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Enterobacter Sakazakii Growth Profile and Tolerance to Chlorine Sanitizers

Diana Carolina Naar Cifuentes
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Diana Carolina Naar Cifuentes entitled "Enterobacter Sakazakii Growth Profile and Tolerance to Chlorine Sanitizers." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Science and Technology.

Francis Ann Draughon, Major Professor

We have read this dissertation and recommend its acceptance:

P. Michael Davidson, Arnold Saxton, Doris D'Souza

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Enterobacter Sakazakii Growth Profile and Tolerance to Chlorine Sanitizers

A Dissertation Presented for

The Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Diana Carolina Naar Cifuentes

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ABSTRACT

Enterobacter sakazakii is considered an emerging opportunistic pathogen associated with sporadic life-threatening bacterial infections in neonates linked to the compsumption of contaminated infant formula [Stoll et al., 2004]. In 2001 a neonate fatal infection associated with the presence of *E. sakazakii* in infant formula occurred in the neonatal intensive care unit (NICU) of the University of Tennessee Hospital [Himelright et al., 2002], as a result of this outbreak, the hospital made several policy changes and requested the Food Safety Center of Excellence of University of Tennessee to analyze the growth pattern of this microorganism at the conditions maintained in the hospital. The objective of this study was to analize *E. sakazakii* growth profile during preparation and administration of formula, as well as *E. sakazakii* tolerance to chlorine sanitizers widely used in hospital settings.Our results showed that if the starting temperature of the formula at the time of administration was 6 ^{o}C, the formula reached 25 ^{o}C in a period of four hours. Once contaminated formula reach 25 ^{o}C the generation times can decrease to less than one hour. We also noted that cells organized in colonies or in contact with solid surfaces had a higher resistance to chlorine sanitizers than those of planktonic cells, this phenomena could be explained by the expression of genes triggered by the physical contact between cell and surface.

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PART 1. INTRODUCTION

Introduction

Enterobacter sakazakii is considered an emerging opportunistic pathogen associated with sporadic life-threatening bacterial infections in neonates particularly affecting low birth weight preterm infants of less than 2500 g [Stoll et al., 2004]. The pattern of disease includes meningitis, bacteremia, necrotizing enterocolitis, necrotizing meningoencephalitis and death in 40-80% of cases [Nazarowec-White and Farber, 1997a]. Recent studies have found that infants in whom meningitis developed tended to be near-term with greater gestational age and birth weight than those with bacteremia alone [Bowen and Braden, 2006].

The reservoir for *E. sakazakii* is still unknown, nevertheless it has been isolated from: milk powder, UHT milk, cheese, meat, vegetable, herbs, chocolate, cereal, potato flour, spices, pasta, rice seeds and from guts of the stable fly: *Stomoxys calcitrans*, and the Mexican fruit fly: A*nastrpha ludens* [Cottyn et al., 2001; Hamilton et al., 2003; Kandhai et al., 2004; Kuzina et al., 2001; Leclercq et al., 2002; Skladal et al., 1993].

Although *E. sakazakii* has been detected in various types of food, special attention has been given to outbreaks linked to the consumption of powdered infant formula milk (IFM). Powdered infant formula is not a sterile product. The analysis of 141 samples of milk-based powdered infant formula products obtained from a number of different countries showed that *E. sakazakii* could be recovered from 20 (14%) of 141 samples [Muytjens et al., 1988], another study

conducted in 2003 found *E. sakazakii* in 2 (2.4%) of 82 powdered infant formula samples [Iversen and Forsythe, 2004]. Results of previous investigations suggest that milk-based powdered infant formula with low levels of *E. sakazakii*, fulfilling the requirements of the Codex Alimentarius, can lead to development of infection [van Acker et al., 2001].

The Food Drug Administration (FDA) has pointed out that powdered milk-based infant formulas are heat-treated during processing, but unlike liquid formula products they are not subjected to high temperatures for sufficient time to make the final packaged product commercially sterile. FDA has noted that infant formulas nutritionally designed for consumption by premature or low birth weight infants are available only in commercially sterile liquid form. However, so-called "transition" infant formulas that are generally used for premature or low birth weight infants after hospital discharge are available in both non-commercially sterile powder form and commercially sterile liquid form [Taylor, 2002]. Some other specialty infant formulas are only available in powder form.

The American Dietetic Association and the FDA have issue guidelines for infant formula preparation, storage and administration and recommend powdered infant formulas not be used in neonatal intensive care settings unless there is no alternative available [Agostoni et al., 2004; Taylor, 2002]. However, to meet the increased nutritional needs of premature infants, some hospitals continue to use powdered infant formulas since certain nutrients are lost during heat processing of liquid infant formulas.

Taxonomy

Enterobacter sakazakii is a member of the phylum proteobacteria, which includes Gram-negative bacteria with an outer membrane composed mainly of lipopopysaccharides. Many of the bacteria pertaining to this phylum move using flagella, but some are non-motile or rely on bacterial gliding. The proteobacteria are divided into five sections or classes: α,β,γ,δ,ε based on rRNA sequences*. E. sakazakii* is a member of the family Enterobacteriaceae included in the γ section or class Gamma-proteobacteria order Enterobacteriales. The family Enteobacteriaceae contains more than 100 species of Gram-negative, oxidase-negative, nonsporing bacilli some of which normally inhabit the intestines of humans and animals and are commonly referred as coliforms. All members of the family degrade sugars by means of the Embden-Meyerhof pathway and cleave pyruvic acid to yield formic acid. Preliminary genera identification is based on motility and biochemical characteristics.

Different genera of the *Enterobacteriaceae* family have earned a reputation placing them among the most pathogenic and most often encountered organisms in clinical microbiology (eg. *Salmonella* and *Escherichia coli*).

E. sakazakii was named using the binomial nomenclature that consists of Genus (*Enterobacter)* and species *(sakazakii). G*enus is a taxonomic category ranking below a family and above a species and generally consisting of a group of species exhibiting similar characteristics.

Species consist of individual organisms which are very similar in appearance, anatomy, physiology and genetics due to having relatively recent common ancestors. *E. sakazakii* was previously known as yellow-pigmented *Enterobacter cloacae* but in 1980 the microorganism was reclassified as a new bacterial species based on differences between *E. cloacae* and *E. sakazaki*i in deoxyribonucleic acid DNA-DNA hybridization, biochemical reactions, pigment production and antibiotic susceptibility [Farmer, 1980]. *E. sakazakii* differs from other species in its ability to produce an extracellular deoxyribonuclease and the inability of some strains to ferment D-sorbitol [Farmer, 1980; Heuvelink et al., 2002].

Distinguishing characteristics of the microorganism also include activity of the enzyme α-glucosidase, utilization of citrate as a sole carbon source, survival of cells in stock cultures stored at 17-30 °C without transfer for up to 8 years and production of yellow pigment. Muytjens et al. [1984] reported that 100% of *E. sakazakii* (n = 129) were positive for α-glucosidase in comparison to 0% of other Enterobacter species (n = 97), however other member of the family *Enterobacteriaceae* have shown to be positive for both yellow pigment and α-glucosidase activity. The yellow pigment production is enhanced at temperatures less than 36 °C, with optimum production at 25 °C, but the production of the diffusible yellow pigment is unstable with repeated subculturing. The occurrence of non-pigmented *E. sakazakii* has also been reported [Farmer, 1980].

Different techniques are used to study the phylogenetic relationships of *E. sakazakii* ; while the microorganism was reported to be 50% related to *Enterobacter cloacae* and *Citrobacter koseri* by using DNA-DNA hybridization [Farmer, 1980], other techniques using 16 S ribosomal DNA (rDNA) and hsp60 sequencing, showed that *E. sakazakii* type strain 16S rDNA sequence was 97.8% similar to that of Citrobacter koseri but 97.0% similar to that of *Enterobacter cloacae* [Iversen et al., 2006; Lehner et al., 2006]. Techniques using DNA hybridization, antibiotic susceptibility and biochemical reactions have further distinguish 57 strains of yellow pigmented *E. sakazakii* [Farmer, 1980], nevertheless the phenotype test has not been proven effective for identification of all species and is not reliable in distinguishing strains within species.

Comparative 16S rDNA and *hsp60* sequencing of 126 strains identified as *E. sakazakii* by biochemical test kits (API20E and ID32E) showed that commercial biochemical test kits identified more than one species as *E. sakazakii*, and that there were at least four clusters conformed by genetically and biochemically distinct subgroups of *E. sakazakii* [Iversen et al., 2004c]. The majority of the 126 strains evaluated (110 strains) were in cluster 1, with 0.1 to 1.2% difference from the type strain. Nine strains exhibited 1.6 to 1.9% sequence divergence from the type strain and formed a second cluster closely related to *E. sakazakii.* The third cluster contained five strains, which were more closely related to *Enterobacter pyrinus*, *Enterobacter hormaech*i, and *C. koseri.* The fourth cluster contained two strains, identified as *E. sakazakii* by API20E and

ID32E, but their 16S rDNA sequences were just 96.5% similar to the type strain of that species.

Other studies in characterization of taxonomic heterogeneity between the strains, have been developed using fingerprinting DNA and RNA techniques such as PCR, pulsed-field gel electrophoresis (PFGE), chromosomal DNA restriction analysis, ribotyping, plasmid typing and randomly amplified polymorphic DNA (RAPD) [Farber, 1996; Grant and Kroll, 1993; Nazarowec-White and Farber, 1999]. Ribotyping analysis with the EcoR1 restriction endonuclease showed to be more discriminatory than restriction endonuclease analysis (REA) [Clark et al., 1990; Nazarowec-White and Farber, 1999]. The application of Artificial Neural Networks (ANNs) to identify key phenotypic characteristics and nucleotide sequences which discriminate *E. sakazakii* from similar, closely related organisms, predicted that testing for the metabolism of glucose-1-phosphate, sucrose and arginine gave the highest discrimination [Iversen et al., 2006; World Health Organization and Food and Agriculture Organization of the United Nations, 2006].

Isolation

Enterobacter sakazakii is a Gram negative, facultative, rod-shaped bacterium, that possesses peritrichous flagella. Initial isolation of the bacterium using the U.S. Food and Drug Administration (FDA) method requires rehydration of infant formula powder in sterile distilled water overnight at 36 °C, enrichment in

Enterobacteriaceae enrichment broth (EE), overnight at 36 °C, direct spreading or streaking method into violet red bile glucose agar (VRBGA) with incubation overnight at 36 °C and subculture of presumptive purple halo *E. sakazakii* colonies onto Trypticase Soy Agar (TSA) for 48-72 hours to detect the production of yellow pigment. Confirmation is performed by using API20E series and oxidase test. This method has the disadvantage that VRBG agar is not sufficiently selective, and that TSA requires 24-72 hours incubation time to produce yellow-pigment colonies. In addition, pigment production in some strains is temperature dependent and not all strains of *E.sakazakii* are yellow pigmented (Farmer et al. 1980) Guillaume-Gentil et al.[2005] reported that pigment production was enhanced by light exposure and the use of artificial white light.

As an alternative for the FDA isolation method, simple and rapid cultural methods for detection of *E. sakazakii* have been developed, including media containing new chromogenic or fluorogenic substrates, such as: DFI developed by Iversen, Drugan and Forsythe (2004), LBDC agar developed by Leuschner, Baird, Donald, and Cox (2004), OK agar developed by Oh and Kang (2004), NES agar developed by Guillaume-Gentil et al. (2005), ISO agar developed by ISO TS 22964, and RF agar developed by Restaino et al (2006). Chromogenic or fluorogenic media are based on the α – glucosidase activity and/or the use of fluorogenic and chromogenic substrates such as 4-methylumbelliferyl (4-MU) and 5-bromo-4-methyl-3-indoxyl (X)- -linked α-D-glucosides in media [Guillaume-Gentil et al., 2005; Iversen et al., 2004a; Leuschner et al., 2004; Oh and Kang,

2004]. The performance of differential selective media for supporting resuscitation and colony development by stressed cells of *E. sakakazakii* previously submitted to heat, freezing, acidic pH, alkaline pH and desiccation indicated that differential selective media vary greatly in their abilities to support resuscitation and colony formation, the general order of growth indices of stressed cells is:TSA agar with 0.1% pyruvate, LBDC > Fecal Coliform Agar > RF > VRBG,OK > DFI, EE [Gurtler and Beuchat, 2005].

The colony morphology follows two distinct phenotypes [Farmer, 1980; Iversen and Forsythe, 2003]. One colony type is described as “matt”, being leathery or rubbery when touched with a wire loop, this type of colony snaps back to the agar when touched. The other colony type is described as “glossy”, it is smooth and creamy, easily removed with a wire loop. It has been found that leathery colonies may revert to typical smooth colonies when subcultured from stock cultures. The production of either matt or glossy morpholgies is related to presence of capsules or production of exopolysaccharides [Harris and Oriel, 1989]. The condition influencing the production of exopolysaccharides was studied by Scheepe-Leberkuhne and Wagner [1986]. Preliminary results of the heteropolysaccharide composition revealed that the exopolysaccharide contained galactose/fucose/glucose/glucuronic acid/acetate in the molar ratio of 2:2:1:0:7:3 [Lehner et al., 2005; Scheepe-Leberkuhne and Wagner, 1986]. The composition of the sugars was independent of the carbon source (Glucose or Glycerol) used during cultivation and the polysaccharide production was

optimized by increasing the carbon/nitrogen ratio in the growth media. Further analysis of heteropolysaccharides produced by *E. sakazakii* 1387-2 indicated the presence of glucose, galactose, fucose and glucuronic acid in the ratio 1:1:1:0.8 [Lehner et al., 2005]. Phenotypic and molecular investigation of cellulose expression suggested cellulose production by certain strains of *E. sakazakii* grown in Luria-Bertani broth (LB) at 28 °C and 37 °C.

Resistance to Stress Conditions

Acid Tolerance

The ability to tolerate acidic environments is an important feature that influences the survival of foodborne pathogens in foods and through the gastrointestinal system. Some members of the family *Enterobacteriaceae*, such as *Escherichia coli* O:157 posses an acid-tolerance response mechanism consisting of an integral membrane protein that pumps glutamate and γ-aminobutyrate in opposite directions [Waterman and Small, 2003]. Glutamate is transported inside the cell where decarboxylation takes place consuming one intracellular proton; the product is then exported out of the cell, thereby helping to maintain a neutral cytoplasmic pH when extracellular pH drops [Waterman and Small, 2003]. While it has being presumed that a mechanism similar to this can be present in *E. sakazakii*, no evidence has been provided. Actually, there is currently little data regarding *E. sakazakii* tolerance to acid environments, some studies report that *E. sakazakii* did not grow on apple juice (pH 3.9) or strawberry juice (pH 3.6); but did grow in tomato juice (pH 4.4), watermelon juice (pH 5.0)

and cantaloupe juice (pH 6.8) [Kim and Beuchat, 2005]. The ability of a microorganism to survive exposure to an acid environment is dependent on a large number of factors such as pH, acidulant identity, acidulant concentration, temperature, water activity, atmosphere, and the presence of other inhibitory compounds [Buchanan and Edelson, 1999; Buchanan and Golden, 1995].

Edelson-Mammel et al. [2006] reported that after 5 hours of exposure at pH 3.5, 10 of 12 *E. sakazakii* strains in stationary phase showed less than 1 log reduction, the most sensitive strain showed a 3.5 log reduction. At pH 3.0, the decline over the 5 hours incubation period ranged from 4.0 to >6.3 log. Their study reports that stationary cells of *E. sakazakii* can withstand transitory exposure to a pH 3.0 and that the acid resistance of some, but not all strains of *E.sakazakii* is enhanced by prior growth in an acidogenic medium that habituated cells to pH 5.0-5.2. This evidence supports the fact that *E. sakazaki*i can survive the acidic conditions of neonates stomach because the pH of infants is rarely below pH 4–5 for the first six months of life.

Another factor that has being linked with acid tolerance in *E. sakazakii* is the synthesis of exopolysaccharides which produces the formation of mucoid colonies. *E. sakazakii* exopolysaccharides contain glucuronic acid [Lehner et al., 2005; Scheepe-Leberkuhne and Wagner, 1986]; the negatives charges of glucuronic acid have the ability to buffer extracellular protons providing protection to the cell, glucuronc acid is also involved in the production of mucoid colonies.

Osmotic and dry stress resistance

The detection of *E. sakazakii* in powdered infant formula suggests that this microorganism can survive for extended periods in low water activity products. Dry infant formula has a water activity (a_w) of approximately 0.2 [Breeuwer et al., 2003]. The ability of *E. sakazakii* to survive in such a dry environment is influenced by the bacterium's osmotic and dry stress resistance.

Desiccation-sensitive bacteria die when the cellular water content is reduced to 0.3 water/g dry weight [Potts, 1994b] while desiccation-tolerant bacteria resist the removal of all but 0.1g water/g dry weight [Billi and Potts, 2000]. Several organisms capable of surviving dehydration have the ability to accumulate large amounts of solutes, such as K+ ions, glutamate, glutamine, praline, glycine betaine, glucosylglycerol sucrose and trehalose.

According to the water replacement hypothesis, trehalose and sucrose hydrogen bond membrane phospholipids and proteins, maintaining their integrity in the absence of water. Another point of view suggests that sugars involved in stabilizing dehydrated microorganisms do so by virtue of the ability to form glasses. A glass is defined as a liquid of such high viscosity that it is capable of slowing all chemical reactions and prevents the complete dehydration of bacterial cells at temperatures below the melting point of glass. An additional mechanism of protection that has been considered is the production of exopolysaccharides of high viscosity around bacterial cell walls, which tend to be hygroscopic thus

decreasing the rate of water loss from the cells. It is widely believed that production of exopolysaccharides provides microorganisms with a means to survive drying; however, little is known about the specific responses of polysaccharide synthesis to drying. Some studies have confirmed the exopolysaccharides protective roles, but others studies have failed to show any obvious correlation [Potts, 1994a]. Recent studies have shown that different carbohydrate derivatives (glycolipids), have subtle effects on the physical properties of membranes, the carbohydrate portion of each derivative appeared to mimic the effects of water; these data align with the water replacement hypothesis [Goodrich et al., 1991].

E. sakazakii resistance to osmotic and dry stress has been related to its ability to accumulate intracellular trehalose [Breeuwer et al., 2003]. In a recent study, it was found that dried stationary cells increased the trehalose concentration more than five folds; this accumulation was not observed in dried exponential phase *E. sakazakii* cells, which are much more sensitive to dry stress [Breeuwer et al., 2003]. Studies conducted with *E. coli* cells demonstrated that the trehalose synthesis genes were induced by stationary phase sigma factor θ^{s} [Hengge-Aronis et al., 1991], these findings help to explain the higher concentration of trehalose in stationary phase and therefore the higher resistance to osmotic and dry stress compared with exponential phase cells.

Edelson-Mammel et al.[2005] studied the survival of *E. sakazakii* inoculated in a batch of infant formula over the course of almost two years. An

approximate 2.3-log decrease in viable counts upon rehydration was observed during the first 150 days of storage. The D-value during this initial period of 150 days, was 73.8 days. In the second phase of the study (153 to 687 days), viable counts declined an additional 1.0 log cycle; the calculated D-value was 684.9 days. Even though a majority of *E. sakazakii* cells were inactivated by storage in dehydrated powdered infant formula, a portion of the cells were highly resistant to the storage conditions and survived for at least 2 years.

In conclusion, stationary phase *E. sakazakii* cells have the ability to survive desiccation and have been found to be more resistant to osmotic and dry stress than *Escherichia coli*, *Salmonella* and other bacteria in the *Enterobacteriaceae*.

Thermal resistance

Effective use of thermal treatments to reduce the risk associated with food borne pathogens requires accurate information on the heat resistance of the targeted microorganism; the thermal treatment should be sufficient to inactivate the microorganism of concern while minimizing the loss of nutrients [Edelson-Mammel and Buchanan, 2003].

It is known that heat resistance is influenced by different factors such as physiological state of the organism, growth temperature and medium composition [Knabel et al., 1990]. The high content of total solids and high amount of fat in infant formula protect microorganisms from heat, making it difficult to compare D-

values. However, Breeuwer et al.[2003] found that for four *E. sakazakii* strains analyzed the type of reconstituted infant formula did not influence the D-value.

Nazarowec-White and Farber [1997b] obtained data on the D and z values of ten Canadian *E. sakazakii* strains (5 clinical and 5 food isolates) in reconstituted dried-infant formula. D-values for clinical strains were not significant different to the D-values for the food strains. D-values of *E. sakazakii* for reconstituted dried-infant formula were $D_{52\,^{o}C}$ 54.8 min , $D_{54\,^{o}C}$ 23.7 min, $D_{56^{o}C}$ 10.3 min, $D_{58^{o}C}$ 4.2 min, $D_{60^{o}C}$ 2.5 min. The z value for the pooled clinical and food strains was 5.82 ºC, which was within the range reported for most non-sporeforming bacteria. Iversen et. al. [2004b] calculated D-values for *E. sakazakii* in a rehydrated powdered milk formula obtaining as result $D_{54\,^{o}C}$ 16.4 min , $D_{56^{o}C}$ 5.1 min, $D_{58^{o}C}$ 2.6 min, $D_{60^{o}C}$ 1.1 min, $D_{62^{o}C}$ 0.3 min. From the limited data obtained, it appears that some strains of *E. sakazakii* are more thermotolerant than many other *Enterobacteriaceae* or *Listeria monocytogenes* in dairy products [Nazarowec-White and Farber, 1997a, Piyasena et al., 1998].

Edelson-Mammel and Buchanan [2003] evaluated the diversity in thermal resistance among 12 *E. sakazakii* isolates and the effect of rehydrating dried infant formula with water at different temperatures on the survival of the microorganism. Substantial variation in thermal resistance was observed among the isolates, with almost a 20 fold differential existing between the most and least heat-resistant strain. The distribution of heat resistance among the various isolates was bimodal, with half of the strains having $D_{58^{o}C}$ -values of less than 50

s and the other half having $D_{58°C}$ -values of more than 300 s. The calculated z value for the most heat resistant strain was 5.6 ºC, and 4-D or more inactivation was observed with water temperatures $\geq$ 70 ºC. On the basis of the 12 strains examined, the thermal resistance of *E. sakazakii* fell into two distinct phenotypes, which suggest that this microorganism might have a relative simple set of genetic determinants for thermal resistance [Edelson-Mammel and Buchanan, 2003].

In order to obtain a 6 or 7 log reduction, the length of heat treatment required at 60 ºC would be 15 and 17.5 min, respectively [Nazarowec-White and Farber, 1997a]. *E. sakazakii* would not survive the pasteurization process; high temperature, short time (HTST) pasteurization schedule (15 s at 71.7 ºC) ensures a greater than 11D kill of *E. sakazakii* in dried-infant formula, which indicates that *E. sakazakii* can not survive a commercial pasteurization process [Iversen et al., 2004b]. However, Skladal et al. [1993] found *E. sakazakii* to be one of the major contaminating bacteria in ultra-high-temperature (UHT) milk cartons, implying that the organism may survive UHT temperatures or post processing contamination may take place.

Determination of D-values of pathogens cannot be easily extrapolated to continuous processes such as HTST, as they do not take into account the possible effects of shear force and other physical stress. By using a pilot scale HTST pasteurizer it was confirmed that treatments at 68 ºC for 16 s can ensure a 5-log reduction of *E. sakazakii* [Nazarowec-White et al., 1999]. Kindle et al. [1996] studied the killing activity of microwaves in milk and reported than heating

150-ml portions of various infant formula for 85 to 100 s achieved a mean temperature of 82 ºC to 93 ºC and lead to more than 4-D inactivation of *E. sakazakii*. However, Edelson-Mammel and Buchanan [2003] suggested the use of rehydration temperatures no higher than 70 ºC to avoid scalding hazard.

Antibiotic resistance

Antibiotics are secondary metabolites produced by microorganisms that inhibit or kill a wide spectrum of microorganisms. *E. sakazakii* is typically susceptible to ampicillin, the aminoglycosides, chloramphenicol, and the third-generation cephalosporins [Lai and 2001]. However the occurrence of antibiotic resistance has been observed during patient treatment. Muytjens el al. [1983] studied eight cases of neonatal meningitis due to *E. sakazakii* which occurred in the Netherlands. Even though all strains were inhibited in vitro by ampicillin, chloramphenicol, gentamicin and kanamycin as measured by an agar disk diffusion procedure, two patients died despite treatment with ampicillin and gentamicin.

It has been reported that strains are much more susceptible to some of the new beta-lactam antibiotics than to ampicillin. Farmer et al. [1980] tested 10 strains of *E. sakazakii* for antibiotic susceptibility and determined that the minimum inhibitory concentrations (MICs) to chloramphenicol and ampicillin were moderate, at 4–8 µg/ml and 2–4 µg/ml, respectively. Examination of 24 strains of *E. sakazakii* using the Kirby–Bauer disk method revealed that 96% were

sensitive to nalidixic acid (30 µg), 100% to gentamicin (10 µg), 92% to streptomycin (10 µg), 100% to kanamycin (30 µg), 87% to tetracycline (30 µg), 100% to chloramphenicol (30 µg), 100% to ampicillin (10 µg) and 87% to carbenicillin (100 µg)[Farmer, 1980]. The same strains were less susceptible to penicillin (0% at 10 U), cephalothin (13% at 30 µg), sulfadiazine (67% at 250 µg), and colistin (71% at 10 µg) [Farmer, 1980].

Results obtained from a study in China where sixteen strains of *E. sakazakii* isolated from powdered infant formula were evaluated showed that all of the isolates were resistant to benzylpenicillin, oxaxillin and vancomycin, r cephazolin and cefpodoxime except one isolate [Pei et al., 2007].

Stock and Wiedemann [2002] analyzed the antibiotic resistance of *35 E. sakazakii* strains and found that all strains were natural sensitive to tetracyclines, aminoglycosides, penicillin (amoxicillin, ampicillin), chephalosporin (cefoxitin, cefaclor), carbapenems, monobactams, quinilones, lincosamides, streptogramins, glycopeptides and antifolates. Some strains were also resistant to azithromycin.

Lai [2001] reported that the case-fatality rate among patients with meningitis before the use of third-generation cephalosporins was 62%; with the introduction of third-generation cephalosporins in 1985, the case-fatality rate was reduced to14%. *E. sakazakii* has developed resistance to the gold standard treatment ampicillin–gentamicin treatment by means of transposable elements

and to β-lactams by the production of β-lactamase [Lai and 2001; Pitout et al., 1997]. Pitout et al.[1997] tested eight strains of *E. sakazakii* for the presence of β-lactamases. Antibiotic susceptibility testing was against ampicillin, ampicillin–sulbactam, amoxacillin–clavulanic acid, ticarcillin, ticarcillin–clavulanic acid, piperacillin, piperacillin–tazobactam, aztreonam, cephalothin, cefazolin, cefoxitin, cefotaxime, cetriaxone, ceftazidime, cefepime and imipenem. Some of the eight strains were sensitive to the three β-lactams, ampicillin, cephalothin, and cefoxitin, while all wild-type *E. sakazakii* strains were susceptible to ampicillin, cefoxitin and cephalosporins. All eight strains tested for β-lactamases were positive for Bush group 1 β-lactamase (cephalosporinase).

Block et al.[2002] examined *E. sakazakii* isolates from six neonatal and childhood infections and reported all as being β-lactamase positive, most likely representing Bush group 1 β-lactamase. Increasing antibiotic resistance among Enterobacter species could lead to consider using the carbapenems or the newer cephalosporins in combination with a second agent such as an aminoglycoside. Lai [2001] also suggested that trimethoprim-sulfamethoxazole may be a useful agent in the treatment of infections caused by the Enterobacter species, especially in view of the production of extended-spectrum beta-lactamases capable of inactivating the cephalosporins and extended-spectrum penicillin.

Willis and Robinson [1988] detailed two cases of *E. sakazakii*-induced neonatal meningitis that, after being unresponsive to ampicillin–gentamicin therapy, resulted in abatement via treatment with moxalactam. Naqvi et al. [1985]

eliminated *E. sakazakii* infection in one patient by using cefotaxime. Block et al. [2002] concluded that general assumptions concerning antimicrobial therapy for *E. sakazakii* cannot be made, and treatment should be guided by clinical judgment and in vitro susceptibility testing.

Mechanisms of Pathogenicity

Little is known about the mechanisms of pathogenicity of *E. sakazakii*. *E. sakazakii* has been implicated in cases of meningitis and neonatal necrotizing enterocolitis originated by the consumption of contaminated infant formula. To cause disease *E. sakazakii*, must be ingested, survive the harsh conditions of the stomach acids and be able to colonize the lower gastrointestinal tract.

E. sakazakii infections are associated with neonates; although the pH of the gastric liquids in the stomach of neonates is higher than in adults, the stomach still provides a harsh condition.

Actual data regarding *E. sakazakii* tolerance to acid environments, confirm the microorganism's ability to stand the acidic conditions of neonates stomach however further research is needed to understand mechanisms used to overcome acidic conditions [Edelson-Mammel et al., 2006].

Adhesion to tissue cells is an essential virulence factor for most bacterial pathogens, once the microorganism adheres, it can colonize tissue and exert pathogenesis. Mange et al. [2006] studied the adherence ability of 50 *E. sakazakii* strains on three cell lines: Epithelial Hep-2 and Caco-2, as well as the

brain microvascular endothelial cell line HBMEC. Their results show that adherence followed two distinctive patterns, a diffuse adhesion and the formation of localized clusters of bacteria on the cell surface. Research on *E. sakazakii* multicellular behavior showed extracellular matrix, cell clumping, pellicle formation and biofilm formation associated with the expression of cellulose and curli fimbrie features which have been related to increased to increased virulence and transmission in other pathogens [Zogaj et al., 2003]. Mange et al. [2006] reported that adherence is maximal during late exponential phase and is mainly non-fimbial based [Mange et al., 2006].

Bacterial mechanisms of pathogenicity include production of endotoxins and exotoxins. Exotoxins are proteins, often enzymes, produced inside the cells that produce damage upon release from the cell. An *enterotoxin* is an exotoxin released by a micro-organism in the lower intestine that disrupts the lining of the gastrointestinal tract. On the other hand, endotoxins are structural components of bacteria such as: lipid A core region of lipopolysaccharide (LPS) which forms the outer membrane of Gram-negative bacteria and exerts their effect after cell lysis.

Endotoxins are heat stable at 100 °C and can remain biologically active after pasteurization. Pagotto et al. [2003] evaluated 18 isolates of *E. sakazakii* for enterotoxin production by the suckling mouse assay, four isolates were found to test positive for enterotoxin production. When comparing the cytopathic effect of the enterotoxin produced by *E. sakazakii LA* to that of *E. coli O157:H7,* it was found that *E. sakazakii LA* enterotoxin was toxic to three cell lines CHO, VERO,

Y-1 while *E. coli O157:H7* was only toxic to Vero cells but to a higher degree. Boiling of the enterotoxin did not eliminate the cytopathic effect either in *E. sakazakii LA* enterotoxin or in *E. coli O157:H7* enterotoxin. These results showed that *E. sakazakii LA* and *E. coli O157:H7* produced an enterotoxin with similar cytotoxicity, which can destroy the tissue in the intestines causing necrotizing enterocolitis.

Further studies determined the level of endotoxin in 75 samples of infant formula collected from seven countries [Townsend et al., 2007]. The endotoxin level ranged from 40 to 55 X 10^3 endotoxin units (EU) per gram, half of the samples had a concentration less than 3000 EU/g. Townsend et al [2007] proposed that endotoxins may contribute to failure of neonatal intestine integrity therefore facilitating bacterial translocation leading to local and systemic infection, and encouraged further research on mechanism which LPS utilizes to enhance bacterial translocation across the gut and blood barrier.

Another factor that contributes to *E. sakazakii* pathogenicity is the synthesis of capsules. The capsule antigens may inhibit phagocytosis, complement, and responses from the host's immunological mechanisms, increasing the organism's ability to breach the blood-brain barrier and to reach the central nervous system. Once the microorganism is established in the central nervous system it can produce meningitis.

Non-thermal Methods of Decontamination

Gamma radiation

The U.S. Food and Drug Administration (FDA) and the World Health Organization consider the use of ionizing (gamma) radiation as an alternative to thermal processes for the preservation of certain foods. Gamma-radiation is an alternative to chemical preservatives. The United States is the 25th nation to endorse irradiation for a wide variety of foodstuffs. Among the countries which have issued unconditional or provisional approval for commercial irradiation of certain foodstuffs are: Belgium, Canada, China, France, Holland, Italy, Israel, Japan, and the USSR [Health Physics Society, 1988].

Lethality due to ionizing radiation, as proposed by the target theory, occurs when the irradiated microorganisms are destroyed by the passage of an ionizing particle or quantum of energy through, or in close proximity to, a sensitive portion of the cell. This direct "hit" on the target causes ionization in this sensitive region of the organism or cell and subsequent death. It is also assumed that much of the germicidal effect results from the ionization of the surroundings, especially water, to yield free radicals, some of which may be oxidizing or reducing and therefore helpful in the destruction of the organisms.

The gamma rays destroy pathogenic microorganisms in the final product after packaging because of its high permeability [World Health Organization,

1999]. Similarly to microwaves in an oven, the gamma rays pass through the food, radioactivity is not added to the product. Gamma radiation does not heat the food, which can be stored or packaged and shipped immediately [Health Physics Society, 1988].

The inactivation effects of Gamma radiation on *E. sakazakii* ATCC 29544 are similar to that of other gram-negative food-borne pathogens. The D10 - values obtained for *E. sakazakii* ATCC 29544 in sterilized tryptic soy broth (TSB) and dehydrated powdered infant formula were 0.27 and 0.76 kGy respectively [Lee et al., 2006]. D_{10} –values in the 0.2 to 0.5 kGy range are typically observed with broth cultures of gram-negative pathogens such as *E. coli* or *Salmonella* Typhimurium. Gamma radiation at a dose of 5 kGy eliminated *E. sakazakii* inoculated at 8.0 to 9.0 log CFU/g onto a dehydrated powdered infant formula without affecting its sensory properties. Given the low contamination levels (1 to 10 CFU/100g) detected in infant formula, lower radiation doses could be used in order to minimize the loss of nutritional properties [Lee et al., 2006].

Pulsed electric field

During the last decade, Pulsed Electric Field (PEF) has been evaluated as alternative process to ensure the quality of liquid products. The process involves applying high-intensity pulsed electric fields to liquid products, the field within the media causes the ions inside any contaminant cell to move along the direction of the field until the ions are held back by the cell membrane. The ions then start to

exert a force on the cellular membrane that builds up causing a potential across the cell. When this potential reaches a critical value electroporation occurs. The size and number of pores formed depends on the strength and duration of the applied electric field [Barbosa-Canovas et al., 1999]. Since PEF do not involve a thermal treatment that can cause denaturation of proteins and degradation of vitamins, the use of this methodology to ensure the safety of liquid infant does not cause detrimental changes in nutritional value.

In a study conducted to analyze the inactivation of *E. sakazakii* in infant formula by PEF, the maximum outlet temperatures reached always remained below 40 ^{o} C. Inactivation of *E. sakazakii* by PEF in buffered peptone water and infant formula milk was studied in a reference medium and in rehydrated infant formula milk; the population of *E. sakazakii* was reduced 2.70 $\log_{10}$ and1.22 $\log_{10}$ units respectively, when processed by PEF technology at 40 kV cm^{-1} for 360 µs [Pina Perez et al., 2007]. The effectiveness of the treatment depended mainly on the treatment time and electric field strength as reported by Barbosa et al.[1999].

High Pressure

The use of high hydrostatic pressure has been accepted as a safe alternative to thermal process for certain foods. The application of high pressure to preserve milk, fruits and vegetables has been investigated more than a hundred years ago [Hite, 1899; Hite et al., 1914]. Japanese companies re-discovered the application of high-pressure in food processing and launched

products using this technology [Rastogi et al., 2007]. Vegetative cells, including yeast and moulds, are pressure sensitive and they can be inactivated by pressures of 300-600 MPa [Knorr, 1995; Patterson et al., 1995]. The application of high hydrostatic pressure affects cell wall structure, leaving the cell more permeable, which leads to significant changes in the tissue architecture [Dornenburg and Knorr, 1993; Farr, 1990; Rastogi and Niranjan, 1998; Rastogi et al., 2005].It was observed that in *Saccharomyces cerevasiae*, at pressures of about 400 MPa, the structure and cytoplasmic organelles were grossly deformed and large quantities of intracellular material leaked out, while at 500 MPa, the nucleus could no longer be recognized, and a loss of intracellular material was almost complete [Farr, 1990].

Studies conducted with milk, showed that the complex physicochemical environment of milk exerted a strong protective effect on *Escherichia coli* against high hydrostatic pressure inactivation, reducing inactivation from 7 logs at 400 MPa to only 3 logs at 700 MPa in 15 min at 20°C. A substantial improvement in inactivation efficiency at ambient temperature was achieved by the application of consecutive, short pressure treatments interrupted by brief decompressions. The combined effect of high pressure (500 MPa) and natural antimicrobial peptides (lysozyme, 400 µg/ml and nisin, 400 µg/ml) resulted in increased lethality for *Escherichia coli* in milk [Garcia et al., 1999].

The effect of high pressure processing on four strains of *E. sakazakii*, was analyzed using pressures at 600 MPa for 1 min at 25 ^{o}C [Gonzalez et al., 2006].

The results showed log reductions ranging from log 3 to 6.84/ml, depending on the strain. It was also observed that reconstituted infant formula had a significant protective effect for certain strains and pressures [Gonzalez et al., 2006]. Even though more research is needed to completely understand the difference in tolerance between the different strains, this research showed that high pressure processing is an effective method to reduce *E. sakazakii* contamination in infant formula.

Bacteriophages

Bacteriophages (phages) are viruses frequently found in the environment which can infect and lyse bacteria. Multiple phages have been isolated from different foods or food processes, which indicates that they are normal inhabitants of foods. Information about the presence of phages in food such as: lettuce, kimchi, chilled and frozen crabs, pork, oysters, mussels, mushrooms, pies, biscuit dough, deli loaf, roast turkey and chicken, chicken, cheese, yoghurt, buttermilk and beef is compiled in a review by Hudson et al. [2005].

The bactericidal activity of bacteriophages against bacteria has been observed in different studies and might pose a novel alternative to preserve the quality of foods. Control of pathogenic foodborne microorganisms by bacteriophages has been successfully demonstrated for several foods, during preharvest and postharvest [Greer, 2005; Hudson et al., 2005]. During preharvest, phage biocontrol strategies have been applied toward the control of

plants and animal pathogens. In postharvest, phages have been successfully used to control spoilage bacteria as well as human pathogens. Greer [2005] provided several references for use of bacterial phages to control contamination in preharvest foods and animals: cultivated mushrooms, tomatoes, apples, stone fruits, sprouts, fish, chicken, beef cattle, calves, piglets, lambs, sheep, dairy cattle, pigs and postharvest food: melon, apple slices, milk, cheese, chicken skin, retail chicken, chicken frankfurters, beef steaks, vacuum-packed beef and pork fat.

Kim et al. [2006a] analyzed the effectiveness of bacteriophages for biocontrol of *E. sakazakii*. In their study two novel *E. sakazakii* bacteriophages were isolated and applied to prevent *E. sakazakii* growth in BHI media and in reconstituted infant formula kept at 12, 24 or 37º C. BHI media was inoculated with a 12h culture of *E. sakazakii* propagating strain to reach an appropriate cell density (OD_{600} approx. 0.5), in addition, powdered infant formula (3.5% fat) was prepared according to the instructions described in the label and inoculated with exponentially growing *E. sakazakii* cells (OD_{600} approx. 0.5), to a final concentration of 10^2 CFU ml^{-1}. Inhibition was dependent upon intrinsic lysis properties and the applied phage concentration. The most significant inhibition was obtained at either 24 or 37°C with the phage at the highest concentration of 10^9 PFU ml^{-1}. A decrease in optical densities was evident 4 h after phage infection. Non-detectable levels of *E. sakazakii* were obtained with

concentrations of 10^{9} PFU ml^{-1}, while lower concentrations 10^{8} -10^{7} PFU ml^{-1} resulted in re-growth of *E. sakazakii* over time.

Monocaprylin

A variety of free fatty acids and their monoglycerides have been reported to exert antimicrobial activity against a wide range of microorganisms. The bactericidal properties of fatty acids are due in general to their function as anionic surface agents. Monocaprylin is a monoglyceride derived from caprylic acid, an eight carbon fatty acid, naturally present in human breast milk, bovine milk and coconut oil. Caprylic acid food grade chemical is generally recognized as safe by the U.S. Food and Drug Administration (FDA).

The antimicrobial effect of monocaprylin against pathogenic bacteria has been studied as a mean to prevent contamination of food by pathogenic bacteria. Nair et al. [2004] investigated the antibacterial effect of low concentrations 2.5 mM or 5 mM of monocaprylin on a five-strain mixture *E. coli* O157:H7 inoculated into apple juice maintained at 23^{o}C and 4 ^{o} C. At room temperature 5 mM and 2.5 mM of monocaprylin reduced the population of *E. coli* for more than 5 logs/ml after 3 and 5 days of storage. When samples were refrigerated, 2.5 mM of monocaprylin reduced the population of *E. coli* for more than 3 logs/ml after 14 days, while 5 mM of monocaprylin reduced the population of *E. coli* by more than 5 logs/ml after 6-7 days.

The antilisterial activity of monocaprylin (MC) in combination con acetic acid (AA) has also been investigated [Nair et al., 2004]. Frankfurters were surface inoculated with a three-strain mixture of *Listeria monocytogenes* and dipped for 35 s in a solution containing 50 mM MC and 1% AA. The solutions were maintained either at 45 or 50 ^{o}C. After 70 days of storage. Overall, results indicated that dipping of frankfurters with MC reduced *L. monocytogenes*, and inclusion of AA further enhanced MC antilisterial activity, without negative effect on sensory attributes (odor or color).

Nair [2004] studied the potential of monocaprylin to inactivate *E. sakazakii* in reconstituted infant formula. A five-strain mixture of *E. sakazakii* was inoculated into infant formula. The samples were incubated at 37 or 23^{o}C. Results showed that monocaprylin (50 mM) reduced the pathogen by >5 log CFU/ml by 1 h of incubation at 37 or 23 degrees^{o} C and by 24 h of incubation at 8 or 4 degrees ^{o} C. This study indicates that monocaprylin could potentially be used to inactivate *E. sakazakii* in reconstituted infant formula; however, sensory studies are still needed before its use can be recommended.

Biofilm Formation

In recent years, it has become evident that, in many cases, the natural mode of growth of bacteria is not as single cells suspended in an aqueous environment (planktonic mode) but as a community of cells living together in an ordered structure knowns as a biofilm [Wilson et al., 2002]. Biofilms are complex

aggregation of microorganisms embedded in a polymeric matrix with growth at interfaces - solid/liquid, liquid/air, solid/air and is often permeated by water channels. Because the polymeric matrix is often negative charged, many nutrients are attracted to the biofilm surface, thus providing the cells with plenty of food in this area compared with the surrounding water and medium [Prakash et al., 2003].

The processes involved in biofilm development include: initiation, maturation, maintenance, and dissolution. Bacteria seem to initiate biofilm development in response to specific environmental cues, such as nutrient deprivation , which promotes the adherence of the microorganisms to surfaces. Factors affecting attachment and biofilm formation include nutrient availability, the pH of the surrounding medium, and the nature of the cell and abiotic surface [Frank, 2001]. Adhesion to a substratum often leads to changes in gene transcription, resulting in an alteration in the phenotype of the organism [Wilson et al., 2002]. If nutrients are available, attached cells grow and reproduce.

Maturation involves the formation of microcolonies and macrocolonies in a complex heterogeneous structure of dormant and actively growing bacteria colonies along with enzymes, extracellular polymers and small channels forming part of the overall structure. It has been suggested that the water channels function as a primitive circulatory system that deliver nutrients to the embedded cells and remove excretory products. There is some evidence that in nutrient-rich environment with a low fluid rate, there are fewer water channels and the biofilms

have a less open structure. Maintenance achieved by stability and resilience against environmental perturbations are critical properties to prevent and delay the dissolution of biofilms on contact surfaces.

E. sakazakii has the ability to form biofilms in equipment used for the preparation of the infant formula [Bar-Oz et al., 2001; Simmons et al., 1989]. This microorganism attaches and adheres to different surfaces, including glass, silicon, latex, polycarbonate, polyvinyl chloride (PVC) and stainless steel [Kim et al., 2006a; Kim et al., 2006b; Kim et al., 2006c; Lehner et al., 2006; Lehner et al., 2005]. Microorganisms seem to attach more rapidly to hydrophobic nonpolar surfaces such as teflon and other plastics than to hydrophylic material such as glass or metals [Bendinger et al., 1993; Characklis et al., 1990]. Once biofilms are developed the microorganisms embedded in the heteropolysaccharide matrix are less susceptible to antimicrobial agents and environmental stresses such as UV light, osmotic stress, heat, starvation, acids, detergent, antibiotics, phagocytes, antibodies and bacteriophages [Costerton and Lappin-Scott, 1995; O'Toole et al., 2000].

Among the explanations that have been offered for the reduced susceptibility of micro-organisms in biofilms are: (i) slow antimicrobial penetration due to binding and slow migration of the antimicrobial agent through the biofilm matrix, (ii) antimicrobial inactivation by enzymes trapped in the biofilm matrix, (iii) altered micro-environment within the biofilm, which can reduce the activity of the agent (iv) formation of a protected subpopulation of ‘persister’ cells in the interior

of a biofilm microcolony, persister cells enter in a slow growth rate when nutrient becomes limiting and reduces the uptake of solutes from the environment including antimicrobials[Spoering and Lewis, 2001; Stewart, 2002; Stewart and Costerton, 2001].

Studies to determine the effectiveness of disinfectants in killing *E. sakazakii* in suspension, dried on the surface of stainless steel, and embedded in biofilms on stainless steel, showed that quaternary ammonium and phenolic disinfectants commonly used in hospitals, food service and childcare settings are ineffective in killing some cells of *E. sakazakii* embedded in organic matrices [Kim et al., 2006a].

Lehner et al. [2005] evaluated several features important for the survival and persistence of *E. sakazakii* in food production units associated with the manufacturing of infant formula. It was found that biofilm formation was influenced by the nutrient availability. It has been suggested that certain components within Brain Heart Infusion (BHI) media promote *E. sakazakii* biofilm formation, this hypothesis can be related to the frequent observation of different colony morphologies (smooth versus rough) in *E. sakazakii* grown on various agars [Lehner and Stephan, 2004]. The presence of cellulose fibrils as part of the extracellular matrix produced by certain *E. sakazakii* strains has been confirmed by HPLC analysis; cellulose appears to play a structural role by facilitating cell adhesion,conferring mechanical, chemical, or biological protection.

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PART 2 *ENTEROBACTER SAKAZAKII* GROWTH PROFILE IN INFANT FORMULA

Abstract

Our environment is vastly populated by microorganisms, which constantly evolve to acquire a better adaptation to the environment. Different microorganisms that in the past were not considered food borne pathogens have entered in this category, due to evolution or to a better application of identification techniques that allow relating the microorganisms with certain reported diseases.

Enterobacter sakazakii is a motile peritrochous, gram-negative rod that has been recognized as a new food borne pathogen implicated in several forms of neonatal meningitis. Urmenyi and Franklin [1961] reported the first two cases of neonatal meningitis caused by *E. sakazakii*, however at that time the microorganism was considered an *E. cloacae* strain. Since 1961 cases have been reported worldwide in different countries. In 2001 a fatal infection was associated with the presence of *E. sakazakii* in a commercial powdered formula fed to a male infant delivered by cesarean section at 33.5 weeks' gestation and hospitalized in a neonatal intensive care unit (NICU) of the University of Tennessee Hospital [Himelright et al., 2002]. As a response to this outbreak, hospital personnel reviewed NICU-infection control practices, policies and procedures for preparation storage and administration of powdered infant formula. The hospital made several policy changes and requested the Food Safety Center of Excellence of University of Tennessee to analyze the growth pattern of this microorganism at the conditions maintained in the hospital.

Preliminary studies showed no significant growth at refrigeration temperatures of 6 ^{o}C for the strains *E. sakazakii* ATCC 29004 and *E. sakazakii* ATCC 29544. However, holding of reconstituted formula at 25 ^{o}C for 4 hours resulted in 0.02 to 2.34 logs CFU increase, depending on the initial temperature of formula. These data are significant since 4 hours is the amount of time the formula bag is maintained at room temperature while applied to a patient.

We concluded that ready to drink formula maintained at room temperature or powdered infant formula reconstituted with sterile water maintained at room temperature constitute the worst scenario for potential growth of *E. sakazakii.* It is critical to refrigerate the ready to drink formula before administration or reconstitute the powdered infant formula with sterile cold water.

Introduction

In 2001 a rare case of neonatal meningitis associated with *E. sakazakii* was detected in a neonatal intensive care unit in the state of Tennessee, Knoxville. As a result of this outbreak a complete investigation of risk factors as carried out in order to determine the source of infections and to prevent future infections in the hospital. The following study set sights on current practices, policies and procedures for preparation, storage, and administration of powdered infant formula and intend to analyze the growth profile of *E. sakazakii* at the particular temperatures maintained in the hospital settings.

The outbreak that leads to this study took place in April 2001. A low birth weight, premature and respiratory distress neonate delivered by cesarean section at 33.5 weeks' gestation was hospitalized in a neonatal intensive care unit (NICU) at the University of Tennessee hospital. At day 11 the infant was diagnosed with fever, tachycardia decreased vascular perfusion and neurological abnormalities. Cerebrospinal fluid (CSF), a normally sterile site, obtained by lumbar puncture revealed the presence of *E. sakazakii*. The infant died 9 days later, despite treatment with intravenous antimicrobials.

Investigations aimed to determine if other patients in NICU were infected lead to the screening of 49 infants present in the NICU. Results from the screening showed than 10 other infants tested positive for the bacterium. Seven of the ten infants were colonized (culture-positive from stool and/or urine), two were suspected to be infected (culture positive from tracheal aspirate) and one was confirmed to be infected (culture positive from CSF).

Of the 49 patients, 9 patients were considered case-patients taking into account confirmed and suspected infections as well as colonization during the study period. Medical records were analyzed to establish risk factors such as, gestational age, birth weight, mechanical ventilator, humidifier incubation, oral medications and feeding time. Analysis of risk factor identified only use of a specific powdered infant formula, Portagen (Mead Johnson Nutritionals, Evansville, Indiana), to be significantly associated with *E. sakazakii* infection or colonization. Cultures of formula taken from opened and unopened cans

revealed the presence of *E.sakazakii* and pulse field gel electrophoresis reveled that isolates from CSF culture and powdered infant formula were identical.

Current microbiological specifications of the Codex code for coliforms in powdered infant formula allow less than 3 cfu/g . The prevalence of E. sakazakii in 141 powdered infant formulas analyzed was estimated as 14 %, yet all formulas found positive complied with the current microbiological specifications (< 3cfu/g) [Muytjens et al., 1988]. Despite the accomplishment of current specification, it is currently assumed that low levels of E. sakazakii in infant formula can lead to infection.

Different studies have analyzed the risk of *E. sakazakii* in infant formula, yet conditions of preparation, storage and administration of infant formula varies between hospitals. Therefore it was considered beneficial to study the growth profile of *E. sakazakii* at the temperatures and times used for the preparation, storage and administration of the powdered infant formula in a specific location.

Materials and Methods

Bacterial strains

Clinical strains used in this study were obtained from the American Type Culture Collection (ATCC). Both strains *E.sakazakii* ATCC 29004 and *E. sakazakii* ATCC 29544 were propagated in Brain Heart Infusion Broth according to ATCC procedures.

Formula

Portagen (Mead Johnson Nutritionals, Evansville, Indiana) a type of formula recommended by the manufacturer for infants with nutritional malabsorption problems was selected to evaluate the growth of *E. sakazakii* in reconstituted infant formula. Formula was prepared following manufacturer's directions under aseptic conditions. For 946.4 ml of prepared product, 203 g of powder were added to 820 ml of water.

Inoculation of the formula:

The prepared formula was divided into sterile flasks containing 100 ml of final product. While some flasks were used as control samples other were inoculated with either *E. sakazakii* ATCC 29004 or *E. sakazakii* ATCC 29544. Inoculated formula and controls were incubated at two temperatures: room temperature 25 ^{o} C and slightly abused refrigeration temperature 6^{o} C.

Sampling

Formula incubated at 25 ^{o}C was plated every hour in Triptose Soy Agar (TSA) and Violet Red Bile Glucose Agar (VBRGA). Formula incubated at 6 ^{o}C was plated every 12 hours in Triptose Soy Agar (TSA) and Violet Red Bile Glucose Agar (VBRGA). The final count of bacteria was recorded; data were analyzed using SAS 9.1 to determine the growth profile over time.

Temperature measurement:

Changes in temperature over time, were measured every 10 min by introducing Barnstead ERTCO time temperature indicators (TTIs) into reconstituted infant formula.

Statistical analysis:

Simple regression analyses are frequently carried out without replication, for each time-value there is only one observed log CFU/ml -value. In this study we used SAS procedure, to apply lack of fit to a regression model. Lack of fit is a technique used when a regression experiment has replicated data [Saxton, 2004]. Since any single analysis is subject to indeterminate error, the mean of several replicate analyses will always provide a better estimate of the true value.The goodness of fit is given by R-square , and diagnosis for normal distribution is given by Shapiro Wilk.

Analysis of variance (SAS procedure) was applied to a RBD model with replication, with the dependable variable: growth , expressed as log CFU/ml and variables strain, media, time.

Results

Formula preparation

This study included a visual inspection of the specific procedures documented in the Hospital's Nursing Manual used for preparing batches of formula from powder. [The University of Tennessee Memorial Hospital, 1999]:

1. Work surfaces were cleaned with Cavicide (Bacto Laboratories).

2. Hands were washed and cleaned with paper towels and covered with gloves. A hat/cap is worn when preparing batch formula.

3. All necessary items for formula preparation were placed readily accessible.

4. The nutritionist's list was checked in order to determine the types and amounts of formula to be prepared.

5. Labels were filled with the expiration date (next day's date).

6. Bottles of sterile water were opened and the desired amount of water poured into the blender according to recipe.

7. The formula can was opened and the lid lay aside “top down” on the counter top. Formula is dated at the time of opening and may be used for 1 month after opening.

8. An sterile ¼ measuring cup was used to scoop the desired amount of formula powder onto the water in the blender container, an sterile tongue depressor was used to dislodge caked formula.

9. Using sterile funnel, prepared formula was poured into the glass sterile water bottles and placed in the refrigerator.

10. Previous day's formula was discarded.

11. Blender was rinsed and washed with sanitizer containing quaternary ammonium compound.

Formula is prepared on daily basis by trained personnel between 10 -11 am, in one large batch. Each patient is assigned an individual 6 oz bottle of formula from which his/her formula are obtained using a sterile syringe which is placed into an automatic syringe pump dispenser. The time from initial preparation of the “batch” to formula administration is listed in table 2.1. Formula is kept in the refrigerator until it is ready to be drawn into the syringe.

Continuous enteral feedings

The protocol used for continuous enteral feeding was explaned, following the procedures described in the University of Tennessee Memorial Hospital Nursing Manual [The University of Tennessee Memorial Hospital, 1999; The University of Tennessee Memorial Hospital, 2000]. Formula contained in the syringe is delivered by the syringe pump through an “Enteral Only” extension set connected to a feeding tube: polyurethane “Indwell” or silastic “Nutri-catch”. The route of continuous feeding (gastric or jejunal), formula type and rate of infusion is ordered by the physician. Syringe pumps with continuous feeding are kept outside of the patient's isolette or radiant warmer to avoid exposure to favorable growth temperature for a prolonged period of time.

Table 2.1 Formula administration: Time from initial preparation [The University of Tennessee Memorial Hospital, 1999]

Start Hang	Time from initial preparation
02:00 pm	3-4 hours
10:00 pm	11-12 hours
06:00 am	19-20 hours
02:00 pm	27-28 hours

Formula is not pre-warmed prior to “hanging”. Syringes and extension sets are replaced every four hours, but the tubing change is performed every 8 hours.

Growth profile at room temperature (25 ºC)

Linear polynomial regressions for the growth profile of *E. sakazakii* ATCC 29004 and *E. sakazakii* ATCC 29544 in infant formula incubated a 25 ºC and plated hourly in VRBGA and TSA were evaluated l (Figure 2.1-Figure 2.2) and ANOVA (Table2.2-Table 2.3).

Growth profile at refrigeration temperature (6 ºC)

Inoculated formula and controls maintained under refrigeration at 6ºC, were plated in VRBGA and TSA every 12 hours. Data were recorded in figure 2.3 and table 2.4.

Changes in population during hanging time and refrigerated storage of ready to use formula Enfamil A.R. Lipil

Changes in population during hanging time and refrigeration of the ready to use formula Enfamil A.R. Lipil are recorded in tables 2.5 and table 2.6 respectively. Results are compared with those obtained for Portagen. In this study Enfamil A.R. Lipil initial temperature was 25 ºC and Portagen initial temperature was 6ºC. Enfamil A.R. Lipil is a ready to drink formula usually kept at room temperature prior to consumption. Portagen as mentioned before is reconstituted and kept refrigerated until time of consumption. (See also tables 2.7 and 2.8 and figure 2.4.)

Table 2.2 Predicted values for the growth profile of *E. sakazakii* ATCC 29004 and *E. sakazakii* ATCC 29544 in Portagen infant formula incubated a 25 ºC and plated in VRBGA and TSA.

Time (hour)	Predicted value Log CFU/ml			
	29004 VRBGA	29544 VRBGA	29004 TSA	29544 TSA
0	0.69	1.38	0.81	1.44
1	0.78	1.29	0.80	1.37
2	0.89	1.27	0.85	1.37
3	1.04	1.34	0.96	1.45
4	1.22	1.48	1.13	1.60
5	1.42	1.69	1.37	1.83
6	1.66	1.99	1.68	2.13
7	1.94	2.35	2.04	2.51
8	2.24	2.80	2.47	2.97
9	2.57	3.32	2.96	3.51
R-square	0.822	0.987	0.801	0.979
Shapiro Wilk	0.976	0.890	0.883	0.960

Predicted values obtained using quadratic regression with lack of fit.

Table 2.3 Analysis of Variance for the growth profile of *E. sakazakii* ATCC 29004 and *E. sakazakii* ATCC 29544 in Portagen infant formula incubated a 25 ºC and plated in VRBGA and TSA.

Effect	Numerator Degrees of Freedom	Denominator Degrees of Freedo	F Value	Pr>F
Strain	1	36	35.1	<.0001
Media	1	36	2.08	0.1577
Time	9	36	34.35	<.0001
Strain*Media	1	36	0.16	0.6912
Strain*Time	9	36	0.47	0.8834
Media*Time	9	36	0.29	0.973
Strain*Media* Time	9	36	0.19	0.9939

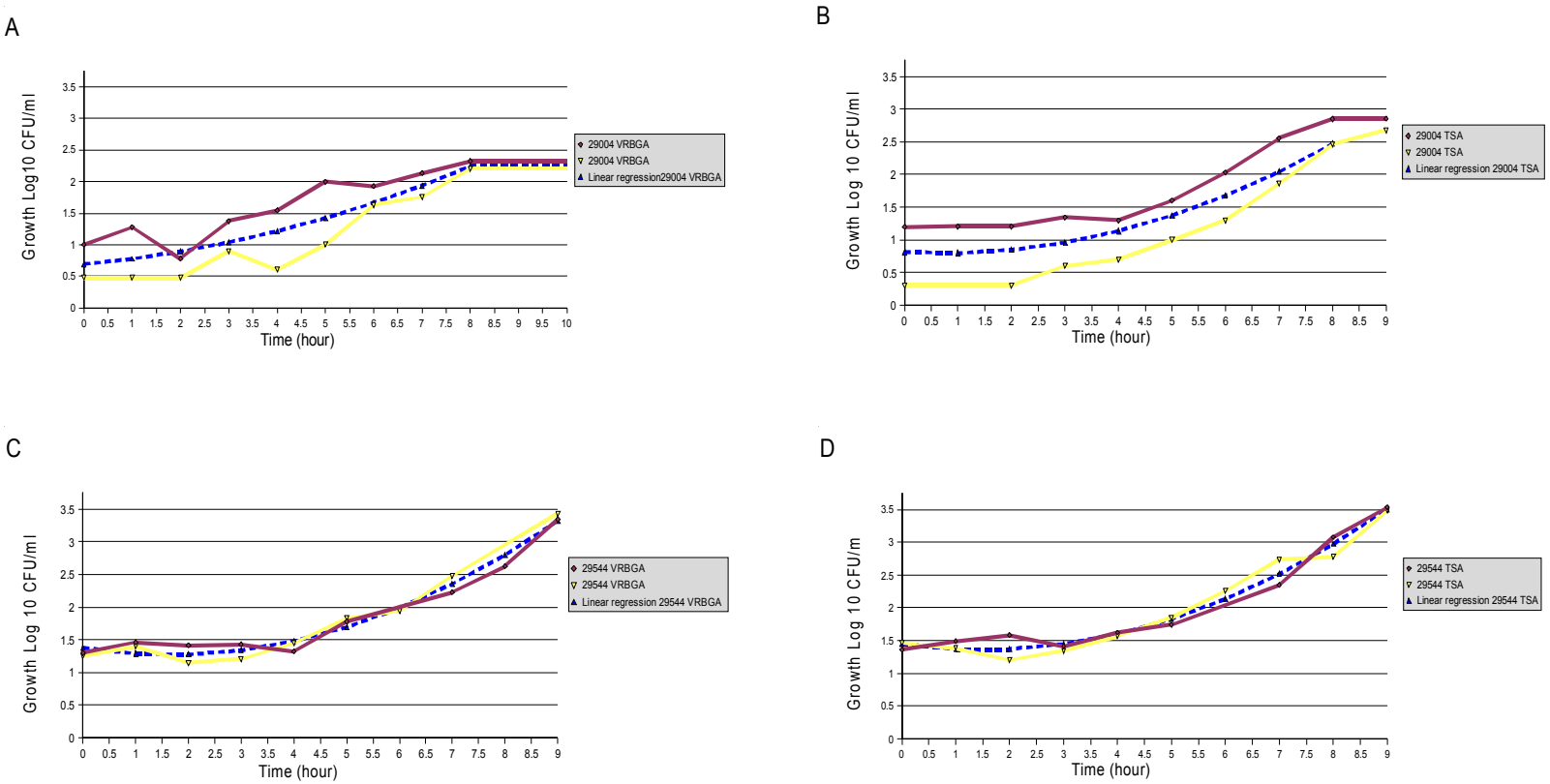

Figure 2.1 Growth profile of *E. sakazakii* in Portagen infant formula incubated a 25 ºC. (A) *E. sakazakii* ATCC 29004 plated in VRBGA (B) *E. sakazakii* ATCC 29004 plated in TSA (C) *E. sakazakii* ATCC 29544 plated in VRBGA (D) E. sakazakii ATCC 29544 plated in TSA.

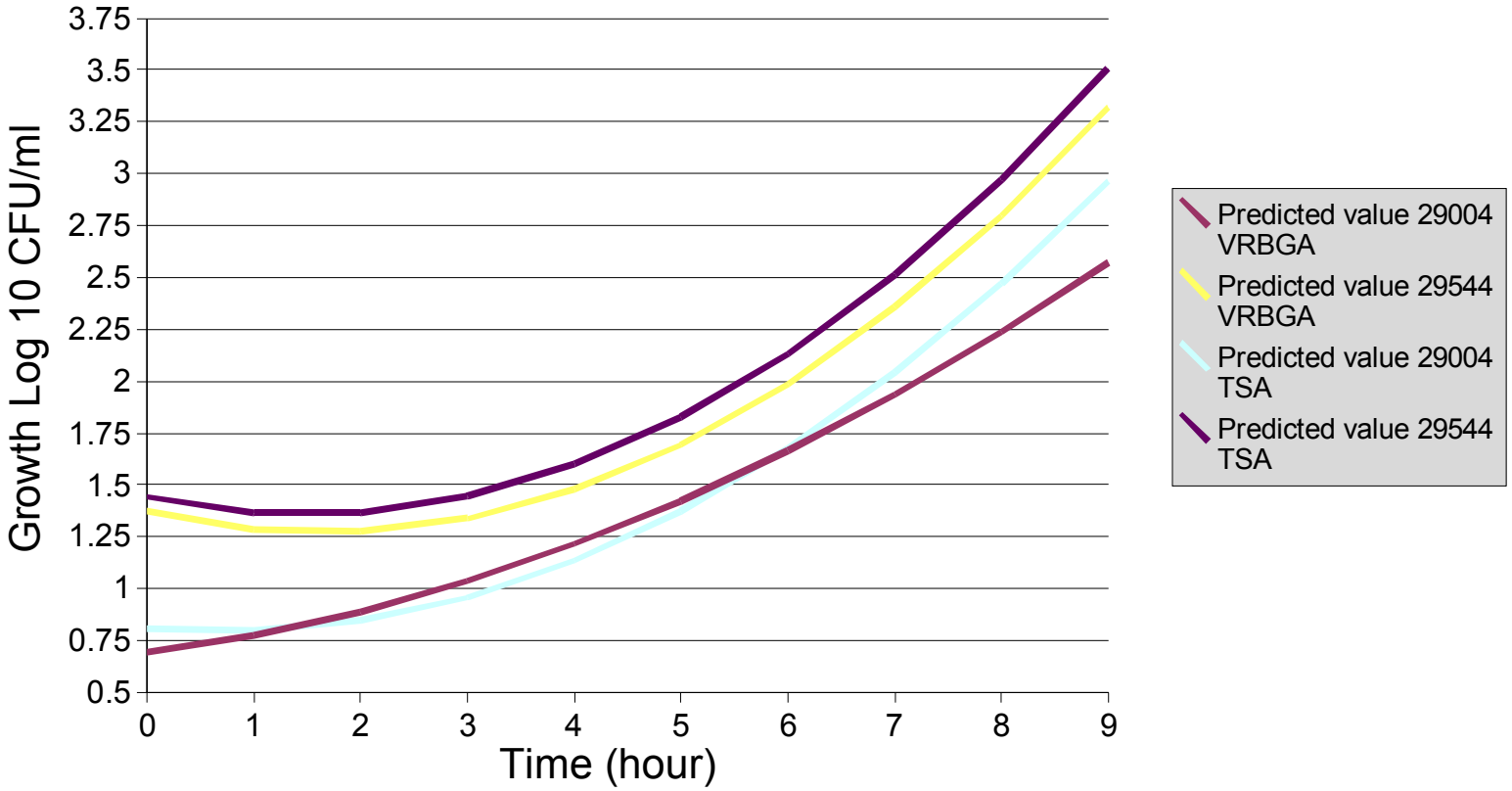

Figure 2.2 Predicted growth curves of *E. sakazakii* ATCC 29004 & 29544 incubated at 25 ºC and plated in VRBGA and TSA

Table 2.4 Analysis of Variance for growth profiles of *E. sakazakii* ATCC 29004 & ATCC 29544 in Portagen infant formula refrigerated at 6 ºC and plated in VRBGA and TSA.

Effect	Numerator Degrees of Freedom	Denominator Degrees of Freedom	F Value	Pr>F
Strain	1	14	6.34	0.0246
Media	1	14	46.35	<.0001
Time	3	14	8.68	<.0017
Strain*Media	1	14	14.13	0.0021
Strain*Time	3	14	11.18	0.0005
Media*Time	2	14	6.98	0.0079
Strain*Media*Time	2	14	0.78	0.4753

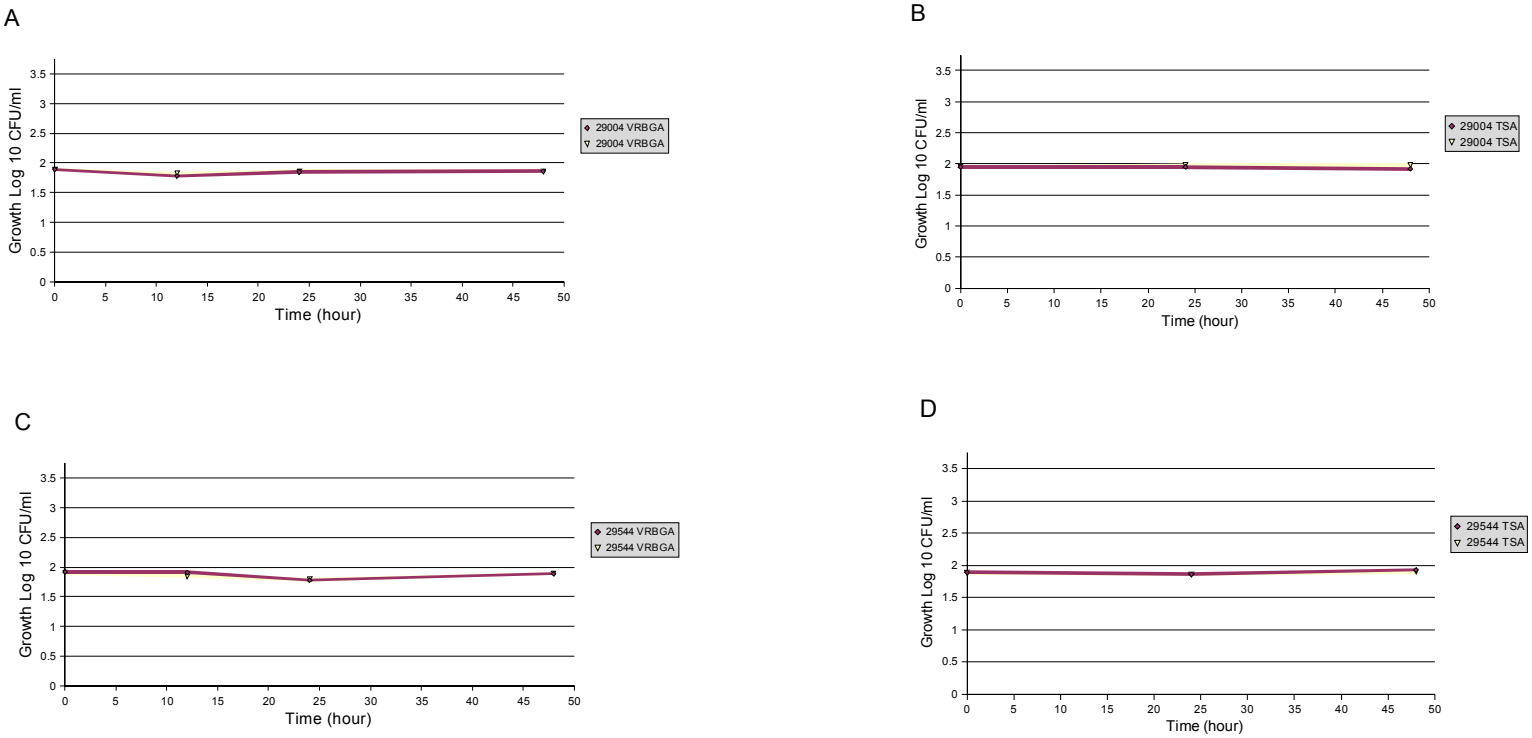

Figure 2.3 Growth profile of *E. sakazakii* in Portagen infant formula incubated at 6 ºC. (A) *E. sakazakii* ATCC 29004 plated in VRBGA (B) *E. sakazakii* ATCC 29004 plated in TSA (C) *E. sakazakii* ATCC 29544 plated in VRBGA (D) *E. sakazakii* ATCC 29544 plated in TSA.

Table 2.5 *E. sakazakii* ATCC 29004 and *E. sakazakii* ATCC 29544 average growth (Log10 CFU/ml) in Enfamil A.R. Lipil and Portagen infant formula after four hours incubation at 25 ºC. Samples were plated in VRBGA and TSA.

Strain	Time (hour)	Log CFU/ml			
		Enfamil		Portagen	
		Plating media		Plating media	
		VRBGA	TSA	VRBGA	TSA
29004	4	1.61	1.67	0.33	0.25
29004	4	2.00	0.89	0.105	0.185

Table 2.6 Analysis of Variance of *E. sakazakii* ATCC 29004 and ATCC 29544 growth in Enfamil A.R. Lipil and Portagen infant formula after four hours incubation at 25 ºC.

Effect	Numerator Degrees of Freedom	Denominator Degreess of Freedom	F Value	Pr>F
Strain	1	11	1.48	0.249
Media	1	11	3.59	0.085
Formula	1	11	91.1	<.0001
Strain*Media	1	11	3.39	0.092
Strain*Formula	1	11	0.03	0.867
Media*Formula	1	11	3.59	0.085
Strain*Media*Formula	1	11	5.85	0.034

Table 2.7 Predicted values for the growth profile of *E. sakazakii* ATCC 29004 and ATCC 29544 in Enfamil A.R. Lipil infant formula incubated a 6 ºC and plated in VRBGA and TSA.

Time hour	Predicted value Log CFU/ml			
	29004 VRBGA	29544 VRBGA	29004 TSA	29544 TSA
0	2.38	2.37	2.70	2.77
12	1.71	2.01	2.55	2.78
24	1.27	1.76	2.44	2.79
48	0.84	1.49	2.28	2.79
54	0.63	1.33	2.15	2.77
60	0.59	1.27	2.07	2.75
90	1.43	1.44	1.75	2.60
R-square	0.78	0.63	0.89	0.36
Shapiro Wilk	0.96	0.89	0.96	0.96

Predicted values obtained using quadratic regression with lack of fit

Table 2.8 Analysis of variance for the growth profile predicted values of *E. sakazakii* ATCC 29004 & ATCC 29544 in Enfamil A.R. Lipil formula incubated a 6 ºC and plated in VRBGA and TSA.

Effect	Numerator Degrees of Freedom	Denominator Degrees of Freedom	F Value	Pr>F
Strain	1	53	34.5	<.0001
Media	1	53	198.21	<.0001
Time	6	53	14.10	<.0001
Strain*Media	1	53	0.29	0.5939
Strain*Time	6	53	1.85	0.1075
Media*Time	6	53	7.64	<.0001
Strain*Media*Time	5	53	2.50	0.0418

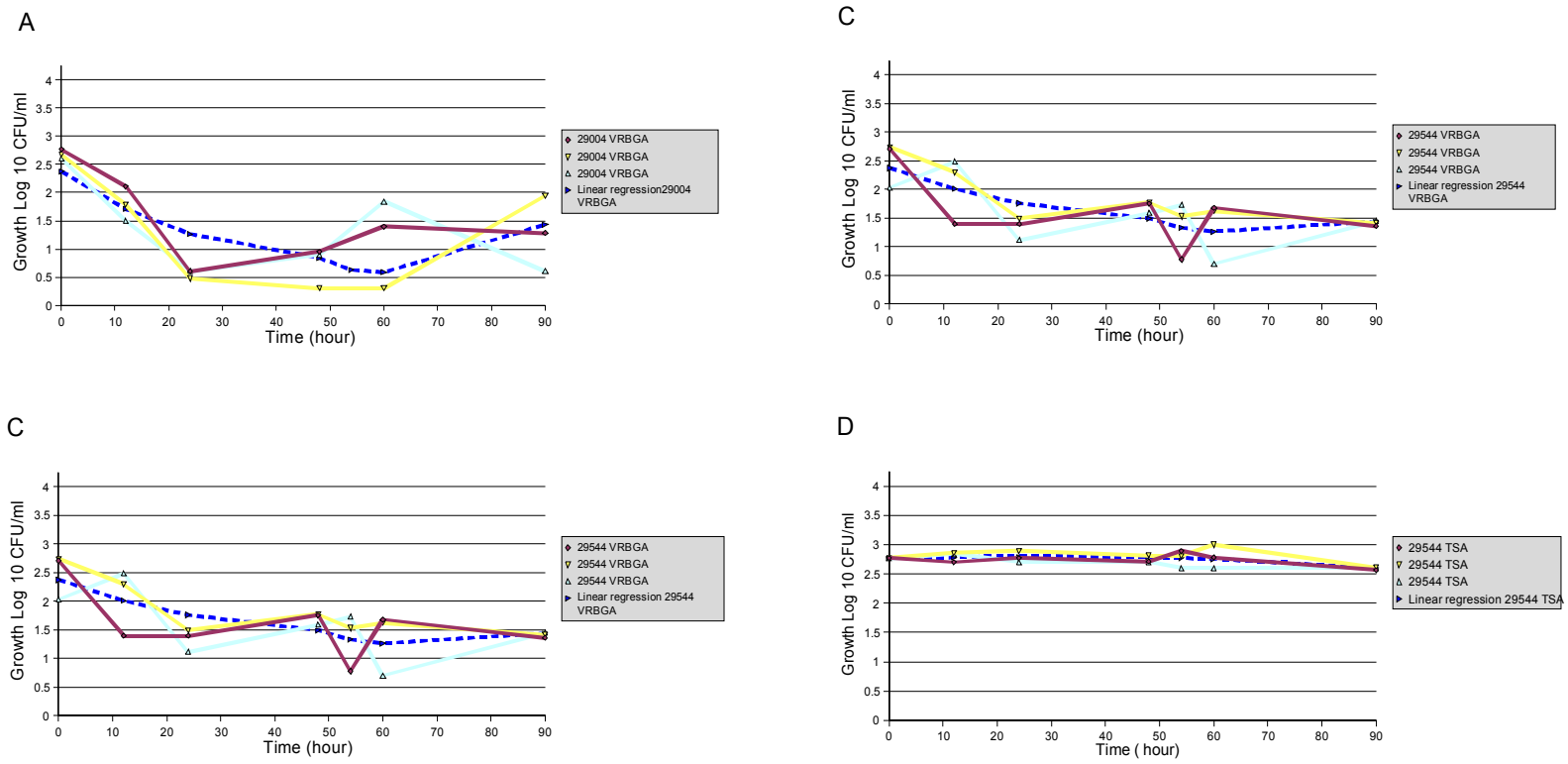

Figure 2.4 Growth profile of *E. sakazakii* in Enfamil A.R. Lipil infant formula incubated a 6 ºC.(A) *E. sakazakii* ATCC 29004 plated in VRBGA (B) *E. sakazakii* ATCC 29004 plated in TSA (C) *E. sakazakii* ATCC 29544 plated in VRBGA (D) *E. sakazakii* ATCC 29544 plated in TSA

Change of temperature over time

In order to reproduce the conditions for formula storage and hanging, a Barnstead ERTCO time temperature indicator (TTIs) was introduced into two (30 ml) samples taken out of the refrigerator (6 ºC) and placed in an incubator at 25 ºC. The change in temperature over time was registered every 10 min; recorded in table 2.9 and plotted in figure 2.5.

Discussion

Formula preparation

Powdered infant formula is not a sterile product and may contain microorganisms that can proliferate after reconstitution, favored by its high nutrient content. Current Codex advisory microbiological specifications for dried and instant products are detailed in table 2.10. The Food and Agricultural Organization have set an accepted maximal limit for powdered milk formula of <3 CFU/g coliform organisms [World Health Organization and Food and Agriculture Organization of the United Nations, 2004]. Even though, identical *E. sakazakii* strains have been isolated from the powdered milk used for feeding and the blood of cerebrospinal fluid of infected infants, only low numbers of *E. sakazakii* (< 3 cfu/g), which are below the accepted maximal limit previously described, have been found in powdered infant formula [Iversen and Forsythe, 2004; Muytjens et al., 1988; Nazarowec-White and Farber, 1997a; van Acker et al., 2001]. Nowadays, the number of cells needed to cause disease has not been

Table 2.9 Changes in temperature over time of Portagen infant formula incubated at 25 ºC, initial temperature 6.1 ºC.

Time(min)	Rep 1	Rep2
0	6.11	6.11
10	8.67	7.33
20	7.00	9.28
30	9.06	11.00
40	11.06	12.56
50	12.89	14.11
60	14.50	15.39
70	15.89	16.61
80	17.11	17.67
90	18.11	18.67
100	19.11	19.50
110	19.89	20.33
120	20.67	21.00
130	21.33	21.61
140	21.89	22.17
150	22.39	22.61
160	22.83	23.00
170	23.22	23.39
180	23.56	23.72
190	23.89	24.06
200	24.11	24.28
210	24.39	24.50
220	24.61	24.78
230	24.78	24.94
240	24.94	25.06
250	25.06	25.22
260	25.22	25.39
270	25.33	25.44
280	25.44	25.44

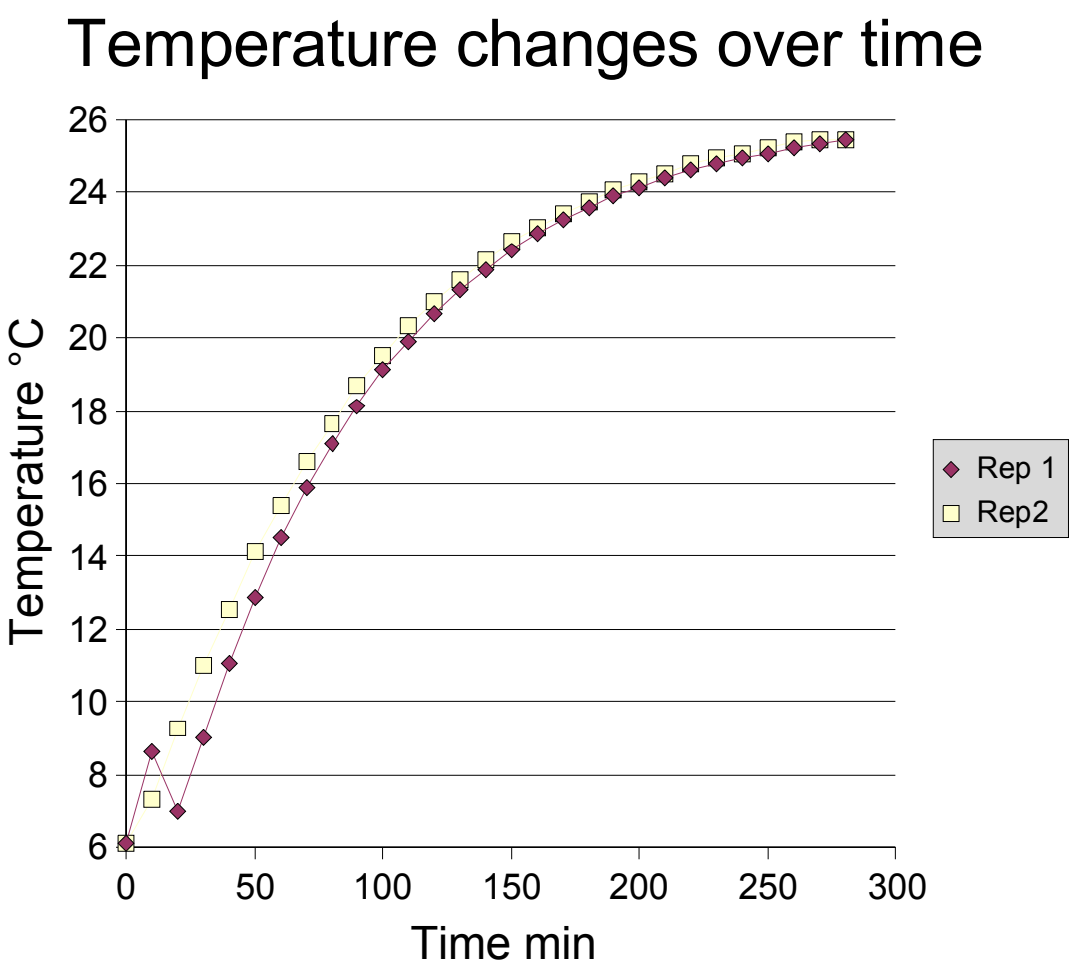

Figure 2.5 Changes in temperature over time of Portagen infant formula incubated at 25 ºC, initial temperature 6.1 ºC.

Table 2.10 Current Codex advisory microbiological specifications for dried and instant products [World Health Organization and Food and Agriculture Organization of the United Nations, 2004].

	Case	Class plan	n	c	Limit per g*	
					m	M
Mesophilic aerobic	6	3	5	2	1000	10000
Coliforms	6	3	5	1	<3	20
Salmonellae	12	2	60	0	0	0

*Including products intended for consumption after the addition of liquid, dried infant formula, instant infant cereal, etc.
< 3 means no positive tube in standard 3 tube MPN (most probable number), method (ICMSF 1978).
For Salmonella, 25 g should be used.

determined, however, it has been noted that poor preparation procedures, temperature abuse and hygienic practices are considered contributing factors to infections .During this study we verified the procedures employed for thepreparation, storage and refrigeration of infant formula followed the recommendations provided by Himelright et al. [2002] and the European Society of Paediatric Gastroenterology [Agostoni et al., 2004]. The “hang time” for continuous enteral feeding does not exceed 4 hours.

Hang time was previously reduced from 8 hours to 4 hours to avoid prolonged multiplication periods; however the optimum growth temperature at which the formula is maintained in the infant bedroom (25 ^{o}C) still makes of this stage a high risk factor. Rosset et al. [2007] recommended the use of a cold syringe cover to maintain the formula at a lower temperature.

The widespread nature of *E. sakazakii* has been detected in food manufacturing facilities, households and clinical [Drudy et al., 2006b; Kandhai et al., 2004]. Contamination of the surface and utensils used for infant formula preparation has also been reported, moreover, linked to outbreaks in neonatal units [Block et al., 2002; Bowen and Braden, 2006; Drudy et al., 2006a]. Kim et al [2006a] noted that *E. sakazakii* had the ability to form biofilms in areas used for the preparation of the infant formula. Once the biofilms are developed some cells can survive the application of disinfectants frequently used in hospital, day-care, and food service kitchen settings [Kim et al., 2006a]. Therefore it is important to

clean the surfaces immediately after the preparation of the formula. Scrubbing of surfaces helps out to break up the biofilm matrix facilitating the penetration of disinfectants to reach and kill the embedded cells. In addition to this, the use of autoclavable blenders and utensils results valuable.

Infant formula time temperature profile

Rosset et al. [2007] registered 6 ^{o}C as the lower initial temperature of infant formula collected from 25 neonatal care units of public hospitals located in Paris and its suburbs. In our study using time temperature indicators, we found out that the small sample volume (30 ml) contained in the syringe was very susceptible to temperature changes.

The samples object of study were maintained under refrigeration conditions and then placed at room temperature (25 ^{o}C) for more than 8 hours; the initial temperature was 6 ^{o}C. The time required for the sample to reach 10 ^{o}C and 25 ^{o}C was in average 30 and 240 minutes respectively (table 2.12). It is well known that changes in temperature affect the growth profile of the microorganisms and the generation time.

Growth profile of E. sakazakii during formula administration at 25^{o}C

For most known bacteria that can be cultured, generation times range from about 15 minutes to 1 hour [Todar, 2006]. The generation time for *E. coli* in the laboratory is 15-20 minutes, but in the intestinal tract, the coliform's generation time is estimated to be 12-24 hours [Todar, 2006].

In this study generation times were calculated from the predicted values recorded in table 2.3. Generation time for the first four hour period, starting from the moment at which the Portagen infant formula was taken out from the fridge (initial temperature 6 ^{o}C) through the four hour administration time (incubation at 25 ^{o}C) was on average 182 min for *E.sakazakii* ATCC 29004 and 590 min for *E.sakazakii* ATCC 29544 . Once the samples reached 25 ^{o}C, the generation time of *E.sakazakii* ATCC 29004 decreased to 46.8 min and for E.sakazakii ATCC 29544 to 38.8 min. These results are in accordance to results obtained by Nazarowec White and Farber [1997a], in which average generation times of 5 hours at 10 ^{o}C and only 40 minutes at 23 ^{o}C were observed.

Data show that previous refrigeration of the formula is crucial to decrease generation times, if the formula is prepared at room temperature and immediately hang at 25 ^{o}C; the generation time will decrease around 3.88 to 15.2 folds. The use of cold water (6 ^{o}C or less) for formula reconstitution is an alternative to be used for the preparation of formula that will be immediately hanged.

The proportion of variability in a data set accounted by the lack of fit statistical model for lineal regression was above 80%. We decided to use a quadratic model because it gave us a closer fit to initial data phase (lag).The regression model complied with the diagnosis requirements , including looking at residual errors for evidence of no pattern, a normal distribution of values and the absence of leverage points. ANOVA analysis showed that over the eight hour period there was statistically significant differences (P0.0246) strains (table 2.4).

Once the growth curve passed the lag phase and the sample reached 25 ^{o}C the generation times and slopes for the two strains became closer. The same logic can be used to compare the results obtained for the population increase during the administration of the ready to drink formula (Enfamil A.R. Lipil) with those obtained for the powdered infant formula (Portagen). There was a statistically significant difference between growth in ready to drink formula (Initial temperature: 25^{o}C) and reconstituted infant formula refrigerated overnight (Initial temperature: 6^{o}C) (table 2.6). Since the ready to drink formula is maintained at an optimum room temperature 25 ^{o}C, the time required for the microorganisms to reach the exponential phase is less, therefore the generation time decreases. The average increase in the population after a four hour period ranged from 0.11 to 0.33 log CFU/ml for Portagen powdered infant formula and from 0.89 to 1.67 for the ready to drink formula A.R. Lipil Enfamil. It is important to highlight that Portagen nutrient content is higher than A.R. Lipil Enfamil's, exception of linoleic acid. However this difference in nutrient content didn't seem to favor growth in Portagen formula, if the formula is kept at an initial temperature of 6 ^{o}C before administration . It was also found that there were no statistically significant differences found in the recovery of bacteria with either VRBGA or TSA.

Growth profile of E. sakazakii during refrigeration

The minimum growth temperature of the *E.sakazakii* type strain (ATCC 29544), was described as 7 ^{o}C [Nazarowec-White and Farber, 1997a]. Nazarowec White and Farber [1997a] noted that *Enterobacter spp*. cultivated

from infant formulae did not growth at temperatures below 5.5 ^{o}C but began to multiply at temperatures between 5.5 ^{o}C and 8 ^{o}C. In our study neither *E. sakazakii* ATCC 29004 nor *E. sakazakii* ATCC 29544 grew under refrigeration temperature (6 ^{o}C) (figure 2.3). Cells inoculated into Enfamil A.R. Lipil and incubated at 6 ^{o}C showed a decrease in population over time (Figure 2.4).

Significant findings

During our study we noted that in hospital settings some of the utensils in direct contact with the formula are washed and disinfected by chemical agents; however quaternary ammonium and phenolic disinfectants commonly used result ineffective in killing some cells of *E. sakazakii* embedded in organic matrices [Kim et al., 2006a]. Ready to drink formula or powdered infant formula kept or prepared a room temperature provide excellent conditions for bacterial replication, therefore it is recommended to autoclave the necessary material used to prepare the formula in order to eliminate the presence of microorganisms resulting from crosscontamination.

There was no statistically significant difference in growth between strains *E. sakazakii* 29004 and *E. sakazakii* 29544 cultured in Portagen formula or between the plating media used to their recovery.

Even though the administration (hang) time of infant formula has been reduced from 8 hours to 4 hours; hang time still constitutes a high risk factor for

the proliferation of *E. sakazakii,* given the temperatures maintained in the infant bedroom (25 ^{o}C). Once contaminated formula reach 25 ^{o}C generation times can decrease to less than one hour. If the starting temperature of the formula at the time of administration is 6 ^{o}C, the formula will reach 25 ^{o}C in period of four hours.

Since growth was no observed under refrigeration (6 ^{o}C) conditions we highly recommend to to keep formula refrigerated or to prepare it with cold water. The use cold cold syringe cover to maintain the formula at a lower temperature during hanging time seems beneficial[Rosset et al., 2007].

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PART 3 *ENTEROBACTER SAKAZAKII* TOLERANCE TO CHLORINE SANITIZERS

ABSTRACT

Enterobacter sakazakii has been recognized as a new food-borne pathogen implicated in a severe form of neonatal meningitis. It is recognized that the same strain of *E. sakazakii* may produce colonies with two distinct morphologies when plated in different media. Typical colonies plated on trypticase soy agar (TSA) produce a smooth and creamy colony with a yellow pigment [Farmer, 1980]. Colonies plated in violet red bile glucose agar (VRBGA) produce two different morphologies either dry, crenated and rubbery or smooth and creamy [Farmer, 1980].

There is also evidence of the ability of *E. sakazakii* to attach to surfaces and form biofilms resistant to the bactericidal effect of a broad range of sanitizers [Kim et al., 2006a].

The objective of this study was to determine the effect of chlorine sanitizers against cells in suspension (planktonic), cells organized in a colony and cells in a biofilm matrix adhered to different surfaces. We were also interested in determining the minimum chlorine level and exposure time required for 3 log/ml inactivation of *E. sakazakii ATCC 29004* and *E. sakazakii ATCC 29544*.

E. sakazakii strains ATCC 29004 and ATCC 29544 were cultivated in brain heart infusion broth- (Difco) and violet red bile glucose broth (Difco ingredients) and exposed to different chlorine concentrations (2 ppm, 4 ppm and

6 ppm) for three minutes. Survival of cells was quantified by direct plating and log CFU/ml reductions calculated. Statistical analyses of variance (SAS 9.1) was used to determine if the medium used for recovery of *E. sakazakii* had an effect on cell survival.

E. sakazakii ATTC 29544 exposed to VRBG broth prior to treatment with chlorine solutions showed a greater resistance to inactivation with chlorine compared to cells grown in BHI. Changes in survival could indicate that metabolic stress of *E. sakazakii* may increase resistance to chlorine sanitizers.

Results showed that exposure to 2 ppm chlorine solutions for 3 minutes produced 1.0 to 2.0 log CFU/ml reductions in *E. sakazakii*, exposure to 4 ppm chlorine solutions for 3 minutes produced 2.1 to 3.3 log CFU/ ml reduction and exposure to 6 ppm for 3 minutes produced 3.5 to 4.5 log CFU/ml reduction in population. Exposure to chlorine solutions for time periods between 3 and 10 minutes did not cause additional increases in the log reduction.

E. sakazakii colonies grown in agar: brain heart infusion agar BHIA (Difco) and violet red bile glucose agar VRBGA (Difco) were more resistant to the effect of chlorine sanitizer than planktonic cells. The application of solutions containing 100 or 200 ppm free chlorine was not an effective method to kill colonies grown in BHIA and VRBGA for 7 days. Even though it has been suggested that the production of extracellular capsule helps to protect bacteria from chlorine, no significant differences ($p > 0.05$) in resistance were found between encapsulated

E. sakazakii ATCC 29004 and non-capsulated *E. sakazakii ATCC 29544.* Expression of cellulose within colonies was analyzed using calcofluor and congo red screening assays.

E. sakazakii ATCC 29004 and *E. sakazakii* ATCC 29544 attached and survived on stainless steel, silicone, polycarbonate and glass surfaces covered by infant formula. Biofilms also were resistant to the bactericidal effect of 100-200 ppm free chlorine solutions. The high resistance of biofilms to chlorine sanitizers can explain the persistence of *E. sakazakii* in the formula processing environment and subsequent sloughing off of cells from surfaces into the formula which can result in foodborne disease outbreaks.

Introduction

Enterobacter sakazakii has become a public health concern since it can cause meningitis or neonatal septicemia with high fatality rates among neonates particularly pre-term infants, low-birth-weight infants or immunocompromised infants. Although no reservoirs for this microorganism have been reported, the bacterium is considered a ubiquitous microorganism, with noticeable presence in the environment. Different studies report that *E. sakazakii* is a widespread microorganism present in a variety of foods and surfaces [Drudy et al., 2006; Forsythe, 2005; Friedemann, 2007; Kandhai et al., 2004a; Kandhai et al., 2004b; Kim and Beuchat, 2005; Muytjens et al., 1988]. Other studies have evaluated the presence of *E. sakazakii* in potable water , Muytjens and Kollee [1990] could not isolate this microorganism from surface water, yet it has been reported that two

strains of *E. sakazakii* were isolated from samples of water collected in Mexico [Cruz et al., 2004] .

E. sakazakii's ability to survive in the environment and in water supplies can be enhanced by the presence of a capsule. The capsule allows the microorganism to attach to surfaces and form biofilms, which increases resistance to cleaning and disinfecting agents. The effectiveness of disinfectants in killing *E. sakazakii* in suspension, dried on the surface of stainless steel, and embedded in biofilm on stainless steel was evaluated by Kim et al [2006a]. Kim et al [2006a] show that quaternary ammonium and phenolic disinfectants commonly used in hospital, day-care, and food service kitchen settings are ineffective in killing some cells of *E. sakazakii* embedded in organic matrices.

Despite the information provided by Kim [2006a], little is known about the effectiveness of chlorine sanitizing agents in killing *E. sakazakii*.

Chlorine is used for the disinfection of our water supply. The Environmental Protection Agency (EPA) has set a limit for drinking water of 4 milligrams of chlorine per liter of water; in Tennessee the chlorine concentration detected in the water distribution systems is on average 2ppm [Environmental Protection Agency, 2006; Knoxville Utility Board, 2006].

Food manufacturing plants, households, day-care and hospitals have established chlorine as one of the disinfecting agents most commonly used. Sanitation with 20 ppm is recommended for 30 to 60 minutes; higher

concentrations or longer soak times will increase effectiveness in sanitation, but repeated sanitation at concentrations higher than 50 ppm can cause corrosion of stainless steel valves, manifolds, and piping [Edstrom Industries, 2003].

Sodium hypochlorite solutions are widely used to disinfect surfaces, equipment and utensils used for food preparation. Sodium hypochlorite dissolved in water ionizes to produce Na^+ and the hypochlorite ion, OCl^-, which remains in equilibrium with hypochlorous acid, HOCl. Between pH 4 and 7, chlorine exists predominantly as HClO, whereas above pH 9, OCl^- predominates. Hypochlorous acid HClO has long been considered the active moiety responsible for bacterial inactivation, the OCl^- ion has a minor effect compared to undissolved HOCl [McDonnell and Russell, 1999].

The actual mechanism of action of chlorine is not fully known; chlorine releasing agents are highly active oxidizing agents, which destroy the cellular activity of proteins [Bloomfield, 1996]; potentiation of oxidation may occur at low pH, where the activity of the chlorine releasing agent is maximal, although increased penetration of outer cell layers may be achieved with chlorine in the unionized state [Bloomfield, 1996; McDonnell and Russell, 1999].

Gram-negative bacteria are generally more resistant to antiseptics and disinfectants than are nonsporulating, nonmycobacterial gram-positive bacteria, thanks to the outer membrane that acts as a barrier that limits the entry of many chemically unrelated types of antibacterial agents [McDonnell and Russell, 1999].

This inherited resistance is added to the ability of *E. sakazakii* to produce exopolysaccharides [Harris and Oriel, 1989; Lehner et al., 2005] and adhere to surfaces forming biofilms [Kim et al., 2006b]. This ability emphasizes the importance of understanding the microorganism's resistance to chlorine sanitizers.

The objective of this experiment was to analyze the effect of chlorine as an antimicrobial against *E. sakazakii* strains in suspension, organized into colonies and in biofilms on different surfaces.

Materials and Methods

Experimental Plan

The study was designed to test two different strains: *E. sakazakii* ATCC 29044 and *E. sakazakii* ATCC 29544, cultivated in different treatments (VRBG or BHI; planktonic or colonies), to three different concentration of chlorine solutions. Cells in suspension cultured either on BHI or VRBG were exposed to 2, 4 and 6 ppm. Colonies growing on BHI agar or VRBG agar for 24 hours were exposed to 2, 4 and 6 ppm free chlorine solutions and colonies grown on BHI agar or VRBG agar for 7 days were exposed to 100 and 200 ppm free chlorine solutions. Preliminary experiments showed that 50 ppm free chlorine solutions did not have an antimicrobial effect on cells growing in colonies. Accurate monitoring of the sanitizing capacity of our solutions was achieved by the use of free chlorine measurements, since total chlorine is the total amount of chlorine in the water,

but free chlorine is the amount of chlorine still available to sanitize the water. The pH of solutions and culture media was continuously monitored; buffered solutions were prepared to maintain pH 7.0± 0.2. Biofilms were grown on four different surfaces: glass, stainless steel, polycarbonate and silicone. Analysis of biofilm growth was performed using crystal violet staining and enumeration of cells by direct plating.

Bacterial strains

Clinical strains used in this study were obtained from the American Type Culture Collection (ATCC). Both strains: *E. sakazakii* ATCC 29004 and *E. sakazakii* ATCC 29544 were propagated in brain heart infusion broth according to ATCC procedures.

Media

Planktonic cells were grown in brain heart infusion broth -BHI (Difco) and violet red bile glucose broth -VRBG (Difco). Colonies were grown on VRBG agar and BHI agar prepared by adding granulated agar (Fisher chemicals, 15 g/L) to BHI broth and VRBG broth. Biofilms were developed on surfaces immersed in Portagen (Mead Johnson) powdered infant formula. Serial dilutions were performed in 0.1% peptone water (Difco) and plated on trypticase soy agar (TSA) (Difco). Expression of cellulose was detected by adding 200 mg/ml $^{-1}$ calcofluor (fluorescent brightener 28, Sigma, St. Louis, Mo.) onto Luria-Bertani Agar (Difco), BHI agar (Difco) and VRBG agar prepared without adding neutral red and crystal

violet. Congo red plates were prepared from TSB plus 2% (w/v) agar containing 0.01% (w/v) congo red, following the procedure described by Smith et al. [1990]

Chemical solutions

Chlorine solutions were prepared from a commercial household product (Clorox) containing 6% sodium hypochlorite. Phosphate Buffer solutions pH 7 and crystal violet solutions 0.41% were obtained from Fisher Chemical**.**

Experimental design

Resistance of *E. sakazakii* planktonic cells to chlorine sanitizers: *E. sakazakii* (ATCC 29004), *E. sakazakii* (ATCC 29554), were grown individually in BHI broth at 35-37°C for 24 h. The 24-h cultures were transferred to VRBG broth and BHI broth and incubated at 35-37 °C for an additional 24 hours. Each culture was transferred again to the same media and incubated for 24 hours at 35-37°C. Once the incubation time was completed the cells were centrifuged for 15 min at 15,000 rpm, the pellet was washed and resuspended in 5 ml of phosphate buffer solution (pH 7), 1 ml of each culture was then submitted for 3 minutes to four different treatments: 0 ppm chlorine solution, 2 ppm chlorine solution, 4 ppm chlorine solution, 6 ppm chlorine solution. After the 3 minute treatment, each culture was transferred to BHI in order to inactive free-chlorine; neutralization of chlorine was verified using Aquacheck total/free chlorine test strips (Hach Company, Loveland, CO). Serial dilutions in 0.1% peptone water were plated in TSA supplemented with pyruvate for recovery of stressed cells.The number of *E. sakazakii* recovered was recorded and analyzed to determine if there was a

significant difference ($p<0.05$) in the log reduction for each of the treatment combinations (strains, chlorine treatments and the media used for the recovery of the microorganisms.)

Resistance of *E. sakazakii* colonies to chlorine sanitizers*: E. sakazakii* (ATCC 29004), *E*. *sakazakii* (ATCC 29554), were grown individually in BHI broth at 35-37°C for 24 h. The 24-h cultures were transferred to VRBG broth and BHI broth and incubated at 35-37 °C for additional 24 hours. Each culture was transferred again to the same media and incubated for 24 hours at 35-37°C. Once the incubation time was completed 0.1 ml of each culture were plated on BHI Agar and VRBG agar and incubated at 35-37 °C for 24 h. The plates were then incubated at 25 °C for either 24 hour or 7 days to allow colonies to grow (Figure 3.1). Colonies with the same diameter were selected and immersed in chlorine solutions of different concentrations: 2 ppm, 4 ppm, 6 ppm (for 24-hour colonies) or 100 ppm and 200 ppm (for 7-day colonies) without disrupting their morphology. After 30 minutes of contact time, BHI broth was added to inactive free chlorine, neutralization of chlorine was verified using Aquacheck total/free chlorine test strips (Hach Company, Loveland, CO). Serial dilutions in 0.1% peptone water were plated in TSA supplemented with pyruvate for recovery of stressed cells.The number of *E. sakazakii* recovered was recorded and analyzed to determine if there was a significant difference ($p<0.05$) in the log reduction for each of the treatment combinations (strains, chlorine treatments and the media used for the recovery of the microorganisms.).

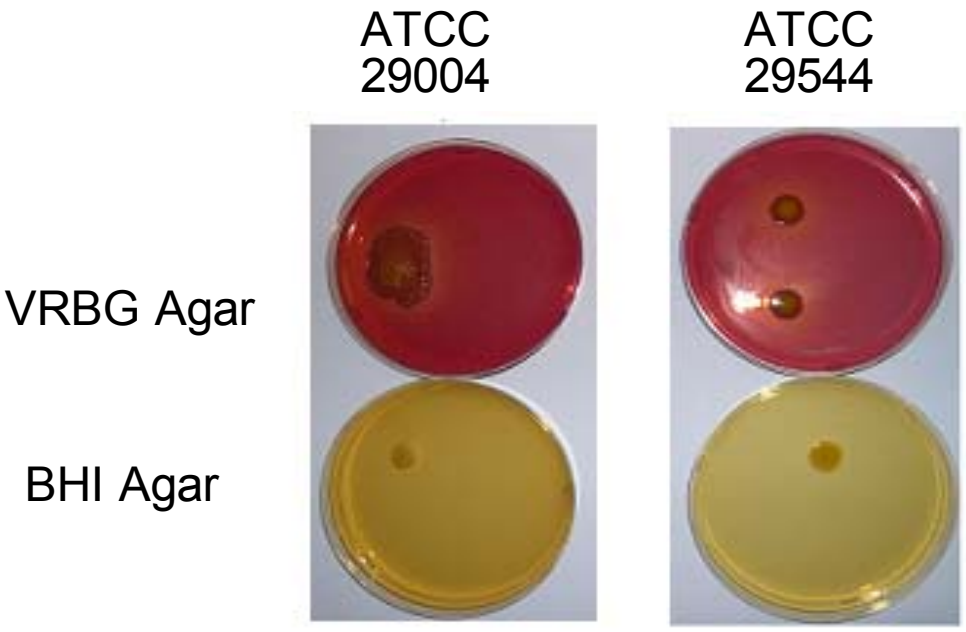

Figure 3.1 *E. sakazakii* ATCC 29004 and *E. sakazakii* ATCC 29544 colony morphology after 7 days.

Expression of cellulose and morphotypes within colonies: *E. sakazakii* (ATCC 29004), *E*. *sakazakii* (ATCC 29554), were grown individually in BHI broth at 35-37°C for 24 h. After incubation microbial outgrowth was collected with a sterile loop and streaked on congo red agar and incubated at 35-37 ^{o}C Colony morphology was compared with the basic morphotypes detected by Zogaj et al. [2003]. *E. sakazakii* (ATCC 29004) and *E*. *sakazakii* (ATCC 29554) were also streaked on LB agar, BHI agar and VRBG agar supplemented with calcofluor, and incubated for 48 hours at 28 ^{o} C. Fluorescent colonies were observed under a 366-nm UV light source.

The Role of Biofilm Formation on the Protection of Enterobacter sakazakii from Chlorination*:* Biofilms for chlorine resistance experiments were grown on coupons of each of the following materials: stainless steel, silicone, polycarbonate and glass. Coupons (4cm ²) were inoculated with 50 µl of a 24 h culture of *E. sakazakii* grown in BHI broth and then incubated at 35 °C for 3 h in sterile petri dishes. Each coupon was rinsed with 100 ml of 0.015 M KH_2PO_4 and adjusted to pH 7.0 using a manual dispenser 10 ml at a time from top to bottom and front to back. After rinsing, the coupons were transferred to sterile plastic petri dishes containing sterile infant formula (Portagen, Mead Johnson) and incubated for 48 h at 35 °C. After biofilm growth, the coupons were washed by using a manual dispenser, directing the stream of sterile phosphate buffer (approximately 50 ml) evenly from top to bottom and front to back to remove the growth media and unattached cells.

Washed coupons were either stained with crystal violet and let dry for 30 minutes to visualize biofilm growth or placed in a petri dish filled with 20 ml of phosphate buffer, 20 ml of 100 ppm or 20 ml of 200 ppm chlorine solution for 30 min. Treated coupons were placed in petri dishes and covered with sterile BHI broth for 5 min to neutralize free chlorine; neutralization of chlorine was verified using Aquacheck total/free chlorine test strips (Hach Company, Loveland, Colo.).Coupons were then rinsed with 50 ml of phosphate buffer three times, and scraped using a cotton swab to remove attached cells. Cotton swabs were immersed in 10 ml of phosphate buffer and vortexed for 15 sec. Coupons were placed in a petri dish, overlaid with TSA+pyruvate and incubated at 30 °C for 48 h to allow colony formation from the attached survivors. Survivors in phosphate buffer solutions (Rinse, swab) and TSA+pyruvate petri dishes were reported as log CFU/ cm^2. Data were transformed to log scale for statistical analysis.

Each experiment was conducted with at least two replications and analyzed with the SAS Mixed Procedures using SAS Software Release 9.1 (SAS Institute Inc., Cary, NC). Significant differences among means were determined by the Least-Squares Means method with the PDIFF (*p*-value for differences) option. We were interested in evaluating:

- Whether or not there was a difference between treatments when applied to the same strain.

- What were the mean estimates for the log reduction obtained for each treatment?

- Whether or not there was a difference between strain or/and media when applying the same treatment.

Data analysis were conducted using RBD factorial model with replicates, blocking by replicate.

Expression of cellulose was analyzed from a qualitative point of view by comparison with previous results obtained from different authors regarding morphotypes and fluorescence on media prepared with the addition of congo red and calcofluor dye.

Results

Exposure of E. sakazakii planktonic cells to 2 ppm , 4 ppm and 6 ppm free chlorine solutions for 3 minutes produced in average1.0 to 2.0 log, 2.1 to 3.3 log and 3.5 to 4.5 log CFU/ml reduction estimates, respectively (Table 3.1, Figure 3.2).

The type 3 test of fixed effect and LSD method ($P < .05$) applied to the overall data showed no statistical differences in the decrease in population between strains, although, there were statistically differences between treatments: 2, 4, 6 ppm and media: BHI broth and VRBG broth (Table 3.2-3.3).

Table 3.1 Average decrease in population after treatment of planktonic cells with 2 ppm, 4 ppm, 6 ppm free chlorine solutions.

Treatment	Log CFU/ml Reduction			
	E.sakazakii ATCC 29004		*E.sakazakii* ATCC 29544	
	Plating media			
	BHI	VRBG	BHI	VRBG
2 ppm	1.99	1.88	1.04	1.07
4 ppm	2.12	2.09	3.29	2.95

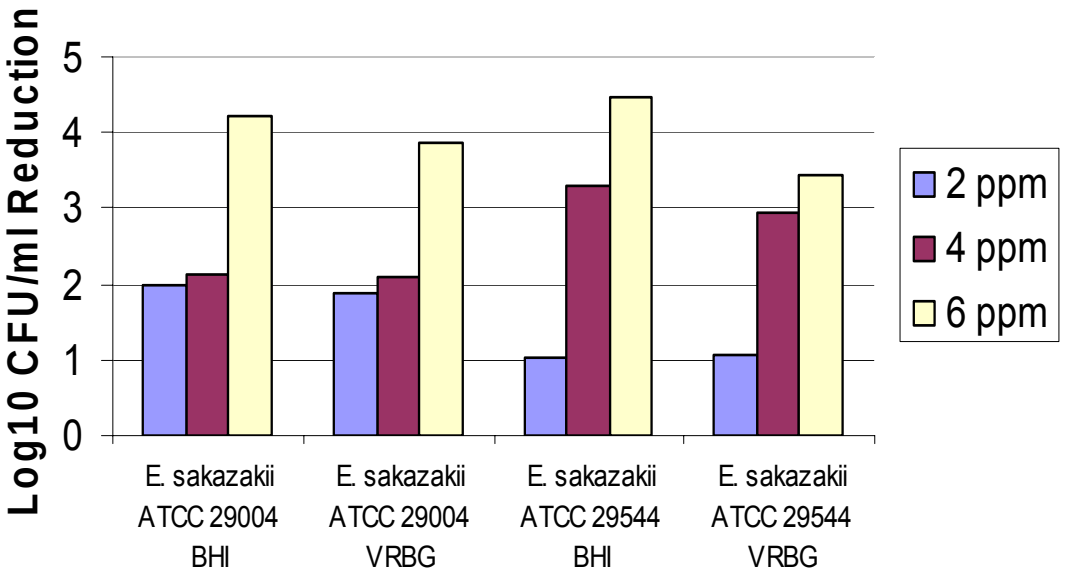

Figure 3.2 Average decrease in population after treatment of colonies with 2 ppm, 4 ppm and 6 ppm free chlorine solutions.

Table 3.2 Analysis of variance for decrease in population means after treatment of planktonic cells with 2 ppm, 4 ppm, 6 ppm free chlorine solution.

Effect	Numerator Degrees of Freedom	Denominator Degrees of Freedom	F Value	Pr > F
Strain	1	34	0.01	0.9347
Media	1	34	5.4	0.0262
Treatment	2	34	109.22	<.0001
Strain * media	1	34	0.93	0.3417
Strain* treatment	2	34	21.39	<.0001
media* treatment	2	34	1.77	0.1861
Strain*media*treatment	2	34	0.78	0.4651

Table 3.3 LSD method (P<.05) for decrease in population after treatment of planktonic cells with 2, 4, 6 ppm free chlorine solution

Strain	Media	Treatment	Log CFU/ml	Standard Error	Letter Group
29004			2.70	0.10	A
29544			2.71	0.08	A
	BHI		2.86	0.10	C
	VRBG		2.55	0.09	B
		2 ppm	1.51	0.10	D
		4 ppm	2.61	0.11	E
		6 ppm	4.00	0.14	F

Mean separation showed that within the same strain and treatment (2ppm or 4ppm) there was no difference in the log reduction between media, however there was a difference between strains (Table 3.4 and 3.5). For the treatment using 6 ppm chlorine solution there was no difference in the log reduction obtained for one strain or the other but *E. sakazakii* ATCC 29544 cells exposed to VRBG prior to treatment with chlorine solutions showed a one log or greater resistance to inactivation with chlorine compared to cells grown in BHI (Table 3.6).

Both strains *E. sakazakii* ATCC 29004 and ATCC 29544 showed a difference in log reduction between treatments with 2ppm and 6 ppm (Table 3.7 and Table 3.8). Treatment with 4 ppm free chlorine was similar to treatments with 2 ppm or 6 ppm free chlorine with the exception of *E. sakazakii* ATCC 29544 grown in BHI for which all treatments differed (Table 3.8).

Exposure of E. sakazakii ATCC 29004 and *E. sakazakii* ATCC 29544 colonies grown for 24 hours to 2 ppm, 4 ppm and 6 ppm free chlorine solutions.

Two different morphologies were observed for cells grown in VRBG agar. *E. sakazakii* ATCC 29544 colonies were smooth, creamy and easy to remove from the agar. *E. sakazakii* ATCC 29544 colonies were dry, crenated, rubbery and difficult to remove from the agar (Figure 3.1).

Exposure of 24 hour colonies grown in BHI agar and VRBG agar to 2 ppm free chlorine solutions did not cause a decrease on the population, exposure to 4

Table 3.4 LSD method (P<.05) for decrease in population after treatment of planktonic cells with 2 ppm free chlorine.

Strain	Media	Log CFU/ml	Standard Error	Letter Group
29004	BHI	2.03	0.28	A
29004	VRBG	1.88	0.19	A
29544	BHI	1.04	0.17	B
29544	VRBG	1.07	0.12	B

Table 3.5 LSD method (P<.05) for decrease in population after treatment of planktonic cells with 4 ppm free chlorine.

Strain	Media	Log CFU/ml	Standard Error	Letter Group
29004	BHI	2.12	0.23	B
29004	VRBG	2.09	0.28	B
29544	BHI	3.29	0.19	A
29544	VRBG	2.95	0.15	A

Table 3.6 LSD method (P<.05) sliced by treatment for decrease in population after treatment of planktonic cells with 6 ppm

Strain	Media	Treatment	Log CFU/ml	Standard Error	Letter Group
29004	BHI	6 ppm	4.23	0.28	AB
29004	VRBG	6 ppm	3.86	0.28	AB
29544	BHI	6 ppm	4.46	0.28	A
29544	VRBG	6 ppm	3.45	0.28	B

Table 3.7 LSD method (P<.05) sliced by media VRBG for decrease in population after treatment of planktonic cells with 2, 4, 6 ppm free chlorine

Srain	Media	Treatment	Log CFU/ml	Standard Error	Letter Group
29004	VRBG	2 ppm	1.88	0.1949	C
29004	VRBG	4 ppm	2.09	0.2757	C
29004	VRBG	6 ppm	3.86	0.2757	A
29544	VRBG	2 ppm	1.07	0.1175	D
29544	VRBG	4 ppm	2.95	0.1474	B
29544	VRBG	6 ppm	3.45	0.2757	AB

Table 3.8 LSD method (P<.05) sliced by media BHI for decrease in population after treatment of planktonic cells with 2, 4 and 6 ppm free chlorine.

Strain	Media	Treatment	Log CFU/ml	Standard Error	Letter Group
29004	BHI	2 ppm	2.03	0.2757	C
29004	BHI	4 ppm	2.12	0.2251	C
29004	BHI	6 ppm	4.22	0.2757	A
29544	BHI	2 ppm	1.04	0.1744	D
29544	BHI	4 ppm	3.29	0.1949	B
29544	BHI	6 ppm	4.46	0.2757	A

ppm produced no more than 0.3 log reduction and exposure to 6 ppm produced averages ranging from 0.2 to 1.0 log reduction(Table 3.9, Figure 3.3).

Planktonic cells of *E. sakazakii* ATCC 29004 and *E. sakazakii* ATTC 29544 had a larger decrease in population after exposure to 2, 4 and 6 ppm free chlorine than cells organized in colonies, grown in VRBG agar and BHI agar for a period of 24 hours.

E. sakazakii ATCC 29544 colonies, grown in VRBG agar for 24 hours were more resistant to the antimicrobial effect of the applied chlorine solutions than colonies grown in BHI agar for the same period of time. However, the opposite occurred for *E. sakazakii* ATCC 29004. Cells of ATCC 29004 growing in BHI agar were more resistant to the antimicrobial effect of chlorine than cells growing in VRBG agar which exhibited the dry, crenated and rubbery morphology.

Exposure of E. sakazakii ATCC 29004 and E. sakazakii ATCC 29544 colonies grown for 7 days to 100 ppm and 200 ppm free chlorine solution

Exposure of 7-day colonies grown in BHI agar and VRBG agar to 100 ppm chlorine solutions for 30 min produced mean reductions ranging from 0.21 to 0.92 log CFU/ml , exposure to 200 ppm chlorine solutions for 30 minutes produced mean reductions ranging from 0.35 to 0.91 log CFU/ml (table 3.10-, figure 3.4). There was no significant difference in population decrease between the strains after treatments were applied (P >0.05) (Table 3.11-3.12), however

Table 3.9 Average decrease in population after treatment of *E. sakazakii* ATCC 29004 and *E. sakazakii* ATCC 29544 24 h colonies with 2 ppm, 4 ppm, 6 ppm free chlorine solutions.

Treatment	Log CFU/ml Reduction			
	E.sakazakii ATCC 29004		*E.sakazakii* ATCC 29544	
	Plating media			
	BHI	VRBG	BHI	VRBG
2 ppm	0	0	0	0
4 ppm	0.07	0.33	0.03	0.02

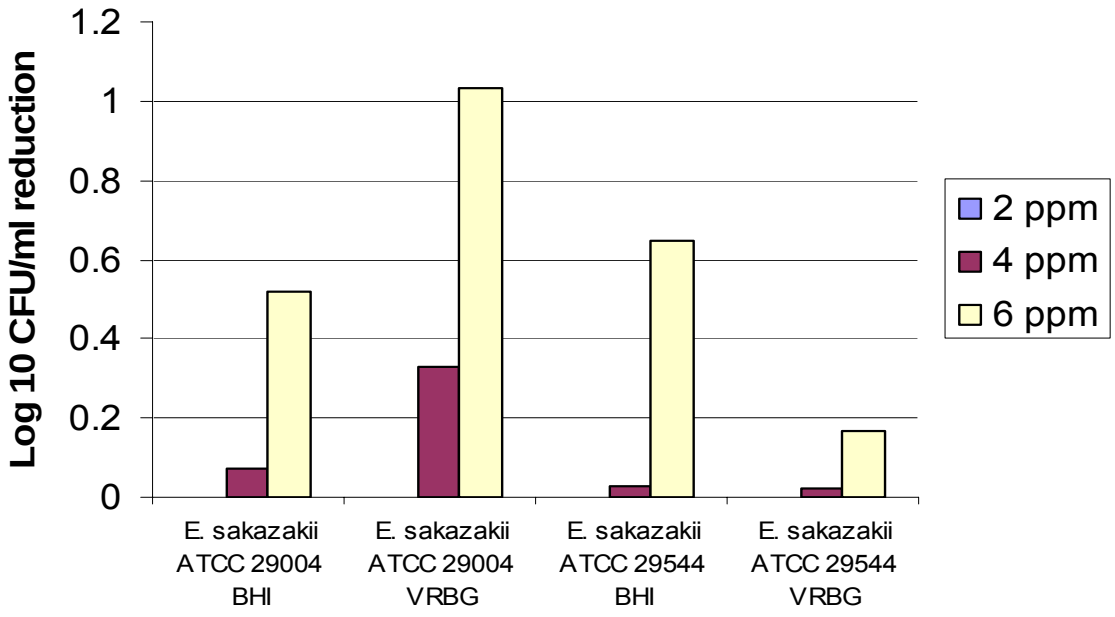

Figure 3.3 Average decrease in population after treatment of *E. sakazakii* ATCC 29004 and *E. sakazakii* ATCC 29544 24 h colonies with 2 ppm, 4 ppm and 6 ppm free chlorine solutions.

Table 3.10 Average decrease in population after treatment of *E. sakazakii* ATCC 29004 and *E. sakazakii* ATCC 29544 7 day colonies with 100 and 200 ppm free chlorine solutions.

Treatment	Log CFU/ml Reduction			
	E.sakazakii ATCC 29004		*E.sakazakii* ATCC 29544	
	Plating media			
	BHI	VRBG	BHI	VRBG
100 ppm	0.87	0.28	0.92	0.21
200 ppm	1.32	0.35	0.91	0.35

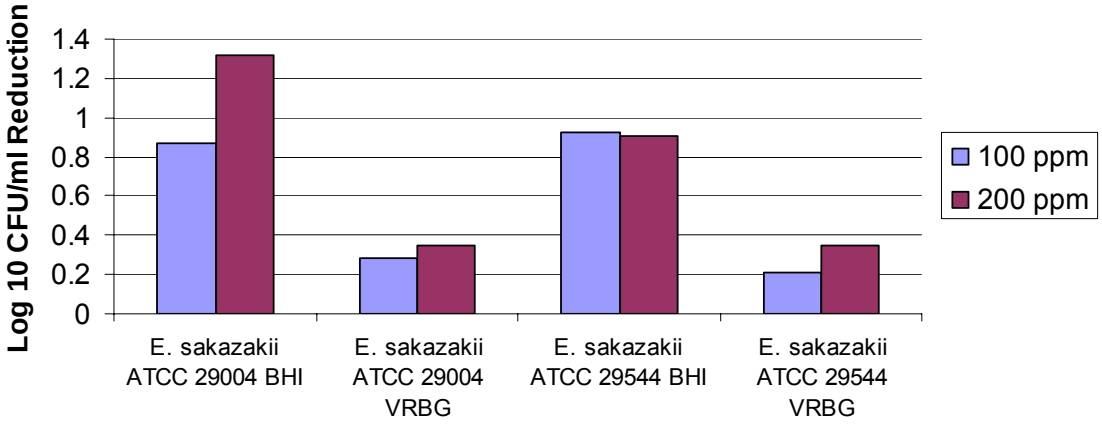

Figure 3.4 Average decrease in population after treatment of *E. sakazakii* ATTC 29004 and *E. sakazakii* ATCC 29544 colonies with 100 and 200 ppm free chlorine solutions.

Table 3.11 Analysis of variance results for decrease in population after treatment of *E. sakazakii* ATCC 29004 and *E. sakazakii* ATCC 29544 colonies with 100 ppm and 200 ppm free chlorine solution

Effect	Numerator Degrees of Freedom	Denominator Degrees of Freedom	F Value	Pr > F
Strain	1	14	4.04	<.0642
Media	1	14	179.87	<.0001
Treatment	1	14	9.25	0.0088
Strain * media	1	14	1.92	0.1873
Strain* treatment	1	14	3.45	0.0846
media* treatment	1	14	1.18	0.2962
Strain*media*treatment	1	14	6.38	0.0242

Table 3.12 Decrease in population after treatment of *E. sakazakii* ATCC 29004 and *E. sakazakii* ATCC 29544 colonies with 100 ppm and 200 ppm free chlorine.

Strain	Media	Treatment	Log CFU/ml	Standard Error	Letter Group
29004			0.7	0.04	A
29544			0.6	0.04	A
	BHIA		1.01	0.04	C
	VRBGA		0.29	0.04	D
		100 ppm	0.57	0.04	F
		200 ppm	0.73	0.04	E

cells grown in VRBG agar showed a lower log reduction (0.29) than cells grown in BHI agar (1.01)(Table 3.12).

There were statistically significant differences ($p<0.05$) in population decrease between 100 and 200 ppm treatments. Colonies treated with 100 ppm free chlorine solution showed an estimate of 0.57 log CFU/ml decrease in population while cells submitted to 200 ppm free chlorine solution showed a 0.73 log CFU/ml decrease in population (Table 3.12); this difference was not seen for colonies recovered from VRBG agar and was only noticed for *E. sakazakii* ATCC 29004 colonies grown in BHI agar and treated with 200 ppm free chlorine (Table 3.15-3.16).

The significant difference in *E. sakazakii* ATCC 29004 population decrease between colonies grown in VRBG agar and BHI agar and exposed to 200 ppm chlorine could be attributed to the metabolic stress produced by the medium (Table 3.15 - 3.16).

Expression of cellulose and morphotypes within colonies

Morphotype and expression of cellulose were tested by screening colonies for congo red and calcofluor binding (Table 3.17, Figure 3.5).

Isolates growing in LB agar supplemented with congo red were compared to the basic morphotypes detected in *S. Typhimurium* (Figure 3.5) and classified in one of the following morphotypes: (i) red, dry, and rough, indicating curli and cellulose production (rdar); (ii) brown, dry, and rough, indicating curli production

Table 3.13 LSD method (P<.05) sliced by treatment for decrease in population after treatment of *E. sakazakii* ATCC 29004 and *E. sakazakii* ATCC 29544 colonies with 100 ppm free chlorine.

Strain	Media	Treatment	Log CFU/ml	Standard Error	Letter Group
29004	BHIA	100 ppm	0.87	0.09	A
29004	VRBGA	100 ppm	0.28	0.07	B
29544	BHIA	100 ppm	0.92	0.07	A
29544	VRBGA	100 ppm	0.21	0.07	B

Table 3.14 LSD method (P<.05) for decrease in population after treatment of *E. sakazakii* ATCC 29004 and *E. sakazakii* ATCC 29544 colonies with 200 ppm free chlorine.

Strain	Media	Treatment	Log CFU/ml	Standard Error	Letter Group
29004	BHIA	200 ppm	1.32	0.09	A
29004	VRBGA	200 ppm	0.35	0.07	C
29544	BHIA	200 ppm	0.91	0.07	B
29544	VRBGA	200 ppm	0.35	0.07	C

Table 3.15 LSD method (P<.05) BHI broth showing decrease in population after treatment of *E. sakazakii* ATCC 29004 and *E. sakazakii* ATCC 29544 colonies with 100 and 200 ppm free chlorine.

Strain	Treatment	Log CFU/ml	Standard Error	Letter Group
29004	100 ppm	0.87	0.09	B
29004	200 ppm	1.32	0.09	A
29544	100 ppm	0.92	0.07	B
29544	200 ppm	0.91	0.07	B

Table 3.16 LSD method (P<.05) sliced by media for decrease in population after treatment of *E. sakazakii* ATCC 29004 and *E. sakazakii* ATCC 29544 colonies with 100 and 200 ppm

Strain	Treatment	Log CFU/ml	Standard Error	Letter Group
29004	100 ppm	0.28	0.07	A
29004	200 ppm	0.35	0.07	A
29544	100 ppm	0.21	0.07	A
29544	200 ppm	0.35	0.07	A

Table 3.17 Morphotype and expression of extracellular matrix component

Strain	Morphotype[1]	Calcofluor binding [2]
E. sakazakii ATCC 29004	pdar	+
E. sakazakii ATCC 29544	pdar	+

[1] Morphotype on CR plates at 28 °C.

[2] Fluorescent phenotype on LB agar, BHI agar and VRBG agar prepared without crystal violet and neutral red

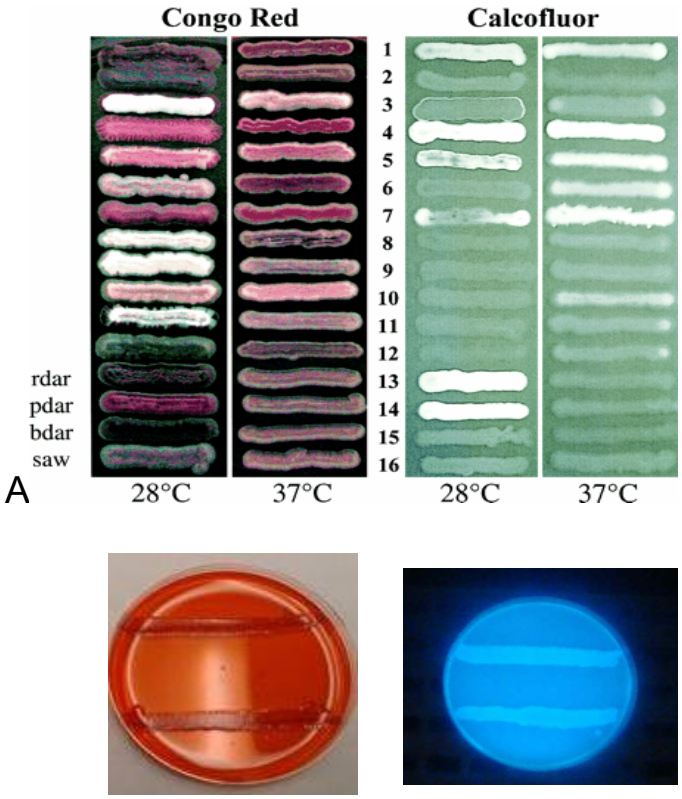

Figure 3.5 Morphotype and expression of extracellular matrix component

A: Congo red and Calcofluor staining of morphotypes of representatives of enterobacterial species isolated from feces. The strains were grown for 48 h at 28°C and for 24 h at 37°C. Morphotypes include the phenotype of serotype Typhimurium UMR1 (ATCC 14028-1s, Nal r) and its mutants, streaked at the lower part of the panel. ROWS: 1, Citrobacter sp. strain Fec2; 2, C. freundii Fec4; 3, C. koseri/farmeri Fec157; 4, Enterobacter sp. strain Fec125; 5, E. aerogenes Fec135; 6, E. cloacae Fec36; 7, *E. sakazakii* Fec39; 8, Klebsiella sp. strain Fec164; 9, K. oxytoca Fec139; 10, K. pneumoniae Fec141; 11, R. ornithinolytica Fec153; 12, P. mirabilis Fec162; 13, serotype Typhimurium UMR1 (cellulose 28+, curli 28+); 14, serotype Typhimurium MAE1 (cellulose 28+, curli -); 15, serotype Typhimurium MAE222 (cellulose -, curli 28+); 16, serotype Typhimurium MAE51 (cellulose -, curli -) (Zogaj et al. 2003). B: Congo red and Calcofluor staining of *E. sakazakii* ATCC 29004 (Top part petri dish) and *E. sakazakii* ATCC 29544 (Bottom part petri dish), incubated at 28 ºC.

but lack of cellulose production(bdar) (iii) smooth and white, indicating lack of both curli and cellulose production (saw); (iv) pink colony, indicating cellulose production (pdar). [Solomon et al., 2005]. Both strains showed a pdar morphotype on congo red incubated at 28 ^{o}C for 48 hour (Figure 3.5), indicating cellulose production but not curli fimbriae.

The screening method for detection of cellulose based on the fluorescence of colonies on calcofluor agar plates, indicated cellulose production after 48 hours incubation at 28 ^{o}C by both *E. sakazakii* ATCC 29004 and *E. sakazakii* 29544. Fluorescence was observed under long-wave UV light, in the three media stained with calcofluor: LB agar, BHI agar and VRBG agar without crystal violet and neutral red.

These results differ from those obtained by Lehner et al. [2005] who didn't report cellulose production on strains *E. sakazakii* 29004 and *E. sakazakii* 29544, using the same calcofluor screening technique.

Exposure of E. sakazakii ATCC 29004 and E. sakazakii ATCC 29544 biofilms to 100 and 200 ppm free chlorine solutions.

From the statistical point of view, there was a significant difference ($p<0.05$) between treatments with 100 and 200 ppm chlorine (Table 3.18-3.20); however log reductions were not greater than 1.04 log CFU/cm^2, with the exception of 3.3 log CFU/cm^2 reductions obtained for *E. sakazakii* ATCC 29544 biofilms formed on glass treated with 200 ppm free chlorine (Table 3.21-3.23, Figure 3.6). There

Table 3.18 Average decrease in population after treatment of biofilms with 100 and 200 ppm free chlorine solutions

Surface	*E. sakazakii* 29004		*E. sakazakii* 29544	
	100 ppm	200 ppm	100 ppm	200 ppm
Glass	0.33	0.75	0.23	3.30
Stainless Steel	0.94	0.76	0.35	0.45
Silicone	0.44	0.15	0.18	0.71
Polycarbonate	0.77	0.83	0.93	1.04

Figure 3.6 Average decrease in population after treatment of biofilms with 100 and 200 ppm free chlorine solutions

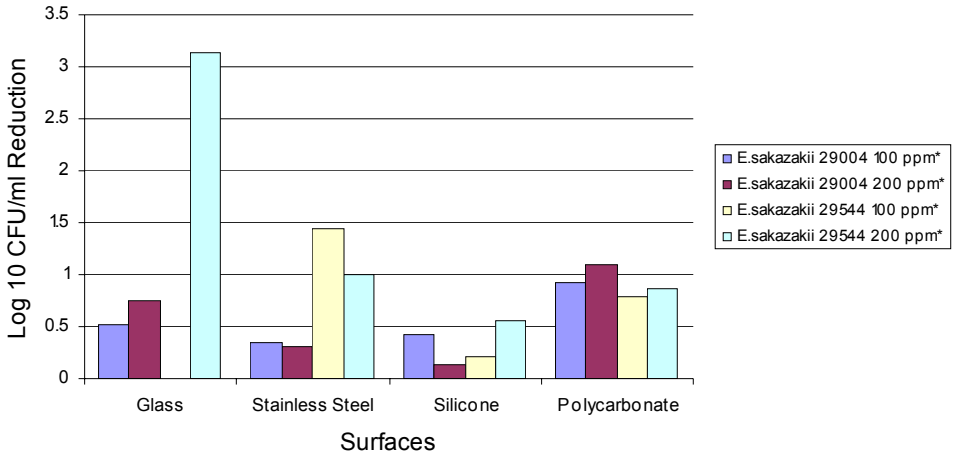

Table 3.19 Analysis of variance results for decrease in population after treatment of biofilms with 100 ppm and 200 ppm free chlorine solution

Effect	Numerator Degrees of Freedom	Denominator Degrees of Freedom	F Value	Pr > F
Strain	1	15	24.53	0.0002
Surface	1	15	34.59	<.0001
Treatment	1	15	71.31	<.0001
Strain *surface	1	15	40.58	<.0001
Strain* treatment	1	15	70.94	<.0001
Surface* treatment	1	15	59.09	<.0001
Strain*surface*treatment	1	15	28.97	<.0001

Table 3.20 LSD method (P<.05) for decrease in population after treatment of biofilms with 100 ppm and 200 ppm free chlorine solution

Strain	Surface	Treatment	Log CFU/ ml	Standard error	Letter group
29004			0.62	0.03904	B
29544			0.90	0.04141	A
	Glass		1.16	0.05522	C
	Polycarbonate		0.89	0.05522	D
	Stainless Steel		0.62	0.05522	E
	Silicone		0.37	0.06173	F
		100 ppm	0.52	0.03904	G
		200 ppm	1.00	0.04141	H

Table 3.21 LSD method (P<.05) sliced by treatment 100 ppm free chlorine for decrease in population after treatment of biofilms with 100 ppm free chlorine solution

Strain	Surface	Estimate	Standard error	Letter Group
29004	Glass	0.33	0.11	B
29004	Polycarbonate	0.77	0.11	A
29004	Stainless Steel	0.94	0.11	A
29004	Silicone	0.44	0.11	B
29544	Glass	0.23	0.11	B
29544	Polycarbonate	0.93	0.11	A
29544	Stainless Steel	0.35	0.11	B
29544	Silicone	0.18	0.11	B

Table 3.22 LSD method (P<.05) sliced by treatment 200 ppm free chlorinefor decrease in population after treatment of biofilms with 200 ppm free chlorine.

Strain	Surface	Log CFU/ ml	Standard error	Letter Group
29004	Glass	0.75	0.11	BC
29004	Polycarbonate	0.83	0.11	B
29004	Stainless Steel	0.76	0.11	BC
29004	Silicone	0.15	0.11	D
29544	Glass	3.33	0.11	A
29544	Polycarbonate	1.04	0.11	B
29544	Stainless Steel	0.45	0.11	CD
29544	Silicone	0.71	0.11	BC

Table 3.23 LSD method (P<.05) sliced by surface glass for decrease in population after treatment of biofilm with 100 and 200 ppm free chlorine solutions

Strain	Treatment	Log CFU/ ml	Standard error	Letter Group
29004	100 ppm	0.33	0.11	C
29004	200 ppm	0.75	0.11	B
29544	100 ppm	0.23	0.11	C
29544	200 ppm	3.33	0.11	A

was an overall significant difference ($p<0.05$) observed between the strains in terms of resistance to chlorine solutions (Table 3.19 -3.20), that could be observed after analyzing each treatment and surface separately (Table 3.21-3.26).

Biofilms formed by *E. sakazakii* ATCC 29004 and *E. sakazakii* ATCC 29544 behaved similarly on all surfaces after treatment with 100 ppm free chlorine with the exception of biofilms formed in stainless steel surfaces, where strain *E. sakazakii* ATCC 29004 showed a 0.94 log CFU/cm^2 decrease in population compare with a 0.35 log CFU/cm^2 decrease observed for *E. sakazakii* ATCC 29544 (Table 3.21-3.25). Biofilms formed by *E. sakazakii* ATCC 29004 and *E. sakazakii* ATCC 29544 behaved similarly in all surfaces after treatment with 200 ppm free chlorine with the exception glass and silicone biofilms (Table 3.22 - 3.23; 3.26) in which E. sakazakii showed a higher decrease in population.

After treatment of glass coupons with 200 ppm free chlorine solutions strain *E. sakazakii* ATCC 29004 showed a 0.75 log CFU/cm^2 decrease in population compare with a 3.33 log decrease observed for *E. sakazakii* ATCC 29544 (Table 3.22-3.23).After treatment of silicone coupons with 200 ppm free chlorine solutions strain *E. sakazakii* ATCC 29004 showed a 0.15 log CFU/cm^2 decrease in population compare with a 0.71 log CFU/cm^2 decrease observed for *E. sakazakii* ATCC 29544 (Table 3.26)

Table 3.24 LSD method (P<.05) sliced by surface polycarbonate for decrease in population after treatment of biofilm with 100 and 200 ppm

Strain	Treatment	Log CFU/ml	Standard error	Letter Group
29004	100 ppm	0.77	0.11	A
29004	200 ppm	0.83	0.11	A
29544	100 ppm	0.93	0.11	A
29544	200 ppm	1.04	0.11	A

Table 3.25 LSD method (P<.05) sliced by surface stainless steel for decrease in population after treatment of biofilm with 100 and 200 ppm free chlorine solutions

Strain	Treatment	Log CFU/ml	Standard error	Letter Group
29004	100 ppm	0.94	0.11	A
29004	200 ppm	0.76	0.11	AB
29544	100 ppm	0.35	0.11	C
29544	200 ppm	0.45	0.11	BC

Table 3.26 LSD method (P<.05) sliced by surface silicone for decrease in population after treatment of biofilm with 100 and 200 ppm free chlorine solutions

Strain	Treatment	Log CFU/ ml	Standard error	Letter group
29004	100	0.435	0.11	AB
29004	200	0.145	0.11	B
29544	100	0.175	0.11	B
29544	200	0.71	0.16	A

Exposure of *E. sakazaki*i to 100 ppm and 200 ppm chlorine solutions produced up to 1.04 log CFU per cm^2 reduction (Table 3.21-3.22), except for the 200 ppm chlorine solution applied to ATCC 29544 biofilm adhered to glass, where the log reduction estimate was 3.3 log CFU/cm^2 (Table 3.24). Even though the mean reduction in *E. sakazakii* on glass (1.16 log CFU/cm^2) is higher than the mean reduction obtained for polycarbonate (0.89 log CFU/cm^2); biofilms growing on polycarbonate showed a higher log reduction after treatment with 100 ppm than biofilms growing on glass (Table 3.21, 3.23, 3.24). ATCC 29004 and ATCC 29544 biofilms treated with 100 ppm showed the highest log reduction when growing on polycarbonate or stainless steel surfaces (Table 3.21). However when 200 ppm chlorine solutions were applied to ATCC 29004 biofilms, no significant difference was found between the surfaces; except for silicone, which showed the lowest log reduction (0.15 log CFU/cm^2) (Table 3.22). For ATCC 29544 biofilms treated with 200 ppm, glass showed the highest log reduction (3.32 log CFU/cm^2) followed by polycarbonate (1.04 log CFU/cm^2) (Table 3.22).

Detachment of *E. sakazakii* ATCC 29544 and *E. sakazakii* ATCC 29004 from the surfaces after application of the treatments was calculated by comparing the initial number of bacteria attached to surfaces before treatment with the final number of cells that remained adhered to the surface after the treatment. The results obtained showed the highest log reduction for *E. sakazakii* ATCC 29544

biofilms formed on glass surfaces and treated with either 100 ppm or 200 ppm free chlorine solutions (Table 3.27).

Discussion

The three most common ways of growing bacteria in vitro are as planktonic cultures, colonies on agar plates, and biofilms [Mikkelsen et al., 2007].

Biofilms were previously defined as “cells irreversibly attached to a surface or interface, embedded in a matrix of extracellular polymeric substances which these cells have produced, and including the noncellular or abiotic components”. This definition should encompass most cell aggregates, including bacterial colonies on agar plates; however, very few studies have mentioned bacterial colonies in a biofilm context [Mikkelsen et al., 2007]. Moreover, Donlan and Costerson [2002], considered that colonies of bacteria growing on the surface of agar behave like planktonic cells "stranded" on a surface and exhibited none of the inherent resistant characteristics of true biofilms, such as: altered growth rate and transcription of specific genes . Conversely, Shapiro [1998] and Kolter and Greenberg [2006] , predict colonies to behave like biofilms, since they are organized communities encased in a polysaccharide matrix with high cell densities and coordinated cellular behavioral patterns.

In a recent review, Kolter and Greenberg [2006] described a *P. aeruginosa* colony as an air-exposed biofilm in which as the community grows;

3.27 Detachment of *E. sakazakii* ATCC 29004 *E. sakazakii* ATCC 29544 from surfaces after treatment with 100-200 ppm free chlorine.

		E. sakazakii 29004		*E. sakazakii 29544*	
Surface	Treatment	Log CFU/ml	Log reduction	Log CFU/ml	Log reduction
Glass	Control	8.0		6.0	
	100 ppm	7.8	0.2	3.0	3.0
	200 ppm	7.3	0.7	3.0	3.1
Stainless Steel	Control	5.2		5.2	
	100 ppm	5.1	0.1	4.4	0.8
	200 ppm	5.1	0.1	4.3	0.9
Silicone	Control	7.5		7.8	
	100 ppm	7.0	0.5	7.7	0.1
	200 ppm	6.9	0.7	7.1	0.7
Polycarbonate	Control	8.7		8.8	
	100 ppm	7.8	0.9	7.9	0.9
	200 ppm	7.8	0.9	7.8	0.9

different cell types appear as the original strain diversifies to allow cells to take on different tasks or to exist in different 'microniches'.

The capability of cells to survive the antimicrobial effect of chlorine sanitizers have been object of study for several years. While some authors report that micro-organisms grow attached to a surface in the form of a biofilm exhibit remarkable resistance to all types of antimicrobials when compared with the same microorganisms grown in suspended cultures [Gilbert and Brown 1995], other authors report that certain biofilms are not different from stationary-phase planktonic cells in their resistance to killing by antibiotics and a biocide [Spoering, and Lewis 2001].

Different hypotheses have been considered to explain tolerance to the antimicrobial effect of chlorine sanitizers [Cochran et al. 2000]:

- Presence of persister cells
- Starving state or low growing activity.
- Failure of the antimicrobial agent to penetrate the full depth of the biofilm.

Presence of persister cells

Keren et al. [2004] emphasized that the presence of a residual fraction of cells die slowly or not at all when exposed to antimicrobials. These survivors (persister cells) are responsible for the high levels of tolerance of biofilms to antimicrobials, yet their increased tolerance is not heritable. It is also thought that persister cells are in a dormant state or are unable to initiate programmed cell death [Massey et al., 2001].

Several studies have presented data to support that biofilm cells resemble planktonic cells in stationary phase; it has also been noted that the proportion of persistent is higher in stationary phase populations [Spoering and Lewis, 2001; Stoodley et al., 2002; Waite et al., 2005].

Planktonic stationary phase cultures of *P. aeruginose* have exhibited a higher resistance to killing by antibiotics than biofilms ({Keren et al., 2004, Spoering and Lewis 2001]. These observations suggest that biofilm survival is based on the presence of persisters regulated by the growth stage of the population and not on expression of possible biofilm-specific resistant mechanisms.

In our study we observed that while exposure of stationary planktonic cells to 2 ppm , 4 ppm and 6 ppm free chlorine solutions for 3 minutes produced in average1.0 to 2.0 log, 2.1 to 3.3 log and 3.5 to 4.5 log CFU/ml reductions ,respectively, the use of the same chlorine solutions against cells growing in

colonies produced in average 0, 0.1 and 0.6 log CFU/ml reduction estimates. Even though *E. sakazakii* ATCC 29004 and *E. sakazakii* ATCC 29544 planktonic cells were in stationary phase after 24 h growth at 35 ^{o}C, they behaved differently than biofilms cells and colonies in term of resistance to chlorine sanitizers. An explanation to this can be related to observations that report that many microbes in contact with solid surfaces, trigger highly sophisticated colonization responses that include expression of specific genes that increase resistance to antimicrobial agents [Lejeune, 2003, Davies et al., 1993]. It is important to highlight that planktonic cells were concentrated and washed, pellicle was disturbed and therefore cells had a greater area of exposure to chlorine. In addition to this, stationary phase in a planktonic culture is merely a descriptive term which is likely to cover a heterogeneous population of cells in different metabolic states [Kolter and Greenberg, 2006].

Starving state or low growing activity

Some authors suggest that cells in the interior of a biofilm microcolony, form a protected subpopulation of cells which enter in a slow growth rate when nutrient becomes limiting and reduces the uptake of solutes from the environment including antimicrobials [Stewart and Costerton, 2001]

In this study we found that colony resistance to chlorine sanitizers increased under starvation stress, being similar to that expressed by biofilms,

and higher than the resistance expressed by planktonic cells or colonies cultivated for 24 hours.

In our study colonies were grown on agar for 7 days. This prolonged period could have generated starvation conditions and triggered genes that are not expressed under normal conditions. Lisle et. al. [1998] reported that *E. coli O157:H7* chlorine resistance progressively increases throughout starvation periods due to the development of chlorine injury-resistant membrane structures. Berg et al [1982] speculated that the increased resistance was due to changes in cell membrane permeability of slow growing bacteria.

In a biofilm, cell densities are substantially higher than in planktonic culture, [Prigent-Combaret et al., 1999] as a consequence, most biofilm cells are likely to encounter nutrient and oxygen limitation as well as higher levels of waste products, secondary metabolites, and secreted factors [Parsek and EP., 2005; Xu et al., 1998]. The development of injury-resistant membranes in starved colonies and cells contained in the biofilm matrix could explain the higher resistance of these cells to chlorine sanitizers.

The effectiveness of 200 ppm free chlorine solutions against *E. sakazakii* ATCC 29544 biofilms can be explained by its effect on increasing the detachment from the glass surface, once the cell detach, the surface area of the cell in contact with the disinfectant is higher which is detrimental for the cell.

Some studies have shown that microorganisms seem to attach more rapidly to hydrophobic nonpolar surfaces such as Teflon and other plastics compared to hydrophylic material such as glass or metals [Bendinger et al., 1993; Characklis et al., 1990]. Different studies [Sharma and Beuchat, 2004; Sharma et al., 2005] suggest a synergistic mechanism between an alkaline cleaner and hypochlorite that results in killing higher numbers of cells of *E. coli O157:H7* in biofilms. These studies suggest the application of alkaline solutions to impart hydrophilic properties to stainless steel which may decrease bacterial attachment to surfaces.

Failure of antimicrobial agent to penetrate the full depth of the biofilm.

The production of extracellular polysaccharides by certain bacteria plays a role in their protection against antimicrobials. Exopolysaccharides which express a negative charge can repel antimicrobials with an anionic charge or attract antimicrobials with cationic charge and neutralized them before they reach the cell. In addition, the penetration rate of antimicrobials in biofilms decreases as the exopolysaccharide content increases. Lisle et. al. [1998] proposed that bacterial resistance to chlorine is a biphasic process, in which the disinfectant first reacts with extrinsic components (e.g., the capsule and outer membrane) and then induces intrinsic components (i.e., the heat shock proteins and redox regulon). However, if the oxidative or disinfectant demand of the extrinsic barriers is met or overwhelmed (e.g., by increased exposure time or increased disinfectant concentration), the disinfectant diffuses to the cell's cytoplasmic

membrane. Oxidative damage to this and other intracellular targets (e.g., nucleic acids) then induces the intrinsic mechanisms in an attempt to repair the resulting damage.

During our experiments, analysis of colony polymorphisms showed that if incubation time of colonies of *E. sakazakii* ATCC 29004 growing on agar was extended to 7 days the cells produced a higher amount of extracellular substances showing strong adhesion to the agar surface and very difficult suspension in liquid medium, even with extreme shaking. On the contrary, *E. sakazakii* ATCC 29544 colonies were easily picked from agar, and evenly suspended in liquid medium, even with very brief, gentle shaking. We also observed the formation of large cell clusters by *E. sakazakii* ATCC 29004 when it was grown under planktonic conditions, which is indicative of enhanced expression of intercellular adhesive properties

Even though *E. sakazakii* ATCC 29004 and *E. sakazakii* ATCC 29544 strains are considered different in terms of the amount of exopolysaccharide produced [Lehner et al., 2005], they showed no significant difference)p>0.05) consistently in terms of resistance to chlorine sanitizers . *E. sakazakii* ATCC 29544 a non-capsulated strain [Lehner et al., 2005] had a similar resistance to chlorine than the capsulated strain *E. sakazakii* ATCC 29004. Therefore, it is reasonable to hypothesize that the resistance of biofilms and colonies against chlorine is explained by mechanisms other than the presence of polysaccharides alone.

Mah and O'toole [2001] noted that the exopolysaccharide matrix does not form an impenetrable barrier to the diffusion of antimicrobial agents, and other mechanisms must be in place to promote biofilm cell survival. Nevertheless, for certain compounds, the exopolysaccharide matrix does represent an initial barrier that can delay penetration of the antimicrobial agent. Aggregation in colonies and biofilms generates a protective barrier against sanitizers that is not observed by cells in suspension. De Beer et al. [1994] reported that chlorine disinfectant, did not reach >20% of the bulk media's concentration within a mixed *Klebsiella pneumoniae* and *P. aeruginosa* biofilm, as measured by a chlorine-detecting microelectrode . The cells located in the internal part of the aggregate are protected by a surrounding layer of cells or organic matter. The surrounding layer will interact with the sanitizer and render it ineffective before reaching the internal cells of the aggregate.

We also discovered that both strains *E. sakazakii* ATCC 29004 and *E. sakazakii* 29544 tested positive for cellulose expression in the screening assays using calcofluor and congo red. The presence of cellulose may contribute to the resistance of both strains to chlorine sanitizers. Solano et al. [2002] described in their studies that bacterial cellulose was responsible for the chlorine resistance of wild-type strains of *Salmonella enteritidis,* when using 30 ppm NaOCl concentrations; after 20 min exposure period, 75% of the wild-type cells survived NaOCl exposure. In contrast, only 0.3% of the cellulose deficient cells survived under the same experimental conditions [Solano et al., 2002]

Significant findings

The results obtained in this study suggest that colony resistance to chlorine sanitizers increased under starvation stress, being similar to that expressed by biofilms, and higher than the resistance expressed by stationary phase planktonic cells or stationary phase colonies cultivated for 24 hours. Cell organization in colonies and growth in solid surfaces confers a higher resistance to chlorine sanitizers than those of planktonic cells, this could be explained by the expression of genes triggered by the physical contact between cell and surface.

We can also hypothesize that tolerance to chlorine occurs due to the production of cellulose and development of chlorine injury-resistant membrane structures and changes in cell membrane permeability of slow growing bacteria as suggested by Lisle et. al. [1998] study on *E. coli O157:H7* and Berg et al [1982].

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