

INACTIVATION OF *CRONOBACTER (ENTEROBACTER) SAKAZAKII* USING DIFFERENT
ANTIMICROBIAL AGENTS AND THE EFFECT OF SANITIZERS ON BIOFILM
FORMATION PROPERTIES

By

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Abstract

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Enterobacter sakazakii is an opportunistic pathogen that represents a health risk to neonates. It is a known contaminant of infant formula milk (IFM) and has been associated with cases of necrotizing enterocolitis and infant meningitis. Many studies have been performed on the growth conditions and inactivation of the microorganism. Control of this microbe is important since it affects highly susceptible populations. The purpose of this study was to investigate the inactivation of *E. sakazakii* (five strains) inoculated into IFM using different antimicrobials, and to evaluate the effectiveness of different disinfectants for the removal of biofilms on plastic surfaces. Results showed that out of all the antimicrobials used, the combination of lactic acid (0.2% v/v) and copper (50 ppm) proved to be the most effective, where the individual use of copper at 50 ppm and lactic acid at 0.2% v/v did not have a significant effect on the growth of *E. sakazakii* ($P > 0.05$), but the combination of both agents produced a significant inhibition of growth in both rehydrated infant formula milk (RIFM) and powdered infant formula milk (PIFM) after 2 hours ($P < 0.05$). The effectiveness of three

different household dishwashing liquids, and a sodium hypochlorite solution in the removal of biofilms formed on plastic surfaces was also studied, and it was found that quaternary ammonium compounds are the most effective, and that treatment time plays an important role in the elimination of the biofilms, since higher reductions in population were observed as the treatment times increased.

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CHAPTER ONE

LITERATURE REVIEW

1.1 *Enterobacter sakazakii* Characteristics

1.1.1 Taxonomy and Biochemical Characteristics of *E. sakazakii*

Enterobacter sakazakii is an emerging opportunistic human pathogen associated with bacterial infections in infants. The microorganism belongs to the family *Enterobacteriaceae*, and is a Gram-negative, motile, facultatively anaerobic, non-sporeforming organism (Drudy and others, 2006). It was known as “yellow pigmented *E. cloacae*” until 1980, when Farmer and others (1980) designated it as a new species based on the results of DNA-DNA hybridization, yellow pigment production, biochemical reactions, and antibiotic susceptibility studies. The organism is named in honor of Riichi Sakazakii, a Japanese bacteriologist (Iversen and Forsythe, 2003).

In the genus *Enterobacter* there are 14 species or biogroups, and *E. sakazakii* is a biovar in the genus (Gurtler and others, 2005). *E. sakazakii* exhibits many biochemical characteristics similar to those of *E. cloacae*, but is D-sorbitol negative, produces an extracellular deoxyribonuclease, and most strains produce yellow pigmented colonies (Farmer and others, 1980). However, some strains of *E. sakazakii* recently isolated ferment D-sorbitol, and do not produce yellow pigment, leading to misidentification as *E. cloacae* (Farber, 2008).

In 1977, researchers suggested that the yellow pigmented *E. cloacae* was a new species, basing this conclusion on DNA-DNA hybridization studies which showed less than 50% homology with non-pigmented strains. Farmer and others (1980) distinguished 57 strains of

yellow-pigmented *E. sakazakii* based on DNA hybridization, antibiotic susceptibility and biochemical reactions, they also observed other characteristics that distinguished the bacterium from *E. cloacae*. Some characteristics were greater pigment production at temperatures less than 36 °C, optimum pigment production at 25 °C, survival of cells in stock cultures stored at 17 – 30 °C without transfer for up to 8 years, the use of citrate as a sole carbon source, a 31 – 49% DNA-DNA homology with *E. cloacae*, and a 57% guanine + cytosine ratio (Farmer and others, 1980; Gurtler and Beuchat, 2007).

Enzymatic profiles of *E. sakazakii* were also used to differentiate the microorganism. A study by Muytjens and others (1984) used the APITM ZYM identification system to evaluate 226 strains of *Enterobacter*, including 129 strains of *E. sakazakii*. Two major differences between *E. sakazakii* and the other *Enterobacter* species were observed, the first is α -glucosidase activity, present in all strains of *E. sakazakii* but absent from the other *Enterobacter* strains (Muytjens and others, 1984). Farmer and others (1985) reported that 53 of 57 strains of *E. sakazakii* were positive for α -glucosidase. The second difference observed by Muytjens and others (1984) was Tween 80 esterase activity unique to *E. sakazakii*.

The use of gas chromatography to differentiate *E. sakazakii* from closely related *Enterobacter* and *Citrobacter* species was recently proposed. A study by Whitaker and others (2007) used capillary gas chromatography with flame ionization detection to compare the cellular fatty acid profiles for 134 *Enterobacter sakazakii* strains, to the cellular fatty acid profiles of closely related *Enterobacter* and *Citrobacter* species. The study demonstrated a clear differentiation in fatty acid composition amongst the species, making this a useful tool to differentiate *E. sakazakii* strains from other closely related species (Whittaker and others, 2007).

1.1.2 Occurrence of *E. sakazakii* in Foods and the Environment

The natural habitat of *E. sakazakii* is not currently well understood, but the microorganism is widely distributed in the environment and foods, with water, soil and vegetables being the most probable sources of the organism. Iversen and Forsythe (2004) stated that because of the organism's physiological features, its most likely habitat is on plant material. These features aid environmental survival, and they include the ability to produce a yellow pigment that protects the cells from UV rays in sunlight, the capsular and fimbriae formation to aid in adherence to surfaces that include other cells, and its remarkable resistance to desiccation over long periods of time (Iversen and Forsythe, 2004; Mullane and others, 2006).

E. sakazakii has been isolated from water, sediment, and soil; but in a study performed by Muytjens and Kolle (1990), the researchers were unable to isolate it from select environmental sources including: raw cow milk, cattle, rodents, grain, bird feces, domestic animals, surface water, soil, mud, and rotting wood (Muytjens and Kollee, 1990). Kandhai and others (2004) evaluated the occurrence of the organism in selected households and food factory environments, isolating *E. sakazakii* from almost all of the environments analyzed; these environments included milk powder manufacturing facilities and common household vacuum cleaners (Kandhai and others, 2004).

The microorganism was also isolated from floor drains, air, a vacuum canister, broom bristles, a room heater and electrical control box, a floor dryer, floor and condensate in a dry product processing environment in the United States. Other environmental sources for *E. sakazakii* reported by Iversen and Forsythe (2003), they include air in a hospital, clinical materials, rats, soil, sediment and wetlands, crude oil and cutting fluids (Gurtler and others, 2005).

Dairy products such as milk powders, cheese products, and baby foods are known reservoirs of *E. sakazakii*, as well as minced beef, sausage meat, and vegetables. There are also documented reports on the isolation of *E. sakazakii* from rats in Kenya and from Mexican fruit flies in California (Farber, 2008). Several reports implicate rehydrated powdered infant formula milk as a source of the organism in neonatal infections. The first outbreak linked to powdered infant formula milk from previously un-opened cans was reported by the CDC in 2001, and in another outbreak the powdered formula tested negative for *E. sakazakii*, but the blender used to prepare the formula was contaminated. It is very likely that the contamination was a result of a previous batch of contaminated powdered infant formula, and even though the blender was washed daily, the cleaning procedure may not have been sufficient to eliminate the contamination (Gurtler and others, 2005).

Out of all the possible sources of *E. sakazakii*, its presence in powdered infant formula milk is of particular concern, due to its use for the feeding of newborn babies. The details regarding the presence of *E. sakazakii* in infant formula milk will be discussed later.

1.1.3 Resistance to Osmotic Pressure and Desiccation

Enterobacter sakazakii is frequently isolated from powdered infant formula milk, which suggests that it has an unusual ability to survive in dry environments. Barron and Forsythe (2007) analyzed the dry stress and survival time of *E. sakazakii* and other *Enterobacteriaceae* in powdered infant formula; they assessed the generation of sub-lethally injured *Enterobacteriaceae* during desiccation, and its persistence in powdered infant formula over a storage period of 2.5 years at room temperature. The study included ten strains of *E. sakazakii*, and the recovery of the organism under dry stress decreased an average of 0.58 log cycles during

the first month, with a decrease of 3.34 log observed during the first 6 months. The average recovery decreased 1.88 log cycles over the next 24 months, resulting in a total decline in viable counts of 4.52 log cycles.

Five out of the ten *E. sakazakii* strains were still recoverable after two years, which shows that some of the microorganism strains can remain viable during long-term storage in powdered infant formula milk (Barron and Forsythe, 2007). The formation of an extracellular polysaccharide may provide *E. sakazakii* with the protection to survive physical and environmental stress; however, during the first 18 mo of this study there was no correlation between capsulation and recovery. After 2 years, four of the five strains recovered from the powdered infant formula milk were capsulated, and after 2.5 years only the capsulated strains were recoverable. Capsulation may play an important role in recovery following long term dessication. *E. sakazakii* can survive in dehydrated environments for long periods of time (Barron and Forsythe, 2007; Lehner and others, 2005).

Edelson-Mammel (2005) studied the survival of a single strain of *E. sakazakii* inoculated at approximately 10^5 CFU/g in powdered infant formula for a period of two years at room temperature, during which the contaminated formula was periodically rehydrated and analyzed for viable *E. sakazakii*. During the initial five mo of storage, there was a decrease in viable count of approximately 2.4 log cycles, and during the next 19 mo the concentration of viable *E. sakazakii* declined an additional 1.0 log cycle.

The calculated *D*-value for the first five months was 73.8 days and 684.9 days for the period between 5 and 19 mo. The concentration of *E. sakazakii* in the infant formula when rehydrated at the end of the study was approximately 300 CFU/mL (Edelson-Mammel and others, 2005). The results were consistent with those observed previously by Breeuwer and

others (2003), they found that *E. sakazakii* was more resistant to osmotic stress and desiccation than other members of the *Enterobacteriaceae* family (Breeuwer and others, 2003). Even when a majority of *E. sakazakii* cells are inactivated by storage in dehydrated powdered infant formula, a portion of the cells are highly resistant to dry storage conditions and can survive for up to 2 years (Edelson-Mammel and others, 2005).

Breeuwer and others (2003) studied the genetic basis for the survival of *E. sakazakii* exposed to dry conditions and concluded that the desiccation of the microorganism leads to an induction of seven genes associated with its heat shock regulation, four genes from the cyclic AMP receptor protein regulon, six genes involved in the stringent response, and a number of genes involved in trehalose synthesis and cell wall functions such as lipid A and lipopolysaccharide biosynthesis. Researchers concluded that the response of *E. sakazakii* to dry stress involves a genome-wide expression of functionally different groups of genes (Gurtler and others, 2005).

The high tolerance of *E. sakazakii* to desiccation provides a competitive advantage in dry environments, such as conditions found in milk powder factories, and increases the risk of post-pasteurization contamination of the finished products. The high desiccation resistance of the microorganism will also allow it to survive in powdered infant formula at water activities as low as 0.2.

1.1.4 Heat Resistance

The thermal tolerance of *E. sakazakii* was first investigated by Nazarowec-White and Farber (1997), they used 10 strains of *E. sakazakii* to inoculate re-hydrated infant formula at selected temperatures to determine the D and z values. They reported that the D-values at five

selected temperatures were: $D_{52^{\circ}\text{C}} = 54.8$ min, $D_{54^{\circ}\text{C}} = 23.7$ min, $D_{56^{\circ}\text{C}} = 10.3$ min, $D_{58^{\circ}\text{C}} = 4.20$ min, and $D_{60^{\circ}\text{C}} = 2.50$ min, with a pooled z-value of 5.82 °C. The comparison of the D-values obtained for *E. sakazakii* with those of other *Enterobacteriaceae* in dairy products, confirms that *E. sakazakii* is one of the most thermo-tolerant microorganisms (NazarowecWhite and Farber, 1997c).

Many thermal processes or pasteurization practices require a 4.0 -7.0 D log reduction of microorganisms as a process control. To obtain a 6.0 – 7.0 log reduction of *E. sakazakii*, a heat treatment of 60 °C for 15 – 17.5 minutes is required. The microorganism is more thermo-tolerant than other *Enterobacteriaceae* in dairy products (NazarowecWhite and Farber, 1997c).

Iversen and others (2004) determined that the D-values for a specific *E. sakazakii* strain were $D_{54^{\circ}\text{C}} = 16.4$ min, $D_{56^{\circ}\text{C}} = 5.1$ min, $D_{58^{\circ}\text{C}} = 2.6$ min, $D_{60^{\circ}\text{C}} = 1.1$ min and $D_{62^{\circ}\text{C}} = 0.3$ min. A capsulated strain showed smaller D values, but the z-values for both strains were 5.7 and 5.8 °C. The authors theorized that a high temperature short-time pasteurization of 15 s at 71.2 °C will result in a 21-D kill when dried infant formula is processed, indicating that *E. sakazakii* will not survive this commercial pasteurization process, therefore the persistence of the microorganism in powdered infant formula milk is attributed to post-process contamination (Iversen and others, 2004b).

A study by Edelson-Mammel and Buchanan (2004) reported a 20 fold difference in thermal resistance between the most and least heat-resistant of 12 strains of *E. sakazakii* in rehydrated infant formula at 58 °C. Half of the strains exhibited a D-value smaller than 50s, and the other half were greater than 300 s, so the authors divided the two thermally resistant groups into two phenotypes. The z-value for the most thermally resistant strain was 5.6 °C. Further examination after inoculation of infant powder formula with the strain and rehydration with de-

ionized water at temperatures of 50, 60, 70, 80, 90 and 100 °C, resulted in no inactivation at 50 °C, but a 4.0-D and greater inactivation was observed at 70 °C. Rehydration of infant formula milk with water at a temperature of 70 °C is a good alternative to reduce the risk of *E. sakazakii* survival in the rehydrated product by at least four orders of magnitude. *E. sakazakii* is seldom isolated at levels above 1 CFU/100g of powdered infant formula milk. A 4.0-D thermal treatment will virtually eliminate *E. sakazakii* (Edelson-Mammel and Buchanan, 2004).

The rates of growth of the organism at ambient and cool temperatures are necessary to predict the possible doses of *E. sakazakii* that might be ingested by an infant fed a rehydrated, stored infant formula. To better understand the thermal behavior of the microorganism, Kim and Park (2007) determined the thermal characteristics of selected strains of *E. sakazakii* at 52, 56 and 60 °C in a saline solution, rehydrated powdered infant formula, and dried baby food.

The study concluded that D-values were larger for rehydrated infant formula in comparison with the saline solution, but smaller than in dried baby food. The overall calculated z-value was 6.0 – 8.0 for saline, 8.0 – 10.0 for powdered infant formula, and 9.0 – 11.0 for dried baby food, and thermal inactivation did not occur for 20 minutes when infant formula milk was rehydrated with water at 50 °C, while a reduction of 1.0 – 2.0 log CFU/g and 4.0 – 6.0 log CFU/g was observed when the rehydration water was at 60 °C and 70 °C, respectively. The conclusions of the study confirm the report of Edelson-Mammel and Buchanan (2004), that to reduce the risk of *E. sakazakii* contamination from powdered infant formula, the water used for rehydration must be at a temperature higher than 50 °C (Kim and Park, 2007).

Breeuwer and others (2003) used five strains of *E. sakazakii* in the stationary phase to perform thermal inactivation studies, they used a disodium hydrogen phosphate/potassium dehydrogenate phosphate buffer with a neutral pH for the heating medium, and reported $D_{58^{\circ}\text{C}}$

values that ranged from 0.39 to 0.60 min, and z values between 3.1 and 3.6 °C for two strains. The z-values are slightly smaller than z-values obtained in the studies performed by Edelson-Mammel and others (2004) and Nazarowec-White and Farber (1997), but the difference in the heating media used may be the reason for the observed difference. The high amount of fat, protein, and carbohydrate in infant formula milk may serve to protect *E. sakazakii* against thermal inactivation, and result in larger D-values (Breeuwer and others, 2003).

Microwave radiation is also being studied as a method to inactivate *E. sakazakii* in rehydrated infant formula milk. Kindle and others (1996) heated infant formula milk inoculated with a population of 5 log₁₀ CFU/mL until the first signs of boiling, and cooled it down and analyzed it for surviving cells. Five samples of heated infant formula milk were analyzed, and four of the five samples tested negative for *E. sakazakii* following the microwave treatment, while the other formula contained 20 CFU/mL.

Based on the results of this study, it is highly recommended that rehydrated infant formula milk be rewarmed using a microwave, instead of more traditional methods. Kinle and others (1996) reported that the microwaving of infant formula milk in baby bottles for 85 – 100 s at 82 - 93°C will achieve a 4 log₁₀ CFU/mL reduction in the *E. sakazakii* population (Kindle and others, 1996).

From the data gathered on thermal resistance of *E. sakazakii*, the World Health Organization concluded that inactivation of the pathogen occurs very rapidly at temperatures above 70 °C. The WHO proposed that infant formula milk be rehydrated at temperatures greater than 70 °C to reduce the risk posed by *E. sakazakii* infection. The data also demonstrates that *E. sakazakii* will not survive the pasteurization step, so factory environments and post-

pasteurization processes should be very carefully monitored to avoid the contamination of powdered infant formula milk (Farber, 2008; Iversen and Forsythe, 2003).

1.1.5 Antimicrobial Resistance

Antimicrobial resistance of *E. sakazakii* is another interesting characteristic of the microorganism. Farmer and others (1980) reported that strains of *E. sakazakii* used in their study were susceptible to gentamicin, kanamycin, chloramphenicol, and ampicillin, while 87% were susceptible to nalidixic acid, streptomycin, tetracycline, and carbenicillin; 71 and 67% were susceptible to sulfadiazine and colistin, respectively, and only 13% were susceptible to cephalothin. The strains of *E. sakazakii* in this study were resistant to penicillin, while only 1 strain showed multiple antibiotic resistance (Farmer and others, 1980; Iversen and Forsythe, 2003). The natural antibiotic susceptibilities of 35 clinical isolates of *E. sakazakii* to 69 selected antimicrobial agents; identified isolates susceptible to most β -lactam and cephalosporin antibiotics, but highly resistant to benzylpenicillin and oxacilin. The results confirmed the observations of Farmer and others (1980) (Iversen and Forsythe, 2003).

Muytjens and Vanderrosvandere (1986) determined the minimum inhibitory concentration (MIC) of 29 antimicrobial agents against 195 strains of *E. sakazakii*, and reported that 90% of them exhibited a lower value than those of *E. cloacae*, but resistance to cephalothin and sulfamethoxazole was observed (Muytjens and Vanderrosvandere, 1986). A study performed by Nazarowec-White and Farber (1999) tested the antibiotic resistance of 17 strains of *E. sakazakii*, and reported that five food isolates and eight clinical strains were resistant to sulphisoxazole and cephalohin, but susceptible to ampicillin, cefotaxime, chloramphenico,

gentamicin, kanamycin, polymyxin-B, trimethoprim-sulphanmethoxazole, tetracycline and streptomycin (Gurtler and others, 2005).

Bacterial meningitis must be effectively treated to prevent death of the patient, so antibiotic resistance of *E. sakazakii* is of extreme concern when treating neonatal infections. The infection resulting from *E. sakazakii* are usually treated with ampicillin in combination with gentamicin or chloramphenicol, with the ampicillin-gentamicin combination being the preferred treatment, but unfortunately *E. sakazakii* developed resistance to both antibiotics by means of transposable elements, as well as resistance to β -lactams by the production of β -lactamase.

The *Enterobacter* inactivates a broad spectrum of penicillins and cephalosporins due to β -lactamase production, and this trend is increasing among strains of *E. sakazakii*. These observations led scientists to suggest a switch to carbapenems or the newer third-generation cephalosporins in combination with an aminoglycoside or trimethoprim-sulfamethoxazole to eliminate *E. sakazakii*. This treatment improved the outcome of *E. sakazakii* meningitis, even though the resistance of *Enterobacter spp.* to these antibiotics is increasing (Gurtler and others, 2005; Iversen and Forsythe, 2003).

Willis and Robinson (1988) reported two cases of *E. sakazakii* associated neonatal meningitis initially unresponsive to ampicillin-gentamicin therapy, but resulted in complete abatement after treatment with moxalactam. Based on the observations on antimicrobial activity, general assumptions regarding antibiotic therapy against *E. sakazakii* cannot be made; treatment should be decided upon clinical observation and *in vitro* susceptibility testing (Gurtler and others, 2005).

1.1.6 Biofilm Formation

Pure cultures of microorganisms often grown on broth media were studied for many years by microbiologists, but it is now accepted that bacteria grow preferentially as bio-films. Biofilms are complex communities that grow on a surface and are surrounded by a polysaccharide material known as glycocalyx. The films contain about 90 – 97% water and vary from a few micrometers to several millimeters in thickness. Biofilms usually consist of mixed species, and the bacterial cells may be accompanied by eukaryotes, a variety of extracellular polysaccharides, enzymes, proteins, bacteriocins, and low mass solutes accompanied with nucleic acids present as a result of lysis (Brooks and Flint, 2008).

Very little was known about biofilms before 1980, but today there is an enormous amount of evidence suggesting food processing equipment is often contaminated by biofilms, often resulting in contamination of food. Some of the hazards related to biofilms include film accumulation on food contact surfaces and microbial colonization of milk storage tanks, fouling of heat exchangers and adhesion of spores on packaging material surfaces. Biofilms can also form on food itself, providing potential for cross-contamination and post-process contamination of foods as well (Brooks and Flint, 2008).

The formation of biofilms on biotic and a-biotic surfaces is suggested as a survival strategy for food associated microorganisms such as *Listeria monocytogenes* and *Escherichia coli*. Biofilms provide the cells with protection to environmental stress factors such as UV light, osmotic stress, heat, starvation, acids, detergents, antibiotics, phagocytes, antibodies, and bacteriophages (Lehner and others, 2005). A study on thermo-tolerance and biofilm formation confirmed that when *E. sakazakii* was grown in infant formula milk, the microorganism was capable of producing biofilms on latex, silicon, and stainless steel; materials commonly used for

processing of infant formula milk, for infant feeding, and in formula preparation areas (Iversen and others, 2004b).

Lehner and others (2005) conducted experiments to identify biofilm formation and the production of cellulose as one of the components of the extracellular matrix, adherence of *E. sakazakii* to hydrophilic and hydrophobic surfaces, the production of extracellular polysaccharides, and the ability of *E. sakazakii* to produce cell-to-cell signaling molecules. Of the 56 strains of *E. sakazakii* used, 44 formed biofilms in nutrient rich media. Lehner and others (2005) also observed that 42.8% of the strains were attached to glass surfaces, while 58.9% of the strains formed biofilms on PVC microtiter wells under the same conditions. Extracellular polysaccharide production was confirmed for 41.8% of the isolates, with greater production by food and environmental isolates than by human isolates (Lehner and others, 2005).

The ability of *E. sakazakii* to form biofilms on stainless steel and enteral feeding tubes at selected temperatures and conditions was studied by Kim and others (Kim and others, 2008). Five strains grown in selected media including infant formula milk were examined at 12 and 25 °C, and larger populations attached at 25 °C on both surfaces. The research also concluded that nutrient availability plays a very important role in the biofilm formation process, since some biofilms were not formed when tryptic soy broth and lettuce juice broth were used to grow the strains (Kim and others, 2008).

1.2 Detection of *E. sakazakii*

1.2.1 FDA Recommended Method

E. sakazakii has a very low infectious dose estimated at around 1000 CFU/mL in re-constituted infant formula milk, which is why a good detection method is of extreme importance

to address the food safety issue. It is possible that due to the lack of an appropriate detection method, there is underestimation of the presence of *E. sakazakii* in the environment and foods, and the number of reported cases related directly to the organism (Al-Holy and others, 2008).

The initial method for the detection of *E. sakazakii* in powdered infant formula was developed by Muytjens and others (1988), and is currently the U.S. Food and Drug Administration (FDA) recommended method. The method consists of three key steps, starting with a pre-enrichment step, where the infant formula is rehydrated in sterile distilled water overnight at 36 °C. The second step is the enrichment of the rehydrated infant formula in *Enterobacteriaceae* enrichment (EE) broth and overnight incubation at 36 °C, after which the rehydrated infant formula is surface plated and streaked onto violet red bile glucose agar (VRBGA), the plates are incubated overnight at 36 °C and then sub-culturing of presumptive positive colonies is performed onto tryptic soy agar (TSA). These plates are incubated for 48 to 72 hours at 25 °C, and only yellow-pigmented colonies are selected. Colony identification is confirmed using the API 20E biochemical identification system, requiring an additional 18 to 24 hours to complete. A summary of the method can be observed in Figure 1 (Mullane and others, 2006; Farber, 2008).

The current method used by the FDA is very time consuming, requiring a minimum of 5 days to complete. In addition to time consumption, the method will not detect non-pigmented *E. sakazakii* strains and uses only one biochemical test kit, whereas other end tests are more reliable. Another problem is that approximately 2% of *E. sakazakii* strains are sensitive to EE broth and will be missed by the procedure. The method is a presumptive, most-probable number test, selective for *Enterobacteriaceae* and not specific for *E. sakazakii*. The final step requires the biochemical identification of presumptive positive *E. sakazakii* colonies. Because of the

problems associated with this method, several other methods are being developed to detect *E. sakazakii* in food and environmental samples (Farber, 2008; Muyltjens and others, 1988; Al-Holy and others, 2008).

1.2.2 Chromogenic and Fluorogenic Media

A variety of media were recently developed for the specific detection of *E. sakazakii* instead of just the *Enterobacteriaceae*. The media include Druggan-Forsythe-Iversen (DFI) medium, OK medium, *Enterobacter sakazakii* isolation agar (ESIA), and *Enterobacter sakazakii* plating medium (ESPM). These media take advantage of one key biochemical characteristic of the microorganism which is the production of α -glucosidase. Muyltjens and others (1984) were the first to report the α -glucosidase enzyme activity of *E. sakazakii*, which is not observed in other *Enterobacter* species (Farber, 2008).

The OK medium was developed by Oh and Kang (2004), it is a selective differential medium that contains a fluorogenic substrate 4-methyl-umbelliferyl- α -D-glucopyranoside, which detects α -glucosidase activity. The fluorogen is an indicator of the production of α -glucosidase by *E. sakazakii*. The media also contains bile salts no. 3 for the selection of enteric bacteria, and ferric citrate along with sodium thiosulfate to differentiate H₂S producing *Enterobacteriaceae* (Oh and Kang, 2004).

Druggan and others (2004) developed the DFI media, a chromogenic agar that contains the substrate 5-bromo-4-chloro-3-indolyl- α -D-glucopyranoside as an indicator of α -glucosidase activity. The substrate is cleaved by α -glucosidase and blue-green *E. sakazakii* colonies are created. The medium was developed as a differential, selective medium for the recovery of *E. sakazakii* in powdered infant formula after pre-enrichment and enrichment in preference to

VRBGA. Sodium desoxycholate, sodium thiosulfate and ferric ammonium citrate are also present in the medium as selective agents, the latter act as indicators of hydrogen sulfide production to differentiate *E. sakazakii* from *Proteus* and *Salmonella*. *Proteus* and *Salmonella* can also grow on the agar and appear as black-colored colonies, while *E. sakazakii* is H₂S negative (Iversen and others, 2004a).

The ESPM medium contains chromogens that produce blue-dark or blue-gray colonies of *E. sakazakii*. ESPM medium contains sorbitol, D-arabitol and adonitol and two chromogens: X- α -D-glucopyranoside and X- α -D-cellobioside. The inclusion of bile salts, vancomycin, and cefsulodin is what achieves the selectivity, and the presumptive isolates are confirmed by the lack of acid production on a second agar that detects acid production from sucrose or melibiose after 6 h at 35 °C (Restaino and others, 2006).

A culture method for the detection of *E. sakazakii* from environmental samples was developed by Guillaume-Gentil and others (2005). The method is based on the selective enrichment for the isolation of *E. sakazakii* from environmental samples. For this purpose a modified enrichment broth containing 0.5 M NaCl and 10 mg of vancomycin/liter in lauryl sulfate broth (mLSB) was tested. The enrichment is carried out at 45 °C for 22 to 24 h, and is followed by streaking onto TSA and incubation at 37 °C for an additional 24 h. The plates are exposed to artificial white light to induce pigment production and yellow colonies are selected for further identification using API 20E and ribotyping. Vancomycin is added to the mLSB in the enrichment stage to suppress the growth of Gram-positive bacteria (Farber, 2008).

The method of Guillaume-Gentil and others (2005) is modified by the vertical ISO method, where chromogenic agar *Enterobacter sakazakii* isolation agar (ESIA) is used after enrichment. The ESIA is incubated at 44 °C for 24 h, and the presumptive *E. sakazakii* colonies

are confirmed by pigment production on TSA at 25 °C, not 37 °C, with enhanced light exposure. ESIA contains 5-bromo-4-chloro-3-indolyl- α -D-glucopyranoside and crystal violet, and colonies appear blue-green after incubation.

There is a great variety of media for the detection of *E. sakazakii*, but there is still considerable variation in the recovery of this organism by the different protocols. Farmer and others (1980) reported that not all strains of *E. sakazakii* are yellow pigmented, and sometimes yellow pigment production depends upon temperature. The FDA method incubates TSA plates at 25 °C, the protocol by Guillaune-Gentil and others (2005) incubated the TSA plates at 37 °C, and some other selection methods use incubation temperatures of 44 to 45 °C. Nazarowec-White and Farber (1997) reported that only 3 of 11 *E. sakazakii* strains grew at 44 to 45 °C, with the maximum growth temperature for 4 of 11 strains being 41 °C. The final biochemical identification of *E. sakazakii* is also problematic, and there are reports of discrepancies between different kits (NazarowecWhite and Farber, 1997c; Farber, 2008).

1.2.3 DNA Based Methods

Studies based on the 16S rRNA gene and *hsp60* indicate that culture methods and biotyping lack necessary discriminatory power to confidently identify distinct phylogenetic lineages among *E. sakazakii* isolates. The sequence analysis of both partial 16S rRNA genes and *hsp60* showed four distinct clusters. More recent full-length 16S rRNA sequence analyses and DNA-DNA hybridization determinations confirmed four lineages of *E. sakazakii*. This diversity must to be considered when implementing DNA-based detection methods (Farber, 2008).

Recently the FDA developed a 5' – nuclease real-time PCR assay for the rapid detection of *E. sakazakii* in infant formula. The method uses a TaqMan approach to specifically amplify

part of the macromolecular synthesis operon, the *rpsU* gene 3' end, and the primase (*dnaG*) gene 5' end. The assay was specific for differentiating *E. sakazakii*, *E. cloacae*, and almost 50 other genera of *Enterobacteriaceae*, allowing detection of as few as 100 CFU of *E. sakazakii* per milliliter of infant formula milk without enrichment (Gurtler and others, 2005).

1.3 *E. sakazakii* Caused Illness

1.3.1 History and Pathogenicity Associated with *E. sakazakii* Infection

Infections caused by *E. sakazakii* lead to important life-threatening meningitis, septicemia, and necrotizing enterocolitis in infants, with premature and low birth-weight infants at higher risk than older infants. Even though the incidence of infection is low, the prognosis is poor with mortality rates of 33 – 80%. About 94% of those infants that survive develop irreversible neurological sequelae which results in quadriplegia, developmental impedance, and impaired sight and hearing. These symptoms are attributed to secondary cerebral infarcts (Drudy and others, 2006).

The epidemiology of *E. sakazakii* is not well understood, since *E. sakazakii* infection is very rare and not commonly reported in most countries. The first reported cases of *E. sakazakii* infection occurred in 1958 in England, where two infants died as a consequence of meningitis produced by the bacterium. However, at the time the microorganism was described as an unusual pigmented strain of *E. cloacae*. Other cases of neonatal and *E. sakazakii* infections were reported in Canada, Belgium, Denmark, Germany, Greece, Israel, The Netherlands, Spain and the United States. At least nine states reported neonatal infections caused by the microorganism in the United States, and at least 76 cases occurred worldwide between 1958 and 2003 (Iversen and Forsythe, 2003).

The incidence of invasive infant infection is very likely increasing according to some reports, but the apparent geographic and temporal trends in *E. sakazakii* infection is probably caused by differences in surveillance and reporting in different countries. The incidence of *E. sakazakii* invasive infection is greater among infants than older groups, and *E. sakazakii* caused meningitis is only reported in infants. A survey conducted by the Foodborne Diseases Active Surveillance Network (FoodNet) identified four cases of *E. sakazakii* meningitis or bacteremia among the catchment population during 2002. A survey of 19 U.S. neonatology centers revealed one episode of *E. sakazakii* bacteremia among 10,660 infants with low birth weight (Farber, 2008).

Although *E. sakazakii* is most associated with infections in neonates, it can also lead to many other types of infections in many age groups. Patients with extended hospital visits, particularly in intensive care units, are at a higher risk of contracting infections from *Enterobacter* species, along with those patients previously treated with antibiotics, the immunocompromised, the elderly, patients with medical implants and those with acute, chronic, or serious illnesses. (Drudy and others, 2006; Farber, 2008).

Very few reports exist of *E. sakazakii* infections in adults, and it is not usually life threatening. In fact most of the adults with reported *E. sakazakii* infections had already experienced serious underlying disease, such as malignancies. In the United Kingdom a total of 173 cases of *E. sakazakii* infection were observed between 2001 and 2003. Out of the eleven detailed adult cases reported in the literature, four were associated with *E. sakazakii* bacteremia, and all occurred in persons over 70 years of age; two of the cases occurred nosocomially following complicated abdominal surgeries, a third case had no clear source, and the fourth patient had a history of urinary retention and developed urosepsis. The remaining seven cases did

not present bacteremia, two had pneumonia, with *E. sakazakii* isolated from sputum, two had *E. sakazakii* isolated from soft tissue infections, and one had diabetes-associated osteomyelitis of the foot with a bone biopsy that yielded *E. sakazakii*, *S. epidermidis*, and enterococci (Farber, 2008).

E. sakazakii was also isolated in association with pneumonia, conjunctivitis, vaginitis and appendicitis, but the central nervous system infection is the best described (Farber, 2008). The pathogenesis in neonates usually involves bacteremia and/or sepsis, cerebrospinal fluid infection and meningitis, brain abscess and infarction, ventricle compartmentalization due to necrosis of brain tissue, or liquefaction of white cerebral matter, cranial cystic changes, fluid collection and dilated ventricles and hemorrhagic and non-hemorrhagic intercerebral infarctions, leading to a softening of the brain, and it has also been associated with necrotizing enterocolitis (Gurtler and others, 2005; NazarowecWhite and Farber, 1997a).

Meningitis is an acute inflammation of the membranes of the brain that surround the brain and the spinal cord, and if left untreated can lead to death. It is the most frequent reported condition associated with *E. sakazakii* infections, and it leads to brain abscesses approximately 90% of the time. The infection increases the inner cranial pressure, which then requires the aspiration of fluid and drainage of cerebral infarction, and even the occasional insertion of a ventriculoperitoneal shunt to prevent cerebral damage (Muytjens and others, 1983).

Low birth weight is a contributing risk factor for contracting the illness, and neonatal meningitis caused by the *Enterobacter* species is very high, with a mortality rate of up to 92% when *E. cloacae* is involved. Meningitis caused by *E. sakazakii* arises between the fourth and fifth day after birth, and it can lead to death within a few hours to several days after the first clinical signs appear. Seizure activity has been reported in approximately one third of the cases

of neonatal *E. sakazakii* meningitis, and some of the physical responses include grunting, bulging fontanelles, convulsions, twitching, and an increase in cranial circumference. The infection can cause hemorrhagic and non-hemorrhagic intercerebral infarctions, which leads to cystic encephalomalacia; and 20% of newborns will develop very serious neurological complications after infection (Muytjens and others, 1983; Gurtler and others, 2005).

Pathogenesis of neonatal meningitis caused by *E. sakazakii* is not well understood. It is believed that there is a translocation by the bacterium through the chordus plexus, and subsequent cellular invasion by means of pathogenic secretory factors like proteases or endotoxins, which increase blood-brain barrier permeability, and with this gaining access to the nutrient-rich cerebral matter (Iversen and Forsythe, 2003). Researchers have also reported a similarity between the tropism of *E. sakazakii* and *C. diversus* for the invasion and infection in the central nervous system (Gurtler and others, 2005).

Some neonatal cases of *E. sakazakii* meningitis may have some level of relation with necrotizing enterocolitis, which is associated with several bacterial pathogens and is the most common gastrointestinal disease in newborns. The disease affects about 2-5% of premature neonates with a mortality rate of 10-55%, and can be characterized by ischaemia, bacterial colonization of the intestinal tract, and increased levels of protein in the gastrointestinal lumen, which has been correlated to the consumption of infant formula milk (Iversen and Forsythe, 2003). Some researchers have suggested that there is a correlation between necrotizing enterocolitis and oral formula feeding; babies fed infant formula instead of milk formula are 10 times more likely to contract necrotizing enterocolitis according to a study by Lucas and Cole in 1990, while another study confirmed that prior to the administration of antibiotics, *Enterobacter* species were the most prevalent bacteria (Gurtler and others, 2005).

The virulence factors of *E. sakazakii* are not known yet, and the minimum infectious dose had not been addressed until a study was performed on the suckling mouse by Pagotto and others (2003). The production of enterotoxins was studied for nine clinical isolates and eight food isolates of *E. sakazakii*, it also included negative and positive controls. One strain of *E. sakazakii* was positive for enterotoxin production, and the minimum lethal oral dose was 10^8 CFU/mouse, while one clinical isolate at a 10^7 CFU/mouse dose was lethal by the peroral route, and one food isolate was lethal by intraperitoneal injection at doses as low as 10^5 CFU/mouse. Extrapolation of the results lead to the conclusion that a minimum lethal dose in neonates would require an unusually high number of viable cells, as might occur over time in temperature-abused infant formula (Pagotto and others, 2003).

There are many possible sources for the transmission of *E. sakazakii*, but the only vehicle which has been epidemiologically and microbiologically associated with infection is powdered infant formula, and the equipment used for its preparation (Iversen and others, 2004b; Muyltjens and others, 1983).

1.3.2. Presence of *E. sakazakii* in Infant Formula Milk

E. sakazakii's ability to survive in powdered infant formula and its potential to multiply in reconstituted formulas are risk factors for the infection of those neonates who are fed with these products. Dried cow milk and milk products are potential sources of pathogenic bacteria for humans, and many pathogenic bacteria are known to have been occasionally isolated from infant formula, such as *Citrobacter*, *Klebsiella*, *Enterobacter*, *Yersinia*, *Staphylococcus* and *Streptococcus*. The FDA has published bulletins highlighting the dangers of bacterial

contamination of formula products which contain powdered milk as the major ingredient (Gurtler and others, 2005).

Powdered infant formula contains a water activity of approximately 0.2, and it is manufactured to mimic the nutritional characteristics of human milk, it is usually manufactured in three different ways: wet-mix, dry-mix and a combination of the processes. The wet mix approach is usually the most popular one; it involves the combination of all the essential ingredients with liquid skimmed milk and fat components, the mixture is then heated at 81 °C for 20 s, and the rest of the components are added to the mixture and heated to 107 – 110 °C for 60 s followed by concentration using a falling film evaporator. The mixture is heated to 80 °C before spray drying (NazarowecWhite and Farber, 1997a; NazarowecWhite and Farber, 1997c). The dry process consists of the dry blending of pasteurized evaporated skim milk with the balance of essential ingredients, such as essential fatty acids, vitamins, whey, stabilizers and emulsifiers, and the pasteurization for 60 s at 110 °C followed by spray drying. This method has a higher probability of post process contamination, ingredient contamination during mixing, and problems due to ingredient separation (Gurtler and others, 2005).

It is difficult to determine which of the two processes is most likely to result in *E. sakazakii* contamination, but it is most likely that contamination from the processing environment is what introduces the microorganism into the product; this could be the result of contaminated materials or surfaces where the powder is manufactured. Since heat treatments like pasteurization are known to eliminate *E. sakazakii*, it has been generally assumed that contamination comes from the processing environment at post-pasteurization steps. The ability of the organism to resist dry and osmotic stress is probably the reason for its survival in the drying stage (Mullane and others, 2006).

Control measures must be implemented in order to reduce the exposure of the manufacturing process to the surrounding environment, in order to avoid contamination from pathogenic organisms including *E. sakazakii*. Examples of these measures are: monitoring raw materials, rejection of contaminated materials, separation of the different processes in the plant (dry and wet processing), cleaning regimes, hygienic zoning around the dry areas of the facility, and maintenance of a low-moisture environment post pasteurization. Continuous monitoring is essential to evaluate the effectiveness of each of the control measures implemented, and since the organism is found in the processing environment, even the strictest hygienic practices will not guarantee complete elimination, which translates into low level sporadic contamination of the finished product. It is important to emphasize that the risk of formula contamination is a function of the factor environment, and not only the manufacturing process (Mullane and others, 2006; Gurtler and others, 2005).

The distribution and production of infant formula is regulated by the 1980 Infant Formula Act, it sets minimum standards for 29 nutrients and mandates adherence to good manufacturing practices and clear labeling, but there are more stringent good manufacturing and sanitation requirements for formula compared to other food, but no requirement for sterility. *E. sakazakii* has been isolated from infant formula in various studies and surveys, for example a study conducted by Muytjens and others in 1988 detected the presence of the microorganism in 14.9 % of 141 samples of infant formula from 35 different countries. The formulas were cultured for *Enterobacter* species, and *E. sakazakii* was the third most commonly isolated bacterium (Table 1). The bacterial numbers obtained from this particular study were under 1 CFU/g of dry powder , which was in accordance at that time with the microbiological guidelines set by The International Commission on Microbiological Specification for Foods (ICMSF), but the

microbiological criteria has been recently revised by the European Union, and they are presently under revision by the Codex Alimentarius Commission (Muytjens and others, 1988).

Four strains of *E. sakazakii* were recovered from powdered milk and two from powdered infant formula in a survey conducted in the former Czechoslovakia, and other reports have confirmed infant powdered formula as the source of bacteria in several cases of neonatal necrotizing enterocolitis. The CDC has reported that a definitive link exists between the presence of *E. sakazakii* in powdered infant formula in an unopened can and an outbreak of infection caused by the microorganism (Gurtler and others, 2005; Baker, 2002). In 1997 a study examined infant formula powder manufactured by different companies, and isolated *E. sakazakii* in 3.3 % of the cans they used, at an average level of 0.36 CFU/100g. (NazarowecWhite and Farber, 1997b).

Due to the increased awareness of the possible contamination of powdered infant formula with *E. sakazakii*, many surveys have been conducted (Table 2), and the presence of the microorganisms ranged from 2 to 14% in the analyzed products. The concentration has never been reported to be higher than 1 CFU/g, but it is important to emphasize that if poor hygienic practices are used in the preparation and handling of infant formula milk, the number of cells can rise quickly due to the short doubling time the organism has at room temperature, and this is why this food safety issue must be addressed (Al-Holy and others, 2008; Farber, 2008).

1.3.3. Risk Management of *E. sakazakii* in Infant Formula Milk

There are a few steps that could prove very beneficial for the prevention of *E. sakazakii* contamination of infant powder formula. It is necessary to control raw materials upon receipt, and although the source of *E. sakazakii* contamination is currently uncertain, it is a known fact

that it is an environmental organism, which can be present in many raw ingredients as well as in milk. This control measure can eliminate the possibility of the microorganism finding its way into the final product, and with the development of better detection methods it could improve the safety of infant formula milk.

It has been stated that *E. sakazakii* has a very high thermal tolerance compared to other members of *Enterobacteriaceae*, and this helps in its prevalence in powders such as infant formula powder, but thermal treatment is an important strategy for its prevention. Kindle and others (1996) have reported for example that the *E. sakazakii* viable count can be reduced from 10^5 to 20 CFU/mL after exposure to microwaves for 85s at 82°C in infant formula milk, while *E. coli* and *Ps. aeruginosa* did not survive this treatment (Kindle and others, 1996).

As stated earlier it is very possible that *E. sakazakii* contamination occurs in the post-processing steps, therefore prevention must be strongly enforced by keeping clean areas and sanitary practices throughout the entire process. The application of microbiological criteria is also an important part of risk management in this particular case. The FAO's code of hygienic practice for foods for infants and children (Codex Alimentarius CAC/RCP 21-1979) requires a minimum of 4-5 samples with ≤ 3 coliforms/g and a maximum of 1/5 control samples with ≥ 3 but ≤ 20 coliforms/g. The criteria has not been exceeded by the numbers of *E. sakazakii* found in infant formula milk, including those associated with outbreaks; so it is probable that infant formula milk associated with *E. sakazakii* outbreaks have met the current statutory criteria. Therefore these criteria must be reviewed and possibly more stringent statutory ones need to be enforced.

It could be argued that criteria of ≤ 1 enteric pathogen cells/25 g should be adopted for the manufacture of infant formula milk, this is a level of microbial contamination that can be

achieved and is already applied for the presence of *Salmonella*. In order to implement these strict microbiological criteria it is also necessary to validate the recommended and newly developed detection methods (Iversen and Forsythe, 2003).

Since powdered infant formulas are not commercially sterile products, the FDA has recommended certain practices for their preparation in neonatal intensive care units. These recommendations include the preparation of the feed by trained personnel, immediate refrigeration, discarding of any food that has not been used in 24 h, continuous enteral feeding should not exceed 4h, and that alternatives to the powdered form should be chosen when possible. Even though these strategies could help in the prevention of infection by the microorganism, there is clearly a need for a review of the accepted criteria for *E. sakazakii* in infant food products, and for a rapid detection test; one that is reliable and feasible for its use in the food manufacturing industry (Iversen and Forsythe, 2003).

1.4. Inactivation of *E. sakazakii*

Due to the risk posed by *E. sakazakii* to a newborn child, health authorities and researchers are exploring ways to eliminate the bacterium. Although heat treatment has been a general method of inactivation or reduction of *E. sakazakii*, studies on its thermal resistance in infant formula indicate that it is one of the most thermo-tolerant bacteria in the *Enterobacteriaceae* (Iversen and others, 2004b). Among the technologies being considered for this purpose, research is being conducted on the inactivation of the organism using gamma radiation, pulsed field and high-pressure processing.

Gonzalez and others (2005) studied the effects of high-pressure processing on strains of *E. sakazakii*, they inoculated four strains (A, B, C, D) of the organism into tryptic soy broth

(TSB) and reconstituted formula, and exposed them to high pressure processing using pressures of 200, 400, and 600 MPa for 1 minute in each medium. Pressures of 600 Pa at 25 °C for 1 minute achieved a 6 log reduction in the population of *E. sakazakii* strains A, C and D in TSB, and in the population of strains A and C in reconstituted infant formula. A 5 log reduction was obtained for strain D in infant formula, while strain B was more pressure tolerant than the other strains in both TSB and infant formula. A 3 log reduction was obtained for this particular strain.

When subjected to pressures of 400 MPa, some of the strains achieved a 3 log reduction in the broth (strains A and D), while a 0.5 to 2 log reduction was observed in infant formula for all strains, where A and D were the most sensitive, and strains B and C the most resistant to pressure treatment in infant formula. The effect of pressurization at 200 MPa was negligible for all strains.

Other research has also examined the effects of pressure treatments on other *Enterobacteriaceae* using longer exposure times. The data collected from all of the different research show that the log reduction achieved for the organism is very similar to the ones obtained for other *Enterobacteriaceae*. Variations in pressure resistance were observed between strains, but the high-pressure processing did effectively inactivate some strains in broth and infant formula, more so at 600 MPa. More research needs to be performed to compare different exposure temperatures and times in combination with different pressures, in order to establish the cause for the pressure resistance variation, and design an optimal treatment for the inactivation of *E. sakazakii* from infant formula milk (Gonzalez and others, 2006).

Pulsed electric field (PEF) is a technology which has potential for use in the food industry, it is a relatively “friendly” technology in relation to thermo-sensitive components of foods, and its effectiveness as a treatment to inactivate *E. sakazakii* was investigated by Perez

and others (2007). Infant formula milk and a reference medium were used in the study; they were inoculated with *E. sakazakii* in order to obtain 10^9 CFU/mL and treated. The electric field intensity and treatment time were varied from 10 to 40 kV cm⁻¹ and from 60 to 389 μ s respectively, and it was found that the population of the organism was reduced by a maximum of 2.70 log units when inoculated into the reference medium and treated by PEF at 40 kV cm⁻¹ for 360 μ s, the same conditions provided a 1.22 log reduction in the population of *E. sakazakii* when infant formula milk was treated (Perez and others, 2007).

Thermal inactivation, pasteurization or ultra-high temperature (UHT) processing are widely used treatments for the inactivation of pathogenic microorganisms and enzymes in milk, but heat can modify components in the product, causing decreases in Ca²⁺, denaturation of serum proteins and degradation of vitamins. The PEF treatment uses temperatures that do not exceed 40 °C. Results from the PEF study show that it is possible to achieve a significant reduction in the population of *E. sakazakii* by the application of different PEF treatment conditions. Normally it is recommended that infant formulas be re-constituted with water at temperatures of 50 and 70 °C, or heated after re-constitution, but this practice may have negative effects on flavor and possible nutrient loss, reasons why the viability of PEF as an alternate technology for processing powdered infant formula before feeding in hospitals should be considered. It could be possible to develop PEF equipment for hospital use because of its relatively small dimensions and easy processing mechanism, reducing the microbial load of *E. sakazakii* present in this type of food (Perez and others, 2007).

Gamma radiation has also been investigated for the possible inactivation of *E. sakazakii*, Lee and others (2007) carried out research to investigate the effect of irradiation on the inactivation of the microorganism in dehydrated infant formula. A particular strain was

inoculated into a commercial dehydrated infant formula purchased at a local supermarket. To determine the D_{10} value the strain was cultured in TSB and irradiated with 0.0 (control) and 0.3 – 2.0 kGy, the infant formula was also irradiated with 0.0 (control) and 1.0 – 4.0 kGy in a powdered state.

Effective reduction of the population of the bacterium was achieved with irradiation in both the TSB and de-hydrated infant formula. A 2.5 log reduction was obtained at 1.0 kGy for the TSB, but only a 1.0 log reduction was seen in the dehydrated infant formula using 1.0 kGy. The D_{10} value for the strain in TSB was found to be 0.27 ± 0.5 kGy, and 0.76 ± 0.5 kGy in the dehydrated infant formula. The difference between the results obtained are possibly due to the fact that factors such as temperature during irradiation, stage of growth, presence of oxygen and availability of water affect the sensitivity of microorganisms to irradiation.

Re-growth experiments were also performed by the researchers, where the infant formula was irradiated again in a powdered state after freeze drying, and the initial population mean in the control samples was around $8.5 \lg$ CFU/g, while irradiation with 5 kGy reduced the population to below the detection level, which means that gamma-irradiation is an effective technique to be considered for the elimination of *E. sakazakii* from infant formula milk (Lee and others, 2007).

Another study on the effect of gamma radiation on the inactivation of *E. sakazakii* found that the D_{10} values of five strains of the microorganisms ranged from 1.06 to 1.71 kGy in dehydrated infant formula milk. The D_{10} values were higher in the infant milk formula when compared to those obtained in brain heart infusion broth, which means that the microbe is more resistant to ionizing radiation in the infant formula, possibly due to a protective effect from the presence of protein, fat, and carbohydrates.

Oral infection doses of 10^3 cells and contamination levels of less than 10^2 CFU/100g of dehydrated infant formula have been approximated for *E. sakazakii*, which suggests that the use of ionizing radiation to reduce the level of the organism by 3 log is a viable alternative for the control of the microorganism (Osaili and others, 2007).

CHAPTER TWO

MATERIALS AND METHODS

2.1. Bacterial Strains

Four strains of *E. sakazakii* from the American Type Culture Collection (ATCC) and one from the FSM collection were used for this study. The cultures were ATCC 29004, ATCC 51329, ATCC 12868, ATCC 29544, and FSM 287. The strains were obtained from the culture collection in the School of Food Science, at Washington State University, Pullman, USA. The five strains were transferred individually from stock aqueous glycerol (15%) solutions stored at -20 °C to slants of tryptic soy agar (TSA) (Difco, Becton Dickinson, Spark, MD) and kept refrigerated at 4 °C.

2.2. Inactivation Studies

2.2.1. Inoculation of Re-hydrated Infant Formula Milk (RIFM)

Prior to the experiments, the five strains were individually transferred to Brain Heart Infusion (BHI) broth (Difco, Becton Dickinson, Spark, MD, USA), and grown for 24 hours at 37 °C to reach the stationary phase of growth. After incubation, the five strains were transferred to a sterilized centrifuge tube, creating a bacterial cocktail. The tubes were then centrifuged for 15 min at 4000 rpm (Fisher Scientific, Fisher AccuSpin™ Model 400 Benchtop Centrifuge, Pittsburgh, PA, USA) to harvest bacterial cells. The pellet was re-suspended in 9 mL sterile 0.1% peptone water solution and centrifuged at the same conditions described earlier. The supernatant was discarded, and the pellet re-suspended in 9 mL of a sterile 0.1% peptone water solution, the initial level of *E. sakazakii* was determined by spread plating on TSA and incubation at 37 °C for

24 h, and was approximately 1×10^9 CFU/mL. Two ten-fold dilutions using 0.1% peptone water were carried out to obtain a final concentration of 1×10^7 CFU/mL to be used for inoculation of the rehydrated infant formula milk.

Milk-based powdered infant formula (Enfamil Lipil Milk Based Infant Formula with Iron-Powder, MeadJohnson Nutrition, Glenview, IL) was bought from a local grocery store, and was rehydrated with sterile distilled water according to the manufacturer's instructions, one scoop (approximately 7.7 grams) was mixed with 60 mL of sterile distilled water under aseptic conditions and mixed with a sterile spatula (this material will be referred to throughout the thesis as RIFM). Nine mL of the RIFM were placed in a sterile test tube, and inoculated with 1 mL of the inoculums solution to give approximately 1×10^6 CFU/mL.

2.2.2 Inactivation of *E. sakazakii* using Lysozyme

A 1000 mg/L solution of lysozyme was prepared by placing 0.1 g of lysozyme (MP Biomedicas, LLC, Solon, OH, USA) into 100 mL of a 10 mM potassium phosphate buffer pH 8. Different volumes of the lysozyme stock solution were added to the inoculated infant formula milk to obtain concentrations of 100, 300 and 400 mg/L of lysozyme. RIFM inoculated with *E. sakazakii* but without treatment was used as a control, every treatment was carried out in triplicate.

2.2.3. Inactivation of *E. sakazakii* using Denatured Lysozyme

The potassium phosphate buffer solution (pH = 8) was heated to 80 °C and 100 mg of lysozyme were added to 100 mL of buffer to obtain a 1 mg lysozyme/1 mL 10 mM buffer solution, the mixture was heated for 20 minutes to achieve thermal denaturation of the lysozyme (Ibrahim and others, 1996). Different volumes of the denatured lysozyme solution were added to

the inoculated RIFM to obtain concentrations of 100, 300 and 400 mg/L of denatured lysozyme. RIFM inoculated with *E. sakazakii* but without treatment was used as a control, every treatment was carried out in triplicate.

2.2.4. Inactivation of *E. sakazakii* using Lactic Acid

Lactic acid was added to inoculated RIFM in concentrations of 0.1, 0.2 and 0.3% v/v. Additions were done from a 10% v/v solution of lactic acid (DL-Lactic Acid 85%, Acros Organics, NY, USA). RIFM inoculated with *E. sakazakii* but without treatment was used as a control, every treatment was carried out in triplicate.

2.2.5 Inactivation of *E. sakazakii* using lactic acid and nisin

A stock solution of 10 000 IU/mL of nisin (Sigma-AldrichTM, Inc., St. Louis,MO,USA) was prepared by dissolving 500 mg of nisin in 50 mL of 0.02M HCl. Different volumes of the stock solution were added to inoculated RIFM to obtain concentrations of 1000, 2000, 3000 , 4000 and 5000 IU nisin/mL RIFM. RIFM inoculated with *E. sakazakii* but without treatment was used as a control, every treatment was carried out in triplicate.

2.2.6 Inactivation using Copper and Lactic Acid

Copper sulfate penta-hydrate (JT Baker Chemical Co., Phillipsburg, N.J., USA) and lactic acid (DL-Lactic Acid 85%, Acros Organics, NY, USA) were added separately and in different amounts to RIFM inoculated with *E. sakazakii*. Concentrations of 0.5, 1, 10, 25, 40, 50, 100 and 200 ppm of elemental copper were used, and concentrations of 0.1, 0.2 and 0.3% v/v of lactic acid were used. Additions were done from an autoclaved 10 000 mg/L solution of copper, prepared from copper sulfate penta-hydrate, and a 10% v/v prepared solution of lactic

acid. RIFM inoculated with *E. sakazakii* but without treatment, was used as a control, every treatment was carried out in triplicate.

A combined treatment was performed which consisted in the addition of both copper (10, 25, 40 and 50 ppm) and lactic acid (0.2% v/v) to 10 mL of inoculated re-hydrated infant milk formula. RIMF inoculated with *E. sakazakii* but without treatment, was used as a control, all treatments were carried out in triplicate.

2.2.7 Inoculation of Powdered Infant Formula Milk (PIFM)

Milk-based infant formula was bought from a local grocery store. *E. sakazakii* cells were desiccation stressed by inoculating 24-h old cultures of the cocktail of the five strains of *E. sakazakii* (ATCC 29004, 51329, 12868, 29544, and FSM 287) onto the surface of the PIFM. PIFM was placed into 1000 ml sterile beaker, and the inoculum (100 µl) was sprinkled onto the dry formula in a drop-wise manner. After inoculation, the PIFM with the inoculum was mixed vigorously with a sterile spatula for 3 min, the initial level of *E. sakazakii* was determined by the overlay method (spread plating on TSA (2 h incubation at 37 °C) followed by applying a layer of VRBA and incubation at 37 °C for additional 22 h) (Al-Holy and others, 2008). The initial level of *E. sakazakii* was ca 1×10^6 CFU/g. Five grams of infant formula powder inoculated with *E. sakazaki* (ca 1×10^6 CFU/g) were mixed with 45 grams of un-inoculated infant powder formula to obtain a concentration of ca 1×10^5 CFU/g, then 1 gram of this mixture is mixed with 99 g of un-inoculated infant powder formula to obtain a population of 1000 CFU/g. A sterile spatula was used for the process, as well as sterile beakers, and the entire procedure was performed under aseptic conditions.

2.2.8 Inactivation of *E. sakazakii* in PIFM using Copper and Lactic Acid

The same treatments used for the RIFM were used for the PIFM. An aliquot of 2,400 µL of the 10% v/v solution of lactic acid were added to 15.5 g of PIFM, the powder was re-hydrated with 120 mL of sterile distilled water (according to manufacturer's instructions) and mixed with a sterile spatula for a final lactic acid concentration of 0.2%v/v. For the copper treatment, 0.02 grams of copper sulfate penta-hydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) were added to 15.5 grams of PIFM powder, re-hydration was carried out the same way as for lactic acid treatment to obtain a final copper concentration of 50 ppm. A final combined treatment was performed, adding both lactic acid and copper sulfate penta-hydrate in the same quantities as specified earlier to 15.5 grams of PIFM powder, and re-hydration was performed in the same way as described for both lactic acid and copper treatments. For control, PIMF inoculated with *E. sakazakii* but without treatment was used, all treatments were carried out in triplicate.

2.2.9 Enumeration of *E. sakazakii*

Ten-fold serial dilutions of the inoculated infant milk formulas with and without treatment were done in 0.1% peptone water, at intervals of 0h, 2h, 4h and 6h. The enumeration of *E. sakazakii* was performed using a minor modification of the overlay method developed by Al-Holy and others (2008). A volume of 0.1 mL of the samples was spread plated on TSA, instead of TSA supplemented with 0.1% (w/v) of sodium pyruvate. The plates were incubated for 2 hours at 37 °C, then a thin layer of violet red bile agar (VRBA) (Difco, Becton Dickinson, Spark, MD) was overlaid onto TSA, the plates were incubated for an additional 22 hours at 37 °C. (Al-Holy and others, 2008).

2.2.9.1 Confirmation of Enumeration Results Using Enrichment Broth

Copper and lactic acid were added to 15.5 g of milk powder containing 10^3 CFU/mL of *E. sakazakii* to obtain concentrations of 50 ppm of copper and 0.2% v/v of lactic acid upon rehydration. The mixture was re-hydrated with 120 mL of sterile de-ionized water (according to manufacturer's instructions) and mixed well, 1 mL of this formula was added to 9 mL of freshly prepared E.E. broth (Difco, Becton Dickinson, Spark, MD, USA) and incubated for 24 h at 37 °C.

After 24 hours two ten-fold dilutions of the E.E. broth were made into 0.1% peptone water to obtain 10^{-2} and 10^{-4} dilutions, 0.1 mL of these dilutions were streaked on OK medium and incubated for 24 hours. The overlay method was also used to test the results of the enrichment.

Copper sulfate penta-hydrate and lactic acid were added in combination to RIMF inoculated with *E. sakazakii* to obtain concentrations of 50 ppm and 0.2% v/v respectively, and the same confirmation procedure used for the PIFM was performed.

For control, PIMF and RIFM inoculated with *E. sakazakii* but without treatment was used, all treatments were carried out in triplicate.

2.3 Effectiveness of Different Sanitizers on *E. sakazakii* Biofilm Formation

2.3.1. Bacterial Strains

Five strains of *E. sakazakii* were used for this study. The cultures were ATCC 29004, ATCC 51329, ATCC 12868, ATCC 29544, and FSM 287. The strains were obtained from the culture collection in the School of Food Science, at Washington State University. The five strains were transferred individually from stock aqueous glycerol (15%) solutions stored at -20 °C to

slants of tryptic soy agar (TSA) (Difco, Becton Dickinson, Spark, MD, USA) and kept refrigerated at 4 °C.

Prior to the experiment, the five strains were individually transferred to sterilized test tubes containing 10 mL of Brain Heart Infusion (BHI) broth (Difco, Becton Dickinson, Spark, MD, USA), and incubated at 37 °C for 24 hours. After incubation, the five strains were transferred to a sterilized centrifuge tube, creating a bacterial cocktail. The tubes were then centrifuged at room temperature for 15 min at 4000 rpm (Fisher Scientific, Fisher AccuSpin™ Model 400 Benchtop Centrifuge, Pittsburgh, PA, USA) to harvest bacterial cells. The pellet was re-suspended in 9 mL sterile 0.1% peptone water solution and centrifuged at the same conditions as described earlier. The supernatant was discarded, and the pellet re-suspended in 9 mL of a sterile 0.1% peptone water solution, to give an approximate concentration of 1×10^9 CFU/mL. The grown cultures were used for inoculation into the infant formula milk, 20 µL of the cultures was poured in the wells of a 96-well flat-bottomed polystyrene microplate (Falcon, Becton Dickinson, NJ, USA) for subsequent determination of effectiveness of different disinfectants on biofilm formation on plastic surfaces.

2.3.2. Biofilm Formation

Biofilm production in the plastic microtiter plates was based on the method developed by Stepanovic and others (Stepanovic and others, 2000), the wells of a sterile 96-well flat bottomed polystyrene microplate were filled with 230 µL of re-hydrated infant formula milk (rehydrated according to manufacturer's instructions). Of the grown cultures 20 µL were added into each well, except for those wells used as negative controls. The plates were incubated for 24 h at 37 °C. The content of the plates was poured off and the wells washed three times with 300 µL of

sterile distilled water. The efficacy of four different common household disinfectants on biofilm formation was tested, they were: Clorox, Sodium Hypochlorite solution, Concentrated Ultra and SunSations (chemical composition of these materials is provided in table 3), 200 μ L of each disinfectant were added in triplicate into different wells that contained the grown overnight culture, except in those wells used as controls (negative and positive). The disinfectants were added for 1, 5 and 10 min at room temperature. After treatment the content of the wells was poured off and washed three times with 300 μ L of sterile distilled water.

The remaining attached bacteria were fixed with 250 μ L of methanol per well for 15 min, and the liquid discarded and then the cells were air-dried. The microplate wells were then stained with 250 μ L of crystal violet for 5 min. Excess stain was rinsed off by placing the microplate under running tap water. After this, the microplates were air-dried, the dye bound to the adherent cells was resolubilized with 250 μ L of 33% (v/v) glacial acetic acid per well. The OD of each well was measured at 570 nm using an automated 96-well microplate reader, PowerWave X (Bio-Tek Instruments, Winooski, VT, USA).

For the efficacy tests, the cutoff OD (OD_c) was defined as three standard deviations above the mean OD of the negative control (infant formula milk with no bacterium added). The adherence capabilities with and without each treatment were classified as follows: $OD \leq OD_c =$ no bio-film was produced, $OD_c \leq OD \leq (2 \times OD_c) =$ weak bio-film formation and $(2 \times OD_c) \leq OD =$ strong bio-film formation. All tests were carried out in triplicate and the results averaged.

CHAPTER THREE

RESULTS AND DISCUSSION

3.1 Inactivation of *E. sakazakii* using Lysozyme and Denatured Lysozyme

Consumers today are more aware than ever about the safety of their foods, and demand safe products with as little artificial chemicals preservatives as possible to control foodborne pathogens. Lysozyme is a good option to fulfill these requirements, since it has antimicrobial properties and is a natural protein.

Lysozymes are a family of enzymes that can damage bacterial cell walls by the catalysis of hydrolysis of the 1,4- β -linkage between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan, and between N-acetyl-D-glucosamine residues in chitodextrins. The protein can be found in secretions such as tears, saliva and mucus; and is also present in egg whites (Ibrahim, 1996).

Both lysozyme and denatured lysozyme were investigated in this study as possible antimicrobials, and the results can be seen in figure 2. The graph shows that concentrations of 300 and 400 ppm of lysozyme had a higher effect on the growth of *E. sakazakii* than 100 ppm concentration; the latter produced an initial 0.1 log reduction in the *E. sakazakii* count, but a strong re-growth was observed after 4 and 6 hours. A higher reduction is observed after 2 hours for both the 300 and 400 ppm (0.4 and 0.46 log respectively) concentrations of *E. sakazakii*, but just the same as in the case of the 100 ppm concentration, re-growth was observed, and after 6 hours the counts almost reach their initial levels.

Denatured lysozyme at 100 ppm had no effect on the growth of the bacteria in the infant formula milk. A 100 ppm concentration produced no reduction on the initial count, while the 300

and 400 ppm concentrations produced initial count reductions of 0.33 and 0.52 log respectively, but as was observed for the lysozyme treatment, re-growth was observed after 4 hours, and the counts almost reached their initial levels after 6 hours.

The results clearly indicate that neither lysozyme nor denatured lysozyme, are good antimicrobial agents against *E. sakazakii* in infant formula milk. A study by Ibrahim and others (1994) had suggested that the antimicrobial action of lysozyme could include Gram-negative bacteria by altering its surface hydrophobicity through genetic and chemical modifications (Ibrahim and others, 1994). To test their hypothesis, the researchers studied the antimicrobial action against Gram-negative and Gram-positive bacteria of irreversibly heat-denatured lysozyme at different pH.

Amongst their many findings, the study revealed that denaturation of lysozyme at increasing temperatures for 20 min at pH 6.0 resulted in progressive loss of enzyme activity, while greatly promoting its antimicrobial actions towards Gram-negative bacteria. Lysozyme when heated at 80 °C and pH 7.0 exhibited strong bactericidal activity against Gram-negative and Gram-positive bacteria, which suggested that its antimicrobial activity is independent of catalytic function. When heated at 80 °C and pH 6.0, lysozyme exhibited the strongest antimicrobial activity towards both Gram-positive and Gram-negative bacteria, retaining 50% of its native enzymatic activity, which exhibited a 14 fold increase in surface hydrophobicity, with two exposed thiol groups.

The results from this study strongly indicated that partial unfolding of lysozyme with the proper acquisition of the hydrophobic pocket to the surface could alter its antimicrobial activity to include Gram-negative bacteria without a detrimental effect on the inherent bactericidal effect against Gram-positive ones (Ibrahim and others, 1996).

Based on the results obtained from the lysozyme studies, it was determined that it could prove to be a good candidate as a natural antimicrobial for the control of *E. sakazakii* in infant formula milk, however it was not the case. One major difference between this research and the one performed by Ibrahim and others (1996), is that they tested the lysozyme and denatured lysozyme antimicrobial activity against Gram-negative bacteria in a peptone broth, and this research used a food matrix (reconstituted infant formula), which has a much different chemical composition, for example a high concentration of simple sugars, fat, and a higher protein concentration.

The study on the peptone broth concluded that the enhanced antimicrobial activity of denatured lysozyme was due to an increased hydrophobicity of the lysozyme, which allowed for a stronger and more disruptive interaction with the bacterial cell wall, and an eventual perturbation of its function. It is very possible that some of the components found in the formula milk (i.e. proteins, fat, carbohydrates etc.) could have provided some protection or interfered with the ability of denatured lysozyme to disturb the cell membrane, resulting in the negligible antimicrobial activity observed.

3.2. Inactivation of *E. sakazakii* using Lactic Acid

Lactic acid is a natural antimicrobial widely used for food treatment, it lacks acute and chronic toxicity and hence has a generally recognized as safe (GRAS) status at high levels of use. It is able to inhibit the growth of many types of food spoilage bacteria, including Gram-negative species of the families *Enterobacteriaceae* and *Pseudomonadaceae*. The antimicrobial activity is largely assigned to its ability in the un-dissociated form to penetrate the cytoplasmic

membrane, which results in a reduced intracellular pH and disruption of the transmembrane proton motive force (Alakomi and others, 2000).

Lactic acid seemed like a good candidate for the control of *E. sakazakii*, and no research was found on infant formula milk concerning the use of lactic acid for the control of *E. sakazakii*. Figure 3 shows the survival curves for the pathogen when lactic acid was used in three different concentrations.

The results show that lactic acid at a concentration of 0.1% v/v apparently does not have an antibacterial effect on the growth of the microorganism, and the number of *E. sakazakii* was not significantly different ($P \geq 0.05$) from the control after 4 and 6 hours of storage at room temperature. Lactic acid at 0.2% v/v imparted a bacteriostatic effect on the growth of *E. sakazakii*, only a subtle increase was noticed after 6 hours of storage at room temperature, which was significantly ($P \leq 0.05$) lower compared to the control. In contrast, lactic acid at 0.3% v/v elicited the most pronounced bactericidal effect against *E. sakazakii* compared to the other concentrations (0.1 and 0.2% v/v). A noticeable gradual reduction in the numbers of the organism was noticed during the whole storage period. In this treatment, the numbers of *E. sakazakii* were significantly ($P \leq 0.05$) lower compared to the other lactic acid concentrations and the control for every single time interval studied.

Besides its antimicrobial activity, lactic acid is a strong outer membrane disintegrating agent. According to a study by Alakomi and others (2000), it permeabilizes the outer membrane of gram negative bacteria, a property that could help other antimicrobials penetrate bacterial cells, and produce a toxic effect. The permeabilizing properties of lactic acid make it both an antimicrobial, and a possible potentiator for other antimicrobial substances (Alakomi and others, 2000).

3.3 Inactivation of *E. sakazakii* using Nisin and Lactic Acid

Nisin was tested in combination with lactic acid for the control of *E. sakazakii* in reconstituted formula milk, the combined effect of the two antimicrobials was observed for any hint of a synergistic effect. The level of lactic acid used was 0.2 % L.A., since it had showed a bacteriostatic effect in the previous experiments, and nisin was used at three different concentrations (1000, 3000 and 5000 IU/mL) to try and find the most useful combination. The results of this trial can be seen in figure 4.

From figure 4 it is quite obvious that the combination of antimicrobials exhibited no synergistic effect. The use of lactic acid alone shows the bacteriostatic effect previously observed when the antimicrobial was tested at different levels for the control of the pathogen, but the combination with nisin did not improve this effect, in fact a higher growth rate can be observed. The lactic acid treatment produced less than a 1 log increase in the count of *E. sakazakii* after a 4 hour period of storage at room temperature, while the control and combinations of nisin with lactic acid showed a count increase greater than 1 log.

As the concentration of nisin is increased in combination with lactic acid, a lower growth rate is also observed, meaning that a 5000 IU concentration of nisin reduces growth more than the 1000 IU concentration of nisin, however; the differences are not significant ($P \geq 0.05$) between themselves or with the control for the entire storage period.

Nisin is a bacteriocin produced by *Lactococcus lactis* spp. *lactis* which is primarily active against Gram-positive bacteria, it is a compound which could be used for the preservation of foods, especially since it is regarded as “natural” by consumers. Its action against Gram-negative bacteria is limited, since the bacterial cell walls are protected by the outer membrane. But it has

been hypothesized that the potency of nisin could be increased if combined with other antimicrobials (Gill and Holley, 2003), which provided the justification for this study.

Branen and Davidson (2004) conducted research to evaluate the enhancement of nisin in the presence of EDTA against strains of *L. monocytogenes*, *E. coli*, *S. enteritidis* and *P. fluorescens*. EDTA is known to release LPS from the outer membrane of Gram-negative bacteria by chelating Ca and Mg salts necessary for LPS to bind to the cell wall, this would eliminate the outer membrane shield protecting the Gram-negative bacteria's cytoplasmic membrane, allowing nisin to form pores and cause depletion of the proton motive force, collapse of the pH gradient, reduction of ATP, as well as other disruptions. This would explain any synergy found by the use of EDTA and nisin.

The results of the study showed that low levels of EDTA acted synergistically with nisin in tryptic soy broth (TSB) against two enterohemorrhagic *E. coli* strains and *L. monocytogenes*, but were ineffective in 2% fat UHT milk stored at mild thermal abuse temperatures. It is possible that the EDTA activity was neutralized by calcium and other cations present in milk (Branen and Davidson, 2004).

Another study tested the use of nisin with EDTA against meat spoilage pathogenic bacteria, and just like in the research conducted by Branen and Davidson (2004) it was found that no enhanced antimicrobial effect against Gram-negative bacteria was present (Gill and Holley, 2003).

No enhanced effect was observed for the combination of nisin and lactic acid evaluated in this study with reconstituted infant formula. Many authors have reported however that an increased antimicrobial activity is observed against Gram-negative bacteria when nisin is combined with membrane disrupting agents, but the majority of these reports are based upon

experiments conducted on organism suspended in buffers or pure broth media, rather than in a rich nutrient media. The reconstituted infant formula provides a nutrient rich environment for *E. sakazakii*, and the experiment effectively evaluated the interactions between the antimicrobial agents of interest without nutrient restriction, and the failure of a synergistic effect could indicate the interaction between antimicrobials and some of the components in the food system.

3.4 Inactivation of *E. sakazakii* using copper and Lactic Acid

The antibacterial activity of copper against *E. sakazakii* in RIFM is shown in figure 5. Six different concentrations (0.5, 1, 10, 50, 100 and 200 ppm) of copper were used, a dose dependent relationship can be observed. As the concentration of copper increased, the extent of inhibition increased accordingly. Copper concentrations of 0.5, 1 and 10 exhibited a slight inhibition on the growth of *E. sakazakii* during the storage period, but the counts were not significantly different from the control ($P \geq 0.05$).

At concentrations of 50, 100 and 200 ppm a significant decrease ($P \leq 0.05$) in the number of *E. sakazakii* cells was observed after 2 hours, but a re-growth occurred in the samples with 50 and 100 ppm of copper, and the *E. sakazakii* cells reached about 5.15 and 3.50 logs respectively after 6 hours of storage. The number of cells is not significantly different ($P \geq 0.05$) between the 50 ppm treatment and the control after 4 hours, only the 100 and 200 ppm dose remain significantly different from the control throughout the entire storage period. Copper at 200 ppm exerted the most remarkable antimicrobial activity against *E. sakazakii*, where a 3.94 log reduction was achieved after the storage period (Table 4).

Copper ions, alone or in combination have been used for centuries to disinfect liquids, solids and human tissue. It is a metallic element which is essential to human health, and along

with amino and fatty acids and vitamins, it is required for normal metabolic processes. The adult body contains between 1.4 and 2.1 mg of copper per kg of body weight, estimates indicate that a human eats and drinks about 1 mg of copper every day.

Copper is considered safe to humans, which is demonstrated by the widespread and prolonged use of copper intrauterine devices, but in contrast to the low sensitivity of human tissue, microorganisms are extremely susceptible to copper. The use of copper has been recently demonstrated as a bacterial inhibitor in various stages of food processing, for example, it has been shown that metallic copper surfaces inhibited the growth of *Salmonella enterica* and *Campylobacter jejuni*, two of the most prevalent bacterial pathogens that cause foodborne diseases. In a similar way, the addition of copper to drinking glasses has been shown to reduce biofilm formation of *Streptococcus sanguis*, reducing the risk of oral infections (Borkow and Gabbay, 2005).

Metals at high concentrations are toxic to microorganism, and toxicity occurs through many mechanisms, such as displacement of essential metals from their native binding sites of enzymes through ligand interactions. The toxic effect of copper on bacteria is possibly attributed to displacement of essential ions, hence inactivating enzymes and obstructing functional groups of proteins, which may lead to the production of hydroperoxide free radicals and affecting membrane integrity (Nies, 1999). For the copper ions to have any physiological or toxic effect, they first have to enter the cell, and it is possible that at concentrations as low as 0.5, 1, 10 and even 50 ppm of copper, not enough ions are reaching the cell membrane to produce a toxic effect.

Based on the results obtained, there is obviously a potential for copper to be used as a preservative agent to inhibit the growth of pathogens in food products, but very little information

is currently available on the effect of copper on the growth inhibition of food-borne pathogens in food products. A study using pigs suggested that the combination of lactic acid and copper may be an effective combination against undesired microbes (Ibrahim and others, 2008).

Beal and others (2004) conducted a study in which the addition of lactic acid (150 mM) and copper sulfate (50 ppm) to liquid pig feed caused a 10-fold decrease in the D_{value} of *Salmonella typhimurium* DT104:30 (Beal and others, 2004). The same combination was used in a study by Ibrahim and others (2008), but this time they tested copper and lactic acid, alone and in combination, to test the survival and growth of *Salmonella* spp. and *E. coli* O157:H7 in laboratory medium and carrot juice. The study found that the growth of bacterial strains was significantly inhibited when both lactic acid and copper were tested in BHI broth and carrot juice within the time frame of the study (Ibrahim and others, 2008).

The results obtained for the combination of lactic acid and copper from the previous studies are of particular importance, since this antimicrobial combinations were used against gram-negative bacteria similar to *E. sakazakii*, which suggests that they could be a good candidate for an inhibition study of the microorganism. Of special importance is the use of carrot juice in the study by Ibrahim and others (2008), since they were using a food matrix containing many components at a near neutral pH, and not a pure laboratory medium.

The studies performed using lactic acid and copper alone on the growth of *E. sakazakii* could be considered preliminary studies to determine the lowest concentration of copper and lactic acid alone that caused slight growth inhibition on the bacteria. Lactic acid was already shown to have a bacteriostatic effect on the growth of *E. sakazakii*, and at levels of 50 ppm of copper a significant reduction ($P \leq 0.05$) was observed after 2 hours of storage. Therefore,

combinations of 0.2% lactic acid and four concentrations of copper (10, 25, 40 and 50 ppm) were used in combination to test for the presence of a synergistic effect between both antimicrobials.

The effects of lactic acid in combination with copper on the growth of *E. sakazakii* in a RIFM sample can be seen in figure 6. In the control samples, the number of *E. sakazakii* increased from 5.19 to 7.71 log CFU/mL after a 6 hour incubation period at room temperature. The addition of 0.2% lactic acid and 10 ppm of copper did not produce a significant growth inhibition ($P \geq 0.05$). The treatment achieved an initial decrease of less than 1 log after 2 hours, and the bacterial population further decreased to 4 log CFU/mL after 6 hours of storage at room temperature. The addition of 0.2% lactic acid and 25 ppm of copper produced a significant inhibition of growth ($P \leq 0.05$), the *E. sakazakii* counts decreased to 3.71 log CFU/mL after 6 hours of storage; a reduction of 4 logs when compared to the counts for the control after 6 hours of storage. The combinations of 0.2% lactic acid with 40 and 50 ppm copper respectively produced the most pronounced antimicrobial activity against *E. sakazakii*, a 2.08 log reduction was achieved after 2 hours when 40 ppm of copper were used, and a 4.42 log reduction was observed after 2 hours when 50 ppm of copper were added. Both treatments produced complete inhibition of the pathogen after 4 hours, and no growth was observed at all after the 6 hour storage period.

As shown in figure 6, after the application of a combined treatment of copper and lactic acid, a synergistic effect was obtained, where a complete elimination of the organism was obtained after the end of the storage period when 40 and 50 ppm of copper were used in combination with 0.2% lactic acid. The validity of the combined treatment against *E. sakazakii* was further explored in PIFM, samples of PIFM were inoculated with *E. sakazakii* and kept

under dry conditions at room temperature to simulate the dry stress conditions that the microorganism may encounter under normal storage conditions.

The PIFM was treated with individual doses of lactic acid (0.2 % v/v) and copper (50 ppm), as well as a combined treatment of both antimicrobials. Figure 7 shows the results of the individual treatment, where the number of *E. sakazakii* increased from 1.68 to 3.25 log CFU/mL after 6 hours at room temperature. The cells increased by about 1 log CFU/mL for both the lactic acid and copper treatments after 6 hours of storage.

As figure 8 shows, the combined treatment achieved complete elimination of the organism after 2 hours, and until the end of the storage period. The numbers in the untreated samples (control) increased by about 1 log after 6 hours of storage at room temperature.

Results for both RIFM and PIFM show a synergistic effect when the two antimicrobials are combined; as stated earlier when 50 ppm of copper are added it is possible that not enough ions are entering the cell in order to produce a toxic effect. The study by Alakomi and others (2000) suggested that lactic acid in addition to its antimicrobial properties, also functions as a permeabilizer of the gram negative bacterial outer membrane, and thus could enable other compounds to penetrate it, and increase the cells susceptibility (Alakomi and others, 2000).

The results seem to indicate that the permeabilizer properties of lactic acid could have facilitated the entry of copper ions into the *E. sakazakii* cells and produce the toxic effect, as well as producing a toxic effect itself. The combination of inhibitory activity and disruption of the outer membrane of *E. sakazakii* by lactic acid may have lead to potentiation of the antimicrobial activity of copper and consequently resulted in the inhibition of *E. sakazakii* growth.

3.5 Biofilm formation and disinfectant efficacy

The results of biofilm formation using the crystal violet biofilm staining method developed by Stepanovic and others (2000) showed that the *E. sakazakii* cocktail used produced a weak biofilm on the plastic surface. The mean OD₅₇₀ for the positive control (infant formula milk with no bacterium or treatment added) was higher than the cutoff OD (OD_c) but less than 2 x OD_c, which classifies the cells as weak biofilm producers.

Three common household dishwashing disinfectants and a solution of sodium hypochlorite were used to test their efficacy in the removal of the biofilms produced on plastic surfaces. It is important to assess this risk, since the production of biofilms protects the cells from being affected by disinfectants, making it harder to wash such bacteria off from the surface they have attached to, increasing the chance of cross-contamination. This is of particular importance for the sanitation of bottles used to feed newborns, since a biofilm presence could lead to many infections and ultimately death.

The results for the disinfectant tests are shown in table 5. The sodium hypochlorite solution was the least effective in removing the biofilm produced by the bacterial cocktail, none of the treatment times were significantly different from the control ($P \geq 0.05$), although a reduction in OD₅₇₀ was observed as the treatment time increased. Treatment with Clorox presented significant difference from the control ($P \leq 0.05$) after 10 minutes of treatment; the OD₅₇₀ was reduced from 0.176 to 0.131 which is below the OD_c, meaning that no bio-film was present. The treatments after 1 minute and 5 minutes with Clorox were not significantly different from the control ($P \geq 0.05$).

The treatment with Concentrated Ultra dishwashing liquid was very similar to the one with Clorox, a significant reduction ($P \leq 0.05$) was observed after 10 minutes of treatment where

the OD₅₇₀ reached 0.122, a value lower than the OD_c. The result shows that biofilm is still present after 1 and 5 minutes of treatment with this disinfectant. The Sun Sations Dishwashing liquid was the most effective against the bacterial biofilms, the OD₅₇₀ reached levels below the OD_c after 5 and 10 minutes of treatment, meaning that it worked the fastest, these values were significantly different ($P \leq 0.05$) from the control.

The disinfectants evaluated in the study showed different levels of efficacy against biofilms of *E. sakazakii* on plastic surfaces, depending on their composition. The results show that the longer the treatment time the more effective the sanitizing step will be, which is very important for the proper cleaning of plastic bottles used to prepare infant formula, otherwise the biofilms could lead to cross-contamination and infection of newborns.

CONCLUSIONS

Combined lactic acid and copper showed a significant inhibitory activity against the growth of *E. sakazakii* in RIF. A combination of sub-lethal concentrations of lactic acid (0.2% v/v) and copper (5 ppm) resulted in a synergistic inhibitory activity against *E. sakazakii* in RIF stored at room temperature; where complete elimination was achieved after 4 hours on RIFM and 2 hours in PIFM. The use of the synergistic interactive combination of lactic acid and copper could be beneficial to control *E. sakazakii* in the infant formula industry, and maybe other combinations of different antimicrobials could result as useful as the ones used in this study.

After the evaluation of different common household disinfectants against *E. sakazakii* biofilms on plastic surfaces, it can be concluded that quaternary ammonium compounds are the most effective. In addition, treatment time plays an important role in the elimination of the biofilms, since higher reductions in population were observed as the treatment times increased. The results provide information useful in reducing *E. sakazakii* embedded on surfaces used for infant formula preparation.

FUTURE RESEARCH

E. sakazakii has caused much concern in the food industry, as well as in regulatory and academic communities. As matter of fact, there has been more research done in the last 2 to 3 years than in the past 25 years, for example; there have been at least 85 publications about the organism in the last 2 years alone (Farber, 2008).

In response to concerns and the potential threat posed by *E. sakazakii* to vulnerable groups (i.e. neonates) by contaminated infant formula milk, the Codex Committee on Food Hygiene (CCFH) organized a working panel to review current knowledge in this area. At the 36th session of CCFH in February 2004, a decision was taken to revise the International Code of Hygienic Practice for Foods for Infants and Children, to include an extensive risk assessment model for *E. sakazakii*. For the completion of this important objective, knowledge on certain key areas regarding this pathogen must be addressed (Mullane and others, 2006).

The key areas prioritized for future research are: the discovery of the sources of *E. sakazakii* in the manufacturing setting, incorporating all environmental and ingredient routes of transmission, effectiveness of different physical and chemical treatments in the management of *E. sakazakii*, and information on possible virulence factors of *E. sakazakii*.

Future research must provide improved detection methods, and standardized subtyping approaches must be implemented to facilitate the identification of reservoirs, and contribute to the elimination of this pathogen from the final food product (Mullane and others, 2006).

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Table 1. Summary of *Enterobacteriaceae* cultured from different infant formulations

Organism	Frequency	Conc. (CFU/100g)
<i>Pantoea</i> ssp. 1 to 4, <i>Escherichia vulneris</i> (<i>Enterobacter agglomerans</i>)	35	0.3 – 14.94
<i>Enterobacter cloacae</i>	30	0.36 – 91.78
<i>Enterobacter sakazakii</i>	20	0.36 – 66
<i>Klebsiella pneumonia</i>	13	0.36 – 46.22
<i>Citrobacter freundii</i>	5	~0.36
<i>Escherichia coli</i>	4	~4.6
<i>Klebsiella oxytoca</i>	4	0.36 – 0.92
<i>Citrobacter koseri</i>	3	0.19 – 0.36
<i>Hafnia alvei</i>	1	0.15

Table 2. Surveys conducted on powdered infant formula for *E. sakazakii* contamination

Reference	Amount Sampled (g)	Sample No.	No. Positive for <i>E. sakazakii</i>
Muytjens et al. (1988)	333 -335	141	20
Nazarowec-White and Farber (1997)	333	120	8
Heuvelink et al. (2002,2003)	25	101	2
Kandhai et al. (2004)	10	68	14
Kress et al. (2005)	333,330	74,20	10,2
Iversen et al. (2004)	25	102	3

Table 3. Disinfectants evaluated for efficacy against *E. sakazakii* biofilms

Disinfectant	Type of Active Ingredient	Active Ingredient listed on label	Conc. Of active ingredient
Clorox Disinfecting Kitchen Cleaner	Quaternary ammonium compounds	Dimethyl benzyl ammonium chloride	0.30%
Concentrated Ultra Dish liquid	Antimicrobial	Triclosan	0.10%
Sun Sations Diswashing Liquid	Quaternary ammonium compounds	Not Specified	0.50%
Sodium Chlorite Solution	Sodium Hypochlorite	Sodium Hypochlorite	100 ppm

Table 4. Antimicrobial activity of copper on the growth of *E. sakazakii* in RIFM as measured by the log CFU/mL. Values with the same superscript within a column are not significantly different from the control ($P \leq 0.05$).

Copper Concentration (ppm)	Time			
	0h	2h	4h	6h
0	6.36 ^a	7.11 ^a	7.08 ^a	7.63 ^a
0.5	6.36 ^a	6.29 ^a	6.24 ^a	6.51 ^a
1	6.36 ^a	6.11 ^a	6.12 ^a	6.18 ^a
10	6.36 ^a	5.98 ^a	6.35 ^a	6.60 ^a
50	6.36 ^a	4.69 ^b	5.17 ^a	5.61 ^a
100	6.36 ^a	2.79 ^b	3.3 ^b	3.49 ^b
200	6.36 ^a	3.58 ^b	2.82 ^b	2.41 ^b

Table 5. Mean OD₅₇₀ of stained biofilms, three replicates. Bold values are significantly lower from control ($P \leq 0.05$). Values with the same superscript across a row are not significantly different ($P \geq 0.05$).

Sanitizer	Treatment Time		
	1 min	5 min	10 min
NaOCl	0.223 ^a	0.206 ^a	0.171 ^a
Clorox Disinfecting Kitchen Cleaner	0.176 ^a	0.171 ^a	0.131^b
Concentrated Ultra Dish liquid	0.218 ^a	0.149 ^b	0.122^b
Sun Sations Dishwashing liquid	0.141^a	0.117^b	0.111^b

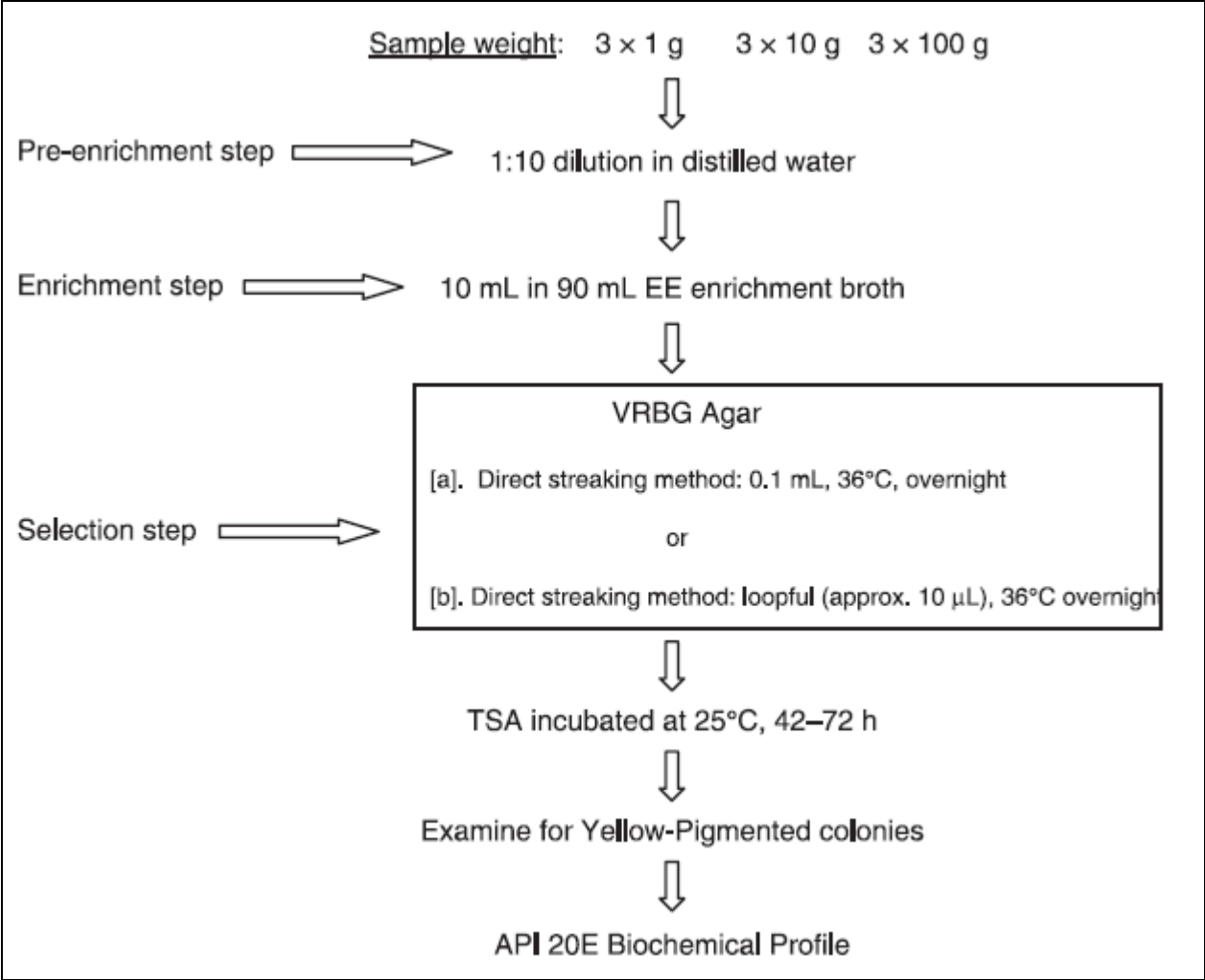
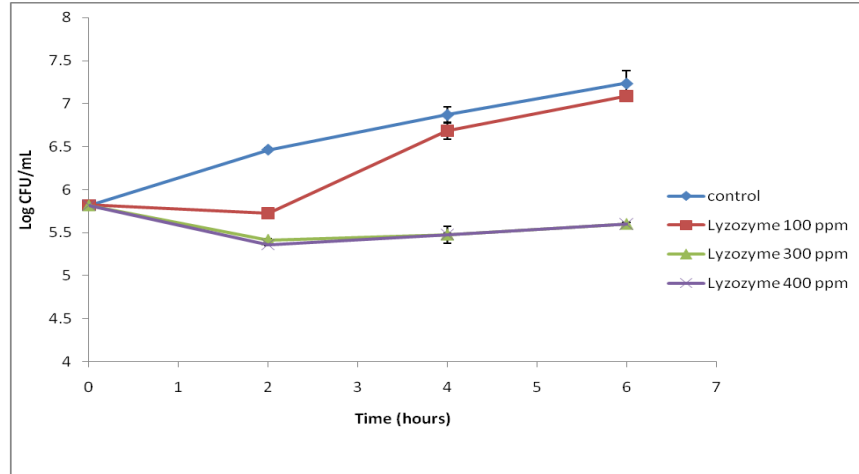


Figure 1. FDA standard protocol for isolation of *Enterobacter sakazakii* from powdered infant formula

A.



B.

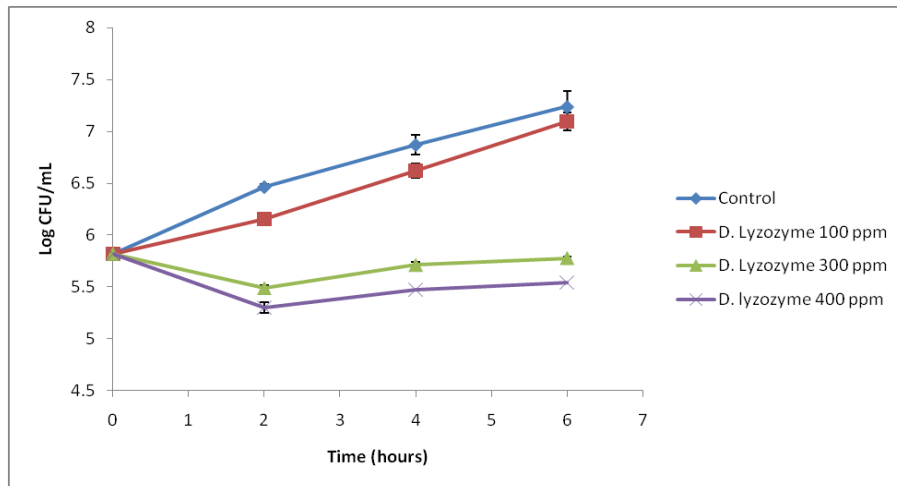


Figure 2. Effect of different concentrations of lysozyme (A) and denatured lysozyme (B) on the growth of *Enterobacter sakazakii* (Log CFU/mL) in reconstituted infant formula milk stored at room temperature.

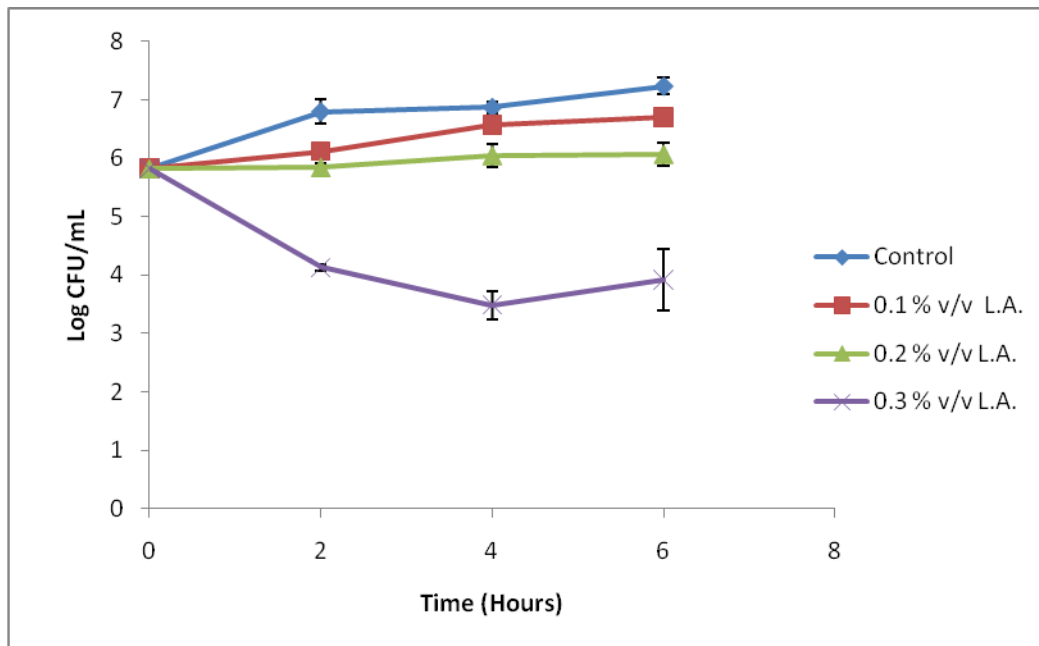


Figure 3. Survival curve of *E. sakazakii* in reconstituted infant formula milk under three different concentrations of lactic acid.

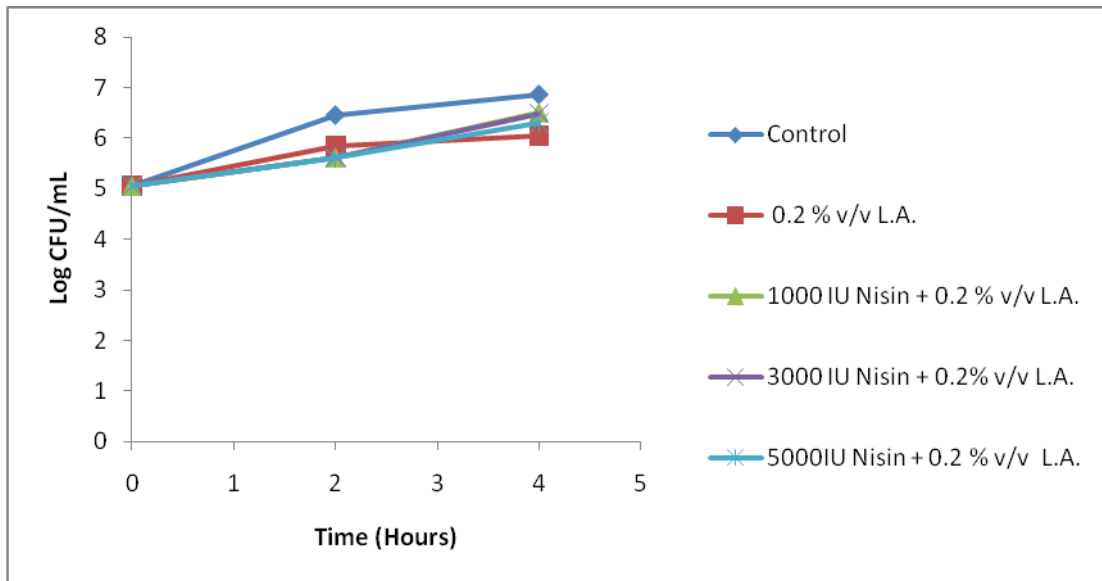


Figure 4. Effect of different concentrations of nisin against *E. sakazakii* in reconstituted infant formula stored at room temperature.

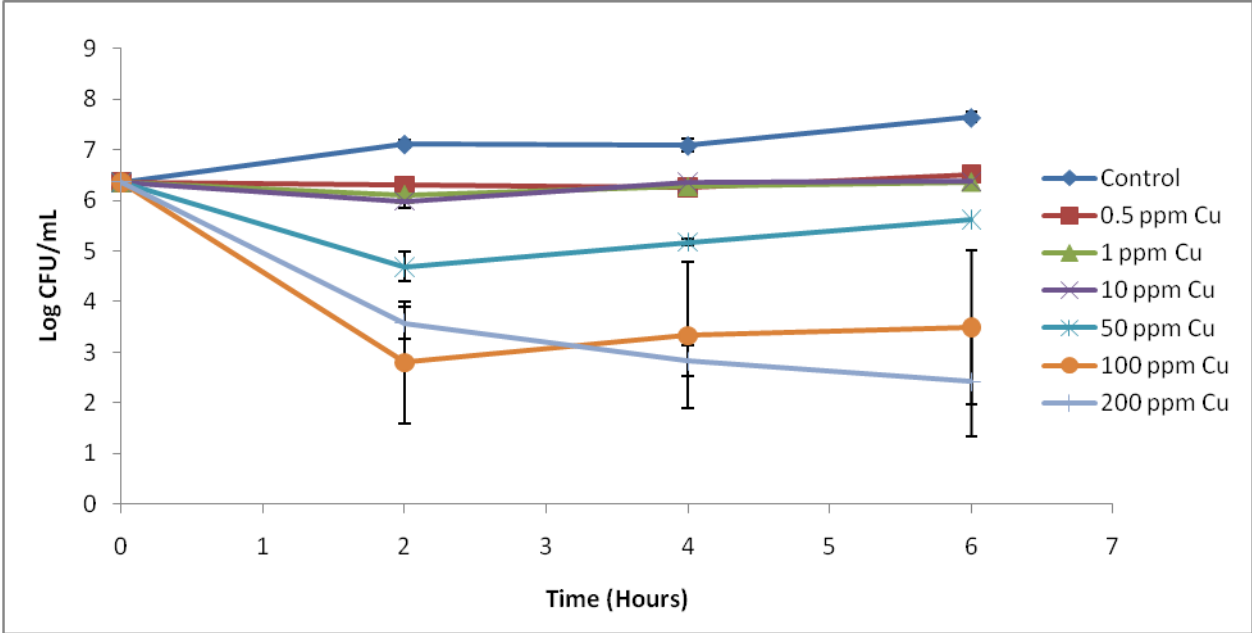


Figure 5. Effect of copper at different concentrations on the growth of *E. sakazakii* in RIFM stored at room temperature.

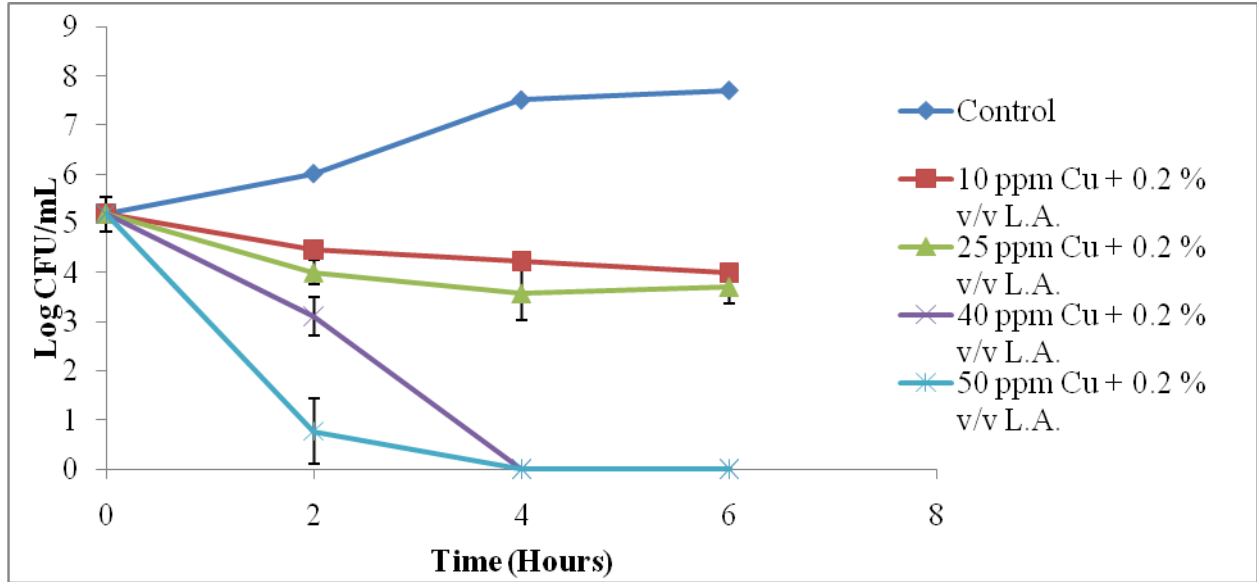


Figure 6. The combined effect of lactic acid and copper on the growth of *Enterobacter sakazakii* in reconstituted infant formula stored at room temperature.

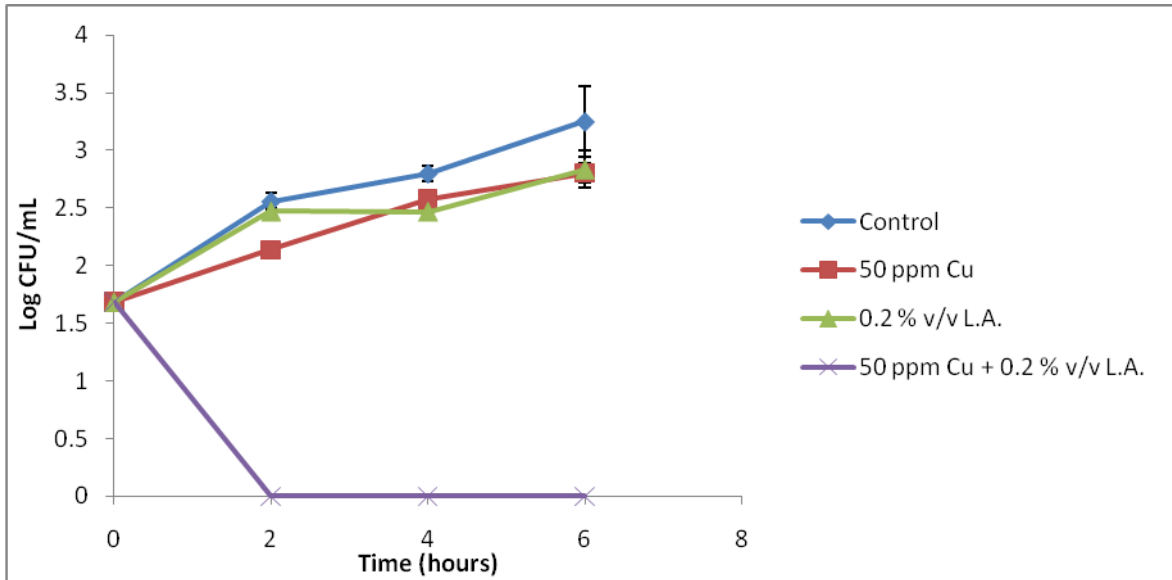


Figure 7. The effect of copper and lactic acid applied individually and in combination on the growth of *Enterobacter sakazakii* in powdered infant formula milk stored at room temperature.

