MICROBIAL SAFETY ASSESSMENT OF COLD BREW COFFEE

by

ANGELA PARRA

(Under the Direction of Hendrik den Bakker)

ABSTRACT

Cold brew coffee samples were brewed with coffee grounds and water (plain) at a 1:8 ratio at 4 °C (n=9) or with additives (cinnamon, nutmeg, and shredded coconut)(n=9) before inoculation with select foodborne pathogens (*L. monocytogenes*, *S. enterica*, *E. coli*, and *B. cereus*). The cold brew samples were then stored at 4°C or subjected to temperature abuse conditions (25°C and 37°C). Under refrigeration, viable cells were not recovered after Day 12 for *E. coli*, *L. monocytogenes* and *S. enterica*. *B. cereus*, populations did not decrease below 4.89 log and persisted past Day 21. Significant growth for *B. cereus* was observed in plain samples and in additive trials (nutmeg and coconut). Coconut aided survivability for all pathogens at some storage temperatures. Initial pH ranged from 5.4 to 5.6 and decreased over time for cold brew stored under refrigeration ($P<.05$). TA increased over time for all pathogens ($P<.05$).

INDEX WORDS: Cold brew, Additives, Coffee quality, Temperature abuse, Storage, pH, Titratable Acidity

MICROBIAL SAFETY ASSESSMENT OF COLD BREW COFFEE

by

ANGELA PARRA

B.S., Gordon State College, 2021

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

MASTER OF SCIENCE

ATHENS, GEORGIA

2024

MICROBIAL SAFETY ASSESSMENT OF COLD BREW COFFEE

by

ANGELA PARRA

Major Professor:	Hendrick den Bakker
Committee:	Faith Critzer
	Govindaraj Dev Kumar

Electronic Version Approved:

Ron Walcott
Vice Provost for Graduate Education and Dean of the Graduate School
The University of Georgia
May 2024

DEDICATION

Thank you to my family, as I could not have done this without their support. Thank you to all my mentors that encouraged me to take this opportunity and watched over me to ensure I succeeded. I will be forever thankful to my friends and to everyone else who has supported me in any way, shape, or form.

ACKNOWLEDGEMENTS

I am thankful to Dr. den Bakker and his lovely family who have given me this opportunity and guided me through this process. Thank you to Amy for passing down her wisdom and having the patience to help with lab endeavors, especially the dishes. I appreciate the guidance from my committee members Dr. Critzer and Dr. Dev Kumar for their knowledge, expertise, and their enthusiasm for teaching.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

Coffee can be prepared using various methods. Hot brewed is the most common however an extraction method known as "cold brew" has been increasing in sales in the U.S. from 2011 to 2016. However, cold brew was introduced in the U.S. in the 1960s. It has roots tracing back to the 17th century largely in Japan and the Netherlands. Cold brew is defined by the extraction method of ground coffee beans brewed in water below body temperature. Extraction methods can include drip filtration, full immersion, and cold press. (Claassen et al., 2021) The popularity of coffee can be attributed to its many benefits such as reducing the risk of type 2 diabetes, cancer, and coronary heart disease. Multiple studies have suggested it was possible coffee consumption had an inverse relationship to developing type 2 diabetes. (Ludwig et al., 2014)

There is currently a lack of agreement for cold brew coffee preparation parameters such as extraction time, temperature, dosage, agitation, water composition, bean type, grind size, and roasting level. An online survey study found a wide range of temperatures, from 0 ℃ to 40 ℃ across at-home, industrial and commercial operations. The general preparation method was found to use a medium roast of ground coffee, around 50-100 g, brewed at 8 ℃ for 16 h. (Claassen et al., 2021) If commercially bagged coffee is used, it may contain different blends of popular species such as *Coffea arabica, Coffea canephora* var robusta, *Coffea liberica* and *Coffea excelsa* which all have varying characteristics impacting the overall beverage quality.

(Cagliani et al., 2013; Kalschne et al., 2021) The resulting coffee composition can ultimately affect the safety of cold brew coffee and potential pathogen growth. (Kwok et al., 2020)

There are increased food safety hazards during cold brew preparation due to its ineffective pathogen suppression capabilities. The absence of a true microbial "kill step" is due to low water temperature during preparation and cold brew's inherently low acidity. Implementing maximum storage times can potentially mitigate the growth of contaminants introduced from the environment during preparation. The guidelines for the addition of cold brew controls to avoid microbial growth are in question; data from microbial challenge studies can be used to apply to a HACCP system for cold brew. (Kwok et al., 2020)

This microbial challenge study aims to provide pathogen survival data for standard cold brew coffee preparation. The main goals of this study were:

i) To determine whether cold brew coffee stored under refrigeration can sustain growth of select foodborne pathogens: *L. monocytogenes, B. cereus, S. enterica, and E. coli.*

ii) To infer if temperature abuse during storage and the addition of natural additives (cinnamon, nutmeg, and coconut) can influence the survival of the foodborne pathogens.

Potential Sources of Contamination in the Production Chain

Coffee Bean Processing. There are several points in the production of coffee where contamination can occur, such as during the harvest and processing of the coffee beans. (CFIA, 2020) The cultivation of coffee beans occurs under a warm, humid tropical climate. This increases susceptibility to microbial contamination from *Enterobacteriaceae* during processing and storage. (Lachenmeier et al., 2021) Subsequent processing of coffee beans such as roasting is modified for the use of cold brew coffee. The beans are roasted for a shorter duration thus

avoiding the reduction of compounds that impact acidity such as chlorogenic acid. (Kwok et al., 2020; Nerurkar et al., 2023)

Cold Brew Preparation**.** Cold brew is a ready-to-eat food that has multiple preparation components that each have the potential to introduce a source of pathogens or alter the composition to allow/hinder its growth. (Brillard et al., 2015) The major ingredient components include coffee and water. (Kwok et al., 2020) The hazard may come from the use of contaminated water for brewing, unsanitary equipment and containers, and infected personnel. (CFIA, 2020) Given that cold brewed coffee lacks a kill step during preparation and is normally consumed "as is", the presence of pathogens could create a potential risk for foodborne illnesses. Once contaminated, pathogen growth is largely influenced by time, temperature, acidity, and available nutrients found later in the production of cold brew. (Claassen et al., 2021)

Storage (Shelf-life). Cold brew coffee is typically consumed immediately after brewing or after only a few days of storage. Storage time, temperature and cold brew access have been shown to influence shelf-life of the product. The amounts of chlorogenic, caffeic and ferulic acids that are attributed to coffee antioxidant activities are not largely impacted by storage time. However, pH decreases occurred in cold brew as a result of storage at higher temperatures, such as 25 °C, and with the presence of oxygen. The shelf-life was decreased to 10 days compared to 20 days if cold brew was stored under refrigeration with no oxygen present. (Pérez-Martínez et al., 2008) Additionally, the increase in popularity has led to commercial cold brew coffee production that sells ready-to-drink products. The products must undergo stabilization techniques to ensure microbial safety throughout their shelf life. A shelf-life study on cold brew showed blast chilling, UV radiation and filtration to be insufficient in decreasing the total bacterial count, mold, and yeast in commercial cold brew. Only pasteurization and high-pressure processing were

enough to ensure microbial safety. (Bellumori et al., 2021) Longer storage periods incur changes such as raised acidity and greater ethanol content. There are also decreases in sugar content/sweetness due to yeast or bacteria activity. Greater effects occur in cold brew stored at 20 °C than at 4 °C. (Kwok et al., 2020)

Coffee Composition and Antimicrobial Effects

Inherit coffee compounds consist of carbohydrates, lipids, melanoidins, organic acids, and nitrogen-containing compounds such as caffeine and trigonelline. (Ludwig et al., 2014) Melanoidins are the products of roasting. They are like dietary fiber in that they are characteristically indigestible and fermented in the gut. (Ludwig et al., 2014) Coffee has antioxidant capacity due to its high content of phenolic acids such as caffeic acid and chlorogenic acid. (Nerurkar et al., 2023; Nosal et al., 2022) Chlorogenic acids are powerful antioxidants in vitro, however they are not as potent once consumed. Coffee compounds such as caffeine and trigonelline contribute to the antimicrobial capacity of coffee.(Al-Janabi, 2011; Gaul & Donegan, 2015) Caffeine content varies between 0.9-1.3% dry matter for arabica coffees. Trigonelline less than 1% dry weight , has antibacterial/hypoglycemic properties. (Ludwig et al., 2014)

Varying post-harvest coffee treatments impact the metabolites, lipids minerals and antioxidant capacity of resulting coffee brew. (Nerurkar et al., 2023) Among these treatments, brewing conditions strongly influence the total phenol content of coffee. It has been observed that cold brewed coffee tends to preserve antioxidant capacity more effectively compared to hot brewed coffee. (Han et al., 2020)

Previous Cold Brew Study Summaries

Potential microbiological hazards have been identified from previous experiments. A German study on coffee shop cold brew tested for *Bacillus cereus, Listeria monocytogenes,* and *Salmonella spp.*, used as hygiene indicators. *Bacillus cereus* was found in cold brew with a storage time of 5 days. *Bacillus cereus* toxins can lead to gastrointestinal disease. The study found that only a small number showed contamination, but compliance to hygiene requirements is necessary to keep a low incidence of microbial contamination. (Lachenmeier et al., 2021)
A challenge study done by Oregon state University using seven day old bottled cold brew found that viable cells were not recovered after 7 days *Salmonella*, 11-days for *E. coli,* and 14 days for *L. monocytogenes*. (Daeschel et al., 2017) They determined cold brew does not favor the survival or growth of non-spore forming bacterial pathogens, likely due to a lack of nutrients and/or the presence of antimicrobial factors from the coffee. (Daeschel et al., 2017) A Canadian study followed up the challenge study by surveying fifty-nine samples of bottled cold brew to determine levels of aerobic colony counts (ACC) and to test for generic *E. coli*, *Salmonella* spp., and *E. coli* O157. (CFIA, 2020) The results supported the conclusion of the original challenge study, however elevated levels of ACC suggest cold brew supports the growth of some bacteria. (CFIA, 2020)

Another study done by Yan et al. (2019) considered microbial survivability during the storage of coffee beans and the use of contaminated beans during the production of cold brew coffee. The coffee beans were inoculated with five strains of *Salmonella*, *Listeria monocytogenes,* and STEC. A significant decrease in *Salmonella* was observed in coffee beans stored at 23 C after 110 days. Cold brewed coffee made from the inoculated beans showed decreased populations for all pathogens after 24 hours; the greatest reductions were observed for

STEC. There was no growth of bacteria, however the die-off of the bacteria was not immediate. An emphasis was placed on the high inoculation levels and how lower levels of contamination would result in faster population reduction. (Yan et al., 2019)

Spoilage organisms, such as moulds and yeasts are not inhibited by the low acidity of cold brew. As they increase during storage and may lead to fermentation. They may increase the pH and promote the growth of pathogens. In ready-to-drink nitro cold brew, this may be particularly risky, filled under pressure with nitrogen, producing an anaerobic environment that was at risk for *C. botulinum* contamination. (Kwok et al., 2020; USDA, 2017) Ochratoxin A produced from mold contamination in coffee beans is used as an indicator for food safety in coffee production. (Febrianto & Zhu, 2023)

B. cereus role in coffee during cultivation, processing, and preparation

B. cereus is a gram-positive, facultative anaerobe that has been identified on occasion to contaminate products such as vegetables, meat products, milk, and rice. It is ubiquitous in nature and has heat resistance due to its endospores. Combined with psychrotrophic properties, it is often a potential contaminant in foods that are cooked then chilled. (Guinebretiere et al., 2003) In a study done in Poland. *B. cereus* was found in 38.8% of samples which included herbs and spices, breakfast cereals, infant formulas, milk, and cheese. In these cases, the ability to hydrolyze casein starch and tributyrin, to ferment lactose, to grow at 7 °C for 10 days, the production of Nhe and Hbl toxin and to possess ces gene was identified and verified. (Berthold-Pluta et al., 2019)

For coffee, the microbiota associated with the coffee plant determines the final quality of the product. (Oliveira et al., 2013) *B. cereus* is one of the endophytic bacteria present in coffee

plants (*Coffea arabica* and *C. robusta*). The pathogen is able to live within the tissues of the plant and control in vitro mortality of nematodes such as *Pratylenchus coffea* and *Radopholus Duriophilus*. (Duong et al., 2022; Nunes & De Melo, 2006)

The fermentation process will influence the natural microbiota present. A study found that after the fourth day of fermentation and drying, three different gram-negative bacteria, six-gram positive bacteria, eight yeasts and fifteen fungi species were identified. Among the gram positive isolates, the *Bacillus* genus was predominate.(Silva et al., 2008)

Common causes of food poisoning by *B. cereus* are the production of emetic and diarrheagenic toxins. Diarrheagenic infections are typically caused by two enterotoxins, HBL and NHE each formed by three components. (Souza & Abrantes, 2011) Some strains produce emetic toxins that are nonprotein based, called cereulide, that is chemically stable and not inactivated by heat. There is no known method to detoxify food contaminated by emetic toxins, however quantitative analysis does exist to test potentially contaminated products. Spores from *Bacillus spp.* have been found to survive high temperatures; however, it is unknown if spores have the ability to produce emetic toxin. In addition, only some strains of *B. cereus* have the ability to produce emetic toxin. (Jääskeläinen et al., 2003)

Salmonella, Listeria, **and** ***E.coli*** **As Potential Contaminates**

Cold brew is generally prepared and consumed as a ready-to-eat product. Contamination from pathogens such as *Salmonella*, *Listeria* and *E. coli* to be introduced to cold brew during preparation unless cross contamination occurs. Cold brew is commonly stored at refrigeration where common food-borne pathogens such as *Listeria monocytogenes* can grow and thrive. (Acharya & Nummer, 2022). Contamination from *Salmonella* mostly comes from poultry, pork,

and egg products. (Ehuwa et al., 2021) Similarly, Listeria has been linked to many food outbreaks which include vegetables, meat and cheese. (Jordan & McAuliffe, 2018) The ubiquitous nature of the pathogens suggest poor handwashing after contact with contaminated food and use of contaminated water during preparation would be the main sources of pathogen transmission in cold brew. (Ehuwa et al., 2021; Jordan & McAuliffe, 2018)

A study done by Dalgia et al. on hot brewed coffee determined roasted coffee showed antibacterial properties against Gram-positive and Gram-negative bacteria. However, the inhibitory effect was dependent on degree of roasting and the coffee species. They determined the antibacterial activity was not affected by brewing procedure, but only traditional hot brewed coffee was used in the study. (Daglia et al., 1994) A follow-up study investigated the potential antibacterial activity of coffee on Enterobacteria. They are a group of bacteria that include many bacteria of food-safety concern such as *Salmonella enterica* and *E.coli.* (Almeida et al., 2006)

Current Food Safety Practices

Despite identifying potential hazards associated with cold brew, there is currently no clear standard available for its production. The food code, which serves as the FDAs best practice guide to ensure uniform procedures and safe products, does not have specific standards for cold brew. Further studies are needed to consider how to effectively regulate cold brew coffee. There have been some previous challenge studies but most have been privately held, or not made available to the public, and have limited parameters. (Lopez, 2021) The National Coffee Association has brought up the issue at the National Conference for Food Protection and has called to create a committee to determine what food safety and compliance standards to follow. (Lopez, 2021; Corey & Murray, 2023)

Overall, the microbial risk of cold brew pathogens and the identification of potential sources of contamination is necessary to the administration of food safety controls for cold brew coffee. (Lachenmeier et al., 2021) Further microbial challenge studies that focus on inactivation would supplement survival and growth data found in this study for cold brew. (Foods, 2010) Research on additive ingredients must be done since available nutrients such as additional carbohydrates from added ingredients during cold brew preparation aid *B. cereus* emetic toxin production. (Jovanovic et al., 2021)

References

Acharya, S., & Nummer, B. A. (2022). Survival of Listeria monocytogenes in Commercially Available Refrigerated Cold-Brewed Coffee. *Journal of Environmental Health*, *85*(2).

Al-Janabi, A. A. H. S. (2011). Potential activity of the purine compounds caffeine and aminophylline on bacteria. *Journal of global infectious diseases*, *3*(2), 133-137.

Almeida, A. A. P., Farah, A., Silva, D. A., Nunan, E. A., & Glória, M. B. A. (2006). Antibacterial activity of coffee extracts and selected coffee chemical compounds against enterobacteria. *Journal of Agricultural and Food Chemistry*, *54*(23), 8738-8743.

Bellumori, M., Angeloni, G., Guerrini, L., Masella, P., Calamai, L., Mulinacci, N., Parenti, A., & Innocenti, M. (2021). Effects of different stabilization techniques on the shelf life of cold brew coffee: Chemical composition, flavor profile and microbiological analysis. *Lwt-Food Science and Technology*, *142*. https://doi.org/ARTN 111043 10.1016/j.lwt.2021.111043

Berthold-Pluta, A., Pluta, A., Garbowska, M., & Stefańska, I. (2019). Prevalence and toxicity characterization of Bacillus cereus in food products from Poland. *Foods*, *8*(7), 269.

Brillard, J., Dupont, C., Berge, O., Dargaignaratz, C., Oriol-Gagnier, S., Doussan, C., Broussolle, V., Gillon, M., Clavel, T., & Berard, A. (2015). The water cycle, a potential source of the bacterial pathogen Bacillus cereus. *BioMed research international*, *2015*.

Cagliani, L. R., Pellegrino, G., Giugno, G., & Consonni, R. (2013). Quantification of Coffea arabica and Coffea canephora var. robusta in roasted and ground coffee blends. *Talanta*, *106*, 169-173.

CFIA. (2020). *Bacterial Pathogens in Cold Brewed Coffee—1 April 2018 to 31 March 2019*. https://inspection.canada.ca/DAM/DAM-food-aliments/STAGING/text-texte/pathogens_cold_brew_apr_2018_mar_2019_1590177174447_eng.pdf

Claassen, L., Rinderknecht, M., Porth, T., Rohnisch, J., Seren, H. Y., Scharinger, A., Gottstein, V., Noack, D., Schwarz, S., Winkler, G., & Lachenmeier, D. W. (2021). Cold Brew Coffee-Pilot Studies on Definition, Extraction, Consumer Preference, Chemical Characterization and Microbiological Hazards. *Foods*, *10*(4). https://doi.org/ARTN 865 10.3390/foods10040865

Daeschel, M. A., Armbrust, N., & Vieru, C. (2017). Survival of non-spore forming foodborne pathogens in cold brewed coffee. *Oregon State University*.

Daglia, M., Cuzzoni, M. T., & Dacarro, C. (1994). Antibacterial activity of coffee: relationship between biological activity and chemical markers. *Journal of Agricultural and Food Chemistry*, *42*(10), 2273-2277.

Duong, B., Marraccini, P., Etienne, H., Villain, L., Hoang, G. T., Khong, G. N., Lebrun, M., & Duponnois, R. (2022). Potential of the coffee endophytic Bacillus cereus sensu lato strain CCBLR15 to control the plant-parasitic nematode Radopholus duriophilus. *Biocontrol Science and Technology*, *32*(8), 971-988.

Ehuwa, O., Jaiswal, A. K., & Jaiswal, S. (2021). Salmonella, food safety and food handling practices. *Foods*, *10*(5), 907.

Febrianto, N. A., & Zhu, F. (2023). Coffee bean processing: Emerging methods and their effects on chemical, biological and sensory properties. *Food Chemistry*, 135489.

Foods, N. A. C. o. M. C. f. (2010). Parameters for determining inoculated pack/challenge study protocols. *Journal of Food Protection*, *73*(1), 140-203.

Gaul, J., & Donegan, K. (2015). Caffeine and its effect on bacteria growth. *The Journal of Biological Sciences, 1*, 4-8.

Guinebretiere, M., Girardin, H., Dargaignaratz, C., Carlin, F., & Nguyen-The, C. (2003). Contamination flows of Bacillus cereus and spore-forming aerobic bacteria in a cooked, pasteurized and chilled zucchini purée processing line. *International Journal of Food Microbiology, 82*(3), 223-232.

Han, J.-W., Boo, H., & Chung, M.-S. (2020). Effects of extraction conditions on acrylamide/furan content, antioxidant activity, and sensory properties of cold brew coffee. *Food Science and Biotechnology, 29*, 1071-1080.

Jääskeläinen, E. L., Häggblom, M. M., Andersson, M. A., Vanne, L., & Salkinoja-Salonen, M. S. (2003). Potential of Bacillus cereus for producing an emetic toxin, cereulide, in bakery products: quantitative analysis by chemical and biological methods. *Journal of Food Protection, 66*(6), 1047-1054.

Jordan, K., & McAuliffe, O. (2018). Listeria monocytogenes in foods. *Advances in food and nutrition research, 86*, 181-213.

Jovanovic, J., Ornelis, V. F., Madder, A., & Rajkovic, A. (2021). Bacillus cereus food intoxication and toxicoinfection. *Comprehensive Reviews in Food Science and Food Safety, 20*(4), 3719-3761.

Kalschne, D. L., Silva, N. K., Canan, C., Benassi, M. d. T., Flores, E. L. M., & Leite, O. D. (2021). Main minerals and organic compounds in commercial roasted and ground coffee: An exploratory data analysis. *Química Nova, 44*, 70-75.

Kwok, R., Lee Wee Ting, K., Schwarz, S., Claassen, L., & Lachenmeier, D. W. (2020). Current challenges of cold brew coffee—roasting, extraction, flavor profile, contamination, and food safety. *Challenges*, *11*(2), 26.

Lachenmeier, D., Noack, D., Röhnisch, J., & Seren, H. (2021). Cold brew coffee–a microbial hazard. *Tea Coffee Trade J*, *193*, 32-33.

Lopez, J. (2021). Cold brew coffee regulations and policies. *Assoc. Food Drug Off. J*, *81*.

Ludwig, I. A., Clifford, M. N., Lean, M. E., Ashihara, H., & Crozier, A. (2014). Coffee: biochemistry and potential impact on health. *Food & function*, *5*(8), 1695-1717.

Nerurkar, P. V., Yokoyama, J., Ichimura, K., Kutscher, S., Wong, J., Bittenbender, H. C., & Deng, Y. (2023). Medium Roasting and Brewing Methods Differentially Modulate Global Metabolites, Lipids, Biogenic Amines, Minerals, and Antioxidant Capacity of Hawai 'i-Grown Coffee (Coffea arabica). *Metabolites*, *13*(3), 412.

Nosal, B. M., Sakaki, J. R., Kim, D.-O., & Chun, O. K. (2022). Impact of coffee preparation on total phenolic content in brewed coffee extracts and their contribution to the body's antioxidant status. *Food Science and Biotechnology*, *31*(8), 1081-1088.

Nunes, F., & De Melo, I. (2006). Isolation and characterization of endophytic bacteria of coffee plants and their potential in caffeine degradation. *Environmental Toxicology.*, *1*, 293-297.

Oliveira, M. N., Santos, T. M., Vale, H. M., Delvaux, J. C., Cordero, A. P., Ferreira, A. B., Miguel, P. S., Tótola, M. R., Costa, M. D., & Moraes, C. A. (2013). Endophytic microbial diversity in coffee cherries of Coffea arabica from southeastern Brazil. *Canadian journal of microbiology*, *59*(4), 221-230.

Pérez-Martínez, M., Sopelana, P., de Peña, M. P., & Cid, C. (2008). Effects of refrigeration and oxygen on the coffee brew composition. *European food research and technology*, *227*, 1633-1640.

Silva, C. F., Batista, L. R., Abreu, L. M., Dias, E. S., & Schwan, R. F. (2008). Succession of bacterial and fungal communities during natural coffee (Coffea arabica) fermentation. *Food microbiology*, *25*(8), 951-957.

Souza, C. d. M. O. d. C. C., & Abrantes, S. d. M. P. (2011). Detection of enterotoxins produced by B. cereus through PCR analysis of ground and roasted coffee samples in Rio de Janeiro, Brazil. *Food Science and Technology*, *31*, 443-449.

CHAPTER 2

PATHOGEN SURVIVAL IN COLD BREW STORED AT REFRIGERATION

1. Introduction

Coffee consumption is estimated to be about 500 billion cups a day and is the second most traded commodity in the world. The preparation of cold brew is becoming a trend worldwide and is rapidly replacing traditional brewing methods. (Czarniecka-Skubina et al., 2021) The method of preparation is diverse and typically consists of varying brewing methods, water quality, extraction time and temperature. A study done by Kwok found that average storage time to be one day, however the maximum was seven days. The study offers a suggestion of a maximum storage time, however no definitive guidance is available to ensure microbial safety and conserve sensory attributes. (Kwok et al., 2020)

There are many points during the cultivation of coffee where contamination may occur. Contamination may occur during harvesting of the coffee berries, which is mostly done by hand, and possibly during transportation. The berries must be cleaned and graded before ultimately being traded. Coffee bean processing involves large vats of water, fermentation tanks, and hulling machines if the wet processing method is used. The dry method involves simply laying the beans out to dry for 3 weeks before hulling, when using this method, the beans are referred to as “unwashed”. (Durocher, 1979) This technique is typically used in countries that do not have sufficient water for wet processing. A risk assessment done for nitro cold brew coffee products by the British Columbia Centre for Disease Control suggests that *B. cereus* is the most common

bacterial species found on coffee. It is associated with the fermentation process. The main mycotoxin concern is ochratoxin A that can occur during prolonged drying. (BCCDC, 2017)

During sorting, the beans are given a grade and eventually shipped out in jute bags. They are stored in warehouses before delivery, sampled by the FDA once it reaches the port, then reaches a final warehouse where it is sampled again before reaching the roaster. Coffee grading has not been standardized.

Then, during roasting, there is an option to blend. This consists of blending several types of coffee with beans from several different sources. Coffee is then roasted at 400 °F, sprayed with water to cool, and cooled quickly with air. At the final stage of production process, the roasted coffee beans are ground and bagged. In the 1970s, ground coffee that was bagged and not meant to be stored longer than a week. The bags contain wax liners to contain the aroma. Otherwise, a nitrogen flush may be used which allows the product to stay fresh for 6 months. Canning may be used to create a vacuum seal and stay fresh for 27 months. (Durocher, 1979) Increased interest in retail coffee can be attributed to an increase of coffee production and trade in Latin America in the late 1970s. (Buzzanell, 1979) For example, in 1989, Starbucks developed FlavorLock bags, which have a one-way valve that allows carbon dioxide to escape and made to prohibit the entry of oxygen. (Starbucks, 2024)

During preparation, contamination of cold brew is likely to occur from bacteria that can naturally live on surfaces. Among the bacteria that may cause illness, include heterotrophic bacteria such as *Escherichia coli*. In addition, *Salmonella* and various types of viruses may remain. Spread of such bacteria can be done from a person's hand or mouth. A study by Yepiz-Gomez isolated *E.coli*, *S. aureus*, *S. faecalis*, and *C. perfringes* from dish cloths and tabletops sampled from the food industry. (Debuisson et al., 2021)

Considering the possible contamination points, this study was done to determine the microbial safety of cold brew as it pertains to *B. cereus, E. coli, S. enterica* and *L. monocytogenes* in short-term storage retail cold brew.

2. Materials and Methods

2.1. Bacteria Strains used.

Pathogens use in this experiment include *B. cereus, E. coli, S. enterica*, and *L. monocytogenes*. Emetic toxin and diarrheagenic producing *B. mosaicus subsp. cereus* isolated from cricket powder was used for this study. Major disease-causing *Salmonella* were used, in which most infectious in warm blooded animals serovars include *S.* Typhimurium and *S.* Enteritidis (Table 3.1). (Kim et al., 2006) The *S.* Enteritidis, *L. monocytogenes* and *E. coli* were obtained from the ATCC. *Listeria monocytogenes* representative of lineage II they are characterized as having more plasmids, resistant to toxic metals, and being more resistant to bacteriocins than lineage I which are virulence attenuated. The strain Li20 (19111) belongs to the 1/2a serotype which has been found to be more robust in biofilm formation. Lineage I *Listeria* are characterized as containing listeriolysin s hemolysin was selected for this experiment. The strain Li2 (19115) belongs to the 4b serotype. Lineages III and IV are rare and normally isolated from animals, so they were omitted from this experiment. *E. coli* serogroups used were representative of the big six and included O26, O45, O103, O111, O121, and O145.

2.3. Inoculum Preparation.

Confirmed, heat-shocked, *B. cereus* culture, stored at -20 °C, was streaked on TSA and incubated for 24 hours at 30 °C. Two 24-hr transfers at 30 °C into TSB was performed before use in experiment or was inoculated into 10 ml of BHI. After transfers each strain was mixed in a 1:1:1 ratio and diluted if necessary to the appropriate dilution to contaminate the product. The same was done for *Salmonella* and *E. coli. Listeria* was transferred into BHI broth and streaked onto BHI agar for enumeration; incubated at 37 °C for 18-24 hours. *Salmonella* may be grown in TSB according to BAM, therefore tryptic soy agar may successfully grow desired *Salmonella* inoculum strains. One colony from each strain was selected from Tryptic Soy Agar (TSA) and cultures in Tryptic Soy Broth (TSB) and incubated at 37 °C for 18-24 h. A lawn plate was prepared, and colonies were dislodged using 0.01% peptone water. 1 ml from each was collected to prepare a mixed inoculum. This was adjusted via dilution to reach target inoculum level of 6 log.

2.4. Plating: Selective Media

Bacillus cereus: MYP agar

Mannitol-Egg Yolk-Polymyxin Agar differentiates *B. cereus* due to its resistance to the antibiotic Polymyxin B, lack of mannitol fermentation and the presence of lecithinase. Mannitol is the carbohydrate source, due to lack of mannitol fermentation by *B. cereus*, the colonies were pink. Phenol red indicator found in the media detects fermentation and turns the colonies yellow. Other bacterial growth requirements, such as nutrients, vitamin and carbon sources are provided by the other ingredients in the agar. The egg yolk emulsion in the agar supplies lecithin. *B. cereus* will hydrolyze the lecithin fold in the egg yolk and form a white halo around.

Salmonella enterica subsp. enterica serovar Typhimurium (LT2) and *S.* Enteritidis were confirmed as *Salmonella* with XLT4 media.

Salmonella spp. can ferment sugars such as xylose, lactose, and sucrose. Due to the presence of phenol red, acid production from fermentation would result in a yellow colony. However, *Salmonella* spp. can decarboxylate lysin, thereby increasing the pH and inhibiting color change. The center of the *S.* Typhimurium and *S.* Enteritidis becomes black as the production of hydrogen sulfide from thiosulfate results in a precipitate with ferric salts. (Clark & Barrett, 1987) The media contains a supplement that inhibits growth of non-*Salmonella*e.

Listeria monocytogenes: Modified Oxford Agar

Modified Oxford Agar (MOX) was used to confirm *Listeria* spp. MOX contains moxakactam as a selective agent for *Listeria monocytogenes* and additionally contains esculin. On this type of selective media, all *Listeria* species hydrolyze esculin and produce black halos surrounding the colonies. (Bergey, 1994; Curtis et al., 1989)

Escherichia coli ('The Big Six'): MacConkey Agar

This is a selective and differential agar that only grows gram-negative bacteria. It further differentiates E.coli from other gram-negative bacteria based on their lactose fermentation capabilities. The fermentation of lactose produces acid which decreases the pH. The pH indicator will turn the colonies pink if they ferment lactose. Non-lactose fermenters will form off-white colonies on this agar. (Jung & Hoilat, 2022)

2.5. Recovery of viable cells procedure.

As there is no defined procedure for testing cold brew, media based on literature review was used to identify presence of pathogen beyond plating under level of detection. A small experiment was conducted to ensure growth of bacteria in respective media with the presence of CB. For *Salmonella*, *Bacillus*, and *E.coli*, TSB was used as growth medium. For *Listeria*, brain heart infusion broth was used to determine presence. 1ml of cold brew was added to 10ml broth tubes incubated for 16-18 hours at 37 °C and subsequently streaked on appropriate selective/differential media to confirm select pathogen presence or absence.

2.6. Cold Brew Preparation Procedure.

For the preparation of the cold brew coffee, 125 grams of pre-ground pre-packaged ground coffee was weighed in a 2 L vessel. One liter of filter-sterilized tap water was added The mixture was agitated to ensure homogenization. This method produces approx. 650 ml of cold brew coffee. The vessel was stored at refrigeration temperature (4 °C) for 12 hours. After 12 hours the cold brew coffee was separated into 250 ml vessels, sealed with a screw cap, and inoculated with representative strains of *L. monocytogenes* (representatives of lineages I, II, III, and IV), *S. enterica* (*S.* Typhimurium LT2, *S.* Enteritidis), *E. coli* (representatives of the 'big six') and *B. cereus*. The control was the uninoculated lab-prepared cold brew coffee. To infer the influence of temperature abuse, we followed the previous preparation methods and stored the cold brew at room temperature (25 °C) and 37 °C.

2.7. Plating Procedure.

Growth was monitored on Day 0, 3, 6, 9, 12, 15 using colony counting on selective media) or all refrigeration trials. For temperature abuse trials, growth was monitored on Day 0, 1, 2, 3, 4, 5. Three biological replicates, via 10 ml aliquots, were performed for each experiment. Dilution factors were adjusted based on colony counts of the inoculum. Recovery procedures as described previously were followed if the counts fall below the level of detection (BLOD). Normally, plates with colony counts are within the range of 25-250 CFU are used for accuracy, however spread plating can detect 1 CFU. The detection limit was calculated using 1 CFU divided by the number of replicate plates used which was 0.50 log CFU/ml.

2.8. pH and titratable acidity.

The pH and titratable acidity (TA) were measured in tandem on each sampling day for the inoculated cold brew. Also, the pH was measured for the negative control samples. The Fisherbrand™ accumet™ AB15 Basic pH meter was used. The measurement of titratable acidity was taken via aliquots of 10 ml cold brew were transferred to a 50 ml canonical tube. Content was titrated using a 0.1 M NaOH solution until pH meter read 8.0.

2.9. Statistical Analysis.

Experiments were repeated three times (biological replicates) with triplicate samples for each trial (technical replicates). The survival of each pathogen in cold brew was compared throughout the duration of two weeks. JMP software (SAS) was used to determine whether temperature abuse and/or additives significantly impact pathogen survival. Statistical analyses were conducted using ANOVA and Tukey's test.

3. Results

3.1. Background microflora of Cold Brew coffee.

The background microflora of cold brew at different temperatures and with select additives was determined on tryptic soy agar (TSA), plate count agar (PCA), and selective/differential media. No colony forming units were observed on plates throughout the duration of plain cold brew refrigeration and temperature abuse trials. Therefore, background microflora would not interfere with the enumeration of select pathogens.

3.2. Survival of B. cereus, E. coli, L. monocytogenes and S. enterica Cold Brew Storage at Refrigeration Trials.

The initial refrigeration experiment involved a plain cold brew recipe which was inoculated at ~ 6 log CFU/ml for each pathogen. *Salmonella* populations enumerated on XLT4 were 5.99 ± 0.12 log CFU/ml after inoculation and were reduced significantly ($P < 0.001$) to 5.36 ± 0.17 CFU/ml on Day 3 and insignificantly declined to 3.79 ± 0.79 CFU/ml on Day 3 and Day 6, log CFU/ml reduction 0.63 and 1.57, respectively, until Day 9 where populations were below the level of detection (BLOD) (Fig 2.1). No *Salmonella* could be recovered on XLT4 from cold brew on Day 9.

Listeria populations enumerated on MOX were 5.67 ± 0.18 on Day 0 and were reduced to 5.54 ± 0.17 log CFU/ml on Day 3, 4.83 ± 0.57 CFU/ml on Day 6, 0.73 ± 0.98 CFU/ml on Day 9, until Day 12 where populations were below the limit of detection (BLOD) (Fig 2.1). Reductions were significant for Day 0, Day 3, Day 6, and Day 9 for *Listeria* samples. Recovery procedures were followed for Day 12 samples and streak plating on MOX confirmed samples were negative for *Listeria*.

E.coli populations enumerated on MAC were 6.10 ± 0.32 initially and were reduced to 5.63 ± 0.46 CFU/ml on Day 3, 5.08 ± 0.42 CFU/ml on Day 6, 4.77 ± 0.12 CFU/ml and Day 9, until Day 12 where populations were BLOD. Reductions were significant between Day 3 ($P < .001$), Day 6 ($P < .001$), Day 9 ($P < .001$), and Day 12 ($P < .001$).

In comparison to *E. coli, L. monocytogenes* and *S. enterica*, for *B. cereus* a small but significant increase in population occurred between Day 9 and Day 12 ($P < 0.01$). Initial populations were 5.38 initially and were 5.09 CFU/ml, 4.89 CFU/ml, 4.89 CFU/ml, 5.02 CFU/ml, and 4.91 CFU/ml. There was no complete inactivation for *B. cereus* samples stored under refrigeration.

3.3. Survival of B. cereus, E. coli, L. monocytogenes and S. enterica Cold Brew Storage during Temperature Abuse.

Plain cold brew was inoculated at ~ 6 log CFU/g for each pathogen and subsequently stored in room temperature (25 °C) and 37 °C incubators. For E.coli, at 25 °C, 5.81 ± 0.09 CFU/ml, 2.88 ± 0.83 CFU/ml, 0.07 ± 0.10 CFU/ml were observed for Day 0, Day 1, and Day 2. Complete inactivation occurred on Day 3, confirmed by recovery procedures. Salmonella populations for samples stored at room temperature were 5.81 ± 0.85 initially and were reduced to 0.82 ± 1.30 CFU/ml on Day 1. For Listeria, initial populations were 5.72 ± 0.29 CFU/ml, samples were BLOD on Day 1 and recovery procedures confirmed complete inactivation occurred on Day 1. Similarly, For *E. coli, S. enterica*, and *L. monocytogenes* at 37 °C, populations were initially 5.56 ± 0.30 CFU/ml, 5.18 ± 0.85 CFU/ml, 5.24 ± 0.81 CFU/ml, complete inactivation occurred.

B. cereus population means were not significantly different across storage temperatures (4 ℃, 25 ℃, 37 ℃) until Day 9, where declines in population were significantly different for all three storage temperatures. Then, mean populations were not significantly different for 4 ℃ and 25 ℃ on Day 12 to Day 21. For *E. coli* and *S. enterica*, values were significantly different until complete inactivation. For S. enterica, storage at 37 ℃ on Day 1-3 and 25 ℃ on Day 3 are statistically similar.

3.4. Comparison of pH and TA of pathogens at various storage temperatures of cold brew.

The pH of cold brew stored at refrigeration averaged 5.59 ± 0.15, 5.54 ± 0.08, 5.59 ± 0.14 , and 5.76 ± 0.16 CFU/ml, initially on Day 0 for *E. coli*, S. enterica, L. monocytogenes, and *B. cereus* (Table 2.5). Refrigerated cold brew pH decreased significantly ($P < 0.001$) from Day 0-Day 6, a significant increase ($P < 0.001$) at Day 9 and decreased significantly from Day 12-15 ($P < .001$) for all inoculated samples (Table 2.5). Mean control values for refrigeration samples remained 5.58 ± 0.18 from Day 0 to Day 15. Final Day 15 pH mean values being 5.32 ± 0.16, 5.60 ± 0.28, 5.60 ± 0.02 , and 5.45 ± 0.05 CFU/ml for *E. coli*, S. enterica, L. monocytogenes, and *B. cereus*, respectively (Table 2.5). Declines of pH were significantly higher for all pathogens at 25℃ and 37℃ compared to storage at refrigeration (4 ℃).

Titratable Acidity mean percentage for 1:8 ratio cold brew was 8.51 ± 0.37 on Day 0 and decreased significantly ($P < .001$) from Day 0-Day 15 for all pathogens at all storage temperatures (Table 2.6).

4. Discussion

There has been little to no survival research done on foodborne pathogen contamination in cold brew. This study focused on contaminating coffee post preparation and storing cold brew under different temperatures to simulate potential retail practices. It is also possible for cold brew coffee grounds to be contaminated by *B. cereus* since its presence can remain during the drying and fermentation process, but populations can be significantly reduced or eliminated during the roasting process. However, contamination may still occur during transport, packaging, storing, grinding, and brewing of the cold brew coffee.

One study that determined potential contaminants in coffee determined presence of *Enterobacteriaceae,* however strains were not typed or identified. (Lachenmeier et al., 2021) Bacterial strains used in previous studies include nonpathogenic strains of *Citrobacter freundii*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Escherichia coli*, *Klebsiela oxytoca*, *Proteus hauseri, Proteus mirabilis, Salmonella enterica*, and *Serratia marcescens* obtained from the ATCC. (Almeida et al., 2006) A multiple strain cocktail with pathogens isolated from different sources was used in this study to represent several potential strains of pathogens (Table 3.1).

Existing literature agrees with lower population results for pathogens in cold brew stored at higher temperatures and indicates that temperature abuse storage conditions reduce pathogen populations more readily than storage at refrigeration. A study done by Cerca et al. (2023) simulated environmental contamination of cold brew by initially inoculating water with *E. coli* and *B. cereus* then using varying preparation methods. They found that cold brew presented higher soluble solids compared to hot brews for most brewing methods, including full immersion for both types of brew. However, the variation in preparation methods between studies showed that hot brews showed higher contents of bioactive compounds. Low water temperatures can

impact the efficiency of the extraction rate since components such as chlorogenic acid (CQA), melanoidins and caffeine are soluble at room temp. This study determined that filters, such as cloth and paper filters, have pores too large to retain tested pathogens (*B. cereus* and *E.coli*) The focus was on comparing extraction times and, similarly, it found that 24h extraction at room temperature is more efficient for microbiological safety than a shorter extraction time at room temperature and storage for 24 h at 4°C, however prolonged storage at 25°C could lead to growth and multiplication, therefore cold brew should only be kept refrigerated for a limited time. (Cerca et al., 2023)

The importance of pH for *E. coli* and *Salmonella* spp. was highlighted in a study that simulated gastric juice pH levels. (Ueno et al., 2020) The antimicrobial activities of the organic acids already available in foods are enhanced at a lower pH as the acids become unionized which penetrate cells more easily than their ionized form. The acid tolerance of bacteria is related to food-borne illness. Both *E. coli* and *Salmonella* have acid tolerance at low pH such as 2-4 due to their ability to excrete and reduce protons from cells. A study with wine found that *E. coli* survival is less impacted by low pH conditions than *Salmonella*. A finding from our study determined a similar effect of *E. coli* being more tolerant to lower pH than *Salmonella*. They were most resistant at stationary phase and more sensitive at exponential. In correspondence to previous findings by Ueno et al. (2020) , longer incubation and concentration of coffee is more effective to reduce or kill *E. coli* and *Salmonella*. (Ueno et al., 2020) The most tolerant was *B. cereus* which along with producing endospores has different physiological responses which initially declines the growth rate, but growth resumes after repairing damage. (Mols & Abee, 2011)

Our observation of growth of *B. cereus* in cold brew at Day 9 and 12 under refrigeration, after a longer storage period of time, agrees with previous study done by Chen et al. (2009) where both acids adapted, and non-acid adapted *B. cereus* were added to commercial acidic beverage (pH 3.7). Chen et al. (2009) observed a rapidly declining population of *B. cereus* within the first 3 minutes after inoculation and the decline of the population was slow throughout the rest of the storage regardless of acid adaptation. Additionally, this study found that survival is less in the beverage stored at 25 °C (Chen et al., 2009)

5. Conclusions

Cold brew is typically brewed ready-to-drink at the desired coffee to water ratio and stored under refrigeration for a short period of time. We analyzed how several common foodborne pathogens survived in cold brew under typical conditions. This study showed that there are significant declines in bacterial populations for all strains tested over the course of the experiment. Compete inactivation occurred for *E. coli*, *S. enterica*, and *L. monocytogenes*; refrigeration prolonged complete inactivation compared to temperature abused trials. Therefore, appropriate sanitation must occur during cold brew preparation to discourage introduction from outside sources such as food-contact surfaces and contamination from human handling. Complete inactivation for *B. cereus* did not occur at refrigeration and room temperature but was observed at 37 °C. Significant growth for *B. cereus* ($P < 0.01$) found in this study suggests cold brew with similar acidity parameters (pH and TA) cannot completely be inhibited. Introducing contaminated ground coffee beans during preparation is the most likely contamination source of *B. cereus* in cold brew coffee. Although there are no tolerance limits for *B. cereus* in coffee, however limits have been established for tea (Souza & Abrantes, 2011). Results of this study

suggest such limits should be adopted to all coffee or a special cold brew coffee bean handling and labeling must be adopted.

References

Almeida, A. A. P., Farah, A., Silva, D. A., Nunan, E. A., & Glória, M. B. A. (2006). Antibacterial activity of coffee extracts and selected coffee chemical compounds against enterobacteria. *Journal of Agricultural and Food Chemistry*, *54*(23), 8738-8743.

BCCDC. (2017). Nitro cold brew coffee food safety risks. http://www.bccdc.ca/resource-gallery/Documents/Educational%20Materials/EH/FPS/Food/Nitro_Cold_Brew_Coffee_Food_Safety_Risks.pdf

Bergey, D. H. (1994). *Bergey's manual of determinative bacteriology*. Lippincott Williams & Wilkins.

Buzzanell, P. J. (1979). *Coffee production and trade in Latin America* (Vol. 288). US Department of Agriculture, Foreign Agricultural Service.

Cerca, N. F., DePaula, J., Calado, V. M., Miguel, M. A. L., & Farah, A. (2023). Bioactive profile and microbiological safety of Coffea arabica and Coffea canephora beverages obtained by innovative cold extraction methods (cold brews). *Food Research International*, *174*, 113667.

Chen, J.-L., Chiang, M.-L., & Chou, C.-C. (2009). Survival of the acid-adapted Bacillus cereus in acidic environments. *International journal of food microbiology*, *128*(3), 424-428.

Clark, M. A., & Barrett, E. L. (1987). The phs gene and hydrogen sulfide production by Salmonella typhimurium. *Journal of Bacteriology*, *169*(6), 2391-2397.

Curtis, G., Mitchell, R., King, A. F., & Griffin, E. J. (1989). A selective differential medium for the isolation of Listeria monocytogenes. *Letters in applied microbiology*, *8*(3), 95-98.

Czarniecka-Skubina, E., Pielak, M., Sałek, P., Korzeniowska-Ginter, R., & Owczarek, T. (2021). Consumer Choices and Habits Related to Coffee Consumption by Poles. *Int J Environ Res Public Health*, *18*(8). https://doi.org/10.3390/ijerph18083948

Debuisson, N., Gurevich, R., & Even, R. (2021). Bacterial and Viral contamination of table forks, table spoons, dessert forks and teaspoons in restaurants, coffee shops, and university/hospital cafeteria. *Int. J. Curr. Microbiol. Appl. Sci*, 1-20.

Durocher, J. F. (1979). A Concise Guide to Coffee from seedling to Cup. *Cornell hotel and restaurant administration quarterly*, *19*(4), 53-66.

Jung, B., & Hoilat, G. J. (2022). MacConkey medium. In *StatPearls [Internet]*. StatPearls Publishing.

Kim, H.-J., Park, S.-H., & Kim, H.-Y. (2006). Comparison of Salmonella enterica serovar Typhimurium LT2 and non-LT2 Salmonella genomic sequences, and genotyping of salmonellae by using PCR. *Applied and environmental microbiology*, *72*(9), 6142-6151.

Kwok, R., Lee Wee Ting, K., Schwarz, S., Claassen, L., & Lachenmeier, D. W. (2020). Current challenges of cold brew coffee—roasting, extraction, flavor profile, contamination, and food safety. *Challenges*, *11*(2), 26.

Lachenmeier, D., Noack, D., Röhnisch, J., & Seren, H. (2021). Cold brew coffee–a microbial hazard. *Tea Coffee Trade J*, *193*, 32-33.

Mols, M., & Abee, T. (2011). Bacillus cereus responses to acid stress. *Environmental microbiology*, *13*(11), 2835-2843.

Souza, C. d. M. O. d. C. C., & Abrantes, S. d. M. P. (2011). Detection of enterotoxins produced by B. cereus through PCR analysis of ground and roasted coffee samples in Rio de Janeiro, Brazil. *Food Science and Technology*, *31*, 443-449.

Starbucks. (2024). Locking in Flavor. https://archive.starbucks.com/record/locking-in-flavor

Ueno, H., Tsutsuura, S., Inoue, A., & Murata, M. (2020). Bactericidal effects of coffee and chlorogenic acid on Escherichia coli and Salmonella spp. under low pH or gastric acid conditions. *Food Science and Technology Research*, *26*(2), 247-256.

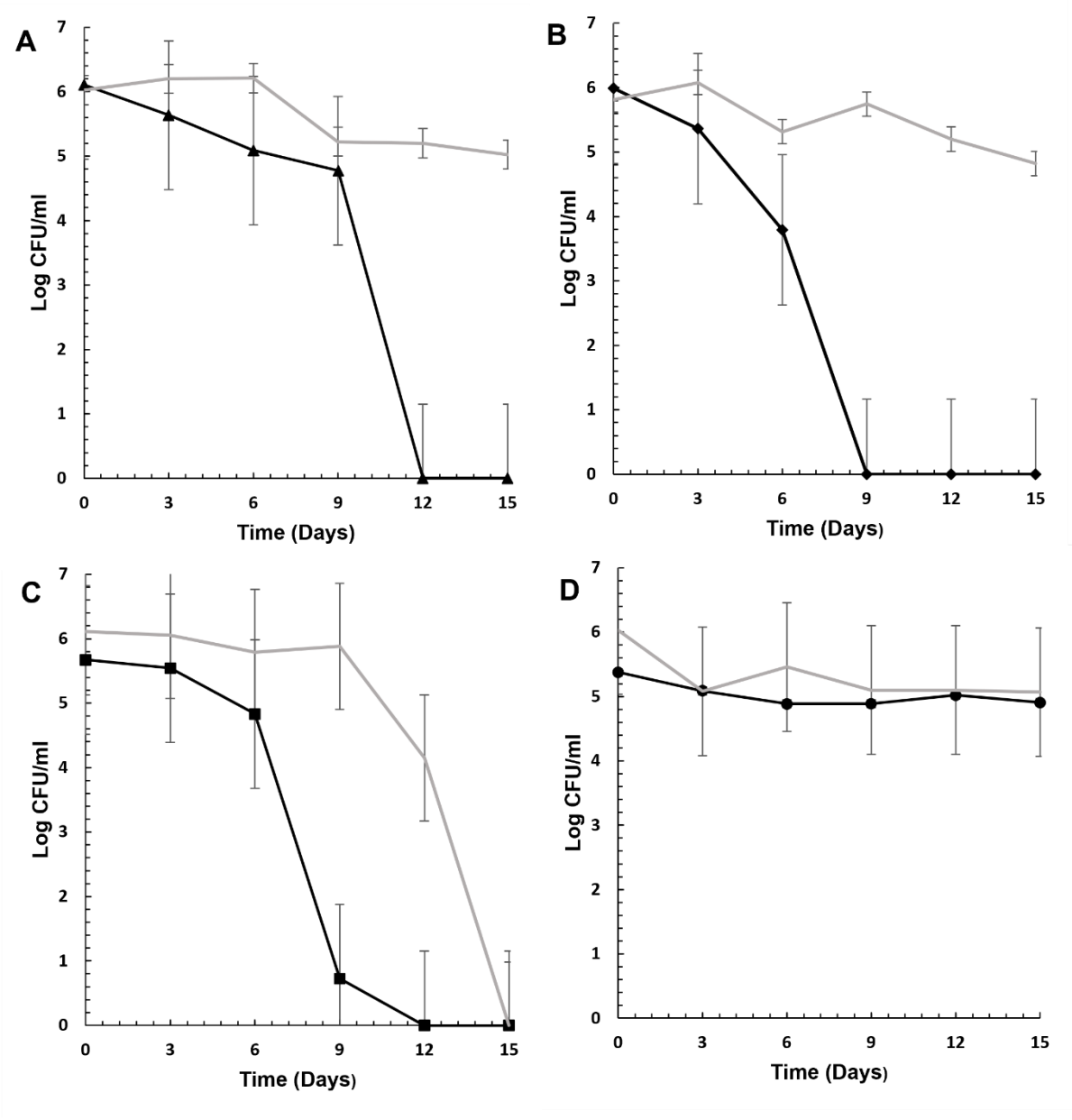

Figure 2.1. Survival of E.coli (graph A), *S. enterica* (graph B), *L. monocytogenes* (graph C), and *B. cereus* (graph D) for plain coffee stored at 4°C. Results are mean ± standard deviation plate counts on MAC, XLT4, MOX, and MYP (n = 9), respectively. Survival of pathogens in PBS is denoted by the gray line with no symbols Survival below 0.50 CFU/ml is reported as below the limit of detection (BLOD).

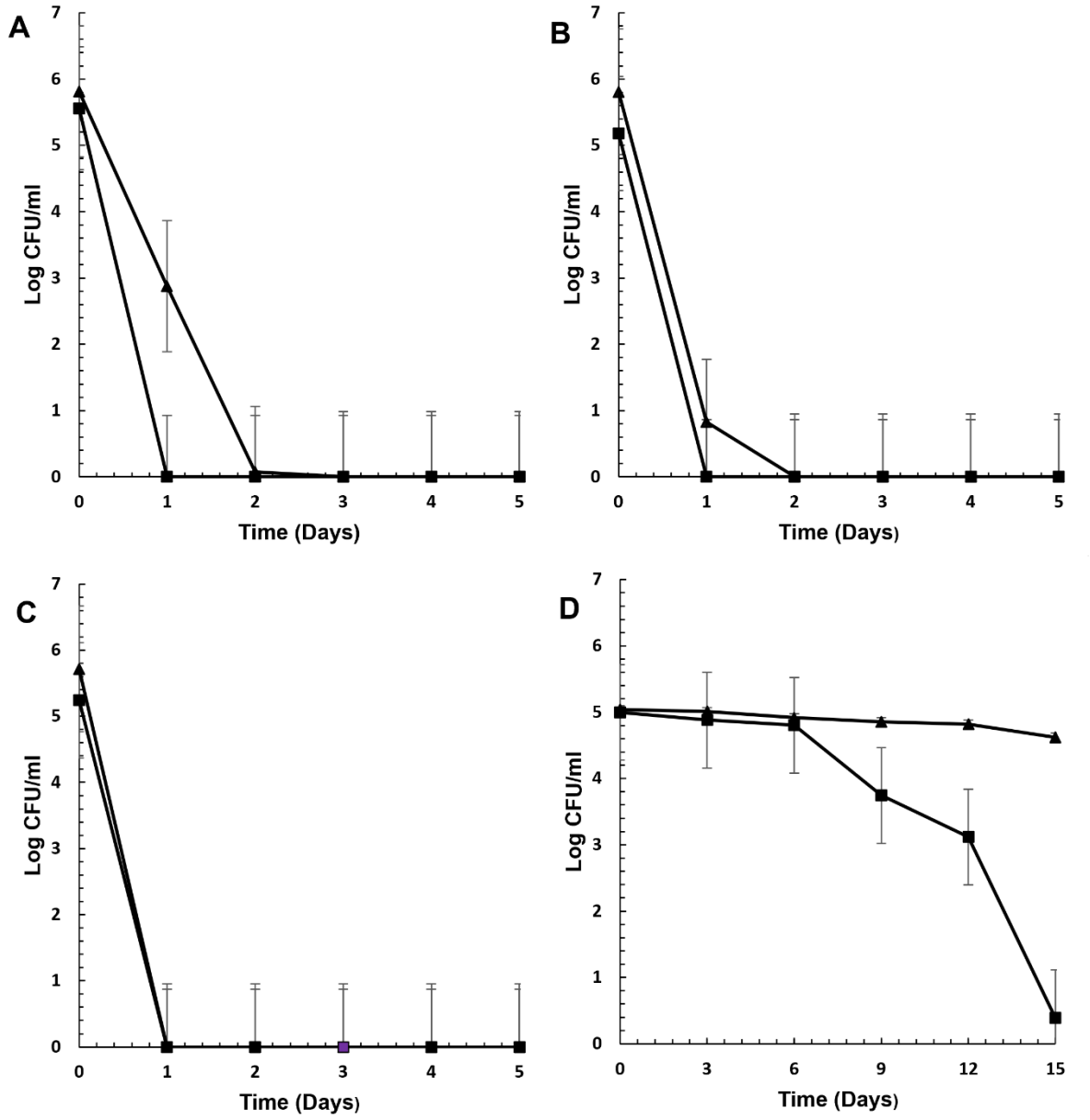

Figure 2.2. Survival of E.coli (graph A), *S. enterica* (graph B), *L. monocytogenes* (graph C), and *B. cereus* (graph D) for plain coffee stored at 25°C (triangle symbol) and 37°C (square symbol). Results are mean ± standard deviation plate counts on MAC, XLT4, MOX, and MYP (n = 9), respectively. Survival below 0.50 CFU/ml is reported as below the limit of detection (BLOD).

Table 2.1. Survival of *E. coli* a during cold brew stored at 4°C, 25°C, 37°C. Also shown in figures (graph A). Populations are listed as mean ± standard deviation.

Storage Temperature	Day	MacConkey (Log CFU/ml)
4°C	0	6.10 ± 0.32
	3	5.63 ± 0.46
	6	5.08 ± 0.42
	9	4.77 ± 0.12
	12	BLOD
	15	BLOD
25°C	0	5.81 ± 0.09
	1	2.88 ± 0.83
	2	0.07 ± 0.10
	3	BLOD
	4	BLOD
	5	BLOD
37°C	0	5.56 ± 0.30
	1	BLOD
	2	BLOD
	3	BLOD
	4	BLOD
	5	BLOD

Table 2.2. Survival of *S. enterica* during cold brew stored at 4°C, 25°C, 37°C. Also shown in figures (graph B). Populations are listed as mean ± standard deviation.

Storage Temperature	**Day**	**XLT4 (Log CFU/ml)**
4°C	0	5.99 ± 0.12
	3	5.36 ± 0.17
	6	3.79 ± 0.79
	9	BLOD
	12	BLOD
	15	BLOD
25°C	0	5.81 ± 0.85
	1	0.82 ± 1.30
	2	BLOD
	3	BLOD
	4	BLOD
	5	BLOD
37°C	0	5.18 ± 0.85
	1	BLOD
	2	BLOD
	3	BLOD
	4	BLOD
	5	BLOD

Table 2.3. Survival of *L. monocytogenes* during cold brew stored at 4°C, 25°C, 37°C. Also shown in figures (graph C). Populations are listed as mean ± standard deviation.

Storage Temperature	**Day**	**MOX (Log CFU/ml)**
4°C	0	5.67 ± 0.18
	3	5.54 ± 0.17
	6	4.83 ± 0.57
	9	0.73 ± 0.98
	12	BLOD
	15	BLOD
25°C	0	5.72 ± 0.29
	1	BLOD
	2	BLOD
	3	BLOD
	4	BLOD
	5	BLOD
37°C	0	5.24 0.81
	1	BLOD
	2	BLOD
	3	BLOD
	4	BLOD
	5	BLOD

Table 2.4. Survival of *B. cereus* during cold brew stored at 4°C, 25°C, 37°C. Also shown in figures (graph D). Populations are listed as mean ± standard deviation.

Storage Temperature	Day	MYP (Log CFU/ml)
4°C	0	5.4 ± 0.58
	3	5.09 ± 0.29
	6	4.94 ± 0.2
	9	4.89 ± 0.08
	12	5.03 ± 0.12
	15	4.92 ± 0.1
25°C	0	4.89 ± 0.02
	3	4.96 ± 0.08
	6	5.04 ± 0.07
	9	5.01 ± 0.13
	12	4.92 ± 0.26
	15	4.86 ± 0.15
37°C	0	4.82 ± 0.13
	3	4.62 ± 0.17
	6	4.56 ± 0.24
	9	4.8 ± 0.16
	12	5 ± 0.11
	15	4.88 ± 0.16

Table 2.5. Mean pH at 4°C, 25°C, 37°C for *E. coli, S. enterica, L. monocytogenes*, and *B. cereus* in plain cold brew. Populations are listed as mean ± standard deviation.

Storage Temperature	Day	pH			
		E. coli	***S. enterica***	***L. monocytogenes***	***B. cereus***
4°C	0	5.59 ± 0.15	5.54 ± 0.08	5.59 ± 0.14	5.76 ± 0.16
	3	5.49 ± 0.01	5.51 ± 0.12	5.35 ± 0.24	5.74 ± 0.25
	6	5.43 ± 0.06	5.32 ± 0.14	5.55 ± 0.06	5.59 ± 0.23
	9	5.65 ± 0.18	5.66 ± 0.07	5.63 ± 0.15	5.60 ± 0.09
	12	5.33 ± 0.27	5.61 ± 0.24	5.61 ± 0.09	5.57 ± 0.02
	15	5.32 ± 0.16	5.60 ± 0.28	5.60 ± 0.02	5.45 ± 0.05
25°C	0	5.60 ± 0.13	5.59 ± 0.11	5.61 ± 0.27	5.62 ± 0.24
	3	5.41 ± 0.09	5.42 ± 0.14	5.55 ± 0.28	5.42 ± 0.15
	6	.	.	.	5.15 ± 0.04
	9	.	.	.	5.12 ± 0.18
	12	.	.	.	5.14 ± 0.14
	15	.	.	.	5.10 ± 0.11
37°C	0	5.58 ± 0.24	5.49 ± 0.36	5.60 ± 0.01	5.68 ± 0.15
	3	5.24 ± 0.11	5.32 ± 0.08	5.43 ± 0.25	5.38 ± 0.13
	6	.	.	.	4.95 ± 0.21
	9	.	.	.	4.92 ± 0.33
	12	.	.	.	4.87 ± 0.15
	15	.	.	.	4.88 ± 0.01

Table 2.6. Mean TA for all three temps for all pathogens at all storage temperatures. Result values are listed as mean ± standard deviation.

Storage Temperature	**Day**	TA (Average % Acid)			
		E. coli	***S. enterica***	***L. monocytogenes***	***B. cereus***
4°C	0	8.90 ± 0.07	8.46 ± 0.09	8.21 ± 0.01	8.50 ± 0.01
	3	8.20 ± 0.09	8.93 ± 0.01	8.61 ± 0.01	8.60 ± 0.10
	6	8.61 ± 0.12	7.81 ± 0.01	8.61 ± 0.01	8.62 ± 0.01
	9	9.01 ± 0.01	8.40 ± 0.01	8.50 ± 0.01	8.51 ± 0.04
	12	9.01 ± 0.05	8.61 ± 0.06	9.21 ± 0.01	9.00 ± 0.04
	15	9.31 ± 0.04	9.81 ± 0.01	9.31 ± 0.01	9.51 ± 0.05
25°C	0	8.60 ± 0.04	8.45 ± 0.12	8.24 ± 0.01	8.21 ± 0.01
	3	8.88 ± 0.01	8.68 ± 0.01	8.99 ± 0.01	8.70 ± 0.01
	6	.	.	.	9.23 ± 0.02
	9	.	.	.	10.21 ± 0.11
	12	.	.	.	11.44 ± 0.01
	15	.	.	.	12.00 ± 0.06
37°C	0	8.77 ± 0.08	8.52 ± 0.38	8.57 ± 0.12	8.66 ± 0.01
	3	9.31 ± 0.28	9.20 ± 0.05	9.33 ± 0.02	9.12 ± 0.17
	6	.	.	.	9.96 ± 0.03
	9	.	.	.	10.92 ± 0.21
	12	.	.	.	13.82 ± 0.43
	15	.	.	.	27.02 ± 0.24

CHAPTER 3

IMPACT OF ADDITIVES ON MICROBIOLOGICAL SAFETY OF COLD BREW

1. Introduction

The use of additives such as shredded coconut and spices (cinnamon and nutmeg) may influence the microbiological stability and shelf-life of cold brew. (Kwok et al., 2020) Initially, lipid content in coffee beans is about 10-17% of their dry weight. (Nerurkar et al., 2023) The addition of shredded coconut introduces sugars and fats that potentially increase nutrient availability for microorganisms. (USDA, 2019) Additionally, low quality raw material for shredded coconut production potentially introduces microbial hazards since the coconut liquid endosperm is conducive to microbial growth. Desiccated coconut is prepared from the white kernel and is processed by paring, pasteurizing, drying, comminuting, and sifting. Post-processing contamination may occur during transport and storage. There have been reported positive cases of *Salmonella spp.* in imported coconut products. (Gabriel et al., 2017)

Similarly. spices such as cinnamon and nutmeg have had incidences of microbial contamination. Dried foods were examined for incidence of *B. cereus* and this species was found to be a common contaminant in spices. However, the study showe a low contamination rate, with no positive sample exceeding 4,000 *B. cereus* per gram. (Kim & Goepfert, 1971; McKee, 1995)

Despite potential contaminants, spices contain natural antimicrobials. The antimicrobial effect of spice is determined by the type of plant. Structural configurations, such as the presence of hydroxyl groups, have varying capabilities that can disrupt microbial cell membranes. (Ali et al., 2021; McKee, 1995). Spices have been added to foods as flavoring agents since ancient

times, often additionally used as medicine and food preservatives. Cinnamon impacts coffee by supplementing flavor and antioxidant activity by adding bioactive compounds such as cinnamic acid and coumarin. Studies on the impact of the nutraceutical potential of coffee supplemented with cinnamon have been done which observed a much higher phenolic compound content,5-caffeoyl-quinic acid, in cinnamon non-digested as opposed to after the digestion processes.(Erskine et al., 2022)

2. Materials and Methods

3.1. Background Microflora of Cold Brew with Additives.

Additional background microflora testing was performed for 1:8 cold brew brewed at refrigeration with either 6 grams of coconut or 1.75 grams spices (nutmeg or cinnamon) added upon extraction per liter of cold brew prepared. Samples were taken on Day 0-15 and tested using both standard plate count agar (PCA) and Mannitol egg-yolk polymyxin agar (MYP) for *B. cereus*, Modified oxford agar (MOX) for *L. monocytogenes*, MacConkey agar (MAC) for *E. coli*, and Xylose lysine tergitol-4 agar (XLT4) for *S. enterica*.

3.2. Bacteria Isolates Preparation and Inoculum Preparation

Bacterial strains for *B. cereus* were isolated from cricket powder (Pal et al., 2024), *E. coli*, *S. enterica*, and *L. monocytogenes* were obtained from the UGA, Center For Food Safety culture collection, and ATCC (see Table 3.1. for further details) . All isolates were maintained as stock cultures at -80 ℃ with Tryptic soy broth containing 15 % glycerol. Each strain selected was cultured in tryptic soy agar overnight at 37 ℃ and tested for purity on selective/differential media. After the second transfer, cultures were streaked onto TSA plates and grown overnight at

37 °C. Colonies were dislodged using 0.01% peptone water and combined in a single tube to prepare a mixed inoculum. Enumeration of inoculum was done using plating technique and dilution with peptone water was calculated using the results.

3.3. Recovery procedure.

A procedure was developed for recovering potential bacterial cells when counts were below the limit of detection after literature review since no guideline currently exists for coffee. Each bacterium was tested to confirm pathogen growth in selected media with the addition of cold brew. The recovery of *B. cereus, S. enterica,* and *E. coli* samples used the same growth medium, tryptic soy agar (TSB). Listeria was grown in brain heart infusion broth (BHI). Aliquots of 1 ml inoculated cold brew, or control additive cold brew was added to 10 ml broth tubes. Recovery samples were tested in duplicate and sterile TSB and BHI blanks were used as a control. Culture media was incubated at 37 °C for 18-24 hrs. and checked for turbidity against control tubes. Selective/differential media was used to confirm presence/absence of select pathogen.

3.4. Cold Brew Preparation Procedure.

The preparation of the cold brew coffee with additives consisted of 125 grams of pre-ground Starbucks Pike Place ground coffee per 1 L of filtered water was prepared in a 2 L vessel via full immersion using a cheesecloth. To infer if additives used during extraction can increase microbial growth, one of three additives: ground cinnamon, ground nutmeg, and shredded coconut, was added. For ground cinnamon and nutmeg 1.75 grams was added to the ground coffee upon extraction, while this was 6 grams for shredded coconut. One liter water filter

sterilized was added and the mixture was agitated with sterilized instruments to ensure homogenization. This method produces approx. 650 ml of cold brew coffee per liter of prepared cold brew. The cold brew was brewed at refrigeration temperature for 12 hours. The coffee was then aliquoted into 250 ml vessels, sealed with a screw cap, and inoculated with representative strains of *L. monocytogenes*, *S. enterica*, *E. coli*, and *B. cereus*. The control was uninoculated lab-prepared cold brew coffee. For samples stored at temperature abuse conditions for additive samples, they were placed in incubators that simulated room temperature (21 °C), and 37 °C.

3.5. Plating Procedure.

Plate growth was monitored on Day 0-15 or 0-5 using traditional microbiology for all refrigeration trials and for temperature abuse trials. Three biological and technical replicates were performed for each experiment. Dilution factors were adjusted based on colony counts. Recovery procedures were followed should the counts fall below the level of detection.

3.6. pH and titratable acidity.

The pH and titratable acidity (TA) were taken on each sampling day after plating of the inoculated cold brew. The pH was measured for the negative control samples for comparison. The Fisherbrand™ accumet™ AB15 Basic pH meter was used and calibrated after each use. The measurement of pH was initially taken with 10 ml of cold brew transferred to a 50 ml canonical tube. Then, titratable acidity was taken via the same aliquot of 10 ml cold brew. Content was titrated using a 0.01 M NaOH solution until pH meter read 8.0.

3.7. Statistical Analysis.

Experiments were repeated three times with triplicate samples for each trial (n = 9). The survival of *E. coli, S. enterica*, *L. monocytogenes*, and *B. cereus* in cold brew brewed at 4°C and stored in temperature abuse conditions were compared throughout the duration of two weeks for B, cereus samples and daily for 5 days for *E. coli, S. enterica* and *L. monocytogenes*. The control, phosphate buffered saline (PBS) inoculated with select pathogens and stored at refrigeration, room temperature and 37°C, was compared to cold brew survival. JMP software (SAS) was used to determine whether temperature abuse and/or additives significantly impact pathogen survival. An analysis of variance (ANOVA), means comparison, and Tuckey-Kramer significant test was performed using the software. The differences are considered significant if p-value < 0.05.

3. Results

3.1. Background microflora of cold brew samples with additives

Background microflora was tested for uninoculated samples of cold brew with additives (coconut, nutmeg, and cinnamon) at refrigeration (4 °C) and temperature abuse (25 °C and 37 °C). Microbiological analysis was done using plating techniques on TSA, PCA and selective/differential media on Day 0 through Day 15. Tryptic soy agar (TSA) and plate count agar (PCA) counts were below level of detection for coconut and cinnamon. Further confirmation was done using recovery techniques and samples streaked on MOX, MAC, XLT4 and MYP were negative for selected pathogens. PCA counts for cold brew with nutmeg samples had a mean value of 1.47 ± 1.05 log CFU/ml. Results of recovery procedures and streaking on MYP agar showed samples were positive for *Bacillus*. This was likely a non-B. *cereus Bacillus* based on colony morphology.

3.2. Additives at refrigeration.

The additive trials included cold brew coffee brewed with cinnamon, nutmeg, and coconut. Populations for inoculated cold brew with cinnamon samples stored at refrigeration were 5.86 ± 0.16 CFU/ml, 5.89 ± 0.06 CFU/ml , and 5.98 ± 0.53 CFU/ml initially for *E. coli, L. monocytogenes* and *S. enterica*, respectively. Significant decreases for *E. coli, L. monocytogenes* and *S. enterica* occurred from Day 0 to Day 15 ($P < .001$).

For additive *E.coli* samples stored under refrigeration, mean log value of 3.58 ± 0.28 log CFU/ml for nutmeg was significantly less than 4.38 ± 0.37 log CFU/ml and 4.41 ± 0.23 log CFU/ml for cinnamon and coconut, respectively, on Day 9 ($P < .001$) *E. coli* populations were 0.45 ± 0.18 log CFU/ml on Day 15 in coconut cold brew and BLOD on Day 12 for cinnamon and nutmeg cold brew. *S. enterica* in cinnamon cold brew was log 3.16 ± 1.1 log/CFU and significantly less compared to coconut and nutmeg which had 4.46 ± 0.04 log/CFU and 4.52 ± 1.42 log/CFU, respectively, and were seen on Day 6 ($P < .001$). Additionally, *S. enterica* nutmeg populations were BLOD on Day 9, compared to 2.39 ± 0.05 log/CFU for coconut and 0.31 ± 0.49 CFU/ml for cinnamon cold brew. *L. monocytogenes* populations stored under refrigeration were significantly different on Day 3, Day 6 and Day 9 for cinnamon, coconut, and nutmeg ($P < .001$). On Day 3 and Day 6 coconut was significantly more than cinnamon. Also, on the same days, cinnamon mean log populations were significantly more than nutmeg cold brew samples ($P < .001$). On Day 9, the order from most to least, log mean values were significantly more for coconut, nutmeg, and cinnamon ($P < .001$). No growth was observed for *E. coli, L. monocytogenes* and *S. enterica* throughout the duration of the experiment (Fig. 3.1).

In additive cold brew inoculated with *B. cereus,* nutmeg average populations were significantly more than cinnamon and coconut populations on Day 6 ($P < .001$). All additives

were found significantly different on Day 9 for *B. cereus* samples stored under refrigeration. Mean log values of 5.92 ± 0.29 log CFU/ml, 5.50 ± 0.17 log CFU/ml, and 5.00 ± 0.65 log CFU/ml for nutmeg, coconut, then cinnamon. Small, but significant growth occurred from Day 3-6 for cinnamon cold brew *B. cereus* samples ($P < .001$). Significant growth occurred from Day 3-6 and insignificant growth from Day 6-9 for nutmeg cold brew ($P < .001$). In coconut cold brew, significant growth occurred from Day 6-9. *Bacillus* populations remained the same with no signs of complete inactivation past Day 21 for samples stored under refrigeration.

3.3. Additives during temperature abuse.

Mean populations for *E. coli* in cold brewed with cinnamon, nutmeg and coconut and stored at 25 °C were significantly different on Day 1 ($P < .001$). *Salmonella* populations were statistically similar on Day 1 when stored at room temperature ($P < .001$). Cold brew with coconut inoculated with *L. monocytogenes* had a mean log value of 3.08 ± 0.27 log CFU/ml, however nutmeg and cinnamon brew were BLOD on Day 1 when stored at room temperature ($P < .001$).

At room temperature, *B. cereus* cold brew with coconut mean 4.75 ± 0.14 log CFU/ml average population is significantly less than 5.02 ± 0.38 log CFU/ml cinnamon and 5.03 ± 0.19 log CFU/ml nutmeg on Day 3 ($P < .001$). Significant growth of 0.12 log CFU/ml *B. cereus* was observed from Day 3-6 for both coconut and cinnamon ($P < .001$). Cinnamon populations were significantly more on Day 3-15 for samples stored at room temperature than coconut and nutmeg ($P < .001$).

Complete reduction of mean log populations for *E.coli* in cold brew stored at 37 °C were found on Day 1 for all additives (cinnamon, coconut, and nutmeg). For *Salmonella* cold brew

stored at 37 ℃, coconut values were significantly less than nutmeg and cinnamon on Day 1 ($P < .001$). Comparatively, coconut values were significantly more than nutmeg and cinnamon on Day 1 for *L. monocytogenes* ($P < .001$).

B. cereus inoculated cold brew additive samples (coconut, nutmeg, and cinnamon) stored at 37 ℃ mean log populations were significantly different on Day 6 and Day 9 ($P < .001$). On Day 15, cold brewed with coconut was significantly less than nutmeg ($P < .001$). Additionally, *B. cereus* populations in cold brew brewed with nutmeg, 3.29 ± 0.25 log CFU/ml, is significantly less than cinnamon, 3.60 ± 0.12 log CFU/ml, when stored at 37 ℃ on the same day ($P < .001$). Coconut allowed the sustained population of *L. monocytogenes* to persist in cold brew coffee stored at 37 ℃ until Day 2 compared to Day 1 for cinnamon and nutmeg samples stored at the same temperature.

3.4. Additives pH and TA

Initial pH ranged from 5.4 to 5.6 and decreased over time for all cold brew with additives stored under refrigeration and temperature abuse conditions ($P<.05$). TA increased over time for all pathogens ($P<.05$).

4. Discussion

In the present study, the impact of additives on the microbiological safety of cold brew coffee was assessed. The results showed that the addition of cinnamon, nutmeg, and shredded coconut to cold brew coffee have a significant effect on the survival of foodborne pathogens (*Bacillus*, *Salmonella*, *Listeria*, and *E. coli*) at 4℃, 25℃, and 37℃. Coconut significantly aided in the survivability of all pathogens at refrigeration. At 25 ℃, *L. monocytogenes* survived an

additional day when coconut was added to the brew. The mean pH of cold brew coffee with additives ranged from 5.18 to 5.35 and decreased over time for cold brew stored under refrigeration and temperature abuse. This decrease in pH may have been due to continued brewing/extraction during storage time. The mean titratable acidity (TA) increased over time for all pathogens additive cold brew samples. This increase in TA may be attributed to the production of organic acids by the coffee itself or by the bacterial population present in the samples

. Overall, the addition of the three additives (cinnamon, coconut, and nutmeg) significantly impacted the microbiological safety of cold brew coffee. However, it is important to note that these results only apply to the specific strains of foodborne pathogens used in this study. Further research is necessary to determine the effects of other additives on the safety of cold brew coffee and to test a broader range of foodborne pathogens.

The effect of cinnamon on *B. cereus* in a ready-to-drink product has been previously tested. A study found that cinnamon showed *B. cereus* suppression as compared to a control in milk, however the reduction was only significant for samples stored at 25 °C. This was true for higher concentrations of cinnamon (1%) during short storage (7-10 days) comparatively to (0.5%) which reduces microbial load, but over a longer storage time (28 days). (Guler & Seker, 2009) Similarly to this study, consistent reduction of *B. cereus* was seen in cinnamon cold brew over time at 4 °C, 25 °C, and 37 °C. The small, but significant, growth that occurred in cinnamon brew on Day 3-6 throughout storage at refrigeration indicates that complete suppression did not occur at the cinnamon concentration used. Additionally, compared to milk, cold brew has a much higher acidity, and the pH increases throughout storage. This likely contributes to the starker increase and decrease in log reduction comparatively to populations in milk. In a study done on

the influence of coffee on microorganisms, it found that gram-positive bacteria appear to be more susceptible to phenolic acids. (Monente et al., 2015)

Despite nutmeg having a compound named that has bactericidal effects against B. cereus, the introduction of *Bacillus spp*. from contaminated dry spices used in cold brew such as nutmeg is also possible. (Rukayadi et al., 2009) Species such as *Bacillus licheniformis* and *Bacillus cereus* are most frequently detected in spices. (Thanh et al., 2018) The nutmeg used in this study was likely contaminated with *Bacillus*. Each species has varying stress responses, therefore *Bacillus* growth data in nutmeg cold brew samples may not reflect selected *B. cereus* strains in this experiment. (Mols & Abee, 2011)

5. Conclusions

No growth was observed for *E. coli, L. monocytogenes* and *S. enterica* in cold brew with additives, however survival of pathogens extended into typical storage times before complete inactivation. *B. cereus* populations remained the same with no signs of complete inactivation past Day 21 for samples stored under refrigeration and room temperature. Complete inactivation of *B. cereus* only occurred at 37 °C at Day 18. There were signs of slight growth, following immediate declines, for *B. cereus* populations for all temperatures. Coconut allowed the sustained population of *E. coli* to persist in cold brew coffee. Results indicated spices such as cinnamon may be used to suppress growth of main pathogen concern *B. cereus* in cold brew coffee. Coconut can aid growth and cause pathogen survival to persist in cold brew coffee throughout storage.

References

Ali, A., Ponnampalam, E. N., Pushpakumara, G., Cottrell, J. J., Suleria, H. A. R., & Dunshea, F. R. (2021). Cinnamon: A Natural Feed Additive for Poultry Health and Production-A Review. *Animals (Basel), 11*(7). https://doi.org/10.3390/ani11072026

Erskine, E., Gültekin Subaşı, B. s. r., Vahapoglu, B., & Capanoglu, E. (2022). Coffee phenolics and their interaction with other food phenolics: Antagonistic and synergistic effects. *ACS omega*, *7*(2), 1595-1601.

Gabriel, A. A., Tongco, A. M. P., & Barnes, A. A. (2017). Utility of UV-C radiation as anti-Salmonella decontamination treatment for desiccated coconut flakes. *Food Control, 71*, 117-123. https://doi.org/10.1016/j.foodcont.2016.06.026

Guler, S., & Seker, M. (2009). The effect of cinnamon and guar gum on bacillus cereus population in milk. *Journal of food processing and preservation*, *33*(3), 415-426.

Kim, H., & Goepfert, J. (1971). Occurrence of Bacillus cereus in selected dry food products. *Journal of Food Protection*, *34*(1), 12-15.

Kwok, R., Lee Wee Ting, K., Schwarz, S., Claassen, L., & Lachenmeier, D. W. (2020). Current challenges of cold brew coffee—roasting, extraction, flavor profile, contamination, and food safety. *Challenges*, *11*(2), 26.

McKee, L. (1995). Microbial contamination of spices and herbs: a review. *Lwt-Food Science and Technology*, *28*(1), 1-11.

Mols, M., & Abee, T. (2011). Bacillus cereus responses to acid stress. *Environmental microbiology*, *13*(11), 2835-2843.

Monente, C., Bravo, J., Vitas, A. I., Arbillaga, L., De Peña, M. P., & Cid, C. (2015). Coffee and spent coffee extracts protect against cell mutagens and inhibit growth of food-borne pathogen microorganisms. *Journal of Functional Foods*, *12*, 365-374.

Nerurkar, P. V., Yokoyama, J., Ichimura, K., Kutscher, S., Wong, J., Bittenbender, H. C., & Deng, Y. (2023). Medium Roasting and Brewing Methods Differentially Modulate Global Metabolites, Lipids, Biogenic Amines, Minerals, and Antioxidant Capacity of Hawai 'i-Grown Coffee (Coffea arabica). *Metabolites*, *13*(3), 412.

Pal, A., Mann, A., & den Bakker, H. C. (2024). Analysis of Microbial Composition of Edible Insect Products Available for Human Consumption within the United States using Traditional Microbiological Methods and Whole Genome Sequencing. *Journal of Food Protection*, 100277.

Rukayadi, Y., Lee, K.-H., Han, S.-H., Kim, S.-K., & Hwang, J.-K. (2009). Antibacterial and sporicidal activity of macelignan isolated from nutmeg (Myristica fragrans Houtt.) against Bacillus cereus. *Food Science and Biotechnology*, *18*(5), 1301-1304.

Thanh, M. D., Frentzel, H., Fetsch, A., Krause, G., Appel, B., & Mader, A. (2018). Tenacity of Bacillus cereus and Staphylococcus aureus in dried spices and herbs. *Food Control*, *83*, 75-84.

Table 3.1. *Bacillus, Salmonella, Listeria* and *STEC* strains used for inoculation

Bacteria	Strain/ID	Isolation Source
E. coli O26: H11	CFS2*	unknown
E. coli O45:H2	MI05-14*	human
E. coli O103:H7	239	unknown
E. coli O121	I2016007842*	flour outbreak
E. coli O145:NT	IH 16*	human
Listeria monocytogenes	ATCC15313	Rabbit, Cambridge, England
Listeria monocytogenes	ATCC51779	Dairy products, Belgium
Listeria seeligeri	F8385	unknown
Listeria monocytogenes	ATCC51780	Belgium cheese
Salmonella enterica	2010K-0316	unknown
Salmonella Typhimurium	96037-1	unknown
B. mosaicus subsp. cereus	IP107*	cricket powder
B. cereus s.s.	IP98	cricket powder
B. mosaicus subsp. cereus; B. cereus	IP71	cricket powder

*Note: Strains have been confirmed as being toxin-producing.

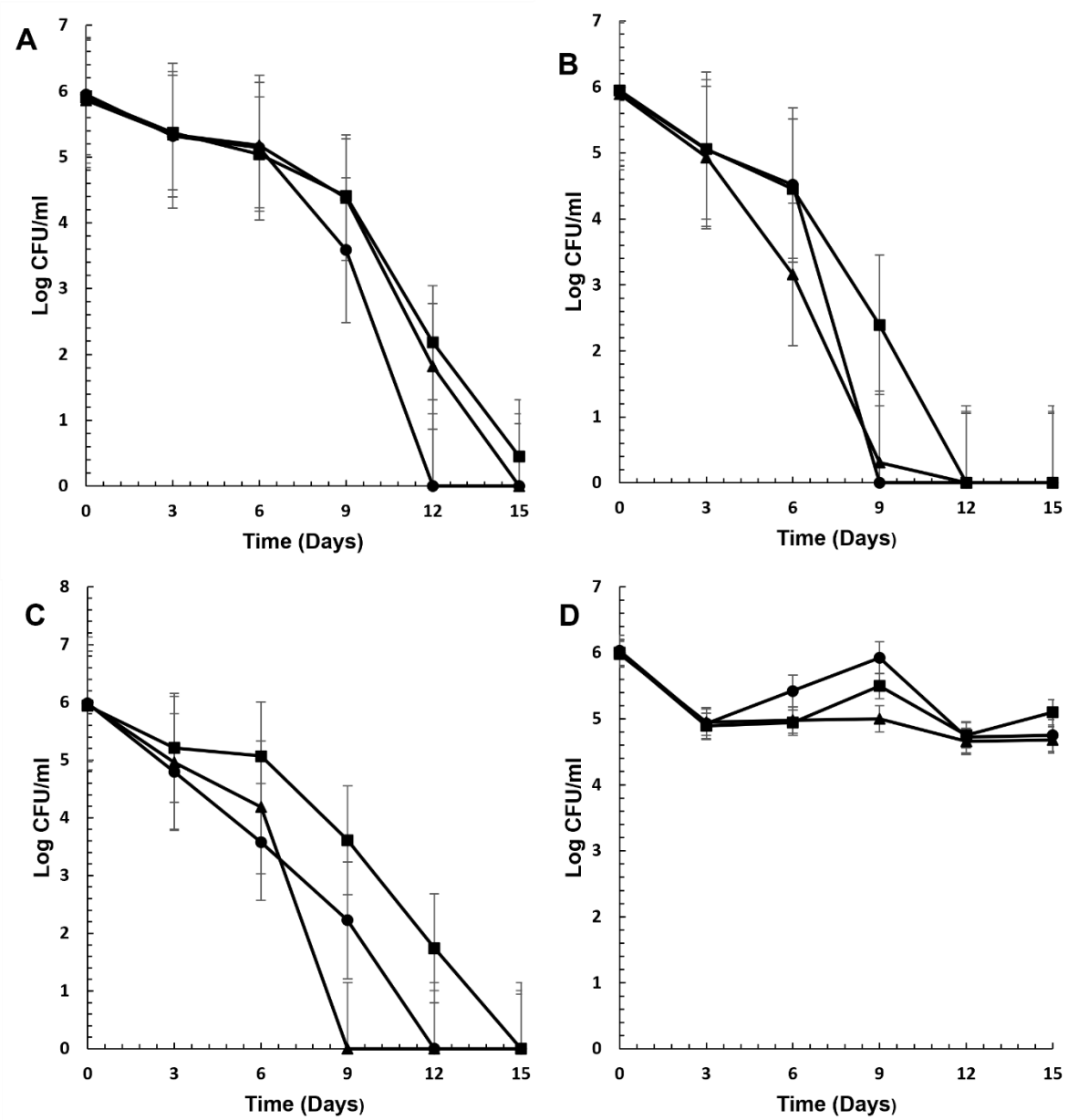

Figure 3.1. Survival of *E.coli* (graph A), *S. enterica* (graph B), *L. monocytogenes* (graph C), and *B. cereus* (graph D) for coffee stored at refrigeration (4 °C) brewed with coconut (square symbol), cinnamon (triangle symbol) and nutmeg (circle symbol). Results are mean ± standard deviation plate counts on MAC, XLT4, MOX, and MYP (n = 9), respectively. Survival below 0.30 CFU/ml is reported as below the limit of detection (BLOD).

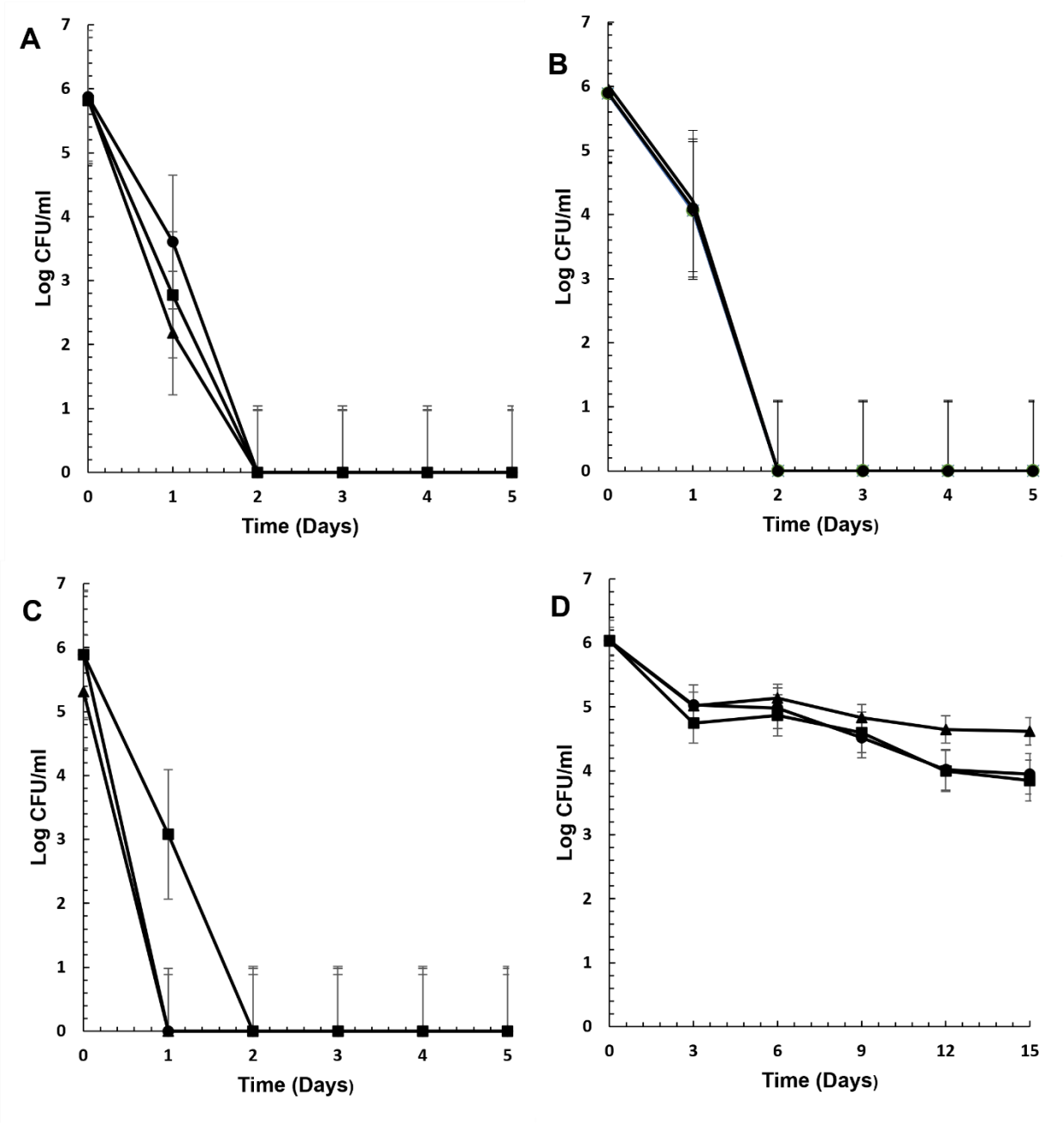

Figure 3.2. Survival of E.coli (graph A), *S. enterica* (graph B), *L. monocytogenes* (graph C), and *B. cereus* (graph D) for plain coffee stored at room temperature (25 °C). brewed with coconut (square symbol), cinnamon (triangle symbol) and nutmeg (circle symbol). Results are mean ± standard deviation plate counts on MAC, XLT4, MOX, and MYP (n = 9), respectively. Survival below 0.30 CFU/ml is reported as below the limit of detection (BLOD).

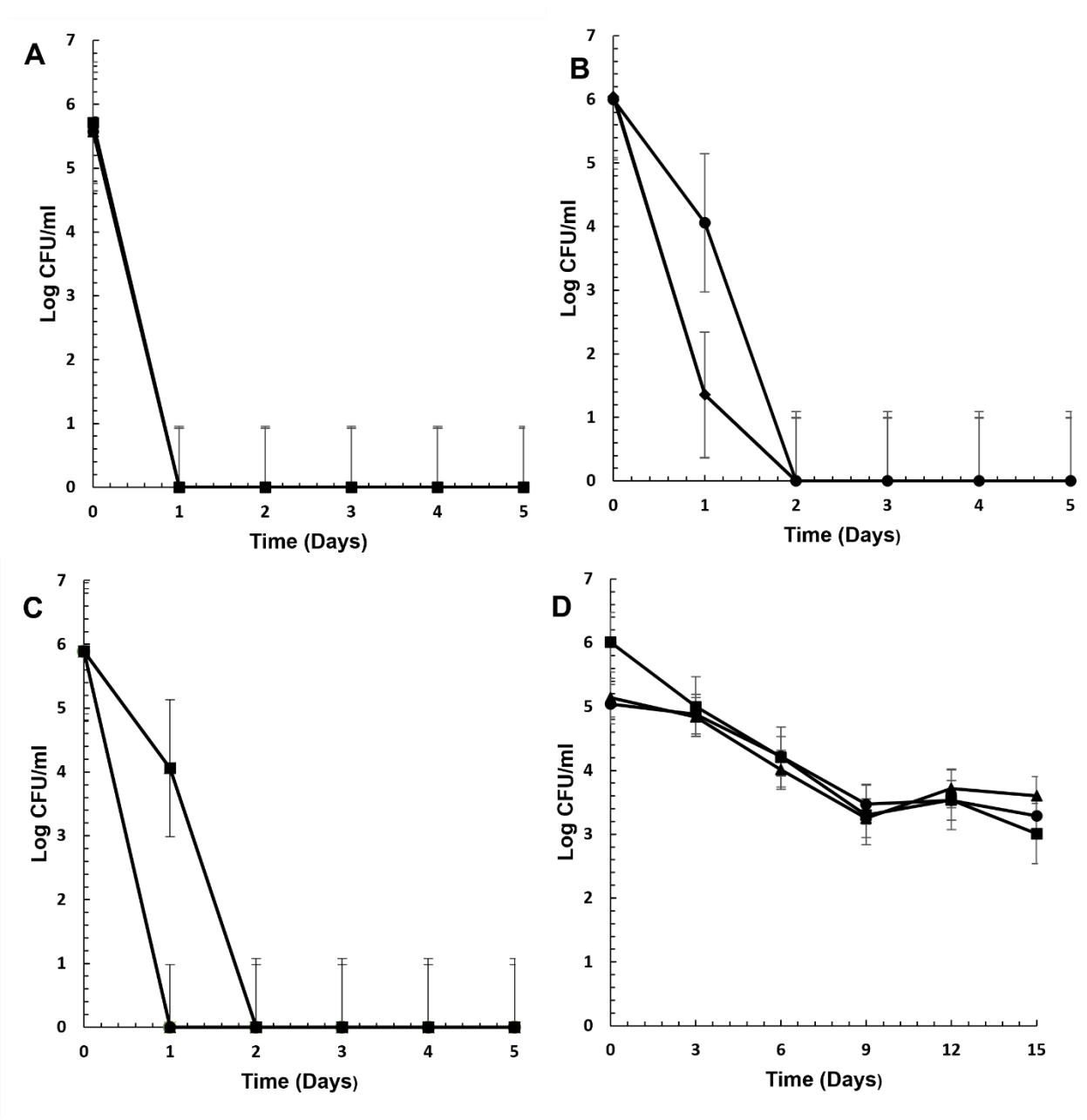

Figure 3.3. Survival of E.coli (graph A), *S. enterica* (graph B), *L. monocytogenes* (graph C), and *B. cereus* (graph D) for plain coffee stored at 37 °C. brewed with coconut (square symbol), cinnamon (triangle symbol) and nutmeg (circle symbol). Results are mean ± standard deviation plate counts on MAC, XLT4, MOX, and MYP (n = 9), respectively. Survival below 0.30 CFU/ml is reported as below the limit of detection (BLOD).

Table 3.2. Pathogen survival in cold brewed with cinnamon during storage at 4 ℃, 25 ℃ and 37℃.

Additive	Storage Temperature	Day	*E. coli* (Log CFU/ml)	*S. enterica* (Log CFU/ml)	*L. monocytogenes* (Log CFU/ml)
Ci	4℃	0	5.86 ± 0.16	5.89 ± 0.06	5.98 ± 0.53
		3	5.34 ± 0.74	4.93 ± 0.66	4.96 ± 2.4
		6	5.32 ± 0.75	3.16 ± 1.1	4.18 ± 1.83
		9	4.38 ± 0.37	0.31 ± 0.49	BLOD
		12	1.82 ± 2.37	BLOD	BLOD
		15	BLOD	BLOD	BLOD
	25℃	0	5.84 ± 0.27	5.89 ± 0.06	5.31 ± 0.03
		1	2.18 ± 0.41	4.06 ± 0.22	BLOD
		2	BLOD	BLOD	BLOD
		3	BLOD	BLOD	BLOD
		4	BLOD	BLOD	BLOD
		5	BLOD	BLOD	BLOD
	37℃	0	5.57 ± 0.35	6.04 ± 0.12	5.89 ± 1.59
		1	BLOD	1.35 ± 1.95	BLOD
		2	BLOD	BLOD	BLOD
		3	BLOD	BLOD	BLOD
		4	BLOD	BLOD	BLOD
		5	BLOD	BLOD	BLOD

Table 3.3. Cold brew with cinnamon stored under refrigeration, room temperature, and 37 °C *B. cereus* microbial counts from Day 0-Day 15. Populations are listed as mean ± standard deviation.

Additive	Storage Temperature	Day	*B. cereus* (Log CFU/ml)
Ci	4°C	0	5.98 ± 0.50
		3	4.95 ± 1.91
		6	4.98 ± 1.2
		9	5.00 ± 0.65
		12	4.73 ± 0.55
		15	4.71 ± 0. 68
	25°C	0	6.03 ± 0.11
		3	5.02 ± 0.38
		6	5.14 ± 1.73
		9	4.83 ± 0.54
		12	4.65 ± 1.43
		15	4.62 ± 0.41
	37°C	0	5.14 ± 0.02
		3	4.84 ± 0.69
		6	4.01 ± 0.84
		9	3.25 ± 0. 33
		12	3.72 ± 1.34
		15	3.60 ± 0.12

Table 3.4. Pathogen survival in cold brewed with coconut during storage at 4 ℃, 25 ℃ and 37 ℃**.** Populations are listed as mean ± standard deviation.

Additive	Storage Temperature	Day	*E. coli* (Log CFU/ml)	*S. enterica* (Log CFU/ml)	*L. monocytogenes* (Log CFU/ml)
Co	4℃	0	5.90 ± 0.04	5.95 ± 0.03	5.95 ± 0.14
		3	5.37 ± 0.23	5.06 ± 0.16	5.21 ± 0.05
		6	5.04 ± 0.27	4.46 ± 0.04	5.07 ± 0.14
		9	4.41 ± 0.23	2.39 ± 0.05	3.61 ± 0.20
		12	2.18 ± 0.28	BLOD	1.74 ± 0.29
		15	0.45 ± 0.18	BLOD	BLOD
	25℃	0	5.82 ± 0.19	6.01 ± 0.20	5.89 ± 0.26
		1	2.77 ± 0.12	4.21 ± 0.12	3.08 ± 0.27
		2	BLOD	BLOD	BLOD
		3	BLOD	BLOD	BLOD
		4	BLOD	BLOD	BLOD
		5	BLOD	BLOD	BLOD
	37℃	0	5.71 ± 0.27	6.08 ± 0.15	5.89 ± 0.16
		1	BLOD	1.35 ± 1.95	1.06 ± 0.19
		2	BLOD	BLOD	BLOD
		3	BLOD	BLOD	BLOD
		4	BLOD	BLOD	BLOD
		5	BLOD	BLOD	BLOD

Table 3.5. Cold brew with coconut additive stored under refrigeration, room temperature, and 37 °C *B. cereus* microbial counts from Day 0-Day 15. Populations are listed as mean ± standard deviation.

Additive	Storage Temperature	Day	*B. cereus* (Log CFU/ml)
Co	4°C	0	6.00 ± 0.23
		3	4.89 ± 0.29
		6	4.94 ± 0.05
		9	5.50 ± 0.17
		12	4.77 ± 0.01
		15	4.67 ± 0.15
	25°C	0	6.04 ± 0.09
		3	4.75 ± 0.14
		6	4.87 ± 0.15
		9	4.60 ± 0.22
		12	4.00 ± 0.21
		15	3.85 ± 0.13
	37°C	0	6.01 ± 0.13
		3	5.00 ± 0.04
		6	4.21 ± 0.17
		9	3.30 ± 0.28
		12	3.54 ± 0.12
		15	3.01 ± 0.25

Table 3.6. Pathogen survival in cold brewed with nutmeg during storage at 4 ℃, 25 ℃ and 37℃. Populations are listed as mean ± standard deviation.

Additive	Storage Temperature	Day	*E. coli* (Log CFU/ml)	*S. enterica* (Log CFU/ml)	*L. monocytogenes* (Log CFU/ml)
N	4℃	0	5.95 ± 0.13	5.92 ± 0.04	5.89 ± 0.02
		3	5.32 ± 0.21	5.05 ± 0.19	4.80 ± 0.29
		6	5.14 ± 0.13	4.52 ± 1.42	3.58 ± 0.14
		9	3.58 ± 0.28	BLOD	2.23 ± 0.19
		12	BLOD	BLOD	BLOD
		15	BLOD	BLOD	BLOD
	25℃	0	5.88 ± 0.05	5.89 ± 0.06	5.89 ± 0.28
		1	3.60 ± 0.30	4.06 ± 0.22	BLOD
		2	BLOD	BLOD	BLOD
		3	BLOD	BLOD	BLOD
		4	BLOD	BLOD	BLOD
		5	BLOD	BLOD	BLOD
	37℃	0	5.58 ± 0.03	5.9 ± 0.09	5.89 ± 0.25
		1	BLOD	4.10 ± 0.29	BLOD
		2	BLOD	BLOD	BLOD
		3	BLOD	BLOD	BLOD
		4	BLOD	BLOD	BLOD
		5	BLOD	BLOD	BLOD

Table 3.7. Cold brew with nutmeg stored under refrigeration, room temperature, and 37 ℃ *B. cereus* microbial counts from Day 0-Day 15. Populations are listed as mean ± standard deviation.

Additive	Storage Temperature	Day	*B. cereus* (Log CFU/ml)
N	4°C	0	6.03 ± 0.27
		3	4.93 ± 0.26
		6	5.42 ± 0.20
		9	5.92 ± 0.29
		12	4.72 ± 0.19
		15	4.62 ± 0.17
	25°C	0	6.04 ± 0.20
		3	5.03 ± 0.19
		6	4.98 ± 0.24
		9	4.52 ± 0.29
		12	4.02 ± 0.28
		15	3.95 ± 0.26
	37°C	0	5.04 ± 0.18
		3	4.88 ± 0.16
		6	4.22 ± 0.23
		9	3.47 ± 0.02
		12	3.53 ± 0.22
		15	3.29 ± 0.25

Table 3.8. Mean pH at 4°C, 25°C, 37°C for *E. coli, S. enterica, L. monocytogenes,* and *B. cereus in cold brew with additives.* Results for pH are listed as mean ± standard deviation.

Storage Temperature	**Day**	**pH**			
		E. coli	***S. enterica***	***L. monocytogenes***	***B. cereus***
4°C	0	5.59 ± 0.15	5.54 ± 0.08	5.59 ± 0.14	5.76 ± 0.16
	3	5.49 ± 0.01	5.51 ± 0.12	5.35 ± 0.24	5.74 ± 0.25
	6	5.43 ± 0.06	5.32 ± 0.14	5.55 ± 0.06	5.59 ± 0.23
	9	5.65 ± 0.18	5.66 ± 0.07	5.63 ± 0.15	5.60 ± 0.09
	12	5.33 ± 0.27	5.61 ± 0.24	5.61 ± 0.09	5.57 ± 0.02
	15	5.32 ± 0.16	5.60 ± 0.28	5.60 ± 0.02	5.45 ± 0.05
25°C	0	5.60 ± 0.13	5.59 ± 0.11	5.61 ± 0.27	5.62 ± 0.24
	3	5.41 ± 0.09	5.42 ± 0.14	5.55 ± 0.28	5.42 ± 0.15
	6	.	.	.	5.15 ± 0.04
	9	.	.	.	5.12 ± 0.18
	12	.	.	.	5.14 ± 0.14
	15	.	.	.	5.10 ± 0.11
37°C	0	5.58 ± 0.24	5.49 ± 0.36	5.60 ± 0.01	5.68 ± 0.15
	3	5.24 ± 0.11	5.32 ± 0.08	5.43 ± 0.25	5.38 ± 0.13
	6	.	.	.	4.95 ± 0.21
	9	.	.	.	4.92 ± 0.33
	12	.	.	.	4.87 ± 0.15
	15	.	.	.	4.88 ± 0.01

Table 3.9. Mean TA at 4°C, 25°C, 37°C for *E. coli, S. enterica, L. monocytogenes,* and *B. cereus* in cold brew with additives. Results for TA are listed as mean ± standard deviation.

Storage Temperature	**Day**	**TA (Average % Acid)**			
		E. coli	***S. enterica***	***L. monocytogenes***	***B. cereus***
4°C	0	8.59 ± 0.01	8.91 ± 0.28	8.81 ± 0.11	8.53 ± 0.17
	3	8.61 ± 0.10	8.95 ± 0.30	8.93 ± 0.03	8.78 ± 0.31
	6	8.71 ± 0.01	8.85 ± 0.18	8.81 ± 0.22	8.79 ± 0.35
	9	9.00 ± 0.04	8.84 ± 0.23	8.77 ± 0.46	8.85 ± 0.28
	12	9.06 ± 0.04	8.99 ± 0.15	9.08 ± 0.23	9.47 ± 0.07
	15	9.52 ± 0.05	9.06 ± 0.01	9.49 ± 0.39	9.56 ± 0.36
25°C	0	8.29 ± 0.01	8.41 ± 0.48	8.24 ± 0.48	8.21 ± 0.31
	3	8.56 ± 0.01	8.62 ± 0.27	9.02 ± 0.16	8.33 ± 0.32
	6	.	.	.	9.20 ± 0.23
	9	.	.	.	10.27 ± 0.07
	12	.	.	.	11.56 ± 0.07
	15	.	.	.	12.15 ± 0.04
37°C	0	8.82 ± 0.11	8.38 ± 0.21	8.62 ± 0.04	8.62 ± 0.06
	3	9.66 ± 0.43	9.64 ± 0.36	9.34 ± 0.31	9.15 ± 0.03
	6	.	.	.	9.98 ± 0.03
	9	.	.	.	10.94 ± 0.11
	12	.	.	.	14.88 ± 0.13
	15	.	.	.	25.02 ± 0.24

CHAPTER 4

FUTURE IMPLICATIONS OF STUDY

The increasing popularity and ease of preparation of cold brew has encouraged retail chains and at- home brewers to make cold brew coffee. Most studies conducted on the survivability of pathogens in coffee have used average preparation procedures, however brewing ratio has been determined to be an important factor of the resulting antimicrobial potential of the coffee. A standard preparation procedure for maximum food safety should be developed to consider compounds that endorse lethality of pathogens and hit target pH for inhibition.

Further research on the survival of potential contaminants must be done to ensure the health of those who consume cold brew. The risk of the use of contaminated product is considerable enough to demand more regulation as it relates to *B. cereus* in coffee. It is important to factor in the influence of additional ingredients such as food additives despite potential antimicrobial properties as they may become the source of contamination or may introduce an extra source of carbohydrates and lipids that persistent bacteria can use as a source of nutrients to grow/survive. However, there is potential to use spices as a form of control for any potential contamination.

Additional hygiene indicators such as yeast and mold should be evaluated at different storage points and maybe how additives may impact the survivability of these organisms as they have been present in coffee. Ochratoxin A is a mycotoxin that has been previously found in contaminated coffee. Detection and quantification of this toxin should be tested.

The indication that *B. cereus* may grow in cold brew calls for follow up research to include the detection of enterotoxins in coffee and the quantification of toxin potentially found in coffee. Spore formation needs to be considered and possibly quantified. Varying concentrations of additives in coffee for increased microbial suppression. Other potential *Bacillus* species can survive in cold brew.

The level of pathogens used does not exist in nature. Strains of bacteria from contaminated cold brew need to be studied. Total solid content has been found to be important and not included in this study, but TA and pH are also indicators of final cold brew coffee parameters.