toole, 2001; Lewis, 2001). Ryu and Beuchat (2005) reported that exopolysaccharide was a major factor enhancing resistance of *E. coli* O157:H7 in biofilms to sanitizers. Oxidizing sanitizers, including hydrogen peroxide, can be neutralized and become less bactericidal upon contact with the surface of biofilms (de Beer et al., 1994; Chen and Stewart, 1996; Xu et al., 1996; Mah and O'Toole, 2001). The production of exopolysaccharide by *E. sakazakii* during biofilm formation may likely have provided a protective barrier against disinfectants. *E. sakazakii* is reported to produce extracellular polysaccharide (Scheepe-Leberkühne and Wagner, 1986; Lehner et al., 2005).

While *E. sakazakii* at or near the surface of biofilms would utilize nutrients and oxygen from the surrounding environment, cells deeply within the biofilm matrix may have undergone starvation, which may increase their resistance to stress. *Pseudomonas aeruginosa* in biofilms showed higher resistance than non-biofilm formers to several antibiotics (Delissalde and Amábile-Cuevas, 2004). Resistance of *E. coli* O157:H7 (Ryu and Beuchat, 2005) and *L. monocytogenes* (Frank and Koffi, 1990; Folsom and Frank, 2006) to sanitizers is significantly greater in biofilms on abiotic surfaces, compared to resistance of planktonic cells. Peracetic acid, mercuric chloride, and formaldehyde at otherwise lethal concentrations were shown to be ineffective in killing microorganisms in biofilms (Carpentier and Cerf, 1993).

In summary, *E. sakazakii* is capable of surviving on the stainless steel for at least 45 - 60 days, depending on type of cell carrier, at refrigeration temperature as well as at temperatures in infant formula preparation and feeding environments. The disinfectants evaluated in this study exhibited various degrees of lethality to *E. sakazakii*, depending on the composition of the carrier used to suspend cells and treatment time. The two test strains, one isolated from a clinical specimen and the other from a food, behaved similarly upon exposure to experimental test

parameters. The presence of infant formula enhanced the resistance of planktonic cells and cells spot inoculated and dried on the surface of stainless steel to the disinfectants. The overall order of resistance of *E. sakazakii* to disinfectants was planktonic cells < cells spot inoculated and dried on stainless steel < cells in biofilms on stainless steel.

Results emphasize the importance of proper cleaning of abiotic surfaces soiled by rehydrated infant formula and other foods. Otherwise, infant formula remaining on these surfaces protects *E. sakazakii* against the lethality of disinfectants or may serve as a source of nutrients, resulting in growth and production of biofilm. Results provide information useful in assessing the efficacy of disinfectants in killing *E. sakazakii* embedded in organic matrices on surfaces in formula preparation and feeding areas in hospitals, day-care centers, and the home.

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CHAPTER 6

SUMMARY AND CONCLUSIONS

The following is a summary of results obtained and conclusions drawn from experiments reported in this dissertation.

1. Survival and growth characteristics of *E. sakazakii* on fresh-cut apple, cantaloupe, strawberry, watermelon, cabbage, carrot, cucumber, lettuce, and tomato and in juice prepared from these fruits and vegetables were determined.
   - *E. sakazakii* can survive and grow on several types of fresh-cut produce and in produce juice.
   - Storage temperature affects survival and growth of *E. sakazakii* on fresh-cut produce as does the type of produce; growth occurs at 12 and 25°C, depending on type of produce, but not at 4°C.
   - *E. sakazakii* is able to grow on fresh-cut produce and in fruit and vegetable juice at a mildly abusive temperature (12°C) and survives at 4°C.

2. Survival and growth characteristics of *E. sakazakii* on the surface of whole (uncut) produce stored at 4, 12, and 25°C for up to 28 days and effectiveness of oxidizing sanitizers for its elimination were studied.
   - *E. sakazakii* was shown to survive on produce stored at refrigerator (4 and 12°C) and ambient (25°C) temperatures for at least 28 days.
   - Treatment of apples, tomatoes, and lettuce with chlorine, chlorine dioxide, and Tsunami 200 causes significant reductions of *E. sakazakii*, although the extent of lethality depends on the type of produce and sanitizer concentration.
   - Chlorine is less effective in killing *E. sakazakii* on lettuce than on apples or tomatoes.

3. The effects of temperature and nutrient availability on attachment and biofilm formation by *E. sakazakii* on the surfaces of stainless steel coupons and enteral feeding tubes were investigated.
    - *E. sakazakii* attached better to the surfaces of enteral feeding tubes and stainless steel in tryptic soy broth (TSB), infant formula broth (IFB), and lettuce juice broth (LJB) at 25°C, compared to 12°C.
    - *E. sakazakii*, pre-attached to stainless steel and enteral feeding tubes and immersed in IFB, formed biofilms at 25°C; biofilms were not formed on stainless steel or tubes immersed in TSB and LJB at 25°C or in IFB, TSB, and LJB at 12°C, indicating that nutrient availability and temperature play major roles in processes leading to the production and accumulation of biometrices on the surfaces of these inert materials. .

4. The efficacy of quaternary ammonium and phenolic disinfectants commonly used in hospitals, day-care centers, and food service kitchens in killing *E. sakazakii* in suspension, dried on the surface of stainless steel, and embedded in biofilm on stainless steel was evaluated.
    - Test disinfectants exhibited various degrees of lethality to *E. sakazakii*, depending on the composition of the carrier used to suspend cells and treatment time.
    - The two test strains, one isolated from a clinical specimen and the other from a food, behaved similarly upon exposure to experimental parameters.
    - The presence of infant formula enhanced the resistance of planktonic cells and cells spot inoculated and dried on the surface of stainless steel to the disinfectants.

- The overall order of resistance of *E. sakazakii* to disinfectants was planktonic cells < cells spot inoculated and dried on stainless steel < cells in biofilms on stainless steel.

To date, produce has not been implicated as a vehicle of *E. sakazakii* infections. However, the potential for infection exists, because the pathogen has been detected on several types of produce not subjected to processing conditions that would cause its elimination before consumption. Results from studies described in this dissertation confirm that fresh produce supports the growth of *E. sakazakii* and demonstrate that treatment with sanitizers reduces but does not eliminate the pathogen. These observations provide valuable information when developing interventions to control the pathogen in produce processing plants.

E. sakazakii was observed to attach to abiotic surfaces and form biofilms, which resulted in an increase in resistance to disinfectants routinely used in infant formula preparation and feeding areas. Cells embedded in infant formula were particularly resistant to treatment with disinfectants.

Findings emphasize the importance of temperature control in produce processing environments to inhibit growth in infant formula preparation areas to inhibit attachment and biofilm formation by *E. sakazakii*. The relative effectiveness of commercial disinfectants used in hospitals, day-care centers, and food service kitchens for the purpose of killing *E. sakazakii* and other microorganisms emphasizes the importance of proper disinfection of abiotic surfaces soiled with rehydrated infant formula and other foods that may contain the pathogen.