Fall 10-2018

A Risk-Based Approach to Evaluate the Impact of Interventions at Reducing the Risk of Foodborne Illness Associated with Wheat-Based Products

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A RISK-BASED APPROACH TO EVALUATE THE IMPACT OF INTERVENTIONS AT REDUCING THE RISK OF FOODBORNE ILLNESS ASSOCIATED WITH WHEAT-BASED PRODUCTS

by

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A DISSERTATION

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Major: Food Science and Technology

Under the Supervision of Associate Professor Andréia Bianchini

Lincoln, Nebraska

October, 2018

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University of Nebraska, 2018
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Pathogens are emerging on previously unrecognized food vehicles, such as wheat flour, which is a widely consumed commodity. Despite its low water activity, wheat-based ingredients and products have been implicated in several foodborne outbreaks over the last few years, resulting not only in the recall of food products but also in consumer illnesses. As a raw agricultural commodity, wheat is exposed to naturally occurring microbiological contamination that may compromise the overall safety of flour and other derived products. To address the increased concern regarding the safety of wheat-based foods, the objectives of the present research study were designed to develop and implement intervention strategies (i.e., acidic saline tempering solutions and high-pressure processing) aimed at reducing foodborne contaminants at different points along the mill-to-table continuum. Additionally, the effectiveness of such interventions at reducing public health risks was assessed using a stochastic, risk assessment model.

Tempering with acidic saline solutions significantly improved the safety of wheat prior to milling, without substantially affecting the functional properties of straight-grade flour. For instance, the combination of lactic acid (5.0% v/v) and NaCl (~26% w/v) reduced the aerobic mesophilic bacteria and *E. coli* O157:H7 counts in soft wheat by 3.1 and 1.8 log CFU/g, respectively. A microbiological survey conducted in a commercial milling facility revealed that, as wheat is milled into flour, there is a substantial risk of cross-contamination by microorganisms potentially inhabiting the milling equipment.

Appropriate cleaning and sanitization regimens should, therefore, be implemented in the mill to reduce such risk. The application of high-pressure processing (600 MPa, 6 min), as a post-packaging intervention, significantly reduced *E. coli* counts in cookie dough by as much as 2.0 log CFU/g, without causing significant changes on product quality parameters. The risk assessment modeling revealed that the application of these interventions along the mill-to-table continuum can significantly reduce the public health risks associated with the consumption of cookie dough contaminated with *E. coli* O157:H7. The findings of this study will lead to better decision-making regarding strategies that could be applied throughout the grain processing chain to safeguard consumers.

To God, my family, my friends and to
all the people who believe in me

ACKNOWLEDGMENTS

There are many people that have earned my deepest gratitude for their contribution and support throughout my journey in graduate school. First, I am especially indebted to Dr. John Rupnow and Dr. Rolando Flores, who offered me the great opportunity to pursue my graduate education in the Department of Food Science and Technology at UNL. Thank you for believing in me, for your endless support and friendship.

Also, I would like to express my warmest appreciation to my advisor, Dr. Andréia Bianchini. Thank you for being instrumental in my professional development. Your constant guidance, motivation, and limitless support throughout my graduate education have been a true blessing, and would definitely be unforgettable. Your hardworking and passionate attitude has certainly inspired me to become better every single day.

Besides my advisor, I would also like to extend my sincerest gratitude to the rest of my dissertation committee members, including Dr. Jayne Stratton, Dr. Devin Rose, Dr. Bing Wang, and Dr. Kent Eskridge. Thank you all for your great support and invaluable advice throughout these years. This dissertation would not have been possible without your intellectual contribution, crucial remarks, and insightful comments.

I would like to thank my lab mates and friends, Rhaisa Crespo, Ana Arciniega, Amy Garrison, Brandon Nguyen, Eric Oliver, and Rodrigo Mendoza for making my experience in graduate school enjoyable and memorable. I am also grateful to Dr. Bismarck Martinez, whose friendship and continued support have been invaluable. Moreover, I am thankful to Marc Walter, in the Department of Plant Science, for providing me technical support when I needed it the most.

Last but not least, I would like to express my deepest gratitude to my parents, Luis Sabillón and Carmelina Galeas; my sister, Alejandra Sabillón; my beloved wife and daughter, Carla Turcios and Victoria Sabillón. This journey would not have been possible without their warm and unconditional love, continued patience, selfless sacrifices, and endless support. I love you all and thank you for always being there for me through thick and thin. I could not say goodbye without thanking you, dear God, for giving me the strength to overcome all hurdles not only during graduate school, but also throughout my life.

PREFACE

The present doctoral dissertation is a holistic study aimed to improve the safety of wheat-based products through the application of several intervention strategies along the mill-to-table continuum. This dissertation is organized in six chapters that provide not only a description of the microbiological hazards and possible sources of contamination, but also potential risk mitigation strategies to safeguard consumers. The first chapter provides a comprehensive literature review that highlights the microbiological hazards along the wheat-based products supply chain and the potential sources and route of entry of pathogenic microorganisms into wheat milled products. It also provides an overview of the effectiveness of the proposed risk mitigation strategies and the use of risk assessment modeling as a tool to evaluate such effectiveness. The second chapter illustrates the effect of adding organic acids and NaCl to the tempering water on the endogenous microbiota of soft wheat grain and the functionality of the resulting straight-grade flour. The third chapter describes the impact of acidic saline solutions applied during wheat tempering on the population of pathogenic microorganisms, including *Salmonella enterica*, *E. coli* O157:H7, and non-O157 Shiga toxin-producing *E. coli*. The fourth chapter reports the findings of a microbiological survey of equipment and end-products conducted in a pilot and commercial wheat milling facilities. In the fifth chapter, the efficacy of high-pressure processing treatments on the reduction of microbial load in sugar-cookie dough and its impact on quality parameters is reported. The last chapter addresses the development of a quantitative microbiological risk assessment model to evaluate the effectiveness of the proposed interventions strategies to mitigate public health risks arising from consumption of unbaked cookie dough contaminated with *E. coli* O157:H7.

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CHAPTER 1

THE WHEAT SUPPLY CHAIN: MICROBIOLOGICAL RISKS AND POTENTIAL CONTROL MEASURES

CHAPTER 1. THE WHEAT SUPPLY CHAIN: MICROBIOLOGICAL RISKS AND POTENTIAL CONTROL MEASURES

1. INTRODUCTION

Wheat milled products, such as flour, fall into the category of low-moisture foods. The low water activity (≤ 0.85) encountered in these products has, for many decades, contributed to the perception that these foods were not of concern from a microbiological standpoint. However, in recent years, wheat flour has been the source of several foodborne disease outbreaks. These food safety incidents have illustrated that, despite the fact that flour does not offer a suitable environment for microbial growth, pathogenic microorganisms do have the possibility to remain viable for long periods of time (Beuchat *et al.*, 2011), which poses potential risks to consumers.

The 2017 multistate outbreak of *Escherichia coli* O121 infections in Canada associated with raw flour represents the most recent example of the potential of low-moisture foods to serve as a vehicle for the transmission of pathogenic microorganisms (BCCDC, 2017). This is the latest in a series of food safety incidents linking flour and flour-based mixes to *Salmonella* and *E. coli* O157:H7 outbreaks (Neil *et al.*, 2011; McCallum *et al.*, 2013; US-FDA, 2016).

Wheat grain, as a raw agricultural commodity, is exposed to multiple sources of microbial contamination throughout the supply chain. Therefore, wheat kernels may harbor a large and varied microbial flora, which may include pathogenic microorganisms (Sabillón and Bianchini, 2016). These microorganisms are located mainly in the surface of the grain, but some may find their way into the inner part of the kernel through the germ or due to mechanical damage during harvesting (Laca *et al.*, 2006).

The physical processes carried out during wheat cleaning and milling have minimal impact on microbial load, resulting more in a redistribution of microorganisms among milled fractions, rather than a true reduction in contamination (Berghofer *et al.*, 2003). This redistribution leads to a higher association of microorganisms with the bran and outer layers of the grain than with the milled endosperm. Additionally, there is the potential for cross-contamination during milling due to the inherent difficulties associated with cleaning and sanitizing milling equipment (Berghofer *et al.*, 2003). Therefore, the initial microbiological quality of the wheat kernel, along with the milling environment, has a strong influence on the ultimate quality and safety of milled products and foods produced from these ingredients, with some being at higher risk than others (i.e., whole-wheat flour versus straight-grade flour).

Although most flour-based foods undergo a microbial-reduction step (e.g., baking) before intended consumption, homemade flour-based mixes or commercial ready-to-bake refrigerated/frozen dough products may represent a potential safety hazard to consumer health, if consumed without proper cooking. A survey in 2010 revealed that, in fact, the practice of eating uncooked dough appears to be popular among consumers (Ardent Mills, 2010).

As a result of the recent food safety events, there has been recognition of the need to more rigorously consider and manage the microbiological hazards associated with wheat-milled products and to develop potential interventions to reduce the risk of pathogen contamination in these products before they reach the consumers.

2. WHEAT PRODUCTION AND CONSUMPTION IN THE UNITED STATES

Wheat production plays an important role in the U.S. economy. In terms of trade, the U.S. is one of the world's leading wheat exporters. In the 2016/17 marketing year, wheat product exports, including grain, flour and pasta, totaled 28.7 million metric tons, representing a share of 15.7% of the world's exports with a value of more than 6.2 billion dollars to the U.S. economy (USDA-ERS, 2018a, 2018b).

2.1. Wheat production in the United States

The U.S. is one of the leading wheat-producing countries worldwide. During the last decade, wheat has been ranked as the third-largest field crop produced in the U.S. in both planted hectares and production. According to the Economic Research Service of the U.S. Department of Agriculture, in 2017, U.S. farmers produced a total of 47.4 million metric tons of wheat, representing a share of 6.2% of the world's wheat production (USDA-ERS, 2018b). The harvested land area in 2017 totaled 15.2 million hectares. The largest wheat producing states in terms of area planted and harvested were Kansas (3.1 million hectares), North Dakota (2.7 million hectares), Texas (1.9 million hectares), and Colorado (0.9 million hectares) (USDA-NASS, 2017b).

The production of wheat in the U.S. is classified into five major classes: hard red winter, hard red spring, soft red winter, white, and durum. Each class has different end-uses, and production tends to be region-specific. In general, the production of wheat is dominated by winter wheat varieties, which represent 70 to 80% of the total U.S. production (USDA-ERS, 2018b). Although, almost half of the U.S. wheat crop is exported, each year the U.S. wheat milling industry utilize more than 25 and 2.7 million metric tons of wheat for food and feed manufacturing, respectively (USDA-ERS, 2018b).

2.2. The role of wheat in the American diet: The impact of a food safety incident

The consumption of cereal-based products in the U.S. has increased over the years. According to a study on food availability, the amount of grains available (e.g., wheat flour, rice, corn products, oat products, and barley products) for consumption in the U.S. increased 28%, from 136.7 pounds per person in 1970 to 174.4 pounds in 2014 (Bentley, 2017). Of the 37.7-pound increase in total grains between 1970 and 2014, wheat-milled products (i.e., white and whole-wheat flour and durum flour) contributed 23.8 pounds, more than any other grain.

The production of wheat flour in the U.S. has increased over the years, from 41.9 billion pounds in 2007 to 42.6 billion pounds in 2017. The U.S. per capita wheat flour consumption has remained above 130 pounds during the last decades, reflecting a mature market and long-term per capita wheat food use trends (USDA-ERS, 2018b). In fact, according to USDA agricultural projections to 2027, food use of wheat in the U.S. will grow marginally over the next decade, indicating that this grain will remain a large fraction of the American diet (USDA-IAPC, 2018). To illustrate this point, in 2014, grains provided more calories for the average American consumer than any other food group, comprising 29% of total caloric intake (Bentley, 2017).

The increase in grain availability and consumption is, perhaps, due in part to the rising popularity of whole-grain foods in their natural form. This popularity emerged from the protective health benefits attributed to whole-grain intake (Schaafsma, 2004). In fact, 8 out of 10 Americans associate whole grains with heart health, weight management and digestive system health, while 6 in 10 connect whole grains to healthy blood sugar (IFIC, 2013).

Results from a U.S. national survey in 2015 indicated that whole-grain and fiber content were top considerations when buying packaged foods for 69% and 68% of consumers, respectively (IFIC, 2015). In response to a growing consumer demand for whole-grain foods, the production of whole-wheat flour has increased over the past few years. For instance, whole-wheat flour production increased from 2.2 billion pounds in 2016 to 2.3 billion pounds in 2017, accounting for a 5.3% of the total U.S. flour production (USDA-NASS, 2017a). As a result of the growing attention for whole-grain consumption, hundreds of new grain-based products are launched every year with claims regarding their fiber content. The majority of these products are sold as ready-to-eat.

Likewise, the segment of ready-to-bake refrigerated / frozen dough products has increased in popularity over the last years. Results from a recent U.S. national survey revealed that, in 2017, 127.7 million Americans consumed refrigerated dough products, while 60.9 million ate frozen dough products (Experian, 2017a). The consumption of these foods in the U.S. is projected to increase from 154.8 million consumers in 2017 to 160.23 million by 2020 (Experian, 2017b).

The items in this product category include canned refrigerated biscuit, pastry, and cookie dough; frozen pizza, breads and rolls; and cake, brownie and other bakery mixes. Unlike ready-to-eat foods, these products have not undergone specific pathogen reduction treatments to decrease the risk of contamination because they are intended to be cooked by the consumer before consumption. This approach poses substantial food safety risks since consumers may not follow proper cooking instructions or may eat some or all of the product without cooking it.

In fact, a nationwide survey in the U.S. revealed that eating raw or partially cooked bakery goods is a popular practice among consumers. The results indicated that 58 and 67% of the 1,032 consumers surveyed have tasted refrigerated store-bought cookie dough and raw homemade dough before baking, respectively. Another 11 and 24% admitted to having eaten pizza dough and biscuit dough, respectively, before it was fully cooked (Ardent Mills, 2010). When these risky eating practices take place, ready-to-bake products can serve as a vehicle of serious and life-threatening foodborne illness, because their manufacturing includes raw agricultural commodities, such as flour, and does not involve a microbial lethality step.

Since flour is a staple ingredient in many commercial products consumed by millions of people and it is an essential item in consumer's pantries, the potential for a pathogen-contaminated lot of flour to have a broad and significant impact on public health and the economy is noteworthy. A number of factors could contribute to a higher incidence of foodborne illness outbreaks caused by flour-based foods, such as new trends in consuming raw or minimally processed grain-based products, the increased demand for ready-to-bake products, and the possibility that consumers may eat raw or undercook products, like pre-made cookie dough.

3. MICROBIOLOGICAL HAZARDS IN WHEAT GRAIN AND FLOUR

As a raw agricultural commodity, wheat is subject to contamination from the environment and usually carries with it a wide variety of microbial contaminants (Laca *et al.*, 2006). The microbial composition of the wheat grain is of great importance for the safety of milling-end products, since the microorganisms present in the grain may be distributed among milled fractions during milling (Berghofer *et al.*, 2003). Once in the

flour, microorganisms may survive for extended periods and, upon rehydration of flour during food preparation, growth may occur; and consequently, the quality and safety of products may be compromised.

3.1. The microbiological profile of wheat grain

Throughout the production chain, from field to harvesting and storage, wheat is exposed to multiple sources of microbial contamination. While in the field, wheat kernels may be contaminated with microorganisms coming from soil, water, insects, and animal feces (Bullerman and Bianchini, 2008). During handling and storage, rodents and insect infestation may be the primary source of microbial contamination (Doyle and Buchanan, 2013).

As a result, the microbial flora associated with wheat kernels is large and varied and may include pathogenic microorganisms. The amount and type of these microorganisms may be influenced by several factors such as the meteorological conditions during the growing season, the conditions during the storage period like moisture and temperature, as well as the activity of pests throughout the supply chain (Doyle and Buchanan, 2013; Sabillón *et al.*, 2016b). Generally, these microorganisms are located in the surface of the grain, although some of them may reach the inner endosperm through the germ or cracks produced during harvesting (Laca *et al.*, 2006).

Yeast, mold, and a wide variety of bacteria, including bacilli, lactic acid bacteria, and micrococci, are among the spoilage microorganisms found on wheat grain. Several studies around the world have reported yeast and mold counts in wheat grain ranging from 1.4 to 6.0 log CFU/g (Berghofer *et al.*, 2003; Manthey *et al.*, 2004; Eglezos, 2010; Sabillón *et al.*, 2016b). Spoilage bacteria counts have oscillated between 0.9 to 8.4 log

CFU/g (Berghofer *et al.*, 2003; Manthey *et al.*, 2004; Eglezos, 2010; Sabillón *et al.*, 2016b). These results suggest that microbial contamination levels are highly variable between geographic locations and harvesting years.

Besides spoilage microorganisms, wheat kernels may also harbor fecal-related microorganisms such as coliforms. In fact, several microbial surveys have detected the presence of coliforms and *E. coli* biotype 1 in wheat kernels (Berghofer *et al.*, 2003; Eglezos, 2010; Sabillón *et al.*, 2016b). Furthermore, a recent survey in the U.S. found high levels of *Enterobacteriaceae* in wheat kernels (Sabillón *et al.*, 2016b). *Enterobacteriaceae* is a large family of Gram-negative bacteria that includes many well-known human pathogens such as *Salmonella*, *E. coli*, and *Shigella*; therefore, the association of these microorganisms with wheat could also indicate the potential presence of enteric pathogens. Indeed, in recent years, *Salmonella* has been isolated from wheat samples in Australia (Berghofer *et al.*, 2003; Eglezos, 2010).

3.2. The microbiological profile of wheat flour

The wheat milling process, including cleaning, tempering, and milling, involved in the production of flour have no direct impact on the level of microbial contamination present in wheat grain; therefore, the majority of these microorganisms are transferred to the milled products (Sperber, 2007). However, milling the wheat grain into flour redistributes the microbial contaminants, concentrating them particularly in the bran and germ fractions, leaving the flour as the less contaminated milling-end product (Richter *et al.*, 1993). Nonetheless, flour may still contain microbial contamination, including life-threatening microorganisms.

As in the wheat grain, yeast and mold are among the common spoilage microorganisms found in flour. Richter *et al.* (1993) conducted a microbiological survey in more than 4,000 commercial wheat flour samples throughout the U.S. and reported average counts of 2.1 and 2.9 log CFU/g for yeast and mold, respectively. Similar fungal counts were reported by Manthey *et al.* (2004) and Sperber (2007) in more recent microbial surveys of wheat flour conducted in North America. Similarly, Berghofer *et al.* (2003) and Eglezos (2010) evaluated the microbiological quality of wheat flour in Australia and reported yeast and mold counts in the range of 2.0 to 3.0 log CFU/g. Considerably higher levels of yeast and mold on the order of 3.7 and 5.3 log CFU/g, respectively, were reported by Batool *et al.* (2012) in flour produced in Pakistan.

In addition to fungi, a wide variety of spoilage bacteria is also present in flour. The North American surveys conducted by Richter *et al.* (1993), Manthey *et al.* (2004) and Sperber (2007) showed aerobic bacterial counts in wheat flour varying from 3.8 to 7.0 log CFU/g. Even though lower aerobic bacteria counts on the order of 2.0 log CFU/g were reported by Berghofer *et al.* (2003) in Australian flour, a sub-sequent survey by Eglezos (2010) reported higher aerobic bacterial counts, with a mean of 4.2 log CFU/g. Besides aerobic bacteria counts, Berghofer *et al.* (2003) also reported *Bacillus* spp. counts ranging from 2.0 to 5.0 log CFU/g. In addition, 93% of 71 flour samples analyzed by this group of researchers tested positive for *Bacillus cereus* (Berghofer *et al*., 2003).

Regarding fecal-related microorganisms, they may also be part of the microbial flora found in wheat flour. In fact, several microbiological surveys in flour have detected the presence of these microorganisms, including enteric pathogens. A survey by Sperber (2003) conducted between 1984 and 1991 in the U.S. revealed that 4 (0.34%) of the

1,170 samples of wheat flour analyzed were positive for *Salmonella*. In another survey also carried out in the U.S., Richter *et al.* (1993) found that 1.32% (3,040 samples) and 12.8% (3,350 samples) of the commercial wheat flour samples analyzed were contaminated with *Salmonella* and *E. coli*, respectively. More recently, a survey by Sperber (2007) conducted in the U.S. during the period 2003-2005, found *Salmonella* contamination in 6 out of 4,358 wheat flour samples analyzed, accounting for a 0.14% prevalence, which is considerably lower than the earlier reported prevalence of 1.32% by Richter *et al.* (1993). In the 2003-2005 survey, Sperber (2007) reported mean counts of 0.7 log CFU/g and maximum values of 3.0 log CFU/g for *E. coli* in the 2,921 samples analyzed; while considerably higher levels of *E. coli* contamination in the order of 3.8 and 4.1 log CFU/g in white and whole grain flour, respectively, were reported by Victor *et al.* (2013) in Lesotho.

As demonstrated by several microbiological surveys, wheat flour harbors a wide variety of microorganisms and enteric pathogens may be among them creating substantial safety risks for consumers. Despite the fact that microorganisms cannot proliferate in low-moisture foods, vegetative cells of certain pathogenic bacteria can survive in a dormant state for prolongated periods of time. For instance, Ray *et al.* (1971) was able to detected *Salmonella* in naturally contaminated dried milk power after 10 months of storage. Similarly, multiple serotypes of *Salmonella* were reported to survived for more than 8 months in paprika powder (Lehmacher *et al.*, 1995). Therefore, if proper handling, sanitation, and cooking practices are not followed, flour can serve as a vehicle for serious and life-threatening foodborne illnesses.

4. POTENTIAL SOURCES AND ROUTE OF ENTRY OF PATHOGENIC MICROORGANISMS INTO WHEAT-MILLED PRODUCTS

In recent years, massive recalls of retail wheat flour have been issued due to *Salmonella* Typhimurium and pathogenic *E. coli* contamination (McCallum *et al.*, 2013; US-FDA, 2016). Although the source of contamination remains largely unknown, it may be traced back to the environment in which the grain is grown, handled, and processed. Given the nature of the wheat supply chain, as well as the milling process, there are multiple opportunities for these pathogenic microorganisms to contaminate the milled products. This contamination may occur, for example, while grains are in the field, during harvesting and transportation, during storage or on-site in the milling facility, from machinery and environment.

4.1. Pre-harvest sources of contamination: Farmland environment

As a field crop, wheat is grown in open environments; therefore, it is prone to microbial contamination from diverse sources. Pre-harvest contamination of wheat grain can occur directly or indirectly via wild or domesticated animals, insects, soil, water and wind (Bullerman and Bianchini, 2008). The climatic conditions, in particular relative humidity and temperature, prevailing during grain ripening may impact significantly the survival, amount and type of microbial contaminants associated with wheat (Sabillón *et al.*, 2016b).

Fecal matter is one of the main sources of microbial contamination that grains may be exposed to while in the field. Pathogenic microorganisms such as *Salmonella* and *E. coli* are commensal bacteria inhabiting the intestine of many wild and domesticated animals (CDC, 2017); therefore, droppings from wild birds, deer, swine, and other animals are a potential source of disease-causing bacteria. The risk posed by animal

droppings, however, depend upon the prevalence, incidence, and amount of pathogen carried by animal hosts; as well as the degree of activity of these animals in and around wheat fields (Alam and Zurek, 2006; Jay *et al.*, 2007).

The application of animal manure to condition soil and increase the availability of nutrients is a common practice in field crops, including wheat. According to the USDA Economic Research Service, in 2009, 2.4% of total U.S. winter wheat planted acres were treated with manure, while manure was applied to 1.1 and 1.2% of the spring and durum wheat crops, respectively (USDA-ERS, 2017). In addition, the manure used to fertilize wheat fields was obtained primarily from beef and dairy cattle operations.

According to MacDonald *et al.* (2009) more than 80% of the farms that incorporated manure into wheat fields produced their own, indicating that wheat growers also raise livestock within the same farmland. Since beef and dairy cattle operations are an important source and reservoir of life-threatening microorganisms (Hancock *et al.*, 1998; LeJeune *et al.*, 2001; LeJeune *et al.*, 2004), wheat fields that were treated with manure or that are adjacent to cattle feedlots, may be at a higher risk of contamination with fecal-related pathogenic microorganisms through direct deposition of animal feces or through deposition of soil, dust, or water previously contaminated with fecal material. Berry *et al.* (2015) noted that airborne transport of *E. coli* O157:H7 from cattle production can occur and the risk is exacerbated when cattle pen surfaces are very dry and cattle behavior or management generates airborne dust.

If not properly managed, the incorporation of cattle manure in crop fields may pose substantial risks to the microbiological quality of harvested grains. For example, in 2002, an outbreak caused by *Salmonella* Agona in Germany was traced back to contaminated

aniseeds imported from Turkey. The source of contamination was attributed to the use of manure as a natural fertilizer (Koch *et al.*, 2005). Epidemiological data indicate that bovine animals are a natural reservoir of enterohemorrhagic *E. coli* O157:H7, other serotypes of Shiga toxin-producing *E. coli*, and *Salmonella* spp. (Huston *et al.*, 2002; Ferens and Hovde, 2011); consequently, these microorganisms are shed asymptomatically in the feces of these animals (Huston *et al.*, 2002; Pao *et al.*, 2005; El-Seedy *et al.*, 2016). Once outside the host, depending on the environmental conditions and initial concentration, enteric pathogens may survive in manure and manure-slurry for lengthy periods of time, ranging from weeks to years (Kudva *et al.*, 1998; Fukushima *et al.*, 1999; Himathongkham *et al.*, 1999). Therefore, the risk of introducing viable pathogenic microorganisms to production fields through the incorporation of manure is noteworthy.

Depending on the abiotic conditions, as well as interactions with indigenous soil microorganisms, enteric pathogens may also survive in manure-amended soils for long periods of time, threatening the safety of agricultural commodities for several production cycles (Gagliardi and Karns, 2002; Jiang *et al.*, 2002; Holley *et al.*, 2006; You *et al.*, 2006; Semenov *et al.*, 2008). In a recent study, Martinez *et al.* (2015) sowed sanitized wheat seeds in soil inoculated with *E. coli* O157:H7. After 9 days, 5 out of 100 wheat seedlings analyzed tested positive for *E. coli*. This result suggests that soil is an important source of contamination for the transmission of pathogenic microorganisms to raw agricultural products and, most importantly, that bacteria may use the root system to translocate internally into plant tissues.

Fecal loading of surface water by livestock and wildlife with subsequent contamination of wells used for irrigation represents another possible route of transmission of enteric pathogens to wheat grain in the field. Heavy rainfall or storm events may facilitate microbial contamination of water sources through lixiviation or run-off from manure-treated soils if adequate control measures are not in place. A clear example of the potential of animal activity to contaminate water sources occurred in Sweden in 2005. A trace-back investigation of an *E. coli* O157:H7 outbreak linked to iceberg lettuce found the outbreak-causing strain in the water used for irrigation. The investigation concluded that grazing cattle or wild animal activity in the surrounding area was the most likely source of water contamination (Söderström *et al.*, 2005).

In a study conducted by Martinez *et al.* (2015), wheat heads at the flowering growth stage were sprayed with water contaminated with *E. coli* O157:H7. The results obtained showed that *E. coli* was able to grow and survive on wheat heads for up to 15 days after flowering. The most important finding of the study was, however, that among the sources of contamination tested, irrigation of wheat plants at the flowering growth stage is the most likely route of contamination under real environmental conditions since *E. coli* O157:H7 may survive on wheat heads long enough to reach the harvesting stage.

Besides surface contamination of grain through irrigation water, there is evidence that bacteria may internalize passively through the movement of contaminated water into the plant from the environment. Research studies have recovered *E. coli* O157:H7 and *Salmonella* spp. from plant seedlings and/or stems after watering seeds with water contaminated with these microorganisms (Itoh *et al.*, 1998; Charkowski *et al.*, 2002; Solomon *et al.*, 2002; Kutter *et al.*, 2006; Sharma *et al.*, 2009). A study on internalization

of *E. coli* O157:H7 in wheat seedlings obtained an internalization rate of 10% (10/100) after exposure of seeds to contaminated water during germination (Martinez *et al.*, 2015). In general, research studies using water as a source of contamination have obtained higher internalization rates of microbes into plant tissues than those studies using soil or seed as sources of contamination, indicating that water mobilize microbes to the plant roots in a more advantageous way than moist soil or inoculated seeds (Erickson *et al.*, 2014).

Pathogenic microorganisms have also been recovered from above-ground portions of developed plants, which indicates that these microorganisms can be taken up through the roots and move freely within the plant. For instance, under experimental conditions and in the absence of competition, *Salmonella enterica* serovar Newport and *E. coli* O157:H7 were able to colonize the roots of *Arabidopsis thaliana* (thale cress), internalize, move within the plant to eventually reach the flower and seeds (Cooley *et al.*, 2003). Although the lack of competition between pathogens and endogenous microbes in farm soils is unlikely, this provides evidence that if colonization of plant tissues by pathogens occurs, it may lead to the production of seeds harboring dangerous microbes under the seed coat. Therefore, a contamination event in the field may later amplify into a more serious problem when grain is milled into flour.

4.2. Post-harvest sources of contamination: Storage and milling

It is clear that agricultural crops are prone to their association with pathogens as open fields may present multiple opportunities for contamination with these microorganisms. Nonetheless, as harvested wheat grain moves through the processing chain, it may also encounter several other sources of contamination including unclean

transport vehicles and storage facilities, improper storage conditions, pest infestation, and cross-contamination during milling.

4.2.1. Grain storage and transportation

During harvesting, millions of metric tons of wheat grain are moved from fields to storage facilities, then delivered to local mills or transferred to port terminals for export, through a complex and dynamic transportation system that includes trucks, railroad hopper-cars, and barges. Transportation vehicles may carry substantial amounts of microbial contaminants due to previous cargoes, accumulation of dust and dirt; as well as birds, rodent and insect activity (Perez-Mendoza *et al.*, 2004; Arthur *et al.*, 2006; Gilbert *et al.*, 2010). Therefore, if proper sanitary measures are not implemented, vehicles used to move harvested grain may serve as a source of contamination.

Before it reaches its final destination, harvested wheat is stored in a network of facilities that encompass farm bins, country elevators, and terminal elevators. Wheat may remain in storage at any of these locations from a few days to several months (Hagstrum *et al.*, 1999). During storage, wheat is vulnerable to attack by stored-product insects, such as rice weevil (*Sitophilus oryzae*). Insect infestation appears to be a common problem not only in farm-stored wheat but also in commercial grain elevators. In previous years, several surveys across the U.S. have detected a significant presence of insects in stored wheat (Storey *et al.*, 1983; Cuperus *et al.*, 1986; Hagstrum, 1987; Lippert and Hagstrum, 1987; Reed *et al.*, 2001; Flinn *et al.*, 2010). Generally, the growth of insect populations during storage can be regulated by controlling the moisture content and temperature of the grain (Storey *et al.*, 1983). However, some insect species may thrive despite the low moisture content of stored grain (Storey *et al.*, 1983).

Insect infestation in stored grain is a concern not only from a quality perspective but also from a safety standpoint. The activity of insects generates heat and moisture due to their metabolic activity, which may create temperature and moisture gradients inside the storage bin (Hagstrum *et al.*, 1999). These gradients will facilitate the development of localized pockets of higher moisture, creating a suitable environment for microbial growth. In addition of causing localized increases in heat and moisture during storage, research studies have shown that common stored-product insects may carry or can become vectors of pathogenic microorganisms (Husted *et al.*, 1969; Julseth *et al.*, 1969; Crumrine *et al.*, 1971; Multon, 1988; Kinde *et al.*, 2005; Hazeleger *et al.*, 2008; Pangloli *et al.*, 2008). For instance, Husted *et al.* (1969) intentionally added rice weevils to wheat kernels previously contaminated with *Salmonella* Montevideo, and let them feed from the kernels for 21 days. The results showed that insects became carriers of the bacterium for at least 5 weeks and were able to contaminate clean, non-inoculated wheat kernels.

In a similar study, Crumrine *et al.* (1971) fed 7 different species of common stored-grain insects for 21 days with wheat grain previously contaminated with *Salmonella* Montevideo. After the initial feeding period, insects were transferred to non-inoculated wheat kernels and were allowed to feed for another 21 days. The progeny was then transferred to another non-inoculated sample. The authors concluded that the 7-species included in the study were able to spread *Salmonella* Montevideo from contaminated wheat to clean wheat. Furthermore, the progeny of the rice weevil, saw-toothed grain beetle, and the red flour beetle were able to transmit the bacterium to clean grain, indicating that the microorganism may be carried through at least one generation.

Spilled grains, residual grain materials and other related debris inside and outside the storage bins and grain conveying equipment may be heavily infested with stored-grain insects, representing a potential source of contamination. Dowdy and McGaughey (1998) surveyed several commercial elevators in Kansas, U.S. and consistently detected populations of stored-product insects, in all surveyed areas, that could potentially migrate into grain in storage. More recently, Reed *et al.* (2003) studied the insect populations inhabiting grain residues in the bottom of elevator silos and from discharge spouts. Several species of stored-grain insects were observed consistently over a 2.5-year period. Similarly, Arthur *et al.* (2006) also showed that grain residues within commercial elevators often contain significant amounts of pest insects.

Furthermore, grain residues may also serve as a food source for pests such as rodents, birds and cockroaches. Rodents and cockroaches are known to act as reservoirs and vectors of pathogenic microorganisms (Tatfeng *et al.*, 2005; Morita *et al.*, 2006; Lapuz *et al.*, 2008; Nkogwe *et al.*, 2011); therefore, their presence in or outside the storage structures represents a serious contamination risk. The presence of rodent excreta in stored grain as well as equipment used to transport grain has been reported in several research studies (Gecan *et al.*, 1980; Stejskal and Aulický, 2014). For instance, Stejskal and Aulický (2014) reported an average contamination of 6.9 feces/m^2 for grain (barley) surfaces and 34.8 feces /m^2 for conveyor belts. Accumulation of fecal matter in conveyor belts can later contaminated clean grain being moved through the conveying system. The prompt removal of build-up of grain residues inside and outside the storage structures along with an integral pest management program is, therefore, a key strategy to prevent microbial contamination and ensure the safety of harvested wheat before its intended end use.

4.2.2. Grain milling

Frequently, prior to arriving at the mill, wheat grain deliveries have gone through a number of aggregation steps including on-farm storage bins, country elevators, and terminal elevators (NAMA, 2011). This extensive handling of wheat grain before milling into flour may create multiple opportunities for microbial contamination. Once wheat enters the milling system, kernels undergo a sequence of complex operations that include cleaning, conditioning, and milling to produce the desired final product (e.g., flour, semolina). As indicated by several microbiological surveys, wheat grain may harbor among its microbial flora not only spoilage microorganisms but also those capable of causing serious illnesses (Berghofer *et al.*, 2003; Eglezos, 2010; Sabillón *et al.*, 2016b).

As wheat passes through the various milling operations, these microorganisms may settle on the surface of the equipment used throughout the process. Therefore, as wheat is milled into flour, there is a substantial risk of cross-contamination by microorganisms inhabiting the milling equipment. Epidemiological and environmental investigations of outbreaks caused by low-moisture foods have, in fact, suggested that cross-contamination is one the most common routes of entry of pathogenic microorganisms into these products (Craven *et al.*, 1975; CDC, 1998; Rushdy *et al.*, 1998; Smith *et al.*, 2004).

The first step in the milling process involves the removal of extraneous matter from wheat kernels (Posner and Hibbs, 2005). Plant-based materials, attached soil, rocks, animal droppings and insects are part of the impurities normally encountered on wheat grain entering the milling system. These impurities may carry significant amounts of microbial contaminants, including pathogenic microorganisms. As a result, machines devoted to removing these impurities in the mill wheat-cleaning section may harbor a

substantially higher microbial load compared to other equipment in the mill. An evaluation of the aerial microbiological contamination in different areas of a grain mill conducted by Dacarro *et al.* (2005) found that the cleaning sector was the most contaminated area in the mill and that the average microbial contamination was 11.4 times higher than in the external environment. Therefore, contaminated cleaning equipment can then serve as a source of cross-contamination as grain moves through the cleaning system.

One of the most critical steps of the milling process, from a microbiological standpoint, is the conditioning of wheat grain after cleaning. This step requires the addition of water to the grain followed by a rest time. Depending on the wheat type and initial moisture content, the wheat can be held in tempering bins for 6 to 18h to enable the water to penetrate the wheat kernel (Posner and Hibbs, 2005). This process makes separation of the grain constituents easier during milling; however, if not handled properly, the addition of water can result in a rapid outgrowth of microorganisms present in the grain as well as on the surface of the tempering bins (Danyluk *et al.*, 2007).

When moisture content increases, bacterial cells that were previously metabolically inactive are given the opportunity to grow, potentially leading to high levels of contamination in milled products. In a survey carried out by Berghofer *et al.* (2003), higher mesophilic aerobic counts were found more frequently in conditioned wheat kernels. In addition, *E. coli* was detected in wheat after conditioning when previously it had been not detected in the grain. In this specific example, the authors concluded that the increase in contamination was likely due to cross-contamination from poorly cleaned conditioning bins and equipment, rather than microbial growth during the conditioning

period. It is important to mention that the water used to condition the wheat grain can also represent a source of microbial contamination if clean, potable water is not used.

Once conditioned, the grain passes through a series of corrugated and smooth rolls, which have defined roles such as breaking-apart the kernels, separating the bran, or reducing the endosperm to flour. As it passes through the breaking rolls, some shattering of the bran occurs resulting in a mixture of endosperm and bran particles. Since the microbial load is mostly located in the outermost layer of the grain (Laca *et al.*, 2006), there is a risk of cross-contamination when the bran particles come in contact with the endosperm during milling.

Moreover, due to the shearing action of the rolls, considerable amount of heat is regenerated when breaking-apart the wheat kernels and reducing the endosperm to flour, hence moisture condensation is likely to occur on the equipment's cool surfaces (Berghofer *et al.*, 2003). This moisture condensation may encourage microbial growth and facilitate the accumulation of broken kernels and flour residues on the inside walls and spouts. Grain/flour debris are likely to remain accumulated for extended periods of time, thus representing a possible reservoir of microorganisms. This mill stock, if not removed periodically, can continuously contaminate the stream of ground material passing through the equipment.

These grain/flour residues in milling equipment were, in fact, the source of a foodborne disease outbreak in England in 1995 linked to the consumption of baby cereal contaminated with *Salmonella* Senftenberg (Rushdy *et al.*, 1998). A trace-back investigation revealed that the bulk cereal was contaminated by the "cleaning remains" from the milling machinery. The investigators concluded that cross-contamination played

an important role in this outbreak since the supplier used common milling machinery to process heat-treated bulk cereal as well as other products that were not heat treated.

After each break and reduction roll, the sheared particles and pulverized endosperm are passed through a series of sieves to separate fine from coarser particles that need further reduction (Posner and Hibbs, 2005). Due to the nature of the sieving process and equipment design, ground material can accumulate in cracks, crevices, and between sieve sections and frames. This ground material may also serve as a reservoir of microbial contaminants and have the potential to contaminate new product passing through the sieves.

Considerable amounts of fine particles of grain, flour, and foreign materials are generated during the wheat cleaning and milling processes. A dust exposure assessment conducted in a grain mill by Dacarro *et al.* (2005) concluded that, among the different milling operations, the cleaning area contains the highest concentration of aero-dispersed dust particles. Control of dust in the flour milling operation is carried out through the use of filtration systems (Posner and Hibbs, 2005). Dust collection systems may be overlooked as an important area to be cleaned and monitored. Because air filtration systems contain dust from grain handling and milling operations, they are a potential reservoir of microorganisms and may also attract insects (Mills and Pedersen, 1990). If air handling systems are not cleaned thoroughly or dust is not regularly disposed of, fumigated, or treated properly it can become a source of microbial contamination and insect infestation within the wheat handling/milling facility.

Not only the mill equipment can be a source of contamination but also the environment where these operations are performed. Open windows, broken window

panes, absence or damaged screens may facilitate the entrance of pests into the milling facility. The presence of pests such as insects and rodents introduce a risk since they are likely carriers of pathogens; therefore, the mobility of pests of any sort could easily aid in widely transferring pathogenic microorganisms throughout the milling facility. Campbell and Arbogast (2004) used a mark-recapture method to evaluate the potential for movement of insects from outside the mill into the mill. Mark-recapture data indicated that Indian meal moth (*Plodia interpunctella*) and other beetles were capable of entering the building from outside. In the same study, red flour beetle (*Tribolium castaneum*) was the predominate species recovered in product samples taken from the mill equipment and trash buckets. The presence of external insects infesting the structure and equipment within the mill may be a potential indicator of unsanitary conditions.

In addition, open or damaged windows will also allow cool air to enter the facility, causing moisture condensation on the inside surface of equipment resulting in conditions suitable for microbial growth. Also, air may serve as a vector by which pathogenic microorganisms can contaminate the final products. Several research studies have noted that dust particles in the air can contain life-threatening microbes such as *Salmonella* and *E. coli* (Morita *et al.*, 2006; Berry *et al.*, 2015). For instance, an investigation by Butcher and Miles (1995) concluded that dust was the major source of *Salmonella* contamination in feed mills. Therefore, air entering the milling facility as well as the dust generated during the wheat cleaning and milling processes, if not properly treated, may increase the risk of cross-contamination.

Other important areas where contamination of flour can occur are the loading and transportation systems. The loading of finished products into transport vehicles often

occurs in areas that are partially open and exposed to microbial threats. In addition, there is a considerable overhead equipment installation as well as roof support structures in loading areas that may harbor pests such as insects, birds, and rodents (Mills and Pedersen, 1990). Because of the nature of the loading operations, flour residues may disperse and accumulate in loading areas, thus serving as a food source for these pests.

Loading of bulk trucks or railcars during cold weather can also result in moisture condensation not only in the flour loading ducts and spouts but also inside the transport vehicles. This condensation may lead to caking of flour, which in turn may facilitate microbial development and serve as a source of contamination. Pests control as well as proper cleaning and sanitization regimens, not only in transport vehicles but also throughout the milling operations, are essential in limiting the risk of cross-contamination in milling-end products.

5. FOODBORNE DISEASE OUTBREAKS CAUSED BY WHEAT FLOUR AND DERIVED FOOD PRODUCTS

Throughout history, wheat flour has been perceived by consumers as one of the safest food ingredients from a microbiological standpoint. This widespread perception of safety has been built on the basis of the erroneous belief that microorganisms will not survive in such a dry environment. Research studies, however, have demonstrated that vegetative cells of pathogenic microorganisms may survive in harsh, dry conditions for lengthy periods of time by using several survival strategies such as biosynthesis of osmoprotectants, use of alternative sigma factors, rRNA degradation, biofilm formation, and entry into a metabolically dormant state (Finn *et al.*, 2013).

During the last decade, wheat flour has been the vehicle of contamination in several foodborne disease outbreaks. These food safety incidents have highlighted the fact that, despite its low moisture content, wheat flour can carry viable cells of microorganisms that can cause disease, such as *Salmonella* and *E. coli*. A list of documented outbreaks of foodborne illnesses that have been traced back to wheat flour contaminated with pathogenic microorganisms is shown in **Table 1-1**. The outbreaks have been clustered into two groups: retail-packaged wheat flour and food products made from contaminated flour.

In **Table 1-1**, the first listed outbreak caused by retail-packaged wheat flour dates back to 2008. A cluster of salmonellosis cases caused by *Salmonella* Typhimurium phage type 42 emerged that year in New Zealand (NZFSA, 2008). Initial investigations indicated that eating uncooked flour-based baking mixture was associated with sixty-six reported illnesses (McCallum *et al.*, 2013). Trace-back investigations recovered the outbreak-causing *Salmonella* strain from retail-packaged wheat flour.

Seven years after that confirmed incident, wheat flour was pointed again as a vehicle of contamination. In 2015, in the U.S., a routine microbial screening performed by the manufacturing company detected the presence of *Salmonella* Thyphimurium in a 5-lb wheat flour sample (US-FDA, 2015). The finding led to recall of all retail-packaged flour produced from the contaminated lot. No illnesses were reported due to this contamination event.

In the subsequent years, wheat flour was implicated once again in several foodborne disease outbreaks, but this time caused by Shiga toxin-producing *Escherichia coli* (STEC) infections. The first of these events occurred in the U.S., where sixty-three

Table 1-1. Foodborne disease outbreaks caused by wheat flour and flour-containing foods.

Product	Manufacturer	Outbreak location	Year	Pathogen	Isolated from product?	Product recalled?	Reported illnesses	Reference
Retail-packaged wheat flour								
Plain flour	Multiple Manufacturers	New Zealand	2008	*Salmonella* Typhimurium phage type 42	Yes	Yes	66	NZFSA, 2008; McCallum *et al.*, 2013
Bleached all-purpose flour	Navajo Pride	USA (4 states)	2015	*Salmonella* Typhimurium	Yes	Yes	Not reported	US-FDA, 2015
Various types of flour	General Mills	USA (24 states)	2015 – 2016	*E. coli* O121 *E. coli* O26	Yes	Yes	63	US-FDA, 2017; CDC, 2016
Various types of flour	Ardent Mills	Canada (6 provinces)	2016 – 2017	*E. coli* O121	Yes	Yes	30	PHAC, 2017
All-purpose flour	Rogers Foods	Canada (1 province)	2017	*E. coli* O121	Yes	Yes	6	BCCDC, 2017
Food products where flour was the suspected or confirmed vehicle of contamination								
Flour-based food	Not reported	Australia (1 state)	1952	*Salmonella* Paratyphi B phage type 1	Not reported	Not reported	Not reported	Dack, 1961; Eglezos, 2010
Cake batter ice cream	Cold Stone Creamery	USA (11 states)	2005	*Salmonella* Typhimurium	Yes	Yes	26	US-FDA, 2005; Zhang *et al.*, 2007
Refrigerated cookie dough	Nestlé USA	USA (30 states)	2009	*E. coli* O157:H7	Yes	Yes	77	Neil *et al.*, 2011; CDC, 2009
Dry dough mix	Not Reported	USA (9 states)	2016	*E. coli* O157:H7 *non-O157 STEC*	Yes	Not reported	13	Gieraltowski *et al.*, 2017

people from 24 states became ill due to STEC O121 or STEC O26 infection (CDC, 2016). The illness onset dates ranged from December 2015 to September 2016. An epidemiologic investigation revealed an association between getting sick with STEC and handling of wheat flour and raw dough.

Further investigations and trace-back evidence indicated that flour produced by a milling company was the likely source of this outbreak. Laboratory testing by health authorities led to the isolation of the outbreak strains from samples of wheat flour. The contamination caused a massive recall, nationwide, of over 45 million pounds of retail-packaged flour. In addition, this contamination event affected at least 5 different food manufacturing companies that used the contaminated flour as ingredient in their products (US-FDA, 2017). Flour-based mixes including jalapeno breader, biscuit, cake, pancake, bread, muffin, and brownie mixes produced by these companies had to be withdrawn from the market due to the potential presence of *E. coli* O121. In total, the downstream recall of products included more than 200 food products and 30 brands.

Shortly after the U.S. outbreak investigation ended, the Public Health Agency of Canada was investigating an outbreak of *E. coli* O121 (PHAC, 2017). In total, thirty cases of *E. coli* O121 infection with matching genetic fingerprints, were reported from 6 provinces on dates ranging from November 2016 to April 2017. During the food safety investigation conducted by health authorities, the *E. coli* outbreak strain was isolated from a package of wheat flour taken from the house of an affected individual.

The investigation also revealed that the genetic pattern of the Canadian outbreak strain was unrelated to the strain of *E. coli* O121 that was responsible for the outbreak in the U.S. in 2016. Several food recalls of various types of retail-packaged wheat flour and

flour-based products distributed across Canada and the U.S. were triggered by the findings of the food safety investigation. In addition, one recalled-flour brand was exported to Hong Kong (Beach, 2017), although no remaining stock of the contaminated flour was found in the market. Also, the Center for Food Safety in Hong Kong did not report any illnesses in connection to the recalled flour.

Curiously, as the nationwide outbreak linked to flour was evolving in Canada, a separate outbreak also related to flour emerged in British Columbia. Six people were infected with *E. coli* O121 between February and April 2017. The public health laboratory of the British Columbia Centre for Disease Control isolated the outbreak-causing strain from a sample of flour obtained from the household of one of the ill individuals (BCCDC, 2017). Canadian public health authorities recalled the contaminated retail-packaged flour from the market. According to public health officials, although both outbreaks were caused by *E. coli* O121, it is unclear whether there was a link between them.

Flour-based products sold at retail level or in restaurants have also been implicated in several food safety incidents over the last decades. The first documented incident caused by a flour-based food occurred in New South Wales, Australia in 1952. Among the ingredients, wheat flour was the suspected carrier of *Salmonella* Paratyphi B phage type 1. Although the bacterium was not isolated from the flour, it remains as the likely source of contamination (Dack, 1961; Eglezos, 2010).

The second food safety incident occurred in 2005 in the U.S. Although wheat flour was not the direct source of contamination, it served as a reminder to the retail and food service industries that flour can carry pathogenic microorganisms. In this outbreak,

twenty-six people across 11 states were infected by a single strain of *Salmonella* Typhimurium after eating cake batter ice cream (Zhang *et al.*, 2007). An epidemiologic investigation linked the cake mix used to prepare the cake batter ice cream as the vehicle of contamination. During the investigation, health authorities reported that dry cake mix was added to pasteurized sweet cream base, but the mixture did not undergo any additional processing prior to freezing.

Because of these findings, the U.S. Food and Drug Administration issued a warning to the food industry reminding them that *Salmonella* can be present in flour and other ingredients used in dry cake mixes (US-FDA, 2005). Flour-based ingredients such as dry cake mixes are design to be rehydrated and then cooked before consumption; therefore, they should not be used in ready-to-eat products without proper cooking. In addition, the U.S. Food and Drug Administration pointed out in their warning that "similar products, such as cookie dough ice creams and cake mix milk shakes, could also pose a serious food safety risk if they are prepared with ingredients that are intended to be cooked."

The next food safety incident was associated with the consumption of uncooked, contaminated baking mixture. In 2009, the United States experienced a multistate outbreak of Shiga toxin-producing *E. coli* O157:H7 infections linked to the consumption of raw refrigerated, prepackaged cookie dough (CDC, 2009). Seventy-seven people were infected with the outbreak strain across 30 states. The epidemiologic study indicated a strong association between eating raw prepackaged cookie dough and becoming ill. Laboratory testing isolated three non-outbreak STEC strains from cookie dough samples (Neil *et al.*, 2011). The investigation led to a nationwide recall of 47 different products and 3.6 million packages of retail-packaged cookie dough. Once again, in 2010, the

manufacturing company announced that 2 samples of cookie dough tested positive *for E. coli* O157:H7 under their routine testing program, but no product had been shipped to stores (Nestlé USA, 2010). The contamination source of the outbreak remains unknown, although suspicions point towards the potential contamination of the flour. As a result, the company reformulated its cookie dough products to use heat-treated flour in an effort to improve safety.

The last food safety event listed in **Table 1-1** implicating flour as the suspected ingredient occurred in 2016 in the U.S. Thirteen STEC O157:H7 outbreak-associated cases were identified in nine states (Gieraltowski *et al.*, 2017). An epidemiologic investigation found that all affected individual had eaten a specific dessert pizza and bread sticks in a national restaurant chain. Trace-back investigations concluded that both products were made from a proprietary dough mix provided by a manufacturing company. Laboratory analysis conducted by health authorities detected the presence of different strains of non-O157 STEC in 7 of the 17 samples of dough mix collected. The investigation also found that dessert pizzas had a very thick dough, which could have been undercooked at some locations leading to health hazards.

Although no conclusive evidence identified flour as the primary source of contamination in this STEC O157:H7 outbreak, the isolation and identification of non-O157 STEC strains in several samples of dry dough mix collected from the restaurant chain, suggests that contaminated flour was the most likely source of pathogen introduction for this outbreak (Gieraltowski *et al.*, 2017). Once again, this outbreak illustrates that flour, a raw agricultural product, may carry pathogenic microorganisms and, when consumed raw or undercooked, might pose a substantial risk to human health.

6. POTENTIAL MITIGATION STRATEGIES

In the light of several outbreaks linked to flour and derived food products, concerns have emerged with regard to the safety of one of the most popular food ingredients of all, wheat flour. Additionally, the increased consumer demand for refrigerated or frozen ready-to-bake products coupled with the consumer's risky habit of eating raw or undercooked flour-based mixes, make ready-to-bake products a potential vehicle of serious and life-threatening foodborne illness.

Commercial methods currently available for ensuring the safety of flour and derived products are proprietary, very limited, and not readily available for the whole food industry. Exploring and developing practical and effective antimicrobial treatments that could be used by the milling industry to deliver safer milled products is, therefore, essential to mitigate the risk of contamination. Moreover, the implementation of post-packaging interventions geared towards reducing any possible microbial contamination before the product reaches the consumer should be considered by food manufacturers to make ready-to-bake products as safe as ready-to-eat products.

6.1. The wheat conditioning process: A promising step to reduce microbial contamination in milling end products

In preparation for milling, the original moisture content of wheat grain is adjusted through a process called conditioning. To adjust the moisture content, water is added to the wheat in precise amounts and distributed as evenly as possible throughout the mass of grain. The moistened wheat is then allowed to rest in bins for a determined period of time or until it reaches the optimum moisture distribution and kernel suitability for milling (Posner and Hibbs, 2005). The objective of this process is to ensure proper separation of the grain constituents (i.e., bran, germ, endosperm) during milling.

The addition of water to the wheat brings an invaluable opportunity to reduce the microbial load of the grain before entering the first grinding stage. Several research studies have, in fact, used the conditioning process as a tool to improve the microbiological safety of wheat prior to milling by adding antimicrobial agents such as chlorine and ozone. A study conducted by İbanoğlu (2001) concluded that tempering soft and hard wheat with ozonated water (11.5 ppm) reduced the aerobic bacteria and yeast/mold counts by 2.1 and 1.1 log CFU/g, respectively, without deteriorating the milling performance and the baking quality of the resulting flour. In a follow-up study, Ibanoğlu (2002) used a quick-wash approach to reduce microbial counts in wheat. Washing wheat kernels with ozonated water (1.5 ppm) for 30 min caused a reduction in aerobic bacteria and yeast/mold counts of 1.0 and 0.4 log CFU/g, respectively. In the same study, the addition of acetic acid (1.0%) to the ozonated water further increased the reduction in yeast/mold counts by 3.7 log CFU/g.

Using a similar quick-wash approach, Dhillon *et al.* (2009) washed durum wheat kernels for 3 min with ozonated water (16.5 ppm) and with a combination of ozonated water (20.5 ppm) and acetic acid (1.0%) at 2 L/kg. Ozonated water alone reduced yeast and mold counts by 0.5 log CFU/g but did not affect aerobic bacteria counts. In contrast, the combination of ozonated water and acetic acid was remarkably effective against yeast/mold and aerobic bacteria, reducing their populations by 4.1 and 3.2 log CFU/g, respectively. In a follow-up study, Dhillon *et al.* (2010) developed a fluidized bed with an automated spraying system to apply gaseous ozone, ozone dissolved in water, or acetic acid solutions to wheat. Among all the treatments used, the combination of gaseous ozone (6 ppm), acetic acid (0.5%), and ozonated water (26 ppm) was the most effective at reducing microbial load with average reductions of 1.7 log CFU/g for aerobic bacteria

and 3.3 log CFU/g for yeast/mold.

In a more recent study, Sabillón *et al.* (2016a) used the water added to the wheat during conditioning as a vehicle to deliver natural antimicrobial agents including acetic, citric, lactic and propionic acid, as well as sodium chloride at concentrations normally used by the food industry. Tempering wheat with solutions containing 5.0% v/v acetic, propionic, and lactic acid reduced the aerobic bacteria, *Enterobacteriaceae* (Eb), and mold counts by 1.7, 2.3, and 3.8 log CFU/g, respectively. The tempering solution containing a combination of lactic acid (5.0% v/v) and NaCl (52% w/v) was the most effective against aerobic bacteria and Eb, with an average reduction of 4.3 and 4.7 log CFU/g, respectively. The substantial reduction observed in Eb counts after tempering with saline organic acid solutions suggests that this approach may be effective against Shiga toxin-producing *E. coli* and *Salmonella* spp.

Furthermore, in a follow-up study, Sabillón *et al.* (2017) showed that tempering wheat with saline organic acids solutions cause a minimal impact on the functional properties and baking performance of the resulting straight-grade flour. The addition of antimicrobial agents to the tempering water can, therefore, serve as a surface sanitizer while kernels reach the optimum milling moisture, thus reducing the risk of microbial contamination in milling end products without compromising functionality.

6.2. High pressure processing: A post-packaging intervention to improve safety of ready-to-bake products

Traditional thermal treatments may cause significant degradation of flavor, nutritional quality, and functionality of flour (Seguchi, 1990; Ozawa *et al.*, 2009; Neill *et al.*, 2012; Mann *et al.*, 2014). Thus, innovative processes like high pressure processing (HPP), which maintain the fresh-like characteristics of food, have attracted much

attention in recent years. HPP consists in treating foods by high pressure with the purpose of inhibiting both pathogen and spoilage microorganisms and of inactivating enzymes that may cause undesirable changes during storage.

A study carried out by Bárcenas *et al.* (2010) showed that 1 min of HPP at 250 MPa reduced the aerobic mesophilic bacteria and yeast/mold counts in wheat bread-dough from 4.2 to 2.0 log CFU/g. Similarly, Barcenilla *et al.* (2016) reported reductions of 0.52 and 0.70 log CFU/g in total aerobic bacteria and yeast/mold counts, respectively, after treating cake batter at 600 MPa for 6 min. Moreover, HPP treatments in wheat, rice, and corn flour mixtures have shown to significantly affect the molecular properties of water in terms of decreasing its proton mobility (i.e., available water) (Vittadini *et al.*, 2004). This may prevent microorganisms from growing, thus enhancing microbial stability of the HPP-treated products. To date, there has been no published research on the application of HPP in wheat flour-based mixtures to inactivate pathogenic microorganisms. However, the results obtained by Bárcenas *et al.* (2010), Barcenilla *et al.* (2016) and Vittadini *et al.* (2004) suggests that HPP has the potential to be used as a post-packaging intervention to improve the safety and shelf-life of ready-to-bake products.

6.2.1. The effect of high-pressure processing on microorganisms: Fundamental aspects

The degree of damage exerted by high pressure on microbial cells depends, in part, on the level of pressure applied. Cellular motility, nutrient uptake, membrane protein functions, cell division and replication can, for instance, be affected or completely suppressed by pressure levels ranging from 10 to 50 MPa. Pressure levels greater than 50 MPa can affect cellular processes such as transcription, protein synthesis and enzyme

functions. Levels of more than 200 MPa can induce irreversible changes to the protein and DNA structures, resulting in a cascade of events that is catastrophic to microbial cell viability (Abe, 2007; Huang *et al.*, 2014). However, besides pressure, factors such as temperature, exposure time, and the composition of the food matrix also impact the degree of microbial inactivation achieved by HPP.

The effects that high pressure has on microorganisms could then be categorized primarily as changes to (1) cell morphology, (2) inhibition of metabolic reactions, and (3) changes on genetic mechanisms. The increase of pressure in the surrendering environment causes a permeabilization of the microbial cell membrane due to irreversible changes in the structure of macromolecules such as proteins and pressure-induced phase transition of the membrane lipid bilayer (Hoover *et al.*, 1989; Balny and Masson, 1993). Disruption of membrane integrity results in morphological changes that can potentially lead to cell death. Electron microscopy observations in high-pressure-treated *E. coli* and *Staphylococcus aureus* cells carried out by Yang *et al.* (2012) showed bacterial cells membranes and walls with a high degree of wrinkles and distortions as well as cellular fluid leakage.

Once high pressure damages the cell membrane structure, the mechanism of nutrient absorption is affected and normal metabolic pathways are disrupted (Torres and Velazquez, 2005). Moreover, denaturation of proteins may lead to a reduction of ATP synthesis, limited proton flow, changes in ribosome configuration, and inhibition of protein repair systems, eventually resulting in cell death (Wouters *et al.*, 1998; Niven *et al.*, 1999; Huang *et al.*, 2014). Regarding the impact on genetic mechanisms, studies have indicated that high pressure inhibits the activity of DNA replication and transcription

enzymes. In addition, high pressure causes genetic materials to condense, resulting in degradation of chromosomal DNA, conformation of nucleoids and cytosolic proteins, leading to cell death (Dubins *et al.*, 2001; Kaletunç *et al.*, 2004; Moussa *et al.*, 2007).

6.2.2. The baroprotective effect of common ingredients used in flour-based products

One of the most important factors that influences the effectiveness of HPP treatments on the inactivation of microorganisms is the composition of the food matrix in which microorganisms are dispersed. Food constituents such as proteins, sugars and lipids could confer a certain degree of protection to microorganisms against high-pressure treatments, which would hinder the effectiveness of HPP (Huang *et al.*, 2014; Georget *et al.*, 2015).

Several studies have reported how high concentrations of solute in a food matrix alters inactivation of microorganisms by HPP. Early investigations on this topic were reported by Raso *et al.* (1998) who demonstrated that high concentrations of sucrose protected *Bacillus cereus* spores from the germinating and the inactivating effect of HPP. Molina-Höppner *et al.* (2004) studied the influence of sucrose (0.5 M) or sodium chloride (4 M) in solutions on the inactivation of *Lactococcus lactis* by pressure between 200 and 600 MPa. The results showed that sucrose protected the membrane integrity and metabolic activity of *L. lactis* cells while salt did not show any degree of protection on metabolic activity. It was also observed that disaccharides may have the potential to interact with the cytoplasmic membrane and maintain its fluidity by retarding the shift from the liquid phase to the gel phase under high pressure environments. The authors concluded that the high concentrations of solutes in food matrices could protect bacteria

cells against pressure-induced inactivation of vital cellular components and metabolic mechanisms.

High pressure requires the presence of a transmitting medium in order to be efficient. The presence of solutes (e.g., sugar, salt) in high concentrations can reduce the amount of the pressure-transmitting fluid (i.e., water) available in the food matrix to cause microbial injury. Koseki and Yamamoto (2007) reported that the inactivating effect of HPP on *Listeria monocytogenes* decreased as the saturation of suspensions (sodium chloride: 0.2–5.0 M, sucrose: 0.9–2.0 M, and phosphate buffer: 0.01–1 M) increased. The authors argued that the poor pressure-induced microbial inactivation observed in over-saturated solutions was due to inhibition of pressure transmission to the water due to the presence of solutes. Other studies have reported that inactivation of microorganisms by HPP was strongly dependent on water activity. Spice-water mixtures with water activity levels of 0.91 required three pressure cycles (30 min at 80 MPa followed by 30 min at 350 MPa) at 70°C in order to achieve complete background flora decontamination, while no inactivation of microorganisms was observed for those mixtures containing a water activity below 0.66 (Butz *et al.*, 1994). These studies, therefore, highlight the importance of the amount of water available in food matrices to achieve significant microbial reductions when subjected to high pressure treatments.

Other food constituents such as fats and oils may exert a baroprotective role against pressure-induced microbial inactivation. Early work carried out by Simpson and Gilmour (1997) showed that inactivation rates of *Listeria monocytogenes* caused by high pressure were substantially lower when the bacterium was suspended in an olive oil/PBS emulsion (30% v/v oil) than when inoculated into PBS buffer alone. The authors suggested that the

low water content in the oil emulsion may have contributed to protect the microbial cells against lethal effects of pressure. Other researches have investigated the inactivation kinetics of *Bacillus amyloliquefaciens* spores in various food systems including fish immersed in different oils and also have highlighted the baroprotective role of oil (Sevenich *et al.*, 2015). The formation of low water activity refuges within the food matrix due to the presence of fat/oil may confer protection against pressure. Bacteria with hydrophobic surfaces may be attracted to these refuges where there is much lower water content and, therefore, lower HPP-induced inactivation (Georget *et al.*, 2015).

Complex food matrices may, therefore, represent a particular challenge to ensure microbial inactivation by HPP due to the potential presence of a number of ingredients that could confer a baroprotective effect. Well-defined differences in microbial reduction between HPP-treated complex and non-complex matrices were observed by Tassou *et al.* (2007). These researchers compared the pressure resistance of *Staphylococcus aureus* inoculated into buffer solution, milk, and sliced ham. The results showed a greater reduction in *S. aureus* counts for the buffer solution and milk than for the meat product. This indicates that solid, complex foods may provide a greater degree of protection for microbial cells than liquid foods.

6.2.3. The effect of high-pressure processing on wheat functional properties

Pressure may induce changes to the structure of food biopolymers such as proteins and carbohydrates. Studies on the effect of high pressure on wheat gluten proteins have reported changes in the rheological properties of gluten upon HPP. For instance, Apichartsrangkoon *et al.* (1998) treated hydrated wheat gluten samples at pressure and temperature levels ranging from 200-800 MPa and 20-60°C, respectively, for 25 or 50

min. A texture profile analysis of HPP-treated gluten revealed that the number of disulphide bonds comprising the gluten structure increased as the treatment variable levels increased, but disulphide crosslinking only became significant when samples were held at 800 MPa for 50 min. Similar structural changes in gluten proteins were also observed by Kieffer *et al.* (2007), who concluded that cleavage and rearrangement of disulphide bonds were responsible for the strength and resistance to extension observed in the HPP-treated gluten.

Besides proteins, wheat starch granules can also undergo structural changes under high pressure environments. Douzals *et al.* (1998) exposed wheat starch suspensions to pressure levels of 600 MPa at 25°C for 15 min. Results showed that pressure induced gelatinization of starch granules, but their granular structure was preserved. In addition, it was also observed a limited retrogradation of HPP-treated gels compared to the control gels. Knorr *et al.* (2006) suggested that Van-der-Waals forces and hydrogen bonds among amylopectin double-helices in starch granules are stabilized due to a restriction in the unwinding and dissociation of amylopectin under high pressure conditions. As a result, unlike heat-induced gelatinization, pressure-induced gelatinization results in incomplete disintegration of crystalline regions within starch granules (Kim *et al.*, 2012).

The effect of HPP on gluten protein structure and starch gelatinization may, subsequently, impact the rheological properties of flour-based doughs. For instance, McCann *et al.* (2013) observed an increase in dough strength, a prolonged development time and greater stability during mixing in HPP-treated wheat-flour doughs (33% moisture; 600 MPa, 5 min). The authors argued that gluten protein aggregation induced by high pressure promoted the formation of a fibril protein network which resulted in

improved mixing properties. Studies using more complex matrices have also been conducted. Bárcenas *et al.* (2010) reported that increasing the pressure levels from 50 to 250 MPa caused a significant increase in hardness and adhesiveness of wheat bread-doughs, while the increase in exposure time reduced the dough stickiness.

Barcenilla *et al.* (2016) studied the effect of HPP on batters and cakes properties. The results showed that HPP increased the density, G', and G'' of batters. Cakes made from HPP-treated batters presented a lower volume, darker crust, and harder texture than cakes obtained from control, untreated batters. Moreover, when the pressure levels were raised, higher values for the specific volume were obtained. Therefore, optimization of HPP parameters such as pressure, time, and temperature, as well as adjustments in product formulations may be required in order to limit negative effects on final product characteristics.

7. QUANTITATIVE MICROBIAL RISK ASSESSMENT: AN EFFECTIVE TOOL TO ASSESS AND CONTROL POTENTIAL MICROBIOLOGICAL RISKS

The field of quantitative microbial risk assessment (QMRA) is relatively young. However, during the last decades, the use of QMRA has increased considerably in popularity among food manufacturers and public health authorities to evaluate the safety of the food supply chain and as a basis for risk management decisions. The increased availability of data on the identification and occurrence of microbial foodborne pathogens, their impact on human health, as well as the development of advanced mathematical techniques, have facilitated the use of the QMRA framework to explore emerging disease threats and to assess pathogen exposure (USDA-FSIS/EPA, 2012).

7.1. The risk assessment framework

A quantitative microbial risk assessment (QMRA) has several components including problem identification, hazard identification and characterization, dose-response assessment, exposure assessment, risk characterization, risk management, as well as communication of risks (Haas *et al.*, 2014d). A risk assessment begins with defining the problem, its boundaries and characteristics, the populations of concern, and the desired level of protection against infection or disease.

Once the problem has been defined, the causative agent(s) must be identified and characterized. Hazard identification is a key component of the QMRA. It refers to the identification of the microbiological agents of interest (e.g., *E. coli* O157:H7) and their possible adverse effects on human health (Haas *et al.*, 2014b). Much of the data used in this step is gathered from available literature on clinical studies from both human and animal subjects. The next step in the QMRA is to examine the relationship between hazard and effect. This is carried out through a dose-response assessment, which aims to mathematically characterize the relationship between the likelihood of adverse health effects (e.g., infection, disease, death) and a known level of microbial exposure (Haas *et al.*, 2014a).

The next step in the QMRA is the exposure assessment, which is considered a critical component. The objective of the exposure assessment is to determine the route of entry and distribution of the microorganisms of interest, the length and magnitude of the exposure, and the size of the population at risk (Haas *et al.*, 2014c). The exposure analysis also takes into account the concentration and the prevalence of the microorganisms of interest. The inactivation rates and survival of microorganisms during

food processing treatments are also considered during the exposure assessment to estimate the final concentration of doses to populations.

Another component of the QMRA is the characterization of the risk. This component is designed to integrate the data collected in the previous steps, including the hazard identification and characterization, dose-response information and exposure assessment with the intent of estimating the magnitude of the risk and the probability of illness from exposure to that risk (Haas *et al.*, 2014e). In this step the uncertainty and variability of the hazards are also estimated. The outcome obtained from the risk characterization step is expressed as the probability of adverse effects on human health (e.g., 1/20 or 1 in a billion) due to exposure to a hazard under a given scenario.

The last component of the QMRA framework if the management and communication of risks. The goal of this step is to identify and evaluate possible risk management strategies to reduce or eliminate the risks (Haas *et al.*, 2014d). In this step the risk characterization is applied to management decision making. Factors such as political, economic, social, and public health may influence the decision on which control strategies will be implemented. An effective risk communication is also essential to accurately and appropriately communicate the results obtained from the QMRA to all stakeholders (USDA-FSIS/EPA, 2012).

7.2. The importance of a quantitative microbial risk assessment for the wheat supply chain

In a quantitative microbial risk assessment (QMRA), a modular process risk model is usually applied to split the food supply chain in basic modules that encompasses all the steps from “farm to table”, including industrial processing, retail and consumer handling (Nauta *et al.*, 2005). These steps are then linked together into a chain model. Stochasticity

is typically incorporated into this chain model by using probability distributions for uncertain model parameters or variables, such as prevalence and concentration of microorganisms (Nauta, 2000). A properly designed QMRA is, therefore, able to objectively and systematically collect relevant scientific evidence that takes into consideration all possible risk factors that may be encountered throughout the supply chain. The resulting model and information may be used to assess the current risk of pathogen contamination and to predict the effect of potential risk mitigation strategies that can be applied at each step of the food supply chain.

Extensive work has been performed on assessing the risk of microbial pathogens in food products of animal origin, such as *E. coli* O157:H7 in beef (USDA-FSIS, 2002), *Listeria monocytogenes* in ready-to-eat meat products (Endrikat *et al.*, 2010), and *Salmonella* Enteritidis in egg products (Hope *et al.*, 2002). However, recent foodborne outbreaks have been linked to novel vehicles of transmission such as wheat flour, thus creating the need to assess the risk of products of plant origin (Neil *et al.*, 2011; US-FDA, 2017). More recently, risk assessments have been applied as a tool to improve the food safety management system for the fresh produce supply chain (Jacxsens *et al.*, 2010). But limited studies have been focused on assessing the risks of microbial hazards on the wheat flour supply chain (Gilbert *et al.*, 2010). Therefore, the establishment of a QMRA for pathogenic microorganisms such as *E. coli* O157:H7, non-O157 STEC, and *Salmonella* spp. along the flour supply chain can be a preliminary model to monitor and prevent microbial food safety risks associated with wheat-based products.

Using risk assessment modeling as a baseline, the impact of pre- or post-milling interventions directed towards reducing microbial load on the ultimate safety of wheat-

based products (e.g., flour and flour-based doughs) derived from potentially contaminated grain can be evaluated. Additionally, the risk associated with consumption of contaminated wheat-based products can be projected by mapping and linking the changes in prevalence and concentration of pathogenic microorganisms along the wheat processing chain using QMRA. Therefore, by using a risk-based assessment that takes into consideration different steps during the wheat processing chain, foodborne hazards associated with wheat-based products could be reduced. A wheat-based QMRA model could then be used by the industry in evaluating preventive control strategies to safeguard consumers.

8. CONCLUDING REMARKS

Pathogens are emerging on previously unrecognized food vehicles, such as wheat flour, which is a widely consumed commodity. Despite its low water activity, wheat flour and derived products have shown the potential to be associated with foodborne outbreaks. In 2017, wheat flour was implicated as a contamination source of *E. coli* O121 in the consumption of flour-based products resulting in dozens of illnesses across six provinces in Canada; consequently, millions of pounds of flour had to be recalled. The increased use of wheat flour to produce refrigerated and frozen products, which have not undergone a validated microbial-reduction step during processing, and the popular practice of eating uncooked dough among consumers, represent a serious public health hazard of great consequences.

As a field commodity, wheat grain is exposed to naturally occurring microbiological contamination throughout the production chain that may compromise the overall safety of flour and other derived products. Microorganisms that may have passed

into flour during milling can survive in a dormant state, retaining their viability and the potential to multiply if flour is incorporated into a more receptive environment for growth, such as dough or batter. Additionally, certain steps employed during the production of flour (i.e., tempering) as well as cross-contamination from mill equipment and flour/grain residues may increase the risk associated with milling-end products. Therefore, the implementation of pre- and/or post-milling interventions geared towards reducing pathogens is essential to mitigate the risk of microbial contamination in wheat-based products. Moreover, the effect of pre- and post-milling interventions on the likelihood that consumers will encounter contaminated wheat flour and flour-based dough products could be predicted under different scenarios through the use of a quantitative microbial risk assessment (QMRA). The information obtained from QMRA may lead to better decision-making regarding microbial control measures that could be applied throughout the wheat processing chain to safeguard consumers.

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CHAPTER 2

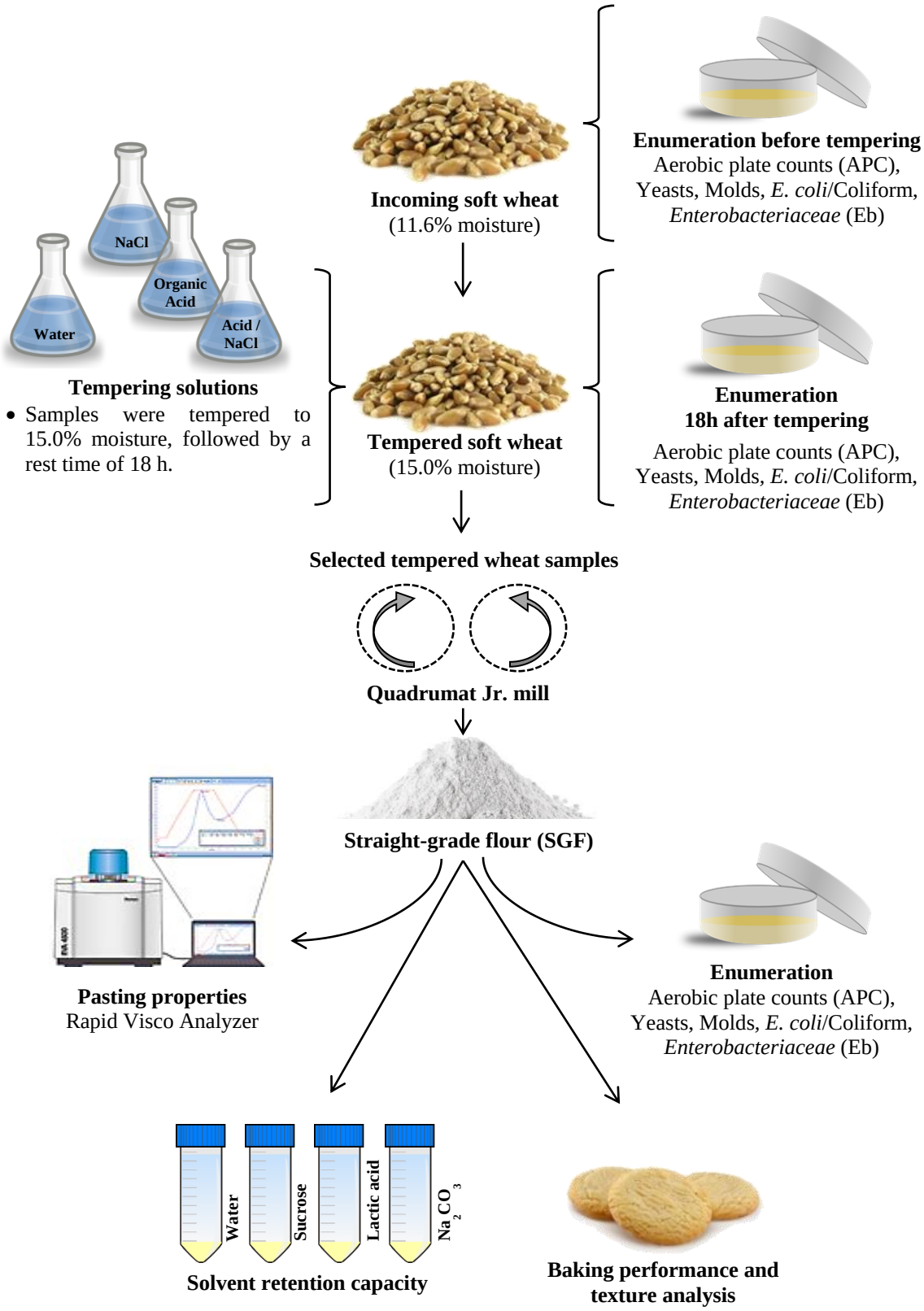
EFFECT OF SALINE ORGANIC ACID SOLUTIONS APPLIED DURING SOFT WHEAT TEMPERING ON MICROBIAL LOAD AND FLOUR FUNCTIONALITY

CHAPTER 2. EFFECT OF SALINE ORGANIC ACID SOLUTIONS APPLIED DURING SOFT WHEAT TEMPERING ON MICROBIAL LOAD AND FLOUR FUNCTIONALITY

ABSTRACT

The initial microbial load of the grain is a safety concern for wheat-based products. Because tempering hard wheat with saline organic acid solutions prior to milling has been shown to reduce the microbial load of wheat without affecting flour functionality, the objectives of this study were 1) to evaluate the efficacy of saline organic acid solutions to reduce the natural microbiota of soft wheat grain and flour, and 2) to assess the impact of saline organic acid solutions on the functional properties of soft wheat flour. Soft wheat was tempered to 15% moisture under controlled (18 h, 22-24 °C, 60% rh), aseptic conditions by adding water (control) or tempering solutions containing acid (acetic or lactic; 2.5% and 5.0% v/v), NaCl (26.6% w/v), or a combination thereof. Tempered wheat and milling streams were analyzed for aerobic plate count (APC), coliforms, *Enterobacteriaceae* (Eb), yeasts, and mold. The microbial load of the tempered wheat was significantly reduced by all saline organic acid treatments. The combination of 5.0% lactic acid-NaCl was the most effective against APC and Eb, with average reductions of 3.1 and 4.5 log CFU/g, respectively. In addition, this tempering solution yielded flour with a significantly lower microbial load compared to the control. Tempering with saline organic acid solutions did result in minor, but significant, variations in the solvent retention capacity profile of some flours. However, the treatments did not cause significant changes in the pasting profile of flours or in the physical characteristics and texture profile of cookies. Therefore, the addition of organic acids and NaCl to the tempering water reduces the risk of microbial contamination with minimal impacts on the functional properties of soft wheat flour.

VISUAL SUMMARY



1. INTRODUCTION

Traditionally consumers have had a perception that wheat flour poses minimal microbiological safety risk. This perception has been based on the erroneous belief that microorganisms cannot survive in the low moisture environment of flour. However, vegetative microbial cells can use several survival strategies such as biosynthesis of osmoprotectants, use of alternative sigma factors, and entry into a metabolically dormant state to subsist in harsh, dry conditions for prolonged periods of time (Finn *et al.*, 2013). Therefore, wheat flour may contain viable microorganisms capable of causing not only food spoilage but also serious illness (Richter *et al.*, 1993; Sperber, 2007).

Once flour is incorporated into a more receptive environment for microbial growth, such as batters or mixes, microorganisms may emerge from dormancy, thereby increasing the risk of foodborne illnesses. The increased demand for refrigerated or frozen, ready-to-bake products, and the popular practice of eating raw or partially-cooked bakery goods among consumers (Ardent Mills, 2010), has resulted in serious public health consequences. Indeed, in recent years, wheat flour and flour-based products have been involved in several foodborne disease outbreaks in various countries (Zhang *et al.*, 2007a; NZFSA, 2008; Neil *et al.*, 2011; PHAC, 2017; US-FDA, 2017).

As a raw agricultural commodity, wheat is prone to microbial contamination not only while it is the field but also as it moves through the supply chain. Therefore, wheat grain entering the milling system may carry a wide and varied microbiota (Berghofer *et al.*, 2003; Eglezos, 2010; Sabillón *et al.*, 2016b). These microorganisms are located mainly on the outer surface of the grain (Laca *et al.*, 2006). Since none of the operations carried out during milling result in substantial microbial reduction, microbial

contaminants in the grain surface may be transferred to flour as wheat passes through the milling process (Berghofer *et al.*, 2003). Therefore, the initial microbial load of the wheat grain entering the milling system has a strong influence on the ultimate safety of wheat flour.

Previous research studies have evaluated several methods to reduce the microbial load of wheat grain prior to milling. Early investigations on this topic included the application of antimicrobial agents such as chlorine, ozone, and acidified ozone by means of washing, soaking, and fluidization systems (Ibanoğlu, 2002; Dhillon *et al.*, 2007; Dhillon *et al.*, 2009; Dhillon *et al.*, 2010); however, these methods resulted in the addition of more moisture to the grain than required for milling, thus introducing the need for a drying step. Alternative methods for wheat decontamination have also been evaluated including atmospheric and low-pressure cold plasma, and pulsed light (Selcuk *et al.*, 2008; Aron Maftei *et al.*, 2014; Los *et al.*, 2018). Nevertheless, these methods may modify the quality and functional properties of the resulting flour, and the practicality and cost-effectiveness of these treatments may be questionable.

Exploring and developing practical and effective antimicrobial treatments that could be used by the wheat milling industry to deliver safer milled products is, therefore, essential to mitigate the risk of contamination. Recently, the value of using the water added to hard wheat during tempering as a vehicle to deliver antimicrobial agents, including organic acids and sodium chloride was evaluated (Sabillón *et al.*, 2016a). The tempering solution containing a combination of NaCl 26% (w/v) and lactic acid 5.0% (v/v) achieved substantial reductions in aerobic bacteria and *Enterobacteriaceae* counts of 3.6 and 4.7 log CFU/g, respectively, without negatively affecting the functional

properties of straight-grade flour (Sabillón *et al.*, 2017). Therefore, these saline organic acid solutions have the potential to improve the microbiological safety of other wheat classes, such as soft wheat, with minimal impact on the functionality of the resulting flour. Soft wheats are used in many flour-based products that are more likely to be consumed raw or undercooked than hard wheats, such as cookie dough, cakes, biscuits, and brownies. Thus, the objectives of this work were 1) to evaluate the efficacy of saline organic acid solutions to reduce the natural microbiota of soft wheat grain and flour, and 2) to assess the impact of saline organic acid solutions on the functional properties of soft wheat flour.

2. MATERIALS AND METHODS

2.1. Materials

One hundred pounds of soft red winter wheat were obtained from a commercial milling facility in the US. Sodium chloride (NaCl) and lactic acid (85%) were obtained from Fisher Scientific™ (Pittsburgh, PA). Glacial acetic acid was obtained from VWR (Radnor, PA). All chemicals used were United States Pharmaceutical (USP) or Food Chemicals Codex (FCC) grade.

2.2. Preparation and application of tempering solutions

The experiment was divided into two phases. The first phase was designed to evaluate the effectiveness of tempering solutions containing a single antimicrobial agent (i.e., organic acid (acetic or lactic; 2.5% and 5.0% v/v) or NaCl (26.6% w/v)) in reducing microbial load. The tempering solutions were prepared as described by Sabillón *et al.* (2016a). Briefly, each organic acid was mixed with sterile distilled water in appropriate

proportions to yield a solution wherein the final acid concentration was 2.5% and 5.0% v/v, respectively. Likewise, a saline solution of 26.6% w/v was prepared.

The second phase of the project was designed to test the hypothesis of whether combining two different mechanisms of microbial inhibition (i.e., organic acid and salt) would be more effective at reducing microbial load during tempering. Therefore, individual organic acid and NaCl solutions were combined together in appropriate amounts to yield a solution wherein the final concentrations were 2.5 or 5.0% v/v for organic acids and 26.6% w/v for NaCl. In total, between the two phases of the experiment, 9 different tempering solutions were prepared (**Table 2-1**). Sterile distilled water was used as a control.

Table 2-1. Antimicrobial solutions prepared and tested during tempering of soft wheat.

Experiment Phase	Treatment ID	Antimicrobial Agent(s)	Concentration
1	Control	None (Water)	0.0%
	1	Acetic Acid	2.5%
	2	Acetic Acid	5.0%
	3	Lactic Acid	2.5%
	4	Lactic Acid	5.0%
	5	Sodium Chloride (NaCl)	26.6%
2	Control	None (Water)	0.0%
	6	Acetic Acid + NaCl	2.5% + 26.6%
	7	Acetic Acid + NaCl	5.0% + 26.6%
	8	Lactic Acid + NaCl	2.5% + 26.6%
	9	Lactic Acid + NaCl	5.0% + 26.6%

Each tempering solution was applied to three 1 kg samples of soft wheat. Wheat was placed in sterile plastic bags and tempered to 15% moisture under controlled (22-24°C, 60% RH), aseptic conditions by adding sterile distilled water (control) or tempering solutions containing organic acid, NaCl, or a combination. Tempering solutions were applied to wheat with an atomizer to ensure even distribution. The

moistened wheat samples where then allowed to rest for 18 h to enable an optimum moisture distribution and kernel suitability for milling. The amount of tempering solution needed (40 mL/kg of wheat) to reach the optimum milling moisture (15.0%) was calculated based on the initial moisture content of the wheat samples (11.6%). The initial moisture content was determined according to AACC forced-air-oven method 44-15.02 (AACCI, 2018). After the resting period, 100 g of each tempered wheat sample were set aside to determine the microbial load before milling.

2.3. Experimental milling

The tempering treatments with the highest overall microbial reduction were selected to evaluate their impact on the microbiological quality and functional properties of flour. Therefore, samples tempered with the selected solutions (three replicates, 900 g each) were milled with a Brabender Quadrumat Junior laboratory roller mill (C.W. Brabender® Instruments, Inc.; South Hackensack, NJ) following AACC standard method 26-50.01 (AACCI, 2018). Milling room temperature and relative humidity were kept at 22-24°C and 60%, respectively, to ensure reproducibility of results. To prevent cross-contamination, before milling each sample the mill interior was brushed and vacuum-cleaned to dislodge and remove any flour/bran residues in openings and passages between roll housing and sifter compartments. In addition, the feed hopper and the flour and bran collection drawers were cleaned and sanitized with 70% ethanol before milling each sample. The flour and bran fractions obtained from milling were transferred, aseptically, into sterile plastic bags. Flour was mixed thoroughly before further analysis.

2.4. Microbiological analysis

To determine the impact of tempering solutions on microbial load, wheat kernels before and after tempering as well as milled fractions (i.e., flour and bran) were analyzed for aerobic mesophilic bacteria (aerobic plate counts [APC]), *Enterobacteriaceae* (Eb), *E. coli*/coliform, yeasts and molds. To perform the microbial analysis, 25 g of grain, flour, or bran were placed along with 225 mL of sterilized 0.1% peptone solution in a sterile plastic bag and mechanically mixed with a stomacher blender (Stomacher® 400, Seward Ltd, Bohemia, NY) for 90 s. After mixing, serial decimal dilutions were prepared using sterilized 0.1% peptone solution.

For APC, dilutions were spread plated in duplicate on standard methods agar (Acumedia; Lansing, MI) and incubated at 35 °C for 48 h, according to standard procedures (Maturin and Peeler, 1995). For yeast and mold counts, dilutions were spread plated in duplicate on dichloran rose begal chloramphenicol agar (Acumedia) and incubated at 25 °C in the dark for 5 d, according to standard procedures (Tournas *et al.*, 1998). *E. coli*/Coliform and Eb counts were determined using appropriate Petrifilm plates (3M Microbiology; Saint Paul, MN), according to official methods 991.14 and 2003.01 (AOAC International, 2013), respectively. Dilutions were inoculated in duplicate on Petrifilm plates and incubated at 37°C for 24 h. The limit of detection for all microbiological tests performed was 10 colony forming units (CFU) per gram.

2.5. Flour functionality tests

2.5.1. pH and acid content

The pH of flour was measured according to AACC standard method 02-52.01 (AACCI, 2018). A 10-g flour sample along with 100 mL of distilled water were placed in

an Erlenmeyer flask and mixed for 15 min with a magnetic stirrer, followed by a 10 min rest. The supernatant liquid was decanted into a clean Erlenmeyer flash and the pH determined with a calibrated Thermo Scientific Orion 2-Star Benchtop pH meter. After measuring the pH, the acid content of the supernatant was determined by titrating with 0.1 N NaOH to a final pH of 7.0. The titratable acidity was expressed as the micromoles of H+ per gram of sample.

2.5.2. Pasting properties

The pasting properties of flour were evaluated by a Rapid Visco Analyzer (RVA) (Model 4S, Newport Scientific; Warriewood, NSW, Australia) following AACC standard method 76-21.01 (AACCI, 2018). A 3.5 g flour sample (adjusted to 14% moisture basis) along with 25 mL of distilled water were added into a test canister and mixed by vigorously plunging the blade through the mixture for 30 s to avoid formation of clumps during the analysis. Samples were analyzed using the Standard-1 test profile in the RVA software. Maximum viscosity before the onset of cooling (peak viscosity), through and breakdown viscosity, minimum viscosity after peak, final viscosity, and time to peak viscosity were recorded for further analysis.

2.5.3. Solvent retention capacity

The solvent retention capacity (SRC) tests were performed according to AACC standard method 56-11.02 (AACCI, 2018). Flour (5 g) was placed in conical centrifuge tubes (50 mL) along with 25 g of each standard solvent (deionized water, 5% sodium carbonate, 5% lactic acid, and 50% sucrose) and kept for 20 min with intermittent agitation in a vortex mixer at 5, 10, 15 and 20 min, followed by 15 min centrifugation at

1,000 x g at room temperature and a 10 min drainage. The pellets were weighed and SRC values were calculated.

2.5.4. Baking performance

The baking quality of flours for the production of cookies was assessed according to AACC standard method 10-50.05 (AACCI, 2018). After thoroughly mixing the ingredients, cookie dough was rolled out to a thickness of 0.7 cm and cut out into 6 cm diameter pieces with a cookie cutter mold. Cookies were baked in a reel type oven (National Manufacturing Corporation; Lincoln, NE) at 205°C for 10 min. After cooling for 30 min, the average diameter (width) and thickness of six cookies was obtained and the spread factor (width/thickness ratio) calculated. Baking experiments were replicated on three different days and the results presented are the average of three trials.

2.5.5. Texture analysis of cookies

The texture of baked cookies was assessed no later than 3 h after baking using a texture analyzer (Model TA-TX2, Texture Technologies; Scarsdale, NY) equipped with a 25-kg load cell and a 3-point bending rig, following the American Institute of Baking (AIB) standard procedure for cookie hardness (AIB, 2012). The gap between the support beams was set to 40 mm to be half the diameter of the cookies. The support rig distance was kept constant throughout the analysis to ensure comparability of results. Each cookie was precisely centered on the support beams to measure the maximum peak force, an index of cookie hardness; and the distance between the trigger force and the maximum peak force, an index of flexibility. Texture experiments were replicated on three different days and the results presented are the average of three trials. Six cookie samples were assessed in each replication.

2.6. Statistical analysis

An analysis of variance (ANOVA) was carried out to determine differences among tempering solutions for microbial load reductions and functional properties of flour. For those tempering solutions with a combination of antimicrobial agents, a factorial type of treatment structure with control was used to test for main effects (i.e., acid type and concentration), interactions, and comparisons to the control. Significant differences ($P < 0.05$) between means were separated by Tukey's test. All statistical analyses were performed using SAS software (version 9.3, SAS Institute; Cary, NC, U.S.) with a significance level of $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Inactivation of wheat natural microbiota by tempering with water, organic acid or saline solutions

The efficacy of saline organic acid tempering against the natural microbiota of soft wheat is presented in **Figure 2-1**. Tempering with water resulted in no significant differences between microbial populations of tempered and incoming wheat samples. Similar results were observed by Dhillon *et al.* (2009) and Sabillón *et al*. (2016a), who reported no significant changes in microbial counts after washing (2 L/kg; 3 min) durum wheat or tempering hard wheat with distilled water, respectively. Nevertheless, other studies have reported a slight increase in total bacteria and mold counts after wheat tempering under controlled conditions (Hu *et al.*, 2016). Moreover, a microbiological survey carried out by Berghofer *et al.* (2003) at a commercial wheat milling facility, also found significantly higher mesophilic aerobic counts in wheat grain after tempering with water. This later finding highlights the fact that under commercial, non-controlled/aseptic

conditions, the traditional tempering process may facilitate microbial growth on wheat grain if proper control measures are not implemented.

Significant reductions in wheat background microbiota were only achieved after adding antimicrobial agents to the tempering water. Adding sodium chloride to the tempering water resulted in 0.68 and 0.97 log CFU/g reduction in yeast and mold counts, respectively (**Fig. 2-1**). No significant changes in APC, Eb and coliform counts were observed after tempering with saline solution. Similar reductions in yeast (1.4 log CFU/g) and mold (1.0 log CFU/g) counts were obtained by Sabillón *et al.* (2016a) after tempering hard wheat with solutions containing 26% w/v salt. Although the ability of microorganisms to tolerate salt stress varies widely among species, many fungi species tend to be more sensitive to osmotic stress than bacteria (Yan *et al.*, 2015).

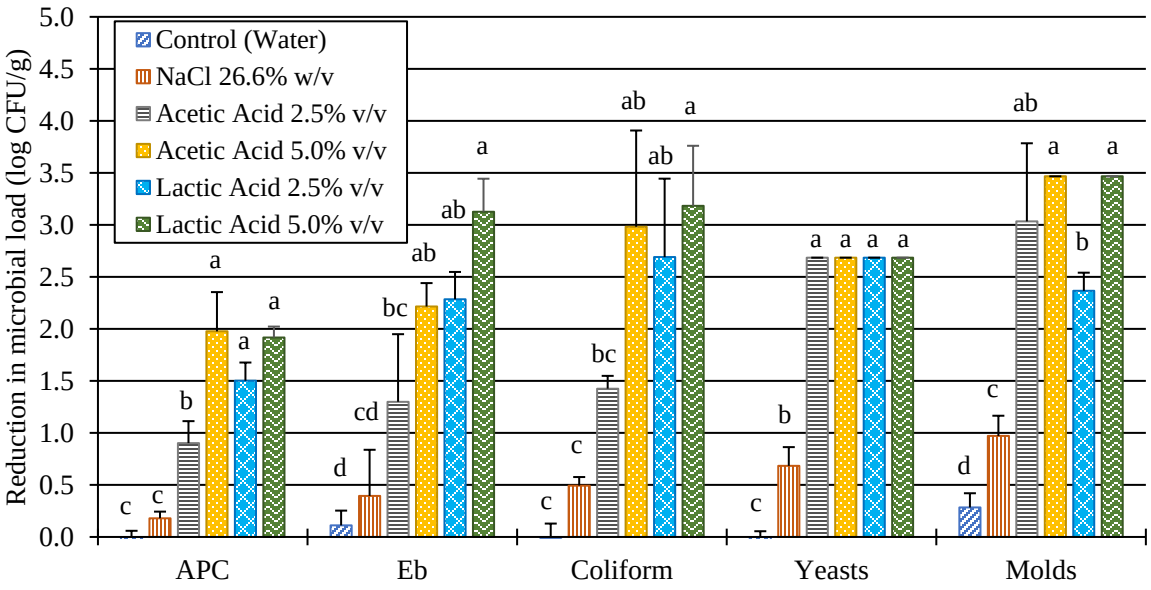


Figure 2-1. Effect of tempering with water or solutions containing salt or organic acids on reducing the microbial load of soft wheat. APC, aerobic plate count; Eb, *Enterobacteriaceae*. Legend: NaCl, sodium chloride; percentage values represent concentration. Error bars denote ± standard deviation. Log reduction values, within the same microorganism, that share the same letter are not significantly different from one another ($P > 0.05$).

The reduction of water activity in the food matrix and the toxicity of Na^+ and Cl^- ions are the primary mechanisms of microbial inhibition exerted by salt (Ravishankar and Juneja, 2000). In addition, the presence of NaCl ions in the environment surrounding the microorganisms causes water efflux through their cell membranes, resulting in dehydration and loss of cell turgor (Davidson *et al.*, 2013). Therefore, to maintain homeostasis, microbial cells must expend significant amounts of energy, which eventually results in reduced growth rates or cell death.

Unlike NaCl, the addition of organic acids to the tempering water significantly reduced the overall microbial counts in the incoming wheat (**Fig. 2-1**). NaCl and organic acids differ in their mechanisms of microbial inhibition. NaCl ions can cause osmotic shock in microbial cells, whereas organic acids in their undissociated form have the ability to freely permeate the cell membrane, resulting in acidification of the cytoplasm, which in turn affects overall cell physiology (Davidson *et al.*, 2013). Tempering solutions containing a 2.5% acetic acid concentration caused a reduction of 0.90 log CFU/g in the aerobic bacteria population. Tempering with lactic acid at 2.5%, however, resulted in a significantly greater reduction (1.50 log CFU/g) than acetic acid at the same concentration. Increasing the concentration of acetic acid from 2.5% to 5.0% led to a significant further reduction in APC counts by 1.08 log CFU/g. In contrast, the increase in lactic acid concentration did not produce a substantial additional reduction effect on APC counts. Similar reduction levels in APC counts were observed in a previous study after tempering hard wheat with organic acid solutions (Sabillón *et al.*, 2016a).

Several research studies have assessed the use of different methods, including washing and atmospheric cold plasma treatments, to reduce the aerobic bacteria counts in

wheat. However, these methods resulted in much lower reductions than in the present study using organic acid solutions. For instance, Dhillon *et al.* (2009) reported that washing durum wheat for 3 min with ozonated water reduced the APC counts by 0.2 log CFU/g. More recently, Los *et al.* (2018) reported that 20 min of high-voltage atmospheric cold plasma (HV-ACP) processing of wheat grain reduced the mesophilic bacteria population by 1.5 log CFU/g.

Although increasing the lactic acid concentration in the tempering water from 2.5% to 5.0% did not significantly improve the reduction in APC counts, it did enhance the efficacy of the solution against Eb, resulting in an average reduction of 3.13 log CFU/g (**Fig. 2-1**). Tempering wheat with solutions containing acetic acid at 2.5% or 5.0%, or 2.5% lactic acid resulted in an average reduction in Eb counts of 1.93 log CFU/g. In a previous study on hard wheat, tempering solutions containing either 5.0% acetic or lactic acids achieved an average reduction in Eb counts of 2.40 log CFU/g (Sabillón *et al.*, 2016a). Reduction trends similar to those obtained for Eb were observed for coliform counts. For instance, the tempering solutions containing 2.5% or 5.0% lactic acid, or 5.0% acetic acid resulted in comparable reduction levels in coliform counts, with an average reduction of 2.95 log CFU/g (**Fig. 2-1**).

Tempering solutions containing organic acids resulted in a much higher reduction in yeast and mold counts than those achieved by saline solutions. The mold population in the incoming wheat was reduced from 3.47 log CFU/g to below detectable levels after tempering with lactic acid (5.0%) or acetic acid solutions (2.5% and 5.0%) (**Fig. 2-1**). Similarly, tempering with organic acids, regardless of acid type and concentration,

resulted in a reduction in yeast counts of 2.69 log CFU/g, which resulted in levels that were below the detection limit of the method for this group of microorganisms.

In a previous study, the greatest reduction in yeast (4.7 log CFU/g) and mold (3.8 log CFU/g) counts were obtained by lactic and acetic acid treatments at the 5.0% level, which also resulted in counts below the limit of detection of the method used for this group of microorganisms (Sabillón *et al.*, 2016a). Decontamination treatments such as washing or tempering with ozonated water have been less effective at reducing the yeast and mold populations of wheat grain when compared to the best treatments described in this study. İbanoğlu (2001) reported that tempering of wheat with ozonated water (11.5 ppm) resulted in a reduction of 1.0 log CFU/g in yeast/mold counts. Similarly, washing wheat kernels with ozonated (16.5 ppm) or chlorinated (700 ppm) water reduced the yeast/mold counts by 0.50 and 1.9 log CFU/g, respectively (Dhillon *et al.*, 2009).

3.2. Inactivation of wheat natural microbiota by tempering with acidic saline solutions

In a previous study, it was reported that combining organic acid and salt in the tempering water was more effective at reducing the microbial load of hard wheat than each antimicrobial agent alone (Sabillón *et al.*, 2016a). Therefore, the tempering assay was repeated with solutions containing a combination of organic acid and NaCl. The reductions of wheat natural microbiota by acidic saline solutions are shown in **Figure 2-2**.

The analysis of variance showed that the level of microbial reduction was determined primarily by the type of organic acid present in the tempering solution, followed by its concentration. In general, microbial reduction levels achieved by solutions containing lactic acid were significantly higher than those containing acetic acid at the same concentration level. Also, increasing the acid concentration resulted in a

higher microbial reduction level. Moreover, depending upon the acid type, some antagonistic and synergistic interactions were observed when NaCl was mixed together with organic acid in tempering solutions.

An enhanced antimicrobial effect was observed when NaCl and lactic acid were combined. Adding NaCl to the tempering solution containing 5.0% lactic acid further increased the microbial reduction from 1.92 to 3.07 log CFU/g for APC and from 3.13 to 4.49 log CFU/g for Eb, which suggests a synergistic interaction (**Figs. 2-1, 2-2**). In fact, the combination of lactic acid at 5.0% and NaCl was the most effective treatment against these two groups of microorganisms. Moreover, this tempering solution reduced the Eb (4.49 log CFU/g), coliform (3.52 log CFU/g), yeast (2.69 log CFU/g), and mold (3.47 log CFU/g) populations to levels that were below the limit of detection of the methods used to quantify these microorganisms. Increasing the lactic acid concentration in the acid/NaCl mixture from 2.5% to 5.0% caused an additional reduction of 1.00 and 1.86 log CFU/g in APC and Eb counts, respectively (**Fig. 2-2**). In a previous study, a strong synergistic interaction between lactic acid and NaCl was observed against the natural microbiota of hard wheat (Sabillón *et al.*, 2016a), which was attributed to the ability of lactic acid to permeate and disrupt the microbial cell membrane, thus facilitating the diffusion of NaCl ions into the cytoplasm.

Unlike the combination lactic acid and NaCl, adding NaCl to the tempering solutions containing acetic acid did not enhance the antimicrobial activity of the solutions. When comparing the microbial reductions achieved by acetic acid with and without NaCl, the reductions in APC and Eb were very similar (**Figs. 2-1, 2-2**). In fact, among the tempering solution containing a combination of antimicrobial agents, the

combination of acetic acid at 2.5% and NaCl was the least effective treatment for reducing the numbers of APC and Eb, with average reductions of 1.17 and 1.72 log CFU/g, respectively (**Fig. 2-2**).

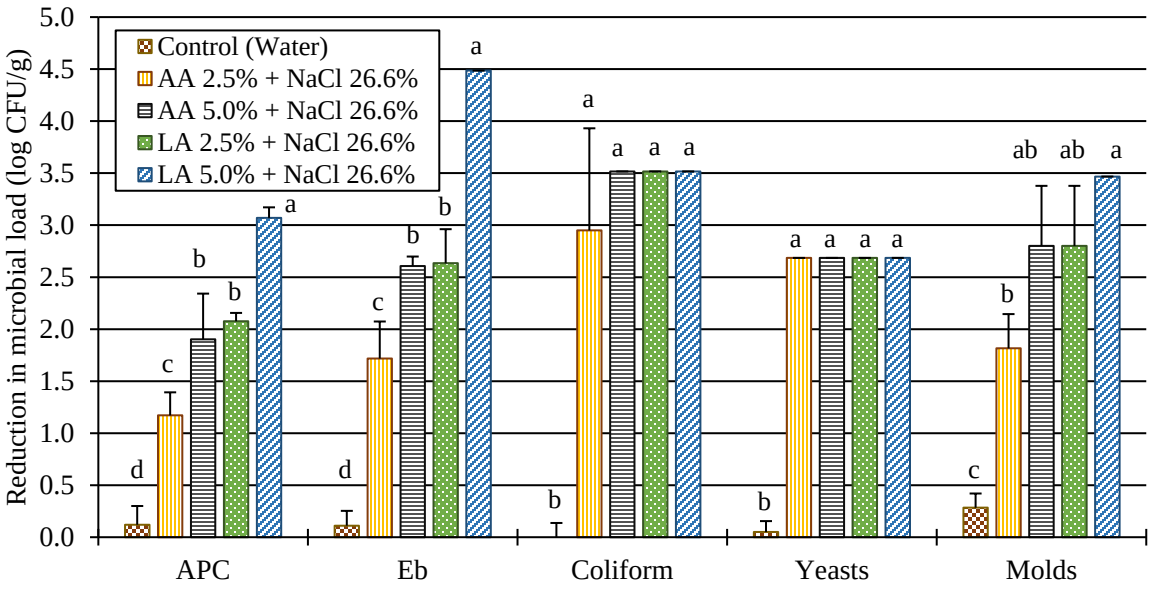


Figure 2-2. Effect of tempering with water or solutions containing a combination of salt and organic acid on reducing the microbial load of soft wheat. APC, aerobic plate count; Eb, *Enterobacteriaceae*. Legend: AA, acetic acid; LA, lactic acid; NaCl, sodium chloride; percentage values represent concentration. Error bars denote ± standard deviation. Log reduction values, within the same microorganism, that share the same letter are not significantly different from one another ($P > 0.05$).

Moreover, the reductions in mold counts obtained by any given combination of acetic acid-NaCl were substantially lower than the reductions achieved by acetic acid solutions alone (**Figs. 2-1, 2-2**), which suggest an antagonistic effect. In fact, in a previous study on hard wheat decontamination, an antagonistic interaction between acetic acid and NaCl was observed for some microorganisms (Sabillón *et al.*, 2016a), which was attributed to an increase in microbial membrane rigidity induced by NaCl ions, thus slowing down the diffusion of protonated acetic acid into the cytoplasm.

With regard to the inactivation of the natural microbial flora of wheat grain, previous studies have also evaluated the efficacy of combining antimicrobial agents. Dhillon *et al.* (2009) evaluated acidified ozonated water (20.5 ppm ozone, 1.0% acetic acid) as an antimicrobial treatment for wheat. After washing wheat kernels with this solution for 3 min, the APC and yeast/mold counts were reduced by 3.2 and 4.1 log CFU/g, respectively. In a follow-up study, Dhillon *et al.* (2010) utilized a fluidized bed system to applied a combination of gaseous ozone (6 ppm), acetic acid (0.5%) and ozonated water (26 ppm) to reduce the microbial load of durum wheat grain. A reduction in APC of 1.7 log CFU/g and 3.3 log CFU/g for yeast/mold counts was noted after the application of this treatment. Also using a fluidized bed, Dhillon *et al.* (2012) sprayed on buckwheat grain a solution containing a combination of acetic acid (5 mL/L) and acidic calcium sulfate (25 mL/L), resulting in reductions of 1.9 and 2.4 log CFU/g in APC and yeast/mold counts, respectively.

More recently, other methods and technologies for wheat decontamination, such as superheated steam and atmospheric cold plasma, have been evaluated. The application of superheated steam at 200°C for 30 seconds reduced the total bacteria population in dry wheat from 5.11 to 3.02 log CFU/g and the fungi population from 3.03 log CFU/g to none-detectable levels (Hu *et al.*, 2016). Los *et al.* (2018) applied high voltage atmospheric cold plasma to barley and wheat grain for 20 min followed by 24 h sealed post-treatment retention time. This treatment resulted in reductions of 2.4 and 2.1 log CFU/g in aerobic bacteria and mold counts, respectively, in barley grain; and reductions of 1.5 log CFU/g for APC and 2.5 log CFU/g for molds in wheat. Although the different methods used in the above-cited research studies have shown potential to reduce the

microbial load of wheat grain, they may have some drawbacks. For instance, the washing treatments employed by some researchers resulted in the addition of more moisture to grain than required for milling. With regards to cold plasma, this treatment has shown to modify the functional properties of flour (Misra *et al.*, 2015; Bahrami *et al.*, 2016) and, its implementation at a commercial scale may prove challenging. In general, higher microbial reduction levels in wheat grain, than those reported by others, were achieved in this study with the tempering solutions containing a combination of lactic acid and NaCl, without altering the target milling moisture of the grain.

3.3. Effect of acidic saline tempering solutions on microbial load of flour and bran

The impact of acidic saline solutions on the microbial load of wheat entering the milling system and the resulting flour and bran is presented in **Figure 2-3**. Tempering with water did not change the microbial load of the grain entering the mill; however, acidic saline solutions significantly improved the microbiological quality of the wheat grain prior to milling. The tempering solution containing a combination of 5.0% lactic acid and NaCl reduced the APC population from 5.56 to 2.37 log CFU/g and Eb, coliform, yeast and mold counts to levels that were below the limit of detection of the methods used to quantify these microorganisms (**Fig. 2-3A-E**). Likewise, tempering solutions containing acetic acid at any given concentration reduced the yeast counts to undetectable levels.

Despite the efficacy of acidic saline solutions to reduce the microbial load of grain prior to milling, various target microorganisms were detected in the bran and flour obtained from clean, non-contaminated wheat. For instance, coliform and mold counts were below the limit of detection in wheat grain after tempering with 5.0% lactic acid-NaCl solution,

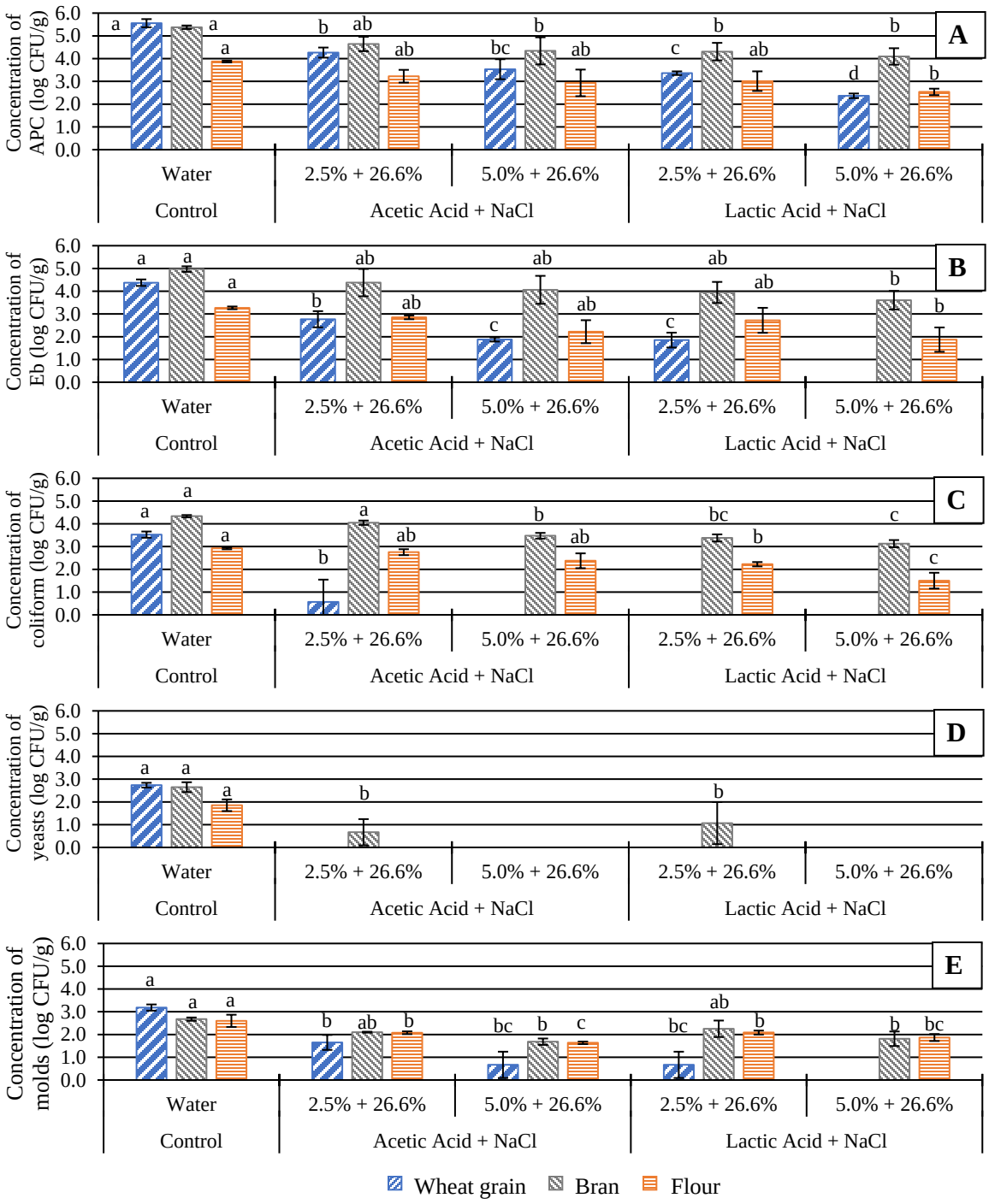


Figure 2-3. Concentration of APC (A), Eb (B), coliform (C), yeasts (D), and molds (E) in wheat grain, bran and flour after tempering with water or acidic saline solutions. APC, aerobic plate count; Eb, *Enterobacteriaceae*. Percentage values represent concentration. Error bars denote ± standard deviation. Mean values not shown were below the assay's limit of detection for that microorganism. Mean values, within the same microorganism and product, that share the same letter are not significantly different from one another ($P > 0.05$).

but the flour and bran obtained after milling exhibited coliform counts of 1.50 and 3.13 log CFU/g, and mold counts of 1.87 and 1.81 log CFU/g, respectively (**Fig. 2-3C and 2-3E**). This rise in microbial counts suggests that recontamination occurred as wheat passed through the various systems in the mill. However, despite the increase in microbial counts after milling, flour and bran fractions obtained from wheat grain tempered with saline lactic acid at 5.0% had a significantly lower microbial load than that observed for the control milled fractions.

A considerable amount of heat is generated by the mill rolls when breaking-apart the wheat kernels and reducing the endosperm to flour; hence, moisture condensation is likely to occur inside the mill equipment (Berghofer *et al.*, 2003). This moisture condensation may facilitate the accumulation of moist kernel/flour residues within the mill, which represents a possible reservoir of microorganisms. This mill stock, if not removed periodically, can continuously contaminate the stream of ground material passing through the equipment. Moreover, as wheat is being milled into flour, some shattering of the bran occurs resulting in a mixture of endosperm and bran particles. Since microbial contaminants are mostly located in the outermost layer of the grain (Laca *et al.*, 2006), there is a risk of cross-contamination when the bran particles come in contact with the endosperm during milling. Therefore, the increase in microbial counts observed in milled fractions was most likely due to cross-contamination from mill equipment and flour/grain residues that could not be removed during the cleaning procedures performed before milling each sample.

Nonetheless, in a previous study, it was reported that a consistent use of wheat tempered with acidic saline solutions helped improve the microbiological quality of mill

streams (Sabillón, 2014). For instance, after milling wheat tempered with saline lactic acid at 5.0% sixteen consecutive times in a Buhler experimental mill, aerobic bacterial counts in the flour streams decreased from 4.0 to 2.1 log CFU/g. In addition, populations of yeast, Eb and coliform were not detected in all milled fractions after sixteen consecutive runs. Therefore, a consistent use of wheat with a lower microbial load may contribute, over time, to reduce microbial establishment within the mill and thus the risk of cross-contamination during milling.

It is also noteworthy that the physical processes carried out during milling causes a redistribution of the microorganisms present in the surface of the grain. In general, microbial contaminants are concentrated in the bran fraction, with the flour being the least contaminated end product. After milling, the populations of Eb and coliform in the bran obtained from wheat tempered with water increased by 0.59 and 0.81 log CFU/g, while in the flour these microorganisms decreased by 1.11 and 0.60 log CFU/g, respectively, when compared to the counts in the grain entering the mill (**Fig. 2-3B-C**). Various studies have also reported higher microbial counts in the bran and germ fractions than in the flour obtained from the inner endosperm (Berghofer *et al.*, 2003; Manthey *et al.*, 2004; Sperber, 2007; Eglezos, 2010).

3.4. Effect of acidic saline solutions on flour functional properties

The pH and acidity profile of flours obtained from treated and control wheat are presented in **Table 2-2**. There were significant differences in pH and acid content among flour samples. In general, tempering with acidic saline solutions decreased the pH and increased the acid content of the resulting flours. Flour obtained from wheat tempered with saline acetic acid had a significantly higher acid content than the flour obtained from

Table 2-2. pH, acidity, pasting characteristics and solvent retention capacity profile of flours obtained from soft wheat tempered with water or acidic saline solutions.

Test Variable	Tempering Solutions [a]				
	Water (Control)	**Acetic Acid 2.5% + NaCl 26.6%**	**Acetic Acid 5.0% + NaCl 26.6%**	**Lactic Acid 2.5% + NaCl 26.6%**	**Lactic Acid 5.0% + NaCl 26.6%**
pH and Acid Content					
Flour pH	6.03 ± 0.03a	5.47 ± 0.04c	5.21 ± 0.02d	5.61 ± 0.02b	5.57 ± 0.03b
Titratable acidity (mmol/g)	0.15 ± 0.01d	0.27 ± 0.01b	0.34 ± 0.01a	0.22 ± 0.00c	0.23 ± 0.01c
RVA Parameters - Viscosity					
Peak (cP)	2254.33 ± 86.15a	2053.00 ± 80.32a	2213.67 ± 199.96a	2178.67 ± 56.57a	2116.00 ± 55.43a
Trough (cP)	1157.00 ± 61.00a	1067.00 ± 20.52a	1122.00 ± 171.72a	1190.33 ± 90.78a	1152.67 ± 20.50a
Breakdown (cP)	1097.33 ± 31.90a	986.00 ± 85.85a	1091.67 ± 28.59a	988.33 ± 47.65a	963.33 ± 48.68a
Final (cP)	2275.00 ± 99.14a	2100.00 ± 34.39a	2238.33 ± 287.11a	2237.33 ± 106.14a	2207.33 ± 36.02a
Setback (cP)	1118.00 ± 38.35a	1033.00 ± 24.43a	1116.33 ± 115.47a	1047.00 ± 23.64a	1054.67 ± 21.96a
Time to peak viscosity (min)	5.73 ± 0.07a	5.78 ± 0.04a	5.80 ± 0.07a	5.84 ± 0.04a	5.80 ± 0.00a
Pasting temperature (°C)	51.73 ± 0.78a	51.92 ± 1.56a	50.92 ± 0.78a	51.87 ± 0.80a	51.65 ± 1.75a
Solvent Retention Capacity					
Water (%)	57.49 ± 0.21a	55.74 ± 0.30b	56.20 ± 0.27b	57.45 ± 0.62a	56.59 ± 0.25ab
Sucrose (%)	108.40 ± 1.36c	111.72 ± 1.00ab	113.92 ± 1.72a	108.34 ± 0.30c	110.29 ± 0.17bc
Sodium carbonate (%)	75.77 ± 0.45a	75.28 ± 0.02a	75.74 ± 0.57a	75.27 ± 0.57a	74.84 ± 0.61a
Lactic acid (%)	120.23 ± 0.35a	103.12 ± 3.04bc	106.32 ± 1.20b	98.04 ± 4.99c	101.20 ± 1.94bc

[a] Mean values with the same letter in the same row are not significantly different from one another ($P > 0.05$).

wheat tempered with saline lactic acid solutions. Additionally, increasing the acetic acid concentration in the tempering solution from 2.5% to 5.0% caused a significant further reduction in pH and a substantial increase in the acid content of the flour. This further increase, however, was not observed in the flours obtained from wheat tempered with saline lactic acid solutions. The less polar nature of acetic acid seems to facilitate its passage through the outer bran layer of the kernel, thereby increasing the acid content of the inner endosperm to a greater extent than lactic acid.

In a previous study, similar trends in pH and acid content were observed in the straight-grade flour obtained from hard wheat tempered with saline organic acid solutions (Sabillón *et al.*, 2017). However, when comparing the acidity profile of flours between the two wheat classes studied, a higher acid content was observed in soft wheat. There are inherent differences in the endosperm characteristics between hard and soft wheat that could help explain the observed differences in acid content. Starch granules in soft wheat endosperm are loosely bound to proteins, while hard wheat endosperm is tightly bound to a dense protein matrix (Posner and Hibbs, 2005). Therefore, the capillary spaces between the endosperm starch granules in soft wheat may have facilitated the diffusion of organic acids, thus reaching a higher internal acid concentration.

The pasting characteristics and the solvent retention capacity profile of flours are also summarized in **Table 2-2**. In general, the pasting behavior of flour was not significantly altered by the tempering treatments. The flour obtained from wheat tempered with saline acetic acid at 2.5% exhibited lower viscosity values for all RVA parameters; however, its pasting behavior was not significantly different from that displayed by other treated or control flours. These results were in agreement with a previous study, where it was reported

that saline organic acid solutions applied during hard wheat tempering to reduce microbial load did not significantly change the pasting properties of the resulting straight-grade flours (Sabillón *et al.*, 2017). Various research studies have, however, reported that the addition of organic acids and NaCl to flour may alter some viscoelastic characteristic such as peak and final viscosity, dough-development time, and dough stability during mixing (Ganz, 1965; D'appolonia, 1972; Ohishi *et al.*, 2007; Wu *et al.*, 2010). Nonetheless, these studies were performed by direct inclusion of acids and NaCl to the flour rather than to the kernels before milling, which could explain the differences between results from this study and those of previous studies.

The solvent retention capacity (SRC) test is widely used to predict commercial baking performance of flours (Ram *et al.*, 2005). Four standard solvents (lactic acid, sucrose, water, and sodium carbonate) provide information on chemical and physical aspects of flour such as gluten strength, starch damage, pentosane content and gliadin characteristics. Small variations in solvent retention capacity values were found among some flour samples (**Table 2-2**). Sodium carbonate SRC, which is associated with starch damage, was not influenced by any tempering solution; whereas lactic acid SRC was significantly altered by all acidic saline solutions applied during wheat tempering. Acidic saline solutions yielded flours with lower lactic acid SRC, which suggested that gluten strength of these flour samples was weaker than in control flour.

Additionally, tempering with acetic acid-NaCl solutions resulted in flours with lower water SRC, which may be related to the reduced gluten strength or altered glutenin characteristics. In contract, sucrose SRC values, which are associated with pentosan characteristics, were slightly increased by tempering with acetic acid-NaCl solutions.

Previous studies have reported that solubility and swelling properties of pentosans increase at low pH values (Hammes and Gänzle, 1997; Lappi *et al.*, 2010).

The physical characteristics and texture profile of cookies made of flour obtained from wheat tempered with acidic saline solutions are presented in **Table 2-3**. The diameter of cookies varied from 9.30 to 9.47 cm with a mean value of 9.36 cm, while the thickness and spread factor ranged from 0.86 to 0.90 cm and from 10.39 to 10.86, respectively. In general, these physical characteristics of cookies were not significantly altered by any of the tempering treatments. Likewise, the hardness and flexibility of baked cookies did not vary significantly among treatments. Dough viscosity plays a key role in defining the degree of spread of the dough during baking as well as the final thickness (Hoseney and Rogers, 1994). No significant changes in the flour viscosity profile were observed among the different treatments (**Table 2-2**), which could explain the observed uniformity in diameter and thickness of baked cookies.

In addition, previous research studies have demonstrated that physical characteristics of cookies such as hardness, diameter, and spread ratio may also be influenced by the amount of damaged starch present in the flour (Gaines *et al.*, 1988; Barrera *et al.*, 2007; Barak *et al.*, 2014). In the present study, no significant differences in sodium bicarbonate SRC, which is related to the damaged starch content, were observed among the different flour samples, which correlates with the observed consistency in cookie diameter, spread ratio, and hardness (**Table 2-3**). Several research studies, however, have reported negative correlations between cookies diameter, spread ratio and lactic acid, water, and sucrose SRC (Guttieri *et al.*, 2001; Barrera *et al.*, 2007; Zhang *et al.*, 2007b; Barak *et al.*, 2014). Despite the variations in lactic acid, water, and sucrose

Table 2-3. Cookie quality characteristics of flour obtained from soft wheat tempered with water or acidic saline solutions.

Tempering Solutions	Cookie Dimensional Characteristics [a]			Cookie Textural Characteristics [a]	
	Diameter (cm)	Thickness (cm)	Spread Factor [b]	Hardness (g)	Flexibility (cm)
Water (Control)	9.30 ± 0.06a	0.90 ± 0.02a	10.40 ± 0.20a	1987.29 ± 29.40a	0.17 ± 0.01a
Acetic Acid 2.5% + NaCl 26.6%	9.30 ± 0.02a	0.87 ± 0.03a	10.71 ± 0.44a	2161.88 ± 251.1a	0.18 ± 0.02a
Acetic Acid 5.0% + NaCl 26.6%	9.29 ± 0.03a	0.86 ± 0.02a	10.87 ± 0.30a	2204.83 ± 132.8a	0.20 ± 0.03a
Lactic Acid 2.5% + NaCl 26.6%	9.47 ± 0.30a	0.88 ± 0.02a	10.80 ± 0.50a	2403.25 ± 194.0a	0.22 ± 0.03a
Lactic Acid 5.0% + NaCl 26.6%	9.41 ± 0.08a	0.87 ± 0.02a	10.86 ± 0.27a	2253.03 ± 132.6a	0.21 ± 0.02a

[a] Values with the same letter in the same column are not significantly different from one another ($P > 0.05$).
[b] Spread factor = Diameter / Thickness.

SRC caused by acidic saline tempering solutions among flours (**Table 2-2**), no significant differences in cookie diameter and spread ratio occurred in the current study.

4. CONCLUSIONS

The natural microbial load of soft wheat grain was significantly reduced by tempering with acidic saline solutions. The tempering solution containing a combination of lactic acid (5.0% v/v) and NaCl (26.6%) was the most effective treatment to reduce the numbers of mesophilic bacteria and *Enterobacteriaceae*. Despite the antimicrobial efficacy of acidic saline solutions, a recontamination of wheat occurred during milling. Nevertheless, the microbial load of flour obtained from lactic acid (5.0%) treated wheat was significantly lower than the control flour. Tempering wheat with lactic acid saline solutions did not alter the pasting profile of the resulting flours; however, it caused minimal but significant changes in their solvent retention capacity profile. Furthermore, no significant differences in the physical characteristics and texture profile of baked cookies were found. Therefore, these tempering solutions could be used by the wheat milling industry to reduce the risk of microbial contamination in milled products, without substantially affecting the functional properties of straight-grade flour. Further research is warranted to evaluate the efficacy of these tempering solutions against pathogenic microorganisms, as well as their impact on the sensory characteristics of baked goods.

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CHAPTER 3

REDUCTION IN PATHOGENIC LOAD OF WHEAT BY TEMPERING WITH SALINE ORGANIC ACID SOLUTIONS AT DIFFERENT SEASONAL TEMPERATURES

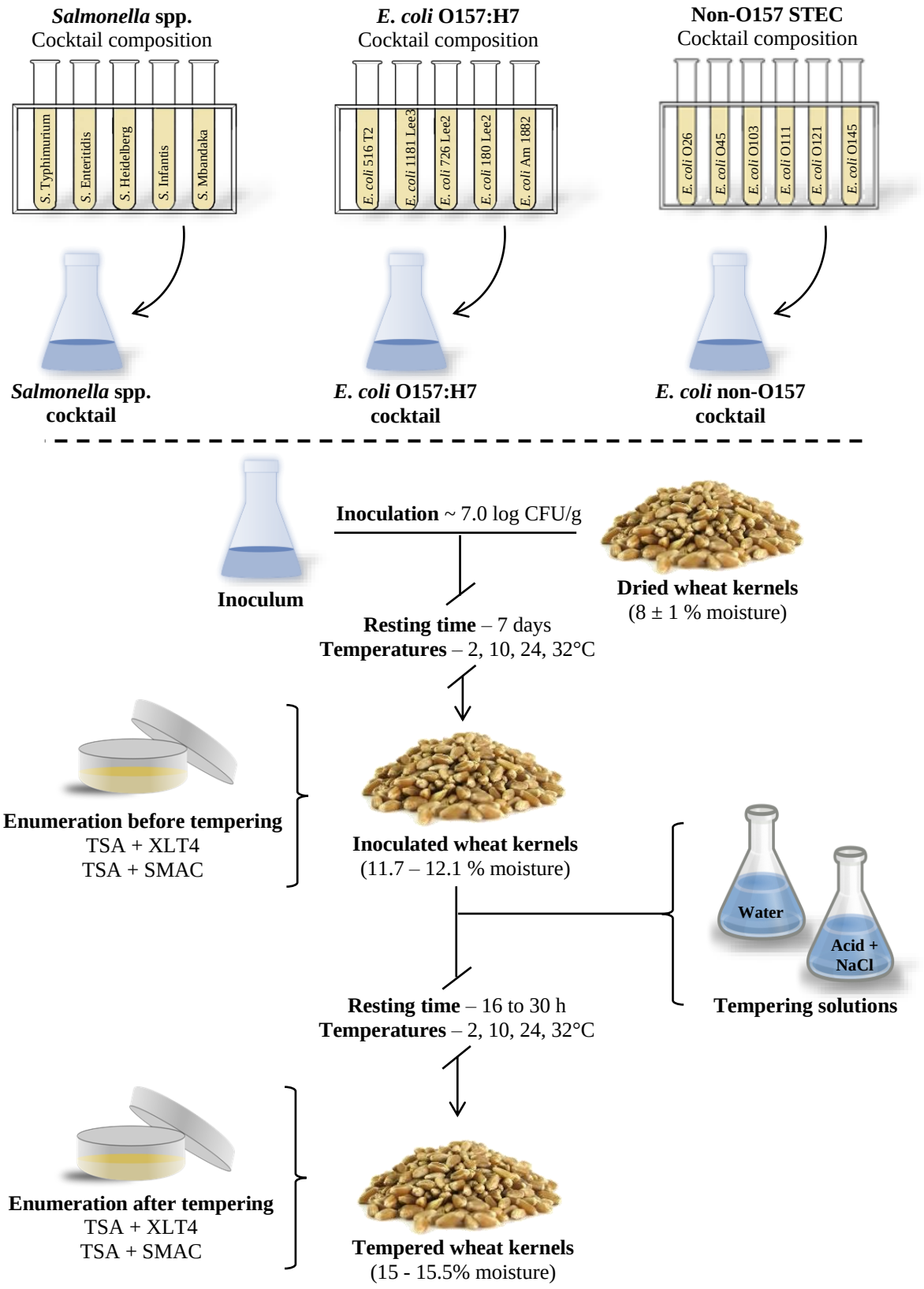
CHAPTER 3. REDUCTION IN PATHOGENIC LOAD OF WHEAT BY TEMPERING WITH SALINE ORGANIC ACID SOLUTIONS AT DIFFERENT SEASONAL TEMPERATURES

ABSTRACT

During production and processing, wheat is exposed to microbial contamination; therefore, enteric pathogens may be among its microflora creating a food safety risk in milled products. The objectives of this research were to determine (1) the effect of seasonal temperature on survivability of pathogens in wheat kernels; (2) the effect of seasonal temperature on pathogen inactivation during tempering with saline organic acid solutions; and (3) the effectiveness of saline organic acid solutions to reduce the counts of pathogens in soft and hard wheat. Wheat samples were inoculated using cocktails of either 5 serovars of *Salmonella enterica*, 5 *E. coli* O157:H7 or 6 non-O157 Shiga toxin-producing *E. coli* (STEC) strains. Wheat samples were inoculated to achieve 7 log CFU/g followed by a 7-day resting period at temperatures (2.0, 10.8, 24.2°C) corresponding to the average temperatures experienced in a period of seven years during the winter, spring/fall, and summer seasons in the major wheat growing regions in the state of Nebraska. The average maximum temperature during the summer season (32.0°C) was also included in this study. Besides water, solutions containing acid (acetic or lactic - 2.5% or 5.0% v/v) and NaCl (~26% w/v) were used for tempering the wheat to 15.0% (soft) and 15.5% (hard) moisture at the different seasonal temperatures. Wheat was plated throughout the resting period, and before and after tempering on Tryptic Soy Agar with an overlay of Xylose-Lysine-Tergitol 4 Agar for *Salmonella enterica* or Sorbitol MacConkey Agar for *E. coli* O157:H7 and non-O157 STEC. Incubation was carried out

at 37°C for 24 h. The survival rate of pathogenic microorganisms on wheat kernels was higher at temperatures experienced during the winter (2.0°C) and fall (10.8°C) months. Regardless of tempering temperature, the initial pathogen load was reduced significantly by all solutions when compared to the control tempered with water ($P \leq 0.05$). The combination of lactic acid (5.0%) and NaCl was the most effective treatment against *Salmonella enterica*, *E. coli* O157:H7 and non-O157 STEC, with average reduction values of 1.8, 1.8 and 1.6 log CFU/g for soft wheat and 2.6, 2.4 and 2.4 log CFU/g for hard wheat, respectively. Implementation of organic acids and NaCl in tempering water may have the potential to reduce the risk of pathogen contamination in milled products.

VISUAL SUMMARY



1. INTRODUCTION

Throughout the production chain, including harvest, storage, and transport, wheat is exposed to multiple sources of microbial contamination; consequently, enteric pathogens such as *Salmonella* and *Escherichia coli*, may be among its microflora creating a food safety risk in milled products (Sabillón and Bianchini, 2016). In fact, microbiological surveys have detected the presence of these pathogenic microorganisms on wheat kernels entering the milling system (Berghofer *et al.*, 2003; Eglezos, 2010). These microbial contaminants are concentrated mainly on the surface of the grain (Laca *et al.*, 2006), and their ability to survive may be determined by environmental conditions, such as temperature and relative humidity, prevailing during grain harvesting and storage (Crumrine and Foltz, 1969; Sabillón *et al.*, 2016b).

Since none of the operations used in the dry-milling process of wheat include either chemical or thermal treatments, the impact of these operations on reducing microbial load of wheat is minimal. Studies have shown that flour with high microbial counts is usually obtained from wheat of inferior microbiological quality (Berghofer *et al.*, 2003). Therefore, if pathogenic microorganisms are present on wheat kernels prior to milling they could eventually reach the final milled products; thus representing a potential risk for consumers' health.

Several microbiological surveys have also indicated the presence of *Salmonella* and *Escherichia coli* in wheat milled products, such as flour (Richter *et al.*, 1993; Berghofer *et al.*, 2003; Sperber, 2003; Sperber, 2007; Eglezos, 2010). Despite the low water activity of flour, pathogenic microorganisms may survive in a dormant state for years and can emerge from dormancy when flour is further processed into food products, such as batters

or mixes (Eglezos, 2010). Therefore, raw flour and ready-to-bake products can serve as vehicles of serious and life-threatening foodborne illnesses. Indeed, wheat-based products, particularly flour, have been involved in several food safety incidents over the last decade (Zhang *et al.*, 2007; Neil *et al.*, 2011; McCallum *et al.*, 2013; PHAC, 2017; US-FDA, 2017).

The increased demand for ready-to-bake products and the possibility that consumers may undercook products like pre-made cookie and pizza crust doughs, or intentionally eat them raw (Ardent Mills, 2010), warrant treatments to make wheat milled products safer for human consumption. Presently, the use of pre-milling interventions to reduce the risk of microbial contamination in milled products is not a widespread practice among the wheat milling industry. Some facilities may use chlorinated water (400-700 ppm) to reduce microbial contamination during tempering. However, chlorine compounds can be inactivated very quickly by organic matter present on wheat kernels and may form various organochlorine compounds that exert toxic effects on human health (Dychdala, 2001).

Published research suggest that typical chlorine concentrations (400-700 ppm) used by the wheat milling industry during tempering may not be enough to reduce the load of pathogenic microorganisms in wheat kernels, since the effectiveness of chlorine may be diminished as soon as it contacts the organic matter present on the kernels. For instance, Taormina and Beuchat (1999) and Lang *et al.* (2000) found that washing alfalfa seeds with 200, 500 and 2,000 ppm chlorine solutions for 3 to 10 min at room temperature did not cause significant reductions in the population of *E. coli* O157:H7 when compared with the corresponding control treatments (distilled water). Only when seeds were treated

with ≥ 2,000 ppm of active chlorine significant reductions were observed. Similarly, Weissinger and Beuchat (2000) did not find a significant reduction in *Salmonella* spp. counts after treating alfalfa seeds with 200 ppm chlorine solution. Alternatives to chlorine-based sanitizers (e.g., ozone, organic acids) to reduce the microbial load of wheat during tempering have been explored (Ibanoğlu, 2002; Dhillon *et al.*, 2010; Sabillón *et al.*, 2016a); however, the efficacy of these treatments to eliminate pathogenic microorganisms from wheat kernels intended for production of flour or sprouts has not been examined.

In a previous study, we reported that tempering wheat kernels with solutions containing a combination of organic acid and NaCl significantly reduced the counts of aerobic mesophilic bacteria and *Enterobacteriaceae* by as much as 4.3 and 4.7 log CFU/g, respectively (Sabillón *et al.*, 2016a). Because of the great effect of these tempering solutions on *Enterobacteriaceae*, which includes enteric pathogens such as *Salmonella* spp. and Shiga toxin-producing *Escherichia coli* (STEC), the objectives of this study were to determine (1) the effect of seasonal temperature on survivability of *Salmonella enterica*, *E. coli* O157:H7, and non-O157 STEC in wheat kernels; (2) the effect of seasonal temperature on the inactivation of *Salmonella enterica*, *E. coli* O157:H7, and non-O157 STEC during tempering with saline organic acid solutions; and (3) the effectiveness of saline organic acid solutions to reduce the counts of *Salmonella enterica*, *E. coli* O157:H7, and non-O157 STEC in hard and soft wheat.

2. MATERIALS AND METHODS

2.1. Materials

Hard red winter wheat samples were provided by Husker Genetics, the University of Nebraska-Lincoln Foundation Seed Division. Soft red winter wheat samples were obtained from a commercial source. Sodium chloride and lactic acid (85%) were obtained from Fisher Scientific™ (Pittsburgh, PA). Glacial acetic acid was obtained from VWR® (Radnor, PA). All chemicals used were United States Pharmaceutical (USP) or Food Chemicals Codex (FCC) grade.

2.2. Test strains and inoculum preparation

Five strains of *E. coli* O157:H7 with resistance to kanamycin (AU516 T2, AU1181 LEE3, AU726 LEE2, AU1809 LEE2, and AU1823) previously employed to study their internalization rate into wheat tissues (Martinez *et al.*, 2015), were used for this experiment. *Salmonella enterica* serovars Typhimurium (ATCC 14028), Enteritidis IV/NVSL 94-13062, Heidelberg/Sheldon 3347-1, Infantis, and Mbandaka were chosen for this experiment based on their role in foodborne outbreaks associated with low-moisture products. Six non-O157 STEC serotypes, including O26:H11 (ATCC 2196), O45:H2 (ATCC 2193), O103:H11 (ATCC 2215), O111 (ATCC 2440), O121:H19 (ATCC 2219) and O145 (ATCC 2192) were also used in this study.

Cultures with ATCC designation were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and propagated according to manufacturer's instructions. For all the other cultures, propagation was carried out in 9 mL Tryptic Soy Broth (TSB; Acumedia, Lansing, MI), incubated at 37°C for 24 h (Heratherm™ IGS 180 Incubator, Thermo Fisher Scientific Inc., Waltham, MA). After propagation, all cultures

were stored individually at -80°C in cryogenic vials containing 20% sterile glycerol (Fisher Scientific™). All cultures were re-activated individually by scraping the frozen broth with a sterile loop and transferring it into 9 mL TSB, followed by incubation at 37°C for 24 h. For *E. coli* O157:H7 strains, TSB was supplemented with kanamycin (50 µg/mL). Cultures were then transferred individually to 15-mL sterile conical tubes to be harvested by centrifugation at 4,000 x g / 4°C for 8 min (Sorvall™ ST 16R Centrifuge, Thermo Fisher Scientific Inc., Waltham, MA). The pellets were then washed once with 9 mL of 0.1% sterile peptone solution (Fisher Scientific™), harvested once again and subsequently re-suspended in 5 mL 0.1% sterile peptone solution. Individual cultures from each group of microorganisms were then mixed by pipetting equal portions of bacterial suspension into a 50-mL sterile conical tube to achieve the five-strain/serovar cocktails of *E. coli* O157:H7 and *Salmonella enterica*, and a six-serotype cocktail of non-O157 STEC.

2.3. Seasonal temperatures

The average seasonal temperatures (i.e., winter, spring, summer, and fall) encountered over a period of seven years (2010-2016) in the major wheat-growing regions in the state of Nebraska, U.S.A. were obtained from the Automated Weather Data Network (AWDN) of the High Plains Regional Climate Center (HPRCC). The average temperature for the spring (10.3°C) and fall (11.4°C) seasons were very similar; therefore, one average temperature was considered to be representative of the ambient temperatures encountered in both seasons. Moreover, the average temperature for the winter months was below freezing point (-3°C), making the absorption of water by the wheat kernels during tempering nearly impossible to occur; therefore, a temperature

slightly above the freezing point was chosen instead. As a result, the average temperatures selected for this study were 2.0, 10.8, and 24.2°C for the winter, spring/fall, and summer seasons, respectively. The average maximum temperature during the summer season (32.0°C) was also included in the study.

2.4. Inoculation of wheat kernels

The initial moisture content of the wheat samples was determined according to AACC approved forced-air oven method 44-15.02 (AACCI, 2018). Before inoculation, wheat kernels were dried overnight (~14 h at 40°C) to 8.5 ± 0.5% moisture using a forced-air oven (Drying Chamber FED 56, BINDER©, Bohemia, NY) to prevent excessive increase in moisture due to inoculation and subsequent tempering. Dried wheat kernels (200 g) were placed in double sterile plastic bags and spread into an even layer inside of a biological safety cabinet. The original moisture content of the wheat kernels (11.7% and 12.1% for soft and hard wheat, respectively) was then restored by adding each cocktail of microorganisms separately. The amount of inoculum added (7.7 mL of inoculum per bag) was calculated to achieve approximately 7.0 log CFU/g in wheat kernels.

The different inocula were applied to wheat by spray misting to ensure even distribution. Upon inoculation, a resting time of 7 days was allowed for microbial adaptation and moisture equilibration. During the resting period the inoculated wheat bags were tightly closed and placed in incubators (Heratherm™ IGS 180 Incubator; Precision™ Low Temperature Incubator, Thermo Fisher Scientific Inc., Waltham, MA) with temperatures set at 2.0, 10.8, 24.2, and 32.0°C. To ensure moisture distribution and thorough homogenization, samples were shaken vigorously by hand every 12 min during

the first 3 h after the addition of the inoculum and then once a day during the resting period. To determine the survivability of the pathogenic microorganisms on wheat kernels stored at different seasonal temperatures over the 7-day resting period, two subsamples of 25 g each were taken at 0, 1, 3, 5 and 7 days after inoculation.

2.5. Preparation and application of tempering solutions

Tempering solutions containing a combination of organic acid (acetic or lactic; 2.5 and 5.0% v/v) and NaCl (~26% w/v) were prepared as described by Sabillón *et al.* (2016a), yielding a total of four different treatment combinations. The tempering solutions were always prepared fresh prior to tempering and under aseptic conditions. The pH and level of protonation of the prepared saline organic acid solutions is shown in **Table 3-1**. Inoculated wheat kernels stored for 7 days were used for tempering experiments. Before the application of tempering solutions, 25 g of each inoculated sample were set aside to determine the pathogen load after the resting period.

Table 3-1. pH and percent protonation of saline organic acid solutions.

Wheat Class	Tempering Solutions [a]	pH	% Protonation [b]
Soft	AA 2.5% + NaCl 26.5%	2.00	99.82%
	AA 5.0% + NaCl 26.5%	1.80	99.89%
	LA 2.5% + NaCl 26.5%	1.36	99.68%
	LA 5.0% + NaCl 26.5%	1.23	99.77%
Hard	AA 2.5% + NaCl 25.4%	2.01	99.82%
	AA 5.0% + NaCl 25.4%	1.84	99.88%
	LA 2.5% + NaCl 25.4%	1.40	99.65%
	LA 5.0% + NaCl 25.4%	1.25	99.75%

[a] AA; acetic acid; LA, lactic acid; NaCl, sodium chloride. Percentage denotes concentration.

[b] Values were calculated using the Henderson-Hasselbalch equation with pKa values of 4.75 and 3.86 for acetic and lactic acid, respectively.

The amount of tempering solution needed to reach the optimum milling moisture was calculated based on the moisture content of the wheat samples after the 7-day resting period. Since the wheat moisture was 11.8% and 12.0% for soft and hard wheat, respectively, the amount of tempering solution needed to reach the target milling moisture was 7.0 mL and 7.7 mL for soft and hard wheat, respectively.

Three replicates (~183 g) for each cocktail of microorganisms and treatment were placed inside of a biological safety cabinet and tempered to 15% moisture for soft wheat and to 15.5% moisture for hard wheat. Tempering was achieved by adding sterile distilled water (control) or tempering solutions containing the combination of organic acid and NaCl. Tempering solutions were applied to wheat by spray misting to ensure even distribution. The temperature of the solutions was 23 ± 1°C when treatments were applied. Tempered wheat samples were placed back in incubators with temperature set at 2.0, 10.8, 24.2, and 32.0°C and were left to stand for a determined period of time until they reached the target milling moisture. Samples were shaken vigorously by hand every 12 min during the first 2 h after the addition of the tempering solutions.

The target tempering time was said to be achieved when samples reached a target flour yield of 69 ± 1% for soft wheat and 72 ± 1% for hard wheat. To determine the tempering time, non-inoculated wheat samples were tempered under different temperatures (2.0, 10.8, 24.2, and 32.0°C) and milled at intervals of 5 h. Soft wheat samples were milled using a Brabender Quadrumat Junior laboratory roller mill (C.W. Brabender® Instruments, Inc.; South Hackensack, NJ), while hard wheat samples were milled with a Buhler MLU-202 laboratory mill (Buhler, Minneapolis, MN).

2.6. Microbiological analysis

To determine the pathogen load present in the wheat during the resting period, and before and after tempering under various experimental conditions, 25 g of wheat were diluted in 225 mL of 0.1% peptone solution in a sterile plastic bag, left to stand for 5 min to soften the kernels, and mechanically mixed with a stomacher blender (Stomacher® 400, Seward Ltd, Bohemia, NY) for 2 min. Original sample dilutions were further diluted (1:10) using 0.1% peptone solution, and spread plated in duplicate on Tryptic Soy Agar (TSA; Acumedia, Lansing, MI). For *E. coli* O157:H7, TSA plates were supplemented with kanamycin (50 µg/ml). Plates were then incubated (Heratherm™ IGS 180 Incubator, Thermo Fisher Scientific Inc., Waltham, MA) at 37.0°C for 3 h to allow the recovery of stressed/injured cells.

To minimize the growth of microorganisms naturally present on wheat kernels, TSA plates were then overlaid with approximately 9 ml of Sorbitol MacConkey (SMAC; Acumedia) agar for the enumeration of *E. coli* O157:H7 and non-O157:H7 STEC, and with Xylose-Lysine-Tergitol 4 (XLT-4; Acumedia) agar for the enumeration of *Salmonella* spp. All plates were then incubated at 37.0°C for 24 h. For the tempering experiment, the reduction in pathogenic load was calculated by subtracting the counts obtained before and after the application of treatments.

2.7. Statistical analysis

Data were analyzed with SAS software version 9.3 (SAS Institute, Cary, NC), using a two-way analysis of variance (ANOVA) to compare the changes in pathogen loads in response to tempering solutions and temperatures. ANOVAs were performed using the GLIMMIX procedure of SAS. Tukey’s multiple comparison test was used to

determine significant differences among means. All statistical analysis was performed with a significance level of $P \leq 0.05$. To estimate the contribution of each independent variable and interactions to the total variance observed in the reduction of pathogen loads, the data were subjected to a variance components statistical analysis using the VARCOMP procedure of SAS.

3. RESULTS AND DISCUSSION

3.1. Survival of pathogenic microorganisms on wheat grain during storage

The effect of temperature (2.0, 10.8, 24.2, and 32.0°C) during a 7-day storage period on the viability of *Salmonella* spp., *E. coli* O157:H7, non-O157 STEC inoculated in soft and hard wheat kernels is presented in **Table 3-2** and **Table 3-3**, respectively. Throughout the 7-day storage period, the moisture content of soft wheat remained at levels of 11.7% ± 0.1%, while for hard wheat kernels was 12.1% ± 0.1%. A significant decrease in the number of viable pathogenic cells on soft and hard wheat kernels occurred within the 7-day storage period, regardless of temperature; however, the reduction was different depending upon the storage temperature.

The association of increased storage temperature with increased rate of death of *E. coli* O157:H7 was similar to those observed for *Salmonella* and non-O157 STEC in both classes of wheat. No significant reduction in the number of viable cells was observed by the end of the 7-day storage period for those samples stored at 2.0 and 10.8°C. In general, pathogenic microbial populations decreased considerably in numbers as the storage temperature and time increased.

Table 3-2. Survival of pathogenic microorganisms on soft wheat kernels as affected by storage temperature.

Wheat Class	Storage Temperature (°C)	Storage Time (days)	*Salmonella* spp. [a]		*E. coli* O157:H7 [b]		Non-O157 STEC [c]	
			Population * (log CFU/g)	Reduction † (log CFU/g)	Population* (log CFU/g)	Reduction † (log CFU/g)	Population * (log CFU/g)	Reduction † (log CFU/g)
Soft Red Winter	2.0	0	6.62 ± 0.06 a		6.59 ± 0.09 a		6.84 ± 0.01 a	
		1	6.21 ± 0.00 b	0.41 ± 0.00	6.43 ± 0.02 b	0.16 ± 0.02	6.73 ± 0.03 ab	0.11 ± 0.03
		3	6.16 ± 0.00 bc	0.46 ± 0.00	6.19 ± 0.01 c	0.40 ± 0.01	6.54 ± 0.07 c	0.29 ± 0.07
		5	6.04 ± 0.04 c	0.59 ± 0.04	6.14 ± 0.00 c	0.45 ± 0.00	6.53 ± 0.05 c	0.30 ± 0.05
		7	6.05 ± 0.00 c	0.57 ± 0.00 c	6.11 ± 0.01 c	0.48 ± 0.01 c	6.60 ± 0.04 bc	0.23 ± 0.04 c
	10.8	0	6.64 ± 0.05 a		6.60 ± 0.07 a		6.86 ± 0.02 a	
		1	6.25 ± 0.01 b	0.38 ± 0.01	6.47 ± 0.02 a	0.13 ± 0.02	6.77 ± 0.04 ab	0.08 ± 0.04
		3	6.24 ± 0.02 b	0.40 ± 0.02	6.10 ± 0.00 b	0.50 ± 0.00	6.62 ± 0.07 bc	0.24 ± 0.07
		5	6.11 ± 0.02 c	0.53 ± 0.02	6.07 ± 0.04 b	0.52 ± 0.04	6.56 ± 0.08 c	0.30 ± 0.08
		7	6.12 ± 0.02 c	0.52 ± 0.02 c	6.17 ± 0.05 b	0.42 ± 0.05 c	6.54 ± 0.02 c	0.32 ± 0.02 c
	24.2	0	6.56 ± 0.01 a		6.65 ± 0.05 a		6.74 ± 0.06 a	
		1	5.99 ± 0.03 b	0.57 ± 0.03	6.55 ± 0.09 a	0.10 ± 0.09	6.68 ± 0.07 a	0.06 ± 0.07
		3	6.00 ± 0.03 b	0.56 ± 0.03	5.97 ± 0.04 b	0.67 ± 0.04	6.06 ± 0.01 b	0.69 ± 0.01
		5	5.71 ± 0.05 c	0.85 ± 0.05	5.84 ± 0.04 b	0.81 ± 0.04	6.01 ± 0.05 b	0.73 ± 0.05
		7	5.64 ± 0.08 c	0.92 ± 0.08 b	5.82 ± 0.03 b	0.83 ± 0.03 b	6.00 ± 0.04 b	0.74 ± 0.04 b
	32.0	0	6.61 ± 0.04 a		6.64 ± 0.03 a		6.66 ± 0.01 a	
		1	5.67 ± 0.03 b	0.94 ± 0.03	5.84 ± 0.04 b	0.80 ± 0.04	6.09 ± 0.00 b	0.57 ± 0.00
		3	5.65 ± 0.02 bc	0.96 ± 0.02	5.54 ± 0.07 c	1.10 ± 0.07	5.99 ± 0.05 b	0.66 ± 0.05
		5	5.49 ± 0.05 cd	1.11 ± 0.05	4.97 ± 0.02 d	1.68 ± 0.02	5.42 ± 0.06 c	1.24 ± 0.06
		7	5.39 ± 0.05 d	1.22 ± 0.05 a	4.75 ± 0.01 e	1.89 ± 0.01 a	5.22 ± 0.03 d	1.44 ± 0.03 a

[a] Five-serovar cocktail of *Salmonella enterica* (Typhimurium, Enteritidis IV/NVSL 94-13062, Heidelberg/Sheldon 3347-1, Infantis and Mbandaka).

[b] Five-strain cocktail of *E. coli* O157:H7 with resistance to kanamycin (AU516 T2, AU1181 LEE3, AU726 LEE2, AU1809 LEE2, and AU1823).

[c] Six-serotype cocktail of non-O157 Shiga toxin-producing *E. coli* (STEC: O26:H11, O45:H2, O103:H11, O111, O121:H19 and O145).

* Pathogenic population is expressed in log CFU/g and ± denotes standard deviation. Means in the same column within the same temperature followed by different letters are significantly different ($P \leq 0.05$).

† Reduction on pathogenic population is expressed in log CFU/g and ± denotes standard deviation. Mean log reduction values at the 7th day within the same microorganism and wheat class followed by different letters are significantly different ($P \leq 0.05$).

Table 3-3. Survival of pathogenic microorganisms on hard wheat kernels as affected by storage temperature.

Wheat Class	Storage Temperature (°C)	Storage Time (days)	*Salmonella* spp. [a]		*E. coli* O157:H7 [b]		Non-O157 STEC [c]	
			Population * (log CFU/g)	Reduction † (log CFU/g)	Population* (log CFU/g)	Reduction † (log CFU/g)	Population * (log CFU/g)	Reduction † (log CFU/g)
Hard Red Winter	2.0	0	6.70 ± 0.09 a		6.62 ± 0.05 a		7.11 ± 0.04 a	
		1	6.62 ± 0.01 ab	0.08 ± 0.01	6.58 ± 0.05 a	0.04 ± 0.05	7.11 ± 0.00 a	0.01 ± 0.00
		3	6.63 ± 0.05 ab	0.06 ± 0.05	6.60 ± 0.01 a	0.03 ± 0.01	7.01 ± 0.04 a	0.11 ± 0.04
		5	6.51 ± 0.02 ab	0.19 ± 0.02	6.60 ± 0.05 a	0.02 ± 0.05	6.80 ± 0.02 b	0.31 ± 0.02
		7	6.39 ± 0.13 b	0.31 ± 0.13 c	6.48 ± 0.02 a	0.14 ± 0.02 c	6.85 ± 0.04 b	0.27 ± 0.04 c
	10.8	0	6.78 ± 0.01 a		6.67 ± 0.02 a		7.08 ± 0.03 a	
		1	6.73 ± 0.06 a	0.05 ± 0.06	6.56 ± 0.02 a	0.11 ± 0.02	7.06 ± 0.01 a	0.02 ± 0.01
		3	6.67 ± 0.01 a	0.11 ± 0.01	6.77 ± 0.10 a	-0.10 ± 0.10	7.07 ± 0.01 a	0.02 ± 0.01
		5	6.32 ± 0.07 b	0.46 ± 0.07	6.27 ± 0.02 b	0.40 ± 0.02	6.92 ± 0.05 b	0.17 ± 0.05
		7	6.45 ± 0.05 b	0.33 ± 0.05 c	6.65 ± 0.07 a	0.02 ± 0.07 c	7.04 ± 0.03 a	0.04 ± 0.03 d
	24.2	0	6.81 ± 0.03 a		6.51 ± 0.08 a		7.06 ± 0.01 a	
		1	6.37 ± 0.05 b	0.43 ± 0.05	6.40 ± 0.03 a	0.11 ± 0.03	6.74 ± 0.00 b	0.32 ± 0.00
		3	6.23 ± 0.02 c	0.58 ± 0.02	6.50 ± 0.08 a	0.01 ± 0.08	6.73 ± 0.06 b	0.33 ± 0.06
		5	6.12 ± 0.00 cd	0.68 ± 0.00	6.08 ± 0.01 b	0.43 ± 0.01	6.55 ± 0.11 b	0.51 ± 0.11
		7	6.01 ± 0.01 d	0.79 ± 0.01 b	6.14 ± 0.01 b	0.38 ± 0.01 b	6.55 ± 0.02 b	0.51 ± 0.02 b
	32.0	0	6.86 ± 0.05 a		6.82 ± 0.06 a		6.55 ± 0.06 a	
		1	5.85 ± 0.03 b	1.01 ± 0.03	5.55 ± 0.07 b	1.27 ± 0.07	6.51 ± 0.05 a	0.05 ± 0.05
		3	5.67 ± 0.07 b	1.20 ± 0.07	5.50 ± 0.08 b	1.32 ± 0.08	5.91 ± 0.02 b	0.64 ± 0.02
		5	5.87 ± 0.03 b	0.99 ± 0.03	5.52 ± 0.13 b	1.30 ± 0.13	5.80 ± 0.02 bc	0.76 ± 0.02
		7	5.72 ± 0.08 b	1.14 ± 0.08 a	5.38 ± 0.06 b	1.44 ± 0.06 a	5.73 ± 0.05 c	0.83 ± 0.05 a

[a] Five-serovar cocktail of *Salmonella enterica* (Typhimurium, Enteritidis IV/NVSL 94-13062, Heidelberg/Sheldon 3347-1, Infantis and Mbandaka).

[b] Five-strain cocktail of *E. coli* O157:H7 with resistance to kanamycin (AU516 T2, AU1181 LEE3, AU726 LEE2, AU1809 LEE2, and AU1823).

[c] Six-serotype cocktail of non-O157 Shiga toxin-producing *E. coli* (STEC: O26:H11, O45:H2, O103:H11, O111, O121:H19 and O145).

* Pathogenic population is expressed in log CFU/g and ± denotes standard deviation. Means in the same column within the same temperature followed by different letters are significantly different ($P \leq 0.05$).

† Reduction on pathogenic population is expressed in log CFU/g and ± denotes standard deviation. Mean log reduction values at the 7th day within the same microorganism and wheat class followed by different letters are significantly different ($P \leq 0.05$).

The retention of cell viability at lower storage temperatures observed in this study are in agreement with other reports describing the death rates of pathogenic microorganisms on dry seeds. For instance, Jaquette *et al.* (1996) reported that after 9 weeks of storage at 8 and 21°C, the initial population of *Salmonella* serovar Stanley on dry alfalfa seeds reduced from 2.53 to 1.81 and 0.92 log CFU/g, respectively. Similarly, Taormina and Beuchat (1999) and Beuchat and Scouten (2002) found that elevated storage temperatures (37-70°C) enhances the rate of death of *Salmonella* spp. and *E. coli* O157:H7 on dry alfalfa seeds. However, despite the reduction in numbers at these higher storage temperatures, cells of *Salmonella* spp. and *E. coli* O157:H7 may remain viable on dry seeds for as long as 2 years (Van der Linden *et al.*, 2013). Therefore, if pathogenic microorganisms are among the microbial flora of wheat kernels, they may survive throughout the wheat flour production chain and represent a serious hazard for consumers' health.

The average temperatures experienced by wheat kernels in storage bins during the winter and fall months may favor the survival of pathogenic microorganisms. In fact, a survey conducted by Richter *et al.* (1993) in over 3,000 wheat flour samples found a higher incidence of *E. coli* (12.8%) and *Salmonella* spp. (3.0%) in samples collected during the fall and winter months than in those collected over the spring and summer months (9.5% and 0.3%, respectively). This indicates that the environmental conditions encountered during the milling process appear to be related to the incidence of these pathogenic microorganisms.

3.2. Effect of tempering temperature on the effectiveness of saline organic acid solutions

Differences in temperature may exist between the outer and inner layers of wheat kernels stored in a bin and such differences may be exacerbated depending upon the season, leading to an uneven temperature distribution across the storage bin. In cold months, for instance, wheat enters the milling facility from the storage bin at temperatures of 6 to 10°C or sometimes near freezing point (Posner and Hibbs, 2005), thus requiring more time to absorb water during tempering than wheat at warmer temperatures. Consequently, this may also have an impact on the efficacy of the treatments applied to reduce the microbial load on wheat kernels prior to milling.

Temperature and tempering solution and their interaction significantly impacted the pathogen load ($P \leq 0.05$), except for the interaction of the variables in hard wheat inoculated with non-O157 STEC (**Table 3-4**). To better understand the impact of the interactions, the contributions of each variable to explain pathogen load were calculated (**Fig. 3-1**). In both wheat classes, the tempering solutions made the largest contribution to explaining the total variance (> 89 and 97% for soft and hard wheat, respectively) in the data set; while the interaction effect accounted for only 0.7 to 2.7%, a value sometimes smaller than the contribution made by the error term (**Fig. 3-1**). Likewise, temperature had a very small level of contribution in the total variance ranging from 0.1 to 6.2%. Due to the low contribution of the interaction, it could be assumed that temperature and tempering solutions may be acting independently of each other.

Table 3-4. Analysis of variance of pathogen reduction in soft and hard wheat.

Wheat Class	Effect	Type III Tests of Fixed Effects					
		***Salmonella* spp.**		***E. coli* O157:H7**		**Non-O157 STEC**	
		F Value	**Pr > *F***	**F Value**	**Pr > *F***	**F Value**	**Pr > *F***
Soft Wheat	Temperature (Temp)	25.96	<0.0001	44.55	<0.0001	21.23	<0.0001
	Tempering Solutions (TS)	326.40	<0.0001	485.84	<0.0001	269.76	<0.0001
	Temp x TS (Interaction)	3.44	0.0016	3.29	0.0023	2.34	0.0219
Hard Wheat	Temperature (Temp)	3.27	0.0308	11.33	<.0001	6.3	0.0013
	Tempering Solution (TS)	778.93	<.0001	1158.8	<.0001	432.39	<.0001
	Temp x TS (Interaction)	2.31	0.0235	3.74	0.0008	0.75	0.6995

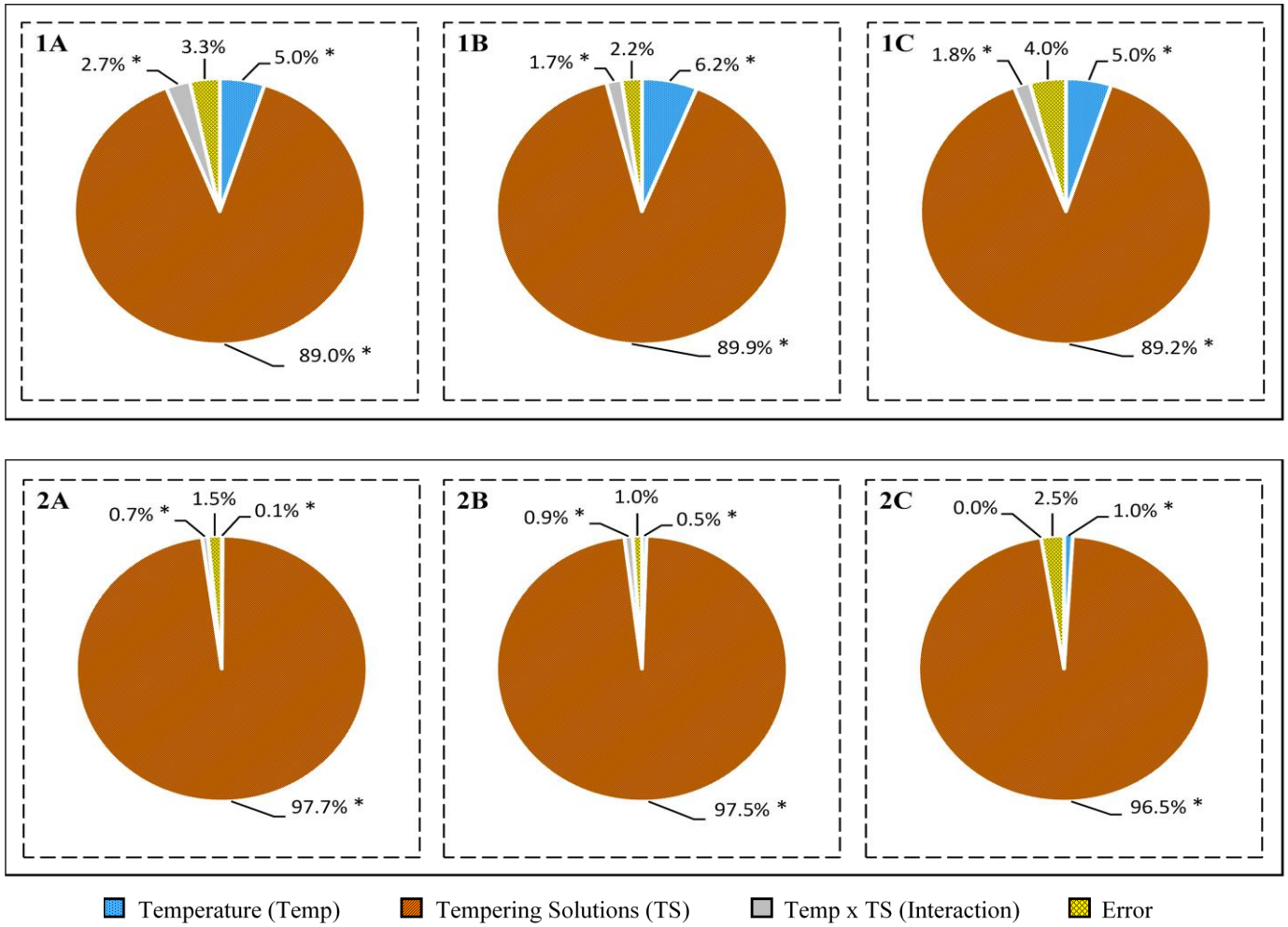


Figure 3-1. Contribution of each independent variable and interactions to the total variance observed in the reduction in counts of *Salmonella* spp. (1A, 2A), *E. coli* O157:H7 (1B, 2B), and non-O157 STEC (1C, 2C) in soft (1) and hard (2) wheat. Independent variables and interactions with an asterisk (*) impacted significantly the pathogen load ($P \leq 0.05$).

When the effect of the tempering solution is removed and the effect of temperature on pathogen reduction is considered alone, some of the temperatures evaluated contributed to the reduction of pathogenic microorganisms during tempering (**Table 3-5**). However, even though some of these temperatures were statistically significant, the reduction values were so close to one another that they may not be practically relevant from a microbiological standpoint.

Table 3-5. Effect of storage temperature on the reduction of pathogenic microorganisms inoculated on soft and hard wheat.

Wheat Class	Storage Temperature (°C)	*Salmonella* spp. [a] Reduction * (log CFU/g)	*E. coli* O157:H7 [b] Reduction * (log CFU/g)	Non-O157 STEC [c] Reduction * (log CFU/g)
Soft Red Winter	2.0	1.18 ± 0.63 a	1.22 ± 0.58 a	1.17 ± 0.55 a
	10.8	0.94 ± 0.50 b	1.23 ± 0.58 a	1.11 ± 0.48 a
	24.2	0.86 ± 0.47 b	1.04 ± 0.44 b	0.91 ± 0.44 b
	32.0	0.93 ± 0.52 b	0.92 ± 0.48 c	0.95 ± 0.42 b
Hard Red Winter	2.0	1.56 ± 0.86 a	1.45 ± 0.69 ab	1.63 ± 0.75 a
	10.8	1.51 ± 0.81 ab	1.39 ± 0.63 bc	1.49 ± 0.73 bc
	24.2	1.44 ± 0.79 b	1.36 ± 0.74 c	1.45 ± 0.69 c
	32.0	1.49 ± 0.71 ab	1.51 ± 0.73 a	1.59 ± 0.72 ab

[a] Five-serovar cocktail of *Salmonella enterica* (Typhimurium, Enteritidis IV/NVSL 94-13062, Heidelberg/Sheldon 3347-1, Infantis and Mbandaka).

[b] Five-strain cocktail of *E. coli* O157:H7 with resistance to kanamycin (AU516 T2, AU1181 LEE3, AU726 LEE2, AU1809 LEE2, and AU1823).

[c] Six-serotype cocktail of non-O157 Shiga toxin-producing *E. coli* (STEC: O26:H11, O45:H2, O103:H11, O111, O121:H19 and O145).

* Reduction on pathogenic population is expressed in log CFU/g and ± denotes standard deviation. Means in the same column within the same wheat class followed by different letters are significantly different ($P \leq 0.05$).

The low variability obtained among replicates may have contributed to the fact that very small differences were detected as significant. This finding is corroborated by the low contribution of temperature to the total variance (**Fig. 3-1**). Therefore, the interaction

and temperature effects were disregarded for further analysis and the average microbial reduction for each tempering solution (main effects) were taken as the best estimate to evaluate for differences in treatments. In addition, it is worth mentioning that independent variables and interactions behaved similarly for both classes of wheat, thus indicating that reduction in pathogen load is more dependent on tempering treatments than wheat classes.

3.3. Effect of saline organic acid solutions on pathogenic microorganisms in soft wheat

Regardless of temperature, tempering with water did not cause a significant reduction in pathogen counts in inoculated soft wheat (**Fig. 3-2**). Mean counts for *Salmonella* spp. (5.8 log CFU/g), *E. coli* O157:H7 (5.9 log CFU/g), and non-O157 STEC (6.2 log CFU/g) after tempering with water remained similar to the levels found before tempering (5.5, 5.7, and 5.9 log CFU/g, respectively). In contrast, pathogen load was reduced significantly by all saline organic acid treatments compared to the control. The antimicrobial effect of saline organic acid solutions has been attributed to the ability of the undissociated form of the organic acids to freely permeate and disrupt the membrane of microbial cells causing a decrease in intracellular pH and facilitating the entrance and accumulation of Na+ and Cl- ions into the cytoplasm resulting in cell death (Sabillón *et al.*, 2016a).

The tempering solution containing lactic acid at 5.0% was the most effective at reducing the numbers of *Salmonella* spp., with an average reduction of 1.8 log CFU/g (**Fig. 3-2**). Increasing the concentration of acetic and lactic acid from 2.5% to 5.0% caused additional reductions of 0.3 and 0.8 log CFU/g, respectively. No significant differences in the reduction of *Salmonella* spp. counts were observed among the

tempering solutions containing acetic acid at 5.0% and lactic acid at 2.5%. These results compare well with a study done by Weissinger and Beuchat (2000), in which increasing the concentration of acetic and lactic acid from 2.0 to 5.0% significantly further reduced the counts of *Salmonella* spp. in dry alfalfa seeds. Also, similarly to the results found here, Weissinger and Beuchat (2000) reported that 5.0% lactic acid was more effective than 5.0% acetic acid. In this study 5.0% lactic acid caused a reduction in *Salmonella* spp. population of 2.98 log CFU/g, while 5.0% acetic acid reduced this pathogen population by 1.74 log CFU/g.

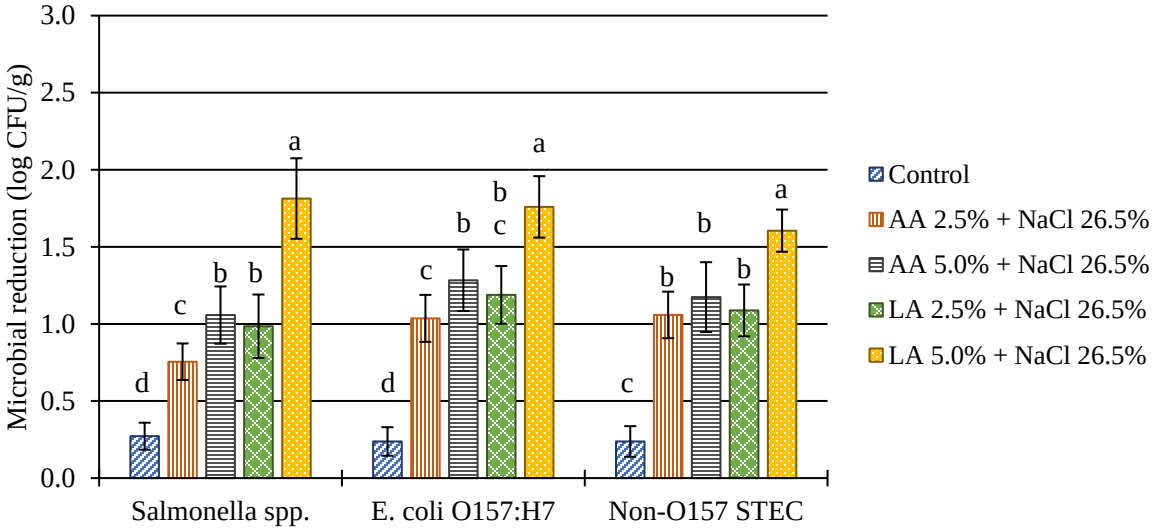


Figure 3-2. Effect of saline organic acid solutions applied during soft wheat tempering on pathogenic microorganisms. Error bars denote ± standard deviation. One log reduction is equivalent to a 90% reduction in colony forming units (CFU) per gram of wheat grain. Log reduction values with different letters within the same microorganism are significantly different ($P \leq 0.05$). Legend: AA, acetic acid; LA, lactic acid; NaCl, sodium chloride; percentage denotes concentration.

As for *Salmonella* spp., the tempering solution containing lactic acid at 5.0% was the most effective at reducing the numbers of *E. coli* O157:H7 and non-O157 STEC, with an average reduction of 1.8 and 1.6 log CFU/g, respectively (**Fig. 3-2**). Increasing the

concentration of acetic from 2.5% to 5.0% caused additional reductions in *E. coli* O157:H7 counts of 0.2 log CFU/g, respectively. Likewise, increasing the concentration of lactic acid from 2.5% to 5.0% caused additional reductions in *E. coli* O157:H7 and non-O157 STEC counts of 0.6 and 0.5 log CFU/g, respectively. No significant differences in pathogen reduction were observed among the tempering solutions containing acetic acid at 5.0% and lactic acid at 2.5%.

The findings reported in this study are in agreement with those described by Lang *et al.* (2000), who washed dry alfalfa seeds contaminated with *E. coli* O157:H7 with pre-warmed (42°C) solutions containing 5.0% lactic or acetic acid for 10 min and reported that solutions containing lactic acid were more effective at reducing the pathogen load (3.0 log CFU/g reduction) than acetic acid solutions (2.3 log CFU/g reduction). These researchers also reported that increasing the temperature of the decontamination solution enhanced its antimicrobial effect, which has been corroborated by other researchers (Huang and Chen, 2011; Dikici *et al.*, 2015). From the perspective of a wheat miller, increasing the temperature of the tempering water may require modifications to the milling facility to accommodate additional unit operations such as heating of the water and subsequent cooling of the grain before milling, thus increasing the overall cost of the milling operation.

As for the differences in counts among pathogenic microorganisms at any given treatment, the overall reductions achieved in *E. coli* O157:H7 were significantly higher than those for *Salmonella* spp., except for the tempering solution containing lactic acid at 5.0% (**Fig. 3-2**). Moreover, no significant differences in the reduction in numbers between *E. coli* O157:H7 and non-O157 STEC were observed at any given treatment.

Previous studies have also observed no differences in the resistance of *E. coli* O157:H7 and non-O157 STEC serogroups to chemical treatments such as lactic acid (Geornaras *et al.*, 2012; Dikici *et al.*, 2015).

3.4. Effect of saline organic acid solutions on pathogenic microorganisms in hard wheat

Similar to results with soft wheat, regardless of temperature, tempering with water did not cause significant changes in pathogen counts in inoculated hard wheat (**Fig. 3-3**). Mean counts for *Salmonella* spp. (6.0 log CFU/g), *E. coli* O157:H7 (6.0 log CFU/g), and non-O157 STEC (6.4 log CFU/g) after tempering with water remained similar to the levels found before tempering (6.3, 6.3, and 6.7 log CFU/g, respectively). In contrast, pathogen load was significantly reduced by all saline organic acid treatments compared with the control. As in soft wheat, the tempering solution containing lactic acid at 5.0% was the most effective at reducing the numbers of *Salmonella* spp., *E. coli* O157:H7, and non-O157 STEC with average reductions of 2.6, 2.4 and 2.4 log CFU/g, respectively.

Increasing the concentration of acetic and lactic acid from 2.5% to 5.0% caused an additional reduction in *Salmonella* spp. counts of 0.6 and 0.9 log CFU/g, respectively. For *E. coli* O157:H7 and non-O157 STEC, the increase in acid concentration further reduced microbial counts by 0.4 to 0.8 log CFU/g depending upon the treatment. No significant differences in the reduction of *Salmonella* spp. counts were observed among the tempering solutions containing acetic acid at 5.0% and lactic acid at 2.5%. The effect observed in this study as a result of tempering hard wheat with saline organic acid solutions is comparable to those reported in the literature for decontamination of *Salmonella* spp. and *E. coli* O157:H7 in dry alfalfa seeds (Lang *et al.*, 2000; Weissinger and Beuchat, 2000), as discussed.

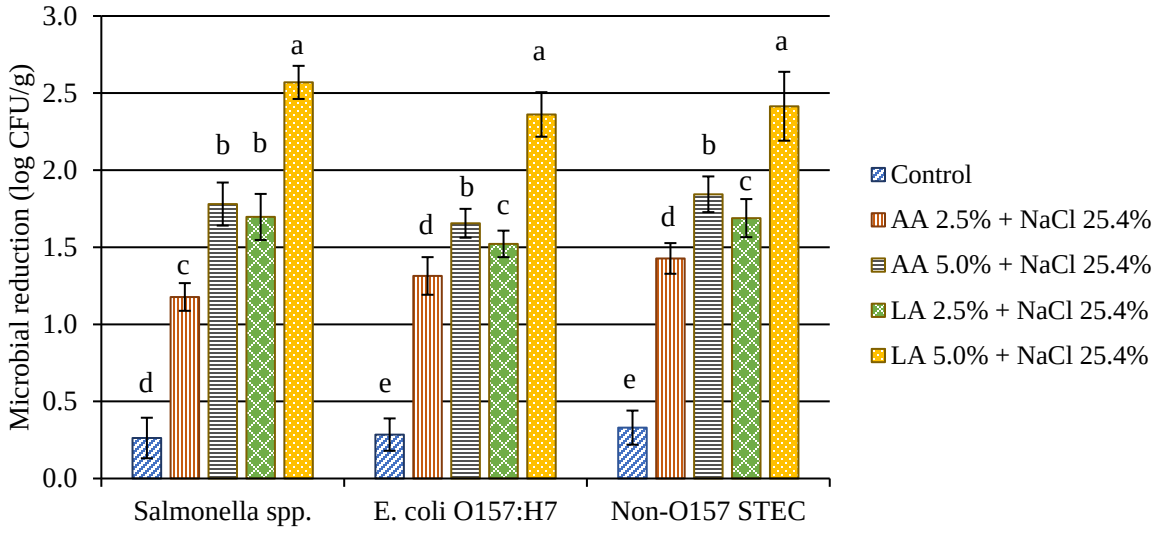


Figure 3-3. Effect of saline organic acid solutions applied during hard wheat tempering on pathogenic microorganisms. Error bars denote ± standard deviation. One log reduction is equivalent to a 90% reduction in colony forming units (CFU) per gram of wheat grain. Log reduction values with different letters within the same microorganism are significantly different ($P \leq 0.05$). Legend: AA, acetic acid; LA, lactic acid; NaCl, sodium chloride; percentage denotes concentration.

The toxicity of organic acids is attributed to the ability of their protonated form to disturb cellular pH homeostasis (Lambert and Stratford, 1999). In this study, the prevalence of the protonated form of the two organic acids did not differ considerably (**Table 3-1**); therefore, the difference in lethality observed between solutions containing acetic or lactic acid may be related to factors other than the concentration of the protonated form of the acid. Factors such as the behavior of undissociated acid molecules inside microbial cells and the intracellular concentration of their protonated form may help explain the observed differences in lethality.

Once inside the cell, the near-to-neutral cytosolic pH causes weak-acid molecules to dissociate into protons and anions, which cannot simply diffuse back through the membrane and therefore are trapped inside the cell (Cherrington *et al.*, 1991). This causes

not only an accumulation of anions but also an acidification of the cytosol until equilibrium is reached such that the internal and external pH values are equal. The pH of the tempering solutions containing lactic acid was considerably lower than their acetic acid counterparts (**Table 3-1**); therefore, microbial cells may have accumulated higher amounts of lactate anions than acetate anions to reach a pH balance, which may explain the higher microbial inhibition observed for lactic acid-containing solutions. By modelling the mode of action of weak-acid in yeast, Lambert and Stratford (1999) demonstrated that microbial inhabitation caused by weak-acids depends more on the degree to which individual acids are concentrated within cells, rather than on undissociated acid concentration *per se*.

In addition, the strength of lactic acid may have contributed to the higher microbial reduction. Lactic acid is a much stronger acid than acetic acid; therefore, it may dissociate to a greater extent inside microbial cells. Furthermore, the toxicity of weak-acids also depends on their chemical structure. Research studies have reported an increase in toxicity of weak-acids as their carbon chain length increases (Sikkema *et al.*, 1995; Stratford *et al.*, 2009); consequently, lactic acid ($C_3H_6O_3$) may have exerted a greater degree of toxicity than acetic acid ($C_2H_4O_2$) since it is a molecule with a larger carbon chain.

Unlike soft wheat, in general, the overall reductions achieved by the different treatments on *E. coli* O157:H7 in hard wheat were slightly lower than those observed in *Salmonella* spp. However, no significant differences in the reduction of *Salmonella* spp. and non-O157 STEC were observed at any given treatment. The discrepancy in the behavior of *E. coli* O157:H7 between soft and hard wheat may be attributed to the ability

of this organism to activate several acid survival systems to protect cells against highly acidic environments. Since acid concentration per gram of wheat might be higher in hard than in soft wheat, it may have triggered the expression of low-pH-inducible acid tolerance response systems thus contributing to a higher acid survival of cells in hard wheat. In fact, it has been previously reported that *E. coli* survive exposure to pH levels as low as 2.0; while *Salmonella enterica* serovar Typhimurium can only tolerate pH values of 3.0 or higher. Additionally, *S.* Typhimurium lacks the acid resistance systems observed in *E. coli*, which contributes to make the cells less acid resistance (Lin *et al.*, 1995).

In general, when comparing between wheat classes, the reduction in pathogen counts achieved by all tempering solutions was higher in hard wheat than in soft wheat. For instance, *Salmonella* spp. counts were, on average, 0.65 log CFU/g lower in hard than in soft wheat after the application of treatments. Similarly, for *E. coli* O157:H7 and non-O157 STEC, the reduction achieved by all tempering solutions was higher in hard than in soft wheat, on average, 0.40 and 0.61 log CFU/g, respectively. The difference might be attributed to a number of factors such as the higher moisture target in hard wheat during tempering (i.e., 15.0% vs 15.5%), thus requiring more volume of treatment solution per gram of wheat. This may not only increase the acid concentration in the sample, but also increases the chances of the solution to reach pathogenic cells trapped or hidden in crevices and within the crease of the kernels. Also, the longer tempering time required to achieve the target milling moisture in hard wheat (e.g., 18 h vs 24 h at 24.2°C) increases the exposure time to the treatment solutions.

4. CONCLUSIONS

The results of this study revealed that tempering soft and hard wheat kernels with solutions containing a combination of organic acid and NaCl, regardless of tempering temperature, effectively reduced the load of pathogenic microorganisms when compared with the traditional tempering process using water. Therefore, these solutions may have the potential to increase the safety of milling end products. For both classes of wheat, the combination of lactic acid (5.0% v/v) and NaCl (~26% w/v) was found to be the most effective treatment to reduce the numbers of *Salmonella* spp., *E. coli* O157:H7, and non-O157 STEC. A variance components statistical analysis revealed that among the variables studied, tempering solution is the main contributing factor to the reduction in pathogen counts, with a contribution ranging from 89 to 97% to the total variance.

Temperatures at which the tempering process may be carried out during different seasons of the year did not significantly influence the effectiveness of the saline organic acid solutions against the pathogenic microorganisms tested. However, the average temperatures experienced by wheat kernels in storage bins during the winter and fall months may favor the survival of pathogenic microorganisms. When comparing among wheat classes, in general, higher reductions in pathogen counts were observed for hard wheat, most likely due to the nature of the kernels, the higher moisture target during tempering (i.e., soft 15.0% vs hard 15.5%) and the longer resting period required to achieve that target milling moisture (e.g., soft 18 h vs hard 24 h at 24.2˚C).

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CHAPTER 4

A MICROBIOLOGICAL SURVEY OF EQUIPMENT AND END-PRODUCTS OF THE WHEAT-MILLING OPERATION

CHAPTER 4. A MICROBIOLOGICAL SURVEY OF EQUIPMENT AND END-PRODUCTS OF THE WHEAT-MILLING OPERATION

ABSTRACT

Microbial contaminants may enter the mill with incoming wheat kernels. Build-up of tempered grain/flour residues in the milling equipment may also serve as a source of contamination during milling. The objectives of this study were to (1) assess the levels of microbial contamination associated with the equipment among the different milling operations and with the wheat as it is processed from grain to packaged flour, and to (2) identify highly contaminated areas or equipment that could serve as a source of contamination during processing. The levels of microbial contamination on the surface of mill equipment were examined using a swab method. A total of 233 contact surface samples of 100 cm^2 were collected from equipment used in a commercial (8.5 million pounds flour/day) and a pilot-scale (40 thousand pounds flour/day) flour milling facility. Sampling points were distributed among equipment involved in cleaning, tempering, and milling wheat grain, and equipment used to handle and store milled products. A total of 44 samples of different wheat milled products, from grain to flour, were also collected. Samples were analyzed for Aerobic Plate Counts (APC), coliform/*E. coli*, *Enterobacteriaceae* (Eb), yeasts and molds. The results indicated that, among the different mill equipment, those used to clean and temper the wheat have the highest microbial load. Generic *E. coli* was found in 2 instances on cleaning equipment at an average of 0.4 log CFU/10 cm^2. Coliform counts increased markedly in wheat kernels and milled fractions after passing through the different milling steps. Flour handling and storage equipment had a considerably higher coliform and Eb counts than other

equipment in the mill. The microbiological quality of the flour streams was greatly influenced by both the microbial load present on the surface of the equipment and the broken kernels/flour residues harbored inside the mill equipment. Appropriate sanitary measures should, therefore, be implemented in the mill, given especial attention to the tempering equipment and grain/flour residues inside mill equipment, to minimize the risk of microbial contamination during processing.

1. INTRODUCTION

Historically, wheat flour has not been a concern from a food safety perspective because of its low moisture content and intended end use. However, over the last few years, the presence of pathogenic microorganisms has been detected in retail-packaged wheat flour in several countries, resulting not only in the recall of flour and flour-based products but also in consumer illnesses (US-FDA, 2015; PHAC, 2017; US-FDA, 2017). Although the source of contamination remains largely unknown, it may be traced back to the environment in which wheat grain is grown, handled, and processed.

As a field crop, wheat is grown in open environments; therefore, it is prone to microbial contamination from diverse sources. As a result, wheat grains usually carry with them a large and varied microbiota (Sabillón and Bianchini, 2016). These microorganisms, which are mostly distributed in the surface of the grain (Laca *et al.*, 2006), are the primary source of contamination for milled products and milling equipment. Once wheat enters the milling system, kernels undergo a sequence of complex operations that include cleaning, conditioning, and milling to produce the desired final product (Posner and Hibbs, 2005b). Since none of these operations includes a microbial-reduction step, as wheat passes through the various milling operations, microbial contaminants may settle on the surface of the equipment. Therefore, as wheat is milled into flour, there is a substantial risk of cross-contamination by microorganisms potentially inhabiting the milling equipment. Epidemiological and environmental investigations of outbreaks caused by low-moisture foods have, in fact, suggested that cross-contamination is one the most common routes of entry of pathogenic microorganisms in these products (CDC, 1998; Rushdy *et al.*, 1998; Smith *et al.*, 2004).

One of the most critical steps of the milling process, from a microbiological standpoint, is the conditioning of wheat grains before milling. This step requires the addition of water to the grains followed by a rest time (Posner and Hibbs, 2005b). This process makes separation of the grain constituents easier during milling; however, when moisture content increases, bacterial cells that were previously metabolically inactive are given the opportunity to grow, potentially leading to high levels of contamination in milled products and equipment surfaces. In a survey carried out by Berghofer *et al.* (2003), higher mesophilic aerobic counts were found more frequently in conditioned wheat kernels. In addition, *E. coli* was detected after wheat conditioning in otherwise previously deemed non-contaminated wheat. The authors concluded that the increase in contamination was likely due to cross-contamination from poorly cleaned conditioning bins and equipment, rather than microbial growth during the tempering period.

Once conditioned, wheat grain passes through a series of rolls and sifters to break apart the kernels, reduce the endosperm to flour, and separate the different grain constituents (Posner and Hibbs, 2005b). Due to the nature of the milling process, build-up of grain or flour residues may be accumulated inside the milling equipment for extended periods of time, representing a potential reservoir of microorganisms and a source of contamination. These grain/flour residues in milling equipment have, in fact, been associated with cross-contamination during milling as well as food safety incidents (Rushdy *et al.*, 1998; Berghofer *et al.*, 2003). The objectives of this study were to 1) assess the levels of microbial contamination associated with the equipment among the different milling operations and with the wheat as it is processed from grain to packaged

flour, and to 2) identify highly contaminated areas or equipment that could serve as a source of contamination during processing.

2. MATERIALS AND METHODS

2.1. Materials

One commercial and one pilot-scale flour milling facility with daily processing capacities of 8.5 million and 40 thousand pounds of flour, respectively, were selected for this study. The microbiological survey was conducted once for the commercial plant and twice for the pilot-scale facility. Surface contact samples were collected using sterile sponges obtained from Fisher Scientific™ (Pittsburgh, PA). Sterilized buffered peptone water and phosphate-buffered saline solution were prepared as indicated by the manufacturer's instructions (Acumedia; Lansing, MI).

2.2. Sampling points and swab method

A total of two hundred thirty-three contact surface samples were collected between the two flour milling facilities. Among the samples collected, sixty-seven were taken from equipment involved in cleaning and tempering of wheat (**Fig. 4-1**). One hundred twenty-eight sampling points were distributed among equipment involved in the milling process (**Fig. 4-2**), while thirty-eight samples were taken from equipment associated with final product handling (**Fig. 4-3**). To obtain the contact surface samples, 100 cm^2 was delimited on the surface of the equipment using a sterile plastic template and the area swabbed using a sterile sponge moistened with 10 ml of 0.1% sterile peptone solution. After swabbing, sponges were placed in sterile plastic bags. In addition to the contact surface samples, forty-four samples of different wheat milled products, from wheat kernels to flour, were collected in sterile plastic bags.

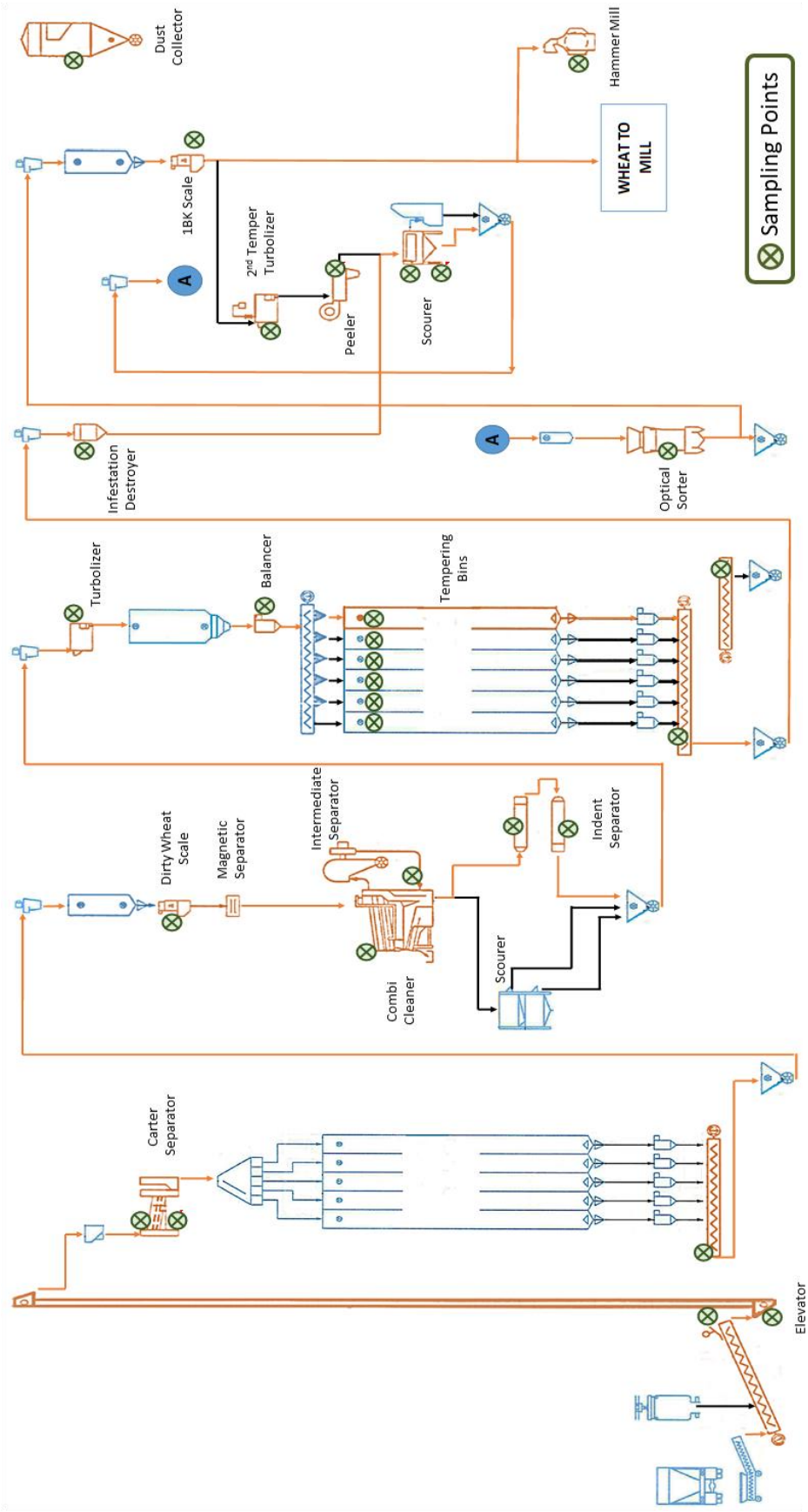

Figure 4-1. Diagram of the wheat cleaning and tempering process, and associated equipment. "X" marks denote locations where contact surface samples were collected.

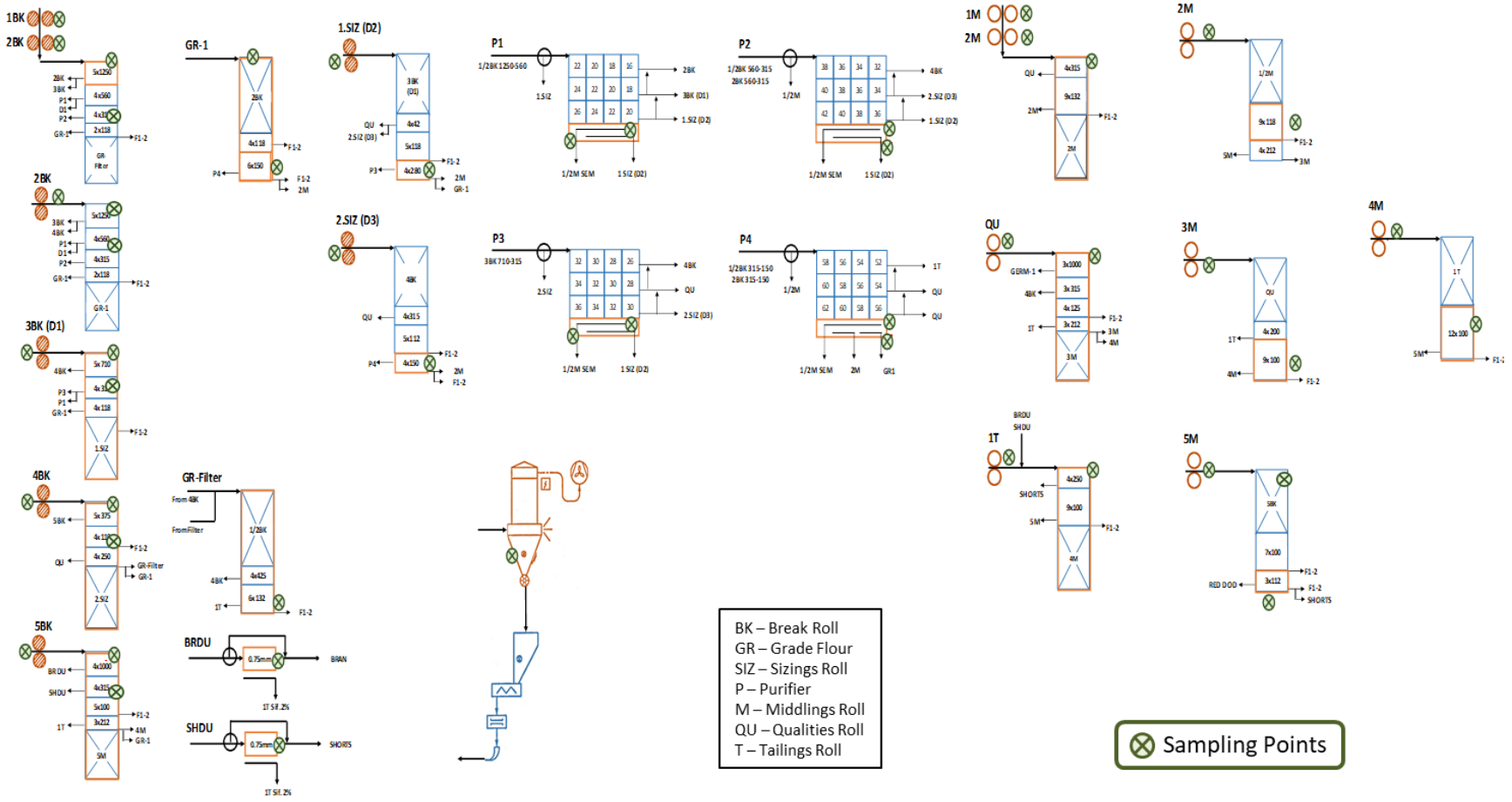

Figure 4-2. Diagram of the wheat milling process and associated equipment. “X” marks denote locations where contact surface samples were collected.

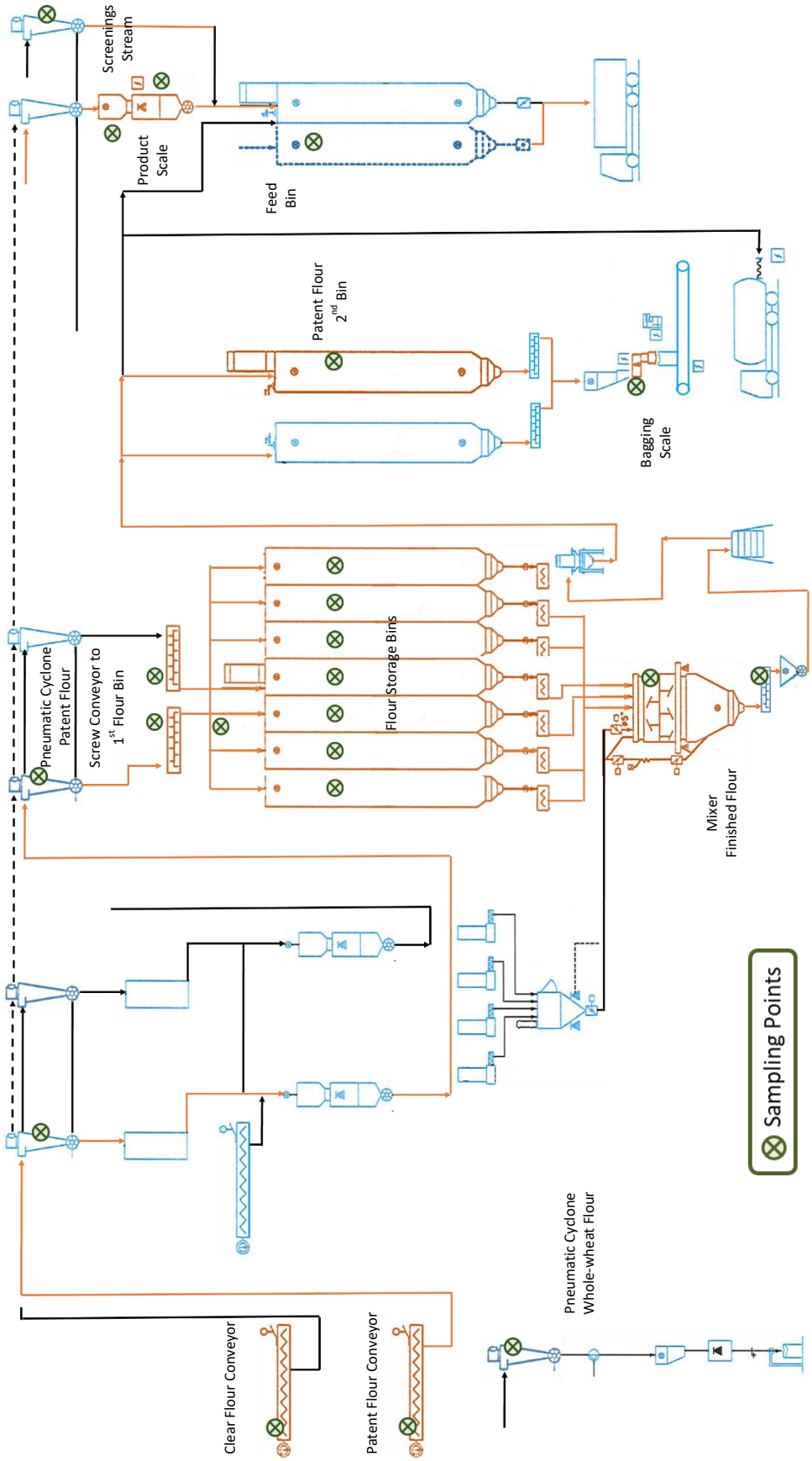

Figure 4-3. Diagram of the flour handling process and associated equipment. "X" marks denote locations where contact surface samples were collected.

2.3. Microbiological analysis

To determine the microbial load of the different milled products, 25 g of sample along with 225 mL of sterilized phosphate-buffered saline solution were placed in a stomacher bag and mechanically mixed with a stomacher blender (Stomacher® 400, Seward Ltd, Bohemia, NY) for 90 seconds. In the case of sponges, the microbial load was determined by adding 90 mL of sterilized phosphate-buffered saline solution to each bag containing the sponge and then mechanically mixed with a stomacher blender (Stomacher® 400) for 90 seconds. Serial decimal dilutions were then prepared from all diluted samples using phosphate-buffered saline solution and analyzed for aerobic mesophilic bacteria [aerobic plate counts (APC)], *Enterobacteriaceae* (Eb), *E. coli* biotype 1/coliform, yeasts, and molds.

For APC, dilutions were surface plated on Standard Methods Agar (SMA, Acumedia, Lansing, MI) and incubated at 35°C for 48 h, according to standard procedures (Maturin and Peeler, 1995). Eb and *E. coli*/coliform counts were determined according to AOAC Official Methods 2003.01 and 991.14, respectively (AOAC International, 2013), using Petrifilm EB and EC plates (3M Microbiology, St. Paul, MN). Dilutions were inoculated on Petrifilm plates and incubated at 37°C for 24 h. For yeast and mold counts, dilutions were spread plated on Dichloran Rose Begal Chloramphenicol agar (DRBC, Acumedia, Lansing, MI) and incubated at 25°C in the dark for 5 days, according to standard procedures (Tournas *et al.*, 1998). The limit of detection for all testing methods was 10 CFU/g or 10 CFU/10 cm^2. Results of bacterial counts were reported as log CFU/g for wheat/flour samples and as log CFU/10 cm^2 for contact surface samples.

2.4. Statistical analysis

An analysis of variance (ANOVA) was carried out to compare the microbial load on the surface of equipment used in the different milling operations. ANOVAs were performed with the SAS software version 9.3 (SAS Institute, Cary, NC). GLIMMIX procedure and Tukey's multiple comparison tests were used to determine significant differences among means. All statistical analysis was performed with a significance level of $P \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Microbial load of wheat milling equipment

The changes in microbial load in the equipment across the different production areas in a flour mill are presented in **Table 4-1** and **Figure 4-1**. The microbial load associated with the equipment in the cleaning and tempering area was significantly higher than in other areas of the mill, with average APC, yeasts and mold counts of 4.7, 2.6, and 4.0 log CFU/10 cm^2, respectively (**Table 4-1**). Slight decreases in microbial load, ranging from 0.8 to 1.3 log CFU/10 cm^2 for APC and from 0.9 to 1.6 log CFU/10 cm^2 for molds, were observed in the equipment used in the following processing areas (i.e., milling and final product handling) (**Fig. 4-1A, 4-1E**). When comparing individual equipment, those that are first in line in the wheat-cleaning system, such as the dirty wheat bucket elevator, dirty wheat bins conveyor, the magnet in conveyor, and the carter separator had, on average, the highest counts for aerobic bacteria (6.4 log CFU/10 cm^2), coliform (2.7 log CFU/10 cm^2), Eb (4.1 log CFU/10 cm^2) and molds (5.1 log CFU/10 cm^2) compared with the rest of the equipment downstream in the cleaning line (4.3, 1.3, 2.4, and 3.7 log CFU/10 cm^2 on average, respectively).

Table 4-1. Microbial load associated with selected equipment used to process the wheat and handle the resulting flour.

Sample Location	Average Microbial Counts (log CFU/10 cm^2) [x]					
	APC [y]	Coliform	Generic *E. coli*	Eb [y]	Yeasts	Molds
Cleaning and tempering equipment						
Cleaning equipment	4.7 ± 1.3 ab	1.5 ± 1.3 b	< 1.0 ± 0.2 [z]	2.6 ± 1.6 ab	2.6 ± 1.3 a	4.0 ± 1.4 ab
Water addition system (Turbolizer)	7.0 ± 1.6 a	4.3 ± 1.2 a	< 1.0 ± 0.0 [z]	4.8 ± 0.7 a	3.9 ± 1.8 a	3.3 ± 0.5 abcd
Tempering bins	4.3 ± 0.9 bc	0.6 ± 0.9 bc	< 1.0 ± 0.0 [z]	1.4 ± 1.5 bc	1.4 ± 1.2 ab	4.9 ± 1.4 a
Milling and sifting equipment						
Roll surfaces - Break system	2.8 ± 1.0 de	0.3 ± 0.6 c	< 1.0 ± 0.0 [z]	1.2 ± 1.0 bc	0.9 ± 0.8 b	2.0 ± 0.9 cd
Roll surfaces - Reduction, sizing, tailing systems	2.3 ± 1.2 e	0.1 ± 0.4 c	< 1.0 ± 0.0 [z]	0.7 ± 1.0 c	0.9 ± 0.8 b	1.4 ± 0.8 d
Inside walls of grinding machines	3.5 ± 0.5 cde	0.7 ± 0.9 bc	< 1.0 ± 0.0 [z]	2.3 ± 0.9 abc	1.9 ± 0.8 ab	2.6 ± 0.6 cd
Walls of sifter boxes	3.3 ± 1.0 cde	0.7 ± 0.9 bc	< 1.0 ± 0.0 [z]	1.5 ± 1.4 bc	0.8 ± 1.0 b	2.2 ± 0.8 cd
Sieves of sifter boxes	3.5 ± 0.9 cde	0.8 ± 1.2 bc	< 1.0 ± 0.0 [z]	2.0 ± 1.4 abc	1.5 ± 1.2 ab	2.5 ± 0.6 cd
Purifiers	3.7 ± 0.9 cd	0.8 ± 0.7 bc	< 1.0 ± 0.0 [z]	2.1 ± 1.2 abc	1.8 ± 1.1 ab	3.0 ± 0.9 bcd
Flour handling and storage equipment						
Flour storage bins	3.8 ± 0.8 bcd	1.2 ± 1.0 bc	< 1.0 ± 0.0 [z]	2.7 ± 0.5 ab	1.6 ± 1.1 ab	3.5 ± 1.1 abc
Twin screw conveyors	3.8 ± 1.1 bcd	1.0 ± 1.1 bc	< 1.0 ± 0.0 [z]	2.4 ± 1.2 abc	2.0 ± 0.6 ab	2.9 ± 0.4 bcd
Product scales	4.2 ± 1.0 bcd	1.1 ± 1.3 bc	< 1.0 ± 0.0 [z]	2.2 ± 1.3 abc	1.9 ± 0.9 ab	3.5 ± 1.0 abc

[x] Values represents average ± standard deviation. Values with different letters within the same microorganism are significantly different ($P \leq 0.05$).

[y] APC, aerobic plate count; Eb, *Enterobacteriaceae.*

[z] Values are below the limit of detection of 1.0 log CFU/10 cm^2.

Wheat arriving in the mill usually contains substantial amounts of foreign matter that must be removed before further processing into flour (Posner and Hibbs, 2005b). These impurities may include not only plant-based materials and soil but also rodent droppings, bird excreta and insects, which may represent a significant source of microbial contamination. As a result, machines devoted to remove these impurities in the mill wheat-cleaning section may carry a considerably high microbial load, which may include pathogenic microorganisms. For instance, generic *E. coli* was found at the bottom of the elevator and in the head of the carter separator at an average of 0.4 log CFU/10 cm^2, while no generic *E. coli* was detected in equipment surveyed in other areas of the mill.

Populations of aerobic bacteria, coliform and fungi were, on average, 1.5, 0.9 and 1.6 log CFU/10 cm^2 higher in machines dedicated to clean the wheat than those devoted to milling and sifting (**Table 4-1; Figure 4-1A-B, 4-1E**). During wheat cleaning, considerable amounts of fine dust particles are likely to be generated and aero-dispersed throughout the cleaning area, thus contributing to increase the levels of microbial contamination on the equipment. An evaluation of the aerial microbiological contamination in different areas of a grain mill conducted by Dacarro *et al.* (2005) found, in fact, that the cleaning section was the most contaminated area in the mill and that the average microbial contamination was 11.4 times higher than in the external environment.

Among the operations involved in preparing the wheat for milling into flour, tempering increases the moisture of the cleaned grain, potentially creating a suitable environment for microbial proliferation either in the wheat kernel or on the equipment. In general, except for molds, microbial populations encountered inside the water addition

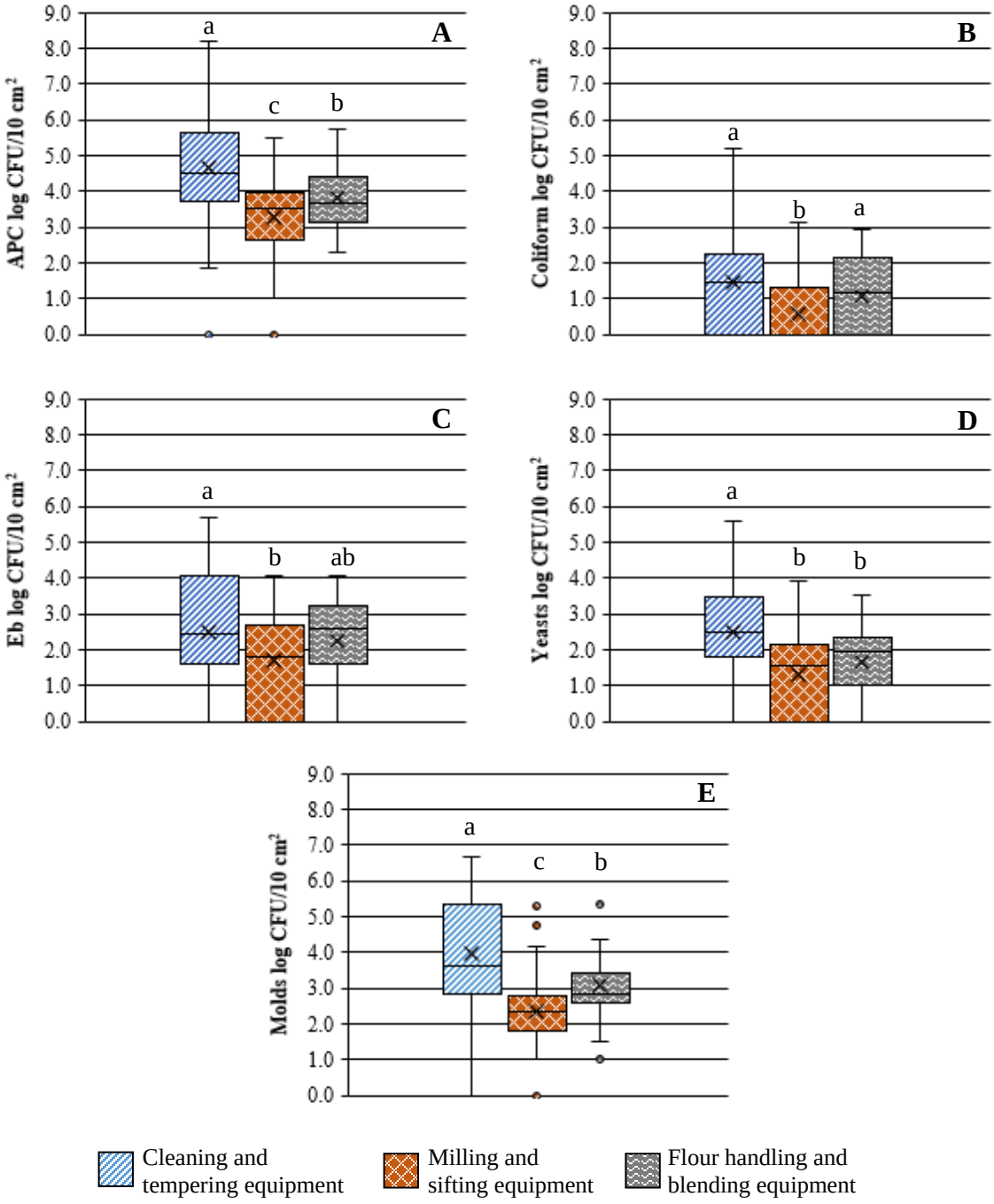

Figure 4-4. Concentration of mesophilic bacteria [APC, (A)], coliform (B), *Enterobacteriaceae* [Eb, (C)], yeasts (D), and molds (E) on equipment used during wheat flour processing. Data are shown by box-and-whisker plots and expressed as log CFU/10 cm^2. Boxes display the lower (25^{th}) and the upper (75^{th}) percentiles, median (horizontal line), and mean (X marker); whiskers indicate the smallest and the highest values; the points below or above the whiskers are outliers. Mean values, within the same microorganism, that share the same letter are not significantly different from one another ($P > 0.05$).

system (turbolizer) were substantially higher than in any other equipment surveyed in this study. For instance, APC, coliform and Eb counts in the turbolizer were, on average, 2.3, 2.8, and 2.2 log CFU/10 cm^2 higher than in the cleaning equipment (**Table 4-1**).

After the addition of water, moistened wheat is held in large tempering bins for a determine period of time to allow the water to penetrate the wheat kernel (Posner and Hibbs, 2005a). The fungi population encountered in the tempering bins was considerably higher than in any other equipment included in this survey. The tempering equipment may, therefore, serve as a source of contamination. In fact, Berghofer *et al.* (2003) reported higher mesophilic bacterial counts and *E. coli* contamination in wheat grain after tempering due to cross-contamination from poorly cleaned water-addition equipment and tempering bins.

Once conditioned, wheat undergoes a series of grinding, reduction, and sifting operations to separate the germ and the bran fractions from the endosperm. This process may be divided into three areas: grinding, sieving and purification (Posner and Hibbs, 2005b). In general, the average microbial counts in the milling and sifting equipment were slightly lower than those observed in the cleaning, tempering and final product handling equipment (**Fig. 4-1**).

The grinding process consists of series of corrugated and smooth rolls, which have defined roles in the milling process such as breaking-apart the kernels, separating the bran, or reducing the endosperm to flour. A lower microbial load was observed in the surface of those rolls dealing with more refined milled products such as middlings (e.g., second and third midds rolls), when compared to those rolls dealing with whole kernels or milled products with high bran content (e.g., first, second or third break rolls) (**Table 4-1**).

Due to the nature of the grinding process, considerable amount of heat is regenerated when breaking-apart the wheat kernels and reducing the endosperm to flour, hence moisture condensation is likely to occur inside the grinding machines (Berghofer *et al.*, 2003). This moisture condensation may facilitate the accumulation of broken kernels and flour residues on the inside walls, which may remain for extended periods of time, thus representing a possible reservoir of microorganisms. In fact, microbial populations associated with the inside wall surfaces of the grinding machines were noticeably higher when compared to microbial populations in the roll surfaces. For instance, APC and Eb counts were respectively, on average, 1.0 and 1.4 log CFU/10 cm^2 higher on the wall surfaces than on the roll surfaces (**Table 4-1**). The constant movement and the heat generated due to friction/abrasion may have contributed to the lower microbial load observed in the roll surfaces when compared to the inside wall of the grinding machines.

After grinding, the next operation is sieving. In this operation the flour produced after each grinding operation is removed and classified for the subsequent step (e.g., further grinding, purification, etc.) (Posner and Hibbs, 2005b). A typical mill gyratory sifter consists of multiple sections, which may contain up to 30 sieves each. Such design may facilitate the accumulation of flour/bran residues on the inside walls, which may remain associated with the equipment for extended periods of time. Microbial contamination levels in the sifter boxes ranged from 3.3 to 3.5 log CFU/10 cm^2 for aerobic bacteria, from 1.5 to 2.0 log CFU/10 cm^2 for Eb, and from 2.2 to 2.5 log CFU/10 cm^2 for molds (**Table 4-1**). These microbial contamination levels were very similar to the levels observed on the walls of the grinding machines.

During the grinding process, some shattering of the bran occurs and results in a mixture of endosperm and bran in the released middlings. The purifiers play an important role in separating these bran particles so that pure middlings go to the reduction system to be reduced to flour (Posner and Hibbs, 2005b). The areas swabbed in the purifiers included the top deck inlet, aspiration channels, and channels conveying semolina, first sizing flour, and grade one flour. The APC and mold counts associated with these areas were slightly higher than the average counts observed for these microorganisms in the milling equipment and in the sifter boxes. For instance, APC counts were, on average, 0.8 and 0.3 log CFU/10 cm^2 higher in the purifiers than in the grinding and sifting equipment, respectively (**Table 4-1**).

To understand why APC and mold counts in the purifiers were higher than in any other piece of equipment analyzed in this section of the mill, an assessment of how this equipment operates may provide some valuable insights. The purifier consists of a reciprocating sieve enclosed in an airtight container with controlled air currents passing through the screens to separate the bran from the middlings (Posner and Hibbs, 2005b). The less-dense materials, such as fine particles of bran, floated by the air current are removed by aspiration in the top section of the purifier, where some surface samples were collected. Considering that microbial contaminants are concentrated in the bran (Laca *et al.*, 2006), the air currents used to remove these bran particles from the system may have contributed, over time, to the dispersion of microorganisms towards the top portion of the equipment.

The flour stream resulting from the grinding and sifting operations is deposited in large storage bins and may go through a flour-blending system to ensure uniform quality

and to treat the flour for special products (Posner and Hibbs, 2005b). Interestingly, the populations of aerobic bacteria, coliform and molds associated with the flour handling and storage equipment were significantly higher than the counts obtained from equipment in the milling section (**Fig. 4-1A-B, 4-1E**). The APC, coliform, and mold counts on the inside wall surface of the flour storage bins were, on average, 3.8, 1.2, and 3.5 log CFU/10 cm^2, respectively (**Table 4-1**). Similar counts were observed on the surface of the product scales and on the surface of the twin screw used to convey the flour. There were no significant differences in the overall coliform and Eb counts between cleaning/tempering equipment and final product handling equipment (**Fig. 4-1B-C**). However, it is noteworthy that average coliform counts in the flour storage bins (1.2 log CFU/10 cm^2) were twice as high as the counts obtained in the tempering bins (0.6 log CFU/10 cm^2). Similarly, Eb counts were 1.3 log CFU/10 cm^2 higher in the flour storage bins than in the tempering bins.

3.2. Microbial load of wheat milled products

As wheat kernels pass through the different steps in the milling process, microbial contaminants located in the outer surface of the kernel are redistributed among the different milled fractions. The microbial load of the different wheat milled fractions is presented in **Table 4-2**. As wheat passes through break, coarse, and fine reduction stages in the mill, microbial contaminants are concentrated in certain milled fractions, particularly in bran and shorts. For instance, when compared to the incoming dirty wheat, coliform counts increased from 1.0 to 3.2 log CFU/g in the bran portion and from 1.0 to 3.0 log CFU/g in the shorts. Similar trends were observed by Berghofer *et al.* (2003) who reported that the bran and germ fractions had higher microbial counts than the incoming

Table 4-2. Microbial load of wheat milled products.

Wheat milled products	Average Microbial Counts (log CFU/g) [x]					
	APC [y]	Coliform	Generic *E. coli*	Eb [y]	Yeasts	Molds
Incoming wheat	5.5 ± 0.2 a	1.0 ± 0.9 b	< 1.0 ± 0.0 [z]	3.2 ± 1.2 a	2.7 ± 0.9 a	2.9 ± 0.6 a
Dry clean wheat	5.3 ± 0.3 a	1.8 ± 1.7 ab	< 1.0 ± 0.0 [z]	3.9 ± 1.1 a	2.5 ± 1.1 a	2.8 ± 0.6 a
Tempered wheat	5.4 ± 0.4 a	1.3 ± 1.2 b	< 1.0 ± 0.0 [z]	3.7 ± 0.8 a	3.3 ± 1.2 a	2.6 ± 0.4 a
1st Break	4.9 ± 0.7 ab	1.9 ± 1.7 ab	< 1.0 ± 0.0 [z]	3.4 ± 0.7 a	1.8 ± 1.5 a	3.1 ± 0.5 a
3rd Break	4.5 ± 0.7 ab	2.3 ± 0.4 ab	< 1.0 ± 0.0 [z]	3.4 ± 1.1 a	2.1 ± 0.6 a	2.5 ± 0.3 a
5th Break	4.8 ± 0.8 ab	3.0 ± 0.1 ab	< 1.0 ± 0.0 [z]	3.6 ± 0.4 a	2.9 ± 1.4 a	2.9 ± 0.4 a
1st Middlings	3.5 ± 0.4 b	2.4 ± 0.3 ab	< 1.0 ± 0.0 [z]	2.7 ± 0.5 a	1.8 ± 0.6 a	2.5 ± 0.1 a
3rd Middlings	3.9 ± 0.3 ab	2.3 ± 0.2 ab	< 1.0 ± 0.0 [z]	2.3 ± 0.5 a	1.6 ± 1.4 a	2.6 ± 0.1 a
5th Middlings	4.9 ± 1.3 ab	2.8 ± 0.5 ab	< 1.0 ± 0.0 [z]	3.2 ± 0.2 a	2.2 ± 0.9 a	2.9 ± 0.9 a
Straight-grade flour	3.9 ± 0.5 ab	2.1 ± 0.5 ab	< 1.0 ± 0.0 [z]	2.2 ± 0.7 a	1.6 ± 0.5 a	3.0 ± 0.6 a
Bran	5.3 ± 0.2 a	3.2 ± 0.5 ab	< 1.0 ± 0.0 [z]	3.4 ± 1.0 a	2.9 ± 1.1 a	2.6 ± 0.6 a
Shorts	5.1 ± 0.4 ab	3.0 ± 0.5 ab	< 1.0 ± 0.0 [z]	3.6 ± 0.8 a	2.7 ± 0.6 a	2.8 ± 0.4 a
Germ	5.1 ± 0.5 ab	3.7 ± 1.0 a	< 1.0 ± 0.0 [z]	4.0 ± 1.1 a	2.6 ± 1.0 a	3.0 ± 1.1 a
Red dog flour	5.2 ± 0.2 ab	3.4 ± 0.5 ab	< 1.0 ± 0.0 [z]	4.9 ± 0.2 a	3.7 ± 0.3 a	2.9 ± 0.2 a

[x] Values represents average ± standard deviation. Values with different letters within the same microorganism are significantly different ($P \leq 0.05$).

[y] APC, aerobic plate count; Eb, *Enterobacteriaceae.*

[z] Values are below the limit of detection of 1.0 log CFU/g.

wheat, with aerobic bacterial count increasing from 6.0 to 7.0 log CFU/g in the bran portion.

When the microbial load of milling-end products is compared, refined flour tends to have lower loads than the incoming wheat kernels and other milled fractions (e.g., bran and shorts). For instance, the aerobic bacteria and Eb counts in the middlings were, on average, 1.4 and 0.5 log CFU/g lower than in the incoming dirty wheat, respectively (**Table 4-2**). A similar trend was observed when comparing middlings with the shorts, bran, or germ fractions. This improvement in microbiological quality, and probably safety, is achieved by the physical removal of the more heavily contaminated outer parts of the kernel (i.e., germ and bran fractions) (Sabillón and Bianchini, 2016). In fact, the impact of bran contamination on the microbial quality of flour is clearly evident when comparing low and high-grade products. For instance, red dog flour, a low-grade product obtained from the tail of the mill, showed higher counts of aerobic bacteria (5.2 log CFU/g), Eb (4.7 log CFU/g), and coliforms (3.0 log CFU/g) than those obtained from straight-grade flour (3.9, 2.2, and 2.1 log CFU/g, respectively).

It is noteworthy that the coliform counts increased markedly in wheat kernels after passing through the cleaning system. For instance, the coliform counts in clean-dry-wheat were 0.8 log CFU/g higher than in the incoming dirty wheat. In fact, in all milled fractions, coliform counts remained at levels that were considerably higher than those present in the wheat kernels before entering the milling system (**Table 4-2**). Mold counts did not vary considerably between milled fractions and incoming wheat. However, in general, the overall microbial counts obtained in the final milled products suggest that

equipment surfaces and build-up of grain/flour residues may serve as a source of contamination as wheat grain is milled into flour.

4. CONCLUSIONS

The results of this microbiological survey revealed that equipment used to remove foreign materials prior to tempering and milling carries a substantially higher microbial load compared to other equipment in the mill. A considerably higher microbial load was found in the tempering equipment when compared to other equipment in the mill, thereby representing a potential source of cross-contamination during processing. Substantial amounts of broken kernels/flour residues were observed on the inside walls of the grinding and sifting equipment. The microbial analysis showed that these residues contain high microbial loads; therefore, if this mill stock is not removed periodically, it can continuously contaminate the stream of ground material passing through the equipment.

The milling process causes a redistribution of microorganisms among the milled fractions, concentrating them in certain fractions such as the bran and shorts, thus leaving the flour as the less contaminated milling end product. However, flour handling and storage equipment showed noticeably higher coliform and *Enterobacteriaceae* counts than other equipment in the mill, thus highlighting the possibility of a potential post-milling microbial contamination before flour reaches the consumer. The potential for post-milling contamination is further increased by the microbial load associated with the product scale – the highest among the product handling equipment for APC, coliforms and molds.

Therefore, the final microbiological quality and safety of wheat flour can be greatly influenced by the microbial load present on the surface of the equipment and by the broken kernels/flour residues within the mill equipment. Appropriate cleaning and sanitization regimens, especially aimed at reducing the microbial load in the tempering equipment and removing the grain/flour residues inside the equipment across the different processing areas, should be implemented in the mill to reduce the risk of cross-contamination during milling.

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CHAPTER 5

THE EFFICACY OF HIGH-PRESSURE PROCESSING TREATMENTS ON THE REDUCTION OF MICROBIAL LOAD IN SUGAR-COOKIE DOUGH AND ITS IMPACT ON BAKING PERFORMANCE

CHAPTER 5. THE EFFICACY OF HIGH-PRESSURE PROCESSING TREATMENTS ON THE REDUCTION OF MICROBIAL LOAD IN SUGAR-COOKIE DOUGH AND ITS IMPACT ON BAKING PERFORMANCE

ABSTRACT

Refrigerated dough products, such as cookie dough, have the potential to be a safety hazard to consumer health because they could be consumed raw or undercooked. This study investigated the efficacy of high-pressure processing (HPP) to reduce the endogenous microbial population and *E. coli* inoculated into sugar-cookie dough and its impact on dough functionality and baking performance. Sugar-cookie dough was prepared at 3 different water activity levels (a_w: 0.80, 0.83, and 0.87), inoculated with a non-pathogenic *E. coli* strain (ATCC 25922) at 7.5 log CFU/g, vacuum-packaged and processed by HPP at 100, 300 and 600 MPa for 1, 3 and 6 min. The experiment followed a split-plot design, with a_w as the main plot factor, combined with a completely randomized design for pressure and time variables. Non-inoculated dough was used to assess the impact of HPP on endogenous microbial populations (i.e., APC, coliform, yeast and mold), as well as functionality and baking performance. HPP treatments significantly reduced all microbial populations tested ($P \leq 0.05$), except for yeasts ($P > 0.05$). Treating the dough at 600 MPa for 6 min significantly reduced E. coli counts by as much as 2.0 log CFU/g. Increasing the water activity of cookie dough from 0.80 to 0.87 did not play a significant role in the reduction of microbial counts ($P > 0.05$); however, it yielded a softer and thicker cookie when baked. Dough and cookie physical characteristics did not differ significantly among HPP-treated and control doughs within the same a_w ($P > 0.05$). These results suggest that HPP could be a useful, post-packaging

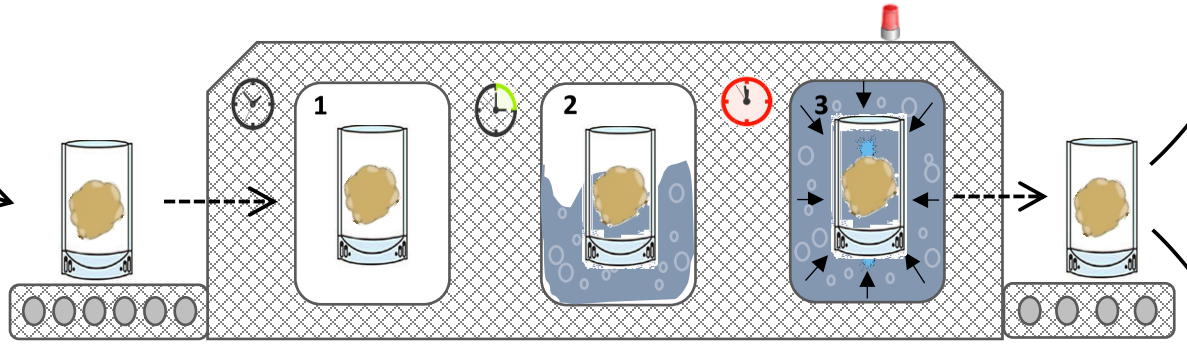
intervention to reduce the risk of microbial contamination in sugar-cookie dough with a minimal impact on product quality parameters.

VISUAL SUMMARY

Preparation
Sugar-cookie dough

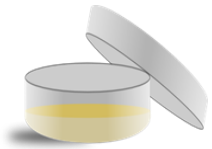
Inoculum
***E. coli* (ATCC 25922)**

Functionality tests



(100-600 MPa, 1-6 min)

Functionality and Baking tests



Enumeration
Aerobic Plate Count, Coliform,
Yeast, Mold, and *E. coli*

Baking performance

1. INTRODUCTION

The potential of commercial ready-to-bake dough products to serve as vehicles of serious and life-threatening foodborne illnesses was illustrated by the outbreak of Shiga toxin-producing *E. coli* O157:H7 infections linked to the consumption of raw refrigerated, prepackaged cookie dough that occurred in the United States in 2009 (Neil *et al.*, 2011). Commercial ready-to-bake cookie dough is a premixed product of various ingredients in which wheat flour, sugar, egg and fat are the major constituents. Many of these ingredients are known for their potential to carry disease-causing bacteria. Shelled eggs and wheat flour, for instance, have been implicated in several foodborne disease outbreaks (Reynolds *et al.*, 2010; CDC, 2016; BCCDC, 2017; CDC, 2018). Premixed cookie dough usually contains a moisture level that does not favor microbial growth (Pareyt and Delcour, 2008); however, pathogenic microorganisms potentially present in their ingredients may survive for lengthy periods of time.

Commercial, refrigerated cookie dough is usually sold without undergoing a pathogen-reduction treatment during processing because the product is intended to be cooked by the consumer before consumption. However, nationwide surveys on risky eating behaviors conducted in the U.S. revealed that eating raw or partially cooked bakery goods is a popular practice among consumers. In a survey conducted by Byrd-Bredbenner *et al.* (2008), 53% of 4,343 young adults surveyed admitted that they regularly consume homemade cookie dough. In another survey, 58 and 67% of the 1,032 consumers surveyed, respectively, indicated that they have tasted refrigerated store-bought cookie dough and raw homemade dough before baking, and another 11 and 24% admitted to having eaten pizza dough and biscuit dough before it was fully cooked

(Ardent Mills, 2010). The investigation of the *E. coli* O157:H7 outbreak linked to commercial cookie dough also concluded that the product was consumed directly from refrigeration without the required baking step (Neil *et al.*, 2011). Relying only on consumers' education about the health risks associated with eating raw dough may not guarantee the absence of food safety incidents. Therefore, further measures should be implemented by manufacturers to eliminate or reduce the risk of pathogen contamination in ready-to-bake dough products prior to their distribution.

Post-packaging interventions, such as high-pressure processing (HPP), can be an alternative to improve the safety level of ready-to-bake dough products. HPP consists in treating foods with high pressure for a determined period of time with the purpose of inactivating both pathogen and spoilage microorganisms and enzymes that may cause undesirable changes during shelf-life. A study carried out by Bárcenas *et al.* (2010) showed that 1 min of HPP at 250 MPa reduced the aerobic mesophilic bacteria and yeast/mold counts in wheat bread-dough from 4.2 to 2.0 log CFU/g. Similarly, Barcenilla *et al.* (2016) reported reductions of 0.52 and 0.70 log CFU/g in total aerobic bacteria and yeast/mold counts, respectively, after treating cake batter at 600 MPa for 6 min. To date, there has been no published research on the application of HPP in wheat flour-based mixtures to inactivate pathogenic microorganisms. Therefore, the objectives of this study were to evaluate 1) the effectiveness of HPP treatments to reduce populations of endogenous microorganisms and a non-pathogenic *E. coli* strain inoculated into sugar-cookie dough as model organism; 2) the impact of dough water activity on the inactivation of microorganisms during HPP; and 3) the impact of HPP on dough functionality and the physical characteristics of the baked cookies.

2. MATERIALS AND METHODS

2.1. Bacterial strain and inoculum preparation

A non-pathogenic *E. coli* strain (ATCC 25922) was used in this study as model organism. This generic strain of *E. coli* is commonly used as quality control strain and has previously been used in HPP inactivation studies (Koseki and Yamamoto, 2006; Lavinas *et al.*, 2008; Viazis *et al.*, 2008). The strain was obtained from the American Type Culture Collection (ATCC; Manassas, VA) and propagated according to manufacturer's instructions. After propagation, the culture was aseptically transferred into cryogenic vials containing 20% sterile glycerol and stored at – 80°C. The culture was re-activated by transferring a small portion of the frozen broth, taken with a sterile loop, into 9 mL tryptic soy broth, followed by incubation at 37°C for 24 h. After incubation, bacterial cells were aseptically transferred into a 50-mL sterile conical tube and harvested by centrifugation at 4,000 x g / 4°C for 8 min (Sorvall™ ST 16R Centrifuge, Thermo Fisher Scientific Inc., Waltham, MA). The pellet was then washed with 25 ml of 0.1% sterile peptone solution, harvested once again and subsequently re-suspended in 50 ml 0.1% sterile peptone solution.

2.2. Preparation and inoculation of sugar-cookie dough

The ingredients and quantities used to prepare the sugar-cookie dough are listed in **Table 5-1**. All ingredients were purchased at a local supermarket. To determine the impact of moisture content on microbial reduction during high pressure processing, the water content of the cookie formulation was adjusted to yield doughs with average water activity (a_w) of 0.80 (Recipe 1), 0.83 (Recipe 2), and 0.87 (Recipe 3). Formulations varied only in the amount of water included in the recipe.

Table 5-1. Ingredients and recipes used to prepare sugar-cookie dough.

Ingredients	Recipe 1	Recipe 2	Recipe 3
	Weight (g)	Weight (g)	Weight (g)
All-purpose wheat flour	750.0	750.0	750.0
Granulated sugar	651.0	651.0	651.0
Salt	12.0	12.0	12.0
Whole egg powder	4.9	4.9	4.9
Baking soda	12.0	12.0	12.0
Non-fat dry milk	13.0	13.0	13.0
All-purpose vegetable shortening	300.0	300.0	300.0
Deionized water (chilled in ice)	204.0	355.0	505.0
Pure vanilla extract	5.5	5.5	5.5
Total Weight	**1952.4**	**2103.4**	**2253.4**

Ingredients were mixed using a 5-quart bowl lift stand mixer (KitchenAid, model KV25G0XER, Benton Harbor, MI) with a flat beater. To prepare the dough, all dry ingredients, except flour, were weighed into the bowl and mixed at low speed (KitchenAid speed 1) for 1 min. Shortening was then added to the bowl and mixed at low speed for 30 s, scraped down, and creamed for 1 min at high speed (KitchenAid speed 4). After creaming, the water and vanilla extract were added and mixed at low speed for 30 s, scraped down, and mixed for 1 min at high speed. Flour was then added to the mixture and mixed for 30 s at low speed and then for 1 min at high speed, with intermittent bowl wall cleaning every 30 s.

To inoculate the dough, the water contained in the formulation was partially replaced by the inoculum to achieve a concentration of approximately 7.5 log CFU/g. To minimize contamination, the mixer was placed inside a biosafety hood throughout the sample preparation process and the bowl was covered with a plastic lid while mixing. The mixer bowl and paddle were autoclaved after each use to ensure sterility. Background microbial flora, functionality and baking performance tests were carried out using non-inoculated dough.

2.3. Packaging and high-pressure processing

Each batch of dough was aseptically divided in ~200 g pieces, placed in 3-mil sterile nylon-polyethylene bags and vacuum sealed at 100 mbar (Model C200, Multivac Inc., Kansas City, MO). Packaged samples were further placed in another vacuum pouch and sealed to avoid any contamination or leakage during high-pressure processing. One double bagged sample was set aside as a control and the rest were placed inside the pressurization vessel of the high-pressure processing device (Hiperbaric 55, Miami, FL) and subjected to either 100, 300 or 600 MPa for 1, 3 or 6 min in a complete 3 x 3 factorial design. High-pressure treatments were conducted at 12°C using water as the pressure-transmitting fluid. The temperature increase due to the adiabatic heating effect during processing was approximately 3°C per 100 MPa.

2.4. Microbiological analysis

To determine the impact of high-pressure processing on the endogenous microbial load, non-inoculated cookie dough was analyzed for aerobic mesophilic bacteria (aerobic plate count [APC]), coliform, yeasts and molds. To determine the number of surviving microbial cells, 25 g of pressure-treated and untreated control doughs were placed in a sterile plastic bag along with 225 mL of 0.1% sterile peptone solution and mechanically mixed with a stomacher blender (Stomacher® 400, Seward Ltd, Bohemia, NY) for 2 min. Original sample dilutions were further diluted (1:10) using 0.1% sterile peptone solution. For APC, dilutions were spread plated in duplicate on standard methods agar (Acumedia; Lansing, MI) and incubated (Heratherm™ IGS 180 Incubator, Thermo Fisher Scientific Inc., Waltham, MA) at 35°C for 48 h, according to standard procedures (Maturin and Peeler, 1995). For yeast and mold counts, dilutions were spread plated in duplicate on

dichloran rose begal chloramphenicol agar (Acumedia) and incubated at 25°C in the dark for five days, according to standard procedures (Tournas *et al.*, 1998). Coliform counts were determined using appropriate Petrifilm plates (3M Microbiology; Saint Paul, MN), according to official method 991.14 (AOACI, 2013). Dilutions were evaluated in duplicate on Petrifilm plates and incubated at 37°C for 24 h.

Surviving *E. coli* populations after high-pressure processing were determined using injury-recovery media to account for sublethally injured cells. Twenty-five grams of inoculated pressure-treated and untreated control doughs were serially diluted as described before and appropriate dilutions were surface plated in duplicate on Tryptic Soy Agar (TSA; Acumedia) plates and incubated at 37.0°C for 3 h to allow the recovery of stressed, injured cells. To minimize the growth of background flora potentially present on cookie dough, TSA plates were overlaid with approximately 9 ml of Sorbitol MacConkey Agar (Acumedia) after the cells-recovery period. All plates were then incubated at 37.0°C for 48 h prior to enumeration. The limit of detection for all microbial enumeration methods was 10 CFU/g. The reduction in microbial load was calculated by subtracting the logarithms of the colony counts obtained from the control and high-pressure treated samples.

2.5. Dough functionality tests

2.5.1. Dough water activity and textural characteristics

High-pressure treated and non-treated (control) dough samples were allowed to reach room temperature before performing the functionality tests. The water activity of the dough was measured at 24°C with an Aqualab 4TE (Decagon Devices, Inc., Pullman, WA). Two water activity measurements were taken for each sample. The stickiness and

strength (cohesiveness) of the dough were evaluated with a TA-TX2 texture analyzer (Texture Technologies; Scarsdale, NY) equipped with a Chen-Hoseney dough stickiness rig. Approximately a 2 g piece was cut from the dough mass and placed in the apparatus. A small amount of dough was extruded through the holes and carefully removed from the lid surface using a spatula. The dough was extruded once again to a length of 1 mm, covered with a plastic cap and rested for 30 s to release the stress produced by extrusion. The dough was then compressed with a 25 mm acrylic cylinder probe to conduct the adhesive test. The test was conducted twice for each dough sample.

2.5.2. Baking performance

Dough samples were rolled out to a thickness of 0.7 cm and cut into circular shapes of 6 cm diameter using a cookie cutter mold. However, due to an excessive stickiness, dough samples with a_w level of 0.87 were weighed and hand-molded to approximately the same dimensions as those obtained with the cutting mold. The cut-out dough pieces were placed at well-spaced points on lightly greased cookie sheets and baked in an electrically pre-heated rotary oven (National Manufacturing Corporation; Lincoln, NE) at 205°C (400°F) for 6.5 min. Each pre-packaged dough sample yielded 6 cookies. After baking, cookies were allowed to cool down at room temperature for 30 min and then packed in high density polyethylene bags until analysis, which was conducted within 7 h. Dough production and baking experiments were replicated on three different days and the results presented are the average of three trials.

2.5.3. Cookie dimensional and textural characteristics

Dimensions of baked cookies (i.e., diameter, thickness, and spread ratio) were measured according to AACC method 10.50.05 (AACCI, 2018). Briefly, to measure the

thickness, 6 cookies were stacked on top of one another, the height (thickness) measured to nearest ½ mm using a caliper, and then restacked in different order and remeasured. In order to measure diameter, 6 cookies were laid edge to edge, the width (diameter) measured, rotated 90° angle, and measured again. The average thickness and diameter of cookies were calculated, separately, by averaging the 2 readings and dividing by 6. Spread ratio was calculated as cookie diameter divided by thickness.

The texture of baked cookies was assessed with a texture analyzer (Model TA-TX2, Texture Technologies; Scarsdale, NY) equipped with a 25-kg load cell and a 3-point bending rig, following the American Institute of Baking standard procedure for cookie hardness (AIB, 2012). The gap between the support beams was set to 40 mm to be half the diameter of the cookies. The support rig distance was kept constant throughout the analysis to ensure comparability of results. Each cookie was precisely centered on the support beams to measure the maximum peak force, an index of cookie hardness, and the distance between the trigger force and the maximum peak force, an index of flexibility. Texture experiments were replicated on three different days and the results presented are the average of three trials. Six cookie samples were assessed in each replication.

2.6. Experimental design and statistical analysis

A split-plot analysis of variance (ANOVA) was used to determine the effect of moisture content, pressure and exposure time on inactivation of microbial cells and functional properties of dough. The experiment was designed with water activity as the whole-plot factor and, pressure and time as the split-plot factors in a randomized 3 x 3 factorial combination. Data were analyzed with SAS software version 9.3 (SAS Institute, Cary, NC). The ANOVAs were performed using the GLIMMIX procedure of SAS.

Significant differences ($P \leq 0.05$) between means were separated by Tukey's test. All statistical analysis were performed with a significance level of $P \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Effect of dough water activity on microbial reduction by high-pressure processing

High pressure requires the presence of a transmitting medium in order to be efficient. The presence of solutes (e.g., sugar, salt) in high concentrations, as well as oils and fats can reduce the amount of the pressure-transmitting fluid (i.e., water) available in the food matrix to cause microbial injury. Wheat flour, sugar and vegetable fats are the major constituents in sugar-cookie dough, which contributes to its low moisture content. To elucidate the influence that the a_w of dough had on the effectiveness of HPP treatments to reduce microbial load, the water content of the cookie formulation was increased to yield doughs with average a_w values of 0.80, 0.83, and 0.87 (**Table 5-1**).

In general, the reduction in counts of the endogenous flora (i.e., APC, coliform, yeasts and molds) present in cookie dough was primarily driven by the level of pressure applied (***P* ≤ 0.05; Table 5-2**). In contrast, the population of *E. coli* inoculated into cookie dough was significantly impacted not only by pressure but also by the holding time, as well as the a_w-pressure interaction (**Table 5-2**). To better understand the impact of this interaction on *E. coli* counts, the contributions of each variable or their combinations to explain the *E. coli* load were calculated through a variance component analysis.

Pressure level made the largest contribution to explaining the total variance (> 72%) in the data set; while the interaction effect a_w-pressure accounted for only 2.0%, a value smaller than the contribution made by the error term (12.8%). Likewise, a_w and time had a very small level of contribution in the total variance, accounting for 4.9

Table 5-2. Analysis of variance of microbial reduction in sugar-cookie dough.

Effect	Type III Tests of Fixed Effects									
	E. coli [a]		**APC** [a]		**Coliform**		**Yeasts**		**Molds**	
	F Value	**Pr > *F***	**F Value**	**Pr > *F***	**F Value**	**Pr > *F***	**F Value**	**Pr > *F***	**F Value**	**Pr > *F***
a_w [b]	2.280	0.184	0.400	0.686	0.130	0.879	0.100	0.905	1.000	0.423
Pressure (Pre)	280.340	<0.0001	8.790	0.001	6.570	0.003	4.610	0.015	14.500	<0.0001
Time	33.040	<0.0001	11.630	<0.0001	1.880	0.164	0.890	0.416	2.260	0.115
a_w*Pre	4.380	0.004	0.030	0.998	0.100	0.982	0.090	0.984	0.320	0.861
a_w*Time	1.380	0.255	0.550	0.698	0.480	0.749	0.140	0.965	0.610	0.661
Pre*Time	0.610	0.659	0.050	0.995	0.130	0.969	0.010	0.999	0.070	0.991
a_w*Pre*Time	1.270	0.281	0.090	0.999	0.030	1.000	0.170	0.994	0.060	0.999

[a] Non-pathogenic *E. coli* strain (ATCC 25922); APC, Aerobic Plate Count.

[b] a_w, water activity.

and 8.2%, respectively. Due to the low contribution of the interaction, it could be assumed that a_w and pressure may be acting independently of each other.

Unlike pressure, the a_w of the dough did not play a significant role at reducing microbial counts (***P* > 0.05; Table 5-2**). In general, increasing the a_w did not cause substantial further reductions in microbial load. For instance, no significant changes in the reduction of APC, coliform, and yeasts were observed when the a_w was increased from 0.80 to 0.87 (**Table 5-3**). However, reduction in *E. coli* counts decreased significantly from 1.37 to 1.07 log CFU/g after increasing the a_w from 0.83 to 0.87. Similarly, the increase in a_w resulted in lower reduction levels of mold counts, ranging from 0.20 log CFU/g at a_w=0.83 to 0.12 log CFU/g at a_w=0.87. Nevertheless, even though some of these a_w levels were statistically significant, the reduction values were so close to one another that they may not be practically relevant from a microbiological standpoint.

Table 5-3. Effect of dough water activity on the reduction of microbial load in sugar-cookie dough.

Dough	Microbial Reduction (log CFU/g) [a]				
a_w	***E. coli*** [b]	**APC** [b]	**Coliform**	**Yeasts**	**Molds**
0.80	1.30 ± 0.42 a	0.40 ± 0.09 a	0.29 ± 0.10 a	0.16 ± 0.11 a	0.13 ± 0.09 ab
0.83	1.37 ± 0.63 a	0.42 ± 0.09 a	0.29 ± 0.12 a	0.15 ± 0.14 a	0.20 ± 0.15 a
0.87	1.07 ± 0.54 b	0.42 ± 0.10 a	0.27 ± 0.10 a	0.13 ± 0.08 a	0.12 ± 0.11 b

[a] Reduction on microbial populations is expressed in log CFU/g and ± denotes standard deviation. Mean values within the same microorganism followed by different letters are significantly different ($P \leq 0.05$).
[b] Non-pathogenic *E. coli* strain (ATCC 25922); APC, Aerobic Plate Count.

Several research studies have reported that inactivation of microorganisms by HPP may be dependent on the level of a_w (Butz *et al.*, 1994; Morales *et al.*, 2006; Hayman *et al.*, 2008); however, in complex food matrices, such as cookie dough, the high concentration of solute (i.e., sucrose) and fat may exert a baroprotective role against

pressure-induced microbial inactivation, thus hampering the effect of a_w. Van Opstal *et al.* (2003) showed that *E. coli* strain MG1655 was pressure-sensitive in the absence of sucrose, but became highly pressure resistant in the presence of 10% to 50% (w/v) sucrose. Numerous research studies have, in fact, indicated that disaccharides (e.g., sucrose) protect microorganisms against pressure-induced inactivation of vital cellular components (Molina-Gutierrez *et al.*, 2002; Molina-Höppner *et al.*, 2004; Koseki and Yamamoto, 2007).

Similarly, fat/oil containing matrices have shown to increase the resistance of microorganisms to HPP destruction due to the formation of local (or global) low a_w refuges. Early work carried out by Simpson and Gilmour (1997) showed that inactivation rates of *Listeria monocytogenes* caused by high pressure were substantially lower when the bacterium was suspended in an olive oil/PBS emulsion (30% v/v oil) than when inoculated into PBS buffer alone. Therefore, due to the presence of high concentrations of sucrose and fat in cookie dough, the net effect of a_w on microbial inactivation after pressure treatment may be difficult to assess. Since a_w and interaction effects did not play a significant role in microbial reduction (**Tables 5-2 and 5-3**), they were disregarded for further analysis and the average microbial reduction for each HPP treatment (main effects) were taken as the best estimate to evaluate for differences in treatments.

3.2. Effect of high-pressure processing on the endogenous microbial population of cookie dough

The increase of pressure in the environment surrounding microbial cells may affect not only their morphology but also may inhibit metabolic reactions and cause changes to genetic mechanisms, resulting in a cascade of events that may be catastrophic to microbial cell viability (Abe, 2007; Huang *et al.*, 2014).

Small but significant reductions in the endogenous microbial population of cookie dough were observed after the application of treatments (**Fig. 5-1**). The initial population of aerobic mesophilic bacteria was reduced significantly by all HPP treatments ($P \leq 0.05$). Treating dough at 100, 300, or 600 MPa for 1, 3 or 6 min reduced the APC counts by an average of 0.5 log CFU/g (**Fig. 5-1A**). Reduction trends of coliform counts in HPP-treated dough were similar to those observed for APC counts. Regardless of the pressure-holding time, HPP at 300 and 600 MPa resulted in about 0.3 log CFU/g reduction in the population of coliform bacteria (**Fig. 5-1B**).

Regarding mold counts, high pressure levels (600 MPa) were required to cause a significant reduction of 0.2 log CFU/g when compared with the initial counts in the control dough (**Fig. 5-1D**). As observed in APC and coliform, no further significant decrease in mold counts was obtained by extending the treatment time. For yeast, no significant changes in counts were observed after the application of treatments when compared with the control (**Fig. 5-1C**). Although usually an increase in pressure is associated with higher microbial inactivation rates, this relationship was not observed in the present study. In addition, increasing the duration of the HPP treatments did not cause significant further reductions in microbial counts, which agree with findings reported by previous research studies on microbial inactivation in high-pressure treated flour-based foods (Bárcenas *et al.*, 2010; Barcenilla *et al.*, 2016; Aguirre *et al.*, 2018).

The pressure resistance of vegetative microbial cells varies considerably among different genera and species. In general, Gram-negative bacteria, yeasts, and molds are more susceptible to pressure than Gram-positive bacteria and bacterial spores (Arroyo *et al.*, 1997). However, the pressure sensitivity of microorganisms is also highly dependent

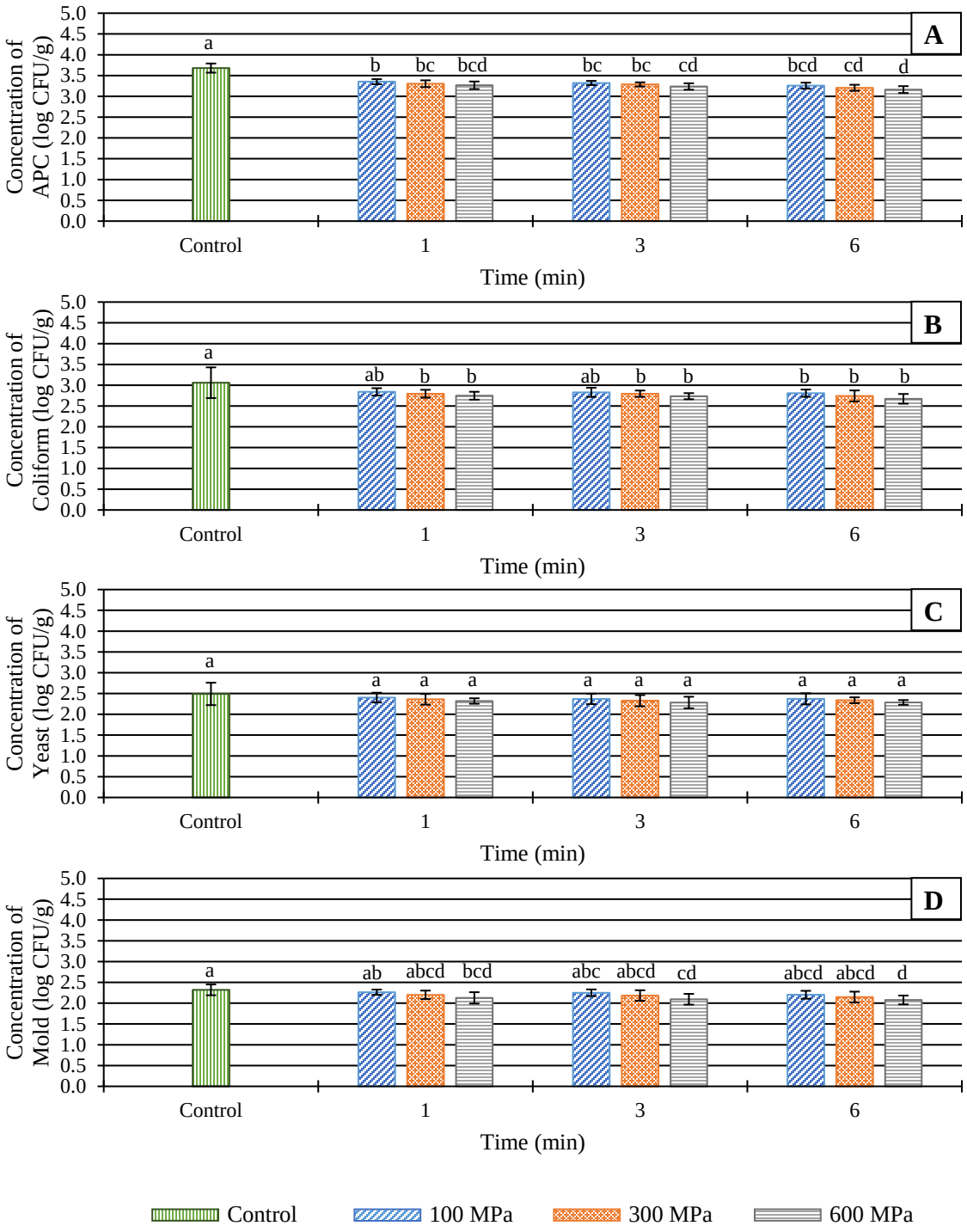


Figure 5-1. Concentration of endogenous microorganisms [APC (A), coliform (B), yeasts (C), and molds (D)] in sugar-cookie dough after high pressure processing treatment. APC, aerobic plate count. Error bars denote ± standard deviation. Mean values, within the same microorganism, that share the same letter are not significantly different from one another ($P > 0.05$).

on the composition of the food matrix. HHP-induced inactivation studies have reported an increase in the pressure resistance of yeasts and molds when present in food matrices with high sugar (e.g., sucrose, fructose, and glucose) concentration (Ogawa *et al.*, 1990; Oxen and Knorr, 1993; Palou *et al.*, 1998; Goh *et al.*, 2007). Hashizume *et al.* (1995) reported that sugars, particularly sucrose, confer a high degree of protection to yeasts against pressure inactivation when the process is applied at ambient temperature.

Similarly, Goh *et al.* (2007) observed an increase in surviving yeast and mold cells when they were pressure-treated in solutions with high concentration of sucrose. In addition, the authors reported that, when suspended in 50 °Brix sucrose solution, yeast cells showed a greater resistance to pressure than molds, which was attributed to the yeast ascospores having greater pressure resistance than vegetative cells (Goh *et al.*, 2007). Therefore, the high sucrose concentration in the cookie dough used in the present study may have created a baroprotective effect against pressure-mediated cell death.

Many research studies have attempted, with varying degrees of success, to reduce the endogenous microbial population in flour-based mixes and batters using HPP. A study carried out by Bárcenas *et al.* (2010) showed that 1 min of HPP at 250 MPa reduced the aerobic mesophilic bacteria and yeast/mold counts in wheat bread-dough from 4.2 to 2.0 log CFU/g. Aguirre *et al.* (2018) reported more than 4.0 and 2.5 log CFU/g reduction in aerobic mesophilic bacteria and yeast/mold counts, respectively, in sugar-snap cookie dough after HPP at 100 or 200 MPa for 2 min. The microbial reductions obtained in the present study were substantially lower than those reported by these authors, most likely due to the composition of the sugar-cookie dough.

As mentioned, food constituents such as proteins, sugars, and lipids could confer a certain degree of protection to microorganisms against high-pressure treatments, which would hinder the effectiveness of HPP (Georget *et al.*, 2015). For instance, considerably lower microbial reductions than those reported by other authors were obtained by Barcenilla *et al.* (2016) using a more complex flour-based matrix. Their results showed reductions of 0.5 and 0.7 log CFU/g in total aerobic bacteria and yeast/mold counts, respectively, after treating layer cake batter at 600 MPa for 6 min. In the same study, Barcenilla *et al.* (2016) reported that lactic acid bacteria and total anaerobic bacteria counts remained unchanged after the application of HPP treatments when compared with the control cake batter. The microbial reduction levels obtained in the present study are similar to those reported by Barcenilla *et al.* (2016), perhaps due to similar composition of the food matrices evaluated.

The total amount of solutes, especially sugar, in the cookie dough formulation used in the present study was considerably higher than in the flour-based products evaluated by Bárcenas *et al.* (2010) and Aguirre *et al.* (2018). Research studies on compressibility of foods under pressure have reported that an increase in solute concentration (i.e., sugar content) results in a decrease of liquid product's compressibility, leading to a poor pressure-induced microbial inactivation (Min *et al.*, 2010; Fauzi *et al.*, 2017). Therefore, a decrease in compressibility due to the high solute concentration in cookie dough may have also contributed to the reduced microbial inactivation observed in the present study.

3.3. Survival of *E. coli* inoculated into cookie dough after high pressure treatment

The numbers of surviving *E. coli* (ATCC 25922) cells in cookie dough after HPP at various pressure levels and holding times are shown in **Figure 5-2**. The initial population

of *E. coli* was reduced significantly by all HPP treatments ($P \leq 0.05$). After treatment at 100 MPa, regardless of the pressure-holding time, the *E. coli* population decreased from 7.5 to 6.8 log CFU/g. Increasing the pressure level to 300 or 600 MPa resulted in significant further reductions in *E. coli* counts. For instance, a reduction of 1.4 log CFU/g was achieved after treatment at 300 MPa, while at 600 MPa the *E. coli* population declined, on average, by 1.8 log CFU/g when compared with the control dough.

Increasing the time of treatment from 1 to 3 min, resulted in no or only negligible further inactivation of *E. coli* cells; however, by extending the exposure time to 6 min, significant further reductions in the order of 0.4 log CFU/g, on average, were observed. HPP of cookie dough at 600 MPa for 6 min decreased the *E. coli* counts by as much as 2.0 log CFU/g when compared with the initial counts in the control dough.

Although numerous studies on pressure-induced inactivation of *E. coli* have been published, direct comparisons between the pressure-inactivation data obtained in the present study and the data reported by other authors may not be appropriate due to remarkable differences in the complexity of the matrices harboring the microbial cells (e.g., phosphate buffer, milk, juice, and meat versus cookie dough). However, HPP inactivation studies that have been carried out using complex food matrices and have used the same or similar model microorganisms may provide the most relevant comparison to the present study.

As mentioned, HPP-induced microbial inactivation may be influenced by both food matrix composition and microbial characteristics. For instance, Garcia-Graells *et al.* (1999) compared the pressure resistance of *E. coli* MG1655 cells inoculated into phosphate buffer and milk. The results showed that *E. coli* inactivation in milk reached

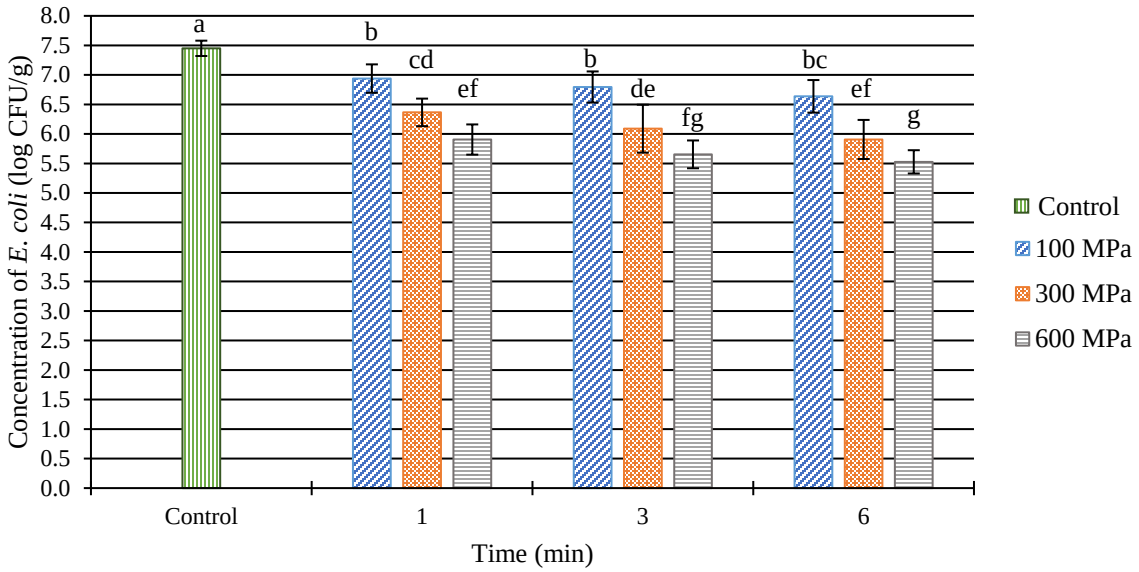


Figure 5-2. Concentration of non-pathogenic *E. coli* (ATCC 25922) in sugar-cookie dough after high pressure processing treatment. Error bars denote ± standard deviation. Mean values that share the same letter are not significantly different from one another ($P > 0.05$).

2.3 log CFU/ml after treatment at 700MPa for 15 min, whereas in phosphate buffer, a pressure of 400 MPa for the same period of time was enough to achieve a reduction in counts of approximately 7 log CFU/ml. Similarly, Viazis *et al.* (2008) reported that *E. coli* ATCC 25922 survival was significantly higher in pressure-treated samples of human milk than in 0.1% peptone solution, with the former requiring more than 46 min at 400 MPa to achieve complete inactivation (9 log CFU/ml), while the later required only 10 min at the same pressure level in peptone solution for total inactivation.

Comparable inactivation levels of *E. coli* to the ones observed in the present study were reported by Lavinas *et al.* (2008), although in cashew apple juice (11.2 °Brix). The reduction in the population of *E. coli* ATCC 25922 in the juice after pressurization for 1.5 min was 0.12, 0.18, and 1.3 log CFU/mL at 250, 300, and 350 MPa, respectively; while a 2.2 log CFU/mL reduction was achieved at 400 MPa for 30 s. In the present study, higher

pressure levels and longer holding times than those used by Lavinas *et al.* (2008) (600 MPa/6 min vs 400 MPa/30 s) were needed to achieve a 2-log reduction in the *E. coli* population (**Fig. 5-2**). This indicates that solid, complex foods may provide a greater degree of protection for microbial cells than liquid foods, which is in agreement with previous published research (Tassou *et al.*, 2007).

The baroprotective effect of high solute concentrations, specially sucrose, has been described by several authors. Studies by Van Opstal *et al.* (2003) reported that *E. coli* MG1655 became highly pressure resistant when sucrose (10% to 50% w/v) was added to the microbial suspension. To illustrate this point, in the presence of sucrose (10% w/v or more), *E. coli* MG1655 acquired almost the same level of pressure resistance as the pressure-resistant mutant *E. coli* LMM1010. Researchers have proposed that disaccharides, such as sucrose, may have the potential to interact with the cytoplasmic membrane and maintain its fluidity by retarding the shift from the liquid phase to the gel phase under high pressure environments, thus protecting vital cellular components and metabolic mechanisms (Molina-Höppner *et al.*, 2004).

Moreover, research studies have suggested that the baroprotective effect of foods with high sucrose concentration is not solely due to the sugar content but also due to the lack of compressibility caused by the excess of solutes (Fauzi *et al.*, 2017). The high sucrose concentration in cookie dough could have, therefore, conferred a certain degree of protection to *E. coli* cells against pressure-induced inactivation.

Other food constituents such as fats and oils may exert a baroprotective role against pressure-induced microbial inactivation. For instance, Garcia-Graells *et al.* (1999) reported that inactivation of *E. coli* cells was significantly higher in skim milk (0.05% fat,

3.0 log CFU/mL reduction) than in whole milk (3.6% fat, 1.6 log CFU/mL reduction), thus suggesting a baroprotective effect of fat. The formation of low water activity refuges within the food matrix due to the presence of fat/oil, it is believed to play a role in the observed protection against pressure. In addition, bacteria with hydrophobic surfaces may be attracted to these refuges where there is much lower water content and, therefore, lower HPP-induced inactivation (Georget *et al.*, 2015).

The surface cell structures, such as lipopolysaccharides, may influence the physicochemical properties of bacterial cells including the degree of hydrophilicity or hydrophobicity. El *et al.* (2002) compared the relative hydrophobicity of several *E. coli* strains expressing different surface structures using a polyethylene glycol-dextran two phase system. The results of their investigation showed that those strains expressing O-antigen exhibited a higher surface hydrophobicity than strains expressing R, K, or H antigens. The *E. coli* strain (ATCC 25922) used in the present study belongs to the serotype O6; therefore, the hydrophobic nature of the bacterium may have facilitated its migration into non-aqueous regions, which in turn may have contributed to protect *E. coli* cells against lethal effects of pressure.

3.4. Effect of water activity and high-pressure processing on dough characteristics and baking performance

Sugar-cookie dough texture related parameters were not significantly affected by any of the HPP treatments applied (**Table 5-4**). Increasing the pressure level and the pressure-holding time did not cause significant changes in the dough physical characteristics analyzed in the present study. In general, the stickiness and cohesiveness did not differ significantly between HPP-treated and control dough samples ($P > 0.05$).

Increasing the moisture content of the dough, however, resulted in a more sticky and cohesive dough.

Slightly contradictory results on the impact of HPP on quality characteristics of flour-based foods have been reported by other researchers. For instance, Bárcenas *et al.* (2010) observed a decrease in bread dough stickiness when increasing the pressure-holding time beyond 1 min, while Aguirre *et al.* (2018) reported an increase in stickiness after treating sugar-snap cookie dough at 200 and 400 MPa for 4 and 15 min, respectively. In the present study, the uniform cohesiveness between HPP-treated and untreated doughs is in agreement with observations made by Bárcenas *et al.* (2010) who detected no significant differences in the cohesiveness of HPP-treated and untreated wheat-bread doughs, and with Barcenilla *et al.* (2016) who reported that cohesiveness of cakes made of either control or HPP-treated batters did not differ significantly.

The dimensional and textural characteristics of cookies made with HPP-treated dough were not significantly different than those observed in control samples (**Table 5-5**). Increasing the a_w of dough yielded thicker cookies when baked. Depending on the dough a_w, the diameter of cookies varied from 8.19 to 8.44 cm, while the thickness and spread ratio ranged from 0.82 to 1.19 and from 6.98 to 9.99, respectively. Previous studies have, however, reported an increase in diameter and a decrease in thickness in cookies made with HPP-treated dough when compared with control cookies, although such differences were not directly related to the intensity or the duration of the HPP treatment (Aguirre *et al.*, 2018).

Table 5-4. Effect of different levels of dough moisture content, pressure and holding times on sugar-cookie dough physical characteristics.

Treatments [a]			Dough Characteristics [b]	
a_w [c]	Pressure (MPa)	Holding Time (min)	Stickiness (N)	Cohesiveness
0.80	0	0	0.18 ± 0.02a	0.31 ± 0.04a
	100	1, 3, 6	0.19 ± 0.01a	0.32 ± 0.03a
	300	1, 3, 6	0.19 ± 0.02a	0.32 ± 0.04a
	600	1, 3, 6	0.19 ± 0.01a	0.31 ± 0.03a
0.83	0	0	0.49 ± 0.02a	1.47 ± 0.08a
	100	1, 3, 6	0.49 ± 0.01a	1.41 ± 0.06a
	300	1, 3, 6	0.49 ± 0.02a	1.47 ± 0.16a
	600	1, 3, 6	0.48 ± 0.02a	1.43 ± 0.19a
0.87	0	0	1.08 ± 0.05a	2.63 ± 0.38a
	100	1, 3, 6	1.07 ± 0.07a	2.69 ± 0.22a
	300	1, 3, 6	1.07 ± 0.08a	2.69 ± 0.19a
	600	1, 3, 6	1.08 ± 0.10a	2.73 ± 0.25a

[a] No significant differences among HPP holding times were observed; therefore, to simplify the presentation of data, results from those dough quality tests were averaged.

[b] Data reported as mean ± standard deviation. Mean values, within the same dough quality parameter and a_w level, that share the same letter are not significantly different from one another ($P > 0.05$).

[c] a_w: water activity of the dough.

The spread ratio, a relationship between diameter and thickness of cookies, was not significantly altered by the application of HPP treatments, although it decreased considerably with the increase in moisture content of the dough (**Table 5-5**). Aguirre *et al*. (2018) reported, instead, an increase in spread ratio when treating dough at high pressures; however, no significant differences were observed among HPP treatments (100-400 MPa, 2-15 min). Dough viscosity plays a key role in defining the degree of spread of the dough during baking as well as the final thickness of cookies (Hoseney and Rogers, 1994). No significant changes in the dough physical characteristics were observed among the different HPP treatments (**Table 5-4**), which could explain the

Table 5-5. Effect of different levels of dough moisture content, pressure, and holding times on cookie dimensional and textural characteristics.

Treatments [a]			Cookie Dimensional Characteristics [b]			Cookie Textural Characteristics [b]	
a_w [c]	Pressure (MPa)	Holding Time (min)	Diameter (cm)	Thickness (cm)	Spread Ratio [d]	Hardness (N)	Flexibility (cm)
0.80	0	0	8.20 ± 0.04a	0.82 ± 0.01a	9.98 ± 0.13a	22.09 ± 0.88a	4.52 ± 0.04a
	100	1, 3, 6	8.20 ± 0.03a	0.82 ± 0.01a	10.04 ± 0.10a	22.72 ± 2.37a	4.52 ± 0.06a
	300	1, 3, 6	8.15 ± 0.04a	0.82 ± 0.01a	9.96 ± 0.09a	22.12 ± 1.48a	4.53 ± 0.03a
	600	1, 3, 6	8.19 ± 0.02a	0.82 ± 0.01a	10.01 ± 0.08a	22.94 ± 1.79a	4.56 ± 0.05a
0.83	0	0	8.49 ± 0.14a	0.93 ± 0.03a	9.17 ± 0.13a	3.68 ± 0.48a	4.33 ± 0.07a
	100	1, 3, 6	8.48 ± 0.13a	0.93 ± 0.02a	9.17 ± 0.28a	3.52 ± 0.43a	4.36 ± 0.05a
	300	1, 3, 6	8.46 ± 0.13a	0.92 ± 0.02a	9.18 ± 0.27a	3.46 ± 0.41a	4.36 ± 0.07a
	600	1, 3, 6	8.34 ± 0.10a	0.93 ± 0.02a	9.00 ± 0.14a	3.52 ± 0.40a	4.37 ± 0.04a
0.87	0	0	8.27 ± 0.07a	1.20 ± 0.02a	6.92 ± 0.18a	1.92 ± 0.52a	4.42 ± 0.08a
	100	1, 3, 6	8.32 ± 0.17a	1.18 ± 0.02a	7.06 ± 0.18a	1.98 ± 0.49a	4.39 ± 0.11a
	300	1, 3, 6	8.28 ± 0.11a	1.19 ± 0.03a	6.96 ± 0.21a	1.99 ± 0.36a	4.48 ± 0.11a
	600	1, 3, 6	8.27 ± 0.05a	1.19 ± 0.03a	6.98 ± 0.19a	2.17 ± 0.27a	4.48 ± 0.06a

[a] No significant differences among HPP treatments were observed; therefore, to simplify the presentation of data, results from cookie quality tests were averaged.

[b] Data reported as mean ± standard deviation. Mean values, within the same cookie quality parameter and a_w level, that share the same letter are not significantly different from one another ($P > 0.05$).

[c] a_w: water activity of the dough.

[d] Spread ratio= Diameter / Thickness.

observed uniformity in diameter and thickness of baked cookies.

Water plays an important role during dough and cookie preparation. The amount of water present in the dough influence its viscosity, which in turn may affect the degree of spread during baking and the final textural characteristics (Hoseney and Rogers, 1994). Miller *et al.* (1997) studied the effect of different water content on the spread of sugar-snap cookies. The results of their investigation concluded that varying the amount of water content in the cookie dough formulation has little, if any impact, on final cookie diameter; however, it did affect cookie dough spread rate during baking. These researchers reported that increasing the amount of water caused the spread rate to increase, presumably by lowering dough viscosity due to a higher water absorption from soluble components like sugar, but at the same time shortened the set time (Miller *et al.*, 1997). In the present study, the increase in water content in the formulation, caused minimal changes to the cookie diameter but substantially decreased the spread factor, which differs with the results previously reported by Miller *et al.* (1997).

The variation in cookie thickness observed in the present study may be related to the so-called collapse phenomenon, which is associated with the water content of the formulation. During baking, the period of dough expansion is followed by a marked structural collapse, which is more pronounced in doughs with low water content because the proteins are not hydrated enough to form a gluten network and the water amount is insufficient to cause enough starch gelatinization (Chevallier *et al.*, 2002). Therefore, the high thickness level observed in the present study for cookies made with dough containing high moisture content may be related to a well-developed protein network and starch gelatinization, leading to a minimal structural collapse after baking.

The flexibility and the maximum force (hardness) to snap the cookies made of HPP-treated and control doughs did not vary significantly (**Table 5-5**). However, increasing the a_w of the dough considerably decreased the hardness of baked cookies. Previous research studies have reported detrimental effects of HPP treatments on quality attributes of flour-based foods, such as hardness. Aguirre *et al*. (2018), for instance, reported a significant increase in hardness of baked cookies when treating dough at 200-400 MPa, although no detrimental effects were observed at mild pressure levels (100 MPa). The diverse composition of food matrices evaluated across different studies may explain why results on HPP-treated dough quality parameters are somewhat contradictory.

4. CONCLUSIONS

Sugar-cookie dough offers a challenging environment for microbial inactivation by high pressure due to the presence of high concentrations of sugar (sucrose) and fat (shortening). The results obtained in the present study nonetheless suggest that the microbial load of sugar-cookie dough could be significantly reduced by the application of HPP treatments. HPP-treated doughs showed reduced levels of endogenous microbial populations including APC, coliform, and molds. In addition, the population of *E. coli* inoculated in dough was reduced by as much as 2.0 log CFU/g when treated at 600 MPa for 6 min. The increase in the moisture content of the dough did not play a significant role in microbial reduction. Furthermore, HPP treatments did not negatively affect the quality characteristics of doughs and cookies. Therefore, HPP could be used as a post-packaging intervention to improve the safety of ready-to-bake sugar-cookie dough. However, variations in food matrix composition must be considered since some food

constituents, such as sugar and fat, can induce an increased pressure resistance in microbial cells. Further research is warranted to evaluate the impact of HPP on the sensory characteristics of baked goods.

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CHAPTER 6

QUANTITATIVE ASSESSMENT OF THE EFFECTIVENESS OF INTERVENTION STRATEGIES TO REDUCE THE RISK OF *E. COLI* O157:H7 INFECTION DUE TO CONSUMPTION OF UNCOOKED READY-TO-BAKE COOKIE DOUGH

CHAPTER 6. QUANTITATIVE ASSESSMENT OF THE EFFECTIVENESS OF INTERVENTION STRATEGIES TO REDUCE THE RISK OF *E. COLI* O157:H7 INFECTION DUE TO CONSUMPTION OF UNCOOKED READY-TO-BAKE COOKIE DOUGH

ABSTRACT

In recent years, wheat flour-based foods have been involved in several food safety outbreaks due to Shiga toxin-producing *E. coli* contamination. A stochastic, quantitative microbiological risk assessment (QMRA) model was developed to evaluate the public health risk associated with consumption of ready-to-bake but raw cookie dough contaminated with *E. coli* O157:H7. The cookie dough production chain was modeled from milling of wheat grain to consumption at home. The effectiveness of several potential intervention strategies (i.e., acidic saline tempering solutions and high-pressure processing) to mitigate public health risks was evaluated using the QMRA model. Monte Carlo simulation coupled with Latin Hypercube sampling method was used to assess the variability and uncertainty of the model parameters. All intervention strategies evaluated significantly reduced the estimated probability of illness per serving and the number of illness cases per year among 100,000 individuals when compared with the baseline model prediction. Combination of interventions applied at different stages of the production chain resulted in the greatest relative risk reductions. The initial concentration of *E. coli* in wheat grain and the potential reduction in contamination caused by the milling process were identified as the main sources of uncertainty and the most important factors affecting the risk estimates. The developed QMRA provides a modeling framework for the grain-based foods production chain to evaluate potential intervention strategies applied at different points along the mill-to-table continuum.

1. INTRODUCTION

In recent years, wheat flour has emerged as a novel food vehicle for transmission of pathogenic microorganisms to humans. In 2016, a multistate outbreak involving retail-packaged wheat flour occurred in the United States and caused 17 hospitalizations and one case of hemolytic uremic syndrome among 63 people infected with strains of *E. coli* O121 or O26 (CDC, 2016). Consequently, multiple recalls of flour and flour-containing foods occurred as a result of the outbreak investigation. Another foodborne illness incident linking to flour contaminated with *E. coli* O121 occurred in Canada in 2017 (PHAC, 2017), highlighting again the potential of this popular ingredient to serve as a vehicle of severe and life-threatening foodborne illness.

Ready-to-bake bakery goods have gained popularity in recent years due to its convenience. Unlike ready-to-eat foods, these products have not undergone specific pathogen reduction treatments to decrease the risk of contamination because they are intended to be "cooked" by the consumer before consumption. This approach poses substantial food safety risks since consumers may not follow proper cooking instructions or may eat some or all of the products without cooking them. A survey in 2010 revealed that, in fact, the practice of eating raw or partially cooked bakery goods appears to be popular among consumers (Ardent Mills, 2010). When pathogen contamination occurs and these risky eating practices take place, consumers health may be at risk as evidenced by the outbreak of *E. coli* O157:H7 infections caused by the consumption of raw refrigerated, prepackaged cookie dough (Neil *et al.*, 2011).

Vegetative cells of foodborne pathogens, including Shiga toxin-producing *E. coli*, are capable of surviving in environments with low moisture content, such as wheat flour,

in a dormant state for prolonged periods of time (Beuchat *et al.*, 2011). Upon rehydration of flour during food preparation, microbial cells may emerge from dormancy and, consequently, the risk of flour-containing foods to cause infection or intoxication can markedly increase.

Microbial contamination can be introduced to wheat flour from a variety of sources along the field-to-table continuum; however, the initial microbiological quality of wheat kernels has the greatest influence on the ultimate safety of milling-end products (Sabillón and Bianchini, 2016). A number of intervention strategies have been developed as an attempt to reduce the microbial load of wheat kernels prior to milling, such as tempering or washing with antimicrobial agents and, atmospheric cold plasma treatments (Ibanoğlu, 2002; Dhillon *et al.*, 2007; Dhillon *et al.*, 2010; Sabillón *et al.*, 2016; Los *et al.*, 2018). Post-packaging intervention strategies, such as high-pressure processing, have also been evaluated to reduce the risk of microbial contamination on flour-based bakery mixes before they reach the consumer (Aguirre *et al.*, 2018).

There is a need not only to evaluate the effectiveness of these potential intervention strategies and their cumulative effect in reducing public health risks, but also to identify candidate steps along the wheat-based food production chain to apply possible control measures. Quantitative microbial risk assessment (QMRA) is a systems approach that can be used to achieve this goal. A QMRA model can objectively and systematically collect relevant scientific evidence on microbial distribution along the field-to-table continuum, which allow the possibility to identify the most significant risk factors of introducing and/or increasing the final risks (Haas *et al.*, 2014a).

In addition, it can provide an objective evaluation of the effectiveness of potential control strategies and allow to predict food safety and public health prevention before actual implementation, which could provide valuable science-based information for risk managers to efficiently allocate limited resources to combat food risks (Haas *et al.*, 2014b). Thus, the objectives of this study were 1) to develop a QMRA model to quantitatively describe the transmission and distribution of *E. coli* O157:H7 in the cookie dough production chain from wheat milling to consumption, 2) to evaluate the effectiveness of different intervention strategies (i.e., tempering solutions, high-pressure processing or combination thereof) aimed at reducing the risk of *E. coli* O157:H7 infection from consumption of uncooked ready-to-bake cookie dough, 3) to identify critical control points along the mill-to-table continuum, and 4) to identify where critical data and/or knowledge were lacking.

2. MATERIALS AND METHODS

2.1. Model overview

The QMRA model described the production chain of refrigerated, ready-to-bake cookie dough in a mill-to-table continuum, starting from initial contamination of the wheat entering the milling system (prevalence and concentration of *E. coli* O157:H7) and ending with risk of illness upon consumption of raw cookie dough (both individual risk per serving and population risks of 100,000 people per year). The conceptual model developed upon which the mathematical model was based is shown in **Figure 1**.

To characterize the production chain, the model was divided into two modules: 1) wheat milling and 2) preparation of cookie dough. Detailed explanation is provided within the text with key equations and assumptions elaborated. The model incorporated

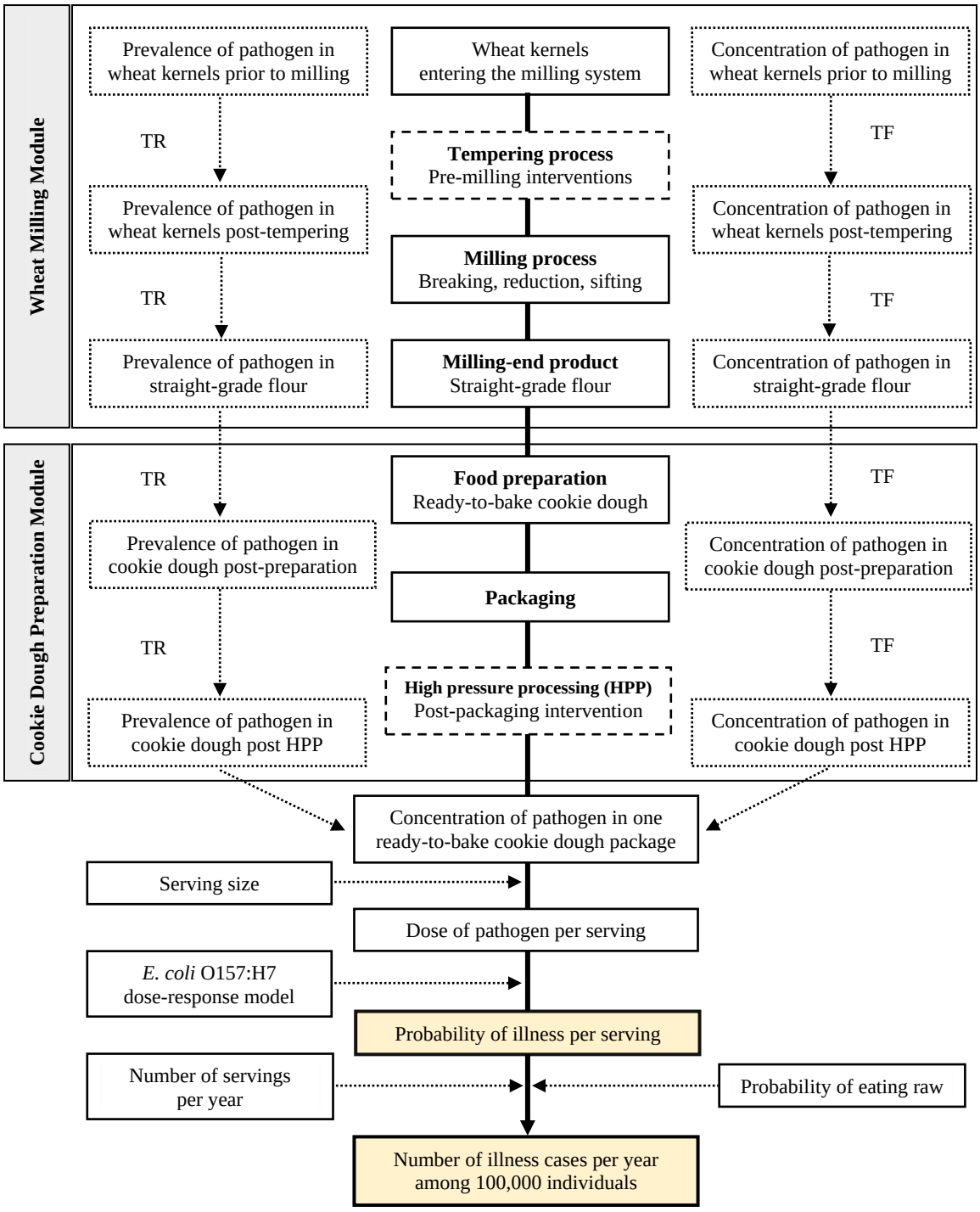

Figure 6-1. Conceptual model for assessing public health risks due to the consumption of prepackaged, ready-to-bake cookie dough contaminated with *E. coli* O157:H7. Bold-dashed boxes indicate points along the production chain where interventions are evaluated in the model. Grey-colored boxes denote module separation. Gold-colored boxes indicate key model outputs. TF, transfer factor; TR, transfer ratio.

several intervention strategies that can be implemented at multiple stages of the production chain to evaluate their impact on reducing public health risk. The risk model was developed using inputs derived from data and experimental trials conducted in the United States, whenever possible. However, where U.S.-specific data were not available, data and information from international scientific literature were consulted to improve the basis for the model. The model outputs were expressed as 1) the probability of illness per serving and 2) number of illness cases per year due to *E. coli* O157:H7 infection in a population of 100,000 individuals.

2.2. Exposure assessment

2.2.1. Scope of the exposure assessment

The exposure assessment was conducted to predict the fate of *E. coli* O157:H7 as wheat grain passes through the various milling operations (**Fig. 6-1**). Previous studies have demonstrated that microbial contaminants present in the grain may be distributed among milled fractions during the milling process (Berghofer *et al.*, 2003). Therefore, pathogenic microorganisms can be introduced by wheat kernels and passed into flour through milling, which may later contaminate popular flour-based products, such as ready-to-bake cookie dough. This type of products is likely to be consumed without the required baking step, thus representing a potential hazard to consumer health.

A chain model was used to describe the transfer of *E. coli* O157:H7 from grain to flour, and ultimately to cookie dough. In this chain model, transfer ratios (TR) were derived to estimate the relationship between pre-step and post-step prevalence, using the following formula:

$$TR = {(P_{aft} - P_{bf} \times P_{aft})}\Big/{(P_{bf} - P_{bf} \times P_{aft})}$$

where P_{bf} and P_{aft} denote the prevalence of pathogens in samples collected prior to and immediately after a specific step, respectively. A beta distribution was used to describe the uncertainty of the pre- and post-prevalence, which were estimated based on data collected from literature. Moreover, changes in *E. coli* concentration were modelled using transfer factors (TF), as follows:

$$TF = \log_{10}(C_{aft}) - \log_{10}(C_{bf})$$

where C_{bf} and C_{aft} denote the concentration of *E. coli* in samples collected prior to and immediately after a specific step, respectively.

2.2.2. Wheat milling module

The wheat milling module includes the operations involved in the production of flour. Also, the module describes the intervention strategies that may have an impact on the transfer of *E. coli* O157:H7 from wheat grain to flour. A summary of the input parameters and simulation variables used in this module is presented in **Table 6-1**.

2.2.2.1. Prevalence and initial contamination level of STEC O157:H7 in wheat grain

Given the nature of the wheat supply chain, it was assumed that there are not controllable factors influencing the prevalence and concentration of *E. coli* O157:H7 in harvested wheat grain. Hence, steps prior to and immediately after harvesting of wheat grain, including transportation and storage, were not considered in the QMRA model. Therefore, the contamination level at the time of harvest is considered the same as when entering the milling plant.

Currently, there is no survey data available in the literature regarding prevalence and concentration of *E. coli* O157:H7 in harvested wheat. Therefore, to overcome this

limitation, it was assumed that the behavior of generic *E. coli* throughout the milling process is not significantly different from that of pathogenic *E. coli*. Thus, the prevalence of generic *E. coli* (2%) reported by Eglezos (2010) in Australian wheat was used as an initial input for the QMRA model. Using the number of *E. coli* positive samples and the total number of samples tested by Eglezos (2010), a beta distribution was used to simulate the uncertainty about prevalence of *E. coli* in wheat grain.

Regarding the concentration, a worst-case scenario was adopted where the initial contamination levels can be as high as those typically used in challenge studies (i.e., 7 log CFU/g). Therefore, the variability in the initial concentration of *E. coli* in wheat entering the milling system was represented by a uniform distribution with a minimum value of 0 and maximum of 7 log CFU/g.

2.2.2.2. Wheat cleaning and traditional tempering

Flour milling begins with removal of unwanted materials from wheat grain, such as plant-based materials, rocks, animal droppings and insects (Posner and Hibbs, 2005). Previous research studies have indicated that cleaning of wheat grain does not have a significant impact on the initial microbial contamination levels. For instance, Sabillón (2014) reported that counts of *Enterobacteriaceae*, a family of microorganisms that includes *Escherichia* spp., did not vary significantly between dirty (5.11 log CFU/g) and clean (4.96 log CFU/g) wheat samples before and after cleaning with a dockage tester.

Similarly, a microbiological survey conducted in commercial wheat milling facilities did not report significant changes in coliform and *Enterobacteriaceae* counts after cleaning incoming dirty wheat (Chapter 4). Therefore, the cleaning step of the milling process was not included in the QMRA model since it was assumed that it does not have a significant

Table 6-1. Overview of simulation variables and parameters used in the wheat milling module.

Notation	Variable Description	Distribution, Value or Formula	Unit	Source
Incoming wheat kernels				
A1	Prevalence of *E. coli* on wheat entering the milling system	=Beta(1+1,50-1+1) [a]	%	Eglezos (2010)
A2	Concentration of *E. coli* on wheat entering the milling system	=Uniform(0,7) [b]	Log_{10} CFU/g	Authors input
Wheat tempering				
A3	Reduction in pathogen load due to tempering with water (traditional process)	=A2	log_{10} CFU/g	Chapter 3
A4	Reduction in pathogen load due to tempering with 5.0% acetic acid	=Lognorm(1.28,0.20) [c]	log_{10} CFU/g	Chapter 3
A5	Reduction in pathogen load due to tempering with 5.0% lactic acid	=Lognorm(1.76,0.20) [c]	log_{10} CFU/g	Chapter 3
A6	Concentration of pathogen on wheat post tempering	=IF(A2>***A3-5***,10^(A2-***A3-5***),0)	CFU/g	Calculated / Scenario Dependent
A7	Transfer ratio - pre/post prevalence of *E. coli*	=((84/90)-(54/58)*(84/90)/((54/58)-(54/58)*(84/90))) [d]	%	Berghofer *et al*. (2003)
A8	Prevalence of *E. coli* on wheat post tempering	=(A7*(A1))/(1-(A1)+(A1)*A7)	%	Calculated / Scenario Dependent
Wheat milling				
A9	Reduction in *E. coli* due to the milling process	=Uniform(0,1.1) [b]	log_{10} CFU/g	Sabillon *et al*. 2014
A10	Concentration of *E. coli* on straight-grade flour post milling	=IF(A6>10^A9,A6/(10^A9),0)	CFU/g	Calculated / Scenario Dependent
A11	Transfer ratio - pre/post prevalence of *E. coli*	=((58/71)-(84/90)*(58/71))/((84/90)-(84/90)*(58/71)) [d]	%	Berghofer *et al*. (2003)
A12	Prevalence of *E. coli* on straight-grade flour post milling	=(A11*(A8))/(1-(A8)+(A8)*A11)	%	Calculated / Scenario Dependent

[a] Beta(s+1, n-s+1); where s = number of positive samples, and n = total number of samples tested. [b] Uniform(minimum,maximum).

[c] Lognorm(mean,standard deviation). [d] Transfer Ratio $(TR) = {(P_{aft} - P_{bf} \times P_{aft})}/{(P_{bf} - P_{bf} \times P_{aft})}$; where P_{bf} = prevalence before, and P_{aft} = prevalence after.

impact on the *E. coli* contamination levels of the wheat entering the milling system.

One of the most critical steps of the milling process, from an operational standpoint, is the tempering of wheat grain after cleaning. This step requires the addition of water to the grain followed by a short rest time with the purpose of making separation of grain constituents easier during milling (Posner and Hibbs, 2005). However, despite the addition of water, research studies and microbiological surveys have reported that the traditional tempering process using water does not significantly affect the counts of the enteric bacterial flora of wheat (Dhillon *et al.*, 2009; Sabillón *et al.*, 2016; Chapter 4). Therefore, it was assumed that the traditional tempering process does not significantly influence the levels of *E. coli* contamination in wheat.

Currently, there is no documented data regarding the impact of the wheat tempering process on prevalence of pathogenic *E. coli*. However, Berghofer *et al.* (2003) reported changes in prevalence of coliform bacteria before and after the tempering process. Therefore, in order to establish changes in prevalence in the QMRA model, coliform bacteria were used as a surrogate for pathogenic *E. coli*. Changes in prevalence of *E. coli* caused by the tempering process were estimated using a TR as previously described. The calculated TR was used as a deterministic value in the QMRA model.

2.2.2.3. Intervention strategies during tempering

Research studies have used the tempering process of wheat as a tool to decrease the microbial load of wheat prior to milling by adding antimicrobial agents to the tempering water (Sabillón *et al.*, 2016). Tempering solutions containing a combination of organic acid (acetic or lactic at 5.0% v/v) and sodium chlorine (~26% w/v) were prepared as described by Sabillón *et al.* (2016) and tested against a five-strain cocktail of *E. coli*

O157:H7 inoculated on soft wheat kernels (Chapter 3). Tempering with solutions containing a combination of lactic acid and NaCl resulted in an average reduction of 1.76 log CFU/g in *E. coli* O157:H7 counts, while tempering with acetic acid/NaCl reduced the *E. coli* population, on average, by 1.28 log CFU/g. Microbial reduction data were fitted using @RISK and the variability in the tempering treatments efficacy was expressed with lognormal distribution using the average reduction and standard deviation as input values. Changes in concentration of *E. coli* caused by the application of the tempering solutions were estimated using a TF as previously described.

Data describing the effects of these tempering solutions on the concentration of *E. coli* O157:H7 were obtained based on challenge studies as opposed of natural exposure trials; consequently, changes in prevalence of the pathogenic microorganism due the application of treatments could not be quantified. Therefore, the present QMRA model assumes a hypothetical, worst-case scenario where no changes in prevalence of *E. coli* occur (i.e., TR = 1) due to the application of tempering solutions.

2.2.2.4. Milling of tempered wheat grain

Once tempered, wheat undergoes a series of reduction, grinding, and sifting operations to separate the bran and germ fractions and to reduce the endosperm in final product (e.g., flour, semolina) (Posner and Hibbs, 2005). As wheat passes through the different stages in the mill, microbial contaminants are redistributed among the different milled fractions. Several microbiological surveys have concluded that this redistribution leads to a higher association of microorganisms with the bran and outer layers of the grain than with the milled endosperm (Richter *et al.*, 1993; Berghofer *et al.*, 2003;

Sperber, 2007). Therefore, when compared with the tempered wheat, flour tends to have a considerably lower microbial load.

Currently, there is no data available describing the impact of the grinding and sifting operations on the concentration and prevalence of pathogenic *E. coli* O157:H7 in milling-end products. However, microbiological surveys of wheat milled products conducted in both laboratory and commercial scale milling facilities have reported reductions in coliform and *Enterobacteriaceae* counts ranging from none to 1.1 log CFU/g in straight-grade flour when compared with the tempered wheat (Chapters 2 and 4).

To account for the variation surrounding the impact of the grinding and sifting operations on levels of *E. coli* O157:H7, it was assumed that reductions in *E. coli* counts would be similar to those observed for coliform and *Enterobacteriaceae*, and that the true reduction would fall uniformly between a minimum and maximum value of 0 and 1.1 log CFU/g, respectively. Moreover, it was assumed that, regardless of the intervention applied before milling, the level of microbial reduction achieved by the grinding and sifting operations will be constant, which means it was assumed that the same log reduction would be achieved regardless of contamination level prior to grinding and sifting. The final concentration of *E. coli* in wheat flour was then calculated using a TF as previously described, considering the reductions achieved by both the tempering and milling processes.

Coliform bacteria were used as surrogate for pathogenic *E. coli* in order to estimate the changes in prevalence due to the milling process. Berghofer *et al.* (2003) measured the prevalence of coliform bacteria in tempered wheat and the resulting flour. The authors

found that 84 out of 90 tempered wheat samples (93%) were positive for coliform, while 58 out of 71 flour samples (82%) showed positive results. These data were used to calculate a TR for the milling steps, which was used as a deterministic value in the QMRA model. Furthermore, the QMRA model assumes a hypothetical, worst-case scenario where no changes in prevalence of *E. coli* (i.e., TR = 1) occur among the straight-grade flour obtained from the different tempering interventions due to the lack of information.

Wheat flour has a considerably low water activity level (~0.6), which prevent microorganisms from growing. At the mill, flour is usually stored in epoxy-coated concrete or metal bins, in which temperature and humidity are controlled with an air-exhaust system to prevent condensation from forming on the interior walls (Posner and Hibbs, 2005). Laminated paper bag is the most commonly used packaging material/container for flour, which prevents moisture loss/gain and keeps the flour free from insect penetration and microbial contamination from the exterior (Posner and Hibbs, 2005). Loading and shipping of packaged flour is usually done in stacked pallets stretch-wrapped in polyethylene. This reduces the risk of physical contamination and moisture uptake while in transit.

Transportation of flour in bulk is carried out in bulk cars or trucks. To prevent moisture condensation and to maintain the microbiological quality of the product during shipment, the flour milling industry has adopted several strategies including insulation of roof and walls of transport vehicles, introduction of desiccant (silica), control of pests and proper cleaning and sanitization regimens (Posner and Hibbs, 2005). For these reasons, a potential microbial growth and cross-contamination during storage or transportation of

flour to the food processing facility were not considered in the present QMRA model. Therefore, it was assumed the contamination level of pathogenic microorganisms in flour at the time being produced at the milling plant would be the same as that at the time of being ready for cookie dough preparation at the processing facility.

2.2.3. Cookie dough preparation module

The cookie dough preparation module begins with the flour immediately before cookie dough preparation. The module describes the preparation steps and potential interventions to improve the safety of ready-to-bake dough. A description of model parameters used in this module is provided in **Table 6-2**.

Table 6-2. Overview of simulation variables and parameters used in the cookie dough preparation module.

Notation	Variable Description	Distribution, Value or Formula	Unit	Source
Cookie dough preparation				
A13	Indicator of a random sample of cookie dough being *E. coli* positive in straight-grade flour	=Bernoulli(A12), 1:positive, 0:negative	No Unit	Calculated
A14	Random concentration of *E. coli* in a cookie-dough sample	=IF(A13=1,A10,0)	CFU/g	Calculated
A15	Reduction in *E. coli* due to High Pressure Processing (HPP)	=Lognorm(1.75,0.27) [a]	Log_{10} CFU/g	Chapter 5
A16	Concentration of *E. coli* on cookie dough post-HPP	=IF(A14<10^A15,0,A14/(10^A15))	CFU/g	Calculated / Scenario Dependent

[a] Lognorm(mean,standard deviation)

2.2.3.1. Preparation of ready-to-bake cookie dough

Commercial ready-to-bake cookie dough is a premixed product of various ingredients in which wheat flour, sugar, egg and fat are the major constituents. Most of

these ingredients do not represent a significant source of microbial contamination. For those ingredients that are traditionally known for their potential to carry disease-causing bacteria, such as eggs, pasteurized products are routinely used by the cookie dough industry.

In addition, the improvement in sanitary designs and the more rigid cleaning and sanitization procedures applied to the dough-handling equipment have considerably minimized the risk of cross-contamination during cookie dough preparation and packaging (Legan, 2000; Harris *et al.*, 2013). Therefore, the present QMRA model assumes that cross-contamination does not occur during cookie dough preparation and that wheat flour is the only potential source of microbial contamination. These assumptions are supported by the findings of the cookie dough outbreak investigation, from which flour remain as the most likely source of STEC contamination among all the ingredients (Neil *et al.*, 2011).

Moreover, the high concentration of solutes (e.g., sugar) and fat contributes to lower the water activity of ready-to-bake cookie dough products to levels (approximately 0.80) that are not favorable for microbial growth. Only those osmotolerant microorganisms, such as some species of yeast, may be capable of growing in such harsh conditions, and growth may only occur after temperature abuse (Harris et al., 2013). Therefore, it was assumed that pathogen growth does not occur during shelf -life of cookie dough, even during cold chain breakdowns. Consequently, since levels of *E. coli* contamination would not be affected during transportation and storage at retail or consumer level, these steps were excluded from the QMRA model.

2.2.3.2. Post-packaging intervention

Commercial, refrigerated cookie dough is usually sold without undergoing a pathogen-reduction treatment during processing because the product is intended to be cooked by the consumer before consumption. Research studies, however, have evaluated the efficacy of high-pressure processing (HPP) as an intervention to reduce the microbial load of flour-based mixes before they reach the consumer. For instance, Aguirre *et al.* (2018) reported more than 4.0 and 2.5 log CFU/g reduction in aerobic mesophilic bacteria and yeast/mold counts, respectively, in sugar-snap cookie dough after HPP at 100 or 200 MPa for 2 min.

To assess the impact of HPP on enteric microorganisms, a commercially formulated sugar-cookie dough was prepared, inoculated with a non-pathogenic *E. coli* strain (ATCC 25922) at 7.5 log CFU/g, vacuum-packaged and treated with HPP at 600 MPa for 6 min. HPP treatments reduced the population of *E. coli* in cookie dough by an average of 1.75 log CFU/g. Microbial reduction data were fitted using @RISK and the variability in the HPP treatment expressed with lognormal distribution using the average reduction and standard deviation as input values. As previously mentioned, the QMRA model assumes a hypothetical, worst-case scenario where no changes in prevalence of *E. coli* (i.e., TR = 1) occur due to the application of interventions.

2.3. Hazard characterization

The dose-response model described by Cassin *et al.* (1998) was used to determine the probability of illness from exposure to *E. coli* O157:H7 in refrigerated, ready-to-bake cookie dough. The Beta-Binomial model developed by Cassin *et al.* (1998) is characterized by two parameters, α and β, which describe the distribution of susceptibility

to the pathogen among the exposed population. The model also assumes a non-threshold level of illness, which means that one cell is capable of causing illness and that each cell is equally infective.

2.4. Risk characterization

The risk characterization is designed to integrate the data collected in previous steps, including the hazard identification and characterization, dose-response information and exposure assessment with the intent of estimating the magnitude of the risk and the probability of illness from exposure to that risk (Haas et al., 2014b). A detailed description of simulation parameters used to estimate both individual and population risks is provided in **Table 6-3**.

2.4.1. Risk estimation

The investigation of the *E. coli* O157:H7 outbreak linked to the consumption of pre-packaged, raw cookie dough revealed that several patients purchased refrigerated dough with the intention of only eating it unbaked (Neil *et al.*, 2011). In fact, a nationwide survey on risky eating behaviors conducted in the U.S. revealed that eating raw or partially cooked bakery goods is a popular practice among consumers (Ardent Mills, 2010). The results showed that 599 (58%) out 1,032 consumers surveyed have tasted refrigerated store-bought cookie dough before baking. However, there is no survey data currently available regarding the amount of raw dough that a typical consumer may eat, neither how many servings in a given year.

To overcome this limitation, it was assumed that a consumer may eat up to 28 g of raw dough, which is the manufacturer's recommended serving size per baked cookie. The variability in the amount of raw dough eaten by a consumer (i.e., serving size) was,

Table 6-3. Overview of simulation variables and parameters used to estimate both individual and population risks.

Notation	Variable Description	Distribution, Value or Formula	Unit	Source
Serving/Consumption				
A17	Mass of uncooked cookie dough ingested	=Uniform(0,28) [a]	g	Estimated / Authors input
A18	Ingested dose of *E. coli* per mass ingested	=Poisson(A16 x A17)	CFU	Calculated / Cassin *at el.* 1998
Individual risk				
A19	Probability of illness from exposure to one *E. coli* cell	=Beta(0.267, Normal(5.435,2.47, Truncate(0,))) [b]	%	Cassin *at el.* 1998
A20	Probability of illness from dose	=1-(1-A19)^A18	%	Cassin *at el.* 1998
Population risk				
A21	Population	100,000	Inhabitants	Assigned
A22	Number of servings consumed annually by a given consumer	=IntUniform(0,24) [c]	No Unit	Estimated / Authors input
A23	Total number of servings consumed by 100,000 individuals in a year	=A21 x A22	Servings	Calculated
A24	Number of illness cases per year per 100,000 individuals	=A23 x A20	Cases	Calculated

[a] Uniform(minimum,maximum)
[b] Beta(α, β) where α = 0.267, and β = Normal(5.435,2.47,Truncate(0,)) where only non-zero values simulated in each iteration.
[c] Uniform(minimum,maximum)

therefore, represented by a uniform probability distribution with minimum and maximum estimated values of 0 and 28 g, respectively. The simulated serving size was used, together with the concentration of *E. coli* O157:H7 (CFU/g) in a mass of unbaked cookie dough determined by the exposure assessment, as an input into the dose-response model to determine the probability of illness per serving, which refers to the individual risk.

Moreover, according to a consumer survey, 30.2 million Americans purchased 24 containers of refrigerated dough in 2017 (Experian, 2017). Using this data as reference, it was assumed that a consumer may have up to 24 servings of raw dough per year, corresponding to the number of purchased containers. Therefore, to simulate the variability about the number of servings consumed annually by a given consumer (*A22*), a discrete uniform probability distribution was used with a lower and upper bound of 0 and 24, respectively, whereby a finite number of servings within the selected range are equally likely to be observed. The total number of annual servings (*A23*) among a population of 100,000 individual was then calculated using the following equation (**Table 6-3**):

$$A23 = A21 \text{ x } A22$$

where, *A21* refers to a population of 100,000 individuals and, *A22* denotes the simulated number of servings eaten by a given consumer in a year. The estimated number of annual servings among a population of 100,000 individuals (*A23*) was then multiplied by the probability of illness (*A20*) predicted by the beta-binomial dose-response model to calculate the annual number of illness cases, which refers to the population risk.

2.4.2. Sensitivity analysis

A sensitivity analysis of the baseline model (i.e., no interventions) was conducted to identify key sources of variability and uncertainty in order to prioritize additional data

collection and research. In addition, insights obtained from the sensitivity analysis were used to prioritize possible critical control points along the mill-to-table continuum in which control measures could be applied to prevent, eliminate, or reduce microbial hazards.

2.4.3. Interventions scenarios

Six intervention scenarios were evaluated using the present QMRA model (**Table 6-4**). Scenarios 1 is the baseline scenarios, which reflects the practices believed to be currently applied along the mill-to-table continuum, from flour production to the manufacture of ready-to-bake cookie dough. The baseline scenario begins with the wheat grain entering the milling systems to be tempered with water and subsequently milled into flour. The resulting flour is then used in the manufacture of ready-to-bake, pre-packaged cookie dough. The risk of *E. coli* O157:H7 infection due to consumption of raw pre-packaged cookie dough was then calculated.

Scenarios 2 and 3 refers to the application of interventions prior to milling with the purpose of reducing pathogen load in wheat grain. In these two scenarios, the effectiveness of acidic saline tempering solutions to reduce public health risks was evaluated. Scenario 4 represents the application of a post-packaging intervention aiming at improving the safety of ready-to-bake cookie dough before it reaches the consumer. In this fourth scenario, the effectiveness of high-pressure processing to mitigate public health risks was assessed. Scenarios 5 and 6 reflects the application of a combined pre-milling and post-packaging intervention. This scenario scheme was selected to determine the effects of single interventions or combinations of interventions on the risk estimate. For each intervention, the model was run for 10,000 iterations, and the process was

repeated three times. Average risk estimates were determined from the average outputs of each model run for each intervention scenario.

Table 6-4. Intervention scenarios evaluated in the QMRA model.

Scenario ID	Intervention Type	Intervention Description
1	None	Baseline – No interventions
2	Single	Tempering solution – Acetic acid + NaCl (AA)
3	Single	Tempering solution – Lactic acid + NaCl (LA)
4	Single	High pressure processing (HPP)
5	Combination	AA + HPP
6	Combination	LA + HPP

It was assumed that none of the interventions assessed herein are currently applied to an appreciable degree in the Unites States. Since no changes in *E. coli* prevalence were adopted in the model, differences among simulation outputs were attributed to effects of interventions on concentration of *E. coli* O157:H7 in ready-to-bake cookie dough.

2.5. Model simulations and statistical analysis

The QMRA model was developed in Microsoft Excel 2016 with the add-on package @Risk (version 7.5.2, 2017, Palisade Corporation, Ithaca, NY, USA). The developed models for all scenarios were simulated using the Monte Carlo simulation technique coupled with the Latin Hypercube sampling method. Simulations of the model representing different intervention application scenarios were run independently with 10,000 iterations each to obtain stochastic estimates of the output variables presented herein. A sensitivity analysis of the baseline model (Scenario 1) was also conducted using the @Risk software.

Resulting data from intervention scenarios and baseline models (i.e., probability of illness per serving, and the annual number of illness cases per year) were subjected to an analysis of variance (ANOVA) in 2 x 3 factorial arrangements to determine significant

differences in the risk output between each scenario and the control (the baseline model). The ANOVA was performed with SAS software (SAS, Version 9.3, SAS Institute Inc., Cary, NC) using the GLIMMIX procedure and the Tukey's multiple comparison test. Differences were reported at a significance level of 0.05.

3. RESULTS AND DISCUSSION

3.1. Comparison of intervention scenarios

A quantitative risk assessment model could be used as a predictive risk tool to estimate and compare the efficacy of risk mitigation strategies before they are implemented (Cassin *et al.*, 1998). Several intervention strategies applied along the mill-to-table continuum were evaluated, either alone or in combination, using the proposed QMRA model to estimate their effectiveness at reducing the probability of illness, and thereby the number of illness cases arising from consumption of unbaked cookie dough contaminated with *E. coli* O157:H7. A summary of model results is presented in **Table 6-5**.

Considering the parameters used in the baseline model (Scenario 1) and the assumptions surrounding them, a population of 100,000 individual would experience an average of 11,447 illness cases per year with a mean probability of illness per serving of 1.0×10^{-2} (95% CI: $8.3 \times 10^{-3} – 1.2 \times 10^{-2}$), which is approximately 1 in 100 servings (**Table 6-5**). These values were set as referent values in order to calculate the percent reduction in the risk estimate achieved by the different intervention scenarios. It is important to highlight that the present QMRA model considered worst-case assumptions where highly contaminated wheat kernels (up to 7 log CFU/g) may enter the flour production chain, as well as no changes in prevalence of *E. coli* contamination occur

Table 6-5. Comparison of the percent reduction in the number of illness cases per year and probability of illness per serving due to consumption of unbaked cookie dough contaminated with *E. coli* O157:H7 predicted by the baseline model and several intervention scenarios.

Intervention Scenarios		Number of illness cases per year [x]	Probability of illness per serving
ID	**Description**	**Percent reduction [y]**	**Percent reduction [y]**
1	Baseline – No Interventions	Referent [z]	Referent [z]
2	Tempering – Acetic Acid (AA)	17% a	23% a
3	Tempering – Lactic Acid (LA)	36% b	38% b
4	High pressure processing (HPP)	36% b	38% b
5	AA + HPP	61% c	63% c
6	LA + HPP	67% c	67% c

[x] Number of illness cases per year among 100,000 individuals.

[y] All intervention scenarios reduced significantly the risk estimate when compared to the baseline scenario (referent value). Percent reduction values with different letters within the same model output are significantly different ($P \leq 0.05$).

[z] The referent value for the number of illness cases per year is 11,447, while for the probability of illness per serving is 1.02E-02.

throughout the production chain; therefore, the mean predicted probability of illness per serving and the resulting number of illness cases are likely to be overestimated.

All interventions evaluated in the present QMRA model significantly reduced the risk estimate from consumption of unbaked cookie dough contaminated with *E. coli* O157:H7 when compared with the no intervention scenario (i.e., Scenario 1) (**Table 6-5**). Scenarios 2 and 3 evaluated the effectiveness of single interventions applied at the beginning of the flour supply chain. Tempering wheat grain with acetic acid-NaCl solutions (Scenario 2) and using the resulting flour in the manufacture of ready-to-bake cookie dough reduced the average probability of illness per serving by 23%, while the estimated number of illness cases per year declined by 17% compared to the baseline scenario.

Tempering solutions containing lactic acid-NaCl (Scenario 3) were significantly more effective at reducing the risk estimate than acetic acid-NaCl solutions ($P < 0.05$). For instance, the reduction in the probability of getting ill from consumption of raw cookie dough prepared with flour obtained from wheat tempered with solutions containing lactic acid was 15% higher than the reduction in the probability of illness obtained with acetic acid-NaCl solution. As a result, the estimated number of illnesses per year was reduced by 36% (**Table 6-5**). Adding antimicrobial agents, such as lactic or acetic acid, to the tempering water with the aim of reducing microbial contamination prior to milling can, therefore, reduce significantly the risk of foodborne disease outbreaks caused by flour-containing foods.

The application of HPP as a post-packaging intervention (Scenario 4) was also highly effective in mitigating public health risk. The average probability of illness per

serving of raw cookie dough following the application of HPP was reduced by 38%, while the annual number of illness cases decreased by 36% relative to the no intervention scenario (**Table 6-5**). In this study, the risk mitigation levels achieved by HPP were not significantly different from those observed for lactic acid-NaCl tempering solution, although a direct comparison may not be appropriate since both interventions are applied under completely different settings and processing environments along the production chain.

Sequential interventions applied at different points along the mill-to-table continuum provided the greatest relative reduction in risks compared to single interventions. For instance, tempering wheat kernels with acetic acid-NaCl solution at the begging of the flour-production chain and subsequently treating the pre-packaged cookie dough with high pressure before it is shipped to the market (Scenario 5) could markedly reduce the probability of illness per serving by 63%, when compared to the baseline scenario (**Table 6-5**). Likewise, the predicted number of illness cases per year was reduced by 61%. Higher reduction levels on public health risk, although no significantly different from the ones obtained in Scenario 5, were observed when combining lactic acid-NaCl and HPP interventions (Scenario 6). The analysis of variance showed that interactions between tempering solutions and HPP treatments were not significant ($P > 0.05$), which suggests that such interventions may be acting independently of each other when applied along the mill-to-table continuum.

It is worthwhile to note that the interventions evaluated using the present QMRA model were conducted based on challenge trails where the food matrix (i.e., wheat kernels, cookie dough) was inoculated with high doses of *E. coli* O157:H7 in order to

measure the decrease in concentration following the application of the intervention; therefore, the results obtained in this study may not adequately represent realistic contamination situations in which population levels of *E. coli* O157:H7 are generally low and thus may overestimate the efficacy of these interventions. Moreover, many other factors, including feasibility, cost, and potential influence on product quality must be taken into consideration when interpreting the effectiveness of these interventions.

Validation is an important part of quality assurance of a model. Validation refers to the comparison of model predictions to independent empirical data for the model output under a known set of conditions (Frey *et al.*, 2004). Historically, cereal-based products have been considered low-risk commodities for microbial contamination; consequently, scientific and empirical data regarding the risks of microbiological hazards along the wheat products supply chain are currently scarce to nonexistent. Therefore, validation of the present QMRA model may prove challenging because "real-world" data are not known for many of the model inputs, and hence it is not possible to determine the degree to which the obtained results overestimate or underestimate the risk. Despite this, the model may be of considerable interest to risk managers because it can provide support for better decision-making regarding strategies that could be applied throughout the grain processing chain to safeguard consumers.

3.2. Sensitivity analysis: Critical control points and data gaps

The sensitivity analysis is a tool extensively used in QMRA to determine not only key sources of variability and uncertainty that affect the model's outcome, but also to identify critical control points along the food production chain and prioritize data collection and research in order to reduce uncertainty (Frey *et al.*, 2004). Therefore, a

sensitivity analysis of the baseline model (Scenario 1) was performed to obtain relevant information regarding which model inputs contribute the most to uncertainty and variability, as well as useful insights regarding critical control points.

A rank ordering of key model inputs based on their contribution to the variation in the output of interest (i.e., number of illness cases per year) is presented in **Figure 6-2**. The analysis revealed that the number of servings of raw dough ingested by a given consumer in a year, was the model input with the largest individual contribution to the variation in number of illness cases. Along with the number of servings, risk estimates were highly sensitive to the concentration of *E. coli* on wheat entering the milling system, as well as the probability of illness from exposure to a single *E. coli* cell, an indicator of host susceptibility. Predicted risks were also impacted by the potential reduction in microbial load caused by the milling process and the amount of unbaked cookie dough ingested.

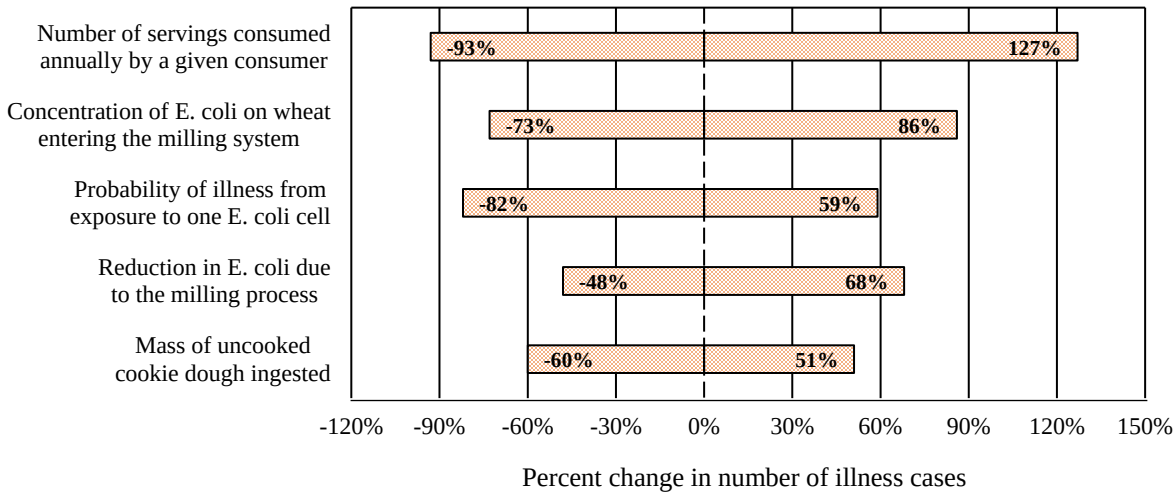

Figure 6-2. Sensitivity analysis showing the most important parameters influencing the estimated number of illness cases among 100,000 individuals per year due to consumption of unbaked cookie dough contaminated with *E. coli* O157:H7. Inputs ranked by effect on output mean. Baseline = 13,079.

The identification of these variables as key sources of uncertainty and variability in the present QMRA model may help to identify, prioritize, and allocate scarce resources for data collection and research, as well as to identify critical control points along the mill-to-table continuum where control measures could be implemented to reduce the risk of *E. coli* contamination in ready-to-bake products. In order to prioritize data collection and research activities, it is useful to determine whether the impact of the model input on the risk estimate is due to variability or uncertainty. Variability refers to real differences in values of a quantity among members of a population (Frey *et al.*, 2004**).** The probability of illness from exposure to a single *E. coli* cell was identified as the main source of variability in the present study (**Fig. 6-2**). This probability is an indicator of the host susceptibility to become ill due to *E. coli* infection, which can vary considerably between individual hosts within the same population. This source of variability may be deemed uncontrollable; however, the collection of additional data regarding the relationship between the likelihood of adverse effects and the level of microbial exposure may help develop more accurate dose-response models and improve the characterization of variation in host susceptibility.

Uncertainty is defined as the lack of knowledge regarding the true value of a quantity (Frey *et al.*, 2004); therefore, additional data collection and research is the only viable method to increase the state of knowledge and thereby reduce uncertainty. The number of raw cookie dough servings was identified as the main source of uncertainty in the present study. The amount of uncooked dough ingested by consumers was also another important factor of uncertainty (**Fig. 6-2**). Although it is known that consumption of unbaked bakery mixes is a popular practice among consumers (Ardent Mills, 2010),

there is a significant lack of knowledge regarding the amounts of raw dough ingested and the frequency with which this happens. Additional research geared towards understanding consumers behavior and household consumption patterns with regards to ready-to-bake bakery mixes may help reduce uncertainty, and thereby improve future QMRA models. In addition, consumption of raw bakery mixes may vary by age, gender, and ethnicity. The dose-response model used in this study did not attempt to differentiate the exposure of different demographic groups but more detailed consumption data may enable more accurate assessment of the relative risk to different types of consumers and the development of better targeted risk mitigation strategies.

Similarly, the initial concentration of *E. coli* O157:H7 in wheat grain entering the milling system and the potential reduction in *E. coli* contamination achieved during milling of wheat grain were other notorious sources of uncertainty in the present QMRA model (**Fig. 6-2**). There is a substantial lack of information surrounding these two parameters; therefore, additional research and data collection should be focused in these input variables to increase the state of knowledge in order to reduce uncertainty and to optimize risk management approaches. Despite the uncertainty surrounding the true level of *E. coli* O157:H7 contamination on wheat grain entering the milling system, it is clear that it can have a substantial impact on the safety of flour-based products. Therefore, the microbiological quality of wheat grain should be considered as a critical control point and thus the implementation of control strategies must be considered early in the mill-to-table continuum to prevent, eliminate, or reduce microbiological hazards to an acceptable level.

In addition to the limitations, data gaps, and critical control points mentioned above, the initial prevalence of *E. coli* O157:H7 in wheat grain entering the milling

system is currently unknown. Further characterization of the prevalence of *E. coli* O157:H7 at various stages throughout the wheat-flour supply chain is, therefore, needed. Data on prevalence would help to validate intermediate model outputs and model assumptions. Moreover, differences between classes of wheat (e.g., soft, hard, or durum) may exists with regards to the milling process, milling behavior, and effectiveness of interventions. Such differences may have a considerable impact on the risk estimate and, therefore, may need to be explored in future risk assessments.

It is possible that cross-contamination may occur as wheat kernels are being milled into flour, as well as during the manufacturing of flour-based foods. However, cross-contamination rates throughout the wheat-based products supply chain are currently unknown. A quantitative analysis to elucidate the fate of *E. coli* O157:H7 during milling and subsequent manufacture of flour-based foods is, therefore, essential to provide more accurate risk estimates. Moreover, the present QMRA model assumes that wheat grain is the only source of *E. coli* contamination; however, other sources of microbial contamination may exist throughout the supply chain. Future research is, therefore, needed to quantify and include other contamination routes, which would improve the model prediction and be helpful in identifying risk factors and evaluating potential intervention strategies at different stages in the mill-to-table continuum.

3.3. Impact of the initial *E. coli* O157:H7 contamination levels on the effectiveness of intervention strategies

As noted by the sensitivity analysis, the microbiological quality of the wheat kernels entering the milling system has a strong influence on the ultimate safety of milled products and foods produced from these ingredients. In fact, Berghofer *et al.* (2003) reported that milled products with high microbial contamination levels are the result of

Table 6-6. Impact of different *E. coli* O157:H7 contamination levels in the incoming wheat on the percent reduction in the number of illness cases per year (among 100,000 individual) predicted by the baseline model and several intervention scenarios.

Intervention scenarios		Initial *E. coli* O157:H7 contamination level (log_{10} CFU/g) [a]						
ID	**Description**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	Baseline – No Interventions	Referent	Referent	Referent	Referent	Referent	Referent	Referent
2	Tempering – Acetic Acid (AA)	100%	68%	31%	14%	6%	7%	11%
3	Tempering – Lactic Acid (LA)	100%	91%	45%	19%	17%	9%	15%
4	High Pressure Processing (HPP)	100%	93%	52%	28%	27%	19%	19%
5	AA + HPP	100%	100%	97%	65%	34%	24%	25%
6	LA + HPP	100%	100%	100%	77%	41%	31%	35%

[a] Values denote percent reduction of illness cases relative to the referent value as predicted by the baseline scenario.

wheat grain with poor microbiological quality entering the milling system. The increase in *E. coli* contamination levels in wheat grain substantially increased the risk estimate (**Table 6-6**, Scenario 1). Therefore, controlling the initial contamination levels of wheat grain is critical to prevent and reduce food safety risks.

Routine microbiological testing of wheat grain prior to entering the milling system can provide valuable insights regarding possible risk management strategies that may be implemented along the mill-to-table continuum. The present QMRA model can be used as a screening tool to determine, based on the initial contamination levels, the number and type of interventions that may need to be applied in order to reduce public health risks associated with the consumption of raw bakery goods.

The risks to public health possessed by wheat grain with an *E. coli* O157:H7 contamination level of up to 2 log CFU/g could be substantially mitigated by the application of one of the interventions evaluated in the present study. Tempering wheat grain with solutions containing a combination of acetic acid and NaCl (Scenario 2) considerably reduced the risk estimate by 68%, while lactic acid solutions (Scenario 3) resulted in a reduction of 91% when compared to the referent value predicted by the baseline scenario (**Table 6-6**). Similarly, the application of HPP to treat pre-packaged cookie dough reduced the number of illness cases by 93% relative to the baseline scenario.

When levels of *E. coli* contamination in wheat grain increase beyond 2 log CFU/g, the application of more than one intervention along the mill-to-table continuum may be necessary to safeguard consumers. The application of acidic saline tempering solutions to wheat grain prior to milling and subsequently treating pre-packaged cookie dough with

high pressure could substantially reduce the risk estimate by more than 97% when the initial contamination level of the incoming wheat grain is 3 log CFU/g (**Table 6-6**).

The combined application of lactic acid tempering solution and HPP (Scenario 6) was markedly more effective at reducing public health risk than the other intervention scenarios when the levels of *E. coli* contamination in wheat grain increased beyond 4 log CFU/g (**Table 6-6**). From a practical standpoint, factors such as feasibility and cost-effectiveness may need to be considered in order to decide which intervention or set of interventions would be implemented.

4. CONCLUSIONS

Using risk assessment modeling as a baseline, the impact of the interventions on the ultimate safety of flour-based foods derived from potentially contaminated grain can be evaluated. All interventions assessed in the present QMRA were effective at reducing the public health risks associated with the consumption of cookie dough contaminated with *E. coli* O157:H7. The most effective strategy to reduce the predicted public health risk was a combination of interventions at several stages along the mill-to-table continuum.

A sensitivity analysis of the model indicated specific input parameters that might warrant more detailed examination to improve the state of knowledge in order to reduce uncertainty. Future research should be directed at collecting more data on prevalence and concentration of *E. coli* O157:H7 on wheat entering the milling system, as well as on the fate of this microorganism during the milling process. Further refinements to the present QMRA model are required to provide a more accurate representation of the milling process, flour transportation, manufacture of flour-based products, and consumption.

Despite the likely possibility that the model under or overestimates the probability of illness and the number of illness cases from consumption of contaminated cookie dough, it nevertheless provides a solid foundation and a comprehensive modeling framework that can be adapted for future risk assessment models as future research elucidates contamination routes and levels along the wheat-based products value chain.

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