FOUL PLAY: THE INFLUENCE OF FOULANT ON THE ATTACHMENT AND THERMAL TOLERANCE OF *CRONOBACTER SAKAZAKII*

by

JAKE RILEY MCGWIN

(Under the Direction of Francisco Diez-Gonzalez)

ABSTRACT

Fouling in heat exchangers presents a significant challenge in the powdered infant formula industry, promoting microbial attachment and hindering heating efficiency. This study evaluated the attachment and thermal resistance of three *Cronobacter sakazakii* strains on stainless steel surfaces fouled with whey (WBF) and soy (SBF) based infant formulas. When bacterial attachment was assessed, attachment on medium foulant (5.63 log CFU/coupon) and high foulant (5.63 log CFU/coupon) was significantly higher than the clean coupon of 5.20 log CFU/coupon Thermal resistance testing revealed that foulant layers could have impaired heat transfer, leading to greater bacterial survival. Clean coupons achieved a significantly greater reduction ($p<0.001$) in all isolates of *C. sakazakii.* D-value increased notably on fouled coupons averaging 4.70 min for WBF and 9.20 min for SBF, compared to 2.94 minutes on control coupons. Results indicate a need for stringent cleaning and sanitation protocols to prevent *C. sakazakii* harborage in processing environments.

INDEX WORDS: *C. sakazakii,* powdered infant formula, attachment, thermal inactivation

# FOUL PLAY: THE INFLUENCE OF FOULANT ON THE ATTACHMENT AND THERMAL TOLERANCE OF *CRONOBACTER SAKAZAKII*

by

JAKE RILEY MCGWIN

BS., Auburn University, 2023

A Thesis Submitted to the Graduate Faculty of the University of Georgia in Partial Fulfillment of the Requirements of the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2025

FOUL PLAY: THE INFLUENCE OF FOULANT ON THE ATTACHMENT AND THERMAL TOLERANCE OF *CRONOBACTER SAKAZAKII*

by

JAKE RILEY MCGWIN

Major professor: Francisco Diez-Gonzalez

Committee: James Gratzek
Abhinav Mishra

Electronic Version Approved:

Ron Walcott
Dean of the Graduate School
The University of Georgia
May 2025

## DEDICATION

To everyone who believed in me – especially my family and Perian – even in moments when I doubted myself

## ACKNOWLEDGEMENTS

First, I want to express my sincere thanks and gratitude to my advisor Dr. Govindaraj Dev Kumar for allowing me to do my graduate studies under his mentorship and providing me with the opportunity to grow as both a person and researcher. None of this could have been done without his support and patience throughout my studies. I also want to thank Dr. Francisco Diez-Gonzalez for stepping in as my advisor towards the end of my program and guiding me in the last months of my Masters degree.

I would also like to thank Dr. Jim Gratzek for his endless support and advice throughout my degree program. His invaluable insights on this and many other projects that I had the opportunity to work on have been instrumental in my growth as a scientist and a person. He allowed me to get out of my comfort zone and exposed me to a wide variety of people and places and made my graduate experience truly diverse and unique. I would further like to thank Dr. Abhinav Mishra for serving on my committee and providing valuable guidance on this project and support throughout my program.

Next, I want to thank all my friends, especially those in the Food Science Department, who made me feel at home and welcome wherever I was and were always there for me. Especially to my lab mates who were always willing to help. Also, a huge thanks to the staff of FoodPIC including Michael Aaron, Alison Payton, and Anna Bragg who always helped we with all the projects I worked on.

Lastly, to my family whose unwavering love and support made this all possible. I couldn't have done it without them.

Table of Contents

## LIST OF TABLES

## LIST OF FIGURES

# Chapter 1

## INTRODUCTION

*Cronobacter sakazakii,* formally characterized as *Enterobacter sakazakii* until 2008, is a gram-negative, rod-shaped bacteria belonging to the *Enterobacteriaceae* family. In recent years, *C. sakazakii* has gained more attention due to the increase is recalls associated with powder infant formula (PIF) and an aseptic facility. It is especially notable that the bacteria can survive in extremely low water activity environments ($a_w < 0.20$) and remain viable in infant formula for 2 years (Barron & Forsythe, 2007). Infants are especially susceptible to *C. sakazakii* infections due to their non-fully developed immune systems. Neonates who are infected with the bacteria can develop neonatal meningitis, necrotizing enterocolitis, and septicemia, all of which has a high probability of death in young and immunocompromised individuals (Farmer et al., 1980). While most of the incidences of infections are associated with infants, elderly and immunocompromised people are also at increased risk.

*C. sakazakii* is known to be a strong biofilm former, and to be able to attach to wide variety of food contact surfaces such as glass, PVC, stainless steel, and feeding tubes (Beuchat et al., 2009; Kim et al., 2006, 2007). The attachment of bacteria to a surface is a complex and multifaceted process that is influenced by many intrinsic and extrinsic factors (Frank, 2001). In the process of forming a biofilm, the first stage is the initial attachment of planktonic bacteria to a material surface. This stage is primarily controlled by physical forces, including van der Waals which promotes the attraction of the bacterium to the material surface based on charge. The

electrostatic interaction begins as the bacterium move closer to the material surface. Due to the net negative charge of most bacterial cells, there are easily attracted to positively charged surfaces, which facilitates increased attachment. Several studies have shown that physically or chemically altering the surface of a material can reduce the attachment of bacteria to the surface. *Escherichia coli* has been shown to elongate into filaments and increase surface density of their flagella, allowing for increased attachment (Renner & Weibel, 2011).

*C. sakazakii* has been shown to have the ability to adapt and evolve to a wide variety of environmental stressors common to food processing and the domestic environment including thermal, chemical, osmotic, and desiccation (Al-Holy et al., 2009; Breeuwer et al., 2003; Dancer et al., 2009). Due to this, the persistent and prevalence of *C. sakazakii* in infant formula and milk powder processing facilities has been extensively studied. Specifically, one study found that in a milk powder processing facility, 32% of samples were positive for *Cronobacter* (Craven et al., 2010). Likewise, tracing of *Cronobacter* throughout a PIF plant found that many isolates that were found in the area surrounding the plant were found in the processing area and eventually in the final product. Another main path of contamination is through air filtration and bioaerosols created during the spray drying process. Prevalence of *C. sakazakii* in spray drying towers was found to be greater than 80% (Jacobs et al., 2011).

PIF is made through two different methods: wet blend and dry blend. In the dry process, plants receive all materials in their dry state and blend and package them in the facility without any heat treatment. In the wet blend process, materials are received and pasteurized and spray-dried in the plant (Masum et al., 2021). Due to the high protein and solids content of infant formula in the wet blending process, the incidence of heat exchanger foulant build-up is much

higher, providing a possible vector of contamination and niche point for bacteria to survive and proliferate if not cleaned adequately (Murphy et al., 2011, 2013).

Fouling in continuous thermal processing is defined as the buildup of proteins, vitamins, biofilms, or any other organic or inorganic substances on the surface of heat exchange surfaces (Bansal & Chen, 2006). It is a paramount issue in the food and dairy industry because it can affect product quality and safety and increase the amount of time and resources required for cleaning. The high nutrient content of fouling provides bacteria with the opportunity to attach to surfaces and resist thermal and chemical treatments. Fouling is very difficult to avoid, especially in the process of a high protein product like infant formula. Methods for avoiding fouling and monitoring cleanliness of heat exchangers is primarily done by validating clean-in-place procedures (Phinney et al., 2017). Because the CIP process is continuous and automated, equipment components are rarely taken apart to visually monitor CIP effectiveness. Due to this, without appropriate monitoring, plants can run for many days with fouling build up that could possibility harbor bacteria such as *Cronobacter.*

The primary research gap is the interaction between the presence of foulant and the attachment of *Cronobacter.* Because typical pasteurization temperatures should eliminate any *Cronobacter* from the product being pasteurized, the presence of foulant on heat exchange surfaces could present an opportunity for the bacteria to attach and find niche points for survival and persistence. If systems are then not adequately cleaned and sterilized prior to running, or are pushed to the limits of processing, *Cronobacter* may remain in the system and contaminate downstream processing. Likewise, if systems are cleaned with sub-lethal amounts of sanitizers, or the heat it not enough to clean the system, the bacteria that are not eliminated can begin to adapt and gain an increased thermos-tolerance and resistance to sanitizers used in the food

industry. If foulant is present in this case, if could present an opportunity for the bacteria to grow to larger numbers if the system is to stay stagnant for extended periods of time.

**References**

Al-Holy, M. A., Lin, M., Abu-Ghoush, M. M., Al-Qadiri, H. M., & Rasco, B. A. (2009). Thermal Resistance, Survival and Inactivation of *Enterobacter Sakazakii* (*Cronobacter* Spp.) in Powdered and Reconstituted Infant Formula. *Journal of Food Safety*, *29*, 287–301. https://doi.org/10.1111/j.1745-4565.2009.00157.x

Bansal, B., & Chen, X. D. (2006). A Critical Review of Milk Fouling in Heat Exchangers. *Comprehensive Reviews in Food Science and Food Safety*, *5*, 27–33. https://doi.org/10.1111/j.1541-4337.2006.tb00080.x

Barron, J. C., & Forsythe, S. J. (2007). Dry Stress and Survival Time of *Enterobacter sakazakii* and Other *Enterobacteriaceae* in Dehydrated Powdered Infant Formula. *Journal of Food Protection*, *70*, 2111–2117. https://doi.org/10.4315/0362-028X-70.9.2111

Beuchat, L. R., Kim, H., Gurtler, J. B., Lin, L.-C., Ryu, J.-H., & Richards, G. M. (2009). *Cronobacter sakazakii* in Foods and Factors Affecting its Survival, Growth, and Inactivation. *International Journal of Food Microbiology*, *136*, 204–213. https://doi.org/10.1016/j.ijfoodmicro.2009.02.029

Breeuwer, P., Lardeau, A., Peterz, M., & Joosten, H. M. (2003). Desiccation and Heat Tolerance of *Enterobacter sakazakii*. *Journal of Applied Microbiology*, *95*, 967–973. https://doi.org/10.1046/j.1365-2672.2003.02067.x

Craven, H. M., McAuley, C. M., Duffy, L. L., & Fegan, N. (2010). Distribution, Prevalence and Persistence of *Cronobacter* (*Enterobacter sakazakii*) in the Nonprocessing and Processing Environments of Five Milk Powder Factories: *Cronobacter* Milk Powder

Factories. *Journal of Applied Microbiology*, *109*, 1044–1052. https://doi.org/10.1111/j.1365-2672.2010.04733.x

Dancer, G. I., Mah, J.-H., & Kang, D.-H. (2009). Influences of Milk Components on Biofilm Formation of *Cronobacter* spp. (*Enterobacter sakazakii* ). *Letters in Applied Microbiology*. https://doi.org/10.1111/j.1472-765X.2009.02601.x

Farmer, J. J., Asbury, M. A., Hickman, F. W., Brenner, D. J., & The *Enterobacteriaceae* Study Group. (1980). *Enterobacter sakazakii*: A New Species of "*Enterobacteriaceae*" Isolated from Clinical Specimens. *International Journal of Systematic and Evolutionary Microbiology*, *30*, 569–584. https://doi.org/10.1099/00207713-30-3-569

Frank, J. F. (2001). Microbial Attachment to Food and Food Contact surfaces. In *Advances in Food and Nutrition Research* (Vol. 43, pp. 319–370). Elsevier. https://doi.org/10.1016/S1043-4526(01)43008-7

Jacobs, C., Braun, P., & Hammer, P. (2011). Reservoir and Routes of Transmission of *Enterobacter sakazakii* (*Cronobacter* spp.) in a Milk Powder-Producing Plant. *Journal of Dairy Science*, *94*, 3801–3810. https://doi.org/10.3168/jds.2011-4318

Kim, H., Ryu, J.-H., & Beuchat, L. R. (2006). Attachment of and Biofilm Formation by *Enterobacter sakazakii* on Stainless Steel and Enteral Feeding Tubes. *Applied and Environmental Microbiology*, *72*, 5846–5856. https://doi.org/10.1128/AEM.00654-06

Kim, H., Ryu, J.-H., & Beuchat, L. R. (2007). Effectiveness of Disinfectants in Killing *Enterobacter sakazakii* in Suspension, Dried on the Surface of Stainless Steel, and in a Biofilm. *Applied and Environmental Microbiology*, *73*, 1256–1265. https://doi.org/10.1128/AEM.01766-06

Masum, A. K. M., Chandrapala, J., Huppertz, T., Adhikari, B., & Zisu, B. (2021). Production and Characterization of Infant Milk Formula Powders: A Review. *Drying Technology*, *39*, 1492–1512. https://doi.org/10.1080/07373937.2020.1767645

Murphy, E. G., Tobin, J. T., Roos, Y. H., & Fenelon, M. A. (2011). The Effect of High Velocity Steam Injection on the Colloidal Stability of Concentrated Emulsions for the Manufacture of Infant Formulations. *Procedia Food Science*, *1*, 1309–1315. https://doi.org/10.1016/j.profoo.2011.09.194

Murphy, E. G., Tobin, J. T., Roos, Y. H., & Fenelon, M. A. (2013). A High-Solids Steam Injection Process for the Manufacture of Powdered Infant Milk Formula. *Dairy Science & Technology*, *93*, 463–475. https://doi.org/10.1007/s13594-013-0116-7

Phinney, D. M., Feldman, A., & Heldman, D. (2017). Modeling High Protein Liquid Beverage Fouling During Pilot Scale Ultra-High Temperature (UHT) Processing. *Food and Bioproducts Processing*, *106*, 43–52. https://doi.org/10.1016/j.fbp.2017.08.007

Renner, L. D., & Weibel, D. B. (2011). Physicochemical Regulation of Biofilm Formation. *MRS Bulletin*, *36*(5), 347–355. https://doi.org/10.1557/mrs.2011.65

Chapter 2

# LITERATURE REVIEW

## Introduction

*Cronobacter sakazakii* (formerly characterized as *Enterobacter sakazakii*) is non-spore forming, gram-negative, rod-shaped opportunistic foodborne pathogen, most associated with infections in newborns and babies born prematurely. The most common infections include neonatal meningitis, necrotizing enterocolitis, and septicemia, all of which has a high probability of death in young and immunocompromised individuals (Farmer et al., 1980). Initially, the bacterium was characterized as "yellow-pigmented *Enterobacter cloacae*" described as early as 1965 by Joker et al in a case of neonatal meningitis in England

*Cronobacter* has gained interest in the recent years due to recalls and outbreaks associated with powdered infant formula (PIF). Since PIF is not a sterile product, contamination with pathogens is not uncommon, and due to the underdeveloped immune system of neonates, infection is much more common and fatal. Because of this, focus by the industry, academic research, and governmental agencies has been directed towards environmental monitoring in production facilities, as well as revised methods for rehydration to eliminate any *Cronobacter* that still maybe present. While there is evidence that incidences of *Cronobacter* have decreased (Al-Holy et al., 2009), further research must be focused on reducing the prevalence and transmission in food processing facilities, healthcare facilities, and the home.

Outbreaks associated with the bacterium *C. sakazakii,* and powder infant formula (PIF) have been increasing in the past years, leading to many deaths and illnesses, especially in infants under 2 months old. While the incidence of infections associated with *C. sakazakii* are relatively low with only 2-4 cases reported every year, the mortality rate has been estimated to be around 40% (Friedemann, 2009). In an analysis done examining the total monetary loss and quality adjusted lifedays (QALD) of different foodborne illnesses in the United States, *Cronobacter* spp. was the highest of all agents investigated with a per illness mean QALD loss of 4,023 and mean monetary loss of $7,013,777 (Minor et al., 2015). While the route of contamination of PIF is largely unknown, research has shown transmission of the bacterium from environmental sources (soil, water, wood) to processing plants is a possibility in spray-drying factories (Forsythe, 2018; Craven et al, 2010). In 2008, the Food and Agriculture Organization (FAO) revised the Codex Alimentarius Commission (CAC) to include direct presence/absence testing for *Cronobacter* spp. in thirty 10-gram samples (CAC, 2008). Additionally, ingredients and environmental samples are subject to the same testing. Another possible source of *Cronobacter* infections is contamination from within the home. Places within the home that *Cronobacter* spp. were isolated include vacuum dust, front entryways, and garage floors (Chan, 2016). Additionally, a high percentage of positive samples tested from a home kitchen sink indicate that this could be a possible source of cross contamination to feeding bottles or utensils that neonates may encounter (Nthenge-Kilonzo et al., 2012).

Specific focus has not been directed on how *Cronobacter* may be transmitted into processing facilities, especially its survival in continuous thermal processing. Research suggests that *Cronobacter* is ubiquitous in the environment, having been found in soil, water, and other crops (Cechin et al., 2023). Additionally, decreasing the aerosolization of the bacteria in spray-

drying plates seems to be a viable method of controlling the widespread of the pathogen. Further transmission of the bacteria by humans on clothes, hands, and shoes is also a dangerous vector of transmission in the home where *Cronobacter* may infect infants on surfaces that may not have as much attention focused on cleaning, such as floors, sinks, and countertops. To address this, this review will focus on environmental and industrial prevalence of *Cronobacter* and contamination into the home, as well as methods of how it can survive in extreme environments.

**Pathogenicity, Taxonomy, and Virulence Factors**

Multiple studies have proven that *Cronobacter* is the most resistant of the *Enterobacteriaceae* family to a wide variety of environmental stressors including thermal, chemical, and desiccation. In the context of the manufacturing the preparation of infant formula, exposure of *C. sakazakii* to sublethal doses of a stressor such as heat or chemical is common. During this process of cross-protection, the bacteria begin to adapt and pre-condition itself if subjected to sub-lethal exposure. Cells that underwent sublethal stressors were then found to be more resistant in rehydrated infant formula (Yang et al., 2015). One specific virulence factor that contributes to the thermal tolerance of *Cronobacter* is the presence of a genomic island (*thrB-Q*, *thrBCD* and *thrOP* genes) which have been reported to give increase thermotolerance to the bacteria as compared to strains that do not have these genes (Phair et al., 2022).

Likewise with desiccation tolerance, many genes have been found that include ones that code for the transport of osmoprotectants glycine, betaine, and trehalose. This function alongside metal ions to support cell membrane maintenance in *Cronobacter.* Virulence factors including RpoS, RpoN, and the Cpx system are genes that have been identified as factors that could contribute to protect against desiccation and osmotic stress in the cell (Phair et al., 2022).

Adaptation of *Cronobacter* to acidic conditions is especially important due to the pH of a newborn's stomach to be as high as 5.17, giving *Cronobacter* a higher chance of survival in the stomach. It has been identified that *rpoS* plays a role in high acid tolerance. Additionally, the envelope response regular CpxR which allows the cell to detect differences in environmental conditions and regulates genes that enhance the integrity of the cell membrane in response to these stressors (Joseph et al., 2012). Curli fimbriae are a component of the extracellular matrix produced by some species of *Cronobacter* that has been shown to play a part in the cell's ability to adhere to each other, different materials, and form biofilms. The specific genes *csgA* and *csgB* are involved in biofilm formation and cell-cell aggregation (Hu, 2018).

**Prevalence in the Environment**

The vector of transmission of *Cronobacter* from the environment to processing facilities, hospitals, or homes is largely unknown. While little research has been done on identifying sources of *Cronobacter* in the environment, it has been isolated from various plant foods and agricultural sources including soil, water, animal feces, rice, wheat, and various other crops (Cechin et al., 2023). Environmental reservoirs of the bacteria are likely sources of transmission. Other members of the *Enterobacteriaceae* family are found normally in water and soil and used as indicators for presence of possible contamination of foodborne pathogens that could cause illness. Unlike other *Enterobacteriaceae* species like *E. coli* that are part of a healthy digestive system and carry probiotic properties, *Cronobacter* is said to not be part of the animal or human gut microflora (Chenu & Cox, 2009). Because of this, soil, food, and water are the most likely sources of transmission and contamination (Iversen & Forsythe, 2003) Rats and flies are a possible secondary source of contamination, having been isolated in their respective digestive tracts (Iversen & Forsythe, 2003). However, sampling of surface water, rotting wood, and cow's

milk yielded no positive *Cronobacter* samples. Due to the rigorous nature of the pathogen, its ability to survive in a wide range of temperatures, water activities, and food matrices is well understood through its various mechanisms of adapting to environmental stressors. It is obvious that the environmental conditions have a large influence on the survival and prevalence of the bacteria.

A study was done by Chan et al (2016) in Georgia to discover the prevalence of *Cronobacter* in domestic environments. They found that higher amounts of precipitation over the course of the sampling was correlated to a higher number of *Cronobacter* isolates recovered. During times where there may be large amounts of rain, the pathogen may be easily adhered to clothes and shoes (likely via soil), providing a vector of transmission into the home. Conversely, there was no effect of average temperature, most likely due to internal temperature being separate from external temperature. Interestingly, sampling done on farm homes found significantly less *Cronobacter* isolates when compared to urban or suburban homes. Due to the relatively proximity to agricultural commodities like crops, animals, and soil, it would have been logical for these values to be higher. It was noted that this is most likely due to boots and jackets being left outside before entering the home. The number of pets in the home did not have an effect either, but the number of residents correlated to an increased number of isolates. Humans working in these facilities or on farms are a notable possibility of cross-contamination of the bacteria into processing plants. Due to the ubiquitous nature of the pathogen in soils, transmission via shoes or dirty vegetables from the field to processing areas is a potential food safety risk and should be monitored accordingly. While procedures are in place to prevent this, transmission by aerosols is also of major interest. *Cronobacter* has also been isolated from

human sources including teeth, saliva, and skin (Forsythe, 2018). Additionally in hospital environments, the bacteria have been found in the air and clinically.

Several studies have focused on the prevalence of *Cronobacter* in processing environments, but research is limited on the transmission of the bacteria from environmental sources to food processing facilities.

**Regulatory**

The contamination of PIF with *Cronobacter sakazakii* is of primary interest to food safety professionals due to the prevalence of recalls associated with the bacterium. To monitor the safety of the product the Code of Hygienic Practices, specifically in 21 CFR 106.55(e), dictates that there is a maximum of zero positives in 30 samples of 10 grams of the PIF and no *Salmonella* spp. in 60 25-gram samples, and any presence of these microorganisms deems the product to be adulterated. In the United States, the Infant Formula Act outlines safe manufacturing processes, as well as specific controls present in Good Manufacturing Practices (GMPs). In 2014, in response to a study done by Jongenburger et al., (2011) the FDA updated their sampling protocols to reflect a more statistical-based approach. It states "when the production aggregate (FDA's new term for a lot or batch) is sampled and the composite is tested, if the pathogen is not detected, the manufacturer has a 95% level of confidence that there would be <1 CFU *Cronobacter* spp. in 100 g powder" (FDA, 2024). This corresponded to lower rates of *Cronobacter*, as well as *Salmonella*, which has also been associated with contamination in PIF.

In the United States, the requirements labeling, nutrition standards, and quality control are governed under 21 CFR Part 107. This gives specific requirements for minimum and

maximum nutrient requirements, as well as how the infant formula should be labeled including directions for preparation, iron content declaration, and use by dates. The regulation also states that manufactures must have a recall plan for formulas that are adulterated (chemically, physically, or biologically) or misbranded. In the European Union, regulations are very similar with the main difference being that advertising of infant formula is highly regulated. More specifically, advertising is restricted to "publications specializing in baby care and scientific publications" as well as no displays, coupons, special sales, free or low-priced products, or samples. This is all due in part to protect breastfeeding promotion. The International Organization for Standardization (ISO) sets the series of standards for the verification of quality of infant formula through standards including Determination of Chloride (ISO 21422), Determination and minerals and trace elements (ISO 21424), fatty acid composition (ISO 16958), vitamin $B_{12}$ (ISO 20634), and pantothenic acid (ISO 20639) and several more (ISO, 2018).

**Aerosols in Processing Environments**

With the prevalence of *Cronobacter* recalls associated with powder infant formula (PIF), much research efforts have focused on isolating sources of the bacteria in PIF and milk powder processing facilities. Both environmental and final product sampling has revealed a high prevalence of the bacteria in these plants. Recently, focus has been given to isolating *Cronobacter* in various locations throughout processing plants and using modern molecular techniques such as PCR and DNA fingerprinting to determine sources within plants. In a study by Craven et al. (2010) *Cronobacter* was isolated from 32% of samples from five milk powder factories. Results of clonal analysis by PFGE found that individual clones were unique to each factory. Notably, a correlation between external and internal areas of the plant was found. Seven

clones were isolated from both the final product and various other areas of the factory, including tankers, evaporator rooms, shoes, and external roofs. This points to the fact that *Cronobacter* is more than likely being transmitted into the factory by workers and being spread throughout the plants. Similarly, when *Cronobacter* was tracked and isolated at a PIF factory in China, results showed that 10 isolates were collected from both hands of employees as well as the environment inside and surrounding the outside of the factory. The majority of strains discovered at the factory were found from the outside area, but the tracing suggested that these were easily transmitted into processing areas, making their way into final products. The ST3 was a predominant strain in one factory and had the same typing as those isolated from soil, providing strong evidence of soil being the source of contamination in this factory.

PIF is made primarily through two processing methods: wet and dry. In wet processing, wet ingredients (milk, whey, etc.) are received and pasteurized and dried by a spray drier. In dry processing (dry blending), powders are brought into plants and combined and packaged without a heating step done in the factory. There are also methods that combine the two and dry some products in house and receive others pre-dried. When comparing sampling at plants following the two separate methods, Lu et al. (2019) found that a specific plant that followed the dry processing method had no incidence of *Cronobacter* in all sampling locations through the plant. This not only indicated exceptional environmental controls and hygienic practices, but suggested that the addition of spray drying (which requires air filters) is a point of contamination in the plant and points to the fact that the bacteria is spread by air movement and movement of employees. While additional supplier verification and ingredient testing is probably needed, the use of dry blending could be an effective way to control the transmission of *Cronobacter* in plants. However, the ingredients subsequently used in a dry-blended process are likely spray-

dried prior to being used in the dry-blending plant, demostrating that the spray drier or processes upstream from this are likely sources of contamination. Due to the high protein content of the materials being processed, the incidence of heat exchanger fouling build-up is much higher, thus providing a possible vector of contamination if not properly cleaned and removed.

Due to the dry nature of the processing of dry milk and infant formula, there is abundant bioaerosols and aerosolized particles in these factories. Mullane et al., (2008) found that the contamination of air filters used in the handling of air in a powder milk protein facility was a significant source of contamination in the final product. Isolation from air filters, environment, and final product revealed the presence of 3 clonal populations throughout the factory. On primary filters of the spray drier, 111 MPN *Cronobacter* per gram dust was found. High numbers both on this filter and identifiable pulsotypes in secondary filters suggested that these filters were unable to eliminate every contaminate in the air. The additional presence of moisture on the primary filter could also provide a stress-response to the bacteria, giving more resilience and further adapting it for survival. The authors concluded that there is an increased risk of contamination with *Cronobacter* in the final process by the air intake and dehumidifier.

Jacobs et at., (2011) found similar results to those previously mentioned during sampling of a milk powder plant and focusing on spray-drying and roller drier areas. By isolating *Enterobacter sakazakii* (as categorized in that article) from filters from two separate drying towers, the prevalence was 82.7% and 93.9%. Seven PFGE types were isolated, which were deduced to have entered the plant through a roller shutter and aperture used to aerate the plant. This equipment was found to be controlled poorly, contaminating the production area, and violating the hygienic design of the plant. An additional route of contamination identified in this plant was the reintroduction of powder found in filters back into the product flow. The roller

driers posed a similar problem in the production area. Because the spray drying and roller-drier areas are separate, isolates different than those found in spray-drying areas were found. The processing used in this area is wet, so there is an increased risk of aerosolization and further contamination of the final product.

**Adaptation and Survival to Environmental Stressors**

Many factors contribute to the survival and persistence of *C. sakazakii* in the environment, clinical setting, and food processing plants. With the intrinsic vigor of this bacteria, exposure to sub-lethal stressors have a notable effect on the survival and persistence in laboratory and real-life environments. The stressors of interest include thermal, desiccation, biofilm formation, and chemical. *C. sakazakii* acquires its ability to adapt and survive to a wide variety of environmental conditions when pre-exposed to those stressors.

**Thermal Stress and Resistance**

The first study to investigate the thermal tolerance of *Cronobacter* was Nazarowec-White and Farber (1997). At the time of publication, research on the bacteria was limited and the virulence factors and mechanism of stress adaptation was not well understood. In testing 10 strains (5 clinical and 5 food) in reconstituted PIF, the pooled D-values were 54.8, 23.7, 10.3, 4.20, and 2.50 minutes at 52, 54, 56, 58, and 60°C, respectively, with a z-value of 5.82°C. D-values of bacteria from clinical sources were noted to be higher than food sources, but not significantly different. When compared to other *Enterobacteriaceae* species, *Cronobacter* was determined to be more thermotolerant. Many factors go into the determination of the D-value of bacteria, so comparing results among different studies may be difficult, as methods, growth medium, and culture state all differ. Osaili et al (2009) investigated the thermal resistance of *Cronobacter* in reconstituted powdered milk and infant formula. D-values were significantly

higher in whole milk versus low-fat and non-fat. Additionally, the thermal resistance of *Cronobacter* in lactose-free formula was significantly higher than that of a soy-protein formula. The overall Z-value in infant formula was 4.01 to 4.39°C; slightly lower than that reported by Nazarowec-White and Farber (1997). The most heat-resistant strain found by Buchanan (2004) found a z-value of 5.6°C, more in line with the original study.

The CDC recommends for babies that are younger than 2 months old, born prematurely, or have a weakened immune system to boil the water to be used, allow to cool for about 5 minutes, mix the formula, cool to body temperature before feeding. Because of the ability of *Cronobacter* to have a high mortality rate in these neonatal groups, it is necessary to ensure that any bacteria are killed prior to feeding. These recommendations are based on published research, including Osaili (2009), that found that that formula inoculated with *Cronobacter* and reconstituted with water at 70°C resulted in a > 4 log reduction. While this is an effective way to ensure the risk of *Cronobacter* infection is reduced, focus should be directed on preventive actions of preventing contamination in processing, rather than reactive measures in the home or clinical setting.

Heat tolerance can also be confounded by other extrinsic factors including pH and water activity. Arroyo et al (2009) found that resistance decreased when the medium had a pH less than 6.0. D-value at pH 3.74 in orange juice at 60°C was found to be 0.026 minutes and at pH 6.68 it was 1.147 minutes. Additionally, when water activity was reduced to 0.96 at pH 4.0, a 32-fold increase in thermal resistance was observed. The effect of pH on the thermal resistance of bacteria has long been studied and generally understood that lower pH values result in decreased thermal resistance, likely due to the interaction with the membrane and the role of the stress response sigma factors *RpoS* and *SigB* (Ait-Ouazzou et al., 2012). The synergistic effect of water

activity and low pH is a likely reason for the high degree of thermal tolerance in this study and should be further investigated. Further research is additionally needed on evaluating thermal resistance on strains implicated in specific recalls for trends in thermal resistance.

Powdered infant formula is not a sterile product, so control of any contamination in the processing must be controlled and monitored as per Hazard Analysis and Critical Control Points (HACCP) methods, as well as Good Manufacturing Practices (GMPs). Because the components (in dry blending) or whole formula (wet blending) must be pasteurized for consumption to ensure a 5 log reduction of the pathogen of interest, per USDA regulations, at common high-temperature short-time (HTST) conditions (15 seconds 70°C/161°F), *Cronobacter* should receive a lethality of greater than 11 log ($D_{72°C}$= 1.3second), eliminating the chance of surviving the pasteurization process (Nazarowec-White & Farber, 1997). While newer thermal resistance data could present higher D-values, a HTST condition should be sufficient to reduce *Cronobacter* to undetectable levels. Because of this, focus has mostly been directly to post-process contamination and other possibilities of transmission of the bacteria in process plants, packaging, environment, and the home.

**Desiccation Tolerance**

Drying of foods is one of the oldest and more traditional methods of preserving foods due to the fact that very few bacteria can survive at water activities below 0.60 (Jay et al., 2005). Lowering water activity stresses bacteria, and they are unable to maintain the nutrients they need to survive, causing them to desiccate and die. However, there are many pathogens that have been found to survive in these extreme environments and acquired the ability to survive low water activities. This is not only limited to *Cronobacter* spp. but also *E. coli* O157:H7 in apple powder, buttermilk powder, Cheddar cheese seasoning, and powdered chicken (Deng et al., 1998) and

additionally *Salmonella* in wheat flour (Archer et al., 1998) and pecan nutmeats (Beuchat & Mann, 2011) Other bacteria of interest include *Bacillus* and *Clostridium* species (Beuchat et al., 2013)

Due to the recalls associated with PIF, the survival of *Cronobacter* over an extended prior was investigated by Barron and Forsythe (2007). In a prolonged study monitoring the viability and survival of multiple *C. sakazakii* strains over 2.5 years, two encapsulated strains survived for the whole length. Of the five strains that survived after 2 years, 3 were not encapsulated and the remaining 2 were the ones that survived for 2.5 years. It was observed that in the first 6 months of storage, viability decreased at a greater rate (3.34 log CFU), while a decrease of only 1.18 log CFU was observed for the remaining 30 months. The encapsulation method used is meant to emulate a bacterium that has first formed a biofilm before being desiccated (Barron & Forsythe, 2007). The difference in viability decreases over storage points to the possibility of an adaptation period of the bacteria to the dry environment. After adaptation, those bacteria that remain viable have a greater persistence. There was additionally no correlation found between encapsulation and recovered during the first 18 months.

To investigate the role of different food matrices and water activities, Lin and Beuchat (2007) found that increases in water activity or storage temperature increased the rate at which *Cronobacter* died in dry infant cereal. When inoculated at a low rate (0.31 CFU/g) into infant rice cereal at water activity range of 0.30-0.69 at temperature of 4, 21, and 30 °C, the bacteria were still recovered after 12 months. The only exception to this was at 30°C with $a_w$=0.46. The survival was not found to be affected by the composition of the foods. These results emphasize the importance of ensuring there is no bacterial contamination because *Cronobacter* can still be recovered and viable after 12 months of storage

**Biofilm Formation and Response to Chemical Interventions**

The formation of *C. sakazakii* biofilms and their ability to adapt and survive under extreme sanitation conditions is an important aspect in analyzing the transmission of the bacteria in the environment. *C. sakazakii* has been found to grow biofilms under various conditions including food processing environments and hospital settings, as well as different food matrices. Once bacteria are attached to a surface, they secrete exopolysaccharide (EPS) primarily composed of polysaccharides, proteins, and nucleic acids. It is this EPS that provides a physical defense layer between the bacteria and cleaning practices used in the food industry (Kumar & Anand, 1998).

In the context of transmission of *Cronobacter* in food and environmental settings, the food matrix and environment are a key factor in biofilm formation. To observe how the components of skim milk affect the growth of biofilms of *Cronobacter sakazakii,* Dancer et al (2009) used 5 different preparations of skim milk as the substrate for biofilm formation: skim milk, skim milk diluted 1:8 with water, 1:8 skim milk plus lactose, 1:8 skim milk plus whey, and 1:8 skim milk plus casein. These preparations exhibited both concentrated sources of protein in the case of casein and whey, and additionally carbohydrates in the case of lactose. While it was concluded that biofilm forming ability was dependent on strain and substrate, the overall trend was that nitrogen source was more supportive of biofilm formation versus carbohydrate. Comparatively, Oh et al (2007) found that reconstituted, undiluted infant milk formula was the best substrate for biofilm production on plastic surfaces, with 56 of 72 strains forming biofilms, and 26 of those being strong formation. Compared to artificial media used in the study, 1:20 diluted TSB had the highest prevalence of biofilm, but significantly lower at 16.7%.

*C. sakazakii* additionally has the *nanAKT* gene that encode for the use of sialic acid as a carbon source. Of the species in the *Cronobacter* genus, *C. sakazakii* is the only species that utilizes sialic acid as growth source, allowing it to remain viable in formulas supplemented with sialic acid (Kalyantanda et al., 2015). Sialic acid, which is added to powdered infant formula, is found naturally in breast milk and plays roles in neural transmission and brain and cognitive development. This has pointed to the possible co-evolution of *C. sakazakii* and sialic acid synthesis in vertebrates, possibly allowing for additionally virulence factors for life-threatening infections in infants (Joseph et al., 2013).

The surface in which biofilms attach to is important in regulating the cleaning and sanitizing of equipment, as well as increase the risk of possibility of transmission and infection into neonates and the immunocompromised. *C. sakazakii* has been reported to attach and form biofilms in various surfaces including silicon, latex, stainless steel, PVC, and enteral feeding tubes (Iversen & Forsythe, 2003; Kim et al., 2007) The temperature at which biofilms are formed is also integral to the strength of the biofilm formed. Kim et al (2006) found that stainless steel and enteral feeding tubes immersed in TSB, IFB, and lettuce juice broth (LJB) attached better to the surfaces at 25 °C as compared to 12°C. LJB provided interesting results as its initial biofilm population increased and remained constant for 2-4 days, then significantly decreased after 10 days of intubation. This again points to the importance of nutrient sources in the formation, because as the formation progresses, nutrients are depleted.

The uses of sanitizing chemicals and disinfectants are integral to ensuring the safety of food contact surfaces and hospitals. The presence of infant formula on *Cronobacter* dried on the surface of stainless steel increased the resistance to disinfectants used (Kim et al., 2007). While efficacy of disinfectants will vary greatly based on pH, active ingredients, and temperature, it is

evident that through this reported data that several sanitizers are effective killing *Cronobacter* biofilms, with reduction levels as low at 0.07 log CFU/coupon. This emphasizes both the importance of sanitizer selection and gross soil removal by other means prior to disinfection.

### ***Cronobacter*** **in the domestic environment**

The main source of interest of this review is to explore possible vectors of contamination of *Cronobacter* into processing environments and infant formula. Further, using the knowledge of bacteria that affect neonates, like *Listeria,* should certain precautions be taken for those working with *Cronobacter*, both intentionally and unintentionally, to prevent contamination into households that have vulnerable populations.

Research focusing on the prevalence of *Cronobacter* in domestic environments is limited. Additionally, comprehensive studies have not been done that trace isolates to find species sources, like those done in milk powder and PIF processing plants described in previous sections. Food safety in the home is paramount in preventing the spread of foodborne illnesses. While most foodborne illness outbreaks are in settings where food safety practices are used, there are also outbreaks associated with food prepared in the home (Holst, 2025). This is likely due to the stringent sanitation and cleaning practices implemented by restaurants, as well as wearing gloves. In the home, cleaning may not be as thorough and often as what is seen in restaurants or processing facilities, so the prevalence of bacteria found on food and non-food contact surfaces is higher. This not only poses a threat to populations at risk such as young, old, immunocompromised individuals, and pregnant, but also aids in the evolution of antibiotic-resistant bacteria (Kilonzo-Nthenge et al., 2012)

The domestic kitchen has been described as “the front line in the battle against food-borne disease” (Redmond & Griffith, 2009). The need for consumers to properly prepare, cook,

and store foods, including infant formula, is very important in the prevention of foodborne disease. Very often, cross-contamination between raw meat and poultry and other foods is a source of foodborne illness in the home, especially with *Salmonella* and *Campylobacter* (Carrasco et al., 2012). Further contamination with dishcloths, sponges, work surfaces, and the fridge provides ways for bacteria to spread throughout the home easily and quickly. Effective controls are not always in place to prevent the spread and contamination in the home. Proper hand washing, as well as keeping raw and ready-to-eat foods separate are important measures in preventing the spread of bacteria (Redmond & Griffith, 2009). Results of swabs on human skin to screen for *Cronobacter* spp. conducted in the Netherlands yielded only 1 positive in 116. This study was done by asking participants to scrub their skin thoroughly in the shower to scrub as much skin area as possible. Because of this, common hand washing procedures should be sufficient to remove any *Cronobacter* that human hands or skin could have picked up, especially in food processing plants (Kandhai et al., 2010).

Much attention has been focused on cross-contamination in the home environment by other pathogenic organisms like *Salmonella, Campylobacter, and E. coli* but only a few studies have focused on the prevalence and isolation of *Cronobacter* in the domestic environment. Understanding routes of contamination of *Cronobacter* in the home provides a way to further control and eliminate this pathogen. Nthenge-Kilonzo et al. (2012) sampled a total of 234 contact sites in 78 separate kitchens to determine the prevalence of *Cronobacter sakazakii* in the home, as well as determining the antimicrobial resistance against various antibiotics used clinically. Results indicated that *C. sakazakii* was present in 26.9% of the homes tested, with the highest prevalence in kitchen sinks (44%) and countertops (20%) followed by dishcloths and fridge door handles (16% and 12%, respectively) and meat drawers (4%). High prevalence in sinks is a point

of concern due to it being a highly trafficked and touched area of the kitchen, possibly cross-contaminating previously cleaned dishes or surfaces. The presence in meat drawers was a point of interest for the authors because previous studies had indicated that *C. sakazakii* had been present in various retail meats. These results point to the importance of hygiene practices in the home, especially those that may have infants being fed PIF and thus more susceptible to infection with *C. sakazakii*. The ability of the pathogen to persist in various environments provides with a strong advantage for persistence and evolution in the home.

In a similar study done by Chan (2016), the effect of home type (urban, suburban, or rural) on presence of *Cronobacter* and *Enterobacteriaceae* was determined. Additionally, various locations throughout the home were determined as high percentage harborage sites for *Cronobacter*. When all the home types were put together, vacuum dust, garage floors, and front entryway floor (50%, 37.8%, and 27%, respectively) had the highest prevalence of *Cronobacter* isolated. The overall category with the highest prevalence was floors. These results point to what has been suggested by other studies that soil and water are main reservoirs of the bacteria, thus being tracked into the home by humans by shoes. The high percentage of isolation from vacuum dust is a point that has been studied by others, especially in food processing environments. Reich et al. (2010) found that *Cronobacter* spp. Prevalence in a PIF processing environment was highest at 28% of from vacuum cleaners in a single area of the plant. The vacuum cleaners used in this specific plant collect powders from across the whole plant, so this could give a good indication of the overall prevalence of *Cronobacter* in the plant, both environmentally and in powders.

One result of particular interest was that in baby bottles (n=86) and bottle nipples (n=95), no *Cronobacter* was isolated from any of the samples. These bottles and nipples which were

collected from day-care facilities the day of use. It was not specified whether they were recently cleaned or how they were cleaned, so given the results and compared to guidance by officials, cleaning strategies are very effective as eliminating *Cronobacter* on these surfaces (Kandhai et al., 2010). Locations where *Cronobacter* was found close to the final product is particularly dangerous because there is no further kill step, thus posing a significant risk to contamination in the final product.

**pGFPuv Tagging**

The green fluorescent protein (GFPuv) is a protein from jellyfish (*Aequorea Victoria*) that is inserted into a plasmid in the bacteria DNA and fluoresces when exposed to UV light at 352 nm. By inserting the protein into the plasmid, the resultant mutant is resistant to ampicillin at 100 micrograms per milliliter. GPF transformation is used to traceability and monitoring of the pathogen and has been used in monitoring the survival of *Cronobacter* in corn starch processing and on corn kernels (Nurjanah et al., 2015). Additionally, GFP labeling has been shown not to change growth rate parameters including lag, log, and stationary phase compared to wild-type strains (Nurjanah et al., 2014)

**Fouling in Thermal Processing**

Heat exchanger fouling is one of the paramount issues facing the dairy processing industry. The bulk of time and cost going into the processing of dairy is devoted to cleaning, breaking down equipment, and sanitizing. Fouling presents many main quality and safety issues for many reasons. To ensure the safety of the products, the specific parameters for time and temperature specific for high-temperature short-time (HTST) and ultra-high temperature (UHT) must be met. While theoretically in an optimized processing scheme there should be no issues with these being met, when another layer that is going to provide a layer of thermal insulation

between the heat source and the product, the heat transfer is disrupted (Sadeghinezhad et al., 2013). In turn, this decrease in the heat transfer process also has additional downstream effects, including the need for increased pumping pressure.

The fouling process is well studied and understood. Milk is the most often studied due to its popularity in stores, as well as it is integral to the safety of the product. There are many factors that go into the process including the proximate analysis of the product, pH, equipment used, flow rates, run times, and temperature. Temperature is of primary interest due to the way the fouling is formed. Fouling can be categorized into two main categories based on temperature conditions. Type A fouling occurs at temperatures between 75-110 °C and are formed primarily of proteins and minerals, with a small amount of fat. Due to the nature of this deposit, if detected early on, can be easily cleaned using traditional clean-in-place (CIP) procedures. At temperatures above 110°C, the deposits (type B) are formed mainly of calcium phosphate with a significantly less portion of protein and fat (Sadeghinezhad et al., 2013). It is possible, however, that more protein-rich foulant can be found at high processing temperatures. A more comprehensive breakdown is given in Figure 2-1. Because of the harder and calcified texture, they are extremely difficult to clean and require harsh chemicals and potential complete disassembly of the equipment, increasing downtime (Gillham et al., 2000).

| Classification | Temperature (°C) | Process | Composition (wt%) | | |
|---|---|---|---|---|---|
| | | | Protein | Mineral | Fat |
| Type A | 75–110 | Pasteurization | 50–70 | 30–50 | 4–8 |
| Type B | 110–140 | UHT treatment | 15–20 | 70–80 | 4–8 |

*Figure 2-1: Types of deposits formed at different process temperature*

Little research has focused on how foulant constituents influence the growth of bacteria, and this is the main aim of this proposed work. Because foulant presents a concentrated source of proteins, vitamins, minerals, and carbohydrates, as well as increased surface area, it is logical

that this will be able to create attachment niches for *Cronobacter.* Additionally, when the fouling layer builds up, there is a lowering of the overall heat transfer, thus lowering the amount of energy that gets transferred to the fluid being processed, creating a potentially non-sterile product. Figure 2-2 gives a cross-sectional design of how fouling occurs. If *Cronobacter* has contaminated the process at a point prior to pasteurization, the continuous build-up will allow the bacteria to fix to the surface of the foulant and continue to grow without being killed due to the thermal resistance created by the formation of biofilms. Further, if equipment is not sufficiently cleaned prior to use, bacteria may harbor in the system and grow vigorous biofilms that will persist on surfaces even if the CIP is done again. This is due to the synergistic effect of bacterial attachment and foulant, which can be very difficult to remove.

In typically CIP processes, there are several specific steps to adequately clean and sanitize continuous processing systems. Because CIP require very minimal or no equipment disassembly, proof of effectiveness is primarily tracked by time, temperature, chemical concentrations, and flow rates. The typical steps are as follows:

1. Pre-rinse with water: removes loose soils
2. Alkaline wash: detach fats, proteins, and other carbohydrates. Typically done with sodium hydroxide (NaOH) or potassium hydroxide (KOH) at 70-80°C for ~30 minutes. Chlorine can also be added in the caustic solution
3. Post rinse with water: remove excess alkaline detergent
4. Acidic wash: remove mineral deposits
5. Sanitizer: chlorine-based, peracetic acid, or hydrogen peroxide
6. Post rinse: remove excess sanitizer and idle mode

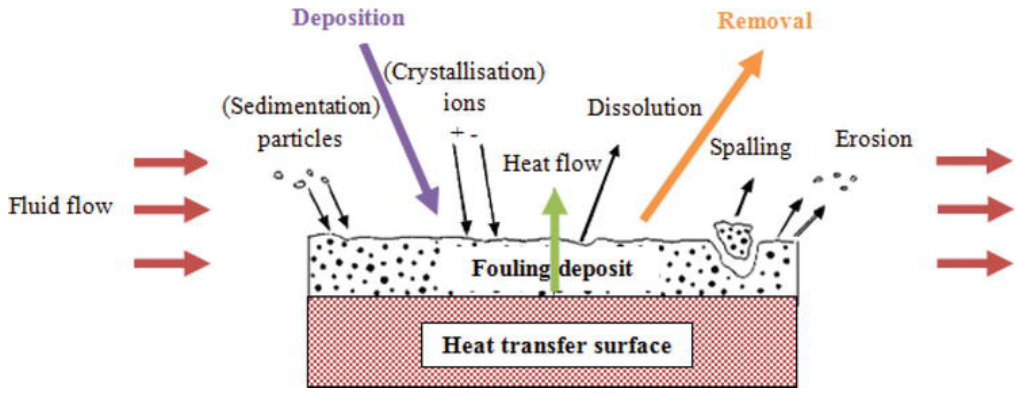


*Figure 2-2: Process of Foulant Build Up*

**Bacterial Attachment**

*Cronobacter* is known to attach to various types of surfaces, including stainless steel, PVC, latex, glass, and silicon (Iversen et al., 2004). As mentioned, the bacterial attachment process is multi-stepped and influenced by many external factors, including surface type. In the case of continuous thermal processing, the fluid flow is a large factor in influencing the attachment of planktonic bacteria. Since *Cronobacter* is a motile bacterium, it can attach to a surface regardless of fluid velocity in a system (Tuson and Weibel, 2013). Given the increased surface area of foulant, *Cronobacter* can easily find harborage sites and be further protected from sanitizers used in food processing. It has been shown that cells that are attached to surfaces not in a biofilm state can be resistant to antimicrobials and antibiotics like that of cells in a biofilm state (Tuson and Weibel, 2013). An additional study found that there was no significant difference in sanitizer effectiveness between cells in an adhered state, biofilm in TSB, and biofilm formed in 100% relative humidity. Because bacterial cells are easily spread throughout processing plants (by movement of people or aerosolization during processing or cleaning), they can easily find their way into harborage sites or niches. Especially is there is standing water or organic matter, these bacteria may be especially difficult to clean and thus may adapt to low sanitizer

concentrations and meet the conditions to continue to grow and further spread throughout a processing line (Carpentier & Cert, 2011).

Likewise, the presence of an additional heat transfer layer present in the form of foulant on a heat exchanger surface only allows for increasing layers to build up due to the negative feedback loop of the system responding to the need for an increase in the media temperature (whether water or steam) required to sufficiently heat the flowing product. If this layer is dramatic enough, not only could it cause blockages and decreased fluid flow but also decrease the overall heat transfer coefficient. This all points to the fact that bacterial cells attached to a surface with foulant may have the ability to proliferate and resist cleaning and sanitation practices in the food industry.

**References**

Ait-Ouazzou, A., Mañas, P., Condón, S., Pagán, R., & García-Gonzalo, D. (2012). Role of General Stress-Response Alternative Sigma Factors Σs (Rpos) And Σb (Sigb) in Bacterial Heat Resistance as a Function of Treatment Medium pH. *International Journal of Food Microbiology*, *153* 358–364. https://doi.org/10.1016/j.ijfoodmicro.2011.11.027

Al-Holy, M. A., Lin, M., Abu-Ghoush, M. M., Al-Qadiri, H. M., & Rasco, B. A. (2009). Thermal Resistance, Survival and Inactivation of *Enterobacter sakazakii* (*Cronobacter* spp.) In Powdered and Reconstituted Infant Formula. *Journal of Food Safety*, *29*, 287–301. https://doi.org/10.1111/j.1745-4565.2009.00157.x

Archer, J., Jervis, E. T., Bird, J., & Gaze, J. E. (1998). Heat Resistance of *Salmonella* Weltevreden in Low-Moisture Environments. *Journal of Food Protection*, *61*, 969–973. https://doi.org/10.4315/0362-028X-61.8.969

Arroyo, C., Condón, S., & Pagán, R. (2009). Thermobacteriological Characterization of *Enterobacter sakazakii*. *International Journal of Food Microbiology*, *136*, 110–118. https://doi.org/10.1016/j.ijfoodmicro.2009.09.013

Barron, J. C., & Forsythe, S. J. (2007). Dry Stress and Survival Time of *Enterobacter sakazakii* and Other *Enterobacteriaceae* in Dehydrated Powdered Infant Formula. *Journal of Food Protection*, *70*, 2111–2117. https://doi.org/10.4315/0362-028X-70.9.2111

Beuchat, L. R., Komitopoulou, E., Beckers, H., Betts, R. P., Bourdichon, F., Fanning, S., Joosten, H. M., & Ter kuile, B. H. (2013). Low-Water Activity Foods: Increased Concern as

Vehicles of Foodborne Pathogens. *Journal of Food Protection*, *76*, 150–172. https://doi.org/10.4315/0362-028X.JFP-12-211

Beuchat, L. R., & Mann, D. A. (2011). Inactivation of *Salmonella* on Pecan Nutmeats by Hot Air Treatment and Oil Roasting. *Journal of Food Protection*, *74*(9), 1441–1450. https://doi.org/10.4315/0362-028X.JFP-11-080

Carrasco, E., Morales-Rueda, A., & García-Gimeno, R. M. (2012). Cross-contamination and Recontamination by *Salmonella* in Foods: A review. *Food Research International*, *45*, 545–556. https://doi.org/10.1016/j.foodres.2011.11.004

Cechin, C. D. F., Carvalho, G. G., Bastos, C. P., & Kabuki, D. Y. (2023). *Cronobacter* spp. in Foods of Plant Origin: Occurrence, Contamination Routes, and Pathogenic Potential. *Critical Reviews in Food Science and Nutrition*, *63*, 12398–12412. https://doi.org/10.1080/10408398.2022.2101426

Chan, M. Y.-K. (2016). Prevalence And Location of *Cronobacter* Species And *Enterobacteriaceae* in Households. *University of Georgia*, *Masters Thesis*.

Chenu, J. W., & Cox, J. M. (2009). *Cronobacter* (*Enterobacter sakazakii*): Current Status and Future Prospects. *Letters in Applied Microbiology*, *49*, 153–159. https://doi.org/10.1111/j.1472-765X.2009.02651.x

Craven, H. M., McAuley, C. M., Duffy, L. L., & Fegan, N. (2010). Distribution, Prevalence and Persistence of *Cronobacter* (*Enterobacter Sakazakii*) in the Nonprocessing and Processing Environments of Five Milk Powder Factories: *Cronobacter* Milk Powder Factories. *Journal of Applied Microbiology*, *109*, 1044–1052. https://doi.org/10.1111/j.1365-2672.2010.04733.x

Dancer, G. I., Mah, J.-H., & Kang, D.-H. (2009). Influences of Milk Components on Biofilm Formation of *Cronobacter* spp. (Enterobacter *sakazakii*). *Letters in Applied Microbiology*. https://doi.org/10.1111/j.1472-765X.2009.02601.x

Deng, Y., Ryu, J.-H., & R. Beuchat, L. (1998). Influence of Temperature and pH on Survival of *Escherichia coli* O157:H7 in Dry Foods and Growth in Reconstituted Infant Rice Cereal. *International Journal of Food Microbiology*, *45*, 173–184. https://doi.org/10.1016/S0168-1605(98)00161-5

Farmer, J. J., Asbury, M. A., Hickman, F. W., Brenner, D. J., & The *Enterobacteriaceae* Study Group. (1980). *Enterobacter sakazakii*: A New Species of "*Enterobacteriaceae*" Isolated from Clinical Specimens. *International Journal of Systematic and Evolutionary Microbiology*, *30*, 569–584. https://doi.org/10.1099/00207713-30-3-569

Forsythe, S. J. (2018). Updates on the *Cronobacter* Genus. *Annual Review of Food Science and Technology*, *9*, 23–44. https://doi.org/10.1146/annurev-food-030117-012246

Friedemann, M. (2009). Epidemiology of Invasive Neonatal *Cronobacter* (*Enterobacter sakazakii*) infections. *European Journal of Clinical Microbiology & Infectious Diseases*, 28, 1297-1304.

Gillham, C. R., Fryer, P. J., Hasting, A. P. M., & Wilson, D. I. (2000). Enhanced Cleaning of Whey Protein Soils Using Pulsed Flows. *Journal of Food Engineering*, *46*, 199–209. https://doi.org/10.1016/S0260-8774(00)00083-2

Hu, L. (2018). Prevalence of Curli Genes Among *Cronobacter* Species and Their Roles in Biofilm Formation and Cell-Cell Aggregation. *International Journal of Food Microbiology*, *265*, 65–73. https://doi.org/10.1016/j.ijfoodmicro.2017.10.031

Iversen, C., & Forsythe, S. (2003). Risk Profile of *Enterobacter sakazakii*, an Emergent Pathogen Associated with Infant Milk Formula. *Trends in Food Science & Technology*, *14*, 443–454. https://doi.org/10.1016/S0924-2244(03)00155-9

Jacobs, C., Braun, P., & Hammer, P. (2011). Reservoir and Routes of Transmission of *Enterobacter sakazakii* (*Cronobacter* spp.) in a Milk Powder-Producing Plant. *Journal of Dairy Science*, *94*, 3801–3810. https://doi.org/10.3168/jds.2011-4318

Jay, J. M., Loessner, Martin, & Golden, David. (2005). *Modern Food Microbiology* (7th ed.). Aspen Publ.

Jongenburger, I., Reij, M. W., Boer, E. P. J., Gorris, L. G. M., & Zwietering, M. H. (2011). Actual Distribution of *Cronobacter* spp. in Industrial Batches of Powdered Infant Formula and Consequences for Performance of Sampling Strategies. *International Journal of Food Microbiology*, *151*, 62–69. https://doi.org/10.1016/j.ijfoodmicro.2011.08.003

Joseph, S., Desai, P., Ji, Y., Cummings, C. A., Shih, R., Degoricija, L., Rico, A., Brzoska, P., Hamby, S. E., Masood, N., Hariri, S., Sonbol, H., Chuzhanova, N., McClelland, M., Furtado, M. R., & Forsythe, S. J. (2012). Comparative Analysis of Genome Sequences Covering the Seven *Cronobacter* Species. *PLoS ONE*, *7*, e49455. https://doi.org/10.1371/journal.pone.0049455

Kalyantanda, G., Shumyak, L., & Archibald, L. K. (2015). *Cronobacter* Species Contamination of Powdered Infant Formula and the Implications for Neonatal Health. *Frontiers in Pediatrics*, *3*. https://doi.org/10.3389/fped.2015.00056

Kandhai, M. C., Heuvelink, A. E., Reij, M. W., Beumer, R. R., Dijk, R., van Tilburg, J. J. H. C., van Schothorst, M., & Gorris, L. G. M. (2010). A Study Into the Occurrence of

*Cronobacter* spp. in The Netherlands between 2001 and 2005. *Food Control*, *21*, 1127–1136. https://doi.org/10.1016/j.foodcont.2010.01.007

Kilonzo-Nthenge, A., Rotich, E., Godwin, S., Nahashon, S., & Chen, F. (2012). Prevalence and Antimicrobial Resistance of *Cronobacter sakazakii* Isolated from Domestic Kitchens in Middle Tennessee, United States. *Journal of Food Protection*, *75*, 1512–1517. https://doi.org/10.4315/0362-028X.JFP-11-442

Kim, H., Ryu, J.-H., & Beuchat, L. R. (2006). Attachment of and Biofilm Formation by *Enterobacter sakazakii* on Stainless Steel and Enteral Feeding Tubes. *Applied and Environmental Microbiology*, *72*, 5846–5856. https://doi.org/10.1128/AEM.00654-06

Kim, H., Ryu, J.-H., & Beuchat, L. R. (2007). Effectiveness of Disinfectants in Killing *Enterobacter sakazakii* in Suspension, Dried on the Surface of Stainless Steel, and in a Biofilm. *Applied and Environmental Microbiology*, *73*, 1256–1265. https://doi.org/10.1128/AEM.01766-06

Kumar, C. G., & Anand, S. K. (1998). Significance of Microbial Biofilms in Food Industry: A Review. *International Journal of Food Microbiology*, *42*, 9–27. https://doi.org/10.1016/S0168-1605(98)00060-9

Lin, L., & Beuchat, L. (2007). Survival of *Enterobacter sakazakii* in Infant Cereal as Affected by Composition, Water Activity, and Temperature. *Food Microbiology*, *24*, 767–777. https://doi.org/10.1016/j.fm.2007.02.001

Lu, Y., Liu, P., Li, C., Sha, M., Fang, J., Gao, J., Xu, X., & Matthews, K. R. (2019). Prevalence and Genetic Diversity of *Cronobacter* Species Isolated from Four Infant Formula Production Factories in China. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.01938

Mullane, N., Healy, B., Meade, J., Whyte, P., Wall, P. G., & Fanning, S. (2008). Dissemination of *Cronobacter* spp. (*Enterobacter sakazakii*) in a Powdered Milk Protein Manufacturing Facility. *Applied and Environmental Microbiology*, *74*, 5913–5917. https://doi.org/10.1128/AEM.00745-08

Nazarowec-White, M., & Farber, J. M. (1997). Thermal Resistance of *Enterobacter sakazakii* in Reconstituted Dried-Infant Formula. *Letters in Applied Microbiology*, *24*, 9–13. https://doi.org/10.1046/j.1472-765X.1997.00328.x

Nurjanah, S., Dewanti-Hariyadi, R., Estuningsih, S., & Suhartono, M. T. (2014). Stability and Growth Characteristics of GFPuv-labeled *Cronobacter sakazakii* Isolated from Foods. *Food Science and Biotechnology*, *23*, 1491–1496. https://doi.org/10.1007/s10068-014-0204-3

Nurjanah, S., Sulistyanti, S. T., & Dewanti-Hariyadi, R. (2015). Application of GFPuv Labeled *Cronobacter sakazakii* for Evaluation of its Survival during Cornstarch Processing. *World Journal of Engineering and Technology*, *3*, Article 3. https://doi.org/10.4236/wjet.2015.33B001

Nutrition, C. for F. S. and A. (2024). Infant Formula. *FDA*. https://www.fda.gov/food/resources-you-food/infant-formula

Oh, S., Chen, P., & Kang, D. (2007). Biofilm Formation by *Enterobacter sakazakii* Grown in Artificial Broth and Infant Milk Formula on Plastic Surface. *Journal of Rapid Methods & Automation in Microbiology*, *15*, 311–319. https://doi.org/10.1111/j.1745-4581.2007.00103.x

Osaili, T. M., Shaker, R. R., Al-Haddaq, M. S., Al-Nabulsi, A. A., & Holley, R. A. (2009). Heat Resistance of *Cronobacter* species (*Enterobacter sakazakii*) in Milk and Special Feeding

Formula. *Journal of Applied Microbiology*, *107*, 928–935. https://doi.org/10.1111/j.1365-2672.2009.04271.x

Osaili, T. M., Shaker, R. R., Ayyash, M. M., Al-Nabulsi, A. A., & Forsythe, S. J. (2009). Survival and Growth of *Cronobacter* Species (*Enterobacter sakazakii*) in Wheat-Based Infant Follow-on Formulas. *Letters in Applied Microbiology*, *48*, 408–412. https://doi.org/10.1111/j.1472-765X.2008.02541.x

Phair, K., Pereira, S. G., Kealey, C., Fanning, S., & Brady, D. B. (2022). Insights into the Mechanisms of *Cronobacter sakazakii* Virulence. *Microbial Pathogenesis*, *169*, 105643. https://doi.org/10.1016/j.micpath.2022.105643

Redmond, E. C., & Griffith, C. J. (2009). The Importance of Hygiene in the Domestic Kitchen: Implications for Preparation and Storage of Food and Infant Formula. *Perspectives in Public Health*, *129*, 69–76. https://doi.org/10.1177/1757913908101604

Reich, F., König, R., Von Wiese, W., & Klein, G. (2010). Prevalence of *Cronobacter* spp. in a Powdered Infant Formula Processing Environment. *International Journal of Food Microbiology*, *140*, 214–217. https://doi.org/10.1016/j.ijfoodmicro.2010.03.031

Sadeghinezhad, E., Kazi, S. N., Badarudin, A., Zubair, M. N. M., Dehkordi, B. L., & Oon, C. S. (2013). A Review of Milk Fouling on Heat Exchanger Surfaces. *Reviews in Chemical Engineering*, *29*. https://doi.org/10.1515/revce-2013-0003

Scott, E. (1996). Foodborne Disease and Other Hygiene Issues in the Home. *Journal of Applied Bacteriology*, *80*, 5–9. https://doi.org/10.1111/j.1365-2672.1996.tb03181.x

Yang, H.-Y., Kim, S.-K., Choi, S.-Y., You, D.-H., Lee, S.-C., Bang, W.-S., & Yuk, H.-G. (2015). Effect of Acid, Desiccation and Heat Stresses on the Viability of *Cronobacter sakazakii*

During Rehydration of Powdered Infant Formula and in Simulated Gastric Fluid. *Food Control*, *50*, 336–341. https://doi.org/10.1016/j.foodcont.2014.09.012

Chapter 3

# Planktonic Attachment of *Cronobacter sakazakii* to Infant Formula Fouled Stainless Steel[1]

[1]McGwin, J., Dev Kumar, G., and Gratzek, J. To be submitted to *Journal of Food Protection*

**Abstract**

*Cronobacter sakazakii* is an uncommon, yet often fatal pathogen commonly linked to recalls in the powdered infant formula industry. Its ability to survive in low water activity environments, attach to a wide variety of surfaces, and form robust biofilms on processing equipment raise food safety concerns. This study evaluated the attachment profiles of three strains of *C. sakazakii* on stainless steel surfaces fouled with infant formula solution, specifically whey (WBF) and soy based (SBF) applied at increasing levels. Strains were transformed with the pGFPuv plasmid to express fluorescence, and fouled surfaces were characterized by quantifying the fouled protein content using the bicinchoninic acid (BCA) assay and measuring browning intensity at 420nm. When bacterial attachment was assessed, attachment on medium foulant (5.63 logCFU/coupon) and high foulant (5.63 log CFU/coupon) was significantly higher than the clean coupon of 5.20 log CFU/coupon. Notably, Alpha (72.59%) and Gamma (70.55%) strains exhibited higher attachment rates than Beta (60.89%). Additionally, Alpha attached significantly higher than the control at the lowest foulant level ($p < 0.05$). Correlation analysis indicated moderate to strong positive correlation between protein content, browning, bacterial attachment and attachment rate with attachment on SBF with Alpha demonstrating the highest correlation ($r = 0.81$) with protein. These findings suggest that even minimal foulant build up on heat exchanger and other processing surfaces can create niches for *C. sakazakii* to attach, proliferate, and contaminate downstream processes. This presents an increased need for monitoring system cleanliness and emphasizing the need for stringent sanitation protocols.

## Introduction

*Cronobacter sakazakii* is a rare but often fatal pathogen found naturally in the environment that is most commonly associated with powdered infant formula (PIF), but also powdered milk, spices, and teas (Cechin et al., 2023). The defining characteristic of this bacteria is the fact that it can survive in low water activity environments and exhibits a range of tolerance to environmental stressors including chemical, thermal, osmotic, and desiccation (Chauhan et al., 2023; Dancer et al., 2009; Kim et al., 2006, 2007). While all age groups are susceptible to infections from *Cronobacter,* infants are most commonly at risk due to underdeveloped immune systems and the consumption of infant formula. While only 2-4 cases are reported a year, incidences are suspected to be higher due to the lack of reporting (CDC, 2024). Over a 20-year time span, the Center for Disease Control and Prevention (CDC) received 76 reports of *Cronobacter* infections in infants and neonates with around 40% of those ending fatally (CDC, 2024; Strysko et al., 2020).

Experts have hypothesized the route of contamination of PIF to be from the environment to processing facilities. More specifically, tracing strains from environments surrounding processing facilities into manufacturing space (Craven et al., 2010). Additionally, since *Cronobacter* can tolerate dry environments, focus has been put on controlling air flow in spray driers used in manufacturing. *Cronobacter* has been found in air filters, vacuums, roller driers, and fans (Jacobs et al., 2011; Lu et al., 2019; Mullane et al., 2008).

PIF is often made through two different methods: wet blend and dry blend. In the dry process, plants receive all materials in their dry state and blend and package them in the facility without any heat treatment. In the wet blend process, materials are received and pasteurized and spray-dried in the plant. Due to the high protein and solids content of infant formula in the wet

blending process, the incidence of heat exchanger foulant build-up is much higher, providing a possible vector of contamination and niche point for bacteria to survive and proliferate if not cleaned adequately. This is also an issue facing the manufactures of the dry ingredients that are spray dried then used in the dry blending process. However, this depends on the ingredient and the product. Although data indicates that *C. sakazakii* should be killed by typical high temperature short time pasteurization (HTST), the questions arise of how the formula is become contaminated, especially in recalls associated with aseptic products as well (Nazarowec-White & Farber, 1997). Because PIF is not a sterile product, contamination with bacteria is not uncommon. However, due to the serious threat of the pathogen, the Food and Drug Administration requires that there by <1 CFU *Cronobacter* spp. in 100 grams of powder (Nutrition, 2024).

The process of bacterial attachment is multifaceted and on relies of several factors including bacterial physiology, growth state, surface medium, temperature, and fluid flow (Frank, 2001; Tuson & Weibel, 2013). The presence of foulant on stainless steel surfaces provides an increased surface area for bacterial attachment, as well as harborage sights for bacteria to further be protected from high temperatures and chemicals used in continuous thermal processing and clean-in-place (CIP) procedures. *Cronobacter* has been shown to attach to a wide variety of surfaces, including stainless steel, glass, feeding tubes, and PVC (Iversen et al., 2004; Kim et al., 2006). Additionally, it can form vigorous biofilms that can become more difficult to remove and clean. The combination of bacteria attachment and foulant from heat exchangers creates the optimal harborage site for *C. sakazakii.*

The objective of this study was to evaluate the attachment profile of three strains of *C. sakazakii* for their ability to attach to surfaces fouled with solutions of powdered infant and determine if heat exchanger foulant could cause an increased risk for manufactures.

## Materials and Methods

Three strains of *C. sakazakii* (see Table 3-1) were obtained from the University of Georgia Center for Food Safety (UGA-CFS) Culture Collection (Griffin, GA, USA) by taking one loopful (10 µL) (Copan, Nurrieta, CA, USA) from the frozen glycerol stock and streaking on tryptic soy agar (TSA; Neogen, Lansing, MI, USA) and incubating at 37°C for 24 h. Stock cultures were maintained at -80°C in 10% glycerol. Cultures were stored at 4°C on TSA plates when not in use. Prior to use in experiments, each strain was cultured on TSA for 18-24 hours at 37°C.

Each strain was confirmed to be *C. sakazakii* by DNA isolation using ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research Corp., Irvine, CA, USA) following the manufacturer's instructions. DNA quantity and purity were measured on a multimode reader using a Take 3 plate (Biotek Cytation 3, Agilent Technologies, Inc., Santa Clara, CA). The extracted DNA was sent for third party sequencing (Plasmidsaurus, Eugene, Oregon, USA).

*Table 3-1: Strains of C. sakazakii used in this study*

| Strain ID | Name | Source | Referred to as: |
|---|---|---|---|
| **3396** | *Cronobacter sakazakii* | Environmental | Alpha (CSE) |
| **4921** | *Cronobacter sakazakii* | Infant formula | Beta (CSIF) |
| **4923** | *Cronobacter sakazakii* | Clinical, infant | Gamma (CSC) |

**pGFPuv transformation**

To transform each isolate of *Cronobacter*, competent cells were first prepared by inoculating 10mL of tryptic soy broth (TSB; Neogen, Lansing, MI, USA) by picking two colonies from a TSA plate and incubating at 37°C for 18-24 h at 150 rpm. After 24 h, each isolate was then sub cultured into 50 mL of TSB by taking 250 µL of the overnight culture and inoculating into the TSB. The cultured were then grown to mid-exponential stage until an $OD_{600}$ of ~0.8 was reached, about 4 h, and then placed on ice to chill. The cultured were then centrifuged for 5 min at 4000 rpm and the supernatant was discarded. The tubes were kept on ice until the next step. The cell pellets were resuspended in 10% ice cold glycerol, pipette mixed and transferred into a 1.5 mL microcentrifuge tube. The tubes were then centrifuged at 12,000 × g for 1 min, supernatant discarded, and glycerol washes and centrifuging repeated three more times, storing the tubes on ice when not being processed. Following the last wash, the pellets were resuspended in 10% glycerol and now ready to be used for electrotransformation. Cells were stored on ice until use.

Electrotransformation protocols were adapted from Dev Kumar et al (2017). To transform the cells, 58 µL of competent cells were added to a sterilized microcentrifuge tube along with 2 µL of pGFPuv plasmid isolated from an *E. coli* O101 isolate. The cells and plasmid were gently vortexed and kept on ice. Next, 60 µL of the cells and plasmid were transferred to the electro transformation cuvette and loaded into the chamber with treatment conditions of 2.5 kV, 20 µF, 200 Ω, and 2 mm gap (Bio-Rad, Hercules, CA). Immediately following electroporation, the cells were transferred to 450 µL of SOC broth (2% Bacto Trytone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) pre warmed to 37°C and incubated while shaking at 100 rpm for 1 h. Following incubation, 100 µL of culture was spread

plated onto TSA supplemented with ampicillin (100 µg/mL) and incubated at 37°C for 18-24 h. Successive transformants were confirmed by viewing the plate under UV at 352 nm and picking fluorescent colonies and re-streaking onto TSA-A. Sub cultures were repeated is TSB-A or TSA-A from 18-24 hours at 37°C to ensure plasmid stability

**Culture Prep**

Before each experiment, a fluorescent isolated colony from a fresh culture plate of TSA-A was streaked onto a new TSA-A plate and incubated at 37°C for 24 h. The inoculum was prepared by taking 2 loopfuls (10 µL) of cells and suspending in 20 mL of sterile 1× phosphate buffered saline (PBS; VWR Chemicals LLC, Solon, OH, USA). The process was repeated for each isolate. The stock concentration was determined by plating serial dilutions using the modified droplet plate method on TSA-A and incubated for 24 h at 37°C.

**Model Fouled Surface**

Stainless steel coupons (Type 304, 4 cm × 2 cm) were washed with lab detergent and air-dried. Coupons were autoclaved at 121°C for 15 min and stored until used. Two brands of PIF were chosen to create the model fouled surfaces, one whey-based (Mead Johnson & Co., Evansville, IN, USA) and one soy-based (Kroger, Lexington, KY, USA) (see Figure 3-1). Model fouling fluid (MFF) was created by mixing 12.5 g of infant formula with 25 g of sterile deionized water to replicate the conditions that would feed a spray drier (Drapala et al., 2017). Formulas were stirred until completely dissolved and used immediately. Coupons were fouled by placing them on a hot plate set to 120°C until the surface was confirmed to reach 90°C with an infrared thermometer. A control coupon was placed on the hot plate as well to ensure consistency in

temperature. Three levels of foulant were used for this study: low, medium, and high. For the low, 200 µL of MFF was pipetted onto the coupon and immediately removed. For medium foulant level, 200 µL was placed on the coupon, allowed to dry, and another 200 µL placed atop and heated until completely fouled, about 3 min. For high foulant level, the process was repeated for the medium with another 200 µL of MFF placed on top for a total of 600 µL. The process was repeated for each formula. Coupons were stored in a dehydrator (Avantco, Meridian, Idaho, USA) at 40°C overnight and stored in a sterile bag until use.

**Whey-Based Formula** **Soy-Based Formula**

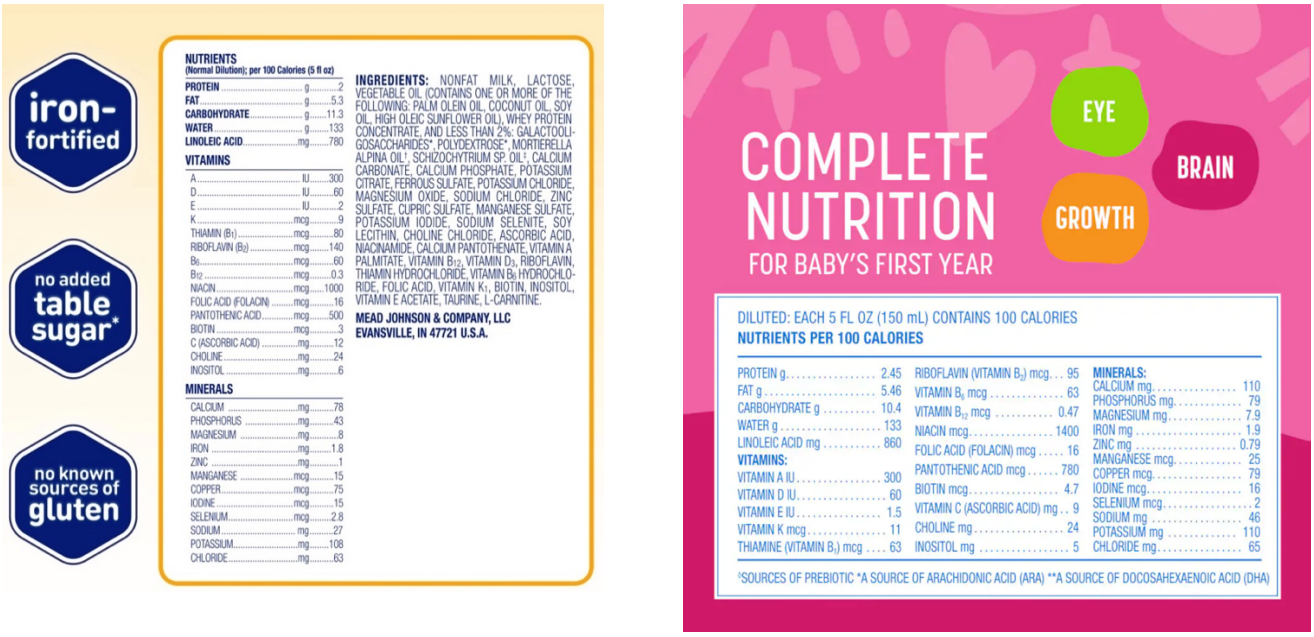


*Figure 3-1: Infant formula nutrition facts of the commercial sources of foulant*

**Protein Quantification**

Protein quantification methods for fouled stainless steel coupons was adapted from Shah et al (2024). Fouled coupons from above were placed into a 7oz WhirlPak® bag (Nasco, Modesto, CA, USA) with 10mL of 2% NaOH and allowed to sit at room temperature for 1 h, agitating every 15 min to ensure all the foulant is detached. Then, 1 mL of fouled NaOH was

aliquoted into microcentrifuge tubes for protein analysis. Protein content on the coupon was quantified using the Pierce™ BCA protein assay kit (ThermoFisher Scientific Inc., Waltham, MA, USA). Briefly, the fouled NaOH was diluted by 1:10 with deionized water. Then, 25 µL of the diluted sample was added to a 96-well polyvinyl chloride microtiter plate (Corning Inc., Corning, NY, USA) in triplicate along with 200 µL of the BCA reagent (proprietary) and manually swirled for 30 seconds. The plate was then incubated at 37°C for 30 minutes and then read on the plate reader (Biotek Cytation 3, Agilent Technologies, Inc., Santa Clara, CA) at 562 nm. Readings were calculated against a standard curve of bovine serum albumin (BSA) to get mg protein/coupon of y = 1.034x – 0.2875. This reaction is based on the ability of peptide bonds in protein to reduce $Cu^{2+}$ ions from the copper (II) sulfate to $Cu^{1+}$. Two molecules of BCA chelate with each $Cu^{2+}$, forming a purple color that absorbs the light at 562 nm.

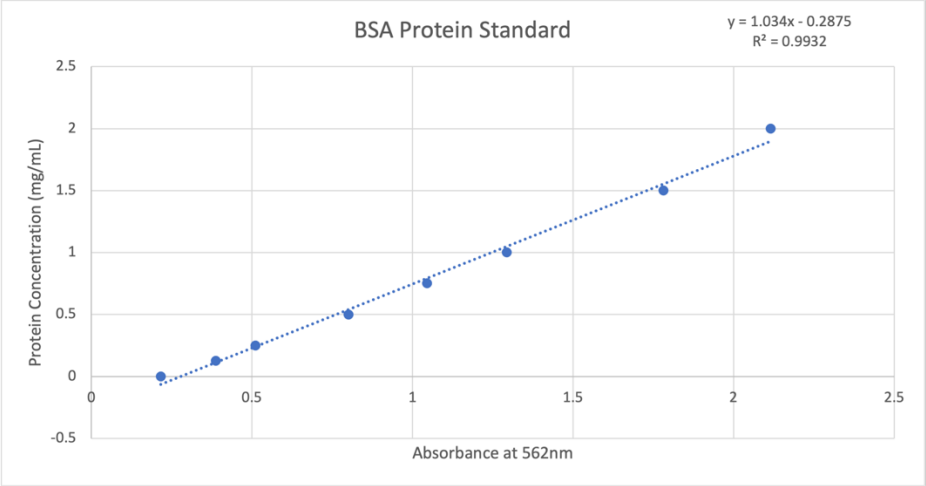


*Figure 3-2: Protein standard curve of the bovine serum protein assay used in this project*

**Foulant Color**

Foulant color was analyzed using a method adapted from Phinney et al (2017). Using the fouled NaOH from above, 200 µL was pipetted into a 96 well plate in triplicate and read at 420 nm. Absorbance at 420 nm has been shown to be indicative of browning (Phinney et al., 2017; Serra-Cayuela et al., 2013).

**Coupon Inoculation**

Fouled coupons from above were inoculated with the *C. sakazakii* suspensions by placing the culture into the bottom of a sterile Petri dish (VWR, Radnor, PA, USA) and placing a coupon fouled side down and placing on the shaking incubator at 20°C for 15 s at 55 rpm. Following inoculation, the coupons are removed from the inoculum with sterile forceps and placed on an absorbent pad, then rinsed with 10 mL of sterile distilled water using a serological pipette; 5 mL on each side. Coupons were then immediately placed into a 24 oz WhirlPak® bag (Nasco, Modesto, CA, USA) bags with 100mL 1× PBS and sonicated for 2 minutes and allowed to sit at room temperature for one hour to detach all foulant. Coupons were hand massaged to detach the foulant.

Suspensions were aliquoted, diluted and drop plated onto TSA-ampicillin (100µg/mg) plates and incubated for 18-24 h at 37°C. Attachment was quantified as log CFU per coupon using the modified droplet plate method. Experiments were repeated in 3 biological and 3 technical reps (n=9).

**Statistical Analysis**

A full factorial design was used to design and preform this experiment (n = 9). All data was analyzed in JMP Pro 17 (SAS Institute Inc., Cary, NC, USA) at a significance level of $p<0.05$. One way analysis of variance (ANOVA) was done to determine the effect of strain, formula, and foulant level on bacterial attachment separately. Tukey HSD was used to compare means for significance. Results for bacterial attachment were reported as both log CFU/coupon and attachment rate, calculated using the following formula:

$$Attachment\ rate\ (\%) = (\frac{logCFU\ attachment\ to\ coupon}{mean\ logCFU\ of\ PBS\ stock}) \times\ 100$$

One way ANOVA was done to determine significant difference among the protein quantification and absorbance at 420 nm. Pearson's Correlation Test was used to determine if there was a significant correlation between protein content, foulant color, and bacterial attachment.

**Results**

**Protein Quantification and Absorbance at 420nm**

The protein content recovered from the fouled coupons when pooled together was 34.90 ± 7.16 mg/coupon for the soy-based formula (SBF) and 40.35 ± 14.09 mg/coupon for the whey-based formula (WBF) (Figure 3-3). These values were not significantly different ($p>0.05$). When split between levels (low, medium, and high), the protein content significantly differed ($p<0.0001$) from each level. When grouped together as a whole between both formulas, low level has 24.56 ± 1.25 mg/coupon, medium 39.59 ± 3.46 mg/coupon, and high 48.73 ± 8.49 mg/coupon. Likewise, when split by formula and level, low for both formulas were significantly

different ($p<0.05$) from medium for both formulas and high for the soy-based formula. All samples were significantly less than high for the whey-based formula ($p<0.05$).

For absorbance at 420nm, values were significantly different ($p<0.001$). For WBF the average was 2.76 ± 0.22 and for SBF it was 2.14 ± 0.30 (Figure 3-4). For the SBF, values between low and medium were significantly different, increasing from 1.73 ± 0.04 for low to 2.32 ± 0.01 for medium. The high level was not significantly different ($p>0.05$) from medium at 2.36 ± 0.02. However, when grouped by level, low level was significantly less than high ($p=0.001$) and medium ($p=0.0173$). High was not significantly different than medium ($p=0.53$). As a whole, all values significantly differed from each other aside from medium and high for the SBF.

### *Cronobacter sakazakii* Attachment to Clean and Fouled Coupons

Both strain of *C. sakazakii* ($p<0.0001$), formula ($p<0.01$), and level ($p<0.0001$) were significantly different variables. As previously stated, bacterial attachment was reported as both log CFU/coupon and as overall attachment rate. This is due in part to normalize the data across differing stock concentrations due to variable physical characteristics of the three strains used. Bacterial attachment for Alpha, Beta, and Gamma were 5.85 ± 0.21, 4.54 ± 0.72, and 6.16 ± 0.31 log CFU/coupon, respectively (Figure 3-5). These values differed significantly ($p<0.05$). When reported as attachment rate, both Alpha and Gamma differed significantly from Beta ($p<0.001$) but not from each other. Values were 72.59 ± 2.51%, 60.89 ± 8.39%, and 70.55 ± 3.54% for Alpha, Beta, and Gamma, respectively (Table 3-3).

### Attachment by Formula

In comparing the clean and fouled coupons, control coupons had an average attachment of 5.21 ± 0.83 log CFU/coupon. Average attachment to coupons fouled with WBF was 5.70 ± 0.67 log CFU/coupon (Figure 3-6), while SBF was 5.44 ± 0.96 log CFU/coupon (Figure 3-7). Overall, attachment rate on the un-fouled coupons and both the WBF and SBF was significantly less ($p<0.0001$) with mean attachment rate on control coupons of 63.17% and 69.77% on WBF and 67.86% on SBF (Table 3-3). On WBF, Alpha and Gamma did not significantly differ in attachment or attachment rate, but both strains did differ in both metrics from the Beta strain. However, on the SBF, each strain was significantly different from one another in terms of attachment. When reported as attachment rate, the same trend as the WBF was seen where Alpha and Gamma did not significantly differ from each other, but both were significantly different from Beta.

For the Alpha strain, all three levels of foulant were significantly greater than the control clean coupon with a mean attachment of 5.50 log CFU/coupon on the clean coupon and 5.88 log CFU/coupon on low level, 5.94 log CFU/coupon on medium, and 5.91 log CFU/coupon on high level. Attachment between the WBF and SBF did not significantly differ for the Alpha strain, as well as between the three levels (Figure 3-8).

For the Beta strain, attachment did not differ between the control coupon and any of the three levels. However, when observed as attachment rate, coupons with high level of foulant had significantly higher attachment than the control, 64.99% compared to 53.76%, respectively ($p<0.05$). Likewise, attachment rate on the Beta strain significantly differed between the WBF (64.42 ± 9.62%) and SBF (59.73 ± 3.41%).

Finally for the Gamma strain, attachment to the clean coupon was 5.83 ± 0.26 log CFU/coupon, which was significantly less than both medium average for WBF and SBF (6.34 ±

0.10 log CFU/coupon) and high average for WBF and SBF (6.18 ± 0.41 log CFU/coupon) (Figure 3-4). A low level of foulant was not significantly different from the control for both formulas. Neither attachment nor attachment rate differed between formulas for the Gamma strain.

**Attachment by Level**

In observing the attachment by level of foulant, all three strain and both formulas did not see any notable trends in increasing attachment or attachment rate with increasing the levels of foulant. However, medium and high level of fouling did see significantly higher attachment and attachment rate ($p<0.05$) than the control (Figure 3-5). When expressed as attachment rate, low level did attach as a significantly higher rate. With the Alpha strain, all levels on both formulas attached significantly higher than the control ($p <0.05$). However, with Beta, only high-level foul on WBF attached significantly more. Likewise for Gamma, the only levels that were significantly more were both formulas for medium level and high-level soy (Figure 3-5).

**Correlation between Protein Content, Absorbance at 420nm, Attachment, and Attachment Rate**

For correlation, attachment and attachment rate values for none, low, medium, and high foul levels were used. For the Alpha strain on WBF and SBF, both attachment ($r = 0.6306$) and attachment rate ($r = 0.6492$) were moderately correlated with absorbance at 420nm. Likewise, with protein content, attachment ($r = 0.5192$) and attachment rate ($r = 0.4984$) were both moderately correlated. Weak correlations were observed for attachment ($r = 0.3533$) and attachment rate ($r = 0.4437$) with the Beta strain and absorbance at 420nm. Similarly with

protein content, very weak correlations were observed for attachment and attachment rate. Lastly with Gamma, weak correlations were observed for all parameters ($r < 0.4200$).

On the WBF, however, correlation for the Alpha and Gamma Strains was notably higher between attachment, attachment rate, and absorbance at 420nm. On WBF, attachment had a strongly positive correlation with absorbance ($r = 0.7260$). With Gamma, attachment was also moderately positively correlated with protein ($r = 0.5683$) and absorbance ($r = 0.6529$).

On the SBF, a strong positive correlation was seen for attachment with Alpha and protein ($r = 0.8123$) and absorbance ($r = 0.8033$). Additionally with Gamma, a weak positive correlation was seen for attachment and protein ($r = 0.5120$) and absorbance ($r = 0.4964$).

**Discussion**

This study sought to compare two different infant formulas fouled with three different levels and the subsequent attachment by *C. sakazakii*. Both formulas did see a higher attachment and attachment rate than the un-fouled coupons. These finding are supported by previous studies that reported *Listeria monocytogenes* attached to stainless steel surfaces with different dairy products (Poimenidou et al., 2009). Likewise, with *Cronobacter*, *L. monocytogenes* is known for its ability to contaminate dairy products, especially in post-heat treatment conditions. Additionally, because *Listeria* is a heat-sensitive organism, the presence is indicative of poor sanitation measures. Differences in attachment to levels of foulant on coupons could be attributed to several different factors. No difference was seen in attachment between the whey-based formula and soy based ($p > 0.05$). Strain and type of formula were however significant with notable significant difference between attachment on the SBF between each strain. So, strain of bacteria plays an important role in the risk associated with bacteria attachment on SBF. Other studies have observed similar differences in attachment to surfaces between strains of *C.*

*sakazakii* with values ranging from 2.92-4.19 log CFU/cm$^2$ at 12°C and 3.71-4.62 log CFU/cm$^2$ for SS coupons immersed in infant formula broth for 4 hours (Kim et al., 2006). Differences in attachment can also be attributed to cell growth state and growth medium (Frank, 2001; Kim et al., 2006).

Interestingly, even with just 15 seconds of inoculation at 20°C in this study, the attachment was significantly higher than observed in other studies. Iversen et al (2004) found that *Cronobacter* grown in infant formula formed more biofilm on latex and silicon compared to stainless steel. This points for the continued need for sanitation and hygiene in the home when preparing infant formula (Chan, 2016; Jo et al., 2010; Kim et al., 2006). Other researchers have shown various disinfectants had varied effects of inactivation on *C. sakazakii* when in the presence of infant formula on SS (Kim et al., 2007). Not only did infant formula increase the resistance of planktonic cells to disinfections, but biofilms also dried to the surface with infant formula were further resistant. In continuous thermal processing, sanitizer is often the final step before a final water rinse in CIP, so if foulant is still present and cells are attached or in the biofilm state, they still can persist the disinfectant treatment.

However, it is of interest that for the Alpha strain, even the coupon with the lowest level of attachment had significantly higher attachment than the un-fouled coupon. Significantly higher attachment was only observed in the medium and high levels for both Beta and Gamma, on both the WBF and SBF. Conversely, Barnes et al (1999) found that SS coupons treated with skim milk was found to reduce the adhesion of *Staphylococcus aureus, L. monocytogenes,* and *E. coli.* This study did only exhibit adhered proteins and not foulant, so likely the surface area created by the foulant is the cause for increased attachment. It is of interest though that a small level of foulant (200 μL liquid formula) did cause a significantly higher attachment. In a study

done with *Listeria,* it was shown that surface roughness of stainless steel is not significantly correlated with initial attachment or biofilm formation (Rodriguez et al., 2008). However, hygienic shortcomings such as bad welds, nicks in surfaces, or dead ends are all possible harborage sights for bacteria in processing environments (Inuwa et al., 2017).

**Protein and Absorbance at 420 nm**

Protein and color analysis were chosen as physical characterizations of the model fouls to give a quantitative representation of increased protein content on the coupons, as well as increased browning as the amount of foulant increased on the coupons. In observing the protein content, all formulas and levels were significantly higher than the low level for the WBF and SBF. The medium level for both formulas were not significantly different. The whey formula on the high level had the higher protein content of all levels with 56.26 mg/coupon.

Increasing the amount of infant formula on each fouled coupon significantly increased the foulant color by reading the absorbance of the fouled NaOH at 420 nm. The WBF had significantly more browning at each level, indicating more Maillard browning taking place while the coupons are being heated. The WBF contained more carbohydrates per gram, as well as included lactose which undergoes caramelization when heated. Additionally, the whey and milk proteins present in the formula can further react with the reducing sugar (lactose) to complete the Maillard reaction. The SBF does not contain lactose and contained less carbohydrates per gram. The formula itself had more a brown-yellow color on the coupon but browned significantly less than the WBF. For this formula, both protein content and browning do not increase as more formula is fouled to the surface. This could be due in part to the plant-based proteins being de-functionalized as the temperature increases and reducing sugars not being present in as high

quantities as the WBF (Liu et al, 2014). Additionally, for the SBF, no significant difference was found between the medium and high levels.

Wang et al (2018) that fouling mechanics between whey and soy proteins differs in both adhesion properties and removal. Soy fouling was shown to more porous and irregular and formed more quickly than whey fouls. However, soy proteins adhered more weakly even though it fouled more severely. This could partly be due in part to higher amounts of proteins available for heating and fouling but has yet to be fully extrapolated.

The Inclusion of the physical characterization of the fouled coupons sought to observe If there was a notable correlation between increased protein content and browned color through and *C. sakazakii* attachment. While no notable differences in bacteria attachment were observed between different foulant levels, the change in both protein content and foulant color were significantly different. Several other research have used these methods to aid in predicting the build of foulant in continuous systems to aid in process optimization and verification of cleaning procedures (Phinney et al., 2017; Shah et al., 2024). Phinney et al (2017) indicated that change in foulant color was a good predictor of remaining foulant in the system. This was achieved by profiling the color of the wash off from the caustic solution used in the CIP process. The results of this study indicant similar findings that increasing the amount of foulant in a system moderately correlates with increased bacteria attachment. Further research should be done to accurately model this approach in continuous systems. While the results indicant the correlation is strongly dependent on strain and formula type, this still could be a more widely adopted method of confirming the effectiveness of cleaning practices.

The limitations of this study include accurately modeling what happens on an industry level. Normally, fluid would be continuously flowing through heat exchangers and pipes and

foulant would build up over time under completely wet and enclosed environments. Because of the difficulty in accurately modeling and constructing that study, we sought to provide some baseline information about the behavior of the bacteria and its attachment properties. Future work should focus on performing a similar experiment on a larger scale using a continuous HTST/UHT system or a lab simulation to determine the effects of fluid flow, homogenization, foulant type, and temperature.

**Conclusion**

The results of this study suggest the presence of foulant causes an increased risk of bacterial contamination in the product of PIF. While no major differences existed between the two formulas, significantly higher attachment was observed from clean coupons to fouled coupon. Even with a preliminary rising step to remove loosely attached cells, the level of bacterial attachment was still high. For certain strains, bacterial was even able to attach significantly higher with even a small film of foulant on the coupon, point to the risk even with small amount of buildup in systems or on food contact surfaces. Good protocols should be implemented to monitor system cleanliness and determine when foulant to too much and placing too much strain on the system. Increased bacterial attachment in the niche points that foulant provides could lead to more contamination downstream.

**References**

Barnes, L.-M., Lo, M. F., Adams, M. R., & Chamberlain, A. H. L. (1999). Effect of Milk Proteins on Adhesion of Bacteria to Stainless Steel Surfaces. *Applied and Environmental Microbiology*, *65*, 4543–4548. https://doi.org/10.1128/AEM.65.10.4543-4548.1999

CDC. (2024, May 20). *About Cronobacter Infection*. *Cronobacter* Infection. https://www.cdc.gov/cronobacter/about/index.html

Cechin, C. D. F., Carvalho, G. G., Bastos, C. P., & Kabuki, D. Y. (2023). *Cronobacter* spp. in Foods of Plant Origin: Occurrence, Contamination Routes, and Pathogenic Potential. *Critical Reviews in Food Science and Nutrition*, *63*, 12398–12412. https://doi.org/10.1080/10408398.2022.2101426

Chan, M. Y.-K. (2016). Prevalence And Location of *Cronobacter* Species and *Enterobacteriaceae* in Households. *University of Georgia*, *Masters Thesis*.

Chauhan, R., Tall, B. D., Gopinath, G., Azmi, W., & Goel, G. (2023). Environmental Risk Factors Associated with the Survival, Persistence, and Thermal Tolerance of *Cronobacter Sakazakii* During the Manufacture of Powdered Infant Formula. *Critical Reviews in Food Science and Nutrition*, *63*, 12224–12239. https://doi.org/10.1080/10408398.2022.2099809

Craven, H. M., McAuley, C. M., Duffy, L. L., & Fegan, N. (2010). Distribution, Prevalence and Persistence of *Cronobacter* (*Enterobacter sakazakii*) in the Nonprocessing and Processing Environments of Five Milk Powder Factories: *Cronobacter* Milk Powder

Factories. *Journal of Applied Microbiology*, *109*, 1044–1052. https://doi.org/10.1111/j.1365-2672.2010.04733.x

Dancer, G. I., Mah, J.-H., & Kang, D.-H. (2009). Influences of Milk Components on Biofilm Formation of *Cronobacter* spp. (*Enterobacter sakazakii*). *Letters in Applied Microbiology*. https://doi.org/10.1111/j.1472-765X.2009.02601.x

Drapala, K. P., Auty, M. A. E., Mulvihill, D. M., & O'Mahony, J. A. (2017). Influence of Emulsifier Type on the Spray-Drying Properties of Model Infant Formula Emulsions. *Food Hydrocolloids*, *69*, 56–66. https://doi.org/10.1016/j.foodhyd.2016.12.024

Frank, J. F. (2001). Microbial Attachment to Food and Food Contact Surfaces. In *Advances in Food and Nutrition Research* (Vol. 43, pp. 319–370). Elsevier. https://doi.org/10.1016/S1043-4526(01)43008-7

Inuwa, A., Lunt, A., Czuprynski, C., Miller, G., & Rankin, S. A. (2017). Hygienic Shortcomings of Frozen Dessert Freezing Equipment and Fate of *Listeria monocytogenes* on Ice Cream–Soiled Stainless Steel. *Journal of Food Protection*, *80*, 1897–1902. https://doi.org/10.4315/0362-028X.JFP-17-178

Iversen, C., Lane, M., & Forsythe, S. J. (2004). The Growth Profile, Thermotolerance and Biofilm Formation of *Enterobacter sakazakii* Grown in Infant Formula Milk. *Letters in Applied Microbiology*, *38*, 378–382. https://doi.org/10.1111/j.1472-765X.2004.01507.x

Jacobs, C., Braun, P., & Hammer, P. (2011). Reservoir and Routes of Transmission of *Enterobacter sakazakii* (*Cronobacter* spp.) in a Milk Powder-Producing Plant. *Journal of Dairy Science*, *94*, 3801–3810. https://doi.org/10.3168/jds.2011-4318

Jo, S.-H., Baek, S.-B., Ha, J.-H., & Ha, S.-D. (2010). Maturation and Survival of *Cronobacter* Biofilms on Silicone, Polycarbonate, and Stainless Steel after UV Light and Ethanol

Immersion Treatments. *Journal of Food Protection*, *73*, 952–956. https://doi.org/10.4315/0362-028X-73.5.952

Kim, H., Ryu, J.-H., & Beuchat, L. R. (2006). Attachment of and Biofilm Formation by *Enterobacter sakazakii* on Stainless Steel and Enteral Feeding Tubes. *Applied and Environmental Microbiology*, *72*, 5846–5856. https://doi.org/10.1128/AEM.00654-06

Kim, H., Ryu, J.-H., & Beuchat, L. R. (2007). Effectiveness of Disinfectants in Killing *Enterobacter sakazakii* in Suspension, Dried on the Surface of Stainless Steel, and in a Biofilm. *Applied and Environmental Microbiology*, *73*, 1256–1265. https://doi.org/10.1128/AEM.01766-06

Kumar, G. D., Williams, R. C., Al Qublan, H. M., Sriranganathan, N., Boyer, R. R., & Eifert, J. D. (2017). Airborne Soil Particulates as Vehicles for *Salmonella* Contamination of Tomatoes. *International Journal of Food Microbiology*, *243*, 90–95. https://doi.org/10.1016/j.ijfoodmicro.2016.12.006

Liu, Q., Li, J., Kong, B., Li, P., & Xia, X. (2014). Physicochemical and Antioxidant Properties of Maillard Reaction Products Formed by Heating Whey Protein Isolate and Reducing Sugars. International Journal of Dairy Technology, 67, 220-228.

Lu, Y., Liu, P., Li, C., Sha, M., Fang, J., Gao, J., Xu, X., & Matthews, K. R. (2019). Prevalence and Genetic Diversity of *Cronobacter* Species Isolated from Four Infant Formula Production Factories in China. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.01938

Mullane, N., Healy, B., Meade, J., Whyte, P., Wall, P. G., & Fanning, S. (2008). Dissemination of *Cronobacter* spp. (Enterobacter *sakazakii*) in a Powdered Milk Protein Manufacturing

Facility. *Applied and Environmental Microbiology*, *74*, 5913–5917. https://doi.org/10.1128/AEM.00745-08

Nazarowec-White, M., & Farber, J. M. (1997). Thermal Resistance of *Enterobacter sakazakii* in Reconstituted Dried-Infant Formula. *Letters in Applied Microbiology*, *24*, 9–13. https://doi.org/10.1046/j.1472-765X.1997.00328.x

Nutrition, C. for F. S. and A. (2024). Infant Formula. *FDA*. https://www.fda.gov/food/resources-you-food/infant-formula

Phinney, D. M., Feldman, A., & Heldman, D. (2017). Modeling High Protein Liquid Beverage Fouling During Pilot Scale Ultra-High Temperature (UHT) Processing. *Food and Bioproducts Processing*, *106*, 43–52. https://doi.org/10.1016/j.fbp.2017.08.007

Poimenidou, S., Belessi, C. A., Giaouris, E. D., Gounadaki, A. S., Nychas, G.-J. E., & Skandamis, P. N. (2009). *Listeria monocytogenes* Attachment to and Detachment from Stainless Steel Surfaces in a Simulated Dairy Processing Environment. *Applied and Environmental Microbiology*, *75*, 7182–7188. https://doi.org/10.1128/AEM.01359-09

Rodriguez, A., Autio, W. R., & Mclandsborough, L. A. (2008). Effect of Surface Roughness and Stainless Steel Finish on *Listeria monocytogenes* Attachment and Biofilm Formation. *Journal of Food Protection*, *71*, 170–175. https://doi.org/10.4315/0362-028X-71.1.170

Serra-Cayuela, A., Aguilera-Curiel, M. A., Riu-Aumatell, M., Buxaderas, S., & López-Tamames, E. (2013). Browning During Biological Aging and Commercial Storage of Cava Sparkling Iine and the Use of 5-HMF as a Quality Marker. *Food Research International*, *53*(1), 226–231. https://doi.org/10.1016/j.foodres.2013.04.010

Shah, U., Rivero, W. C., Wang, Q., Zheng, H., & Salvi, D. (2024). Exploration of Plasma-Activated Water (PAW) as a Cleaning-In-Place (CIP) Solution for Fouling Removal and

Microbial Reduction. *Journal of Food Process Engineering*, *47*, e14669. https://doi.org/10.1111/jfpe.14669

Strysko, J., Cope, J. R., Martin, H., Tarr, C., Hise, K., Collier, S., & Bowen, A. (2020). Food Safety and Invasive *Cronobacter* Infections during Early Infancy, 1961–2018. *Emerging Infectious Diseases*, *26*, 857–865. https://doi.org/10.3201/eid2605.190858

Tuson, H. H., & Weibel, D. B. (2013). Bacteria–Surface Interactions. *Soft Matter*, *9*, 4368–4380. https://doi.org/10.1039/C3SM27705D

Wang, J., Li, L., Fu, N., Mercade-Prieto, R., & Chen, X. D. (2018). A Comparative Study on Fouling and Cleaning Characteristics of Soy Protein Isolate (SPI). *International Journal of Food Engineering*, *14*, 20170381. https://doi.org/10.1515/ijfe-2017-0381

## Figures

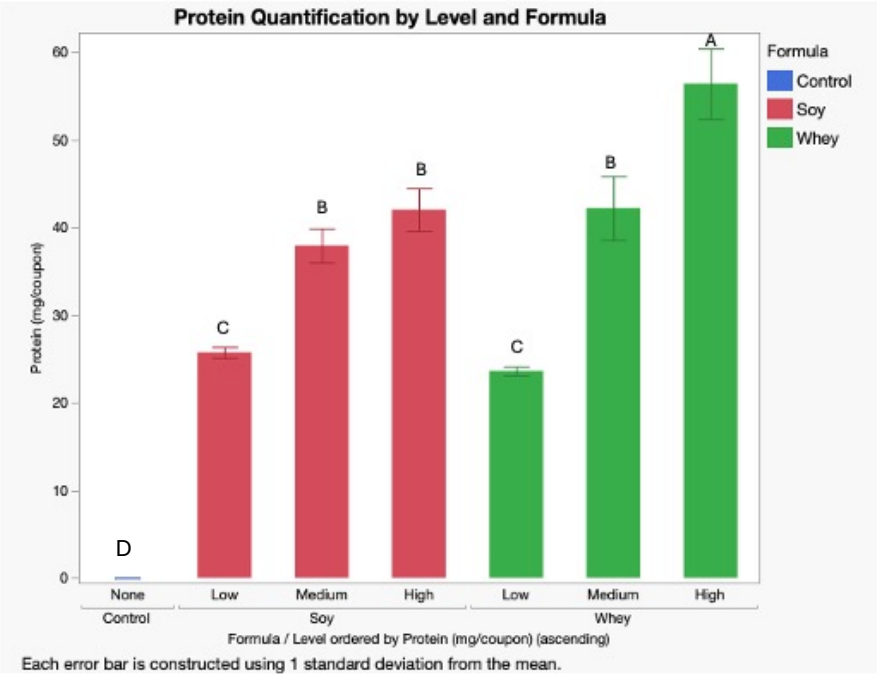


*Figure 3-3: Quantification of protein on fouled stainless steel coupons containing soy or whey-based formula*

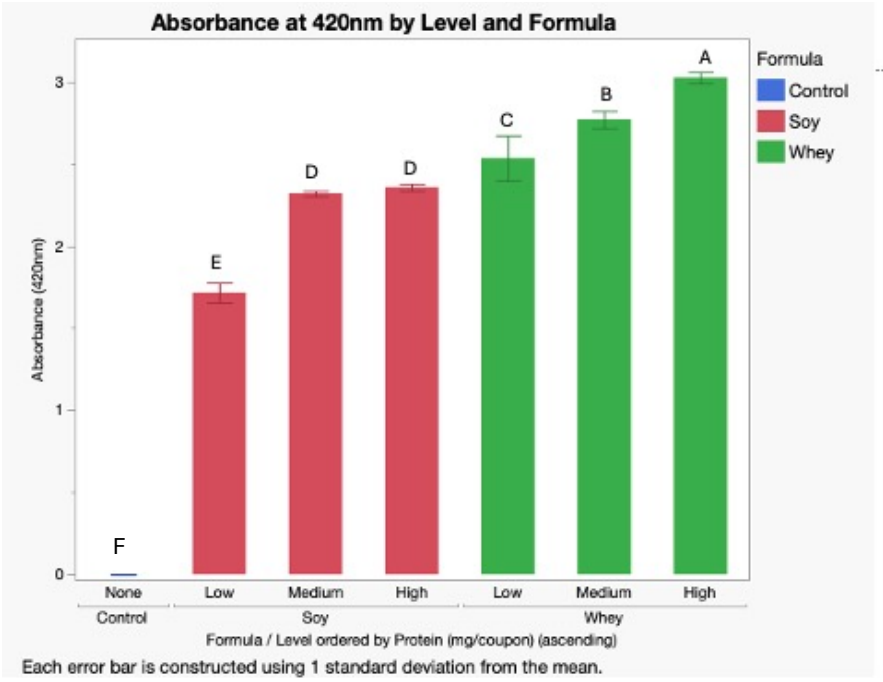


*Figure 3-4: Profiling the color of the wash of from fouled coupons*

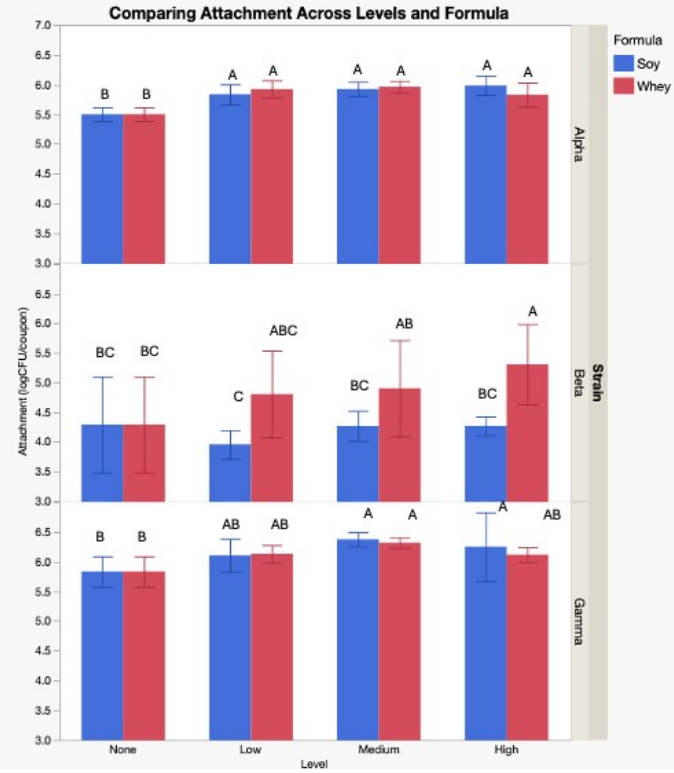


*Figure 3-5: Attachment of 3 strains of C. sakazakii to stainless steel coupons by formula and foulant amount*

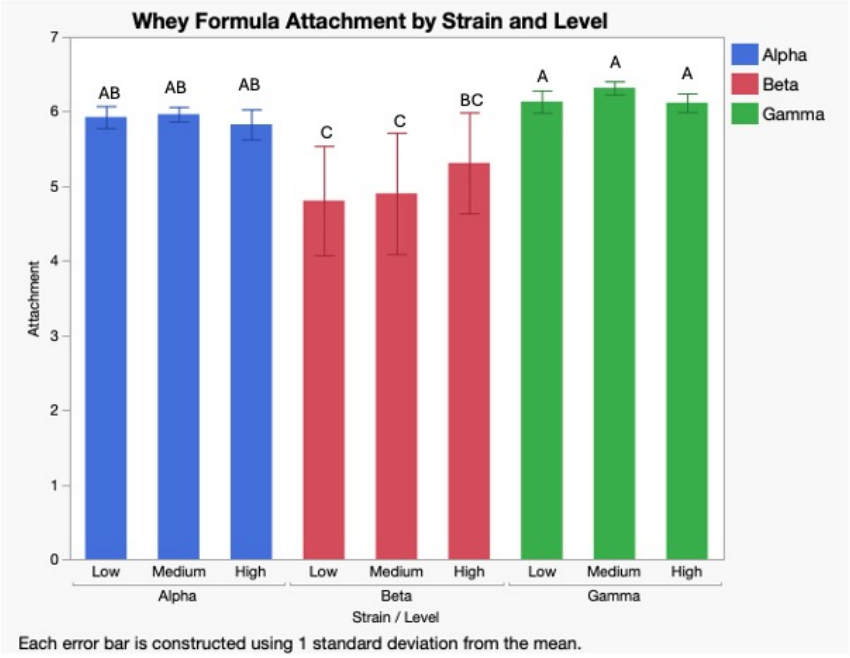


*Figure 3-6: Attachment of C. sakazakii on stainless steel coupons with whey-based formula (WBF)*

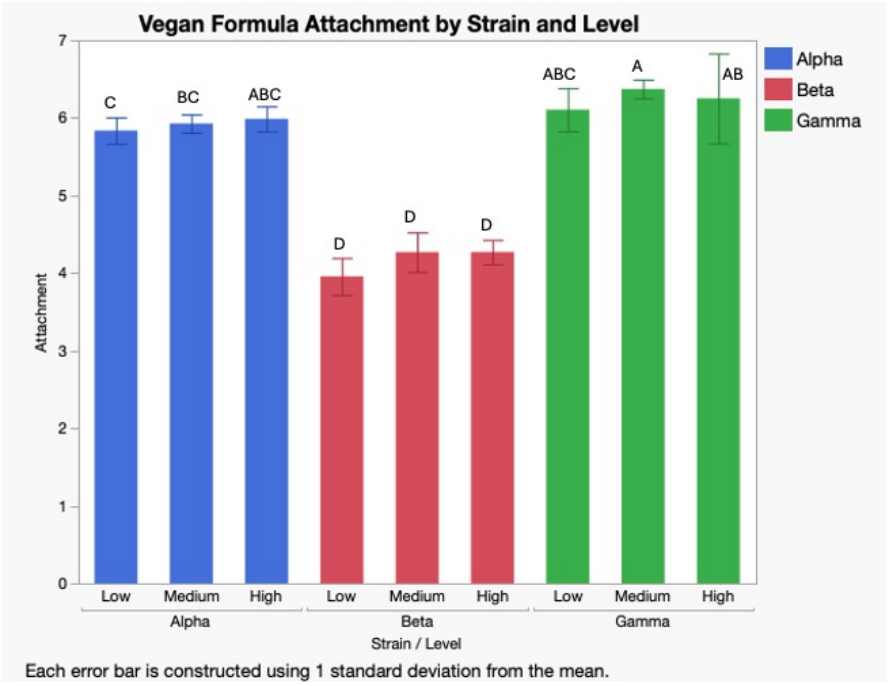


*Figure 3-7: Attachment of C. sakazakii strains to stainless steel coupons on soy-based formula foulant*

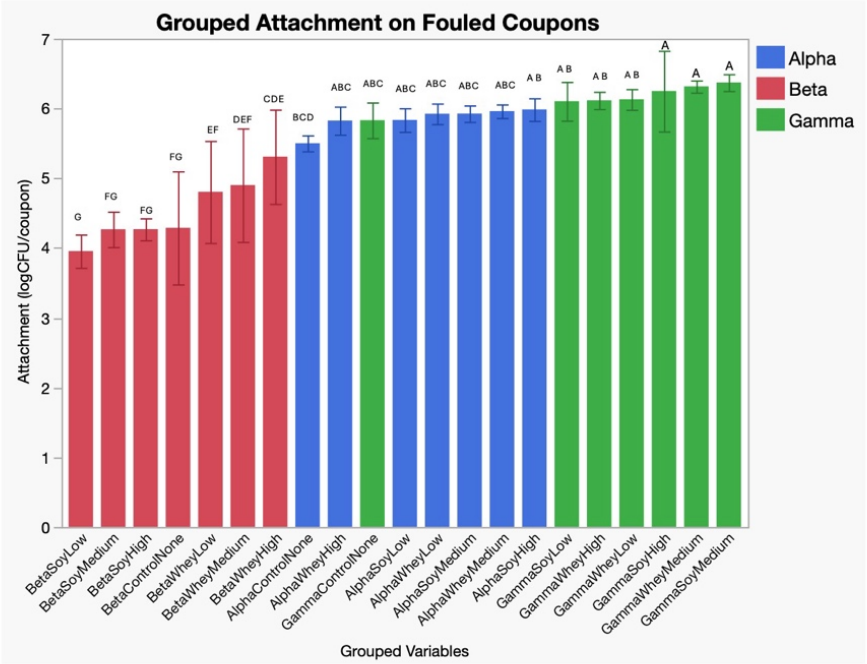


*Figure 3-8: Differences in attachment by C. sakazakii strains to stainless steel coupons affected by strain, foulant type, and amount*

*Table 3-2: Attachment of C. sakazakii to stainless steel coupons fouled with soy-based infant formula (SBF) and whey-based infant formula (WBF)*

| | Population of Alpha (log CFU/coupon) | | Population of Beta (log CFU/coupon) | | Population of Gamma (log CFU/coupon) | |
|---|---|---|---|---|---|---|
| **Level** | **WBF** | **SBF** | **WBF** | **SBF** | **WBF** | **SBF** |
| None | $5.50 \pm 0.11_{Ab}$ | | $4.29 \pm 0.81_{Bbc}$ | | $5.83 \pm 0.26_{Ab}$ | |
| Low | $5.92 \pm 0.15_{Aa}$ | $5.84 \pm 0.17_{Aa}$ | $4.80 \pm 0.73_{Babc}$ | $3.96 \pm 0.24_{Cc}$ | $6.13 \pm 0.15_{Aab}$ | $6.10 \pm 0.28_{Aab}$ |
| Medium | $5.96 \pm 0.10_{Aa}$ | $5.92 \pm 0.12_{Aa}$ | $4.90 \pm 0.81_{Bab}$ | $4.27 \pm 0.25_{Cbc}$ | $6.31 \pm 0.09_{Aa}$ | $6.37 \pm 0.12_{Aa}$ |
| High | $5.83 \pm 0.20_{Aba}$ | $5.98 \pm 0.16_{Aa}$ | $5.31 \pm 0.68_{Ba}$ | $4.27 \pm 0.16_{Cbc}$ | $6.12 \pm 0.12_{Aab}$ | $6.25 \pm 0.58_{Aa}$ |

*Values followed by different uppercase letters within each level are significantly different ($p \leq 0.05$)

** Values followed by lowercase letters within each strain are significantly different ($p \leq 0.05$)

*Table 3-3: Attachment Rate of C. sakazakii to stainless steel coupons fouled with soy-based infant formula (SBF) and whey-based infant formula (WBF)*

| | Percentage Attachment of Alpha | | Percentage Attachment of Beta | | Percentage Attachment of Gamma | |
|---|---|---|---|---|---|---|
| **Level** | **WBF** | **SBF** | **WBF** | **SBF** | **WBF** | **SBF** |
| None | $68.87 \pm 1.43_{Ac}$ | | $53.76 \pm 10.15_{Bb}$ | | $66.89 \pm 2.93_{Ab}$ | |
| Low | $74.16 \pm 1.84_{Aab}$ | $71.53 \pm 2.07_{Ab}$ | $61.85 \pm 9.41_{Bab}$ | $57.89 \pm 3.49_{Bab}$ | $70.32 \pm 1.70_{Aab}$ | $69.78 \pm 3.17_{Aab}$ |
| Medium | $74.62 \pm 1.22_{Aa}$ | $72.64 \pm 1.45_{Aab}$ | $63.09 \pm 10.47_{Bab}$ | $59.67 \pm 3.55_{Bab}$ | $72.44 \pm 1.00_{Aa}$ | $72.83 \pm 1.38_{Aa}$ |
| High | $72.94 \pm 1.52_{Aab}$ | $73.36 \pm 1.98_{Aab}$ | $68.34 \pm 8.70_{Aa}$ | $61.63 \pm 2.27_{Bab}$ | $70.15 \pm 1.42_{Aab}$ | $70.15 \pm 1.42_{Aab}$ |

*Values followed by different uppercase letters within each level are significantly different ($p \leq 0.05$)

** Values followed by lowercase letters within each strain are significantly different ($p \leq 0.05$)

# Chapter 4

## The Presence of Foulant Increases the Thermal Tolerance and Inactivation Rate of *Cronobacter sakazakii*[2]

[2]McGwin, J., Muthiah, JLJ., Dev Kumar, G., and Gratzek, J. To be submitted to *Journal of Food Process Engineering*

**Abstract**

Heat exchanger fouling is a persistent challenge in the continuous thermal processing industry. Fouling layer build up presents an increased risk for microbial attachment and decreased rate of heat transfer. This study sought to investigate the thermal inactivation of three strains of *Cronobacter sakazakii* sources from clinical, environmental, and infant formula origins, on stainless steel coupons fouled with whey based (WBF) and soy based (SBF) infant formulas. Coupons were then inoculated with pGFPuv transformed *C. sakazakii* and subjected to thermal treatments at 90°C for 0, 1, 5, and 10 min. Bacterial survival was quantified as log CFU/coupon and decimal reduction times (D-value) were determined from the inactivation curves. Results revealed that clean coupons achieved a significantly higher reduction, averaging 4.55 log CFU/coupon, compared to 1.87 log CFU/coupon on fouled surfaces. D-value increased notably on fouled coupons averaging 4.70 min for WBF and 9.20 min for SBF, compared to 2.94 minutes on control coupons. Strain dependent differences were observed, with the Gamma strain exhibiting increased thermal tolerance. These findings suggest that foulant can act as a protective barrier, potentially impairing heat transfer and reducing the efficacy of standard thermal processing schemes. This study highlights the importance of robust cleaning and sanitization procedures in powdered infant formula processing and indicates that further research incorporating precise heat transfer models is needed to better simulate industrial conditions and improve microbial safety.

## Introduction

Heat exchanger fouling is one of the paramount issues facing many continuous thermal processing industries. A significant time and cost in with dairy processing is associated with the cleaning, breaking down, and sanitization of equipment (Gillham et al., 2000). Fouling present may cause food safety and quality issues and has been implicated in FDA reports following recalls of aseptic processing facilities (FDA, 2022). In aseptic processing, the requirement is that the product and packaging is sterile and free from any bacterial contamination. However, the lack of commercial sterility has been seen in several plant-based and dairy products, with the presence of *Bacillus subtilis* and *Cronobacter sakazakii.* Additionally, the incidence of *C. sakazakii* in infant formula has been increasing as of recent.

In the manufacturing of powdered infant formula (PIF), several different processes are used. Commonly the wet blending or dry blending process are used, and sometimes re combination of both methods. The wet blending is said to be more commonly used due to more effective control of the process from receipt of raw ingredients to final product (Masum et al., 2021). While the wet blending does allow for more control over every step of production, Lu et al (2019) found that a plant that followed the dry processing method had no incidences of *Cronobacter* contamination compared to a wet blend plant. Ultimately, the cleanliness and hygienic design is going to vary from plant to plant and implementing strict cleaning procedures is Imperative In both manufacturing methods. In the heating of liquid Infant formula, both HTST and UHT treatments are used to pasteurize or sterilize the mix prior to evaporation and spray drying. Because of heat sensitive vitamins and minerals included in PIF, these are either added after heat treatment or thermal processes are used to limit the degradation of the bioactive components (Murphy et al., 2011). Additionally, because of the high solids content of infant

formula, foulant is expected to form and compromise heat transfer properties and potentially safety and quality (Murphy et al., 2011).

Typically, the methods of heat treatment for liquid infant formula are direct or indirect heating (Masum et al., 2021). In direct, steam is injected directly into the liquid, causing a rapid rise in temperature and is suitable for high solid level formulas. This method also has been seen to cause less denaturation of whey proteins (Murphy et al., 2013). Most commonly though, indirect heating is used with mixes at lower solids levels. However, indirect processing is more highly prone to fouling and thus creates increased risk for bacterial survival and time for cleaning (Masum et al., 2021; Murphy et al., 2011, 2013).

*Cronobacter sakazakii* has been found to have a wide variety of thermal and chemical stress resistance. With $D_{58°C}$ values ranging from 30.5 to 591.9 s (z = 5.6°C), the thermal tolerance across the species is expansive and several extremely heat-resistant strains exist (Edelson-Mammel & Buchanan, 2004). Data does suggest that standard HTST and UHT treatments should inactivate *Cronobacter*, but under the presence of foulant, heat transfer is reduced, and sufficient heat loads may not be delivered to the product is fouling layers are sufficiently large (Iversen et al., 2004). Additionally, if the foulant is not cleaned and removed, bacteria can persist in the niches of the foulant and contaminate downstream processes and possibly develop further chemical and thermal stress tolerance.

To explore the thermal tolerance of *C. sakazakii,* three strains of the bacteria from clinical, environmental, and infant formula sources were investigated for their resistance on coupons fouled with infant formula. This study sought to determine if foulant is a cause of persistence and contamination in the manufacturing of PIF.

## Materials and Methods

Three strains of *C. sakazakii* that had been transformed to express the pGFPuv plasmid as described previously were used in this study. Before each experiment, a fluorescent isolated colony from a fresh culture plate of TSA-A was streaked onto a new TSA-A (100 µg/mL) plate and incubated at 37°C for 24 h. The inoculum was prepared by taking 2 loopfuls (10 µL) of cells and suspending in 20 mL of sterile 1× phosphate buffered saline (PBS; VWR Chemicals LLC, Solon, OH, USA). The process was repeated for each isolate. The stock concentration was determined by plating serial dilutions using the modified droplet plate method on TSA-A and incubated for 24 hours at 37°C. Experiments were repeated in 3 biological reps (n=3).

## Model Fouled Surface

Stainless steel coupons (Type 304, 4 cm × 2 cm) were washed with lab detergent and air-dried. Coupons were autoclaved on a dry cycle at 121°C for 15 minutes and stored until used. Two brands of PIF were chosen to create the model fouled surfaces, one whey-based and one soy-based (see Figure 3-1). Model fouling fluid (MFF) was created by mixing 12.5 grams of infant formula with 25 g of sterile deionized water to replicate the conditions that would feed a spray drier (Drapala et al., 2017). Formulas were stirred until completely dissolved and used immediately. Coupons were fouled by placing them on a hot plate set to 120°C until the surface was confirmed to reach 90°C. One level of foulant was used for this study with a total of 600 µL of MFF added per coupon in additions of 200 µL. First, 200 µL of MFF was pipetted onto the coupon and allowed to adhere, then another 200 µL was placed on the coupon, allowed to dry, and lastly 200 µL placed atop and heated until completely fouled, about 5 m. The process was repeated for each formula. Coupons were stored in a dehydrator (Avantco, Meridian, Idaho, USA) at 40°C overnight and stored in a sterile bag until use.

**Coupon Inoculation**

Fouled coupons from above were inoculated with the *C. sakazakii* suspensions by placing the culture into the bottom of a sterile Petri dish (VWR, Solon, OH, USA) and placing a coupon fouled side down and placing on the shaking incubator at 20°C for 15 seconds at 55 rpm. Following inoculation, the coupons are removed from the inoculum with sterile forceps and placed on an absorbent pad, then rinsed with 10mL of distilled water using a serological pipette; 5mL on each side. Coupons were placed on a new absorbent pad until the next step. At this point, 0-minute coupons were added to a 24 oz WhirlPak® bag (Nasco, Modesto, CA, USA) with 100mL of 1 × PBS. Filtered bags were used for fouled coupons to eliminate large pieces of foulant.

**Thermal Inactivation**

To perform the thermal inactivation, the hot plate was set to 90°C and an uninoculated and un-fouled coupon was placed on the plate to monitor temperature. When the coupon was confirmed to reach >90°C, the inoculated coupons (both fouled and un-fouled control) were placed on the hot plate and the timer started for 1 min. Following the treatment, the coupons were removed and immediately a 24 oz WhirlPak® bag (Nasco, Modesto, CA, USA) with 100mL of 1 × PBS. This was repeated for the 5 and 10-min time points. The temperature of the hot plate was monitored continuously in order to maintain a coupon surface temperature of >90°C. Each coupon was sonicated in a water bath for 2 min. Coupons were hand massaged to remove foulant.

Coupons were enumerated using the droplet plate method at each time point and reported as log CFU/coupon. For coupons below the limit of detection, spread plate and pour plate were done on TSA plates supplemented with 100 µg/mL ampicillin.

### Statistical Analysis

A full factorial design was used to design and complete this experiment. The experiment was performed in 3 biological replicates (n=3). Data was plotted as a continuous data set graphed as log CFU/coupon versus time. An interpretation of the decimal reduction time (D-value) was calculated by taking the negative reciprocal of the slope of the trendline and reported as minutes.

One-way analysis of variance (ANOVA) was used in JMP Pro 17 (SAS Institute Inc., Cary, NC, USA) to determine the effect of isolate, formula, and time point separately at a significance level of $p<0.05$. Further, the means of bacterial populations for each experimental unit were compared using Tukey's HSD to observe differences separately between isolates and across time points.

## Results

### Thermal Inactivation of *Cronobacter sakazakii* on Clean and Fouled Coupons

In total, clean control coupons have a significantly higher log reduction over the 10 minutes heat period when compared to the fouled coupons. Average log reduction for un-fouled coupons was 4.55 ± 0.34 log CFU/coupon, whereas average reduction on the fouled coupons was only 1.87 ± 0.34 log CFU/coupon (Table 4-1).

### Comparison between Strains on WBF

In comparing the inactivation across strains of *C. sakazakii* on the WBF, attachment at 0 minutes (control) was 5.47 ± 0.05 log CFU/coupon for Alpha, 5.20 ± 0.33 log CFU/coupon for

Beta, and 6.01 ± 0.19 log CFU/coupon for Gamma on fouled coupons (Figure 4-1). On the control un-fouled coupon, attachment was 4.91 ± 0.16 log CFU/coupon, 4.24 ± 0.47 log CFU/coupon, and 5.87 ± 0.12 log CFU/coupon for Alpha, Beta, and Gamma, respectively. These values for attachment on control un-fouled coupons were significantly higher ($p<0.05$). These values for initial starting attachment on fouled coupons were significantly different ($p<0.05$). After 1 minute of heating, values reduced to 4.43 ± 0.48 log CFU/coupon (Alpha), 4.52 ± 0.47 log CFU/coupon (Beta), and 5.91 ± 0.23 log CFU/coupon (Gamma) on fouled coupons. On control coupons, attachment was significantly reduced to 1.65 ± 1.32 log CFU/coupon and 1.81 ± 1.54 log CFU/coupon for Alpha and Beta. However, for Gamma, attachment was not significantly reduced ($p>0.05$) with a recovery of 5.91 ± 0.22 log CFU/coupon after 1 minute on WBF. On the control coupon for Gamma, attachment was not significantly reduced to 4.98 ± 0.34 log CFU/coupon. After 5 minutes, on the un-fouled coupon, both Beta and Gamma were significantly reduced to 0.33 ± 0.82 log CFU/coupon and 2.76 ± 0.89 log CFU/coupon, respectively. Alpha on the control coupon was not significantly reduced from 1 minute to 5 minutes. On fouled coupons, both Beta and Alpha were significantly reduced ($p<0.05$), while Gamma was not. Finally, after 10 minutes, both Alpha and Beta were reduced to undetectable levels on the control coupon. Gamma was significantly reduced, although 1.38 ± 1.08 log CFU/coupon was still recovered after 10 minutes on control coupons. For all three strains, values were significant reduced from 5 minutes to 10 minutes to 3.36 ± 0.32 log CFU/coupon for Alpha, 2.43 ± 1.93 log CFU/coupon for Beta, and 4.12 ± 0.18 log CFU/coupon for Gamma. The Gamma strain had significantly more recovered after 10 minutes than Alpha and Beta ($p<0.05$). In total for all three strains on the WBF, though, all values were significantly reduced after 10 minutes to

3.34 ± 1.28 log CFU/coupon for fouled coupons and 0.46 ± 0.89 log CFU/coupon for un-fouled coupons (Table 4-1).

**Comparison between Strains on SBF**

On the soy-based formula, initial average attachment across all three strains at zero minutes was 5.37 ± 0.80 log CFU/coupon (Figure 4-2). At each time point, values were significantly reduced ($p<0.05$) from the previous with counts at 10 minutes averaging to 2.36 ± 2.25 log CFU/coupon. Initial attachment on the SBF for the Gamma strain was significantly higher than Alpha and Beta with a mean attachment of 6.12 ± 0.56 log CFU/coupon. After 1-minute, significant reduction was seen for Gamma and Beta, but not Alpha. From 1 minute to 5 minutes, Alpha and Beta were both significantly reduced from 5.82 ± 0.26 log CFU/coupon to 5.41 ± 0.06 log CFU/coupon and 4.12 ± 0.21 log CFU/coupon to 2.48 log CFU/coupon, respectively. Gamma was not significantly reduced ($p>0.05$). However, it had been reduced significantly from 0 minutes to 5 minutes. Lastly from 5 to 10 minutes, only Gamma was significantly reduced, still with 5.17 ± 0.19 log CFU/coupon being recovered (Table 4-1).

**$Log_{10}$ Reductions**

On the WBF, the average log reduction for the Alpha strain was 2.11 ± 0.37 log CFU/coupon, Beta was 2.77 ± 1.67 log CFU/coupon, and Gamma 1.89 ± 0.12 log CFU/coupon. On the SBF, average log reductions were 0.77 ± 0.12 log CFU/coupon (Alpha), 2.57 ± 0.13 log CFU/coupon (Beta), and 1.25 ± 0.22 log CFU/coupon (Gamma). Whereas on un-fouled coupons, log reduction for all three strains was >4 log CFU/coupon.

**Comparison between WBF and SBF**

In comparing the $\log_{10}$ reductions between the two formulas, mean reduction after 10 minutes between the two formulas was not significantly different ($p>0.05$) with the WBF 2.26 ± 0.45 log CFU/coupon, while the SBF was 1.49 ± 0.93 log CFU/coupon. Average reduction after 10 minutes on the un-fouled coupon was 4.55 ± 0.34 log CFU/coupon. Reduction on the un-fouled coupon was significantly higher ($p<0.01$) than both the WBF and SBF (Figure 4-3).

To interpret the rate of inactivation between formulas and strains, an interpretation of the decimal reduction time (D-value) was determined to observe a theoretical time at 90°C for a one $\log_{10}$ cycle reduction under the experimental conditions. To do this, each log CFU data point from each formula/strain pair was plotted continuously against time and the line of best fit calculated. From the line, the negative reciprocal of the slope was taken to calculate the D-value. Average decimal reduction time on the WBF was 4.70 ± 0.83 minutes, while the SBF was 9.20 ± 5.10 minutes. Due to the large standard deviation on the SBF, these values were not significantly different. However, on the un-fouled coupons, d-value was averaged to 2.94 ± 0.86 minutes.

Between individual strains, the d-value interpretation for Alpha on WBF was calculated to be 5.36 minutes ($R^2$=0.66) and 14.20 minutes ($R^2$=0.70) on SBF. Values for Beta on both formulas were very similar with 3.77 minutes ($R^2$=0.52) on WBF and 4.01 minutes ($R^2$=0.81) on SBF. Lastly for Gamma, values of 4.97 minutes ($R^2$=0.90) and 9.39 minutes ($R^2$=0.83) for the WBF and SBF, respectively (Table 4-2).

**Discussion**

Heat exchanger foulant is a paramount issue facing the continuously thermal processing industry and its interaction with *Cronobacter* has yet to be determined (Gillham et al., 2000). Both fouling and *C. sakazakii* pose significant threats to the infant formula industry, and the

increase in recalls of PIF has shown there is need for further investigating the route of contamination. The results of this study demonstrate that foulant could provide a protective barrier to bacteria and increases the thermal tolerance. Lower $\log_{10}$ reductions were observed for all strains on coupons fouled with WBF and SBF compared to clean, un-fouled coupons. Fouled coupons still had 5.31 log CFU/coupon, and 5.17 log CFU/coupon present after heating for 10 minutes on SBF for Alpha and Gamma, respectively.

*C. sakazakii* is not generally known as a thermophilic bacterium but previous studies have found it to be the most thermotolerant species of the *Enterobacteriaceae* family. (Nazarowec-White et al., 1999; Nazarowec-White & Farber, 1997). Pooled D-values from this study as determined in reconstituted PIF were 54.8, 23.7, 10.3, 4.20, and 2.50 minutes at 52, 54, 56, 58, and 60°C, respectively, with a Z-value of 5.82°C. Because many different factors go into D-value determination, results across studies maybe difficult to compare, but consensus suggests that *Cronobacter* should be inactivated by standard HTST and UHT pasteurization (Iversen et al., 2004). *C. sakazakii* has also been observed as one of the major bacteria contaminated UHT milk, suggesting its ability to survive high temperatures and contaminate aseptic environments and products (Nazarowec-White et al., 1999; Skladal et al., 1993). As such, the main gap lies in the explanation for the Lyons Magnus, LLC recall of 2023 in which approximately 35 million aseptically processed low-acid canned foods (LACF) such as nutritional drinks and plant-based milks were recalled for the presence of *Cronobacter sakazakii, Clostridium botulinum*, and *Bacillus subtilis*, indicating a lack of commercial sterility required by 21 CFR 113 (Thermally Processed Low-Acid Canned Foods Packaged in Hermetically Sealed Containers). These findings are contradictory to previously established thermal tolerance data, indicating a failure in cleaning and sanitization programs. Specifically, investigation as part of the recall stated:

> "Your technicians reported finding a portion of the **(b)(4)** in the **(b)(4)** and heating sections of Processor **(b)(4)** partially blocked with residual product…there was visible residue in the **(b)(4)** of Processor **(b)(4)**, and there was what appeared to be a dried residual "film" at the **(b)(4)** flow panel at valve (**(b)(4)**). This was not the first time in 2022 where your firm had identified product fouling in Processor **(b)(4)** after the minimum wash flow rates could not be reached during CIP…"

The observations of this study support the fact that layers of foulant from milk-based products have been shown to have a protective effect of bacteria against thermal stress and the importance of validating the completion and efficacy of CIP processes (B. Li et al., 2024; P.-T. Li et al., 2013; Wedel et al., 2020).

The presence of dairy components may also allow *Cronobacter* to enhance its tolerance to desiccation. In studying thermophilic spores, Wedel et al (2020) found that fouling layers can harbor these spores. Scanning electron microscope analysis has shown that the slight cracks on the surface of both pea and whey based foulant were suitable niche points for bacteria to form biofilms (Ghevariya & Salvi, 2025). If the foulant is not sufficiently removed, as demonstrated in our study, bacteria could remain viable and contaminate downstream production and, without a further kill step, the hygienic environment of spray drying and packaging are of utmost importance.

Likewise, the presence of milk proteins can also aid as a protective measure against chemical sanitizers (B. Li et al., 2024). Flint et al (2002) demonstrated that attachment medium (milk and water) were significant factors in the heat sensitivity of *Streptococcus thermophilus* attached to stainless steel. Cells attached to stainless steel in milk were found to have

significantly higher D-values than cells in water. D-values determined at 62°C were 4.16 min in milk, while in water only 1.83 min. Planktonic cell D-value (60°C) in water was 2 minutes and in milk was 14 minutes. This is confirmatory of the above results, demonstrating that milk proteins, both attached and in solution have a protective effect of bacteria, as the hypothetical D-values determined were 1.6 to 3 times higher when determined on fouled coupons (S. Flint et al., 2002; S. H. Flint et al., 1997). Additionally, Kim et al (2007) found that cells attached to stainless steel with infant formula broth were more protected against inactivation compared to M9 media. As observed in both the WBF and SBF, a more protective effect against thermal inactivation was see compared to an un-fouled coupon.

On the contrary, Osaili et al (2009) found that rehydrated feeding formula did not indicate any appreciable thermal stress protection for *Cronobacter* in traditional D-value determination. A slight resistance was observed in a lactose-free formula compared to soy protein formula, possibly due to types of proteins present, as well as different carbohydrates present. In terms of fouling, soy-based fouling deposits are known to be easier to remove compared to whey-based due to its ability to swell under the presence of water and cleaning chemicals commonly used in the industry (Wang et al., 2018).

While inactivation on SBF was generally higher than WBF, there was no significant difference ($p>0.05$) due to deviations between strains. However, a significantly higher D-value was observed for the Alpha and Beta strains. Results point to Alpha being the most thermoresistant strain on fouled coupons, but more research would need to be done to fully extrapolate this. Several studies have pointed at the wide range of D-values for *Cronobacter* strains. Edelson-Mammel and Buchanan (2004) found D-values at 58°C of 30.5 seconds on the low end to 591.9 seconds (9.865 minutes) with a z-value of 5.6°C. Interestingly, this most

resistant strain was from a clinical isolate. Another point made here is that thermal resistance tended to fall into 2 distinct groups, suggesting a simple genetic marker that determines the thermal resistance.

Heat transfer in continuous thermal processing is governed by the overall heat transfer coefficient given by the equation

$$\frac{1}{U_i A_i} = \frac{1}{h_i A_i} + \frac{\ln\left(\frac{r_o}{r_i}\right)}{2\pi L k} + \frac{1}{h_0 A_o}$$

Fouling resistant from deposited layers on the inside of a pipe is $R_{fi}$. Resistance due to fouling is given by

$$R_{ft} = \frac{A_o}{A_i} R_{fi} + R_{fo}$$

Overall heat transfer with fouling taken into account is

$$\frac{1}{U_{fo}} = \frac{A_o}{h_i A_i} + \frac{A_o \ln\left(\frac{r_o}{r_i}\right)}{2\pi L k} + R_{ft} + \frac{1}{h_0}$$

Thus the cleaning factor is defined as the ratio between the overall heat transfer coefficient for a clean system by the overall heat transfer coefficient for a fouled system

$$C_F = \frac{U_{fo}}{U_{co}}$$

All of this is to say that as fouling increasing on a heat exchanger surface, the rate at which heat is transferred to the fluid flowing is significantly decreased. To compensate for this, the system controls will adjust the temperature of the heat exchange medium so that the product reaches the required temperature for pasteurization. However, if left unmanaged, this fouling may become too much for the system to compensate for, and parameters may not be met due to insufficient ability to reach the required steam or water temperature.

Some similar studies have looked at efficacy of novel cleaning and sanitizing solutions used in the clean-in-place process to both inactivate bacteria and remove foulant (Ghevariya & Salvi, 2025; Shah et al., 2024). In testing the combined use of plasma-activated water (PAW), caustic, acid, and sanitizer it was found that PAW was as effective as traditional CIP chemicals due to its ability to remove foulant and reduce biofilms to the same ability as the CIP chemical controls. While this study only looked at the thermal effect through conductive heating, Ghevariya & Salvi (2025) observed similar results to this study when creating a model CIP loop to observe differences in *E. coli* O157:H7 and *Listeria innocua* biofilm inactivation. At a temperature of 45°C and time of 5 minutes, only a 1.2 log CFU reduction was observed with RO water, and an alkaline cycle saw the higher reduction at 5.8 log CFU. Therefore, high velocity and high temperature water will be able to physically remove biofilms but not inactivate them. Moreover, this study also suggested the similar conclusion that plant-based proteins such as pea or soy are easier to remove compared to whey. This is possibly due to stronger disulfide bonds in whey proteins that may it more difficult to remove (Ghevariya & Salvi, 2025; Wang et al., 2018).

The major limitations of this study lie in the fact that this study was performed on a small scale and does not accurately represent the industrial conditions of thermal processing. While this does give a glimpse into the survival and thermal tolerance that foulant provides, future work should focus on modeling fouling and bacterial inactivation in continuous systems. By doing this, the factor of fluid flow and turbulence can be introduced to determine if flow rate and temperature from both sides influences inactivation. Instead of using a hot plate, studies could be done by submerging coupons into preheated water or buffer to provide more of a convective heat as opposed to conductive heat seen here. Additional studies to predict and monitor foulant build

up for specific products would be valuable to allow for proactive measures to stop processing and clean the system before the foulant becomes too difficult to clean.

Future studies of this nature should focus on modeling a traditional CIP cycle and observing the combined effects of cleaning chemicals, higher temperatures, and fluid velocity on foulant removal and inactivation of *C. sakazakii*. An additional factor to observe could be the removal and inactivation of *C. sakazakii* biofilms compared to planktonic attachment to further explore the effect of physiological state on the resistance and persistence of the pathogen.

**Conclusion**

In conclusion, the fouling of infant formula on stainless steel surfaces could pose a major risk to the in industry. The inactivation of *C. sakazakii* on SS coupons depends on the type of formula being processed, the strains of bacteria that are present, and the overall cleanliness of the system. A significantly higher reduction of *C. sakazakii* was observed on clean coupons as compared to coupons with fouled infant formula. This study highlights the importance of the validation and monitoring of cleaning and sanitizing procedure used in the thermal processing industry. Especially in the processing of high protein and high solids products, fouling will be more prevalent, and manufactures must create procedures to ensure that foulant is removed and corrective actions are issued if the conditions for running production are not met.

**References**

Drapala, K. P., Auty, M. A. E., Mulvihill, D. M., & O'Mahony, J. A. (2017). Influence of Emulsifier Type on the Spray-Drying Properties of Model Infant Formula Emulsions. *Food Hydrocolloids*, *69*, 56–66. https://doi.org/10.1016/j.foodhyd.2016.12.024

Edelson-Mammel, S. G., & Buchanan, R. L. (2004). Thermal Inactivation of *Enterobacter sakazakii* in Rehydrated Infant Formula. *Journal of Food Protection*, *67*, 60–63. https://doi.org/10.4315/0362-028X-67.1.60

Flint, S., Brooks, J., Bremer, P., Walker, K., & Hausman, E. (2002). The Resistance to Heat of Thermo-Resistant streptococci Attached to Stainless Steel in the Presence of Milk. *Journal of Industrial Microbiology & Biotechnology*, *28*, 134–136. https://doi.org/10.1038/sj/jim/7000229

Flint, S. H., Bremer, P. J., & Brooks, J. D. (1997). Biofilms in Dairy Manufacturing Plant-Description, Current Concerns and Methods of Control. *Biofouling*, *11*, 81–97. https://doi.org/10.1080/08927019709378321

Ghevariya, D., & Salvi, D. (2025). Assessing Plasma-Activated Water as an Acidic and Sanitizer Solution in Clean-In-Place (CIP) and Comparing Efficacy with Other Traditional CIP Chemicals. *Journal of Food Science*, *90*, e17632. https://doi.org/10.1111/1750-3841.17632

Gillham, C. R., Fryer, P. J., Hasting, A. P. M., & Wilson, D. I. (2000). Enhanced Cleaning of Whey Protein Soils using Pulsed Flows. *Journal of Food Engineering*, *46*, 199–209. https://doi.org/10.1016/S0260-8774(00)00083-2

Iversen, C., Lane, M., & Forsythe, S. J. (2004). The Growth Profile, Thermotolerance and Biofilm Formation of *Enterobacter sakazakii* Grown in Infant Formula Milk. *Letters in Applied Microbiology*, *38*, 378–382. https://doi.org/10.1111/j.1472-765X.2004.01507.x

Kim, H., Ryu, J.-H., & Beuchat, L. R. (2007). Effectiveness of Disinfectants in Killing *Enterobacter sakazakii* in Suspension, Dried on the Surface of Stainless Steel, and in a Biofilm. *Applied and Environmental Microbiology*, *73*, 1256–1265. https://doi.org/10.1128/AEM.01766-06

Li, B., Zhang, J., Fouhy, K., & Lindsay, D. (2024). Developing a Method for Dry-Stressed *Cronobacter* spp. Cells for Subsequent Sanitizer Efficacy Testing. *International Dairy Journal*, *150*, 105850. https://doi.org/10.1016/j.idairyj.2023.105850

Li, P.-T., Hsiao, W.-L., Yu, R.-C., & Chou, C.-C. (2013). Effect of Heat Shock on the Fatty Acid and Protein Profiles of *Cronobacter sakazakii* BCRC 13988 as Well as its Growth and Survival in he Presence Ofo Various Carbon, Nitrogen Sources and Disinfectants. *Food Microbiology*, *36*, 142–148. https://doi.org/10.1016/j.fm.2013.04.018

Lu, Y., Liu, P., Li, C., Sha, M., Fang, J., Gao, J., Xu, X., & Matthews, K. R. (2019). Prevalence and Genetic Diversity of *Cronobacter* Species Isolated from Four Infant Formula Production Factories in China. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.01938

Masum, A. K. M., Chandrapala, J., Huppertz, T., Adhikari, B., & Zisu, B. (2021). Production and Characterization of Infant milk Formula Powders: A Review. *Drying Technology*, *39*, 1492–1512. https://doi.org/10.1080/07373937.2020.1767645

Murphy, E. G., Tobin, J. T., Roos, Y. H., & Fenelon, M. A. (2011). The Effect of High Velocity Steam Injection on the Colloidal Stability of Concentrated Emulsions for the

Manufacture of Infant Formulations. *Procedia Food Science*, *1*, 1309–1315. https://doi.org/10.1016/j.profoo.2011.09.194

Murphy, E. G., Tobin, J. T., Roos, Y. H., & Fenelon, M. A. (2013). A High-Solids Steam Injection Process for the Manufacture of Powdered Infant Milk Formula. *Dairy Science & Technology*, *93*, 463–475. https://doi.org/10.1007/s13594-013-0116-7

Nazarowec-White, M., & Farber, J. M. (1997). Thermal Resistance of *Enterobacter sakazakii* in Reconstituted Dried-Infant Formula. *Letters in Applied Microbiology*, *24*, 9–13. https://doi.org/10.1046/j.1472-765X.1997.00328.x

Nazarowec-White, M., McKellar, R. C., & Piyasena, P. (1999). Predictive Modelling of *Enterobacter sakazakii* Inactivation in Bovine Milk During High-Temperature Short-Time Pasteurization. *Food Research International*, *32*, 375–379. https://doi.org/10.1016/S0963-9969(99)00100-3

Osaili, T. M., Shaker, R. R., Al-Haddaq, M. S., Al-Nabulsi, A. A., & Holley, R. A. (2009). Heat Resistance of *Cronobacter* Species (*Enterobacter sakazakii*) in Milk and Special Feeding Formula. *Journal of Applied Microbiology*, *107*, 928–935. https://doi.org/10.1111/j.1365-2672.2009.04271.x

Shah, U., Rivero, W. C., Wang, Q., Zheng, H., & Salvi, D. (2024). Exploration of Plasma-Activated Water (PAW) as a Cleaning-In-Place (CIP) Solution for Fouling Removal and Microbial Reduction. *Journal of Food Process Engineering*, *47*, e14669. https://doi.org/10.1111/jfpe.14669

Skladal, P., Mascini, M., Salvadori, C., & Zannoni, G. (1993). Detection of Bacterial Contamination in Sterile UHT Milk Using an L-lactate Biosensor. *Enzyme and Microbial Technology*, *15*, 508–512. https://doi.org/10.1016/0141-0229(93)90084-F

Wang, J., Li, L., Fu, N., Mercade-Prieto, R., & Chen, X. D. (2018). A Comparative Study on Fouling and Cleaning Characteristics of Soy Protein Isolate (SPI). *International Journal of Food Engineering*, *14*, 20170381. https://doi.org/10.1515/ijfe-2017-0381

Wedel, C., Konschelle, T., Dettling, A., Wenning, M., Scherer, S., & Hinichs, J. (2020). Thermally Induced Milk Fouling: Survival of Thermophilic Spore Formers and Potential of Contamination. *International Dairy Journal*, *101*, 104582. https://doi.org/10.1016/j.idairyj.2019.104582

## Figures

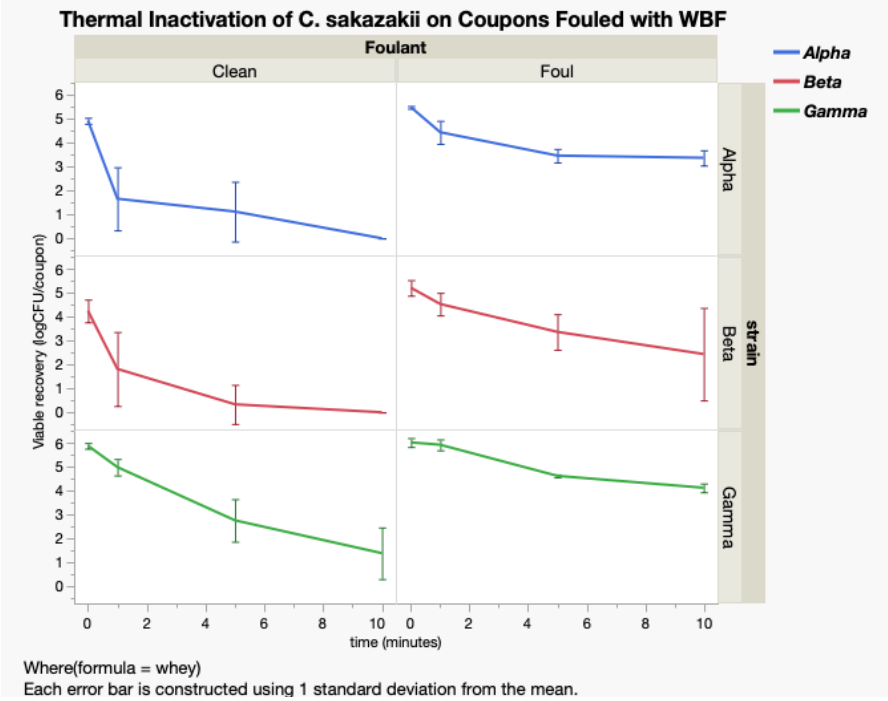


*Figure 4-1: Thermal inactivation of C. sakazakii on stainless steel coupons containing whey-based formula (WBF) foulant heated at 90°C*

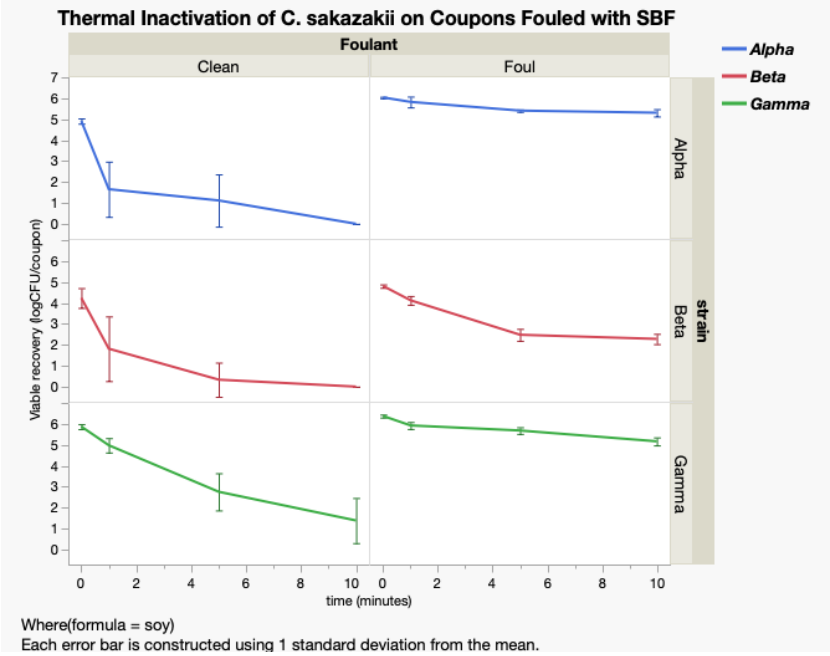


*Figure 4-2: Thermal inactivation of C. sakazakii on stainless steel coupons containing soy-based formula (SBF) foulant heated at 90°C*

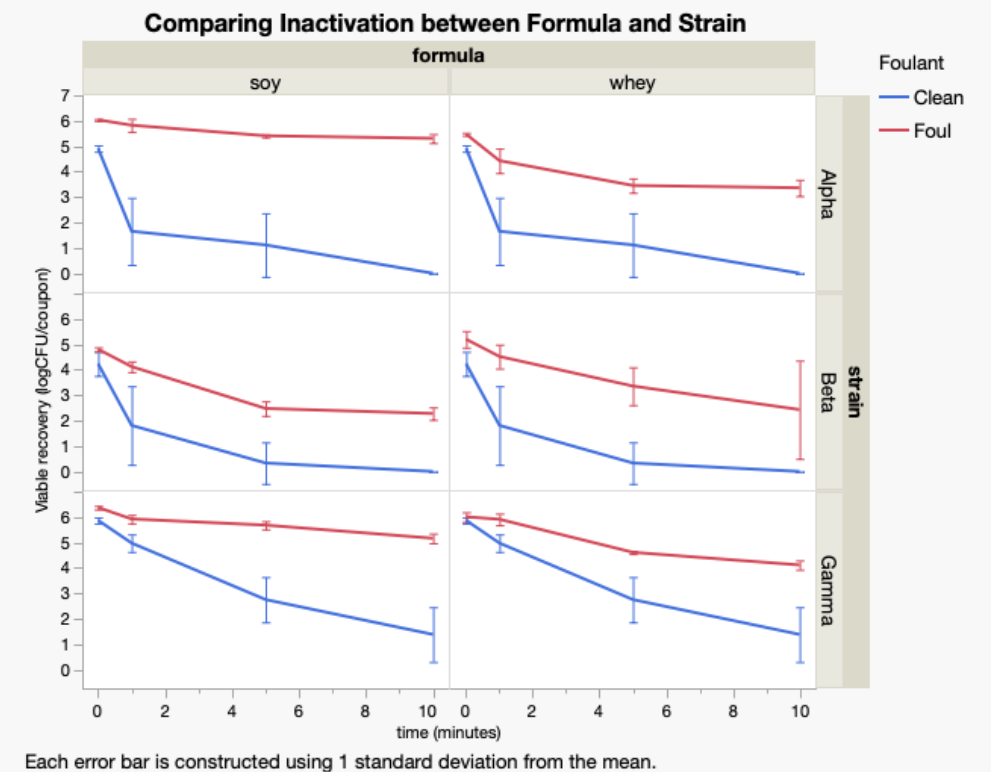


*Figure 4-3: Comparing the thermal inactivation of 3 strains of C. sakazakii on stainless steel coupons contained whey-based formula (WBF) and soy-based formula (SBF) foulant heated at 90°C*

*Table 4-1: Comparing the decimal reduction times of C. sakazakii on stainless coupons containing whey and soy-based infant formula foulant*

| Strain | Variable | D-value (minutes) | $R^2$ | RMSE |
|---|---|---|---|---|
| Alpha | Whey | $5.45 \pm 0.80_{c}$ | 0.685 | 0.55 |
| | Soy | $14.51 \pm 2.31_{a}$ | 0.699 | 0.19 |
| | Control | $2.72 \pm 0.54_{de}$ | 0.552 | 1.4 |
| Beta | Whey | $4.95 \pm 2.40_{cd}$ | 0.519 | 1.05 |
| | Soy | $4.05 \pm 0.43_{cde}$ | 0.807 | 0.5 |
| | Control | $2.99 \pm 0.81_{de}$ | 0.565 | 1.28 |
| Gamma | Whey | $5.02 \pm 0.54_{cd}$ | 0.902 | 0.27 |
| | Soy | $9.52 \pm 1.20_{a}$ | 0.835 | 0.19 |
| | Control | $2.37 \pm 0.53_{e}$ | 0.842 | 0.78 |

*Values followed by different lowercase letters are significantly different ($p \leq 0.05$)

*Table 4-2: Thermal Inactivation of C. sakazakii on Fouled and Un-fouled Coupons*

| | **Population of Alpha (log CFU/coupon)** | | | **Population of Beta (log CFU/coupon)** | | | **Population of Gamma (log CFU/coupon)** | | |
|---|---|---|---|---|---|---|---|---|---|
| **Time** | **WBF** | **SBF** | **Un-fouled** | **WBF** | **SBF** | **Un-fouled** | **WBF** | **SBF** | **Un-fouled** |
| 0 min | $5.47 \pm 0.06_{ABcd}$ | $6.04 \pm 0.04_{Aab}$ | $4.91 \pm 0.13_{ABe}$ | $5.20 \pm 0.32_{Ade}$ | $4.81 \pm 0.08_{ABe}$ | $4.24 \pm 0.47_{ABf}$ | $6.01 \pm 0.19_{ABab}$ | $6.38 \pm 0.07_{Aa}$ | $5.87 \pm 0.12_{ABbc}$ |
| 1 min | $4.43 \pm 0.48_{BCab}$ | $5.83 \pm 0.26_{Aa}$ | $1.65 \pm 1.32_{Dc}$ | $4.52 \pm 0.4_{ABab}$ | $4.12 \pm 0.21_{ABCb}$ | $1.81 \pm 1.54_{DEc}$ | $5.91 \pm 0.23_{ABa}$ | $5.93 \pm 0.17_{ABa}$ | $4.98 \pm 0.34_{BCDab}$ |
| 5 min | $3.46 \pm 0.28_{Cbc}$ | $5.41 \pm 0.06_{ABa}$ | $1.12 \pm 1.25_{DEde}$ | $3.36 \pm 0.74_{BCDbc}$ | $2.48 \pm 0.29_{CDcd}$ | $0.33 \pm 0.82_{EFe}$ | $4.61 \pm 0.06_{CDab}$ | $5.68 \pm 0.16_{ABCa}$ | $2.76 \pm 0.89_{Ec}$ |
| 10 min | $3.36 \pm 0.32_{Cbc}$ | $5.31 \pm 0.18_{ABa}$ | $BLOD_{Ee}$ | $2.43 \pm 1.93_{CDcd}$ | $2.28 \pm 0.26_{Dcd}$ | $BLOD_{Fe}$ | $4.12 \pm 0.18_{Dab}$ | $5.17 \pm 0.19_{BCDa}$ | $1.38 \pm 1.07_{Fde}$ |
| **Log reduction** | **2.11 ± 0.37** | **0.77 ± 0.12** | **≥ 4.91 ± 0.13** | **2.77 ± 1.67** | **2.57 ± 0.13** | **≥ 4.24 ± 0.47** | **1.89 ± 0.12** | **1.25 ± 0.22** | **4.49 ± 1.01** |

*Values followed by different uppercase letters within each isolate are significantly different ($p \leq 0.05$)

**Values followed by different lowercase letters within each time point are significantly different ($p \leq 0.05$)

WBF: whey-based formula

SBF: soy-based formula

BLOD: below limit of detection

Chapter 5

# Conclusion and Future Prospectives

The risks associated with *C. sakazakii* in the manufacturing, processing, and packaging of powdered infant formula are not only limited to environmental contamination, but also contamination due to improper cleaning procedures. While incidences of fatalities from *Cronobacter* infections are low, recalls of powdered infant formula are still prevalent. Given the strict regulations surrounding the microbial safety of infant formula, several studies have focused on how the pathogen infiltrates processing environments. This research evaluated the impact of fouled infant formula on the attachment and survival of *C. sakazakii* from a perspective of food processing and sanitation.

The first study sought to evaluate the attachment profiles of three strains of *C. sakazakii* to stainless steel surfaces fouled with two varieties of powdered infant formula. Through this, results suggested that heat exchanger foulant could present an increased risk for contamination with the bacterium, independent of if the formula is whey based, or soy based. By introducing foulant, a significantly more attachment was seen compared to clean coupons by providing niche points for the bacteria. The attachment was similar across the increasing levels of foulant and mild correlations between increasing protein content and foulant color was observed. Attachment profile differed significantly across strains of *C. sakazakii.*

The second study analyzed the effect of heat on the survival and thermal tolerance of *C. sakazakii.* It was previously shown that the bacteria can attach significantly more to fouled

surfaces, so it was then imperative to explore how that effects the cleanability and survival of the surfaces that are contaminated with *C. sakazakii*. Through this, it was observed that populations of *C. sakazakii* on clean stainless-steel surfaces could be successfully reduced to undetectable levels following 10 minutes of heating at 90°C. However, on surfaces fouled and inoculated with *C. sakazakii,* populations were only reduced by 1-3 log CFU/coupon. Differences in inactivation were observed based on infant formula used and strain of bacteria. Likewise, rates of inactivation were significantly different between strains and between fouled and unfouled coupons.

The combined results from these studies point to the need for infant formula processors to be very strict about cleaning procedures for their heat exchangers and processing systems. Even small amounts of foulant induced attachment of *C. sakazakii*, so manufactures must validate their clean-in-place procedures and ensure that run times are not pushed to their limits such that heat transfer rates are dramatically changed. Further research is needed on the exact mechanisms of attachment and inactivation is systems that accurately model industrial processes so that further guidance can be issued to the industry to reduce the number of recalls associated with infant formula or aseptically processes products.

Future research in this area should be focused on scaling up to pilot-scale level to more precisely model industrial conditions of continuous thermal processing. On this level, scenarios could be run to show the challenges that the dairy, alternative dairy, protein, and infant nutrition industries face when it comes to fouling, microbial contamination, effective cleaning, sanitization, and process optimization. By doing this, focus can be directed on all aspects that go into the processing of a pasteurized or aseptic food product from development and formulation, process development, equipment selection, cleaning procedures, packaging, and monitoring. On a pilot scale, this could be modeled with a pilot HTST/UHT system. From an industrial point of

view, studies could be designed around monitoring of each step from receiving to packaging, with specific focus on monitoring temperature and pressure fluctuations across the system, indicative of foulant build up. From there, robust models could be built to better understand the complexity of foulant build-up and how to catch it before it is too late. Likewise, monitoring the effectiveness of the CIP process is another effective solution that has been used by researchers in the past. Combining processing data received from the systems and microbiological data could present robust results that can provide guidance to the industry on how to carry out safe and optimized thermal processes.

# APPENDIX

**Abbreviations:**

TSB – tryptic soy broth

SDW – sterile distilled water

TSA – tryptic soy agar

TSA-A – tryptic soy agar with 100μg/mL ampicillin

EPS – exopolymeric substances

WBF – whey-based formula

SBF – soy-based formula

MFF – model fouling fluid

CIP – clean in place

BCA – bicinchoninic acid

GFP – green fluorescent protein