Intervention steps for controlling *E. coli* contamination in wheat flours

by

Jared Lou Del Rosario Rivera

B.S., University of the Philippines Los Baños, 2017

A THESIS

Submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Grain Science and Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2021

Approved by:

Major Professor
Dr. Kaliramesh Siliveru

Copyright

Abstract

The frequency of recalls involving Shiga toxin-producing *E. coli* (STEC) – contaminated wheat flours has increased in recent years. Such events have led to the need for food safety intervention strategies for wheat flours. This is an overlooked area of food safety research in flour milling.

To better understand the issue, the initial objective was to characterize the transfer of *E. coli* into the mill fractions and equipment through consecutive milling steps. To evaluate this, 40 batches (900 g / batch) of wheat were prepared and split into inoculated (n = 20) and non-inoculated (n = 20) groups. Inoculated wheat (~3 log CFU/g) was prepared by tempering (16% moisture, 24 h) wheat using an *E. coli* cocktail (ATCC 1427, 1429, 1430, and 1431) while non-inoculated wheat was tempered with sterile distilled water. The inoculated wheat batches were milled first (20 batches) followed by the non-inoculated batches (20 batches) using a Chopin lab-scale roller mill (2 break, 1 sizing, and 3 reduction rolls). *E. coli* counts of the mill surfaces (log CFU/ 100 cm^2), flour (break, sizing, reduction, and straight-grade flour), and non-flour (bran, fine bran, shorts, rough middling, and fine middling) fractions (log CFU/g) were enumerated after milling. *E. coli* counts for each flour fraction were then plotted against the cumulative wheat quantity milled (kg). A best-fit model describing the *E. coli* counts trend for each flour fraction was then selected. Results show that *E. coli* counts were higher ($P \leq 0.05$) on the rolls (break and reduction rolls), feeders, hoppers, and break sifter surfaces after the inoculated wheat milling. In the initial milling batch, the bran, fine bran, rough middling fractions (2.3 – 2.7 log CFU/g) had higher ($P \leq 0.05$) *E. coli* counts than all the flour fractions (1 – 1.9 log CFU/g). *E. coli* counts of all flour (3.4 – 3.9 log CFU/g) and non-flour (3.7 – 4.2 log CFU/g) fractions from the last inoculated wheat batch milled were not different ($P > 0.05$). For the non-inoculated milling sample,

E. coli surface counts declined after milling (0.4 – 1.9 log CFU/ 100 cm^2). *E. coli* counts (log CFU/g) of all flour and non-flour fractions declined to levels below detection limit (< 1 log CFU/g) as the quantity of wheat milled increased. Break, sizing, reduction, and straight-grade flours (n = 20 / fraction) obtained from non-inoculated wheat were positive for *E. coli*. The model describing the *E. coli* transfer to flour fractions (break, sizing, reduction, and straight-grade) as a function of wheat quantity milled (kg) had good fit ($P < 0.001$; se < 0.43). Results from this study could provide insights to mill managers on how *E. coli* contaminates equipment and mill fractions produced in wheat milling operations.

The second objective evaluated the efficacy of adding sodium bisulfate (SBS) to the tempering water to reduce STEC O121 and O26 loads of wheat before milling. The minimum inhibitory concentration (MIC) of SBS was evaluated. Wheat samples were inoculated (~6 log CFU/g) with *E. coli* O121 (ATCC 2219) or O26 (ATCC 2196) and tempered (17%, 24 h) with increasing SBS concentrations (0, 0.5, 0.75, 1.0, 1.25, and 1.5% SBS). Tempering concentrations that resulted in ≥ 3 log reductions were evaluated for their effects on flour quality. The MIC of SBS against both *E. coli* serotypes was 0.32% w/v. After tempering, the 1.25 (O121 – 3.5 logs; O26 – 3.2 logs) and 1.5% SBS (> 4 logs for O121 and O26) resulted in wheat STEC load reductions ($P \leq 0.05$). The concentrations of 0, 1.25, and 1.5% SBS were used for subsequent flour quality evaluations. Wheat flours produced with 1.25 and 1.5% SBS were more acidic (pH = 5.41 – 5.60; $P \leq 0.05$) and had comparable composition, size, pasting, and baking quality to the control treatment.

Overall, wheat grains and milling equipment were observed to be viable routes of *E. coli* contamination of wheat flours and SBS tempering was a feasible intervention step for controlling STEC contamination in wheat prior to milling.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ACP – Atmospheric Cold Plasma

BK - break

BPW – Buffered Peptone Water

CFU – Colony forming units

GMP – Good Manufacturing Practices

FSMA – Food Safety Modernization Act

FM – Fine Middling

HRW – Hard Red Winter

HRS – Hard Red Spring

HWW - Hard White Wheat

HUS – Hemolytic Uremic Syndrome

LOD – limit of detection

MPN – Most Probable Number

RD - Reduction

RM – Rough middling

SRW – Soft Red Winter

SWW – Soft White Wheat

SG – Straight Grade

SZ – Sizing

SBS – Sodium bisulfate

STEC – Shiga toxin-producing *Escherichia coli*

SS – Superheated steam

TSA – Tryptic soy agar

Acknowledgements

This research would not be possible without the support of various people from the Department of Grain Science and Industry. I would like to thank my advisor Dr. Kaliramesh Siliveru for his constant support and patience in conducting this study. I would also like to thank my committee members, Dr. Greg Aldrich and Dr. Randall Phebus for their inputs and support given during the conduct of this study. I would also like to extend my gratitude to the GSI faculty members and their graduate students for sharing their lab spaces and allowing me to work on the tests needed for this research in their labs. I would also like to thank the GSI staff for assistance in the purchase and procurement of materials needed for this study. I also extend my thanks to Dr. Janak Dhakal for his help in the microbial analysis parts of this research. I'd also like to thank my lab mates: Manoj, Anu, Mohana, Rania, and Roselle for their support, and encouragements.

Chapter 1 - Introduction

1.1 Problem Statement

Wheat-based foods have been one of the most consumed foods in the world for many years. Wheat grains are considered a raw agricultural product and could harbor a wide range of microorganisms including pathogenic microorganisms such as Shiga toxin-producing *E. coli* (STECs). Wheat grains are commonly milled into wheat flours prior to being used as a food ingredient. The current wheat milling process is comprised of physical processes that involve a series of size reduction, sieving, and blending operations. The milling process currently does not involve specific steps designed for controlling microbial hazards (e.g. foodborne pathogens) that could be introduced during milling. Thus, there is a significant chance for the pathogens present in the wheat to contaminate the resulting wheat flour.

Wheat flours were historically regarded as one of the safest kinds of food due to their low water activity. This characteristic has led to the common belief that pathogenic microorganisms such as STECs would not be able to proliferate in wheat flours. Hence, wheat flours have been regarded as one of the least common vehicles for foodborne illnesses in humans. However recent recalls and outbreaks linked to pathogen-contaminated wheat flours have caused renewed interest in the food safety aspect of the wheat milling process. Such events pose a food safety risk for consumers as well as a potential financial burden for flour manufacturers due to flour recalls. Therefore, studies aimed at antimicrobial interventions and an understanding the cross-contamination of relevant microorganisms of concern during milling are needed in order to improve the food safety of wheat flours.

1.2 Research Objectives

This research focused on the application of intervention steps and understanding the transfer of microorganisms during the milling process, Specifically, the objectives for this research were to:

1. Quantify the distribution of *E. coli* (non-pathogenic) from artificially contaminated wheat into the milling fractions and onto the processing equipment
2. Evaluate the efficacy of sodium bisulfate (SBS) on reducing the *E. coli* O121 and O26 wheat contamination during the tempering process and assess its effects on wheat flour quality

1.3 Outline

This thesis is composed of four chapters excluding this chapter. Chapter 2 covers the review of literature regarding on wheat grains, the wheat milling process, microbiological quality of wheat (pathogens and spoilage), recalls and outbreaks involving wheat flours, and potential intervention strategies to improve wheat flour safety that have been reported. Chapter 3 describes the transfer of *E. coli* from contaminated wheat kernels during the milling process into the mill fractions and equipment. Chapter 4 describes the significance of sodium bisulfate tempering as an intervention step for reducing the pathogenic STEC (O121 and O26) load of wheat during tempering as well as its effects on wheat flour quality. The summary of findings for this research work and recommendations for future work based on the findings from this research are then discussed in chapter 5.

Chapter 2 - Literature Review: Wheat and Wheat Flour Food Safety

This chapter addresses some of the relevant information regarding wheat including its varieties, wheat grain description, wheat flour production and consumption, and the wheat milling process. It also covers the microbiological quality of wheat, wheat flour food safety, and previous antimicrobial intervention steps introduced for wheat milling. The goal of the chapter is to review current information regarding wheat grains and its current food safety status which would help in understanding the current needs for improving wheat and wheat flour food safety.

2.1 Wheat

Wheat (*Triticum spp.*) is a grass plant primarily grown for its kernels which has become a staple food for majority of the people worldwide. Different wheat species comprise its genus with the common wheat (*Triticum aestivum*) being the most commonly grown among its different species. Its plant is composed of root (nodal and seminodal) and shoot system with roots developing from the lower nodes of the shoot (Kirby, 1996). The shoots are made up of the node, leaf, internode, and a bud in the leaf axil. The tillers which grow from the crown found below ground level further develops into flowering heads which are self-fertilized to start the grain development of the plant. The plant is a cool season crop growing best at temperatures near 60^{o}F. It is an annual grass being mainly cultivated as a winter annual in milder climates. Seeding is usually done in the fall and harvest is generally done from June through August depending on the duration of winter (Magness et al., 1971). On areas with extreme winters, wheat is spring seeded with harvest being done in the late summer and early fall seasons.

2.2 Wheat Classes

In the United States (US), wheat is classified based on their kernel hardness, color, and growth season (McCluskey, 2011). Six wheat classes are recognized in the US which are: durum

(*Triticum durum* Desf.), hard red spring (*Triticum aestivum L.*), hard red winter (*Triticum aestivum L.*), soft red winter (*Triticum aestivum L.*), hard white wheat (*Triticum aestivum L.*), and soft white wheat (*Triticum aestivum L.*) (Kansas State University, n.d.). These classes have different physical and chemical properties which make each class suitable for different applications. The wheat classes except durum are hexaploid plants (AA, BB, and DD genomes) with 42 chromosomes while durum is a tetraploid (AA and BB genomes) with 28 chromosomes (Anderson, 2018). An overview of the characteristics of the six wheat classes is shown in the table below:

Table 2.1 Summary of characteristic differences of 6 U.S. wheat classes (adapted from U.S. Wheat Association, 2017)

Wheat Class	Test Weight (lb/bu)	Moisture (%)	Protein (%)	Ash (%)	Falling Number (s)
Hard Red Spring	61.70	12.20	14.00	1.50	374
Hard Red Winter	60.10	11.20	12.40	1.53	393
Hard White	63.20	9.60	12.00	1.52	400
Soft Red Winter	58.20	13.00	9.70	1.47	304
Soft White	60.50	9.20	10.30	1.36	341
Durum	60.40	11.70	13.60	1.57	374

Hard wheat classes include hard red winter (HRW), hard red spring (HRS), and hard white wheat (HWW) varieties which are cultivated at different regions in the US (U.S. Wheat Associates, 2013). According to USDA ERS (2019), approximately 35 million acres of land are used for hard wheats with an average production of 1200 million bushels in 2018. The HRS is mainly cultivated in Minnesota, North and South Dakota, and Montana (Anderson, 2018). Wheat flours from this class are usually used for making bagels, pizza crusts, and croissants. The HRW is mainly grown in the Great Plains states of Colorado, Kansas, Nebraska, Oklahoma, Texas, Montana, North Dakota, South Dakota, Wyoming, and the Pacific Northwest (Plains Grains Inc., 2011). These class is widely used to produce bread, all-purpose flours, Asian-style noodles, hard rolls, and

tortillas (Rohrich, 2014). The HWW class is the most recent wheat class to be cultivated in the US with milling and baking qualities similar to the red wheat classes although it has a milder and sweeter flavor (Rohrich, 2014). It has a protein content ranging from 10-14% and is best applied for noodles, whole wheat flours, tortillas, and yeast-raised breads.

Soft wheat classes include soft red winter (SRW) and soft white wheat (SWW). These classes are planted at approximately 10 million acres with 285.86 million bushels being produced in 2018 (USDA ERS, 2019). SWW is grown in numerous states including Missouri, Arkansas, Louisiana, Mississippi, Alabama, Illinois, Indiana, Ohio, Kentucky, Tennessee, Georgia, South and North Carolina, and Virginia (Anderson, 2018). This wheat class is primarily used for pastries, crackers, and pretzels production. SWW on the other hand is cultivated in the North-Western U.S. regions including Washington, Oregon, and Idaho (U.S. Wheat Associates, 2013). The class generally has a low protein content (8.5 to 10.5%) and low water absorption capacity making them more suited for cakes and pastries (Rohrich, 2014).

Durum wheat is mainly cultivated in North Dakota and Montana (U.S. Wheat Associates, 2013). In 2018, approximately 2 million acres of land is used for its plantation producing 77.29 million bushels of wheat (USDA ERS, 2019). It has amber-colored kernels and has the highest hardness among the wheat classes. It has a protein content of 12 to 15% making them more suitable for pasta production. The classes of wheat are determined through several tests which are summarized in the table below:

Table 2.2 Summary of tests conducted for the 6 U.S. wheat classess (adapted from Anderson, 2018).

Test	Wheat Class					
	Hard Red Spring	Hard Red Winter	Hard White	Soft Red Winter	Soft White	Durum
Flour/semolina quality						
Milling extraction	x	x	x		x	x
Moisture	x	x	x		x	x
Ash	x	x	x	x	x	x
Protein	x	x	x	x	x	x
Color	x	x	x	x	x	x
Gluten Index	x	x	x	x	x	x
Wet Gluten	x	x	x	x	x	x
Falling number	x	x	x	x	x	
Starch pasting profile	x	x	x	x	x	
Starch damage	x	x	x	x	x	
Solvent retention capacity		x	x	x	x	
Dough quality						
Farinograph	x	x	x	x	x	
Alveograph	x	x	x	x	x	x
Extensigraph	x	x	x	x	x	
Mixograph						x
Baking /cooking quality						
Pan bread	x	x	x	x	x	
Sponge cake					x	
Cookies				x	x	
Steamed bread			x		x	
Noodles			x		x	
Pasta						x

2.3 Wheat Grain

The tillers of the wheat plant further develop into flowering heads which are then self-fertilized triggering the development of grain kernels (New Zealand Flour Millers Association, 2016). The grains then reach their maximum size one month after fertilization. The kernels serve as the storage site for starch and protein compounds which can either be used by the new plant or

processed (e.g. milled) to be converted into food. Once the grains are fully developed, the grains start to dry out and once they are dry enough the plant is harvested using a combine harvester separating the grains from the main plant (Bushuk and Rasper, 2012).

The cereal grain is a one-seeded fruit wherein the fruit coat adheres to the seed as the fruit starts to ripen (Pomeranz, 1982). A typical wheat grain has a rounded structure at the dorsal side while on the ventral side it has a crease along the entire longitudinal axis. Brush hairs can be found at the apex of the grain. The grain is comprised of three main parts which are the bran, endosperm, and the germ. The bran layer accounts for 14.5% of the grain and is the outermost layer of the grain protecting the seed. This layer is also partially waterproof which helps prevent the grain from sprouting immediately after harvest (New Zealand Flour Millers Association, 2016). It is composed of several sub-layers namely epidermis, hypodermis, cross-cells, and tube cells (Pomeranz, 1982). The germ on the other hand is another part of the wheat berry which germinates into a new plant when grains are planted and nurtured in suitable growth conditions. The endosperm typically constitutes 83% of the grain which is comprised mainly of carbohydrates and protein compounds with small amounts of other essential nutrients such as pantothenic acid, riboflavin, niacin, pyridoxine, and thiamine (Kent and Evers, 1994). Other important parts of the wheat kernel are the aleurone and scutellum. The aleurone separates the bran and endosperm while the scutellum is a thin layer between the germ and endosperm. These two parts contain significant quantities of essential nutrients to humans although many are lost during the milling process. This makes the addition of vitamin additives essential in the milling process in order to compensate for the loss of these nutrients (Bushuk and Rasper, 1997).

2.4 Wheat Flour Production and Consumption

Wheat is one of the earliest plants to be domesticated and has served as a staple food for major civilizations in Europe, West Asia, and North Africa (Curtis, 1996). In terms of grain acreage, wheat is the most important crop globally with 220.4 million hectares being used for its cultivation while ranking third on total crop production volume (Statista, 2019). Wheat production has also been increasing with approximately 532 million metric tons produced in the 1990s to approximately 757.92 million metric tons in 2017/2018 (Sosland, 2017). This total production is led by the European Union (EU) with a production of 137 million metric tons followed by China, India, and Russia (USDA, 2019). The United States (US) is also among the leaders with 51 million metric tons produced in 2017/2018. In terms of actual consumption, wheat (735 million tons) is the second most consumed crop in the world next to maize (1024 million tons) in 2018 (International Grains Council, 2019). In the US, wheat-based products are the most consumed food with a per capita consumption of 134.68 pounds. In the US, hard red winter wheat has the highest production among the hard wheat classes in 2019 (22.7 million metric tons) followed by hard red spring wheat (US Wheat Associates, 2020). For the soft wheat classes, soft white (6.6 million metric tons) and soft red winter (6.5 million metric tons) had relatively similar productions for 2019. Production of durum wheat was at 1.6 million metric tons while hard white wheat had the lowest production among the wheat classes at 0.9 million metric tons (US Wheat Associates, 2020). The same trends were observed in terms of the demand for these wheat classes for 2019.

2.5 Wheat Milling Process

Wheat is primarily grown for its kernels which are primarily converted to food through the milling process. Wheat flour milling is a continuous gradual process of extracting the endosperm from the raw wheat berry. The resulting flour is then utilized as an ingredient by the manufacturing

industry and domestic consumers in making wheat-based foods such as breads, cakes, and bagels (Owens, 2001). The aim of milling is to extract the maximum amount of endosperm from the wheat berry with minimal bran and germ contamination. The bran is generally removed because of its negative effects to the flour color and texture. The germ on the other hand is separated because of its high fat content which could undergo lipid oxidation causing rancidity to the flours (Bushuk and Rasper, 1994). The presence of these components (bran and germ) in wheat flours can negatively impact flour quality throughout its shelf life. The separated bran and germ layers are then utilized as an ingredient for animal feeds adding to the financial capability of the flour mill. Although in making whole wheat flours, the germ and bran are mixed together with the flour giving a more nutritious flour (added fiber, protein, and vitamins) in exchange for a shorter shelf life.

The wheat milling process starts with the delivery of the grains. Incoming wheat is tested for specifications such as moisture content, test weight, impurities, and enzyme activity. Wheat lots that satisfy specifications undergo a cleaning process to remove coarse impurities (e.g. stones, nails, twigs, etc.). Clean wheat lots are then stored based on various quality indicators such as hardness, protein content, and gluten quality. Wheat lots undergo another cleaning process aiming to remove lighter impurities and metallic foreign materials (e.g. sticks, straws, metals, and dust). Several machines are used in the cleaning process namely magnetic separator, aspirator, de-stoner, disc separators, impact entoleters, and color separators which are collectively known as cleaning houses (Owens, 2001).

Prior to milling, cleaned wheat lots are conditioned to the target milling moisture content in a process called tempering. Tempering is done with the precise addition of water into the wheat to increase its moisture content to the target specifications. The wheat is then rested for 6 to 24

hours depending on wheat hardness to allow the water to penetrate further into the interior parts of the kernel. This process toughens the bran and mellows the wheat endosperm allowing a more efficient separation of the bran and endosperm layers during milling giving a higher flour extraction rate and better milled flour quality (Hashimi, 2019).

The tempered wheats are then blended to produce wheat flours of varying quality characteristics. The blended tempered wheats are then milled to extract the flour from the wheat kernel. The milling process is a gradual size reduction process of the wheat kernel through a series of grinding, purifying, and sifting process (North American Millers Association, 2019). The grinding process is a critical step as the way kernels are grind affect the subsequent purifying and sifting steps (Posner, 2005). The grinding system is composed of four systems which are the break, sizing, reduction, and tailings system. Wheat is first fed into the break system consisting of a series of corrugated rolls with varying parameters (e.g. roll gap, roll speed, and speed differential). The crushing forces of the break rolls opens the kernels scraping the endosperm away from the bran in larger sizes with minimal bran powder generation (Sebastian, n.d.). The ground kernels are then sieved to separate it into bran, middling, flour, and coarse endosperm particles attached to bran. The coarse endosperm particles are then forwarded to the sizing system which further separates the coarse endosperm from the bran (Posner, 2005). The middling particles proceed to a purifying system to remove lighter bran particles (Besant, 1982). The purifier moves the middling to a series of vibrating screens to be more finely ground while air currents remove the finer bran particles. The middling particles are then further ground in the reduction system using a series of pairs of smooth rollers. Ground middling are then sieved to separate them according to particle sizes. The materials commonly used for the sieves are metal wires for coarse particles and nylon/silk or finer flours (Kent, 1975). This allows different grades of flours to be produced to achieve the target

flour specifications. Bleaching agents and nutritional additives (vitamins and minerals) are then added into the flour according to regulatory requirements. Finished flours are then packed in either cloth bags or large metal bins and are aged for up to two months (Wrigley, 1996).

2.6 Microbiological Quality of Wheat

Wheat flours and wheat-based foods are historically considered as microbiologically safe foods due to its low water activity nature and the application of validated thermal process (e.g., baking and extrusion) in the production of wheat-based foods. However, these conditions do not always guarantee their food safety as foodborne pathogens could still contaminate the product at any point in the supply chain. Proper management of cereals is critical for limiting the occurrence of foodborne pathogens and mycotoxins in the grain. Mycotoxin from molds is one of the most prevalent hazards occurring in wheat and wheat flours which is often caused by improper handling and storage throughout the supply chain. This hazard is a major concern for wheat handlers as they negatively affect the wheat grains both quantitatively and qualitatively (Los et al., 2018). As for wheat flours, the presence of bacterial pathogens (e.g. *Salmonella* and pathogenic *E. coli)* in addition to mycotoxins is a major concern as it could lead to foodborne illness outbreaks and food recalls. The typical wheat milling process is mostly comprised of physical processes which have been reported to be ineffective in controlling foodborne pathogens during milling. Thus, the initial microbiological quality of the grain has a significant impact on the safety and quality of milling products (Sabillon, 2016).

The sources of these microbiological hazards could be traced back to the environment where the wheat plants are cultivated and handled. Environmental factors such as air, soil, water, insects, animals, human handlers, and processing equipment are all potential vehicles for microbiological contamination in the wheat (Bullerman and Bianchini, 2009). Furthermore,

weather factors such as temperature, humidity, and drought levels influence the amount of contamination and persistence of the wheat microflora. Pathogenic bacteria that have public health significance have also been reported to be present in wheat. This includes *Bacillus cereus, Escherichia coli, Salmonella, Staphylococcus aureus, Clostridium perfringens* and *Clostridium botulinum* (Bullerman and Bianchini, 2009). These pathogens are not viewed as major causes of grain spoilage instead they are viewed as biological hazards which could cause health problems to consumers when consuming raw flour products contaminated by these pathogens. Their presence in the wheat end products could potentially cause massive recalls and foodborne illness outbreaks. Grain spoilage on the other hand is mainly associated with filamentous fungi present in the grains. Improper storage conditions (e.g. moisture, humidity, insect infestation) allow these fungi to grow and produce compounds (e.g. mycotoxins) that damage the quality of the stored grains.

Numerous studies have aimed to evaluate and enumerate the microbiological quality of wheat from different parts of the world. Berghofer et al. (2003) reported the high prevalence of *Bacillus spp.* (10^4 to 10^2 CFU /g), coliforms (1 to 10 MPN/g), yeast and molds (10^3 to 10^2 cfu/g) in Australian wheats. The presence of pathogenic bacteria (*Salmonella, E. coli, and B. cereus*) were also detected although they are present in low levels with majority of the samples giving counts close to the minimum detection limit. Eglezos (2010) studied the microbiological quality of wheat grains and flour from two mills in Queensland, Australia. In the wheat samples, a single isolation of *Salmonella* was detected among the samples (n = 350) in addition to the presence of *E. coli* (2.0%) and *Bacillus cereus* (4.0%). Wheat flour samples (n = 350) also tested positive for *E. coli* (0.7%) and *Bacillus cereus* (< 0.3%). Yeast, molds (3.5 log CFU /g), and coliforms (3.9 log CFU /g) were also found to predominate (Peles et al., 2012). Similar trends were also reported for wheat samples in Morocco where fungi and coliforms were the most prevalent microorganisms in

wheat flours (Ennadir et al., 2012). Furthermore, higher counts were also recovered from household samples compared to samples from retail stores demonstrating the risk of cross-contamination in wheat flours throughout its supply chain. Aydin et al. (2009) reported the high prevalence of coliforms and molds in addition to the presence of *E. coli*, *C. perfringens*, and *B. cereus* in wheat flour samples from Thrace, Turkey. In the US, Richter et al (1993) evaluated the microbiological quality of wheat flour with the highest aerobic counts observed in durum wheat flours while soft red winter wheat flours had the highest mold counts. Furthermore, 12.8% of the samples were positive for *E. coli* and 1.3% were positive for *Salmonella* with the highest incidence occurring in the fall and winter months (Richter et al., 1993).

2.7 Pathogenic and Spoilage Microorganisms Found in Wheat

During wheat cultivation, it is exposed to multiple sources of contamination which includes soil, water, insects, and feces (Deibel and Swanson, 2001). Microbiological contamination could also occur during storage due to cross-contamination with handlers and processing equipment. Both conditions provide ideal routes for contaminating wheat grains. The microflora found in wheat is diverse and includes bacteria belonging to the families of *Micrococcacae*, *Pseudomonadaceae*, *Enterobacteriaceae, Lactobacillaceae* and *Bacillaceae* while fungal species include *Alternaria*, *Cladosporium*, *Fusarium*, *Helminthosporium*, *Aspergillus*, *Penicillium* and *Eurotium* (Laca et al., 2006). These microorganisms mainly colonize the surface of the wheat grain with some fungal species able to colonize the inner kernel parts through the germ or physical damage to kernels (International Commission on Microbiological Specifications for Foods, 1980).

Grain spoilage is mostly associated with mold growth in the kernels. These spoilage molds could be classified as either field or storage fungi (Bullerman and Bianchini, 2009). Field fungi colonize the wheat during the time it is grown in the field where high moisture and relative

humidity conditions are more prevalent. This includes species such as *Alternaria*, *Cladosporium*, *Fusarium*, and *Helminthosporium*. Storage fungi on the other hand colonizes the wheat during the grain storage step where lower moisture and humidity conditions exist and includes species such as *Eurotium*, *Aspergillus*, and *Penicillium*. Berghofer et al. (2003) reported the high prevalence of yeast and molds in Australian wheats. Furthermore, Eglezos et al. (2010) reported counts of 3 to 3.7 log CFU /g for yeasts and 2.7 to 2.8 log CFU /g for molds in Australian wheats. Aydin et al (2009) reported mold counts ranging from 1.5 to 3.2 log CFU /g for wheat samples obtained from various regions in Thrace, Turkey while Riba et al. (2010) reported mean fungal counts ranging from 2.4 to 3.1 log CFU /g in Algerian wheats.

In addition to fungi, bacterial species such as *Bacillus*, lactic acid bacteria, and *Micrococci* in wheat were also reported (Sabillon and Bianchini, 2014). An average aerobic count of 4.9 log CFU /g was reported for 54 US wheat samples (Rogers and Hesseltine, 1978). Berghofer et al (2003) reported the presence of aerobic bacteria (10^5 to 10^2 CFU /g), coliforms (1 to 10 MPN/g), aerobic, and thermophilic spores (1 log CFU/g) for Australian wheats. Furthermore, aerobic counts for various wheat samples were reported with 4.2 log CFU/g for Australian wheats (Eglezos et al., 2010), 2.89 to 5.59 log CFU/g for Turkish wheats (Aydin et al, 2009), and 4.9 log CFU/g for Algerian wheats (Peles et al., 2012). The presence of *Bacillus* spp. which causes rope spoilage in breads was also reported in wheats (Berghofer et al., 2003; Aydin et al., 2009).

Pathogenic microorganisms could also be part of the wheat microflora. This includes enteric pathogens such as *Salmonella* spp. and *Escherichia coli* (STECs) which pose a food safety risk for wheat flours and wheat flour-based products (Sabillon, 2014). In addition to this, the presence of spore forming pathogens such as *Bacillus cereus* and *Clostridium perfringens* were also reported for wheats. The levels of these pathogens are mostly below or slightly above the

minimum detection levels of common detection and enumeration tests as reported by various studies. Berghofer et al. (2003) and Eglezos et al. (2010) reported *Salmonella* isolations in addition to the presence of *E. coli* (3 MPN/g) and *B. cereus* (0.3 MPN/g) although their levels in Australian wheats are near or below the minimum detection limits of the tests. The prevalence of *E. coli* and *B. cereus* in wheats were reported to be at 2.0% (n=50) and 4.0% (n = 50) respectively for grains and 0.7% (n = 300) and <0.3% (n = 350) for wheat flours (Eglezos et al., 2010). In Turkish wheats, *E. coli* (0.27 to 1.37 MPN/g) was present in majority of the samples with some samples testing positive for pathogenic spore formers *B. cereus* (0.13 to 0.20 MPN/g) and *C. perfringens* (0.13 to 0.92 MPN/g) (Aydin et al., 2009). In U.S. wheat samples, the presence of fecal streptococci, aerobic thermophilic spore formers and flat sour bacteria were detected although counts for these were generally low in positive samples (Roger and Hesseltine, 1978). In another study by Richter et al. (1993), 12.8% (n = 3350) and 1.32% (n =3040) of US wheat samples tested positive for *E. coli* and *Salmonella* respectively with coliform counts of 1.2 MPN/g (n=1447). The highest prevalence of these pathogens occurred in the fall and winter months while the lowest incidences occurred in samples obtained during the summer season (Richter et al., 1993). Coliform counts of 1.6 MPN/g in wheats were reported by Sperber (2007) for US wheats. On the other hand, Victor et al (2013) reported *E. coli* (coliform) levels of 3.8 (3.4) and 4.1 (3.7) log CFU /g in white and whole grain flour samples respectively from Lesotho. Spicher (2005) reported a mean coliform count of 2.0 log CFU /g in German flours. Khanom (2016) reported coliform counts (3 to 4 log CFU /g) for wheat flour samples from Pakistan.

Aside from these bacterial pathogens, mycotoxin-producing molds also pose a food safety risk for wheat and wheat-based products. These molds could either be classified as field or storage fungi depending on the handling step in which they predominate. The most prevalent molds

isolated in Australian wheats are species of *Aspergillus*, *Penicillium*, *Cladosporium* and *Eurotium* (Berghofer et al., 2003) while *Aspergillus* and *Fusarium* species dominate Algerian wheats (Riba et al., 2008). Birck et al. (2006) evaluated the prevalence of fungal species during wheat storage (180 days) with *Aspergillus* (96.7%) being the most prevalent followed by *Penicillium* (80%), and Fusarium (46.7%). Benassi et al. (2011) reported the dominance of *Alternaria* spp. followed by *Fusarium* on the mycobiota of Tunisian wheat grains. Furthermore, Gallo et al. (2008) evaluated the mycobiota of wheat after harvest and storage (3 months) and reported the predominance of *Alternaria*, *Cladosporium* and *Fusarium* in wheat during harvest and *Aspergillus* and *Penicillium* during storage.

The colonization of these molds poses a food safety risk in wheat as it increases the chance of mycotoxin formation in the wheat. Mycotoxins are secondary mold metabolites that have chronic adverse effects on cell functions of humans and animals when ingested in sufficient amounts (Zain, 2011). Mycotoxins of concern in wheat grains include deoxynivalenol (DON) and zearalenone (ZEA) produced by field fungi Fusarium *graminearum* and *Fusarium culmorum* at the wheat flowering stage and ochratoxin (OTA) produced by *Penicillium verrucosum* and *Aspergillus ochraceus* during wheat storage (Magan et al., 2010). Among these mycotoxins produced by *Fusarium* spp., the most prevalent and economically important type for grain production is DON. Maximum limits for these toxins have been established by various regulatory agencies to reduce the risk for humans and animals to contract its toxic effects.

Several studies have noted that the levels of these mycotoxins in grains are directly related to the growth levels of fungi (Nishio et al., 2010). Furthermore, their growth rates are influenced by various factors such as geographical location, climate, and storage conditions. Seiler (1986) reported that wheat grown in wetter conditions had relatively higher bacterial (8.1 log CFU/g) and

mold (6.0 log CFU /g) counts than wheat samples grown in warm, dry conditions (5.7 log CFU/g for total bacteria and 5.1 log CFU/g for molds). Manthey et al. (2004) also reported similar observations in durum wheat harvested from different regions in US North Plains as a result of different precipitation levels in each growing region. Kriss et al. (2010) concluded the positive correlation of relative humidity to mold infection and mycotoxin production leading to lower quality and more toxic wheats.

2.8 Effect of Wheat Milling on the Microbial Quality of Wheat/Wheat Flours

The milling process is reported to have no significant impact on the level of microbiological contamination present in the wheat (Sabillon, 2014). It only involves physical processes with the aim to extract the maximum amount of flour possible. Due to this, the presence of spoilage and pathogenic microorganisms in wheat flour can be expected (Sperber, 2007). Hence, the microbiological quality of grains used in milling has a big impact on the contamination level of the milling end products (e.g., flours, brans). Typical milling processes are only composed of a series of cleaning, tempering, and grinding procedures lacking steps specifically aimed to control microbial contamination in wheat flour. This process only redistributes the microbial contaminants in the subsequent milling fractions. This could potentially cause a lower contamination level in the wheat flours although pathogens could still be present which compromises wheat flour safety (Sabillon, 2014).

Wheat milling begins with the removal of foreign materials (e.g., sticks, stones, unsound kernels etc.) from sound grains. The cleaning process removes impurities based on shape, density, and size (Posner, 1997). Several studies have suggested that the removal of these contaminants improves the microbial quality of grains prior to milling. Manthey et al. (2004) reported an average of 1.0 log CFU/g reduction in aerobic bacterial counts and yeast/mold counts after cleaning wheat

with dockage testers and cyclone grain cleaners. On the other hand, Riba et al. (2008) have reported no significant difference in the fungal counts (2.7 to 2.9 log CFU /g) of clean and unclean wheat. A proper cleaning process could potentially reduce mycotoxin levels in wheat by removing the highly contaminated and damaged kernels from the wheat batch (Bullerman and Bianchini, 2007). Proper wheat cleaning has been of crucial importance in lowering economically important mycotoxins (DON, NIV, ZON, (H) T-2, and OTA) although the extent of reduction is highly variable (Schaarschmidt and Fauhl-Hasek, 2018). Abbas et al. (1985) reported a DON reduction of 5.5 to 19% after scab-infected wheats have been cleaned. Furthermore, Lancova et al. (2008) observed an average reduction of 48% in DON levels after sieving, scouring, and polishing wheat grains and Pascale et al. (2011) have observed reductions in T-2 and HT-2 levels of 54 and 89% respectively for cleaned wheat. Tibola et al. (2015) have shown significant reduction in DON levels by as much as 70% after cleaning and sorting wheat. The cleaning methods incorporating sieving, high air pressure, and gravity separator were also efficient in removing *Fusarium* damaged grains and foreign materials (Tibola et al., 2015). Although these studies have reported reductions in the microbial and mycotoxin content of wheat after cleaning, their effectiveness in controlling the microbial and mycotoxin contamination of wheat can be highly variable. Therefore, wheat kernels might still contain significant levels of these toxins and microorganisms.

The cleaned wheats undergo tempering which involves the addition of water to the wheat to increase its moisture content to the optimum level for milling (Posner, 1997). The moistened wheats are then held at conditioning bins for set times (6 – 24 h) to allow moisture penetration in the wheat kernels. This is a vital step as incorrect tempering could encourage microbial growth on either the wheat or the milling equipment (Sabillon, 2014). Berghofer et al. (2003) reported that increased mesophilic counts ($> 10^4$ CFU/g) are more frequent in Australian wheats after tempering.

Furthermore, the presence of *E. coli* was detected in previously non-contaminated wheat after tempering. The corresponding water activity levels of the wheat moisture content after tempering is still not enough to support bacterial growth (Posner, 1997). This contamination after the tempering process could be caused by unclean conditioning bins and containing grain residue build ups harboring substantial amounts of microorganisms (Berghofer et al., 2003). Thus, the sanitary conditions and cleaning protocols of the milling facility should be properly implemented.

The tempered wheat then undergoes a series of grinding, reduction, and sifting operations to separate the flour from the bran and germ layers (Posner, 1997). This process only redistributes the microorganisms and toxins present in the wheat into the resulting milling fractions (Pascale et al., 2011). The flour fractions obtained usually have reduce microbial load compared to the tempered grains. Berghofer et al. (2003) showed a decrease in the overall load of flour relative to the tempered grains for aerobic bacteria (7 to 5 log CFU/g), yeast (6 to 3 log CFU/g), and molds (6 to 4 log CFU/g). The bran and germ layers on the other hand had higher mesophilic counts compared to the wheat (6 to 7 log CFU/g). The wheat milling process concentrates at least 90% of the aerobic bacteria into the wheat and germ fractions (Sperber, 2007). The same redistribution can also be observed for the mycotoxins present in the wheat (Park, 2002). The extent of the redistribution is also affected by several factors such as wheat variety, penetration of contamination (mycotoxin and bacterial) and flour extraction rate (Pinson-Gadais et al., 2007).

2.9 Pathogenicity of Wheat Related Pathogens and Toxins

The prevalence of pathogenic microorganisms has been reported in wheat by several studies which includes bacterial pathogens and toxigenic molds. One commonly detected bacterial pathogen in wheat is *Escherichia coli*. This is a gram-negative, rod shaped, motile, non-spore forming, facultative anaerobic enteric bacteria belonging to the *Enterobacteriaceae* family (Todd

and Baker, 2006). This bacterium is commonly found in the environment and gastro-intestinal tracts of both humans and animals (Centers for Disease Control, 2019). Many serotypes of *E. coli* exist, and some are part of the natural microflora found in the digestive systems of humans and animals. Over 200 of these serotypes are classified as Shiga-toxin producing *E. coli* (STEC) in which half of its serotypes are pathogenic to humans (Sondi and Salopek-Sondi, 2004). Pathogenic *E. coli* also be classified according to their pathogenicity mechanisms. This includes Shiga-toxin producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) (Centers for Diseases Control, 2015). Among these classifications, the STEC type is the most commonly associated with foodborne illness outbreaks with the *E. coli* O157:H7 accounting for 36% of the cases. In addition, the "Big Six" non-O157 serovars which include O26, O45, O103, O111, O121, and O145 have been increasingly related with *E. coli* linked foodborne illnesses (Centers for Disease Control, 2015). As for wheat flours, the O121 and O26 serogroups have been increasingly linked to wheat flour products involved in recalls and foodborne illness outbreaks.

Infectious doses of *E. coli* can be as low as 10 cells for some serovars (Schmid-Hempel and Frank, 2007). The STEC group also referred to as "verocytotoxin-producing" *E. coli* are zoonotic pathogens considered as one of the most dangerous among *E. coli* pathotypes which can cause a variety of foodborne illnesses (Karmali, 2004). The STEC pathotype causes illnesses by attaching to the intestinal walls of the host and producing Shiga toxins (Karmali, 2004). The toxins produced are mainly referred to as Shiga toxin 1 (Stx 1), Shiga toxin 2 (Stx 2) with some types classified as variations between the two types (Anderson, 2018). The severity of the illnesses caused by *E. coli* is mainly affected by the immune status of the infected individual with pregnant, infants, elderly, and immune-compromised individuals being the most susceptible. The illnesses

can range from the common diarrhea and stomach cramps to a potentially fatal hemolytic uremic syndrome (HUS) which is a form of kidney failure (Fitzpatrick et al., 1991). HUS is often characterized by the following symptoms: kidney failure, thrombocytopenia, and hemolytic anemia (Melton-Celsa, 2014). *E. coli* related illness is more likely to progress to HUS in young children, older adults, and those with immune system-weakening diseases (Centers for Disease and Control, 2017).

2.10 Foodborne Outbreaks and Recalls Involving Wheat Flours / Products

Wheats flours are generally regarded as a safe ingredient due to the belief that its dry nature will not allow any pathogens to proliferate or survive in them. However, wheat is a raw agricultural commodity and is highly prone to contamination of pathogens while in the field or in storage through various routes (Bullerman and Bianchini, 2009). Studies have shown that pathogens such as *Salmonella* and *E. coli* are able to survive in the dry environment of wheat or wheat flours for significant periods of time through various mechanisms (Forghani et al., 2018). These mechanisms include accumulating osmoprotectants, use of various sigma factors, rRNA degradation, and reverting into a dormant state (Finn et al., 2013; Sabillon, 2016). Furthermore, the wheat milling process only redistributes the microbial load of the wheat into the milling fractions. Thus, there is a significant chance for the pathogens present in the wheat to contaminate the wheat flour produced after milling. These factors contribute to the occurrence of food recalls and foodborne illness outbreaks linked to wheat flours. Wheat flours act as vehicle for foodborne illnesses through the usage of pathogen - contaminated flours in baking mixtures (e.g., doughs, batters) and products. Validated thermal processes (e.g., baking, and extrusion) are used in processing flour-based products. However, outbreaks still occur due to the risky consumer behavior of eating raw baking mixtures (e.g., cookie dough, cake mixes, etc.). The pathogens present could be activated due to

the increased moisture and sugar levels present in the product. In addition, deviations in the thermal processes employed during processing can also lead to these pathogens persisting in the finished product causing recalls and outbreaks. Table 2.3 shows the documented outbreaks linked to pathogen-contaminated wheat flours and its derivatives while table 2.4 shows the recalls involving wheat flours and subsequent wheat flour-based products.

Table 2.3 Outbreaks of foodborne illnesses associated with wheat flours and its derivatives

Product	Pathogen	Outbreak Location	Reference
	Wheat Flours		
Plain Flour (New Zealand; 2008-09)	*Salmonella* Typhimurium phage type 42 (c)	New Zealand; 67 cases, product recalled	McCallum et al, 2013
Bleached all-purpose flour (Navajo Pride; 2015)	*Salmonella* Typhimurium (c)	USA (4 states), cases unknown, product recalled	US-FDA, 2015
Various wheat flours (General Mills; 2015-2016)	*E. coli* O121 and O26 (c)	USA (24 states), 63 cases, product recalled	US-FDA, 2017
Various wheat flours (Ardent Mills; 2016-2017)	*E. coli* O121 (c)	Canada (6 provinces). 30 cases, product recalled	PHAC, 2017
All-purpose bleached flours (Rogers Foods; 2017)	*E. coli* O121 (c)	Canada (1 province), 6 cases, product recalled	CFIA, 2017
Pillsbury Flours (ADM Milling Co.; 2019)	*E. coli* O26 (c)	USA (8 states),	FDA, 2019
	Wheat Flour-Based Baking Mixes		
Cake mix in raw ice cream (USA; 2005)	*Salmonella* Typhimurium (c)	USA (11 states), 26 cases, product recalled	Zhang et al, 2007
Frozen pot pies (flour as suspect ingredient) (USA; 2007)	*Salmonella* Typhimurium (c)	USA (41 states), 401 cases, product recalled	Roos, 2008
Prepacked refrigerated cookie doughs (Nestle, 2009)	*E. coli* O157:H7 (c)	USA (30 states), 72 cases, product recalled	US-FDA, 2009
Dry dough mix (USA; 2016)	*E. coli* O157:H7 (c)	USA (9 states). 13 cases, product recalled	Gieraltkowski et al, 2017
Cake Mix (Duncan Hines; 2018)	Salmonella Agbeni (c)	USA (5 states), 7 cases, product recalled	US FDA, 2019

*(c) indicates that the pathogen was isolated/confirmed to be present from the suspected food causing the outbreak

Table 2.4 Food recalls involving wheat flours and its derivatives

Product	Recall Year/Issue	Brand and Company	Reference
Unbleached all-purpose flours	Potential presence of pathogenic *E. coli* after routine testing (2019)	Hodgson Mill; Effingham IL	FDA, 2019
Organic unbleached all-purpose flour	Confirmed presence of *E. coli* after US FDA testing (2019)	Wild Harvest; United Natural Foods Inc., Providence RI	FDA, 2019
Unbleached all-purpose flour	Potential presence of pathogenic *E. coli*, part of a previous recall by ADM (2019)	King Arthur Flour ADM Milling Co., Chicago IL	Sorto, 2019
Unbleached all-purpose flour	Possible pathogenic *E. coli* contamination in flour (2019)	Robin Hood Flours; J.M. Smucker Company, Orville OH	FDA, 2019
Unbleached all-purpose flour	*E. coli* O26 contamination (2019)	Gold Medal Flours; General Mills MN	FDA, 2019
Bread flour	Potential presence of pathogenic E. coli (2019)	Pillsbury BEST; Hometown Food Company, Chicago IL	FDA, 2019
All-purpose flour	Presence of pathogenic *E. coli* (2019)	Baker's Corner; ALDI, Essen GER	Wicks, 2019
Unbleached all-purpose flour	Presence of *Salmonella* (2019)	Pillsbury Flour Hometown Food Company, Chicago IL	FDA, 2019
Unbleached all-purpose flour	Presence of *Salmonella* (2019)	Gold Medal Flours. General Mills, MN	FDA, 2019
Various cake mixes	*Salmonella* was isolated from retail samples linked to outbreak (2018)	Duncan Hines Cake Mixes, ConAgra Brands, Chicago IL	FDA, 2019
Bread flour	Possible E. coli O121 contamination (2017)	Robin Hood, Super Strong Bakers, Ardent Mills, Canada	CFIA, 2017

… continued Table 2.4

Cookie dough, pie, and tart shells	Suspected contamination of *E. coli* O121 (2017)	Apple Valley, Midmay Cheese Haus, Canada	Harris and Yada, 2019
All-purpose flour	*E. coli* O121 contamination (2017)	Robin Hood Flours; Country Bulk Inc. and Country Pantry Bulk Foods (ON, Canada)	CFIA, 2017
All-purpose flour	Suspected *E. coli* O121 contamination (2017)	Robin Hood Flours; Ardent Mills, Canada	CFIA, 2017; Harris and Yada, 2019
All-purpose flour	Confirmed contamination of *E. coli* O121 (2017)	Rogers Flours; Roger's Foods Canada	CFIA, 2017
Durum wheat flours	Possible contamination of *E. coli* O121 (2017)	Golden Temple, Swad, and Maya flours; Smucker Foods of Canada	FDA, 2018
Pie and Tart Shells	Possible contamination of *E. coli* O121 due to use of contaminated flour (2017)	Great Value, Apple Valley, and Western Family	Harris and Yada, 2019
Various flour variants	Confirmed *E. coli* O121 contamination in baking mixes linked to outbreaks (2017)	Brodie, Creative Baker, Golden Temple, and Robin Hood Flours; Ardent Mills, Canada	Harris and Yada, 2019
All Purpose Flour	Detected *E. coli* O121 in routine testing of flours (2017)	Robin Hood Flours; Smucker Foods, Canada	CFIA, 2017
Cake Mix	*E. coli* contamination due to use of contaminated wheat flours in the mix (2016)	Betty Crocker Delights; General Mills, MN	FDA, 2016
Pancake Mix	*E. coli* contamination due to use of contaminated wheat flours in the mix (2016)	Krusteaz; Continental Mills, WA	FDA, 2016
All-purpose flour	*E. coli* O121 and O26 isolated from outbreak samples (2016)	Gold Medal Flours; General Mills, MN	FDA, 2016
Refrigerated cookie and brownie doughs	*E. coli* O157:H7 isolated from contaminated food linked to outbreak (2009)	Various cookie and dough products; Nestle Toll House	FDA, 2009

The recalls and outbreaks shown in tables 2.3 and 2.4 shows that wheat flours are potential vehicles for foodborne pathogens such as Shiga toxin-producing *E. coli* (STEC) and *Salmonella*. These pathogens can then further contaminate subsequent wheat flour-based products (e.g. baking mixes, pie crusts, pre-made doughs) through the use of contaminated wheat flours as raw ingredient. The manufacturing process for wheat flour-based products such as bake mixes and prepackaged doughs usually lack sufficient antimicrobial interventions increasing the risk of the pathogens initially present in the ingredients to persist in the final product. These are usually sold raw to consumers accompanied with labels detailing instructions for proper use and safety (e.g. proper baking temperature). Powdered wheat flour products need to be properly hydrated to make doughs or batters while premade doughs need minor preparation steps (e.g. cutting, fermentation) before being cooked or baked. Generally, the cooked products from these flour-based products are safe as the thermal methods employed are validated to be effective in controlling these pathogens. However, illness outbreaks still occur due to the risky consumer habit of eating raw dough or batter before baking. The hydrated state of these batters or doughs and the presence of a viable food source in them could activate dormant microorganisms and increase their numbers increasing the risk of contracting foodborne illnesses from consumption of raw baking products.

The risk of wheat flours harboring foodborne pathogens prompted the U.S. Food and Drug Administration (FDA) to issue warnings to the food industry and consumers stating that pathogens such as *Salmonella* and pathogenic *E. coli* could be present in flour and other ingredients used in these wheat-based products (FDA, 2005). These products are designed to be cooked prior to consumption therefore consumption should only be done after sufficient cooking is done. Furthermore, the US FDA warnings also included that these products could also pose a serious food safety risk if ingredients that are intended to be cooked are included (Sabillon, 2016; and

FDA, 2005). Thus, raw wheat flours and wheat-based products have also included labels in their products prohibiting consumption of the product in its raw state. However, there is no guarantee that consumers will always follow the safety instructions stated in these products, therefore the responsibility of controlling these pathogens and producing safer wheat flours fall to the manufacturers.

2.11 Potential Strategies for Improving Food Safety of Wheat Flours

Due to the increased relevance of the food safety of wheat flour and wheat flour-based products various methods for improving flour safety have been reported over the years. In addition, the high demand for wheat-based products in addition to the risky consumer behavior of eating raw doughs or batters strengthen the need for improving the food safety aspect of wheat flours. The common wheat milling process have been shown to have minimal effect in reducing the microbial load of the flour (Richter et al., 1993; Berghofer et al., 2003; Eglezos, 2010). Evaluating the microbiological quality of incoming wheat is implemented by testing for the presence of significant foodborne pathogens (e.g., *E. coli* and *Salmonella*) and hygiene indicator microorganisms (coliform, aerobic counts, and yeast/molds counts). Regulations pertaining to the microbiological quality of wheat grains are not yet set in place by regulatory agencies making the tolerance limits for these microorganisms different for each miller. Safety guidelines for other hazards such as mycotoxins, heavy metals, and pesticide residue have been set in the Codex guidelines. However, incoming wheat lots are often delivered in large quantities with small fractions of it being tested (Sperber, 2007). In addition, the pathogens present in wheat are usually not homogenously distributed in the wheat but are instead clumped together in groups (Ardent Mills, n.d.). This can be addressed by the use of valid statistical sampling methods and increasing

the sample size and tests for the incoming wheat lots although this can become cost-prohibitive to millers.

Flour millers also implement quality management programs such as adhering to Good Manufacturing Processes (GMPs). GMPs refer to regulations enforced by the US FDA requiring food manufacturers to take proactive steps to ensure the production of safe and quality products (FDA, 2019). These practices help in establishing good quality management systems, raw material procurement, development of reliable processes, detection, and investigation of quality deviations, and maintaining reliable testing procedures. Companies are also mandated to use current and updated systems in implementing these practices ensuring minimal quality and process deviations. The implementation of food safety plans such as Preventive Control (PC) plans can also help improve the food safety of wheat flours. These plans allow the accurate determination of hazards to be controlled by the millers helping them devise procedures that can properly control the occurrence of these hazards. Proper implementation of these plans helps reduce the likelihood of hazards (physical, chemical, and biological) occurring in wheat flours reducing the risk of producing unsafe foods. In addition to this, the implementation of proper and validated sanitation practices for the milling facility also helps reduce the likelihood of foodborne pathogens occurring in the wheat flour. This is because the milling equipment can harbor wheat residues that can support microbial growth and contaminate the wheat and milling fractions when it comes in contact with contaminated surfaces.

The increase in the incidence of recalls and outbreaks related to wheat and wheat flours lead to a need for an effective antimicrobial step to improve control the pathogens present in wheat. Furthermore, the implementation of FSMA (Food Safety Modernization Act) have mandated the need for effective preventive control steps to be implemented in order to prevent food hazards

from entering the food supply chain. Modifications in the milling process aiming to improve wheat flour safety have been explored. The earliest method used is flour pasteurization which involves heating the flour at different time-temperature combinations to reduce the number of pathogenic microorganisms to acceptable levels. Russ and Doe (1970) patented a flour pasteurization process by heating the flour at temperatures ranging from 110 to 115^{o}C for 60 minutes. Another heat treatment procedure was patented by Cauvain et al. (1976) wherein whole wheat and semolina where first dried to 6% moisture followed by heating at a specific time and temperature combination before milling. This was followed by another patent by Hanamoto and Bean (1979) wherein flour is heated to 71^{o}C for 4 to 5 days. Guy and Mair (199) then introduced another heat treatment process which includes drying the flour to <5% moisture (w/w) followed by heating the flour between 130 and 140^{o}C for 30 minutes. This process was stated to help reduce protein denaturation helping the flour reach optimum performance. Other patented heat treatments for flour include suspending the flour in heated gas (50-130^{o}C) at a holding time of 5 to 8 seconds and heating the flour in an aqueous state using hydration prevention agents to 130^{o}F at a holding time of 60 minutes (Wolt et al., 1994; Hankinson et al., 1967). These heat treatment processes were noted to cause up to 5-log reductions in the flour microbial population with flours having the least amount of bran contamination having the greatest reduction in microbial populations (Ardent Mills, n.d.). These heat treatment processes also cause moisture loss in the flour which induces changes in the flour's physical and rheological properties. These changes include protein denaturation, reduction/inactivation of α-amylase enzyme, and partial starch gelatinization (Schlauri, 1999). The changes in the surface protein and lipid properties can also lead to modification of the starch granules potentially improving its starch properties. Although effective, flour pasteurization was also reported to have negative effects to flour properties. Mann et al.

(2013) reported that heat treatments (50 to 90^{o}C) caused the formation of gluten aggregates resulting in decreased protein solubility, lower dough strength, and lower baking functionality in the flour. The effect was reported to be more pronounced in heat treated flours with higher moisture contents causing higher mobility of molecules. In addition to this, economic factors also limit the use of heat treatments in flour. The wheat milling process is primarily designed to keep the moisture levels of wheat flour as low as possible to increase shelf life and improve yield. The low moisture and humidity conditions present in wheat flour milling causes an increase in the thermal resistance of the pathogens present and lower heating rate of the wheat flour. Due to these factors, a longer heating time and higher heating temperature is needed for wheat flours to achieve significant pathogen reductions. The higher energy requirement of the heating process increases the capital costs of the process. This coupled with the costs of procuring and setting up the equipment necessary for flour pasteurization presents an economic restraint for smaller scale millers to incorporate it in their milling process.

The effectiveness of incorporating antimicrobial agents in the milling process to improve wheat food safety was also explored although most of the processes involved are not fully adapted in the milling industry. The use of strong oxidizing agents such as chlorine and ozone in improving wheat safety was first explored. Both chemicals have been mainly used by the food industry in either gas or liquid forms for sanitizing plant facilities and processing equipment. As for the wheat industry, these chemicals are often used as fumigants during bulk storage of wheat grains (Bonjour et al., 2011). Several studies have demonstrated the effectiveness of these chemicals as an antimicrobial agent in wheat. Ibanoglu (2001) have reported a 1-log reduction (yeast/mold) and 2.1 (aerobic bacteria) log reductions in wheat microbial counts through washing with ozonated water (1.5 ppm) for 30 minutes followed by drying to 16.1 and 17% moisture for soft and hard

wheats respectively prior to milling. In a subsequent study, the addition of 1% acetic acid in ozonated water increased the microbial reduction by 3.7 log CFU /g (yeast/mold) (Ibanoglu, 2002). Dhillon et al (2010) washed wheat using ozonated (16.5 mg/L) and chlorinated (700 mg/L) water for 3 minutes. The treatments were reported to be effective in reducing the yeast/mold counts of durum wheat but did not affect its aerobic counts. In a follow-up study, deploying a fluidized bed system to treat wheat with ozone improved the log reductions to 4.1 (yeast/mold) and 3.2 (aerobic bacteria) (Dhillon et al., 2010). Although these chemicals have strong oxidative properties, majority of the oxidation processes happen only on the outer surface of the wheat kernel thus having minimal effects on the functional properties of wheat flour (Tiwari et al., 2010; Rose et al., 2012). Furthermore, the treatments were also reported to have minimal impact on the flour extraction rate, dough rheological properties (farinograph and extensograph), protein functionality, falling number, and sedimentation volume properties of the flour (Ibanoglu, 2002). Despite the effectiveness of these chemicals, limitations exist in a way that they are known to have adverse health effects to humans when residue concentrations are high. Both of these chemicals are known to be toxic to humans when inhaled thus proper knowledge and training in handling these chemicals should be considered before implementation. Furthermore, regulatory limits on chlorine residues in foods are set (e.g. 4 ppm in drinking water) making its use more practical for sanitizing processing equipment and facility surfaces as high concentrations (200 ppm max) are needed for it to be effective. Although chlorine is also used as a bleaching agent in wheat flours, the allowable concentration of chlorine in flours is set at 2.5 ppm while for chlorine dioxide is set at 30 ppm (Al-Dmoor and El-Qudah, 2016). Furthermore, the effectiveness of chlorine is affected by the organic load present in the surface or food. Due to wheat kernels being raw and minimally processed before

tempering, the high organic load potentially present in wheat grains could limit the efficacy of chlorine in controlling pathogens present in wheat.

The addition of organic acids in reducing wheat microbial load was also explored. Organic acids lower the pH of the tempering water with this increased acidity being able to control the pathogens present in wheat. Dhillon et al. (2010) reported that using acidified water in washing wheat (1% acetic acid) gave reductions similar to wheat treated with ozonated water while their combination showed an improved microbial load reduction. Sabillon (2014) studied the microbial reduction capability of incorporating different organic acids (acetic, citric, lactic acid, and propionic acids) and salt in the tempering water. Results from the study indicate significant reductions in the wheat microbial load after tempering (aerobic bacteria, yeasts/molds, and *Enterobacteriaceae* counts). Maximum reductions were observed at the highest acid concentrations used (5%) for all microbial counts enumerated. Furthermore, the combination of salt and acids showed a synergistic effect in reducing the microbial load of wheat with the salt (1% wheat basis)-lactic acid (5%) combination showing the highest reductions (Sabillon, 2014). This same treatment has been used in a subsequent study evaluating their effects in reducing pathogenic *E. coli* (O157 and non-O157 strains) and *Salmonella* in wheat. Results showed a 1.8 (O157) and 1.6 (non-O157) log reductions in *E. coli* counts and a 1.8 log reduction in *Salmonella* counts.

Aside from these methods, less common methods have been explored in various studies. The use of superheated steam (SS) in treating wheat was reported to cause significant reductions in the microbial load of the wheat. Latiful Bari et al. (2015) reported that SS treatment of cereal grains at 250^{o}C for 15 seconds decontaminated the molds present in wheat with no visible mold growth after 26 months of storage. Furthermore, Hu et al. (2016) reported a 3-log reduction in total bacteria and an 81.1% reduction in *Bacillus* spp. after SS treatment of 200^{o}C for 80 seconds while

molds were not detectable after treatment of 110^{o}C for 30 seconds or longer. Los et al. (2017) studied the effects of using atmospheric air cold plasma (ACP) for controlling the bacterial loads of wheat. Based on their study, significant reductions (> 3 logs) in microorganisms (*E. coli* and *Lactobacillus*) associated with cereal grains were observed although their effectiveness was affected by several intrinsic factors such as bacteria type, mode of existence, substrate composition, and surface hydrophobicity (Los et al., 2018). However, ACP is noted to have safety concerns as its method of generation (e.g., dielectric barrier discharge) increases the risk of dust explosion. Thus, stringent safety practices should be implemented in the mill before it could be adapted. Irradiation of wheat/wheat flours to reduce their microbial loads was also explored as it is was previously used to kill insects during bulk wheat storage (Rose et al., 2012). Hanis et al. (1988) reported a 2-log reduction of viable bacteria in wheat flour by treating it with gamma irradiation at 1 kGy while treatment at 10 kGy completely decontaminated the sample. Laszlo et al. (2008) reported the effectiveness of UV irradiation of wheat flours in reducing the wheat flour microbial load. However, this treatment also yielded a darker flour color compared to ozone and chlorine treated wheat. Although effective, changes in the physical and chemical properties of the wheat flour can still occur due to oxidation. Koksel et al. (1998) observed a decrease in the dough quality parameters after exposure to high doses of radiation (>2.5 kGY). Other forms of treatment were also explored in some studies namely microwave and radio-frequency irradiation. These are non-ionizing in nature which causes minimal changes in the physical and chemical properties of the flour (Rose et al., 2012). These treatments work by heating the product more rapidly compared to conventional methods. However, heating using these methods are uneven requiring more heating time to achieve the same lethality as conventional methods (Hui et al., 2004). Weaver et al. (2011) developed a radio frequency irradiation treatment wherein flour is heated to a

temperature range of 75 to 100^{o}C followed by cold air cooling. The treatment was reported to cause 4 to 7 log reductions in bacterial load with minimal effects on flour functionality. On the other hand, microwave radiation treatment of flours did not achieve any significant reduction in the wheat flour microbial load as reported by MacArthur and D' Appolonia et al. (1981). Maximum flour temperature only reached 61^{o}C after heating and baking quality losses were also reported when flour was heated for more than 4 minutes.

2.12 Research Gap

The increased incidences of food recalls involving wheat flours/wheat flour derivatives contaminated with pathogens such as STEC *E. coli* increased the attention given to the food safety aspect of wheat flours. This led to the development of the antimicrobial intervention steps mentioned above that is applicable for the wheat milling process. Among the processes mentioned, modifying the tempering process to include antimicrobial effects are fewer in comparison compared to the other antimicrobial treatments proposed in wheat. In addition, the incorporation of antimicrobials in the tempering water does not need significant equipment additions to potentially reducing the implementation costs to millers compared to other processes such as heating and irradiation. In addition, tempering usually requires long periods of resting time (6 to 24 hours) which can give these antimicrobial compounds ample time to reduce the wheat microbial load which allows for minimal changes in the milling processing time. Furthermore, research can be done on exploring the effects of adding various food-grade antimicrobials such as food acidulants in the tempering process. These compounds are generally regarded as safe (GRAS) therefore potential hazards due to exposure and mishandling are minimized as they have no adverse effects.

The microbiological quality of wheat flours is a relatively new area of research. Thus, few researches have been done describing how the initial microbial contamination of wheat is being transferred into the resulting milling fractions and milling equipment. Further understanding on how the microorganisms of concern in wheat behaves throughout the milling process can help in properly implementing potential antimicrobial steps as well as improve the sanitation practices involved in milling. This will be beneficial as milling equipment surfaces can also act as a route for pathogens to contaminate wheat flours through direct contact therefore properly cleaning and sanitizing milling equipment more prone to contamination could be able to reduce the risk of producing contaminated wheat flours. With this, this research aimed to develop a food safety intervention and understand how *E. coli* contaminates wheat flours and processing equipment which would help improve wheat flour safety.

2.13 References

Al-Dmoor H., and El-Qudah J. (2016), Cake Flour Chlorination and Alternative Treatments (Review), Current Research in Nutrition and Food Science, 4 (2) pp. 127 - 134

Anderson C. (2018), Presence of *Escherichia coli* and *Salmonella enterica* on Wheat and Possible Control Measures, Master's Thesis, North Dakota State University

Ardent Mills (n.d.), Flour Food Safety by Ardent Mills Food Safety Team, accessed from https://www.ardentmills.com/media/1090/ardent_mills_flour_food_safety_white_paper.pdf on April 24, 2020

Bensassi F., Mahdi C., Bacha H., and Hajlaoui M. (2011), Survey of the mycobiota of freshly harvested wheat grains in the main production areas of Tunisia, African Journal of Food Science 5 (5) pp. 292-298

Berghofer L., Hocking A., Miskelly D., and Jansson E. (2003), Microbiology of wheat and flour milling in Australia, International Journal of Food Microbiology (85) pp. 137-149

Besant L., and Sparks P. (1982), Grains Production, Processing and Marketing, Chicago Board of Trade, Food and Agriculture Organization - AGRIS

Birck N.M.M., Lorini I., and Scussel V.M. (2006), Fungus and mycotoxins in wheat grains at post-harvest, 9th International Working Conference on Stored Product Protection PS2-12-6281

Bonjour E.L., Opit G.P., Hardin J., Jones C.L., Payton M.E., and Beeby R.L. (2011), Efficacy of ozone fumigation against major grain pests in stored wheats, Journal of Economic Entomology 104 (1) pp. 308-316

Bullerman L., Bianchini A. (2009), Food Safety Issues and the Microbiology of Cereals and Cereal Products, Microbiologically Safe Foods, John Wiley & Sons, Hoboken NJ

Bushuk W., and Rasper V.F. (2012), Wheat: Production, Properties, and Quality, Springer Science Business Media, pp. 7, 15, 25-26

Canadian Food Inspection Agency (2017a), Updated food recall warning - Robin Hood brand Baker's Hood All Purpose Flour recalled due to *E. coli* O121, accessed from: http://www.inspection.gc.ca/about-thecfia/newsroom/food-recall-warnings/complete-listing/2017-06-15/eng/1497583899498/1497583903666 on April 21, 2020

Canadian Food Inspection Agency (2017b), Food recall warning - Rogers brand All Purpose Flour recalled due to *E. coli* O121 accessed from: http://www.inspection.gc.ca/about-the-cfia/newsroom/food-recallwarnings/complete-listing/2017-06-07/eng/1496896334668/1496896336875 on April 21, 2020

Canadian Food Inspection Agency. (2017c), Updated food recall warning - Various brands of flour and flour products recalled due to *E. coli* O121, accessed from http://www.inspection.gc.ca/about-the-cfia/newsroom/foodrecall-warnings/complete-listing/2017-04-12/eng/1492038924857/1492038929165.

Cauvain, S.P., Hodge, G., Muir, D.M. and Dodds, N.J. (1976), Treatment of Grain, U.S.: A23B 4/04

Centers for Disease Control and Prevention. (2015), *E. coli* (*Escherichia coli*), accessed from https://www.cdc.gov/ecoli/general/index.html on April 21, 2020

Centers for Disease Control and Prevention (CDC) (2009), Multistate outbreak of E. coli O157:H7 infections linked to eating raw refrigerated, prepackaged cookie dough. Updated August 7, 2009 (final web update), accessed from https://www.cdc.gov/ecoli/2009/0807.html on April 21, 2020

Curtis B.C. (1996), "Wheat in the World", Food and Agriculture Organization accessed from http://www.fao.org/3/y4011e04.htm on April 20, 2020

Debes J. (2014), "Which Wheat for What?"- Kansas Wheat accessed from http://kswheat.com/news/2014/12/02/which-wheat-for-what on April 20, 2020

Deibel K.E., and Swanson, K.J. (2001), Cereal and cereal products. Microbiological Examination of Foods (Ed PF Downes, K), American Public Health Association, Washington DC pp. 549- 552.

Dhillon B., Wiesenborn D., Wolf-Hall C., and Manthey F. (2009), Development and evaluation of an ozonated water system for antimicrobial treatment of durum wheat, Journal of Food Science 74(7) pp. 396-403

Eglezos S. (2010), Microbiological quality of wheat grains and flour from two mills in Queensland, Australia, Journal of Food Protection (73) 8 pp. 1533-1536

Ennadir J., Hassikou R., Ohmani F., and Hammamouchi J. (2012), Microbiological quality of wheat flour consumed in Morocco, Canadian Journal of Microbiology 58 (2) pp. 145-150

Food and Drug Administration (2019), Current Good Manufacturing Practices (CGMP) Regulations, accessed from https://www.fda.gov/drugs/pharmaceutical-quality-resources/current-good-manufacturing-practice-cgmp-regulations on April 24, 2020

Food and Drug Administration (2021), Recalls, Market Withdrawals, and Safety Alerts, accessed from https://www.fda.gov/safety/recalls-market-withdrawals-safety-alerts on April 21, 2020

Finn S., Condell O., McClure P., Amezquita A., and Fanning S. (2013), Mechanisms of survival, responses, and sources of *Salmonella* in low-moisture environments, Frontiers in Microbiology 4 pp. 331 - 343

Fitzpatrick M. (1999), Hemolytic uremic syndrome and *E. coli* O157, British Medical Journal, 318 (7185) pp. 684-685

Food Safety News (FSN) (2017), "Robin Hood flour recall expands amid *E. coli* outbreak". accessed from https://www.foodsafetynews.com/2017/04/139082/ on April 21, 2020

Forghani F., den Bakker M., Liao J.Y., Futral A., and Diez-Gonzalez F. (2018), Long-term Survival and Thermal Death Kinetics of Enterohemorrhagic *Escherichia coli* Serogroups O26, O103, O111, and O157 in Wheat Flour, Applied Environmental Microbiology (10) pp. 11-28

Gallo G., Lo Bianco M., Bognanni R., and Saimbene G. (2008), Mycotoxins in durum Wheat grain: Hygienic-health quality of Sicilian production, Journal of Food Science 73 pp. 42 - 47.

Gieraltowski L., Schwensohn C., Meyer S., Eikmeier D., Medus C., Sorenson A., Forstner, Madad A., Blankenship J., Feng P., and William I. (2017), Notes from the field: Multistate outbreak of Escherichia coli O157:H7 infections linked to dough mix – United States, 2016. Morbidity Mortality Weekly Report 66 (3) pp. 88–89.

Guy R.C.E., and Mair C. (not published), The improvement of wheat flours for use in high-ratio cakes product-heat moisture treatments, Campden BRI

Hanamoto M. and Bean M. (1979), Process for Improving Baking Properties of Unbleached Cake Flours. U.S.: A21D6/00

Hanis T., Mnukova J., Jelen P., Klir P., Perez B., and Pesek M. (1988), Effect of gamma irradiation on survival of natural microflora and some nutrients in cereal meals, Cereal Chemistry 65 pp. 381 - 383

Hankison C., Linke E., and Seleen W. (1967), Method for pasteurizing flour, Hexion Inc. Patent No. US3554772A

Harris L. J., and Yada S. (2019), Flour and cereal grain products: foodborne illness outbreaks and product recalls [Tables and references], In Flour & Cereal Grains – Outbreaks and Recalls, accessed from http://ucfoodsafety.ucdavis.edu/Low_Moisture_Foods/

Hashmi I. (2019), Step by Step- Wheat Farming, Milling, and Quality Requirements, GrainCorp Storage and Handling, International Association of Operative Millers -MEA Conference

North American Millers Association (2019), Wheat Milling Process. accessed from https://www.namamillers.org/education/wheat-milling-process/ on April 20, 2020

Hui Y.H., Lim M.H., Nip W., Scott J., and Yu P. (2004), Principles of food processing, pp. 3-25 in: Food Processing Principles and Applications, J. Scott, and Y. Hiu, eds. Blackwell Press, Ames, IA

Ibanoglu S. (2002), Wheat washing with ozonated water: effects on selected flour properties, International Journal of Food Science and Technology 37 (5) pp. 579-584

International Grains Council (2020), Wheat Supply and Demand accessed from https://www.igc.int/en/markets/marketinfo-sd.aspx on April 20, 2020

Karmali M.A. (2004), Infection by Shiga toxin-producing *Escherichia coli*: An Overview, Molecular Biotechnology 26(2) pp. 117-122

Kent N.L., and Evers A.D. (1994), Technology of Cereals: An Introduction for Students of Food Science and Agriculture, Woodhead Publishing pp 165 pp. 170-173

Khanom A., Shammi T., and Kabir M.S. (2016), Determination of microbiological quality of packed and unpacked bread, Stamford Journal of Microbiology 6 pp. 24-29

Kirby E.J.M. (1993), Effect of sowing depth on seedling emergence, growth and development in barley and wheat, Field Crops Research 39 pp. 101-111.

Koksel H., Sapirstein H.D., Celik S., and Bushuk W. (1998), Effects of gamma-irradiation of wheat on gluten proteins, Journal of Cereal Science 28 (3) pp. 243-250

Kriss A.B., Paul, P.A., and Madden, L.V. (2010), Relationship between yearly fluctuations in Fusarium head blight intensity and environmental variables: A window-pane analysis, Phytopathology 100 pp. 784–797

Laca A., Mousia Z., Diaz M., Webb C., and Pandiella S. (2006), Distribution of microbial contamination within cereal grains, Journal of Food Engineering 72 pp. 332-338

Lancova K., Hajslova J., Kostelanska M., Kohoutkova J., Nedelnik J., and Moravcova H. (2008), Fate of trichothecene mycotoxins during the processing: milling and baking, Food Additives and Contaminants 25 pp. 650-659.

Laszlo Z., Hovorka-Horvath Z., Beszedes S., Kertesz S., Gyimes E., and Hodur C. (2008), Comparison of the effects of ozone, UV, and combined ozone/UV treatment on the color and microbial counts of wheat flour. Ozone: Science and Engineering 30 pp. 413 - 417

Latiful Bari Md., Hisaharu O., Nagakuwa K., and Ukai M. (2015), Application of Ultra Superheated Steam Technology (USST) to Food Grain Preservation at Ambient Temperature for Extended Periods of Time, Advances in Food Technology and Nutritional Sciences Open Journal SE 1 pp. 14-21

Los A., Ziuzina D., Akkermans S., Boehm D., Cullen P., Impe J., and Bourke P. (2018), Improving microbiological safety and quality characteristics of wheat by high voltage atmospheric cold plasma closed processing, Food Research International 106 pp. 509-512

MacArthur L.A., and D'Appolonia B.L. (1981), Effects of microwave radiation and storage on hard red spring wheat flour, Cereal Chemistry 58 pp. 53 - 58

Magan N., Aldred D., Mylona K., and Lambert R.J. (2010), Limiting mycotoxins in stored wheat, Food additives & contaminants. Part A, Chemistry, analysis, control, exposure, and risk assessment 27 (5) pp. 544-650

Magness J.R., Markle G.M., and Compton C.C. (1971). Food and Feed Crops of the United States Interregional Research Project IR-4, IR Bul. 1 (Bul. 828 New Jersey Agr. Expt. Sta.)

Mann J., Schiedt B., Baumann A., Conde-Petit B., and Vligis T. (2014), Effect of heat treatment on wheat dough rheology and protein solubility, Food Science and Technology International 20 (5) pp. 341 - 351

Manthey F.A., Wolf-Hall C. E., Yalla S., Viajayakumar C., and Carlson D. (2004), Microbial loads, mycotoxins, and quality of durum wheat from the 2001 harvest of the North Plains region of the United States, Journal of Food Protection 67 pp. 772-780

Marler B. (2018), Step away from the batter – Duncan Hines cake mixes recalled due to Salmonella outbreak, Marler Blog, 5 November, accessed from: https://www.marlerblog.com/legal-cases/step-awayfrom-the-batter-duncan-hines-cake-mixes-recalled-due-to-salmonella-outbreak

McCallum L., Paine S., Sexton K., Dufour M., Dyet K., Wilson M., Campbell D., Bandaranayake D., and Hope V. (2013), An outbreak of Salmonella Typhimurium phage type 42 associated with the consumption of raw flour, Foodborne Pathogens and Disease 10 pp. 159–164

Neil, K. P., G. Biggerstaff, MacDonald J. K., Trees E., Medus C., Musser K. A., Stroika S. G., Zink D., and Sotir M. J. (2012), A novel vehicle for transmission of Escherichia coli O157:H7 to humans: multistate outbreak of E. coli O157:H7 infections associated with

consumption of ready-to-bake commercial prepackaged cookie dough—United States, 2009. Clinical Infectious Diseases 54 pp. 511– 518

New Zealand Flour Millers Association. (2011). Wheat- Structure and Physical Characteristics. Accessed from http://www.flourinfo.co.nz/nzfma-knowledgebase/wheat/wheat-the-structure-a-physical-characteristics on April 20, 2020

Nishio Z., Takata K., Ito M, Tanio M., Tabiki T., Yamauchi H., and Ban T. (2010), Deoxynivalenol distribution in flour and bran of spring wheat lines with different *Fusarium* head blight resistance, Plant Diseases 94 pp. 335-338

Owens G. (2001), Cereals Processing Technology, Woodhead Publishing Limited, Abington Hall, Cambridge pp. 30-33,38

Park D.L. (2002), Effect of processing on aflatoxin, Advances in Experimental Medicine and Biology 504 pp. 173-179

Pascale M., Haidukowski M., Lattanzio V. M. T., Silvestri M., Ranieri R., and Visconti A. (2011), Distribution of T-2 and HT-2 toxins in milling fractions of durum wheat, Journal of Food Protection 74 pp. 1700-1707

Peles F., Gyori Z., Backsai T., Szabo Z., Murvai M., and Kovacs B. (2012), Microbiological quality of organic wheat grains and sprouts, Analele Universităţii din Oradea, Fascicula Protecţia Mediului 18

Pinson-Gadais L., Barreau C., Chaurand M., Gregoire S., Monmarson M., and Richard F. (2007), Distribution of toxigenic *Fusarium* spp. and mycotoxin production in milling fractions of durum wheat, Food Additives and Contaminants 24 pp. 53-62

Plains Grains Inc. (2011), Hard Red Winter Regional Quality Report, accessed from https://www.plainsgrains.org/wp-content/uploads/2018/10/2018-HRW-Regional-Quality-Report.pdf on April 20, 2020

Pomeranz Y. (1982), Grain Structure and End-Use Properties, Food Structure 1(2) Article 2

Posner ES and Hibbs AN (2005), Wheat Flour Milling 8th Ed, AACC Inc., St. Paul Minnesota, USA

Public Health Agency of Canada (PHAC) (2017), Public Health notice - Outbreak of E. coli infections linked to various flours and flour products, June 2, 2017 – Final update., accessed from: http://www.phac-aspc.gc.ca/phn-asp/2017/outbreak-ecoli-eng.php.

Thacker S., (2015), Navajo Pride voluntarily recalls flours, accessed from https://navajotimes.com/biz/navajo-pride-voluntarily-recalls-flour/#:~:text=A%20voluntary%20recall%20of%20bleached,Manager%20of%20Navajo%20Pride%20LLC. on April 20, 2020

Riba A., Mokrane S., Mathieu F., Lebrihi A., and Sabaou N. (2008), Mycoflora and ochratoxin A producing strains of Aspergillus in Algerian wheat, International Journal of Food Microbiology 122 pp. 85–92

Richter K.S., Dorneanu E., Eskridge K.M., and Rao C.S. (1993), Microbiological quality of wheat flours, Food and Agriculture Organization 38(5) pp. 367-369

Rogers, R.F., and Hesseltine C.W. (1978), Microflora of wheat and wheat flour from six areas of the United States, Cereal. Chemistry 55(6) pp. 889-898.

Rose D.J., Bianchini A., Martinez B., and Flores R.A. (2012), Methods for reducing microbial contamination of wheat flour and effects on functionality, AACC International 57 (3) pp. 105-106

Russ, J.V., and Doe, C.A. (1970), Heat Treatment of Flour as an Alternative to Chlorination, Journal of Food Technology 5 pp. 363-374

Sabillon L. (2014), Understanding the factors affecting microbiological quality of wheat milled products: From wheat fields to milling operations, Master's Thesis. Digital Commons @ University of Nebraska-Lincoln

Sabillon L., and Bianchini A. (2016), From Field to Table: A review on the Microbiological Quality and Safety of Wheat-Based Products, Cereal Chemistry AACC International 93 (2) pp. 105-115

Schaarschmidt S., and Fauhl-Hassek C. (2018), The fate of mycotoxins during the processing of wheat for human consumption, Comprehensive Reviews in Food Science and Food Safety 17 (3) pp. 556-593

Schlauri M., Buhler A.G., and Uzwil K. (1999), Alteration of flour quality by heat treatment, AGRIS FAO

Schmid-Hempel P., and Frank S. (2007), Pathogenesis, Virulence, and Infective Dose, PLOS Pathogens 3(10) pp. 147

Sebastian R. (2020). "The importance of the break system in flour milling process". Miller Magazine accessed from https://www.millermagazine.com/english/the-importance-of-break-system-in-flour-milling-process/.html on April 21, 2020

Seiler D.A.L. (1986), The microbial content of wheat and flour. International Biodeterioration and Biodegradation 22 pp. 35-40

Sondi I., and Salopek-Sondi B. (2004), Silver nanoparticles as antimicrobial agent: A case study on *E. coli* as a model for Gram-negative bacteria, Journal of Colloid, and Interface Science 275 pp. 177-182

Sorto G. (2019), "King Arthur Flour recalls some unbleached all-purpose flour for possible *E. coli*, CNN News accessed from https://www.cnn.com/2019/10/03/health/king-arthur-flour-recall-trnd/index.html on April 21, 2020

Sosland J. (2019). "U.S. per capita flour consumption ticks up in 2018". World-Grain. Accessed from https://www.world-grain.com/articles/12083-us-per-capita-flour-consumption-ticks-up-in-2018 on April 20, 2020

Sperber WH. (2007), Role of microbiological guidelines in the production and commercial use of milled cereal grains: A practical approach for the 21st century, Journal of Food Protection 70 pp. 1041-53

Spicher G. (1986), Judging the microbiological-hygienic quality of white flour. Die Muhle & Mischfuttertechnik 33 pp. 449

Tibola C., Guarienti E., Guerra Dias A., Nicolau M., Devos R., and Teixeira D. (2015), Effect of de-branning process on deoxynivalenol content in whole-wheat flours, Cereal Chemistry 96 (4) pp. 717-724

Tilley M., Chen Y.R., and Miller R.A. (2012), Breadmaking 2nd edition. Chapter 9- Wheat Breading and Quality Evaluation in the US pp. 218-220, Woodhead Publishing Series in Food Science, Technology, and Nutrition

Tiwari B.K., Brennan C.S., Curran T., Gallagher E., Cullen P.J., and O'Donnell C.P. (2010), Application of ozone in grain processing, Journal of Cereal Science 51 (3) pp. 248-255

Todd E., and Baker N. (2006), *E. coli* Infection in Canada. The Canadian Encyclopedia. accessed from https://www.thecanadianencyclopedia.ca/en/article/hamburger-disease on April 21, 2020

U.S. Food & Drug Administration. (2005), Bulletin to the Food Service and Retail Food Store Industry Regarding Cake Batter Ice Cream and Similar Products, accessed from http://www.foodprotect.org/media/reportdate/8-05FDA5.pdf on May 5, 2020

U.S. Food and Drug Administration: Silver Spring, MD (2019), accessed from U.S. Wheat Associates, 2019 Crop Quality Report accessed from https://www.uswheat.org/wp-content/uploads/2019/10/2019-USW-Crop-Quality-Report-English.pdf on April 20, 2020

United States Department of Agriculture- Economic Research Service (USDA-ERS) (2018), Wheat Data accessed from https://www.ers.usda.gov/data-products/wheat-data/ on April 20, 2020

Victor N., Bekele M., Ntseliseng M., Makotoko M., Peter C., and Asita A. (2013), Microbial and Physicochemical Characterization of Maize and Wheat Flour from a Milling Company in Lesotho 15 pp. 11-19

Weaver G., Akins-Lewenthal E., Allen B., Baker S., Hoerning D., Peterson A., Schumacher R., and Warren B. (2011), Microbial reduction in a processing steam of a milled product. U.S. Patent Application 20110177216

Wicks L. (2019), "Two Flour Recalls Issued Nationwide Due to Potential *E. coli* Contamination" accessed from http://www.eatingwell.com/article/2060046/flour-recall-ecoli-hodgson-mill/ on April 21, 2020

Wolt M., Chigurupati S., and Pulverenti J. (1994), Method for heat treating a bread baking wheat flour and resulting flour and dough, United States Conagra, Inc. (Omaha, NE). 5433966

Wrigley C.W. and Nirmal R.C. (2017), The Major Cereal Grains: Corn, Rice, and Wheat. John Wiley & Sons

Zain M. (2011), Impact of mycotoxins on humans and animals, Journal of Saudi Chemical Society 15 (2) pp. 129-144

Zhang, G., L. Ma, N. Patel, B. Swaminathan, S. Wedel, and M. P. Doyle. (2007), Isolation of *Salmonella* Typhimurium from outbreak-associated cake mix, Journal of Food Protection 70 pp. 997–1001

Chapter 3 - Quantitative Transfer of *E. coli* (Non-Pathogenic) from Wheat into Milling Fractions and Equipment during Lab Scale Milling

Abstract

Cross-contamination during processing has been a major cause of pathogen contamination of food leading to recalls and foodborne illnesses. Knowledge concerning *E. coli* transfer in the mill fractions and equipment during milling could help improve wheat flour safety. The objectives of this study were to characterize the transfer of *E. coli* into the mill fractions and equipment through consecutive milling steps. To evaluate this, 40 batches (900 g/ batch) of wheat were prepared for every milling run and this was split into inoculated (n = 20) and non-inoculated (n = 20) batches. Inoculated wheat (~3 log CFU/g) was prepared by tempering (16% moisture content, 24 h) wheat using an *E. coli* cocktail (ATCC 1427, 1429, 1430, and 1431), while non-inoculated wheats were tempered using sterile distilled water. The entire batch of inoculated wheats prepared (n = 20 batches) were milled first followed by the non-inoculated batches (n = 20 batches) using a Chopin lab-scale roller mill (2 break, 1 sizing, and 3 reduction rolls). *E. coli* counts on the mill surfaces (log CFU/ 100 cm^2), flour (break, sizing, reduction, and straight-grade flour), and non-flour (bran, fine bran, shorts, rough middling, and fine middling) fractions (log CFU/g) were enumerated after milling. *E. coli* counts (log CFU/g) of each flour fraction were plotted against wheat quantity milled (kg) for the inoculated and non-inoculated wheat milling runs. A best-fit model describing the *E. coli* count trends for each flour fraction was then selected. Results indicate that *E. coli* surface counts were higher ($P \leq 0.05$) in the rolls (break and reduction rolls), feeders, hoppers, and break sifter surfaces. In the initial milling batch, the bran, fine bran, rough middling

fractions (2.3 – 2.7 log CFU/g) had significantly higher ($P \leq 0.05$) *E. coli* counts than all the flour fractions obtained (1.0 – 1.9 log CFU/g). *E. coli* counts of all flour (3.4 – 3.9 log CFU/g) and non-flour (3.7 – 4.2 log CFU/g) fractions from the last inoculated wheat batch (20^{th}) were similar ($P > 0.05$). For the non-inoculated milling, *E. coli* surface counts declined after milling (0.4 - 1.9 log CFU/ 100 cm^2). *E. coli* counts (log CFU/g) of all flour and non-flour fractions declined to levels below detection limit (< 1 log CFU/g) as the quantity of wheat milled increased. Break, sizing, reduction, and straight-grade flours (n = 20 / flour fraction) obtained from non-inoculated wheat were positive for *E. coli*. The model describing the *E. coli* transfer to flour fractions (break, sizing, reduction, and straight-grade) as a function of wheat quantity milled (kg) had good fit ($P \leq 0.05$, se < 0.43). Results from this study could provide insights to mill managers on how *E. coli* contaminates the equipment used and mill fractions produced in wheat milling operations.

3.1 Introduction

The occurrence of recalls linked to pathogen contaminated wheat flours has increased in recent years (Harris and Yada, 2019). The entry of contaminated wheat flours into the supply chain has also led to recalls of wheat-based foods. Majority of these recalls were reported to be caused by Shiga toxin – producing *E. coli* (STEC) contamination of wheat flours (FDA, 2021). An outbreak linked to *E. coli* O157:H7 contaminated cookie dough occurred in 2009 wherein 72 foodborne illnesses were recorded in 30 US states which resulted in a large recall of contaminated products (Neil et al, 2012). More recent foodborne illness outbreaks have occurred wherein non-O157 STECs were identified as the contaminant in raw wheat flours. An outbreak linked to *E. coli* O121 and O26- contaminated wheat flours occurred in 2016 which resulted in 63 illness cases across 24 states in the US (FDA, 2017). These illness outbreaks also resulted in the recall of significant volumes of affected wheat flour products.

Due to the physical nature of the milling process, the microbiological quality of the raw wheat entering the milling facility has been shown to have significant impacts on the microbial quality of the wheat flour (Bullerman and Bianchini, 2009). Previous studies have demonstrated that milling redistributes the microflora of the wheat into the resulting milling fractions (Sabillon et al., 2019; Laca et al., 2006). Microbial contamination of wheat flours due to cross-contamination from processing equipment has also been viewed as a potential route for foodborne pathogens (Eglezos, 2010; Berghofer et al., 2003). This is due to the formation of microbial reservoirs in the milling equipment as a result of moisture and accumulation of mill residues within the mill.

The cross-contamination or transfer of foodborne pathogens from various sources (e.g., processing equipment, humans, environment, etc.) into food products during processing has been a major concern for consumers and manufacturers alike (Sheen and An-Hwang, 2011). Research

efforts aiming to describe, simulate, and model potential pathogen contamination in different processing scenarios are useful in providing insights for improving control of these pathogens during processing and improving equipment sanitation practices (Sheen and Hwang, 2011). Such studies have been previously pursued for foods recognized as potential vehicles of foodborne illness such as ground meat (Mustapha, 2007), fresh produce (Jensen et al., 2015; Bucholz et al., 2012), and deli meats (Sheen and An-Hwang, 2011; Perez-Rodriguez et al., 2007). These studies evaluate the transfer of relevant foodborne pathogens (e.g., STECs, *Salmonella*, and *Listeria*) on surfaces or finished products from various routes such as introducing contaminated products (e.g., meats, produce) in the processing equipment or using a contaminated piece of equipment (slicers, washers, etc.) to process non-contaminated products. The findings from such studies could then be used as basis for improving control of foodborne hazards during different stages of processing (Gallager et al., 2003).

As wheat flours were somewhat overlooked as a potential vehicle for foodborne illness in humans, knowledge gaps describing the extent of cross-contamination of pathogens during the milling process exist. Such gaps should be addressed to help improve sanitation practices in wheat mills by identifying probable "hotspots" in the equipment where microbial contamination is likely to occur. Such studies could also provide insights on the extent of microbial contamination in the wheat flours produced. Therefore, the first objective of this study was to quantify the *E. coli* transferred during a series of mill runs in two processing scenarios, namely from contaminated wheat into the milling fractions and processing equipment and from contaminated mill equipment to non-contaminated milling fractions. The second objective was to construct mathematical models that aim to predict the *E. coli* counts (log CFU/g) of each batch of flour fraction produced as a function of the wheat quantity milled (kg) in consecutive milling runs for the two scenarios.

3.2 Materials and Methods

Materials

Hard red winter (HRW) wheat samples were used for this study. The grains were provided by the pilot plant facility of the Department of Grain Science and Industry- Kansas State University in Manhattan, KS. The background microflora of the wheat lots (n = 30 samples) was enumerated by plating on appropriate 3M petri films giving the following microbial counts (log CFU/g): aerobic count – 4.0, coliform - 3.4, *E. coli* – negative (by enrichment), *Enterobacteriaceae* – 4.0, yeast and mold – not detected (< 1 log CFU/g). Wheat lots that tested negative for *E. coli* (after enrichment) were used for the milling experiments.

Inoculum Preparation

Non-pathogenic *E. coli* cultures (ATCC 1427, ATCC, 1429, ATCC 1430, and ATCC 1431) were used. The cultures were maintained on TSB: glycerol (7:3) mixture at -80^{o}C. Thawed cultures were streaked in tryptic soy agar plates and incubated (37^{o}C; 24 h). Incubated streak plates were refrigerated until use. A well-isolated colony from the streak plates of each strain was then picked and inoculated into individual TSB tubes (10 ml) and incubated (37^{o}C, 24 h). Cells for each strain were then harvested by centrifugation (2795 x g; 10 minutes) (Fisher Scientific-Sorvall, Hampton, NH) and resuspended in fresh TSB (10 ml). 1 ml of each *E. coli* cell suspension was inoculated individually in fresh TSB (9 ml) and incubated (37^{o}C, 24 h). This resulted in an *E. coli* inoculum concentration of ~ 9 log CFU/ml for each strain. Cells from each strain were harvested by centrifugation after incubation and re-suspended in 10 ml of 0.1% peptone water. The individual cell suspensions in 0.1% peptone water were then combined in equal proportions to obtain the *E. coli* cocktail (~ 9 log CFU/ml) to be used for inoculation. This cocktail was further diluted to ~ 6

log CFU/ml using 0.1% peptone water to achieve the target wheat inoculation level of ~ 3 log CFU/g when applied during tempering.

Wheat Tempering and Inoculation Procedure

For preparing the wheat grains, 40 batches of wheat (~900 g/ batch) were weighed into individual resealable bags for each milling experiment. The number of batches prepared was determined based on the efficiency of the lab mill used (approximately 2 kg / hr). The number of batches prepared corresponds to a day of milling. The prepared wheat was split into 20 batches each for the inoculated and non-inoculated wheat milling runs. Wheat batches were tempered to 16% moisture (wet basis) with the amount of tempering solution (75 ml) required calculated based on the initial wheat moisture content (9-10%). The tempering solutions were dispensed into the wheat (900 g / batch) using sterile 25 ml pipettes. Tempering solutions were gradually dispensed across the wheat grain surface and bags were continuously massaged during the application of the tempering solutions. After applying the tempering solutions, the resealable bags containing the wheat grains were sealed and massaged by hand for 5 minutes to allow even distribution of the tempering solution. For the inoculated wheat batches, tempering was done using the prepared inoculum (~ 6 log CFU/ml) resulting in an inoculation level of ~ 3 log CFU/g wheat. For the non-inoculated wheat batches, sterile distilled water was used for tempering instead of the *E. coli* cocktail inoculum. Wheat kernels were tempered statically for 24 hours (20-23^{o}C) prior to milling.

Wheat Milling and Cleaning Procedure

Tempered wheats were milled using a Chopin Laboratory Mill (Chopin Technologies, France) consisting of 2 break, 1 sizing, and 3 reduction steps. The feed rate for the 1st break step was set at 5 g /s while for the rest of the rolls a feed rate of 2.5 g / s was set. 2 kg of non-inoculated wheat (tempered with sterile water) was milled to warm up the mill rollers before the actual milling

runs. The entire batch (20 batches) of inoculated wheat tempered were milled first followed by the non-inoculated wheat batches (20 batches). As for the milling process, one batch (900 g) of wheat was milled at a time, with the mill fractions to be sampled taken after milling before introducing the next batch of wheat to be milled. This was done until all prepared wheat batches (inoculated and non-inoculated) was milled. The individual flour fractions (break, sizing, and reduction) produced after milling were combined to obtain the straight-grade flours. This milling procedure also produced the following non-flour fractions: bran, fine bran, shorts, rough middling, and fine middling.

For the cleaning procedure, the mill fractions inside the mill was vacuum cleaned, and its surfaces were sprayed and wiped with 70% ethanol. This was done prior to and after each independent milling run replication. One milling replication consists of milling the 40 batches of wheat prepared consisting of 20 batches of inoculated wheat and 20 batches of non – inoculated wheat.

Sampling Procedure for Milling Fractions

Milling fractions from the inoculated and non-inoculated milling runs were aseptically sampled during the sampling intervals for each milling fraction. A stainless-steel scooper/ sampler was disinfected with 70% ethanol prior to each sampling procedure. Prepared wheats (inoculated and non-inoculated) were sampled at the start (30 min) and end of the tempering procedure (24 h) (n = 5; 1^{st}, 5^{th}, 10^{th}, 15^{th}, and 20^{th} batch were sampled). Flour milling fractions (break, sizing, reduction, and straight-grade flours) were sampled after each batch of wheat was milled (n = 20). The non – flour milling fractions (bran, fine bran, shorts, rough, and fine middling) were sampled after every 5 batches of wheat milled (n = 5; 1^{st}, 5^{th}, 10^{th}, 15^{th}, and 20^{th} batch were sampled).

Milling samples were stored in sterile sampling bags (VWR, Radnor, PA) and refrigerated (4 - 7^{o}C) until *E. coli* enumeration analysis.

Swabbing Procedure

Mill surfaces (34 total surfaces) were swabbed using a sterile cotton swab moistened with sterile 0.1% peptone water. The swab locations were determined based on the product contact surfaces observed during the preliminary milling experiments. Used swabs were snapped and resuspended in 10 ml 0.1% peptone water until further microbial analysis. Swabbing of the mill surfaces were conducted at the following time points in the duration of the milling experiment: a) after cleaning and disinfection, b) after mill (roller) warm-up, c) end of inoculated wheat milling run (after the 20th batch of inoculated wheat), and d) end of non-inoculated wheat milling run (after the 20th batch of non-inoculated wheat). Due to the small size of the surfaces to be sampled in the mill, the use of a 100 cm^2 template was not suitable, instead swabbed surface areas were estimated based on the shape of the mill surfaces sampled. For example, the mill fraction container surface was rectangular shaped, hence the formula for calculating the area of a rectangle (length x width) was used. The dimensions of the mill surfaces sampled were taken using a standard ruler and caliper. Swab counts were expressed as CFU/cm^2. Locations of the mill surfaces swabbed are shown in Figures 3.1 to 3.7.

Figure 3.1 Chopin (Chopin, France) lab-scale roller mill used in the milling experiment

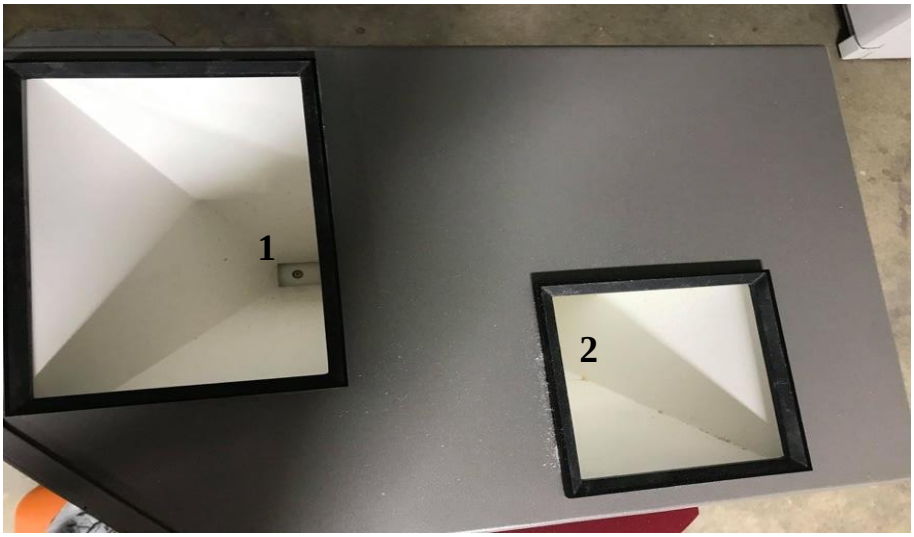

Figure 3.2 Hopper surface of the lab mill; 1 - BK hopper and 2 - RD hopper

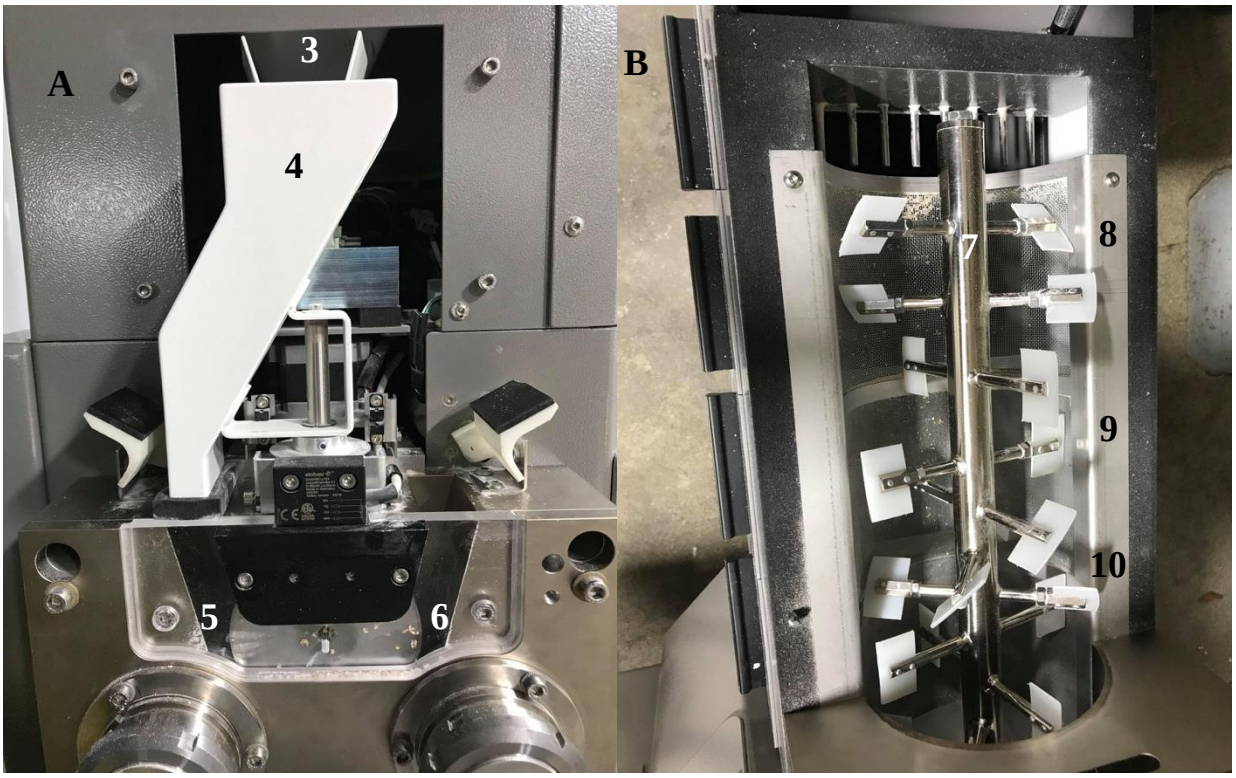

Figure 3.3 BK Feeder (A) and BK Sifter system (B); 3 – BK Feeder, 4 – BK Feeder Guide, 5 – 1st BK roll, 6 – 2nd BK roll, 7 – BK Beater Paddle (n = 2), 8 – RM sieve, 9 – FM sieve, and 10 – BK flour sieve

Figure 3.4 Sifter Covers; 11 - BK sifter cover, 12 - RD sifter cover, 13 - SZ sifter covers

Figure 3.5 SZ and RD system; 13 - SZ roll, 14 - RD feeder, 15 - RD feeder guide, 16 - RD roll

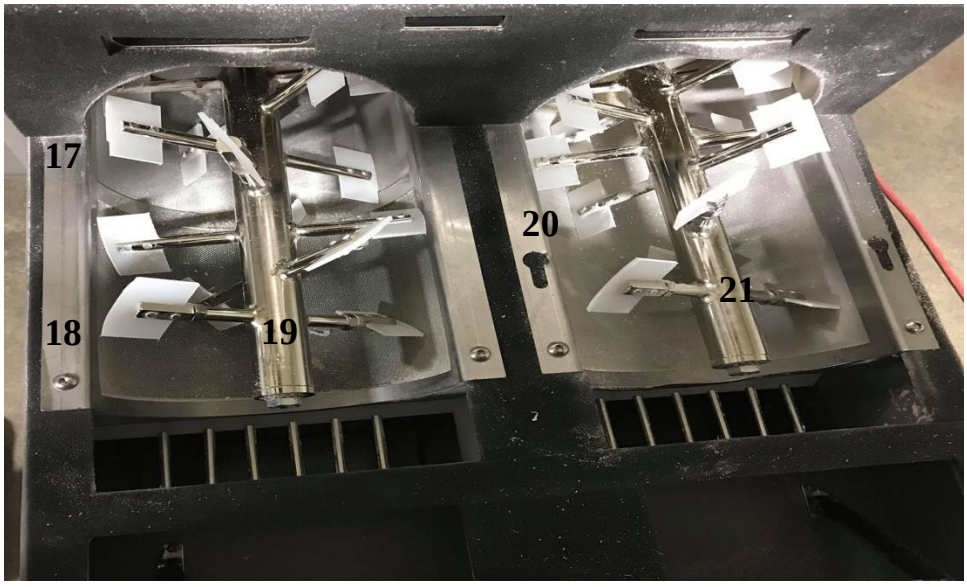

Figure 3.6 SZ and RD Sifter system; 17 - SZ flour sifter, 18 - FM sifter, 19 – SZ beater paddle (n = 2), 20 - RD flour sifter, and 21 - RD beater paddle (n = 2)

Figure 3.7 BK containers (A), SZ containers (B), and RD container; 22 – bran, 23 – RM, 24 – FM, 25 – BK flour, 26 – SZ flour, 27 – FM, 28 – Fine bran, 29 – shorts, and 30 – RD flour

Microbiological Analysis

For the milling samples, 25 g of the mill fraction (wheat, flour, or non-flour fractions) was mixed with 225 ml of buffered peptone water (BPW). Sample suspensions were homogenized (2 mins) using a stomacher (Seward, Davie, FL). Homogenized samples were serially diluted in 0.1% peptone water and appropriate dilutions were plated on 3M *E. coli* / coliform petri film plates (Saint Paul, MN). For samples that gave no countable *E. coli* colonies after incubation, homogenized samples were incubated (as an enrichment) for an additional 24 h (37^{o}C) followed by serial dilution and plating. The limit of detection (LOD) for the *E. coli* microbial analysis of the milling fractions was 1.0 log CFU/g. Samples that gave positive *E. coli* colonies after enrichment were assigned *E. coli* count values of 0.5 log CFU/g (half of detection limit) in the data used for comparisons and curve fitting analysis. For the swab samples, tubes containing the suspended swabs were vortexed (Vortex Genie, Scientific Industries, Bohemia, NY) for 10 seconds (Vortex Genie, Scientific Industries, Bohemia, NY) followed by serial dilution in 0.1% peptone water and plating in 3M *E. coli*/ coliform petri film plates. A single plating procedure was used for the enumeration of both *E. coli* counts in the mill fractions and surface swab samples. Plates were incubated for 48 h at 37^{o}C and enumerated. Blue to bluish-red colonies with surrounding gas bubbles that formed after incubation were counted as *E. coli* colonies.

Data analysis

Transfer rates (%) of *E. coli* from the wheat into its resulting milling fractions were calculated using the equation below:

$$Transfer\ rate\ (\%) = \frac{logCFU\ milling\ fraction}{logCFU\ wheat}\ x\ 100$$

The counts obtained from the flour milling fractions were plotted with the microbial counts (log CFU/ g) as the dependent variable and the quantity of wheat processed (kg) as the independent

variable. Models describing the trends in the *E. coli* counts of each flour fraction were fitted using TableCurve 2D v5.0 (Systat Software, San Jose, CA). An empirical approach was used in determining the best-fit model which was done by selecting the appropriate model from all the fitted models given based on the following criteria: significance of the model ($P \leq 0.05$), standard error of prediction, simplicity of the model (fewer model coefficients), and R^2 value.

Surface swab counts were transformed into log CFU/ 100 cm^2 prior to statistical analysis. Mean mill fraction counts (log CFU/g), surface counts (log CFU / 100 cm^2), and mean transfer rates (%) were analyzed using the GLIMMIX procedure with mean comparisons done using the LSMEANS procedure ($P \leq 0.05$). Analysis was conducted using SAS statistical software 9.3 (SAS Institute, Cary, NC). Three independent replications of each milling experiment were conducted. One replication of the milling experiment consists of milling the entire batches of wheat prepared (40 batches) with inoculated wheat (20 batches) being milled first followed by the non-inoculated wheat batch (20 batches).

3.3 Results and Discussion

The microbial quality of the wheat samples used in this experiment were (log CFU/g): aerobic count – 4.0, coliform - 3.4, *E. coli* – negative (by enrichment), *Enterobacteriaceae* – 4.0, yeast and mold – not detected (< 1 log CFU/g). The counts obtained for the wheat samples used were typical for incoming wheat grains based on previous studies where they reported coliform (3.0 – 4.0 log CFU / g), and aerobic (2.9 – 5.59 log CFU/ g) counts in wheat grains (Khanom, 2016; Aydin et al., 2009). These counts were expected as wheat grains could be contaminated at any point in its supply chain. The grains being negative for *E. coli* was also typical as it was reported to have low prevalence (0.44%, n = 3891) in wheat grains (Myoda et al., 2019). The yeast and mold count on the other hand were not typical as Eglezos (2009) reported yeast and mold counts for wheat of 3.7 and 2.8 log CFU/ g respectively. These low counts (yeast and molds) in the wheat samples used could be due to proper management and storage of the wheat grains.

Wheat samples were tempered to 16% (15.85 ± 0.39%) moisture using the *E. coli* inoculum (inoculated batches) or sterile water (non-inoculated batches). This tempering moisture target is within the range of common moisture levels used in commercial hard wheat milling which ranges from 15 to 17%. The use of the *E. coli* cocktail inoculum for tempering resulted in a wheat inoculation level of 4.1 (± 0.5) log CFU/g 30 minutes after application. After tempering (24 h), the *E. coli* load of the wheat decreased by ~0.54 log CFU/ g units giving *E. coli* counts of 3.5 (± 0.2) log CFU/ g prior to milling. Inoculation of the wheat batches (~ 3 log CFU/g) was done to have quantifiable *E. coli* counts in the mill fractions as natural *E. coli* loads of wheat were reported to be near or below detection limits (< 1 log CFU/g) of common enumeration methods (Myoda et al., 2019). Myoda et al. (2019) also reported that *E. coli* prevalence in wheat is very low with only 0.44% of raw wheat berries (n = 3891) being positive. Thus, inoculation was needed to be able to

properly quantify *E. coli* counts transferred into mill fractions and equipment. Furthermore, this also provides a closer approximation of how wheat milling equipment is contaminated. As wheat milling is typically done in a closed system, microbial contamination of the equipment surfaces usually occurs because of the entry of contaminated grains in the system. These contaminated grains when milled results in the accumulation of contaminated mill residues inside the mill which serves as a source of microbial contamination for the incoming mill fractions (Eglezos et al., 2010). Milling the non-inoculated wheat batch immediately after the inoculated wheat batches in each milling experiment assumes that only one type of wheat will be milled in a single day which eliminates the need for equipment cleaning when shifting from one batch of tempered wheat (inoculated) to another batch of wheat (non-contaminated) during each independent milling replication. This also creates the condition that the non-contaminated wheat grains tempered were milled using a contaminated milling equipment to produce the mill fractions.

Table 3.1 *E. coli* swab (log CFU/ 100 cm^2) counts of lab mill surfaces after the inoculated milling experiment (average of 3 independent milling replications)

Swab Location	**Swab Counts (log CFU / 100 cm^2)**	
	End of inoculated wheat milling run	End of non-inoculated wheat milling run
	Break System	
Hopper (n = 1)	3.8 (0.2) ^{ab}	1.1 (1.0) ^{a}
Feeder (n =1)	3.2 (0.8) ^{ab}	1.4 (1.2) ^{a}
Feeder Guide (n =1)	3.7 (0.3) ^{ab}	0.4 (0.8) ^{a}
1st BK Roll (n = 1)	3.5 (0.2) ^{ab}	0.6 (1.0) ^{a}
2nd BK Roll (n = 1)	3.7 (0.5) ^{ab}	ND
BK Beater Paddle (n = 2)	1.0 (0.8) ^{cde}	ND
RM Sieve (n = 1)	ND	ND
FM Sieve (n = 1)	ND	ND
BK Flour Sieve (n = 1)	3.7 (0.1) ^{ab}	ND
BK Sifter Cover (n = 1)	3.8 (0.1) ^{a}	1.4 (1.2) ^{a}
Bran Container (n = 1)	1.8 (1.5) ^{bcde}	ND
RM Container (n = 1)	2.8 (1.0) ^{ab}	0.9 (1.5) ^{a}
FM Container (n = 1)	1.9 (1.4) ^{bcde}	1.7 (1.4) ^{a}
BK Flour Container (n =1)	2.0 (1.6) ^{bcde}	1.8 (1.6) ^{a}

… continued Table 3.1

Reduction and Sizing System		
RD Hopper (n = 1)	3.6 (0.3) [ab]	ND
RD Feeder (n = 1)	1.9 (1.7) [bcde]	1.1 (1.0) [a]
RD Feeder Guide (n = 1)	2.7 (0.2) [abc]	1.0 (1.8) [a]
SZ Roll (n = 1)	1.7 (1.6) [abcd]	1.9 (0.4) [a]
SZ Beater Paddle (n =2)	0.9 (0.8) [bcde]	ND
SZ FM Sifter (n = 1)	ND	ND
SZ Flour Sifter (n = 1)	ND	ND
SZ Sifter Cover (n =1)	1.4 (1.3) [cde]	ND
SZ Fine Bran Container (n =1)	1.3 (1.1) [cde]	0.6 (1.0) [a]
SZ Flour Container (n = 1)	0.7 (1.3) [e]	ND
SZ FM Container (n =1)	1.5 (1.3) [cde]	ND
RD Roll (n = 1)	3.2 (0.2) [ab]	0.6 (1.0) [a]
RD Beater Paddle (n = 2)	1.1 (0.9) [e]	ND
RD Flour Sifter (n =1)	1.5 (1.3) [cde]	ND
RD Sifter Cover (n =1)	1.4 (1.3) [cde]	ND
RD Flour Container (n =1)	0.3 (0.4) [de]	ND
Shorts Container (n = 1)	1.4 (1.2) [cde]	ND

[1] mean swab counts (log CFU / 100 cm^2) that has different superscripts within each column are significantly different ($P \leq 0.05$) due to lab mill surface

[2] values enclosed in parentheses correspond to standard deviation: BK – break, SZ – sizing, RD – reduction, FM – fine middling, and RM – rough middling

[3] ND indicates that no *E. coli* colonies were recovered after direct plating (LOD – < 1 log CFU / 100 cm^2) of swab samples in all three milling run replications

[4] n corresponds to the number of swab samples taken from the mill surface during each independent milling replication

Milling using the lab scale mill used in this study generally proceeds by pouring the mill fraction into the mill hopper. The mill fraction to be milled are then fed into the roller mills using a vibrating feeder for milling. The milled fractions are then separated by sieving using the beater paddles into the resulting mill fractions. The mill fractions are then collected in the mill fraction containers.

The break system of the mill consists of the 1st and 2nd BK milling steps. In the 1st BK milling step, the wheat grains (inoculated or non-inoculated) are milled first producing the bran, rough middling, fine middling, and break flour milling fractions after sieving. For the 2nd BK step, the bran fraction is further milled and subsequently sieved to further separate the wheat into its bran, rough middling, fine middling, and BK flour components.

For the sizing and reduction system, the rough middling produced from the break (1st and 2nd BK) milling steps are milled in the SZ roll and subsequently sieved producing the fine bran, fine middling, and SZ flour fractions. The fine middling fractions produced from the break and sizing milling steps are then milled in the 1st RD milling step and sieved producing the shorts and RD flour milling fractions. The shorts fraction is milled (2nd and 3rd RD milling step) in the same RD roll and subsequently sieved producing more RD flour and shorts (reduced amount).

No *E. coli* cells were recovered (< 1 log CFU / 100 cm^2) from the surfaces sampled after the first two swab points (after cleaning/ disinfection, and after lab mill warm up). This observation indicates that no initial *E. coli* contamination was present in the mill surfaces sampled prior to the inoculated wheat milling runs. Table 3.1 shows the *E. coli* counts of the mill surfaces at the end of the inoculated wheat milling run. More *E. coli* counts were recovered in the break system surfaces especially on the BK rolls (1st and 2nd), feeders, hoppers, and BK flour sieve and sifter covers. These higher counts could be explained by the milling flowsheet used such that the inoculated

wheat is milled first in the break system before being milled in the subsequent rolls. The highly contaminated milling fractions produced (bran and middling) by milling the wheat in the break steps could have caused the higher transfer of *E. coli* in the break system. As for the hoppers, and feeder surfaces (feeder, and feeder guide), the higher amount of *E. coli* in them could be explained by their more frequent contact with contaminated milling fractions such as the inoculated wheat and the bran.

For the sizing and reduction system surfaces sampled, more *E. coli* cells were recovered ($P \leq 0.05$) from the hopper, and RD rolls. For the RD rolls, this could be due to the higher frequency of contact with contaminated milling fractions (fine middling, and shorts) as the 1^{st}, 2^{nd}, and 3^{rd} reduction milling steps are all conducted in a single RD roll. As for the hopper surface, its high *E. coli* surface contamination is because it had the most frequent contact with mill fractions (rough middling, fine middling, and shorts) as they need to pass through the hopper before milling.

Cross-contamination events often involve certain interactions between the bacterial cells and surfaces (e.g., product, and equipment) allowing cell attachment (Perez-Rodriguez et al., 2007). The transfer of bacteria from food to equipment occurs when the attachment of these cells fails allowing transfer of cells from one source to another (Dickson, 1990). Based on this, cross-contamination could be enhanced when cells attached to the mill fractions are exposed to forces (e.g., shear and frictional forces) present during milling operations such as feeding, roller milling, and sieving. This could also explain why *E. coli* contamination is lower in the other mill surfaces sampled such as the mill containers and beater paddles.

Table 3.2 *E. coli* counts (log CFU/g) recovered in the milling fractions after the inoculated wheat milling run (average of three independent milling replications)

Milling Fraction	***E. coli* counts (log CFU/ g)**				
Wheat Batch	1	5	10	15	20
			Flour Fractions		
Break	1.9 (0.3) [bcB]	2.6 (0.9) [abB]	2.2 (0.2) [aB]	3.4 (1.3) [aAB]	3.8 (1.2) [aA]
Sizing	0.9 (0.7) [dC]	2.2 (0.3) [abBC]	2.1 (0.2) [aB]	2.4 (0.3) [aB]	3.4 (0.8) [aA]
Reduction	1.2 (0.6) [cdC]	2.0 (0.9) [abBC]	2.2 (0.3) [aBC]	2.7 (0.4) [aB]	3.9 (0.7) [aA]
Straight- grade	1.9 (0.3) [bcB]	2.2 (0.8) [abB]	3.1 (0.9) [aB]	3.3 (1.0) [aB]	3.6 (1.2) [aA]
			Non-flour fractions		
Bran	2.3 (0.8) [abA]	3.3 (1.3) [aA]	3.2 (0.3) [aA]	3.8 (1.3) [aA]	4.2 (1.3) [aA]
Fine Bran	2.7 (0.2) [aA]	2.9 (0.8) [abA]	2.8 (0.8) [aA]	3.7 (1.8) [aA]	3.8 (2.0) [aA]
Shorts	2.2 (0.4) [abA]	2.7 (0.9) [abA]	2.9 (0.7) [aA]	3.3 (1.2) [aA]	4.1 (1.6) [aA]
Rough Middling	2.7 (0.9) [abA]	2.2 (0.2) [abA]	2.2 (0.2) [aA]	3.4 (1.5) [aA]	3.7 (1.3) [aA]
Fine Middling	2.0 (0.4) [abcA]	1.9 (0.2) [bA]	2.2 (0.6) [aA]	3.2 (1.4) [aA]	3.7 (1.3) [aA]

[1] mean counts with different lowercase letter superscripts in each column are significantly different ($P \leq 0.05$) due to mill fraction in each wheat batch
[2] mean counts in each row with different uppercase letter superscripts are significantly different ($P \leq 0.05$) due to wheat batch sampled
[3] values enclosed in parentheses correspond to standard deviation

Based on Table 3.2, *E. coli* counts of the non-flour mill (bran, fine bran, shorts, rough, and fine middling) fractions produced from the 1st batch of wheat were higher than the flour fractions (break, sizing, reduction, and straight-grade flours) from the same batch. The highest *E. coli* counts were recovered from the fine bran, rough middling, and bran fractions respectively. This observation is consistent with the results previously reported by Laca et al. (2006) wherein they reported that microbial contamination is highest/concentrated in the outer layers of the grain such as the bran. As milling separates the bran from the wheat endosperm, wheat flour fractions are expected to contain less amounts of microbial (e.g., *E. coli*) contamination compared to the initial wheat and non- endosperm layers such as the bran and germ. Furthermore, in the initial batch milled (batch 1), reduction and sizing flours had lower *E. coli* counts compared to the break and

straight-grade flours. These observations could be explained by the exposure of the break flour to more contaminated fractions (wheat and bran) as it is extracted after 1st and 2nd break milling steps where the inoculated wheat and coarse bran particles are milled. The straight-grade flour on the other hand is made by combining all the flour fractions recovered (break, sizing, and reduction) and therefore it is more likely to contain higher *E. coli* loads as it accumulates the *E. coli* cells from the individual flour fractions.

As the amount of contaminated wheat milled increased (5th to 20th batch), the *E. coli* counts recovered from all of the flour fractions increased. Similar *E. coli* ($P > 0.05$) counts were obtained for all the flour fractions (break, sizing, reduction, and straight-grade) and non-flour fractions (bran, fine bran, shorts, fine middling, and rough middling) starting from the 10th up to the 20th inoculated wheat batch milled. In addition, *E. coli* counts recovered from all the flour fractions obtained from the 20th batch were significantly higher ($P \leq 0.05$) compared to the *E. coli* counts of the flour fractions from the 1st batch of wheat milled. For all the non-flour fractions (fine bran, bran, shorts, rough and fine middling), no significant differences in *E. coli* counts ($P > 0.05$) due to quantity of wheat milled (batch) was observed.

During milling, heat is generated by the rollers which encourages moisture condensation within the mill (Berghofer et al., 2003). This moisture generated helps facilitate the accumulation of milled contaminated kernel / flour residues within the mill resulting in a viable reservoir for microorganisms (Sabillon et al., 2019). The accumulated milling residues could then contaminate the subsequent batches of flour milling fractions which could explain the increased *E. coli* counts recovered from all the flour fractions produced from the latter batches of inoculated wheat milled. *E. coli* counts of all the flour fractions from the last wheat batch (20th) were higher by 1 to 2 log units compared to the 1st batch of wheat milled ($P \leq 0.05$). This difference indicates that *E. coli*

counts from the later batches of wheat flours produced after milling could increase by as much 10 to 100 times the initial microbial load of wheat flours from the initial batches of wheat. Furthermore, this increase in flour *E. coli* counts also reflect a potential increase in the risk of *E. coli* contaminating the wheat flour during milling which could result into foodborne illnesses if pathogenic *E. coli* is present in the wheat flours. According to FDA (2012), the infective dose of STECs could be as low as 10 to 100 cells. Therefore, the increased *E. coli* counts of the flour fractions due to cross-contamination during milling could pose an increased risk for wheat flours being contaminated at infectious levels of STEC. The observations show the importance of controlling the contamination levels of incoming wheat lots through antimicrobial interventions as this could lead to equipment contamination during milling. Furthermore, the introduction of contaminated grains in the mill could also lead to an increased *E. coli* load of the flour fractions (break, sizing, reduction, and straight- grade flours) produced.

Table 3.3 Transfer rates (%) of *E. coli* in the milling fractions during the inoculated wheat milling run (n = 3)

Milling Fraction	Transfer Rate (%)	
	TR_I	TR_T
Flour Fractions		
Break	53.8 (10.2) [bcB]	108.2 (30.9) [aA]
Sizing	24.72 (17.1) [dB]	96.3 (19.7) [aA]
Reduction	34.1 (17.5) [cdB]	112.4 (18.4) [aA]
Straight- grade	54.9 (8.7) [bcB]	102.7 (32.0) [aA]
Non – flour Fractions		
Bran	64.7 (19.6) [abB]	121.5 (35.0) [aA]
Fine Bran	78.9 (10.2) [aB]	110.9 (56.7) [aA]
Shorts	61.9 (8.5) [abB]	119.4 (44.5) [aA]
Rough Middling	75.3 (20.1) [abB]	108.6 (39.2) [aA]
Fine Middling	56.4 (8.2) [abcB]	105.8 (37.9) [aA]

[1] mean transfer rates (%) with different superscripts in each column (TR_I and TR_T) are significantly different ($P \leq 0.05$) due to mill fraction
[2] TR_I correspond to transfer rates at the start of milling (1st batch) while TR_T correspond to the transfer rates to the mill fractions after the 20th batch of the inoculated wheat was milled
[3] values enclosed in parenthesis correspond to the standard deviation

The observations made from the *E. coli* counts of the milling fractions were also in agreement with the transfer rates (%) calculated shown in Table 3.3. Transfer rates (%) are expressed as the percentage of *E. coli* recovered in individual milling fractions relative to the *E. coli* load of the wheat batch that was milled. Transfer rates are often included in quantitative microbial risk assessments (QMRA) modelling to estimate the number of pathogens being transferred to different substrates (e.g., food, surfaces, etc.) during certain processing steps (Perez-Rodriguez et al., 2011). Based on the TR_I (%) values, significantly higher ($P \leq 0.05$) *E. coli* transfer occurred in the non-flour fractions (fine bran, bran, and rough middling) compared to flour fractions (break, sizing, reduction, and straight-grade flours after the 1st batch of wheat was milled.

For the flour fractions, both the straight-grade and break flours had higher amounts of *E. coli* transferred into them during the first batch of milling which agrees with the observations made in Table 3.3. This observation is similarly explained by the milling flow sheet wherein the break flour is exposed to highly contaminated mill fractions (bran and wheat) during milling while straight- grade flours are produced by combining all the flour fractions obtained therefore accumulating the individual *E. coli* cells from the individual flour fractions produced. The transfer rates (TR_T) for the mill fractions from last batch of inoculated wheat milled (20^{th} batch) show that the amount *E. coli* cells that could be transferred to the flour fractions are similar or greater than the *E. coli* load (3.5 log CFU/g) of the wheat milled. The transfer rates (TR_T) above 100% could be explained by the continuous milling of contaminated wheat grains which led to the concentration of *E. coli* cells into the mill fractions. This is also due to the accumulation of contaminated mill residues inside the mill which eventually served as a source of contamination for incoming mill fractions. High variability in the TR_T was also observed especially on the non-flour fractions. This variability could be related to the bulk nature of wheat flour milling such that its microbial contamination (*E. coli*) could be heterogeneously distributed in the milling fractions sampled. Furthermore, similar ($P > 0.05$) transfer rates were observed for the flour and non-flour fractions milled from the last batch of inoculated wheat. This suggests that the quantity of wheat that has been milled could have a significant impact on the amount of *E. coli* that could be transferred in the wheat flours produced after milling.

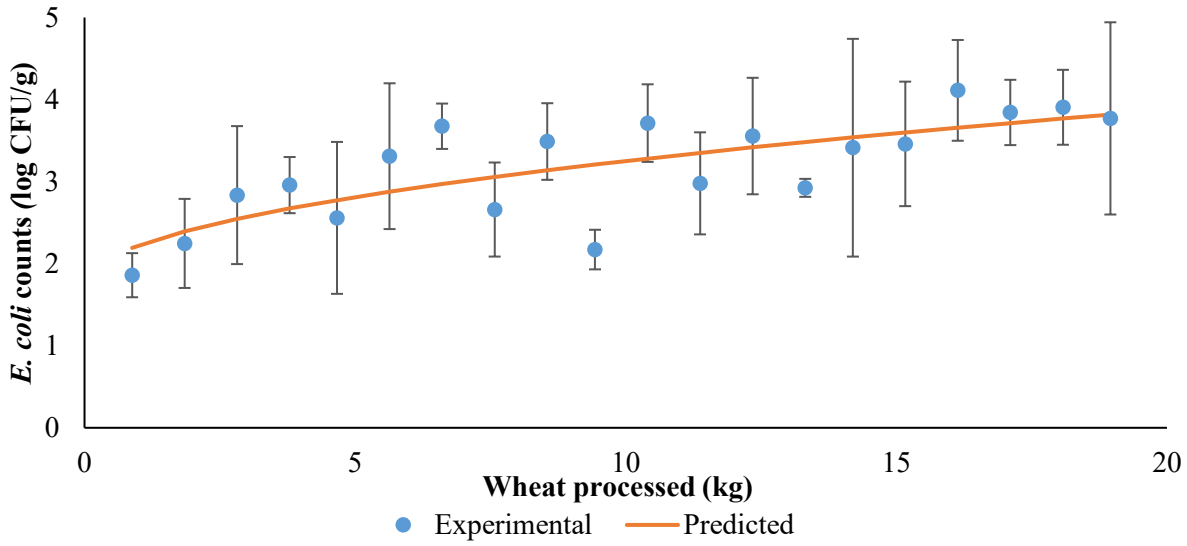

Figure 3.8 *E. coli* counts recovered in the break flour fraction during the inoculated wheat milling experiment (average of three independent milling replications)

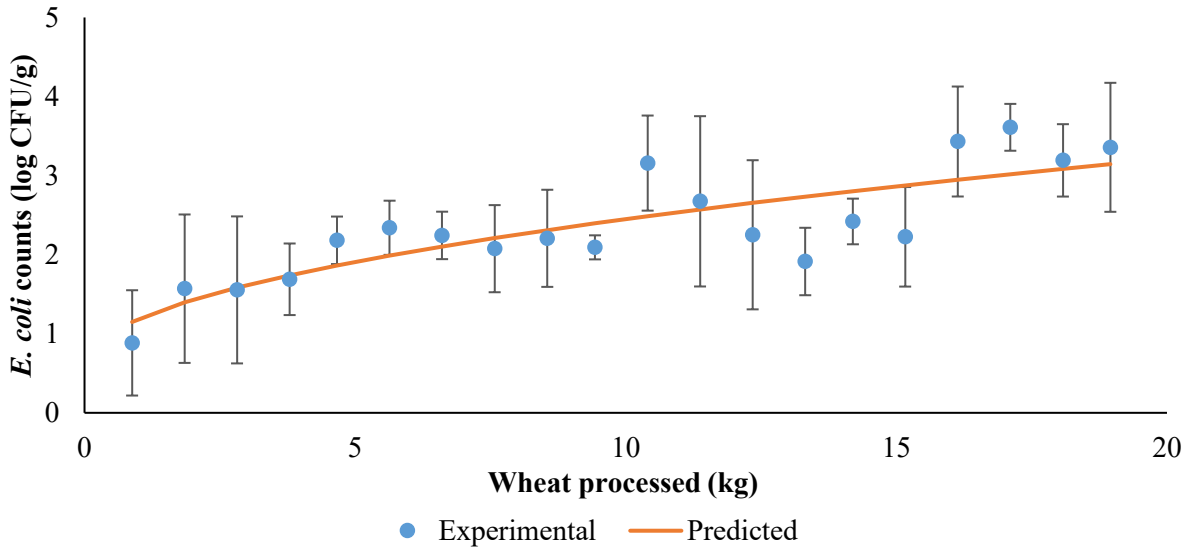

Figure 3.9 *E. coli* counts recovered in the sizing flour fraction during the inoculated wheat milling experiment (average of three independent milling replications)

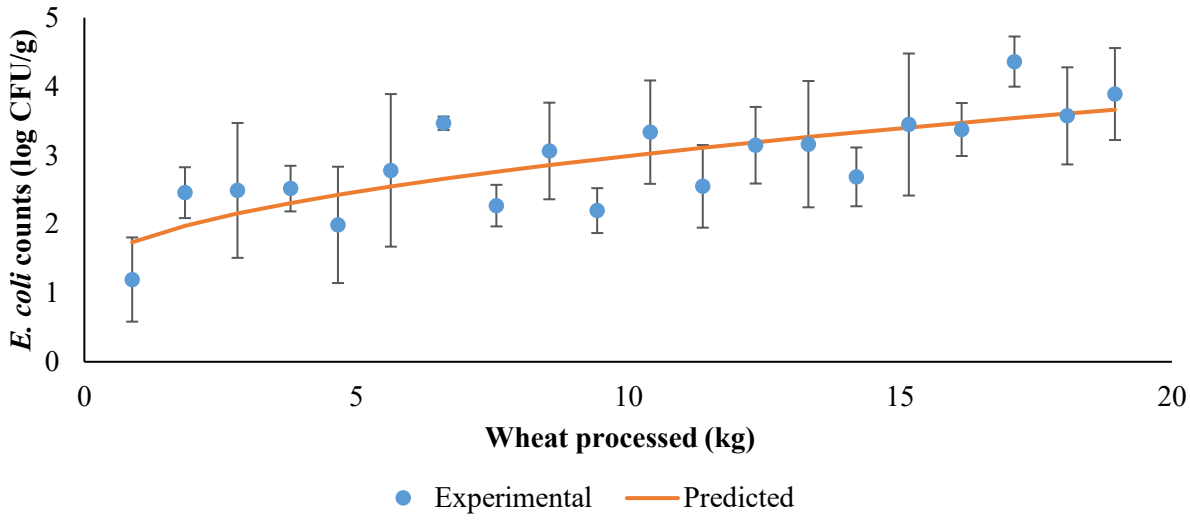

Figure 3.10 *E. coli* counts recovered from the reduction flour fractions during the inoculated wheat milling experiment (average of three independent milling replications)

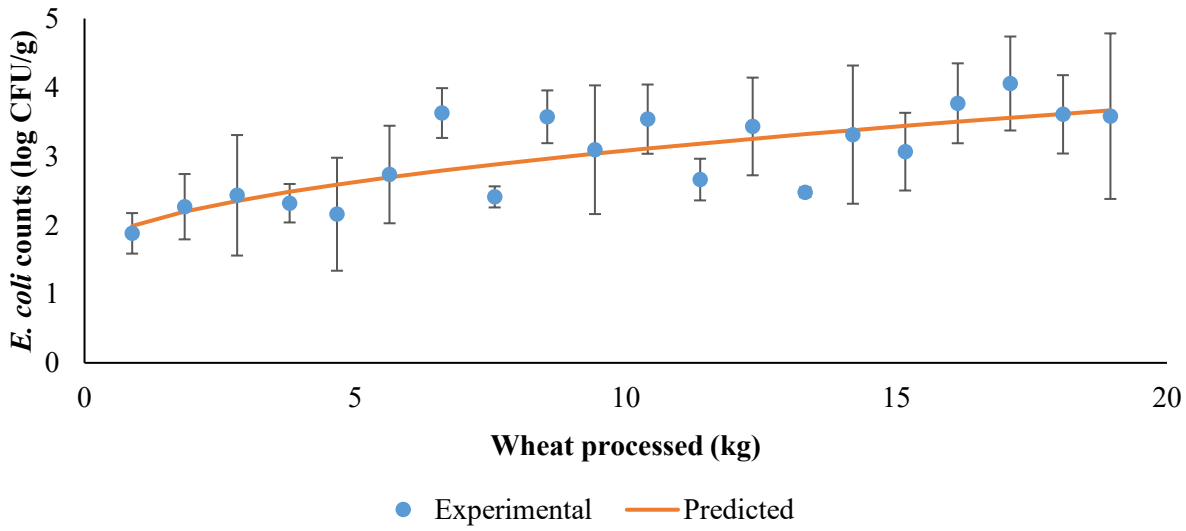

Figure 3.11 *E. coli* counts recovered from the straight- grade flours obtained during the inoculated wheat milling experiment (average of three independent milling replications)

Table 3.4 Model coefficients and goodness-of-fit indices obtained from the *E. coli* counts of flour milling fractions during the inoculated wheat milling experiment

Flour Fraction	Coefficients		Goodness-of-Fit Indices	
	A	B	R^2	Standard Error
Break	1.75	0.48	0.51	0.43
Sizing	0.60	0.58	0.54	0.41
Reduction	1.21	0.56	0.56	0.41
Straight-grade	1.53	0.49	0.54	0.42

[1] model coefficients and goodness of fit indices were obtained using TableCurve 2.0 v5.0
[2] A and B are regression parameters obtained from model fitting

Figures 3.8 to 3.11 shows the plots of *E. coli* counts recovered from the break, sizing, reduction, and straight-grade flours obtained against the cumulative weight of the inoculated wheat batches milled. Based on the figures, there is an increasing trend for the *E. coli* counts recovered in the flour milling fractions. The model that satisfies the criteria mentioned is shown below:

$$Y = A + Bx^{0.5}$$

The model coefficients and goodness of fit indices are shown in Table 3.4. The model and its coefficients obtained for all the flour fractions were significant ($P \leq 0.05$). In the model, A and B are the regression coefficients fitted with x being the amount of wheat processed (kg) and Y as the *E. coli* load of the flour fraction (log CFU/g) produced. The model fitted for predicting the *E. coli* counts present in each flour fraction indicate that *E. coli* counts of the flour fractions will increase according to the product of the B coefficient and the square root of the wheat quantity milled (kg). Based on the A coefficient, the break flour fraction is predicted to have the highest initial *E. coli* load among the flour fractions followed by the straight-grade, reduction, and sizing flours. This agrees with the *E. coli* counts of the flour fractions reported in Table 3.2. When using the model in predicting the *E. coli* counts of flour fractions, the fitted model had an estimated error of around 0.4 log CFU/g for all the flour fractions. R^2 values for all the models ranged from 0.51-0.56. This

low R^2 value could be explained by the fact that the *E. coli* counts recovered for all the flour fractions are not consistently increasing with higher quantities of wheat milled. The model selected was still deemed acceptable as it had a relatively low standard error of prediction value which is important for assessing the predictive capabilities of a model. The counts shown in Figures 3.8 to 3.11 were shown to be in an alternating pattern for each wheat batch. For example, in the straight-grade flours (Fig. 3.11), the count for the 7^{th} batch was lower, while the count for the 8^{th} batch increased followed by lower *E. coli* counts in the 9^{th} batch and so on. This could also be related to the variable distribution of contamination in wheat flours as the *E. coli* cells are not homogenously distributed but instead are concentrated in various parts of the flour sample giving the inconsistent increase in *E. coli* counts of the flour fractions produced.

Uninoculated Wheat Milling Run

Wheat lots used for the non-inoculated runs were negative prior to the tempering step. The wheat samples (n = 5) were also negative for *E. col* (after enrichment) at the end of tempering step. Table 3.1 shows the swab counts of the mill surfaces sampled at the end of the non-inoculated wheat milling run. *E. coli* counts for all the surfaces sampled approached values close to non-detectable levels (< 1 log CFU / 100 cm^2) after the last batch of non-inoculated wheat was milled. This observation indicates that *E. coli* cells have transferred into the mill fractions from the contaminated surfaces during milling. Transfer of *E. coli* from surfaces to the mill fractions involves horizontal movement as the mill fractions move and come into contact with the contaminated surfaces. The detachment of the cells attached in the surfaces could be explained in the same way as the inoculated milling experiment. The shear and frictional forces that occurred between the contaminated surface (rolls, hoppers, and feeders) and mill fractions break the surface attachments of *E. coli* allowing transfer of cells into the mill fractions lowering the mill surface

counts. The decrease in counts suggest that milling non- *E. coli* contaminated wheat batches could improve the *E. coli* contamination levels present inside the mill.

Table 3.5 *E. coli* counts recovered from milling fractions during non-inoculated wheat milling (average of three independent milling replications)

Milling Fraction	***E. coli* counts (log CFU/ g)**				
Wheat Batch	1	5	10	15	20
Flour Fractions					
Break	2.6 (0.2) ^{aA}	0.5 (0.0) ^{bB}	0.5 (0.0) ^{aB}	0.5 (0.0) ^{aB}	0.5 (0.0) ^{aB}
Sizing	2.3 (0.4) ^{bA}	0.9 (0.7) ^{bB}	0.7 (0.1) ^{aB}	0.5 (0.0) ^{aB}	0.6 (0.0) ^{aB}
Reduction	2.7 (0.4) ^{aA}	1.2 (0.5) ^{abB}	0.9 (0.2) ^{aB}	0.7 (0.1) ^{aB}	0.5 (0.0) ^{aB}
Straight- grade	2.6 (0.3) ^{abA}	1.4 (0.7) ^{abB}	0.5 (0.0) ^{aB}	0.9 (0.6) ^{aB}	0.5 (0.0) ^{aB}
Non-flour fractions					
Bran	2.8 (0.2) ^{aA}	0.5 (0.0) ^{bB}	0.5 (0.0) ^{aB}	0.8 (0.5) ^{aB}	0.5(0.1) ^{aB}
Fine Bran	3.4 (0.6) ^{aA}	1.3 (0.7) ^{abB}	0.9 (0.2) ^{aB}	1.5 (0.6) ^{aB}	0.7 (0.1) ^{aB}
Shorts	3.0 (0.3) ^{aA}	1.9 (0.2) ^{aB}	0.8 (0.2) ^{aB}	0.96 (0.3) ^{aB}	0.8 (0.2) ^{aB}
Rough Middling	3.3 (0.4) ^{aA}	0.9 (0.6) ^{bB}	1.4 (0.8) ^{aB}	0.5 (0.0) ^{aB}	0.5 (0.0) ^{aB}
Fine Middling	2.0 (1.0) ^{bA}	1.4 (0.7) ^{abB}	1.5 (0.7) ^{aB}	0.7 (0.1) ^{aB}	0.5 (0.0) ^{aB}

[1] mean counts with different lowercase letter superscripts in each column are significantly different ($P \leq 0.05$) due to mill fraction

[2] mean counts in each row with different uppercase letter superscripts are significantly different ($P \leq 0.05$) due to sampling interval

[3] fractions with counts (log CFU / g) of 0.5 (0.0) indicate that the fraction was *E. coli* positive only after enrichment in all three milling replications

[4] values enclosed in parenthesis correspond to the standard deviation

Table 3.5 shows the counts of *E. coli* transferred from the mill surfaces into the mill fractions. After the 1st batch of non-inoculated wheat was milled, at least 2 log CFU/g (2.0 – 2.7 log CFU/g) of *E. coli* have been transferred into all flour and non-flour milling fractions. The *E. coli* counts of the milling fractions produced from the 1st batch of non-inoculated wheat was higher compared to the mill fractions produced from the 1st batch of inoculated wheat milled (Table 3.2). These higher counts (2.0 – 3.4 log CFU/g) recovered from the mill fractions of the 1st batch of non-inoculated wheat milled could be due to the bulk transfer of the loosely accumulated

contaminated milling residues left in the mill. After the 5th batch was milled, the *E. coli* load for both flour and non-flour fractions decreased by 1.1 – 2.3 log CFU/ g except for the break and bran fractions wherein enrichment of samples was needed to detect *E. coli*. As more amounts of non-inoculated wheat have been milled using the contaminated lab-scale mill, *E. coli* counts of all the milling fractions declined to levels below the detection limit. This decrease could be explained by the continuous reduction of *E. coli* levels inside the mill as it gets transferred into the preceding batches of non-contaminated wheat milled. Enrichment of the mill fraction samples was needed to detect the *E. coli* contamination present in the samples as no *E. coli* colonies were recovered after the initial direct plating procedure. Overall, all flour fractions (n = 20 samples) and non-flour fractions (n = 5) from the 20 batches of non-inoculated wheat milled immediately after the inoculated wheat milling run were positive for *E. coli*. The observations show the need for proper cleaning and sanitation of wheat mills as it is a viable route for *E. coli* contamination of wheat flours.

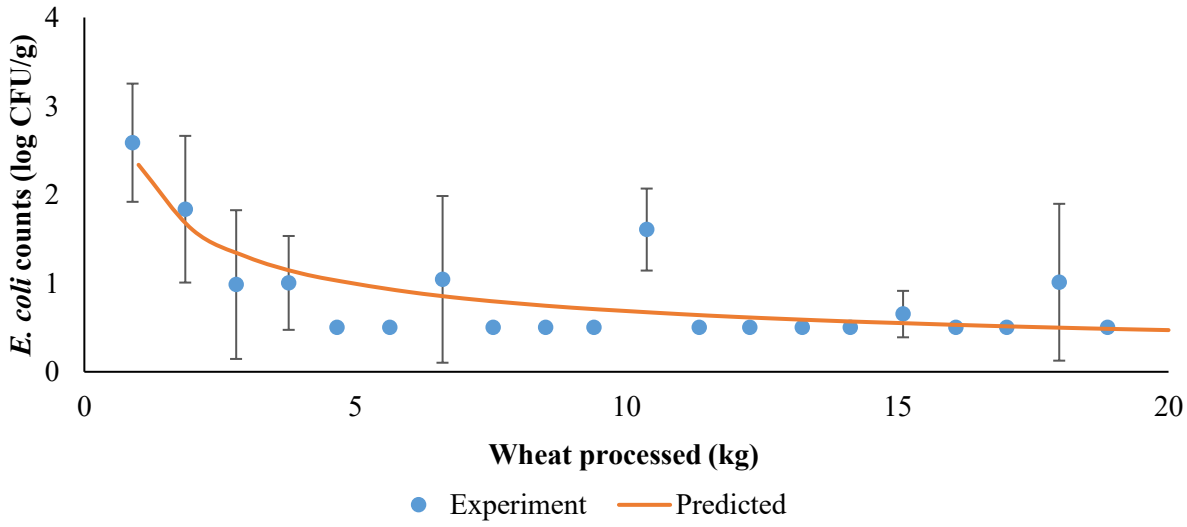

Figure 3.12 *E. coli* counts recovered from the break flour fraction during non-inoculated wheat milling experiment (average of three independent milling replications)

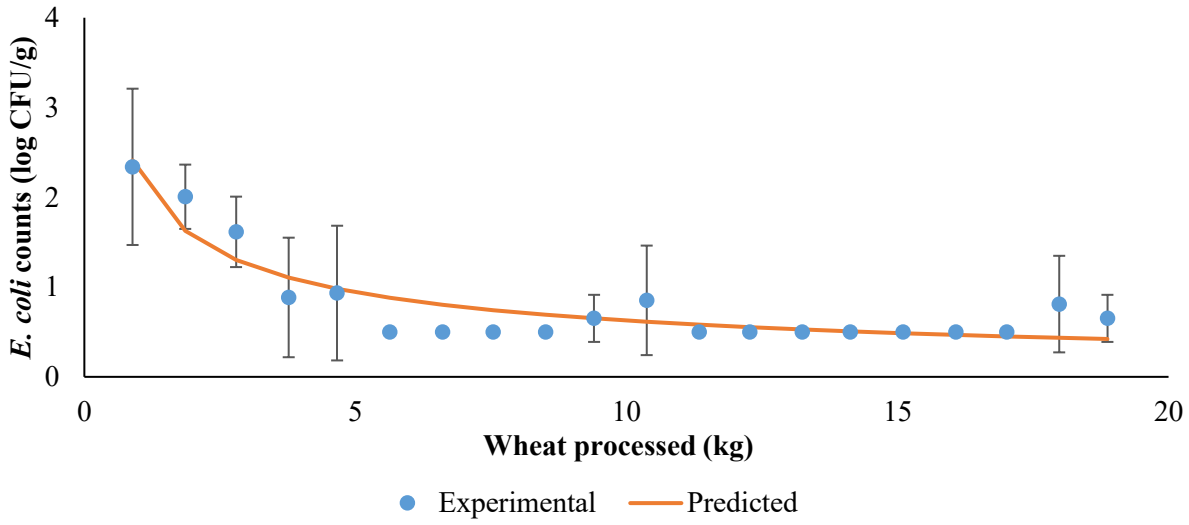

Figure 3.13 *E. coli* counts recovered from sizing flour fractions during the non-inoculated wheat milling experiment (average of three independent milling replications)

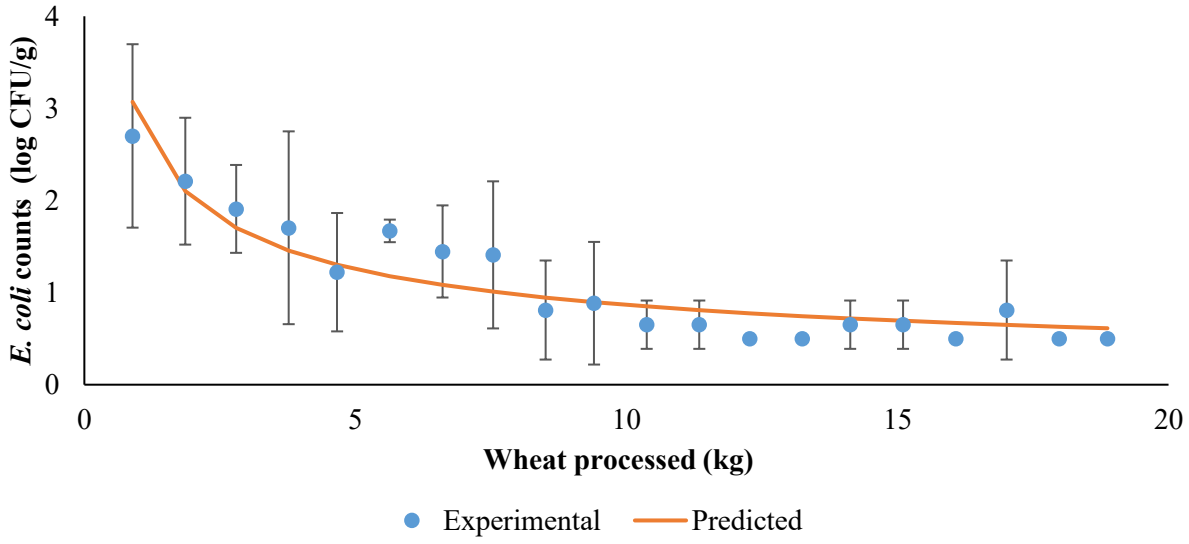

Figure 3.14 *E. coli* counts recovered from reduction flour fractions during the non-inoculated wheat milling experiment (average of three independent milling replications)

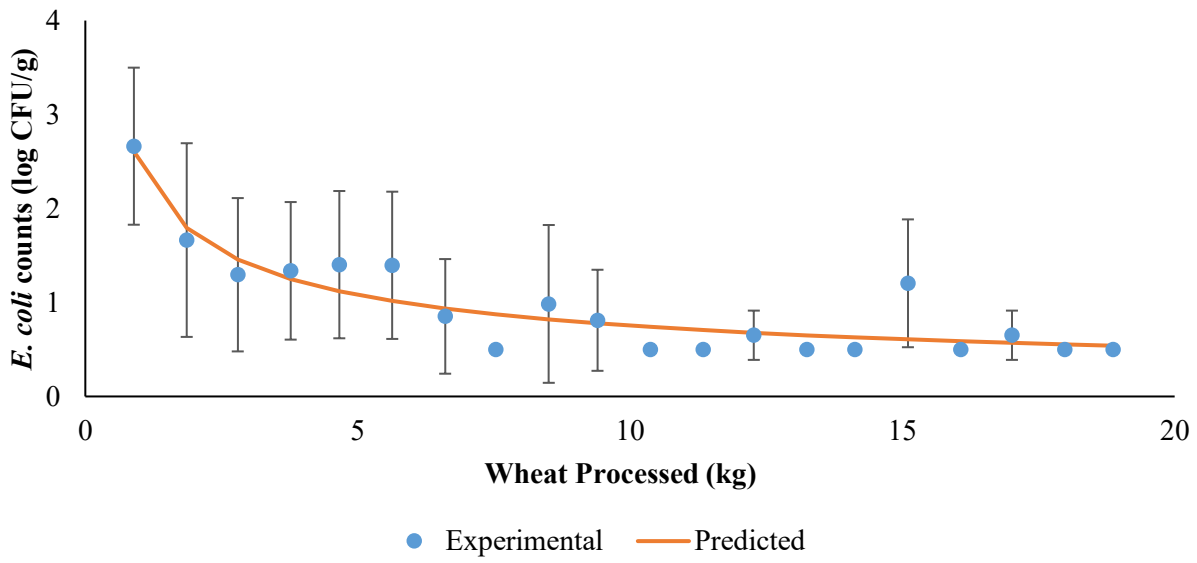

Figure 3.15 *E. coli* counts recovered from the straight-grade flour fractions during the non-inoculated wheat milling experiment (average of three independent milling replications)

Figures 3.12 to 3.15 show the plots of *E. coli* counts recovered from the different flour fractions obtained against the cumulative weight of the wheat batches milled using the contaminated mill. *E. coli* counts of all the flour fractions exhibited similar trends such that they all declined to non-detectable levels (needing enrichment to detect *E. coli*) with increasing quantities of non-inoculated wheat milled. The *E. coli* counts of the break, sizing, reduction, and straight-grade flour declined to non-detectable levels after 5, 6, 8, and 9 batches respectively. The relatively higher number of batches with quantifiable *E. coli* counts without enrichment for the reduction flour could be due to its longer contact time with the mill. All the milling steps (2nd break, 1 sizing, and 3 reduction rolls) are required before the reduction flour is extracted which increases the chance of *E. coli* contamination for the reduction flours. The break and sizing flours on the other hand are produced only after the 2nd and 3rd milling steps respectively. These results are consistent with the findings of Miranda and Schaffner (2016) wherein their study reported that higher contact times led to an increased cross-contamination rate of *E. aerogenes* transferred from

contact surfaces to the food. As for the straight-grade flours produced, the greater number of batches that are above detection limits could be due to the effect of combining the individual flour fractions which concentrates *E. coli* cells from the individual flour fractions in the straight-grade flour. Overall, all the samples of each flour fraction (n = 20 / fraction) produced by milling non-contaminated grains using contaminated equipment were positive for *E. coli* although majority of the samples needed enrichment to detect *E. coli* presence.

Overall, the decreasing trend in the *E. coli* counts of all flour fractions agree with the results observed by Sheen and An-Hwang (2011), and Perez-Rodriguez et al. (2007) for slicing operations. In their study, the microbial counts of meat slices decreased with higher slice numbers in a contaminated slicer (equipment) and non-inoculated food (deli meat) process scenario. The observations also agree with the reports by Buchholz et al. (2012) for leafy green processing wherein *E. coli* O157:H7 counts recovered in the shredded greens decreased with increasing amounts of non-contaminated leafy greens processed. In a contaminated mill- non-inoculated wheat milling scenario, this could be explained by the event that the number of *E. coli* cells decline inside the mill with increasing amounts of wheat milled which lowers the chance of *E. coli* transfer from the equipment to the processed mill fractions.

Table 3.6 Model coefficients and goodness-of-fit indices obtained from the *E. coli* counts of flour milling fractions during the non-inoculated wheat milling experiment

Flour Fraction	**Coefficients**		**Goodness-of-Fit Indices**	
	A	B	R^2	Standard Error
Break	-0.04	2.25	0.63	0.34
Sizing	-0.13	2.40	0.82	0.22
Reduction	-0.07	2.96	0.85	0.25
Straight-grade	-0.03	2.49	0.82	0.23

[1] model coefficients and goodness of fit indices were obtained using TableCurve 2.0 v5.0
[2] A and B are regression parameters obtained after model fitting

Table 3.6 shows the coefficients and goodness of fit indices of the best-fit model describing the *E. coli* counts of the flour fractions during the non-inoculated milling experiment. The best fit model is shown below:

$$Y = A + B / x^{0.5}$$

Wherein A and B are regression coefficients with x as the amount of wheat milled (kg) and Y as the *E. coli* count (log CFU/ g) of the flour fraction. The model selected was significant ($P \leq 0.05$) for all the flour fractions. According to the model obtained, an inverse relationship occurs between the *E. coli* load of each flour fraction and wheat quantity milled wherein *E. coli* load decreases with increasing amount of wheat milled. This relationship suggests that for non-inoculated wheat batches milled, *E. coli* contamination would be lower for wheat flours obtained from latter wheat batches. Furthermore, the A coefficients obtained for the models of each flour fraction were not significant ($P > 0.05$), therefore the model could be further simplified and expressed as:

$$Y = B / x^{0.5}$$

High variability exists in the *E. coli* counts of the flour fractions (BK, SZ, RD, and SG) obtained from batches not requiring pre-enrichment as shown by the larger error bars. This variability could be related to the different extent of cross-contamination in each milling system as well as the variability of the traditional plating enumeration methods at low (near detection limits) *E. coli* levels. The model obtained from the non-inoculated wheat milling run had a better prediction performance compared to the model obtained for the inoculated milling run as evidenced by the higher R^2 (0.63- 0.85) and standard error (0.23 – 0.34) values. However, most of the flour samples obtained needed enrichment to be able to detect *E. coli*. A constant log count value (0.5 log CFU /g) was assigned for the *E. coli* positive samples which could have led to the higher R^2 and lower standard error values. Hence, the proper quantification of counts below the

detection limit could be employed to better assess the fit of the selected model in this study. Overall, *E. coli* counts of the flour fractions declined with increasing wheat quantity milled up to levels below detection limits (< 1 log CFU/g). Furthermore, all flour fractions obtained from every batch (n = 20) of non-inoculated wheat milled tested positive for *E. coli*. These observations show the importance of proper cleaning and sanitation of milling equipment as equipment contamination could also be a major route for *E. coli* contamination of wheat flours which could result to regulatory actions if detected.

3.4 Conclusion

The results from this study show that both the *E. coli* load of the wheat and milling equipment affect the *E. coli* levels present in the resulting wheat flour fractions. Observations from the inoculated milling run indicate that *E. coli* from the wheat generally contaminates the resulting milling fractions. Furthermore, *E. coli* cell numbers recovered from the wheat flours (break, sizing, reduction, and straight-grade flours) increased as more quantities of contaminated wheat were milled. The *E. coli* counts of the flour fractions (break, sizing, reduction, and straight-grade) obtained from the last batch of inoculated wheat milled increased by 1 to 2 log CFU/g compared to the flour fractions from the initial batch (1^{st}) of inoculated wheat milled. By the end of the inoculated wheat milling run, no significant differences ($P > 0.05$) were observed with the *E. coli* load of the flour fractions (break, sizing, reduction, and straight- grade) and the non-flour fractions (bran, shorts, fine bran, rough, and fine middling) obtained from the last batch of inoculated wheat that was milled. These findings suggest that cross-contamination of *E. coli* from previous batches of wheat milled is highly likely and that wheat flour *E. coli* counts could reach levels similar to the non-flour fractions (e.g., bran). This also suggests that the amount of *E. coli* present in the wheat flour is affected by the quantity of contaminated wheat milled. Lastly, these observations demonstrate the importance of controlling the microbial load (*E. coli*) of wheat prior to milling.

As for the milling surfaces swabbed, *E. coli* contamination was higher in the break system. Among the break system surfaces sampled, the rolls (1^{st} and 2^{nd} BK, RD rolls), sifter surfaces (cover and BK flour sieve), hoppers, and feeders had higher *E. coli* loads compared to the other surfaces sampled. These findings show that specific parts of the milling equipment could become likely “hotspots” for *E. coli* contamination after milling and that greater attention should be given to these parts during the mill cleaning and sanitation operations.

For the non-inoculated wheat milling run conducted, *E. coli* counts of the milling fractions that were produced declined with increasing wheat quantity milled. All the milling fractions produced from the 20 batches of non-inoculated wheat tested positive for *E. coli* although a majority needed enrichment to be detected. Swab counts (*E. coli*) of the mill surfaces significantly decreased after the non-inoculated wheat milling with a majority of the surfaces giving swab direct plating counts below detection limits (< 1 log CFU / 100 cm^2). Findings from this study suggest that cross-contamination of *E. coli* from the wheat and milling surfaces is likely to occur which could have a significant impact on the food safety of wheat flours. Furthermore, the time point/ stage of milling has a significant impact on the amount of *E. coli* contamination present in wheat flours wherein less contamination was present in the wheat flours from the latter batches of non-inoculated wheat milled. The empirical models fitted in this study could be used in predicting the potential *E. coli* load of wheat flour fractions in a lab-scale milling scenario.

Food processing scenarios often involve various parameters in terms of the nature of processing and product characteristics which could all affect how the microorganisms are transferred throughout processing. In this sense, the effects of different factors relevant to wheat milling such as grain hardness, wheat variety, and wheat inoculation level / method could be tested in future works. The effects of these parameters could then be fitter in the model by evaluating how model coefficient change with response to different process parameters. The application of the model in bigger scale milling operations could also be done to verify its accuracy as bigger mills would have more mill fractions produced, roller milling steps, surface area, and different surface materials which could all affect how much *E. coli* is transferred into the mill fractions. Lastly, as high variability in the *E. coli* counts were observed for the mill fractions and mill surface

counts, a bigger sample size (quantity and area) could be used to improve the variability of the *E. coli* counts obtained in both milling fractions and surfaces.

3.5 References

Aydin A., Paulsen P., and Smulders F. (2009), The physic-chemical and microbiological properties of wheat flour in Thrace, Turkish Journal of Agriculture and Forestry 33 (5) pp. 445-454

Berghofer L., Hocking A., Miskelly D., and Jansson E. (2003), Microbiology of wheat and flour milling in Australia, International Journal of Food Microbiology 85 pp. 137-149

Buchholz A., Davidson G., Marks B., Todd E., and Ryser T. (2012), Transfer of *Escherichia coli* O157:H7 from equipment surfaces to fresh-cut leafy greens during processing in a model pilot-plant production line with sanitizer-free water, Journal of Food Protection 75 (11) pp. 1920-1929

Bullerman L., and Bianchini A. (2009), Food Safety Issues and the Microbiology of Cereals and Cereal Products, Microbiologically Safe Foods, John Wiley & Sons

Dickson J.S. (1990), Transfer of *Listeria monocytogenes* and *Salmonella* typhimurium between beef tissue surfaces, Journal of Food Protection 53 pp. 51-55

Eglezos S. (2010), Microbiological quality of wheat grains and flour from two mills in Queensland, Australia, Journal of Food Protection 73(8) pp. 1533-1536

Food and Drug Administration (2012), Bad Bug Book, Foodborne Pathogenic Microorganisms and Natural Toxins, 2nd ed., Enterohemorrhagic *E. coli* pp. 75 – 78

Food and Drug Administration (2017), FDA Investigated Multistate Outbreak of Shiga toxin-producing *E. coli* Infections Linked to Flour. In. U.S. Food and Drug Administration: Silver Spring, MD. Accessed from https://www.fda.gov/Food/RecallsOutbreaksEmergencies/Outbreaks/ucm504192.htm March 6, 2021

Food and Drug Administration (2021), Recalls, Corrections, and Removals, accessed from https://www.fda.gov/medical-devices/postmarket-requirements-devices/recalls-corrections-and-removals-devices on March 6, 2021

Gallagher D., Eric E., and Kause J. (2003), FSIS Risk Assessment for *Listeria monocytogenes* in Deli meats.

Harris, L. J., and S. Yada. (2019), Flour and cereal grain products: foodborne illness outbreaks and product recalls [Tables and references]. In Flour & Cereal Grains – Outbreaks and Recalls. Accessed from http://ucfoodsafety.ucdavis.edu/Low_Moisture_Foods/

Jensen D., Friedrich L., Harris L., Danyluk M., and Schaffner D. (2015), Cross-contamination of *Escherichia coli* O157:H7 between lettuce and wash water during home-scale washing, Food Microbiology 46 pp. 428-433

Khanom A., Shammi T., and Kabir M.S. (2016), Determination of microbiological quality of packed and unpacked bread, Stamford Journal of Microbiology 6 pp. 24-29

Laca A., Mousia Z., Diaz M., Webb C., and Pandiella S. (2006), Distribution of microbial contamination within cereal grains, Journal of Food Engineering 72 pp. 332-338

Miranda R., and Schaffner D. (2016), Longer contact times increase cross-contamination of *Enterobacter aerogenes* from surfaces to food, Applied and Environmental Microbiology 82 (21) pp. 6490 – 6496

Mustapha A. (2007), Real-time monitoring of cross-contamination of *Listeria monocytogenes* between equipment and ready-to-eat meat products via a GFP reporter, University of Missouri, accessed from https://www.beefresearch.org/resources/beef-safety/project-summaries/2006/realtime-monitoring-of-cross-contamination-of-listeria on March, 12, 2021

Myoda S., Gilbreth S., Akins-Leventhal D., Davidson S., and Samadpour M., (2019), Occurrence and Levels of Enterohemorrhagic *Escherichia coli*, and *Listeria* in Raw Wheat, Journal of Food Protection 82 (6), pp. 1022-1027

Neil K., Biggerstaff G., MacDonald J.K., Trees E., Medus C., Musser K., Stroika S., Zink D., and Sotir M. (2012), A novel vehicle for transmission of Escherichia coli O157:H7 to humans: multistate outbreak of E. coli O157:H7 infections associated with consumption of ready-to-bake commercial prepackaged cookie dough--United States, 2009, Clinical Infectious Diseases 54 pp. 511-518

Perez-Rodrigues F., Valero F., Todd E.C.D., Carrasco E., Garcia-Gimeno R.M., and Zurera G. (2007), Modeling transfer of *Escherichia coli* O157:H7 and *Staphyloccocus aureus* during slicing of a cooked meat product, Meat Science 76 pp. 692-699

Perez-Rodriguez F., Campos D., Ryser E.T., Bucholz A.L., Posada-Izquierdo G.D., Marks B. P., Zurera G., and Todd E. (2011), A mathematical risk model for *Escherichia coli* O157:H7 cross-contamination of lettuce during processing, Food Microbiology 28 pp. 694 - 701

Sabillon L., Stratton J., Rose D., and Bianchini A. (2019), Effect of saline organic acid solutions applied during soft wheat tempering on microbial load and flour functionality, Cereal Chemistry 96 (6) pp. 1048 – 1059

Sheen S., and Hwang C.A. (2011), Modeling the surface cross-contamination of *Salmonella* spp. on Ready-to-eat meat via slicing operation, Food and Nutrition Sciences 2 pp. 916-924

Sperber W.H. (2007), Role of microbiological guidelines in the production and commercial use of milled cereal grains: A practical approach for the 21st century, Journal of Food Protection 70 pp. 1041-53

Chapter 4 - Significance of Sodium Bisulfate (SBS) Tempering in Reducing *E. coli* O121 (ATCC 2219) and O26 (ATCC 2196) Load of Wheat and its Effects on Flour Milling and Baking Quality

Abstract

The recent recalls and foodborne illness outbreaks linked to Shiga toxin producing *E. coli* (STEC) – contaminated wheat flours prompt the need to control enteric bacterial contamination in the wheat milling process. Tempering is a front-end milling step wherein water is added to the wheat kernels to improve yield and milling quality. Modifications to this step could provide a route to control STEC in wheat prior to milling. The objectives of this study were to evaluate the efficacy of adding sodium bisulfate (SBS) to the tempering water to reduce STEC O121 and O26 loads in wheat before milling. Wheat samples were inoculated (~6 log CFU/g) with *E. coli* O121 (ATCC 2219) or O26 (ATCC 2196) and tempered (17%, 24 h) with increasing SBS concentrations (0, 0.5, 0.75, 1.0, 1.25, and 1.5% SBS). Tempering concentrations that resulted in ≥ 3 log reductions were evaluated for their effects on flour quality. The minimum inhibitory concentration (MIC) of SBS against both *E. coli* serotypes was 0.32% w/v. After tempering (24h), the 1.25 (O121 – 3.5 logs; O26 – 3.2 logs) and 1.5% SBS (> 4 logs for O121 and O26) treatments resulted in significant reductions ($P \leq 0.05$) in the STEC load of wheats. SBS concentrations of 0, 1.25, and 1.5% SBS were used to evaluate treatment effects on flour quality. Wheat flours produced using 1.25 and 1.5% SBS tempering treatments resulted in more acidic flours (pH = 5.41 – 5.60; $P \leq 0.05$) with comparable properties in terms of composition, size, pasting, and baking quality to the control treatment (0% SBS). Results from this study show that SBS tempering could be a viable food safety intervention step for controlling STEC contamination in wheat prior to milling.

4.1 Introduction

Wheat is a raw agricultural commodity that can harbor substantial microbial contamination from various sources (e.g., soil, water, insects, wildlife, harvesting, transport equipment etc.). The presence of pathogenic microorganisms such as STEC and *Salmonella* were also reported in published literature (Aydin et al., 2009). The standard wheat milling process redistributes the wheat microflora into the milling fractions. This presents a risk of pathogenic microorganisms contaminating the wheat flour produced. (Pascale et al., 2011).

Wheat flours were historically perceived as a microbiologically safe ingredient due to its low water activity (a_w) (Sabillon et al., 2019). However, pathogenic microorganisms such as STECs were reported to be able to survive dry environments through various mechanisms such as the accumulation of osmoprotectants and reverting to a dormant state (Finn et al., 2013). This survival capability of pathogens reinforces the risk of wheat flours being a potential vehicle for foodborne illness in humans.

Wheat flours are not intended to be consumed raw and the subsequent wheat-based food products often undergo validated thermal processes. Despite this, foodborne illness outbreaks still occur because of the risky behavior of consuming raw / undercooked doughs or baking mixes. The potential of wheat flours as a vehicle for foodborne illnesses was also demonstrated by the increased occurrence of recalls and outbreaks linked to *Salmonella* and STEC-contaminated wheat flours in recent years. The first outbreak related to *E. coli* O157:H7-contaminated wheat flours occurred in 2009 wherein a prepackaged cookie dough caused 77 illnesses (35 hospitalizations with 8 cases proceeding to hemolytic uremic syndrome) in the US (Neil et al., 2012). Another multi-state outbreak linked to STEC (*E. coli* O121 and O26) contaminated wheat flour occurred in 2016 wherein 63 cases across 24 US states were recorded (CDC, 2016). These outbreaks

typically lead to massive recalls of affected flour products presenting a significant economic loss for manufacturers.

These events prompted the need for improving the food safety of wheat flours through the addition of antimicrobial interventions in the milling process. Over the years, thermal and non-thermal processes (ozone, chlorination, and cold plasma) have been reported to be effective in improving wheat flour safety (Los et al., 2018; Dhillon et al., 2009). However, these processes involve further modification of the milling process while also having some issues with regards to worker safety (e.g., chemical and dust explosion hazards). These factors limit the practicality and cost-effectiveness of adapting such technologies into the wheat milling process. (Sabillon et al., 2019).

The inclusion of antimicrobial agents such as organic acids (e.g., lactic, acetic) and saline solutions in the tempering step were reported to be effective food safety interventions for wheat prior to milling (Sabillon et al., 2016). Sodium bisulfate (SBS) is a GRAS acid salt that is commonly used in foods as an acidifier and anti-browning agent. It was hypothesized in this study that the increased acidity brought by adding SBS in the tempering water could be a viable intervention step for controlling *E. coli* O121 and O26 contamination in wheat prior to milling. Therefore, the objectives of this study were to: a) evaluate the efficacy of SBS in reducing the *E. coli* O121 and O26 loads in wheat during tempering, and b) evaluate the impact of effective SBS treatments that resulted in ≥ 3 log reduction on wheat flour milling and baking quality.

4.2 Materials and Methods

Materials

The hard-red winter (HRW) wheat grain used for this study was obtained from Indigo Agriculture (Boston, MA). The microbiological quality of the wheat grains used was enumerated by plating in appropriate 3M petri films (Saint Paul, MN). The counts (log CFU/g) obtained for the samples used in this study are: aerobic counts – 4.6, coliforms – 4.1, *E. coli* – negative (after enrichment), yeast and molds – not detected (< 1 log CFU/g), and *Enterobacteriaceae* – 3.5. The granular sodium bisulfate (SBS) powder used was provided by Jones-Hamilton Co. (Walbridge, OH).

Inoculum Preparation

E. coli O121 (ATCC 2219) and O26 (ATCC 2196) cultures were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). Cultures were maintained in tryptic soy broth (TSB): glycerol (7:3) solution at -80^{o}C. Thawed cultures were streaked on tryptic soy agar (TSA) plates and incubated at 37^{o}C for 24 h. Incubated plates were refrigerated until use. A well-isolated colony of each *E. coli* strain from the streak plate was inoculated in TSB (10 ml) and incubated (37^{o}C, 24 h). The cells were harvested by centrifugation (2795 x g; 10 mins) and were inoculated in 800 ml TSB followed by another incubation for 24 h at 37^{o}C. Cells from the incubated TSB (800 ml) *E. coli* (O121 and O26) culture were harvested by centrifugation (2795 x g; 10 mins) and re-suspended in 10 ml of sterile 0.1% peptone water (for SBS tempering experiments) or sterile TSB (MIC determination). This resulted in an inoculum concentration of ~ 9 log CFU/ml.

Minimum Inhibitory Concentration (MIC) Assay

The minimum inhibitory concentration (MIC) of SBS was determined using broth microdilution in TSB based on the method describe by the Clinical Laboratory Standards Institute in TSB (CLSI, 2012). One hundred microliter (100 µl) of *E. coli* (O26 and O121) inoculum in TSB (~6 logs CFU/ml) were added to each well (three wells / concentration) containing 100 µl of decreasing concentrations (two-fold dilutions) of SBS solutions (10% - 0.16% w/v) yielding a final volume of 200 µl per well. Positive (*E. coli* inoculum only) and negative (TSB only) controls were also maintained. The microdilution plate was incubated at 37^{o}C for 24 h. The MIC was determined by as the lowest concentration that inhibited visible (visually checked) *E. coli* (O121 and O26) growth after incubation. MIC was determined individually for *E. coli* O121 and O26. The MIC determination study was independently replicated twice.

Wheat Preparation, Inoculation, and Tempering Process

For the first objective, wheat grains (200g) were placed in loosely capped metal containers and sterilized by autoclaving (121^{o}C, 15 mins) to reduce background microflora. Autoclaved wheats were cooled at ambient room temperature (24h) before the tempering experiments. The moisture content (% wet basis) of sterile wheats were determined based on ASAE 352.2 method (ASAE, 1988). Sterile wheat (200g) was tempered to 17% moisture with the amount of tempering solution required (16 ml) calculated based on the initial moisture content (10.4%). The tempering liquid was comprised of the prepared STEC inoculum and SBS tempering solutions applied in 1:1 ratio. SBS solutions were prepared by diluting a 50% (w/v) SBS stock solution to the required concentrations that yielded the target tempering concentrations of 0.5, 0.75, 1.0, 1.25, and 1.5% SBS (wheat basis) when applied. These final concentrations correspond to the following SBS solution concentrations (% w/v): 13.4, 20.3, 26.9, 33.7, and 40.5%. The SBS solutions were

prepared fresh prior to the tempering experiments. The required SBS solution concentrations (% w/v) required to achieve the SBS tempering concentrations (% wheat basis) were calculated using the equation below:

$$\%SBS\ \left(\%\frac{w}{v}\right) = \frac{[(\%SBS\ wheat\ basis)\ x\ (weight\ of\ tempered\ wheat)]}{volume\ of\ tempering\ solution}$$

Sterile wheat (200g) in metal containers were spray-inoculated with the *E. coli* O121 or O26 inoculum (~9 log CFU/ml, 8 ml) giving wheat inoculation levels of ~ 6 log CFU/g. Inoculated wheats were rested at ambient temperature for 30 minutes to allow cell attachment. The sprayer used was washed with 70% ethanol and rinsed with adequate amounts of sterile water before each inoculation procedure. Inoculated wheats were then treated using the prepared SBS solutions (8 ml) by spraying. Wetted kernels were mixed manually by shaking the metal containers vigorously for 5 minutes. Containers were then sealed, and wheat grains were allowed to temper for 24 h (22-25^{o}C). Positive (inoculated wheat + sterile water) and negative controls (sterile wheat + sterile water) were also maintained throughout the duration of the tempering experiments. Inoculation and tempering of the wheats were conducted in aseptic conditions within a Biosafety Cabinet.

Microbiological Analysis

For the first objective, treated wheat was aseptically sampled (25 g) at the following time intervals: 0.5, 2, 6, 12, 18, and 24 h upon application of the tempering treatments. 25 g of wheat were mixed with 225 ml buffered peptone water (BPW) in stomacher bags (VWR, Radnor, PA) and homogenized (2 mins) using a stomacher (Seward, Islandia, NY). Serial decimal dilutions in 0.1% peptone water were made followed by spread plating on TSA plates (single plating). Spread plates were incubated (37^{o}C, 24 h) and STEC (O121 or O26) colonies were counted and enumerated. This test resulted in a detection limit of 2.0 log CFU/g.

Flour Quality Evaluation

Tempering Procedure for Flour Quality Evaluation

Non-sterile wheat samples (2 kg per treatment, 3 replications) were tempered using SBS concentrations of 1.25 and 1.5% SBS (wheat basis). The SBS concentrations used were selected based on the observed *E. coli* O121 and O26 reductions from the tempering experiments (≥ 3 log CFU/g). Wheat samples tempered with water (0% SBS) only were maintained as control. The SBS tempering solutions (33.7 and 40.5% w/v SBS) were prepared by dissolving the required amounts of SBS (grams) in corresponding amounts of distilled water. The required amount of tempering solution (160 ml) for tempering wheat to 17% moisture was calculated based on the initial wheat moisture content (10.3%) and was comprised of water (80 ml) and SBS solutions (80 ml) applied in 1:1 ratio for the SBS tempering treatments. Tempering was done by mixing the wheat kernels (30 mins) using rotary tempering mixing bins. For the tempering treatments with SBS (1.25 and 1.5% SBS), water was poured first (80 ml) into the wheats followed by mixing (15 mins) before the application of the prepared SBS solutions (80 ml) which correspond to %SBS concentrations (wheat basis) of 1.25 (33.7% w/v) and 1.5% (40.5% w/v). Wetted kernels were mixed for another 15 mins inside the tempering bins after application of the SBS tempering solutions. For the control treatment, 160 ml of water (tempering solution) was poured in the wheat and mixed for 30 mins using rotary tempering bins. Treated/ sufficiently mixed wheats (30 mins mixing time) were placed in resealable bags and tempered statically (24 h, 22-25^{o}C, 17% moisture) prior to milling.

Experimental Milling

Tempered wheats (2 kg per treatment, 3 replications) was milled using a Chopin LabMill (Chopin, France). The mill had 6 pairs of rolls comprised of 2 break, 1 sizing, and 3 reduction rolls. The feed rate was set at 5 g/sec for the 1st break roll and 2.5 g/sec for the subsequent roller

milling steps. The lab scale mill was brushed and vacuum-cleaned before each mill run. The flour fractions obtained (break, sizing, and reduction flours) were blended to make the straight- grade flours for evaluation. The yields (%) of the mill fractions were expressed in % as-is basis and calculated as:

$$\%yield\ (as-is) = \frac{weight\ of\ milling\ fraction\ (grams)}{weight\ of\ wheat\ (grams)}\ x\ 100$$

Particle Size Analysis

The particle size distributions (PSD) of the wheat flours were established by vacuum sieving using a Hosokawa Alpine Jet Siever (Hosokawa, Augsberg, Germany). One hundred grams (100 g) of flour were sieved through increasing sieve sizes ranging from 20 to 250 µm based on the ASTM sieve standard sizes. The flour quantity that passes through the sieve was used for establishing the PSD of the wheat flours produced.

Proximate Analysis

Moisture content (%wet basis) of straight-grade flour and wheat was measured according to ASAE 352.2 (ASAE, 1988). For the wheat flours, ash (%) was measured according to AOAC 923.03. Nitrogen content was measured according to AACC method 46-30.01 (AACC, 2010) with protein content (%) determined as N x 5.7 for wheat or wheat flour. The fat and fiber contents (%) were measured based on AOAC 922.06 and AOAC 962.09 (AOAC, 2019) methods respectively. Carbohydrate content was determined based on calculation [100 – (%ash + %moisture + %fat + %protein)]. Measurements were expressed in as-is (%) basis.

Total Starch, Damaged Starch, and Falling Number

Total starch (%) content of the wheat flours obtained were measured according to AACC method 76-13.01 (AACC, 2010). Damaged starch (UCD) was measured according to AACC method 76-33.01 (AACC, 2010) using a SDMatic (Chopin, France). Falling number (sec) values

were measured according to AACC method 56-81.04 (AACC, 2010) using a Foss Alphatec^{TM} FN^{o} machine (Foss; Eden Prairie, MN).

pH analysis

The pH measurement was conducted using a calibrated pH meter (Mettler Toledo, Columbus, OH) with a combined electrode pH probe (LE438, Mettler Toledo, Columbus, OH). For the wheat, wheat flours, and non-flour milling fraction samples, pH was measured by making a 1:9 suspension of the analyte and distilled water. Wheat pH was taken at the start (0.5 h) and end (24 h) of tempering. The suspensions were mixed for 15 minutes using a magnetic stirrer and rested for 10 minutes. The supernatant was decanted, and its pH was measured. For the tempering solutions, an aliquot of the freshly prepared solution was taken for analysis.

Solvent Retention Capacity (SRC)

The SRC tests for the wheat flours were conducted based on AACC method 56-11.02 (AACC, 2010). The SRC values of the flour were tested against the following solvents: deionized water, 5% lactic acid, 50% sucrose, and 5% sodium bicarbonate.

Gluten Properties

Gluten properties of the wheat flours were measured according to AACC method 38-12.02 (AACC, 2010) using a Glutomatic apparatus (Perten Instruments, Springfield, IL). Gluten characteristics (%) such as wet gluten, dry gluten, gluten index, and water binding capacity were calculated.

Pasting Properties

The pasting properties of the wheat flours were measured using a Rapid Visco Analyzer (RVA) Model-4 (Newport Scientific; Warriewood, NSW). The test was conducted according to STD2 of AACC method 76-21.02 (AACC, 2010). The flour quantity was adjusted to 14% moisture

basis before testing. The STD2 RVA test includes the following parameters: Stage 1 – 50°C, 0 sec; Stage 2 – 960 rpm, 0 sec; Stage 3 – 160 rpm, 10 secs; Stage 4 - 50°C, 1 min; Stage 5 - 95°C, 8.5 min; Stage 6 - 95°C, 13.5 mins, Stage 7 - 50°C, 21 mins, and end of test – 23 mins. The time between readings was 4 seconds. Peak viscosity, peak time, breakdown, minimum viscosity, pasting temperature, and final viscosity values were measured.

Dough Rheology

Dough rheology tests were conducted using MixoLab (Chopin, France) according to AACC method 54-60.01 (AACC, 2010). The test was conducted according to the "Chopin +" protocol in 14% moisture basis. The water absorption (%) of the flour samples were optimized prior to testing by adjusting the water absorption until the C1 value was within 1.10 ± 0.05 Nm after 8 mins of mixing. Once this criterion was met, the test was allowed to proceed with the following parameters: 80 rpm mixing speed, initial equilibrium (30°C, 8 mins), heating to 90°C (15 mins, 4°C/min), holding (90°C,7 mins), cooling to 50°C (5 mins, -4°C/min) and holding (50°C, 5 mins). Mixolab values: C1, CS, C2, C3, C4, C5, absorption, and development time were taken.

Flour Color Analysis

Color analysis of the wheat flours obtained was done using a MiniScan EZ 4500 Colorimeter (HunterLab, Reston, VA). Flours were placed in a cell holder and analyzed for its L* (-black to +white), a* (-green to +red), and b* (-blue to +yellow) color values.

Bake test

Bake test was conducted according to the formulation in AACC method 10-10.03 consisting of (% flour basis): 100% flour, 5.3% yeast, 6% sugar, 1.5% salt, 3% shortening, and water (% based on mixolab water absoprtion). Doughs were made by mixing the ingredients in a 100g-pin mixer (National Manufacturing Co., Lincoln, NE) for 5 mins. Doughs were rounded and

fermented (26-27^{o}C, 90 %RH, 30 mins) before sheeting and molding. Molded doughs were then placed in pup loaf bake pans and proofed (26-27^{o}C, 90%RH, 60 mins). Baking (204^{o}C, 24 mins) was conducted in a reel type oven. Loaves were cooled prior to the bread analysis part. Duplicate loaves (2 independent applications of wheat flour per treatment) were made from the wheat flours of each tempering treatment (0, 1.25, and 1.5% SBS).

Bread Analysis

Loaves were cooled at ambient room temperatures. Bread volume was determined by rapeseed displacement (AACC, 2010). The rapeseed apparatus was calibrated twice with a wooden block (500cc) before analysis. Loaf weight was measured and used for calculating specific bread volume (cc/g). Loaves were sliced (~15mm thickness) using a slice regulator and an electric slicer (Black + Decker, Towson, MD) in preparation for c-cell and texture analysis. Slices were stored in polyethylene bags at ambient temperature (22-25^{o}C) before further bread analyses.

For c-cell analysis, two center slices (4 total slices per treatment) were analyzed using the C-cell image analyzer (CCFRA Tech Ltd., UK) with c-cell software 2.0. Images were taken with cell wall thickness, cell volume, and number measurements taken.

Texture profile analysis was conducted using a TA. XT texture analyzer (Stable MicroSystems, UK) with a 2 in. diameter cylindrical probe. Each bread slice (8 total slices per treatment) was analyzed with the following test parameters: 1.0 mm/s pre-test, 1.0 mm/s test, 5.0 mm/s posttest speed, 50% strain, and 20g trigger force. Hardness, cohesiveness, chewiness, springiness, and resilience values were obtained.

Data Analysis

The first objective of the study followed a 7x6 factorial design with seven treatments (positive, negative, 0.5, 0.75, 1.0, 1.25, and 1.5% SBS) and 6 sampling intervals for each *E. coli* serogroup. Mean *E. coli* (O121 or O26) log reductions were analyzed using the GLIMMIX procedure with mean comparisons done using the LSMEANS procedure ($P \leq 0.05$). Three independent experimental replications were conducted for the SBS tempering experiments.

The second objective followed a completely randomized design (CRD) with three treatments (0, 1.25, and 1.5% SBS). Flour quality tests were conducted in duplicates (2 replicate samples tested / tempering treatment) with values expressed as means (SD). Mean values were analyzed using the GLM procedure with mean comparisons done using Tukey's HSD ($P \leq 0.05$). All statistical analyses were conducted using SAS statistical software 9.3 (SAS Institute, Cary, NC).

4.3 Results and Discussion

E. coli O121 and O26 Load Reduction in Wheat during Tempering

Sodium bisulfate ($NaHSO_4$; pKa =1.99, MW= 120.0 g/mol) is a dry acid salt that readily dissolves in water and dissociates into sodium, hydrogen, and sulfate ions (Sun et al, 2008). The MIC of SBS against both *E. coli* O121 and O26 was found to be 0.32% (%w/v, pH=2.37) in TSB. This shows the inhibitory effects of SBS against STECs (O121 and O26) which could be related to the acidifying properties of SBS which lowers the pH of the growth media. The *E. coli* O121 and O26 serotypes were used for this study as they were the most frequent cause of recalls involving STEC-contaminated wheat flours (FDA, 2020).

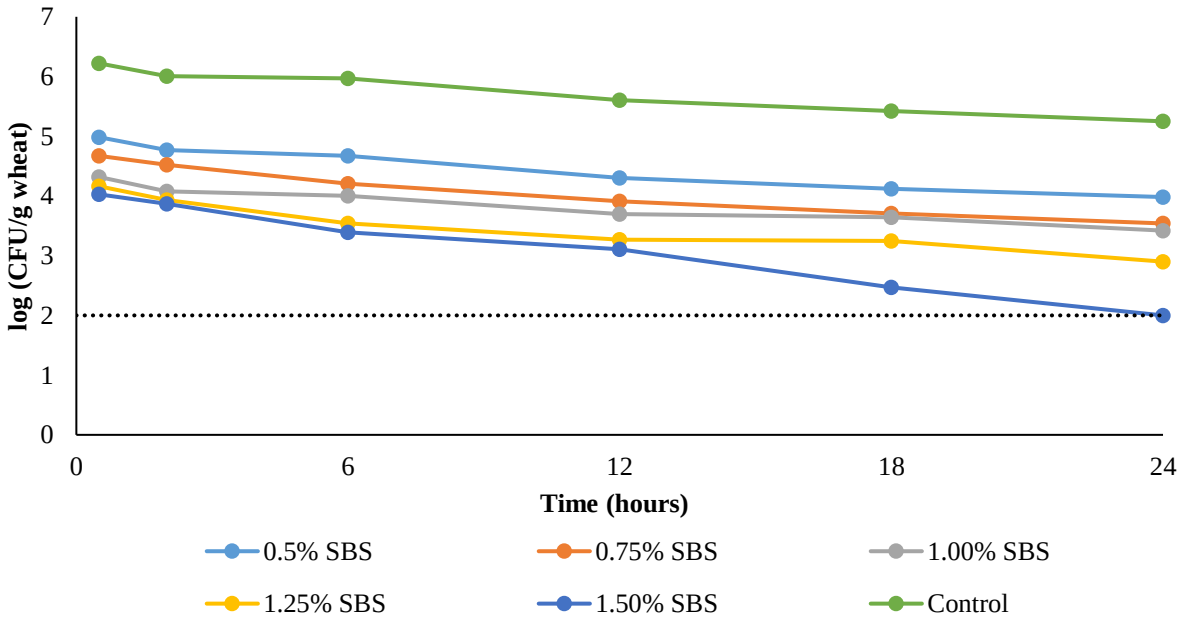

Figure 4.1 *E. coli* O121 load reduction in wheat during tempering (24 h) using different concentrations of SBS; control corresponds to 0% SBS. Dashed line corresponds to the detection limit (2.0 log CFU/g) for the study (average of three experimental replications)

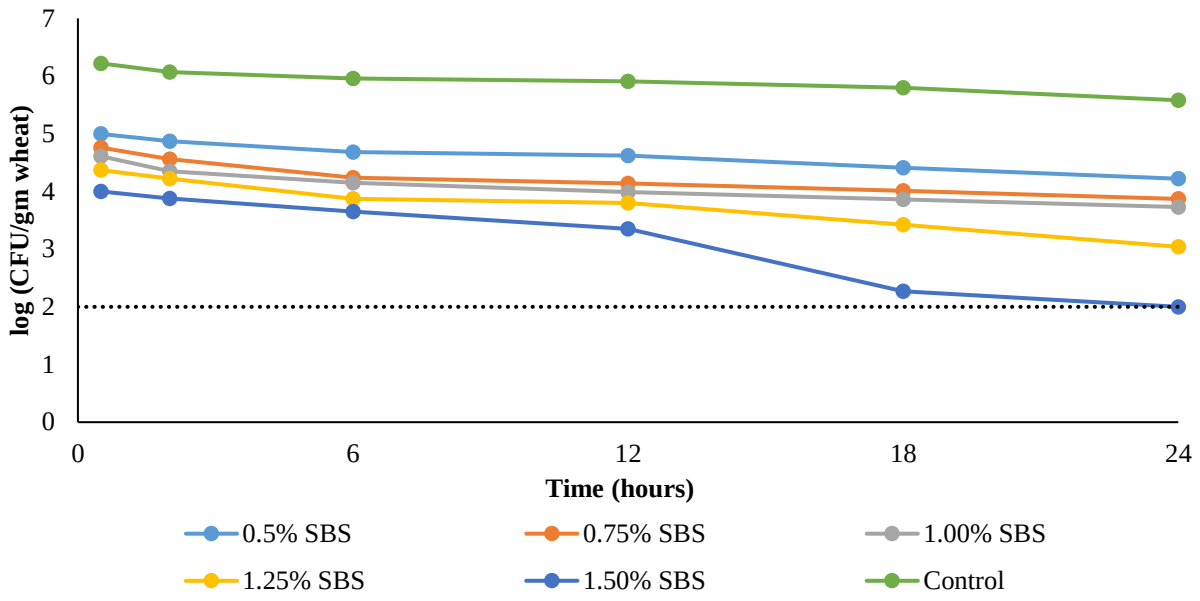

Figure 4.2 *E. coli* O26 load reduction in wheat during tempering using different concentrations of SBS; control corresponds to 0% SBS. Dashed line corresponds to the detection limit (2.0 log CFU/g) for the study (average of three experimental replications)

Figures 4.1 and 4.2 show gradual reductions in the *E. coli* O121 and O26 loads of the wheat samples during the tempering step. At the end of the tempering phase (24 h), small but significant reductions ($P \leq 0.05$) were observed for the *E. coli* O121 (0.84 log CFU/g) and O26 (0.64 log CFU/g) loads of the wheat samples tempered with the control treatment (0% SBS). This indicates that the moisture levels used during tempering are not sufficient to promote the growth/survival of *E. coli* cells in wheat. The tempering moisture used (17%) usually corresponds to water activity (a_w) levels around 0.70 (Carter, 2015). This a_w level is known to inhibit multiplication of for bacterial cells such as *E. coli* which requires a_w levels of ≥ 0.95 for growth. For the negative control maintained during the tempering experiment, no colonies were recovered after plating throughout the sampling intervals.

The addition of SBS in the tempering water used was shown to be effective based on Figures 4.1 and 4.2. The lowest SBS concentration used (0.5%) gave a 1-log reduction for both

STEC serotypes 0.5 h after application of the tempering treatment. Greater reductions in STEC load were observed with increasing concentrations of SBS with a maximum of 2-log reductions observed with the use of 1.25 and 1.5% SBS at 0.5 h of tempering. By the end of the tempering step (24h), the 1.5% SBS concentration was able to reduce *E. coli* O121 and O26 loads of the wheat samples below detection limits (< 2.0 log CFU/g) ($P \leq 0.05$). *E. coli* load reductions (log CFU/g) observed after tempering were 2.2 (0.5%), 2.5 (0.75%), 2.7 (1.0%), and 3.5 (1.25%) for *E. coli* O121 ($P \leq 0.05$). As for *E. coli* O26, load reductions (log CFU/g) observed were 2.0 (0.5%), 2.4 (0.75%), 2.5 (1.0%), and 3.2 (1.25%) at the end of tempering ($P \leq 0.05$). The reductions were calculated based on the pre-treatment inoculation levels of the control sample at 0.5 h tempering. An interaction between the tempering time and SBS concentrations was also observed ($P \leq 0.05$) wherein longer tempering times were needed to achieve maximum *E. coli* (O121 and O26) load reduction in wheat due to SBS tempering prior to milling.

Table 4.1 Mean (SD) pH values measured during the tempering experiments

Treatment (% SBS)	**pH (mean ± SD)**		
	pH^1	pH^2	pH^3
0	6.93 (0.15)[a]	6.87 (0.06)[a]	6.96 (0.12)[a]
0.5	1.28 (0.04)[b]	3.64 (0.04)[b]	3.62 (0.04)[b]
0.75	1.18 (0.02)[c]	3.46 (0.08)[cd]	3.46 (0.06)[c]
1.0	1.10 (0.01)[d]	3.29 (0.02)[de]	3.25 (0.02)[d]
1.25	1.02 (0.02)[d]	3.20 (0.01)[e]	3.20 (0.01)[d]
1.5	0.85 (0.01)[e]	3.11 (0.01)[e]	3.13 (0.02)[d]

[1]mean values for each column with different superscripts are significantly different ($P \leq 0.05$) due to treatment
[2] pH^1 of tempering solution, pH^2 of wheat at the start of tempering (30 mins), and pH^3 of wheat at the end of tempering (24h)

Sodium bisulfate is commonly used as an acidifier for various applications such as farm litter management and food applications (Pope and Cherry, 2000). As a food ingredient, SBS is

recognized as a GRAS (Generally Recognized as Safe) substance. This classification means that it can be used by manufacturers without the need for a pre-market review verifying its safety. Furthermore, the use of GRAS substances is permitted as long as it is used in accordance with the manufacturers' Good Manufacturing Practices (GMPs). The MIC (0.32% w/v) observed in this study was also comparable with several common organic acids such as lactic (0.5%), and acetic (0.5%) for *E. coli* (Wali et al., 2019; Wang et al., 2015). The lower MIC of SBS against STEC (O121 and O26) indicate that lower amounts of SBS could be used to inhibit STECs (O121 and O26) which could represent an economic benefit to manufacturers. Furthermore, the acidification property of SBS was reported to be effective in controlling the enteric microbial load of animal manure with reported 2 to 5 log reductions upon application (McGarvey et al., 2011). Table 4.1 shows that adding SBS significantly lowered ($P \leq 0.05$) the tempering water and kernel pH of the wheats. These lowered pH values contributed to the reduction of the *E. coli* O121 and O26 load of wheat during tempering. Higher pH values were observed for the treated wheat compared to the prepared SBS solutions showing that wheat kernels have some buffering capabilities giving them the capability to resist acidification (Nayeri et al., 2013). In addition, the decrease in pH of the wheat kernels become smaller as the SBS tempering concentration used increases. Despite this observation, higher STEC load reductions in wheat were still observed after tempering. The reductions could be explained by the increased acidity and osmolarity caused by the higher concentration of SBS added in the tempering solutions. The antimicrobial activity of acids is usually linked to the movement of acidic ions across the cell membrane which lowers the pH of internal cell. This lowered pH then disrupts necessary cellular activities for survival which eventually results into cell death (Brul and Coote, 1999; Russel, 1992). Another explanation is that the cell acidification could be resisted by certain pathogens such as STECs through various acid

resistance mechanisms, however these mechanisms consume additional cell ATPs which eventually also leads to cell death (Booth, 1985). As SBS is considered as an acid salt, the ion components of SBS changes the osmolarity of the cell environment (wheat grain) which could lead to cell dehydration. Bacterial cells expel water from their cells in order to balance out the solute concentrations between the cell and the environment (Davidson, 2001).

The maximum reductions observed upon addition of SBS were comparable to previous non-thermal intervention steps applied for wheat products such as lactic acid tempering (1.6 log- non-O157 STECs, 1.8 logs - O157 STECs), ozone (3.2 logs- aerobic counts), and gamma irradiation (2 logs- aerobic counts) (Sabillon, 2018; Weaver et al., 2011; and Dhillon et al., 2009). STEC load reductions due to SBS tempering were also comparable to the reductions from the thermal treatment of wheat flour (70^{o}C, 60 minutes) which yielded 4.1 log reductions for *E. coli* O26 (Forghani et al, 2018). Furthermore, the observations from this experiment were consistent with previous studies demonstrating the antimicrobial activity of SBS. The application of SBS was found to cause a 5 log-reduction in the *E. coli* load of chicken litter although the effect was found to be temporary due to recontamination with fresh litter (Hunolt et al., 2015). The reductions were also comparable to other SBS challenge studies reported such as 1.7 log reduction in the *Salmonella* load of chicken drumsticks dipped in 3% SBS solution (Dittoe et al., 2019).

Overall, SBS was found to be effective against *E. coli* O121 and O26. The microbial reductions of $\geq$ 2 logs after tempering observed with the addition of SBS to the tempering water could be considered effective as normal levels of *E. coli* in wheat were reported to be only around 1.0 log CFU/g (Sperber, 2007). The improved contamination levels of wheat due to SBS water treatment during tempering could also limit the *E. coli* cross-contamination of mill fractions and equipment that could occur during the milling process.

Flour Quality Evaluation

The higher SBS concentrations (1.25 and 1.5% SBS) were selected for the flour quality evaluation part as they resulted in an *E. coli* (O121 and O26) load reduction of ≥ 3 log units after tempering. A log reduction of ≥ 3 logs is usually accepted by most flour industry consumers if a microbial reduction step is applied in wheat milling (Sterk, 2017). No mandatory performance standards are required as of not for pathogen reduction steps applied in the wheat milling process. Table 3.2 summarizes the milling yields (% as-is) of the wheats tempered with different SBS concentrations (0, 1.25, and 1.5% SBS).

Table 4.2 Mean (SD) milling yields (% as-is) of tempered wheats

Milling Fraction	**Treatments (% SBS)**		
	0	1.25	1.5
Wheat Moisture (%)[1]	16.10 (0.03)[b]	16.21 (0.06)[b]	16.39 (0.04)[a]
Bran	21.16 (0.57)[a]	20.06 (0.30)[b]	18.92 (0.21)[c]
Shorts	5.17 (0.147)[c]	5.91 (0.09)[b]	6.36 (0.20)[a]
BK Flour	27.86 (0.21)[a]	26.50 (0.11)[b]	26.35 (0.30)[b]
SZ Flour	14.01 (0.15)[b]	16.51 (0.19)[a]	17.18 (0.11)[a]
RD Flour	31.80 (0.67)[a]	31.01 (0.50)[a]	31.18 (0.27)[a]
SG Flour (BK + SZ + RD)	73.66 (0.62)[a]	74.02 (0.50)[a]	74.72 (0.44)[a]

[1] mean values in each column with different superscripts are significantly different due to treatment (P ≤ 0.05)
[2] wheat moisture after the tempering step (24 h)
[3] BK – break, SZ – sizing, RD- reduction, and SG -straight- grade

Based on table 4.2, moisture levels (16.10 – 16.39%) of the tempered wheats were within the acceptable levels commonly used in commercial hard wheat milling which are between 15 to 17% (wet basis) (Posner and Hibbs, 2005). Bran yields decreased while the shorts fraction yields increased with higher SBS concentrations used (P ≤ 0.05). This observation suggests that adding SBS in the tempering water could have increased bran friability which increased the amounts of

fine bran produced based on the shorts yield. These finer bran particles could then be transferred into the flour fractions during milling. No significant differences ($P > 0.05$) were observed for the straight-grade flour yields (73.66 – 74.72%) of the tempered wheats. For the individual flour yields increasing SBS tempering concentrations resulted in an increased SZ flour yield and decreased BK flour yield ($P \leq 0.05$). No significant differences were observed for the RD flour yield. Quantifying individual flour fraction yields (BK, SZ, and RD) is important as each fraction possesses different characteristics such as particle size, protein, and damaged starch content which could influence the quality of the straight-grade flours depending on their proportions after blending. The lower extraction rate for the break flour could be an indicator of increased wheat hardness as harder wheat classes tend to have lower extraction rates from the break (1st and 2nd BK) milling step with most of the flour extracted in the latter milling steps (e.g., sizing and reduction) (Dubat and Bock, 2019).

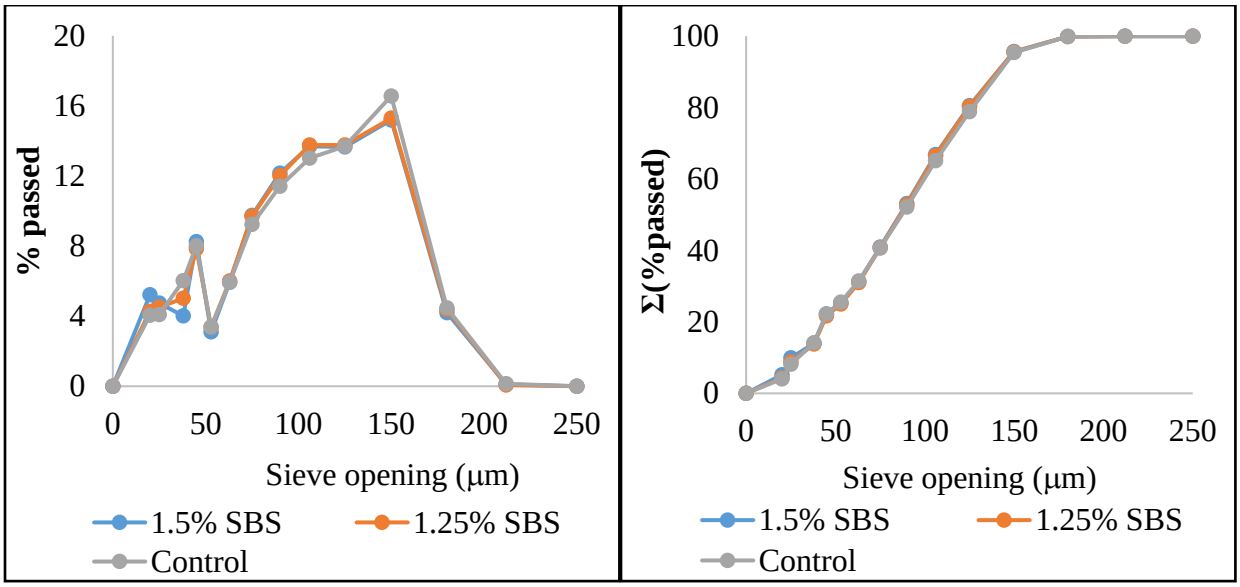

Figure 4.3 Particle size distribution (left) and cumulative particle size distribution (right) of wheat flours obtained; control corresponds to 0% SBS treatment

Based on Figure 4.3, the wheat flours obtained from the different tempering treatments (0, 1.25, and 1.5% SBS) had relatively similar particle size distributions. A majority of the particles had sizes ranging from 50 to 150 μm satisfying the regulatory requirement for wheat flours wherein at least 98% of particles should pass through a 212 μm sieve (Codex Alimentarius, 2019). Particle size is an important characteristic as it affects the hydration and mixing properties of wheat flours.

Table 4.3 Mean (SD) pH values for the wheat milling process

Test Variable	Treatment (% SBS)		
	0	1.25	1.5
Tempering solution	6.25 (0.02)[a]	1.52 (0.12)[b]	1.05 (0.05)[c]
Wheat[1]	6.65 (0.04)[a]	3.12 (0.02)[b]	3.00 (0.02)[c]
Wheat[2]	6.59 (0.18)[a]	3.11 (0.02)[b]	3.00 (0.03)[b]
Bran	7.36 (0.04)[a]	4.05 (0.16)[b]	3.87 (0.06)[b]
Fine Bran	7.40 (0.01)[a]	4.28 (0.03)[b]	4.08 (0.06)[b]
Shorts	7.31 (0.17)[a]	4.77 (0.06)[b]	4.61 (0.08)[c]
BK Flour	6.74 (0.03)[a]	5.44 (0.01)[b]	5.23 (0.03)[c]
SZ Flour	6.51 (0.01)[a]	5.75 (0.02)[b]	5.56 (0.01)[c]
RD Flour	6.59 (0.03)[a]	5.56 (0.04)[b]	5.32 (0.01)[c]
SG Flour	6.67 (0.05)[a]	5.60 (0.04)[b]	5.41 (0.01)[c]

[1] mean values in each row (test variable) with different superscripts are significantly different ($P \leq 0.05$) due to treatment
[2] wheat[1] pH measured 30 mins after application of tempering solutions, wheat[2] pH measured after tempering (24h)
[3] BK – break, SZ – sizing, RD- reduction, and SG -straight- grade

Tempering with SBS resulted in more acidic milling fractions (Table 4.3). Milling at 1.5% SBS tempering concentration yielded the most acidic milling fractions ($P \leq 0.05$). pH values for the flour fractions (BK, SZ, RD, and SG flours) obtained from both SBS concentrations ranged from 5.23 to 5.75. These values were significantly lower ($P \leq 0.05$) than the usual pH of untreated wheat flours which ranges from 6.0 to 6.7 (Egan et al., 1981). This observation indicates that part of the SBS-acidified water added to the kernels diffused from the bran layer into the inner

endosperm layer during tempering. The bran and shorts fractions on the other hand had lower pH values compared to the flour fractions indicating that more SBS molecules were retained on the surface of the wheat grain relative to the endosperm.

Table 4.4 Mean (SD) physico-chemical characteristics of wheat flours

Test Variable	Treatment (% SBS)		
	0	1.25	1.50
Moisture (%wet basis)	15.57 (0.13)[a]	15.02 (0.09)[b]	14.99 (0.05)[c]
Ash (%)	0.44 (0.03)[c]	0.62 (0.01)[b]	0.71 (0.01)[a]
Protein (%)	10.81 (0.03)[c]	10.94 (0.03)[ab]	10.92 (0.09)[bc]
Fat (%)	0.54 (0.02)[b]	0.48 (0.01)[b]	0.65 (0.02)[a]
Fiber (%)	0.18 (0.04)[c]	0.25 (0.01)[bc]	0.37 (0.04)[ab]
Carbohydrates (%)	72.59 (0.07)[c]	72.88 (0.05)[ab]	72.68 (0.04)[bc]
Total Starch (%)	66.28 (1.42)[c]	71.27 (0.38)[ab]	68.74 (0.95)[bc]
Damaged Starch (UCD)	14.63 (0.51)[b]	14.73 (0.70)[b]	16.57 (0.45)[a]
Falling Number (sec)	587.3 (15.6)[a]	522.0 (12.7)[b]	517.3 (30.4)[b]
L* (0- black to 100-white)	89.21 (0.13)[a]	89.76 (0.42)[a]	88.45 (0.43)[b]
a* (-green to +red)	0.58 (0.04)[b]	0.92 (0.02)[a]	0.93 (0.02)[a]
b* (-blue to +yellow)	9.70 (0.17)[b]	10.17 (0.05)[a]	10.25 (0.06)[a]

[1] mean values in each row (test variable) with different superscripts are significantly different ($P \leq 0.05$) due to treatment
[2] values are expressed in as-is (%) basis

The proximate compositions of the wheat flours obtained from both SBS concentrations (1.25 and 1.5%) were comparable with the control treatment based on Table 4.4. The moisture values for all the flours obtained were higher than the common moisture content (14%) for wheat flours. This could be explained by the reduced workflow as fewer rolls are present in the lab scale mill used resulting in less heat generation. Moisture loss during milling is driven by the heat produced from the successive roller milling steps. This moisture values however do not have

significant impacts on the wheat properties evaluated in this study. Higher ($P \leq 0.05$) ash and fiber contents were observed for wheat flours obtained from SBS- tempered wheats (1.25 and 1.5% SBS). This could be due to the increased amount of fine bran particles produced that could have contaminated the wheat flours during milling. Furthermore, ash content is also related to the amount of minerals present in the flour. Hence the increased ash content could also be linked to the SBS salts that diffused into the wheat endosperm during tempering. Significantly higher flour protein and carbohydrate contents were observed with increasing SBS (1.25 and 1.5%) tempering concentrations although the changes were relatively small (< 0.3%). As for the starch properties, tempering with SBS (1.25 and 1.5%) generally resulted into higher total and damaged starch contents. These changes in the starch quantities could have implications on the pasting and dough properties of the wheat flours. The FN values of all wheat flour samples were above 250 secs indicating sound grains with minimal amylase activity were used. In addition, FN values decreased with increasing SBS concentrations. This could be linked to the increased hydrolysis of starch molecules into smaller glucose units due to acid and heat stresses (Hirashima et al., 2005). The wheat flours produced from SBS tempered wheats had lower L* and higher a* and b* values indicating lower brightness, higher red, and higher yellow tones. The color changes could be attributed to the higher ash and fiber contents of the 1.25 and 1.5% SBS wheat flours. These indicate the presence of bran particles in the flour which could have influenced the flour color to have a higher reddish and darker color values.

Table 4.5 Mean (SD) values of gluten properties (%) and solvent retention capacity (%) of wheat flours

Test Variable	Treatment (% SBS)		
	0	1.25	1.50
Gluten properties (%)			
Wet gluten	26.17 (0.43)[a]	26.38 (0.29)[a]	24.66 (0.28)[b]
Dry gluten	9.12 (0.14)[a]	9.75 (0.50)[a]	9.10 (0.42)[a]
Gluten Index	99.42 (0.28)[a]	99.04 (0.26)[a]	99.80 (0.28)[a]
Water-binding capacity	17.17 (0.43)[ab]	16.64 (0.21)[b]	15.56 (0.14)[c]
Solvent retention capacity (%)			
Water	54.60 (0.86)[b]	57.01 (0.61)[a]	58.53 (0.34)[a]
50% sucrose	73.40 (0.67)[b]	75.68 (0.39)[a]	75.49 (0.86)[a]
5% lactic acid	106.22 (0.35)[a]	84.52 (0.40)[b]	80.29 (0.41)[b]
5% sodium bicarbonate	62.95 (0.45)[c]	66.22 (0.59)[b]	67.71 (0.23)[a]

[1] mean values in each row (test variable) with different superscripts are significantly different ($P \leq 0.05$) due to treatment
[2] values expressed in 14% moisture basis

No significant differences ($P > 0.05$) were observed with the gluten index and dry gluten values of the wheat flours (Table 4.5). These indicate that tempering with SBS did not affect gluten strength (gluten index) and quantity (dry gluten) of the wheat flours. Decreasing wet gluten and water-binding capacity values were observed with increasing SBS tempering concentrations. The differences could be explained by the lower pH of the wheat flours which could have affected the gluten formation and structure lowering the flour water-binding capacity (Garg et al., 2019). Solvent retention capacity (SRC) values are used for assessing the baking performance of flours (Ram et al., 2005). The use of lactic acid, sodium carbonate and sucrose solvents provide insights on the glutenin, damaged starch, and gliadin characteristics respectively. Water being a universal solvent shows the overall quality of the wheat flour. Lower lactic acid SRC values were observed for SBS wheat flours relative to the control indicating lowered gluten strength. Significant

differences ($P \leq 0.05$) were also observed for the water, sucrose, and sodium bicarbonate SRC values wherein SBS tempered wheat flours had higher SRC values (1.25 and 1.5% SBS) than the control (0% SBS).

Table 4.6 Mean (SD) rapid-visco analyzer characteristics of wheat flours

Test Variable	Treatment (% SBS)		
	0	1.25	1.50
Peak viscosity (RVU)	3936.3 (83.7)[b]	4304.0 (59.5)[a]	4288.7 (34.2)[a]
Trough (RVU)	2005.3 (25.1)[b]	2068.0 (34.9)[ab]	1966.0 (7.5)[c]
Breakdown (RVU)	1931.0 (86.5)[b]	2236.0 (27.5)[a]	2322.7 (27.8)[a]
Final viscosity (RVU)	4902.0 (85.3)[a]	4735.7 (62.6)[b]	4561.7 (24.0)[c]
Setback (RVU)	2896.7 (82.3)[a]	2667.3 (30.5)[b]	2595.7 (20.8)[b]
Peak time (mins)	8.98 (0.10)[a]	8.94 (0.03)[ab]	8.87 (0.04)[b]
Pasting temperature (^oC)	65.3 (0.20)[a]	64.8 (0.80)[a]	64.2 (1.30)[a]

[1] mean values in each row (test variable) that have different superscripts are significantly different ($P \leq 0.05$) due to treatment

[2] breakdown (RVU) is calculated as the difference between peak viscosity and trough; setback (RVU) is calculated as the difference between final viscosity and trough

Significantly higher peak viscosities and breakdown values ($P \leq 0.05$) were observed for flours from SBS-tempered wheats. The increased peak viscosity could be due to the increased salt concentration in the flour due to the added SBS. Zhou and Hou (2012) reported an increase in peak viscosity (RVA profile) of wheat flour suspensions with the addition of phosphate salts. The higher breakdown values could be explained by the increased rate of hydrolysis of the leeched amylopectin and amylose components due to heat and acid (lower flour pH) stresses that occur during testing (Hirashima et al., 2004). The lower trough values observed also indicate lower hot gel strength for flours from SBS-tempered wheats. Lower final viscosity, and setback values were observed for wheat flours from SBS-tempered wheat (1.25 and 1.5% SBS) showing lower retrogradation tendencies. This could be explained by the acidic pH and increased salt

concentration brought by SBS tempering into the flour as these are recognized methods for limiting starch retrogradation (Wang et al., 2015). Pasting temperatures for the wheat flours produced were not significantly different ($P > 0.05$) although slightly lower peak times were observed with increasing SBS tempering concentrations.

Table 4.7 Mean (SD) mixolab characteristics of wheat flours

Test Variable	Treatment (% SBS)		
	0	1.25	1.50
Water absorption (%)	54.5 (0.24)[a]	54.4 (0.29)[a]	54.7 (0.13)[a]
Development time (min)	2.21 (0.26)[a]	1.90 (0.24)[a]	2.00 (0.19)[a]
Stability (min)	8.90 (0.20)[a]	9.30 (0.21)[a]	8.98 (0.22)[a]
Amplitude (Nm)	0.06 (0.01)[a]	0.07 (0.01)[a]	0.08 (0.01)[a]
C1 (Nm)	1.10 (0.03)	1.10 (0.04)	1.10 (0.03)
CS (Nm)	1.05 (0.02)[a]	1.02 (0.02)[a]	1.04 (0.02)[a]
C2 (Nm)	0.47 (0.01)[a]	0.46 (0.01)[a]	0.46 (0.01)[a]
C3 (Nm)	2.17 (0.02)[a]	2.16 (0.02)[a]	2.11 (0.03)[b]
C4 (Nm)	2.03 (0.03)[a]	2.01 (0.02)[a]	1.94 (0.04)[b]
C5 (Nm)	3.28 (0.07)[a]	3.12 (0.04)[b]	3.07 (0.02)[b]

[1] mean values in each row (test variable) that have different superscripts are significantly different ($P \leq 0.05$) due to treatment
2 Mixolab tests were conducted using "Chopin +" protocol at 14% moisture basis

Based on Table 4.7, no significant differences ($P > 0.05$) were observed for the water absorption, development time (time to C1), mixing stability, and amplitude (dough elasticity) of SBS-wheat flours relative to the control. These observations indicate the wheat flours produced from SBS tempered wheats had similar mixing properties relative to the control treatment (0% SBS). C2 values for the wheat flours were also not significantly different ($P > 0.05$) indicating similar protein qualities of the wheat flours produced from the tempering treatments used. The C3 and C4 values for the 1.5% SBS were lower compared to the control (0%) indicating less dough

viscosity and gel stability during heating which was also reflected in its RVA characteristics (Table 4.6). C5 values were lower for the SBS-tempered wheats (1.25 and 1.5% SBS) which is an indicator of decreased starch retrogradation during the cooling phase. The mixolab behavior of the flours during the heating phase are relatively similar to their RVA profile. With that, the differences in mixolab characteristics (C3, C4, and C5) could also be explained by the difference in salt and pH levels of the SBS wheat flours.

Table 4.8 Mean (SD) baking and bread characteristics of wheat flours

Test Variable	**Treatment (% SBS)**		
	0	1.25	1.50
Bread Characteristics			
Bread volume (cc)	545.0 (7.1)[b]	562.5 (10.6)[a]	542.5 (3.5)[b]
Specific bread volume (cc/g)	3.90 (0.82)[a]	3.90 (0.08)[a]	3.70 (0.04)[a]
C cell analysis			
Number of cells (n)	2354.0 (167.8)[b]	2990.5 (134.6)[a]	2930.3 (132.2)[a]
Cell diameter (mm)	2.48 (0.20)[a]	2.00 (0.09)[b]	1.77 (0.06)[b]
Wall thickness (mm)	0.48 (0.02)[a]	0.44 (0.01)[b]	0.43 (0.01)[b]
Cell volume (cc)	8.72 (0.79)[a]	6.58 (0.43)[b]	5.75 (0.25)[b]
Texture Properties			
Hardness (N)	5.55 (0.60)[c]	7.41 (0.74)[b]	8.90 (1.54)[a]
Resilience (%)	23.56 (1.96)[b]	29.29 (2.02)[a]	27.98 (1.83)[a]
Cohesion	0.51 (0.02)[b]	0.62 (0.06)[a]	0.59 (0.02)[a]
Springiness (%)	102.70 (5.22)[a]	111.76 (14.49)[a]	120.98 (24.26)[a]
Chewiness (N)	2.92 (0.41)[c]	5.11 (0.94)[b]	6.54 (1.21)[a]

[1] mean values in each row (test variable) row that have different superscripts are significantly different ($P \leq 0.05$) due to treatment

The bread making characteristics of the wheat flours obtained from different tempering treatments are shown in table 4.8. No significant differences were observed ($P > 0.05$) for the bread

and specific volume of SBS wheat flour (1.5%) compared to the control (0% SBS). Bread making qualities such as bread volume are mostly dependent on the protein attributes (gluten quality and quantity) of the wheat flour (Collado – Fernandez, 2003). The observations from Table 4.8 agree with the observations made for the flour gluten qualities (Table 4.5).

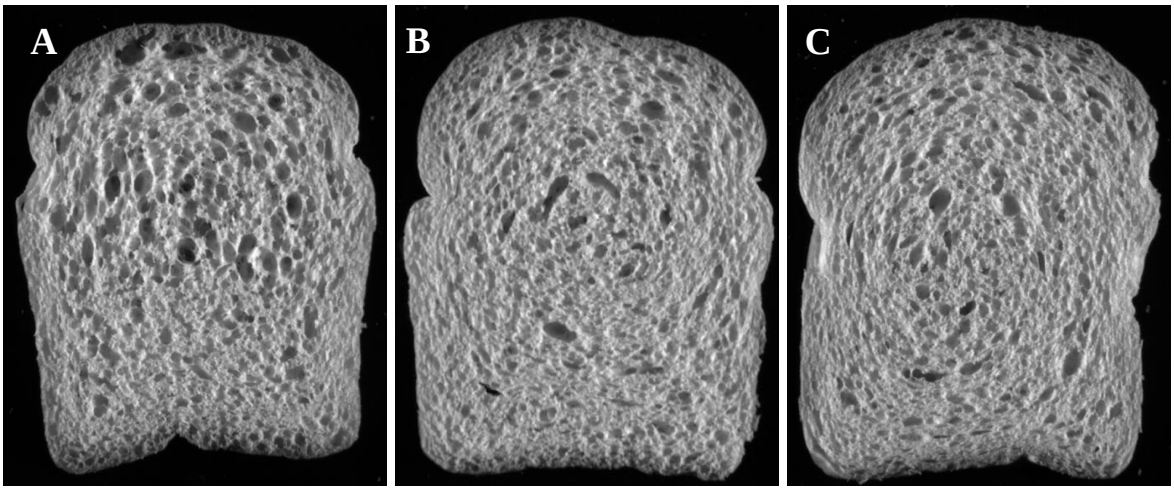

Figure 4.4 C-cell images of bread slices; control (A), 1.25% SBS (B), and 1.50% SBS (C)

The crumb structure of the bread slices made were also summarized in table 4.8. Crumb structures of breads form when the water and alcohols evaporate from the dough during baking which forms the cells. The gluten - starch structure then sets as water is removed from the dough which gives the bread its structure. Wheat flours from SBS tempered wheats (1.25 and 1.5% SBS) yielded breads with higher ($P \leq 0.05$) cell counts and finer crumb structure (lower cell diameter and volume) compared to the control. Breads made from SBS tempered wheat also had significantly lower ($P \leq 0.05$) wall thickness compared to the control tempering treatment (0% SBS). The significance of the c-cell properties of breads (e.g., loaf bread) is usually based on the type of bread that is required. In this study, the fine structure of the breads made from wheat tempered with SBS and water indicate that they would be suitable for sandwich breads wherein a fine structure, smaller cells and narrow cell wall characteristics are preferred.

The texture properties of the breads made were also given in Table 4.8. Hardness is an important quality for bread crumb structure as it describes the bread's resistance to deformation. Based on Table 4.8, hardness increased with increasing amounts of SBS used for the tempering with the 1.5% SBS giving the highest bread hardness value among the treatments. Furthermore, the resilience (ability of bread to spring back to original shape), springiness (elastic recovery of bread after removal of compression), chewiness (rubbery texture of bread during chewing), and cohesion (resistance to deformation of breads) also increased with higher SBS concentrations used for tempering. Overall, the SBS wheat flours resulted in breads with finer crumb structure and higher texture profile values (e.g., hardness and chewiness). This could be explained by the increased salt concentration (in addition to table salt added in the formula) and acidity of the bread due to the SBS wheat flours used in the bread formulation. Salts are known to help tighten and stabilize the gluten structure of breads allowing production of more bread structure (Nahar et al., 2019). Higher acidity on the other hand is also beneficial to breads as it improves the structure and solubility of gluten in the dough (Garg et al., 2019).

4.4 Conclusion

The addition of SBS during tempering lowered the pH value of wheat grains during tempering. This lowered pH resulted in significant reductions in the *E. coli* O121 and O26 load of wheat during tempering. These reductions ranged from 2.0 logs up to ≥ 4.0 logs CFU/g (1.5% SBS) after 24 hours of tempering. In addition, longer tempering times were needed (24 h) in order to achieve maximum reductions in the *E. coli* load of wheat. The SBS tempering concentrations of 1.25 and 1.5% SBS were used for the flour quality evaluation study as they yielded > 3 log CFU/g reductions in the *E. coli* (O121 and O26) load of wheat. For its effects on wheat flour quality, SBS tempering yielded more acidic wheat flours (lower pH) which had implications on the flour milling and baking quality. SBS tempering lead to higher ash and fiber contents which resulted in slightly darker wheat flour color. Higher starch (total and damaged) content, lower pasting properties (breakdown and trough), better retrogradation properties (C5, setback, and final viscosity), and decrease in water-binding capacity of wheat gluten. The bread making quality of the SBS-wheat flours (1.25 and 1.5% SBS) were comparable to the control (0% SBS) as it yielded comparable volume, crumb structure, and textural properties. These observations indicate that the changes seen in the physico-chemical properties and functionality of SBS-tempered wheat flours were not enough to negatively impact its bread making qualities. Future work can be conducted on the sensory evaluation of the breads made from SBS-tempered wheat flours to assess its effects on product characteristics and acceptability. Scaling up the process to bigger quantities of wheat tempered could also be done to better evaluate SBS effectiveness. Researches on testing the feasibility of SBS tempering on other types of wheat flour such as cookie and cake flours could also be done as the acidity brought by SBS could have more significant impacts on cookie and

cake flour quality. This is because cookie and cake flours (soft wheat flour) are usually chlorinated after milling. Bread flours (hard wheat flours) on the other hand often include acidulants such as citric and ascorbic acid which function as dough improvers during breadmaking. Overall, SBS wheat tempering could be a viable intervention step for wheat milling as it reduced STEC (O121 and O26) load in wheat and produced wheat flours with comparable properties to conventional wheat flours produced from wheats tempered with water only.

4.5 References

AACC International. (2010), *Approved methods of analysis (11th ed.)*, Method 46-30.01, Crude Protein-Combustion method; Method 76-13.01, Total Starch Assay Procedure; Method 76-33.01, Damaged starch – Amperometric Method; Method 56-81.04, Determination of Falling Number; Method 56-11.02, Solvent Retention Capacity Profile; Method 38-12.02, Wet Gluten, Dry Gluten, Water-Binding Capacity, and Gluten Index; Method 76-21.02, General Pasting Method for Wheat or Rye Flour or Starch Using the Rapid Visco Analyzer; Method 54-60.01, Determination of Rheological Behavior as a Function of Mixing and Temperature Increase in Wheat Flour and Whole Wheat Meal by Mixolab; Method 10-10.03, Optimized Straight Dough Bread-Making Method; Method 10-05.01, Guidelines for Measurement of Volume by Rapeseed Displacement, St. Paul, MN: AACC International

AOAC International (2019, Official methods of analysis *(21st ed.)*, Method 923.03 – Ash Determination; Method 922.06-Fat in flour acid hydrolysis method; Method 962.09 – Fiber in Animal Feed and Pet Food, Association of Official Agricultural Chemists, Rockville, MD

ASAE International (1988), Method S352.2- Moisture Measurement-Unground Grain and Seeds. American Society of Agricultural and Biological Engineers, St. Joseph, Michigan

Aydin A., Paulsen P., and Smulders F. (2009), The physic-chemical and microbiological properties of wheat flour in Thrace, Turkish Journal of Agriculture and Forestry 33 (5) pp. 445-454

Brul, S., and Coote, P. (1999), Preservative agents in foods. Mode of action and microbial resistance mechanisms. International Journal of Food Microbiology 50 pp. 1–17

Carter B., Morris C., Weaver G., and Carter A. (2015), The Case for Water Activity as a Specification for Wheat Tempering and Flour Production, AACCI Publications accessed from https://aaccipublications.aaccnet.org/doi/pdf/10.1094/CFW-60-4-0166 on October 31, 2020

Centers for Disease Control and Prevention (2016), Multistate outbreak of Shiga toxin-producing Escherichia coli infections linked to flour (final update), accessed from https://www.cdc.gov/ecoli/2016/o121-06-16/index.html on October 28, 2020

Clinical and Laboratory Standards Institute (2012), Performance standards for antimicrobial susceptibility testing, 22nd Information supplement, Clinical and Laboratory Standards Institute, Wayne, PA.

Codex Alimentarius -International Food Standards, (2019), Standard for Wheat Flour, accessed from http://www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXS%2B152-1985%252FCXS_152e.pdf on March 16, 2021

Collado-Fernandez M. (2003), Encyclopedia of Food Sciences and Nutrition (2nd ed.), Bread-Breadmaking Process, In: Caballero B., Elsevier Science Ltd., New York, USA

Davidson P.M., and Taylor M.T. (2006), Food Microbiology: Fundamentals and Frontiers (3rd ed.), Chapter 33 – Chemical Preservatives and natural Antimicrobial Compounds, In: Doyle M.P. and Beuchat L.R., ASM Press, Bel Air, MD

Dhillon B., Wiesenborn D., Wolf-Hall C., and Manthey F. (2009), Development and evaluation of an ozonate water system for antimicrobial treatment of durum wheat, Journal of Food Science 74 pp. 396-403

Dubat A., and Bock J. (2019), Impact of the wheat tempering procedure on the grain behavior during milling and on the flour quality at the laboratory Part 1: Effect of final tempering moisture content, Project: Understanding & Mastering Laboratory Milling

Egan H., Kirk R., and Sawyer R. (1981), *Pearson's Chemical Analysis of Foods*, Churchill Livingstone, London UK

Finn S., Condell O., McClure P., Amezquita A., and Fanning S. (2013), Mechanisms of survival, responses, and sources of *Salmonella* in low-moisture environments, Frontiers in Microbiology 14 pp. 331- 343

Food and Drug Adminstration. (2020), Recalls, Market Withdrawals, and Safety Alerts, accessed from https://www.fda.gov/safety/recalls-market-withdrawals-safety-alerts on November 2, 2020

Forghani F., den Bakker M., Futral A., and Diez-Gonzalez F. (2018), Long-term survival and thermal death kinetics of Enterohemorrhagic *Escherichia coli* serogroups O26, O103, O111, and O157, Applied and Environmental Microbiology 84 pp. 283-18

Garg S., Cran M., and Mishra V. (2019), Effect of heating and acidic pH on characteristics of gluten suspension, International Journal of Food Science and Technology 54 pp.1892-1900

Guttieri M., Bowen D., Gannon D., O'Brien K., and Souza E. (2001), Solvent retention capacities of irrigated soft white spring wheat flours, Crop Science 41 pp. 1054-1061

Hirashima M., Takahashi R., and Nishinari K. (2005), Effects of adding acids before and after gelatinization on the viscoelasticity of cornstarch pastes, Food Hydrocolloids 19 pp. 909-914

Los A., Ziuzina D., Akkermans S., Boehm D., Cullen P., Van Impe J., and Bourke P. (2018), Improving microbiological safety and quality characteristics of wheat and barley by high

voltage atmospheric cold plasma closed processing, Food Research International 106 pp. 509-521

McGarvey J.A., Stackhouse K.R., Miller W.G., Stanker L.H., Hnasko R., and Mitloehner F. (2011), Effects of sodium bisulfate on the bacterial population structure of dairy cow waste, Journal of Applied Microbiology 111 pp. 319-328

Nahar N., Madzuki I., Izzah N., Ab Karim M., Ghazali H., and Karim R. (2019), Bakery Science of Bread and the Effect of Salt Reduction on Quality: A Review, Borneo Journal of Sciences and Technology 1 pp. 9-14

Nayeri M.D., MdTahir P., Harun J., Abdullah L.C., Bakar E.S., Jawaid M., and Namvar F. (2013), Effects of temperature and time on the morphology, pH, and buffering capacity of bast and core kenaf fibers, Biological Research 8 pp.1801-1812

Neil K., Biggerstaff G., MacDonald J.K., Trees E., Medus C., Musser K., Stroika S., Zink D., and Sotir M. (2012), A novel vehicle for transmission of Escherichia coli O157:H7 to humans: multistate outbreak of *E. coli* O157:H7 infections associated with consumption of ready-to-bake commercial prepackaged cookie dough--United States, 2009, Clinical Infectious Diseases 54 pp. 511-518

Pascale M., Haidukowski M., Lattanzio V., Silvestri M., Ranieri R., and Visconti A. (2011), Distribution of T-2 and HT-2 toxins in milling fractions of durum wheat, Journal of Food Protection 74 pp. 1700-1707

Pope M.J., and Cherry T.E. (2000), An evaluation of the presence of pathogens on broilers raised on Poultry litter treatment-treated litter, Poultry Science 79 pp. 1351-1355

Posner E.S. and Hibbs A.N. (2005), Wheat Flour Milling (8^{th} ed), AACC Inc., St. Paul Minnesota, USA

Ram S., Dawar V., Singh R., and Shoran J. (2005), Application of solvent retention capacity test for the prediction of mixing properties of wheat flour, Journal of Cereal Science 42 pp. 261-266

Russell, J. B. (1992), Explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling, Journal of Applied Bacteriology 72 pp. 363–370

Sabillon L., Stratton J., Rose D., and Binachini A. (2019), Effect of saline organic acid solutions applied during soft wheat tempering on microbial load and flour functionality. Cereal Chemistry 96 pp.1048-1059

Sabillon L. (2018), Dissertations - A Risk-Based Approach to Evaluate the Impact of Interventions at Reducing the Risk of Foodborne Illness Associated with Wheat-Based Products, Digital Commons - University of Nebraska-Lincoln

Sabillon L., Stratton J., Rose D., Flores R., and Bianchini A. (2016), Reduction in microbial load of wheat by tempering with organic acid and saline solutions, Cereal Chemistry 93 pp. 639-646

Sterk R. (2017), "Millers await advancements in controlling pathogens in flour", Food Business News, accessed from https://www.foodbusinessnews.net/articles/10823-millers-await-advancements-in-controlling-pathogens-in-flour on April 10, 2021

Sun H., Pan Y., Zhao Y., Jackson W.A., Nuckels L.M., Malkina I.L., Arteage V.E., and Mitloehner F.M. (2008), Effects of sodium bisulfate on alcohol, amine, and ammonia emissions from dairy slurry, Journal of Environmental Quality 37 pp. 608-614.

Wali M.K., and Abed M.M. (2019), Antibacterial activity of acetic acid against different types of bacteria causes food spoilage, Journal of Food Technology and Preservation 3 pp. 1-4

Wang, C.; Chang, T.; Yang, H., and Cui, M. (2015), Antibacterial mechanism of lactic acid on physiological and morphological properties of *Salmonella* Enteritidis, *Escherichia coli* and *Listeria monocytogenes,* Food Control 47 pp. 231–236

Wang S., Li C., Copeland L., Niu Q., and Wang S. (2015), Starch Retrogradation: A comprehensive review, Comprehensive Reviews in Food Science and Food Safety 14 pp. 568-585

Weaver G., Atkins-Lewenthal E., Allen B., Baker S., Hoerning D., Peterson A., Schumacher R., and Warren B. (2011), Microbial reduction in a processing stream of a milled product, U.S. Patent Application 20110177216

Zhou Y., and Hou G. (2012), Effects of phosphate salts on the pH values and Rapid Visco Analyser (RVA) pasting parameters of wheat flour suspensions, Cereal Chemistry 89 pp. 38-43

Chapter 5 - Summary and Future Work

5.1 Summary

The findings from these studies could be used as a basis for improving the food safety of wheat flours during the milling process. In the findings from Chapter 3, *E. coli* was found to be more prevalent on the rollers, feeders, hoppers, and break sieve surfaces of the mill. These observations could be used as basis for improving sanitation practices in the mill. Furthermore, the models developed in predicting the *E. coli* counts in flour fractions during lab scale milling. This could then be used as a basis for conducting risk assessments related to *E. coli* for the milling process. However, the model should be validated for large scale milling operations as the milling equipment could vary in terms of material, size, surface area, and grinding intensity which could all affect how *E. coli* is transferred into the resulting milling fractions and equipment.

Based on the results from Chapter 4, the lowest concentration of SBS (0.5% SBS) used was able to reduce the *E. coli* (O121 and O26) load of wheat by 2 logs at the end of the tempering step (24 h). The highest concentration used (1.5% SBS) was able to reduce STEC loads to non-detectable levels (> 4 log reduction). Furthermore, the quality of flours from SBS-tempered wheats (1.25 and 1.5% SBS) were comparable to the conventionally tempered wheats (water-tempered) in terms of flour characteristics and baking performance. These findings show that SBS could be a viable intervention step to control STEC contamination in wheat prior to milling.

5.2 Future Work

For the *E. coli* contamination experiment (Chapter 3), the effects of other factors such as inoculation protocol, organism type, wheat variety and grain hardness could also be explored. This is due to the fact that the milling process is highly variable in terms of its parameters and the inclusion of these parameters could improve the accuracy of the models constructed for predicting *E. coli* contamination levels in wheat flours. Also, as the *E. coli* counts from the non-inoculated wheat mill run involved low levels of *E. coli* with most of them below detection limits, research aimed at developing prevalence-based (positive/negative) models for *E. coli* contamination of wheat flours could also be pursued. Scaling up the milling process could also be done to have a better approximation of *E. coli* contamination of the milling fractions and equipment in commercial mill scenarios.

For the SBS tempering experiments (Chapter 4), the intervention could be extended to other wheat varieties such as soft wheats as well as other foodborne pathogens relevant to wheat flours such as *Salmonella* in order to evaluate their effectiveness. This would be important as foodborne illness outbreaks were more common in non-bread products such as cake and cookie mixes which involve the use of soft wheat flours while some of the recalls and outbreaks involving wheat flours or wheat-based products were caused by *Salmonella*. Research involving scaling-up the process to a bigger scale (e.g., pilot scale) could also be pursued in order to better approximate the effectivity of the intervention (SBS tempering) used. Finally, a sensory evaluation study of the flours produced from SBS-tempered wheats could also be done to further evaluate the viability of the intervention in the wheat milling process from a quality and consumer acceptance viewpoint.