BEHAVIOR OF ENTERIC PATHOGENS ON WHEAT GRAINS DURING TEMPERING AND EVALUATION OF INACTIVATION METHODS PRIOR TO MILLING

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ABSTRACT

Outbreaks linked to *Salmonella* and Shiga toxin-producing *E. coli* (STEC) in wheat flour led to increased interest in characterizing the fate of these pathogens on wheat grains during processing and exploring potential inactivation methods. Despite the increase in water activity of wheat grains to ~0.85, the changes in the pathogen populations on inoculated soft wheat grains remained relatively stable during tempering. Using confocal microscopy, no significant redistribution of pathogen was found based on the fluorescent intensity measurement.

Chlorinated water had been used in the tempering solution in mills to control mold and yeast growth. When hard wheat grains were treated with 800ppm chlorinated water as a tempering over 18 hours, pathogen population changed from -2.35 to -0.30 log CFU/g, without negatively impacting the flour quality or functionality. Treating soft wheat at 75˚C using vacuum steam pasteurization, resulted in $D_{75°C}$ of *Salmonella* strains of 2.8 and 3.2 min of STEC ranges from 3.1 to 4.6 min, and of *E. faecium* was 3.3 min.

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LIST OF ABBREVIATIONS

AACC ..... American Association of Cereal Chemistry

APC..... Aerobic plate count

CFU..... Colonies forming unit

CDC ..... Center of Disease Control and Prevention

FDA ..... Food and Drug Administration

HRS..... Hard red spring

HUS ..... Hemolytic uremic syndrome

LMF ..... Low moisture food

NIR..... Near-infrared spectrometer

RIF ..... Rifampicin

RVA ..... Rapid Visco Analysis

SRW ..... Soft red winter

STEC..... Shiga-toxin producing *E. coli*

TSA ..... Trypticase soy agar

TSAYE..... Trypticase soy agar with Yeast Extraction

USDA..... United States Department of Agriculture

VSP ..... Vacuum steam pasteurization

1 INTRODUCTION

In the past, low moisture food such as wheat kernel was less of a food safety concern, as the low water activity environment does not support the growth of microorganisms. And we started to see continuously low moisture food-related outbreaks, especially those associated with flour in the past decade (*7*, *8*, *9*).

On wheat grains, contamination could happen both pre-and post- (*42*), and there is no kill step before the wheat grains are milled into flour. Lack of consumer awareness of flour safety by associating the risk of eating raw cookie dough or cake batter makes the milling industry seek solutions for flour safety (*17*).

Tempering involves adding water to wheat grain while mixing to gradually increase the moisture content, therefore toughen the bran and soften the endosperm, making milling wheat grains easier. During this process, the water activity of wheat grains also increases, potentially facilitate the growth of bacteria on the wheat grains, and survival in the flour after milling.

There is currently limited research on quantifying changes in the pathogen population during wheat tempering. During milling, the outer bran layer of the wheat kernel is removed, where the majority of the contamination is located. The wheat kernel also has a unique physical characteristic, crease, which extends inside close to the endosperm (*46*). Therefore, any contamination of the crease during tempering could increase the risk of bacterial contamination in flour. Thus, the study's objective included quantifying the bacterial population changes during tempering and determining if contamination shifts to the crease during tempering by utilizing fluorescent-tagged pathogen. Answering these questions helps understand pathogen behavior during tempering and provides the groundwork for future study.

Various methods to inactivate enteric pathogens have been tested, mainly those focusing on replacing water with disinfectant solutions for wheat tempering. Traditionally, chlorinated water was used to control mold and yeast growth during (*15*). No previous research has assessed the efficacy of chlorinated water in inactivating pathogens on wheat grains. Understanding the effectiveness of using chlorinated water as a tempering aid for pathogen inactivation, in addition to the quality check, could provide helpful information to the milling industry.

Moreover, thermal inactivation of the *Salmonella* and STEC was also studied. Vacuum steam pasteurization (VSP) at 75 ˚C reduced the pathogen population inoculated on wheat grain by 5-log after 8 min, without comprmising the resulting flour quality(*59*). The study used a newly designed VSP unit, a different approach to determine the inactivation efficacy using modeling and thermal resistance of additional serovars of *Salmonella* and STEC, also testing if *Enterococcus faecium* could be a reasonable surrogate for commercial processing.

2 LITERATURE REVIEW

2.1 Low moisture food safety

Low moisture food (LMF) is defined as food that has water activity lower than 0.85. Some common LMF includes flour, almond, peanuts, meat jerky, dried fruits, etc. Water activity is an important intrinsic factor that measures the availability of unbound water in a food, that supports microbial growth.

Most bacterial growth is inhibited when the water activity is lower than 0.90, while molds can grow at water activity as low as 0.65 (*62*). However, bacteria can persist in low water activity environments for many months(*24*). Therefore, the increasing number of outbreaks associated with low moisture food have raised food safety concern.

2.2 Flour outbreaks

Harris and Yada (*27*) summarized the outbreaks and recalls associated with flour and cereal grains; most of which were reported in the past decade. However, numerous other outbreaks related to other LMFs such as almonds, peanuts, and pistachios had also been documented.

Flour is not a ready-to-eat food and is traditionally used to make other products such as bread, cake, noodles, pasta, etc. Pathogens in the product will be inactivated after proper cooking. However, certain consumer behavior, such as consuming raw cookie dough or cake batter, significantly increase the risk of illness. Feng and Archila-Godinez (*17*) found that most flour consumers were unaware of the food safety risk of consuming raw dough and batter, with more than half of flour-using eaten raw dough or batter. Therefore, major flour manufactures added warning messages in the packages of flour. However, only 22% of consumers reportedly

paid attention to the warning. Base on the findings, additional consumer education on flour safety is clearly needed.

2.3 Enteric pathogens

The two pathogens most found associated with these outbreaks were *Salmonella* and Shiga-toxin producing *Escherichia coli* (STEC), both considered enteric pathogens typically found in the intestines of animals and humans.

Salmonella is a non-spore forming, rod-shaped, gram-negative bacterium that is responsible to estimate 1.35 million infections and 420 deaths in the U.S each year (*11*). There are two main species of *Salmonella*: *Salmonella enterica* and *Salmonella bongori*. *S. enterica* includes all the outbreak serovars listed in Table 2-1. *S.* Typhimurium is the most common serovar found in flour outbreaks. However, other serovars should not be ruled out, including *S.* Agheni found in the cake mix outbreak in 2018, S. Agona in 2008 puffed rice outbreak, *S.* Mbandaka from the 2018 puffed wheat cereal outbreak, *S.* Montevide*o* found in Pistachios from a 2016 outbreak, and *S.* Tennessee found in 2017 peanut butter outbreak.

Table 2-1 Flour and cereal grain product outbreaks.

Product (source)	Pathogen	Year	Number of cases	Source
Wheat flour				
Flour (New Zealand)	*Salmonella* Typhimurium phage type 42	2008 – 2009	67	McCallum et al., 2013
Flour	*E. coli* O121, *E. coli* O26	2015 – 2016	63	CDC, 2016; Crowe et al., 2017; US FDA, 2017
Flour	*E. coli* O121	2016 – 2017	30	Gill et al., 2019; Morton et al., 2017, 2020; PHAC, 2017; Robertson et al., 2018
Flour	*E. coli* O121	2017	6	BCCDC, 2017; Beach, 2017

Table 2-1 (cont'd)

Flour	*E. coli* O26	2018 – 2019	21	CDC, 2019b; Marler, 2019; US FDA 2019b
Wheat flour product				
Cake mix, raw – in ice cream (USA)	*Salmonella* Typhimurium	2005	26	Zhang et al., 2007
Frozen pot pies [flour could not be ruled out] (USA)	*Salmonella* serotype I 4,5,12:i:-	2007	396	CDC, 2008a; Mody et al., 2014
Prepackaged, refrigerated cookie dough (USA)	*E. coli* O157:H7	2009	80	CDC, 2009; Neil et al., 2012
Dough mix, dry (USA)	*E. coli* O157:H7	2015 – 2016	13	Gieraltowski et al., 2017
Cake mix (USA)	*Salmonella* Agbeni	2018	7	CDC, 2019a; US FDA, 2019a
Cake mix (USA)	*E. coli* O121	2021	16	CDC, 2021
Cereal grain product				
Infant cereal [with oats] (Sweden)	*Salmonella* Muenchen	1961	110	Silverstolpe et al., 1961
Toasted oats cereal (USA)	*Salmonella* Agona	1998	400+	CDC, 1998; Russo et al., 2013
Puffed rice or puffed wheat cereals (USA)	*Salmonella* Agona	2008	33	CDC, 2008b; Hoffman et al., 2015; Russo et al., 2013
Puffed wheat cereal (USA)	*Salmonella* Mbandaka	2018	135	CDC, 2018

Reprinted from Harris, L.J., and S. Yada. 2022. Last updated 2/1/2022, with column "Isolated from products" removed.

STEC is a group of *Escherichia coli* (*E. coli*) that causes disease by producing Shiga toxin, is responsible for estimated 265,000 infections in U.S each year (*6*). The most common pathogenic STEC is *E. coli* O157:H7, however, other 'big six' non-O157 STEC including *E. coli* O26, O45, O103, O111, O121, and O145, are also prevalent. Three serovars of STEC identified in flour or cereal related outbreaks: STEC O157:H7, O26, and O121. The *E. coli* gene *rpoS* is a

37.8 kD protein in *E. coli* found to be the regulator gene of stress response. The gene not only enhances the survival of *E. coli* under stress, but it can also cross-protect the cell when exposed to other stresses. In LMF safety, bacteria undergo desiccation stress that may trigger cross-protection, against sanitizers and lead to increased survivalbility.

2.4 Wheat and its end products

The wheat plant belongs to the genus *Triticum*, one of the most widely growth cereal crops in the world. Wheat flour is a staple food in many cultures and an essential carbohydrate source.

Currently, there are six main categories of wheat harvested in the United States, based on the season planted, color, and hardness of the grain as stated by their names: hard red winter, hard red spring (HRS), soft red winter (SRW), soft red spring, hard white, and durum wheat. The hardness of wheat is the main parameter to determine what product to make from the wheat variety. The harder the wheat, the higher the protein content. Therefore, hard wheat varieties are usually used to make bread flour that requires higher gluten content, and soft wheat is used to make cake flour. Durum wheat is the hardest variety and is mainly used to make pasta.

2.5 Structure of wheat

In brief, wheat flour milling involves three parts of the wheat kernel: bran, endosperm, and germ. Bran is the outmost protective layer around interior components. The internal components include the endosperm and germ. Endosperm contributes more than 80% of the grain mass (*32*), and is the most decisive element of flour yield. The germ contains the embryo and the scutellum, which together conposed of 2-3% of the total kernel weight (*46*).

Wheat kernel have a unique morphology, the crease, that extends inward to the center of a wheat grain (*46*). Figure 2-1 shows the 3D structure by dorsal and ventral views. The crease is

located on the ventral side of the kernel, which is also referred as the “crease side” of a kernel. And no crease in the middle of the dorsal view, therefore referring as the “non-crease side” of the kernel, where the embryo is located. Due to its depth, the crease can potentially harbor microorganism contaminants, that may move downstream with the endosperm during milling and separation.

The Brush is a cluster of hairs that located on the opposite side of germ, about 0.5mm in length with some grains having longer brush hairs than others. The brush hairs also collect

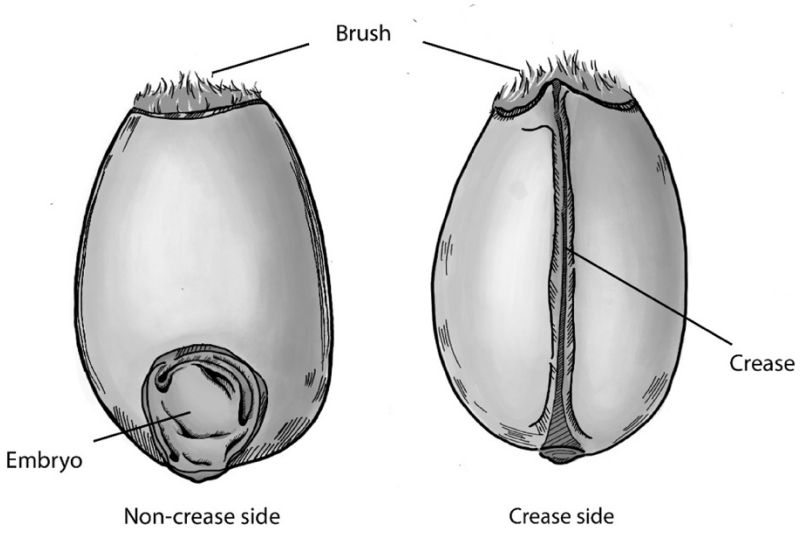

Figure 2-1 Dorsal and ventral view of a wheat grain.

foreign material including microorganisms that may eventually contaminate the flour (*46*) depending on the milling practice.

2.6 Native microflora on wheat

Native microflora abundance on wheat grains shift, at different point pre- and post-harvest, and can be different geographically (*42*). Wheat microbiomes contains bacterial and fungal genera as these are the two major food safety risks. In brief, *Bacillus spp.* noticeably as spore-forming bacteria that could survive under stress, such as chlorine concentration of 100ppm

or greater, had been the focus in wheat. Recent outbreaks raised the concern of enteric pathogens on wheat grains. Myoda et al. (*45*) surveyed 3891 wheat samples in the US on their occurrence rate of *Salmonella* and STEC. The prevalence rate of *Salmonella* and STEC are 0.44% and 1.23%, respectively. A previous study in Australia (*3*) found the percentage of wheat that tested positive for coliform to be 93%, and coliform could serve as an indicator of potentially pathogenic *E. coli* contamination.

2.7 Potential sources of pathogen contamination on wheat

Los et al. (*42*) conducted a literature review on the potential route of contamination of wheat grains from field to table. Environmental sources are the primary cause of microbial contamination on wheat grains. Rainfall that raised the moisture content of wheat grains greatly enhances the chance of bacteria and mold contamination, raising food safety concerns regarding pathogens and mycotoxins.

The other main concern is cross-contamination that happens both during harvesting and transportation by tools, equipment, and improper handling.

2.8 Wheat storage and enteric pathogen survival during storage in wheat and flour

After harvest, wheat grains are usually transported to a storage facility, also known as an "elevator", and stored from several months to a year before milling.

Several studies have shown that *Salmonella* and STEC can survive on wheat for an extensive amount of time. Lauer et al. (*36*) showed that even though the D-value was significantly different for STEC and *Salmonella*, they could survive for extensive periods. When hard red spring wheat was inoculated with fours serovars of Salmonella, and six serovars of STEC at 6 log CFU/g, under room temperature storage, STEC remained quantifiable after 44 weeks, and *Salmonella* remained quantifiable for 52 weeks.

Both *Salmonella* and STEC could also survive in flour. Flour from 2016 Canadian outbreak that was contaminated with STEC O121:H19, after additional 18-months of storage, STEC O121:H19 remained quantifiable in the flour (*24*). In a lab-scale study, after storing flour inoculated at 8 log CFU/g with STEC and *Salmonella*, two species remained quantifiable after 84 and 112 days, respectively (*22*). This later study used a broth inoculum, which had been proved in other LMF studies that it likely shortened the survival of *Salmonella* in LMF (*64*).

These studies showed the extended survival of *Salmonella* and STEC in wheat and flour, even as a low water activity food, neither wheat grains nor flour provide an ideal environment for bacterial growth. Similar finding have been reported for other low water activity food matrixes such as peanuts, pistachios, almonds, etc (*4*, *33*, *64*). Therefore, a killing step should be implemented to enhance flour safety.

2.9 Wheat processing

Wheat processing starts after receiving wheat grains from storage. Upon the arrival at the milling facility, wheat grains will go through a "cleaning" process, where foreign materials are removed. The foreign materials include stones, dirt, and other cereal grains from the field. Even though the cleaning step is not intended to decrease the bacterial load on wheat grains, some contaminants from the foreign materials are removed. A past study reported about a 1 log CFU/g in aerobic mesophilic bacteria from wheat grains with an initial load of approximately 8.2 log CFU/g (*43*).

After cleaning, the wheat grains are tempered by adding 1-3% of water according to the weight with mixing. Tempering allows moisture to penetrate the bran layer, toughening the bran for easier removal during milling. At the same time, the endosperm is mellowed, which allows greater flour extraction. Tempering times vary due to kernel hardness, protein content, the

wheat's initial and target moisture content. Usually, the harder the wheat grain, the longer the tempering time. Typical tempering time and target moisture content for different wheat types are summarized in Table 2-2 (*34*).

Table 2-2 Tempering time and target moisture content of different wheat varieties.

Wheat type	Recommended tempering time	Target moisture content
Hard wheat	24-36 (48) hours	16.0-17.0%
Semi-hard wheat	18-24 hours	15.5 -16.0%
Semi-soft wheat	12-18 hours	15.0-15.5%
Soft wheat	6-12 hours	14.5-15.0%

Reprinted from Kunz.S.K., 2016

After adding moisture, the wheat grains will remain in the tempering bin for the remainder of the tempering time, with optimum temperature of wheat in the tempering bin not lower than 25˚C (*46*). The increase in water activity during tempering enhaces opportunity for microbial growth. Previous studies have found an increase in wheat grain aerobic plate counts after tempering (*3*, *53*).

Milling is a complicated process that aims to separate endosperm, germ, and bran. The milling process involves series of breaking and sorting steps to purifying the endosperm particles that are eventually reduced to flour (*32*).

Table 2-3 Major steps of the mill flow sheet.

Stages	Input	Output	Objective
Break system	Cleaned conditioned wheat	Bran, sizings, and germ to purifiers; pure endo - sperm to reduction system; some flour	Clean the bran from endosperm; classify remaining material for further processing
Grading system	Mixture of sizings and flour from breaks	Sizings to purifiers and sizing rolls, and flour	Separate flour from sizings

Table 2-3 (cont'd)

Purification system	**Mixture of pure endosperm, compound endosperm/bran, bran, and germ**	**Germ, large compound bran/endosperm, small compound bran/ endosperm, pure endosperm**	**Maximize pure endosperm particles to reduction; divert fine breaks material and sizings for further processing**
Sizings system	Compound particles of bran/endosperm from breaks, purifiers, and middlings	Pure bran and germ as by-products; pure endosperm to reduction, compound endosperm/ aleurone to low-grade system; some flour	Reduce endosperm particles to a required size and separate between endosperm particles and adhering bran
Reduction system	Relatively clean endosperm from the breaks, grading, and purification systems	Flour; germ fragments; mixture of endosperm/ aleurone/bran to low grade	Reduce pure endosperm to flour; divert unpure endosperm to sizings and low-grade systems
Low-grade system	Compound endosperm/ aleurone/bran particles from reduction, sizings, and tail of the breaks systems	Low-grade flour, compound aleurone/ bran/endosperm	Remove the last flour from by-products

(Reprinted from Khan and Shrewry, 2009, without changes)

As discussed earlier, wheat is prone to cross-contamination, with pathogen generally confined to wheat kernel surface. During milling, bran is usually removed during the first break, decreasing the microbial level. In one study, the aerobic plate count from wheat grains to flour dropped from 5 log CFU/g to 2 log CFU/g (*3*). A recent publication (*49*) ran a 20 batches continuous test of inoculated wheat with nonpathogenic *E. coli* through a laboratory scale roller mill equipped with two breaks, one sizing, and three reduction steps, populations of *E. coli* in flour increased throughout the process. Immediately after the experiment, another 20 batches of

uninoculated wheat were run through the same system. *E. coli* remained quantifiable in both flour and non-flour fractions.

2.10 Microbial decontamination of wheat and flour

Contaminated wheat grains could cross-contaminate the facility and other non-contaminated wheat grains. Therefore, cleaning of milling equipment is suggested (*51*). However, as a fine particle, flour must avoid contact with liquid. Therefore, after tempering, milling is considered a dry process. To address increasing concerns about flour safety, several wheat grain disinfectant treatments have been suggested, either focused on wheat grains before milling, such as during tempering, or on the final product, using dry methods such as radiofrequency to disinfect flour. López and Simsek (*40*) conducted a comprehensive review to summarize the current advancement in technology to disinfect wheat grains on flour. Earlier studies mainly focusing on removing mold and yeast from wheat grains, with increasing occurrence of flour outbreaks associated with enteric pathogen, more studies were conducted targeting reducing bacterial density on wheat grains.

2.10.1 Non-thermal treatments

Non-thermal treatments have been implemented for wheat grains in the milling industry. About 85% of mills in the United States use chlorine of different forms in their tempering water (*63*). No previous study had evaluated how tempering water with added chlorine could be used to decrease bacterial levels on wheat grains.

Tempering with 16 ppm of ozonated water resulted in <0.5 log reduction in yeast , mold and aerobic plate counts (*15*). Slightly acidic electrolyzed water led to a 0.93 log CFU/g reduction in the total aerobic count(*12*). However, neither of these studies involved pathogen testing.

Recent studies have focused on tempering water containing both organic acid and saline (*52*, *54*, *55*). The combination of 5% lactic acid with 26.6% NaCl was found to be the most effective. Reducing *Salmonella*, *E. coli* O157:H7, and non-O157 STEC count by 1.8, 1.6, and 1.6 log CFU/g on inoculated soft wheat, respectively. Greater reductions were observed on hard wheat, of 2.6, 2.4, and 2.4 log CFU/g for *Salmonella*, *E. coli* O157:H7, and non-O157 STEC, respectively (*52*). In flour functionality test, no significant changes were found in the viscosity or solvent retention capacity, nor changes in cookie dimension size during baking. However, the flour's pH dropped significantly after the tempered soft wheat was milled into flour.

Expanding upon the idea of combining organic acid to prepare tempering solution, Rivera et al. (*47*, *48*) tested using 5% and 10% sodium bisulfate solution (SBS) to inactivate STEC inoculated on hard wheat, reported a reduction in STEC load by 2.0, and 2.6 log CFU/g, respectively (*48*). Quality tests showed a significantly lower pH for the resulting flour tempered with 5% SBS solution.

Since tempering is part of the milling process, and solutions such as acidic water are relatively inexpensive, solutions to inactivate microbial loads on wheat grain had mostly been focus on tempering solution. However, some other newer technologies that involves adding extra unit operation leading to higher cost also been studied. For example, pulsed light-emitting diode (LED) at 395nm (UV) led to a 2.91 log reduction in *Salmonella* on wheat flour when exposed for 60 min (*16*). Atmospheric cold plasma at a high voltage output of 80kV applied to wheat grains led to 1.5 log CFU/g reduction in total aerobe count after 20 min (*41*).

2.10.2 Thermal treatments

Thermal treatment involves heating at high temperatures, which usually leads to a more significant reduction in the bacterial population. Radiofrequency (RF) heating (0.5kW, 27MHz,

90mm electrode) for less than 9 minutes until the minimum temperature of 75˚C was reached, decreased 7 log CFU/g of *Salmonella* inoculated on flour surrounded by polystyrene material (*68*). In other work, RF pasteurization using a 6kW, 27.12 MHz, 35mm electrode gap heating unit yield a 5 log CFU/g reduction in *Salmonella* after heating flour at 85 ˚C for 33 min of flour in aluminum test cells (*38*). Finally, using the same heating unit, found a 4.9 log CFU/g reduction in *Enterococcus faecium,* a common *Salmonella* surrogate in LMF thermal studies was achieved, when the flour was heated at 85 ˚C for 27 min (*71*).

The temperature of steam can be raised to above 100 ˚C upon saturation by applying pressure, where different temperatures (110, 140, 170, and 200 ˚C) of superheated steam were applied to wheat grain, at 200 ˚C led to an 81.8% decrease in Bacillus spp. populations after 80 seconds (*29*).

On the other hand, saturated steam can also be maintained at temperatures below 100 ˚C under a vacuum environment. Vacuum steam pasteurization (VSP) was conducted on 1 kg of hard wheat (*60*), treated at three different temperatures for either 4 or 8 min. The hard wheat flour quality found to be significant damaged after treated at 75 ˚C. Therefore, the bacterial inactivation trials focused on the VSP temperature at 65 ˚C. When treated at 65 ˚C for 8 min, *E. coli* O121:H19 and *Salmonella* PT30 population reduced 3.57 log CFU/g and 3.21 log CFU/g, respectively, without compromising flour quality. Another study (*59*) focusing on soft wheat, reported that the resulting flour quality was not impacted when soft wheat grains were treated at 75 ˚C, which led to 7.34 and 5.09 log CFU/g reduction in *E. coli* O121:H19 and *Salmonella* PT30, respectively.

During thermal treatment, pathogen inactivation efficacy has been found to be associated with the water activity of the food. Different thermal inactivation studies have shown that

Salmonella survival increases as the water activity of the food matrix decrease. (*23*, *69*). Inversely, wetting almonds before pulse light treatment led to a greater reduction in *Salmonella* inoculated on almonds (*39*).

The D-value measures the time required to achieve a one log reduction in the bacterial population at an isothermal temperature. However, at the start of heating, some time is required for the food sample to rise to the target treatment temperature, referred to as come-up time. During the come-up time, which usually is non-isothermal, some degress of bacterial inactivation may also occur along with changes in the chemical or physical properties of the food. For example, raising the water activity of a low-moisture food, as described earlier, may change the bacterial stress response. At the same time, some lab-scale studies use a smaller sample size to reduce the come-up time and use the data collected during isothermal heating to establish an inactivation model. However, how well such a model could perform in real-life scenarios may be questionable, especially for complicated food matrices like wheat, which later need to be processed without negatively impacting the resulting flour quality and gluten functionality.

3 CHANGES IN ENTERIC PATHOGEN POPULATION, AND MIGRATION OF PATHOGEN DURING WHEAT TEMPERING

3.1 Abstract

Outbreaks of Salmonella and Shiga-toxin producing Escherichia coli (STEC) linked to wheat flour led to increased interest in characterizing the fate of Salmonella and STEC on wheat during processing. This study aimed to quantify changes in the numbers of bacteria on STEC and Salmonella inoculated soft red winter wheat, and to observe potential changes in the distribution of the pathogens on the surface of kernels, during tempering. Lab-scale tempering experiments were conducted to quantify the water activity of and bacterial populations on wheat grain at various time points during 16 h of tempering. The highest water activity observed throughout 16 hours of tempering was 0.88. There was no significant change ($p>0.05$) in numbers of Salmonella, STEC, or native mesophiles. Using confocal microscopy, observation of Salmonella and STEC cells expressing mCherry on wheat kernels showed an even distribution of inoculated cells, though the localization of cells on kernels did not change significantly after tempering. Even though the environment was not favorable for pathogen replication on grain, the population remained stable, suggesting that contamination on kernels needs to be effectively reduced to minimize food safety concerns.

3.2 Introduction

Recent outbreaks of *Salmonella* and Shiga toxin producing *Escherichia coli* (STEC) (*7*, *8*, *9*) in flour and flour products have increased concerns regarding the safety of this low moisture food. Low moisture foods (LMF), defined as those having a water activity equal to or lower than 0.85, generally will not support the bacterial growth, with the exception of halophilic bacteria (*62*). However, several studies on LMF, such as almonds, pistachios, and flour, have

demonstrated the ability of *Salmonella* and STEC to survive in a low water activity environment for extended periods of time (*4*, *22*, *24*, *33*, *36*). On wheat grain, *Salmonella* and STEC survived and were detected above the limit of detection for 52 and 44 weeks when inoculated at 6 log CFU/g, respectively (*36*).

Wheat flour milling consists of several processes. Grain is transported from a storage facility and goes through a cleaning process before milling, which removes the non-wheat materials (*42*). Then the wheat grain is tempered, a process in which water is added to increase the moisture content of the kernels, making the separation of the bran and endosperm easier during milling. The amount of water added is generally 1 to 3% of the weight of wheat grain and depends on the moisture content, wheat variety, and milling practices. After the addition of water, the grain remains in conditioning bins for hours before milling to allow for water absorption by the bran layer.

The addition of water during tempering leads to increased water activity, potentially providing a more favorable environment for bacteria to grow. The milling industry mainly uses moisture content during tempering as the metric to assess whether the grain is ready to be milled. Previous studies evaluating microbial numbers on wheat before and after tempering found that the aerobic plate count of wheat grain increased slightly after tempering (*3*, *53*).

Even though the water to grain ratio used is relatively low during tempering, the water coats the surface of the grain and could potentially lead to cross-contamination of other kernels, or lead to changes in bacterial distribution on the same kernel.

Bran is the outer layer of wheat grain that protects tissues beneath from exposure to moisture, and microbial contamination (Figure 3-1). The bran is usually removed in a manner intended to minimize damage and fragmentation of the bran during wheat flour milling, therefore reducing

the chance of microbial contamination. The crease is an irregular-shaped gap that exists on one side of the kernel that extends inward to a depth just beyond the center of the grain (*46*). The crease could potentially harbor pathogens that may be more difficult to remove, and eventually contaminate the milled flour. The brush of the grain is a cluster of hairs generally about 0.5mm long (*32*, *46*), which may also harbor microbes. Limited research has been done to assess the redistribution of brush hairs after milling. Debranning or pearling is conducted prior to milling in order to remove the outer bran layers, which may also include the brush hairs. Depending on the milling practice, the brush hairs could end up in the flour (*46*) and therefore contaminate the flour.

We are unaware of previous studies that have evaluated if individual pathogen populations are altered by the change in water activity during tempering, or whether the addition of water would facilitate bacterial movement to the creases of kernels. To answer these questions, the goals of the current study were to (1) quantify the changes in the numbers of native mesophiles, inoculated *Salmonella*, and STEC on kernels of soft red winter wheat during tempering and (2) assess the distribution of fluorescently labeled pathogens attached on soft red winter wheat grains during wheat tempering using confocal microscopy.

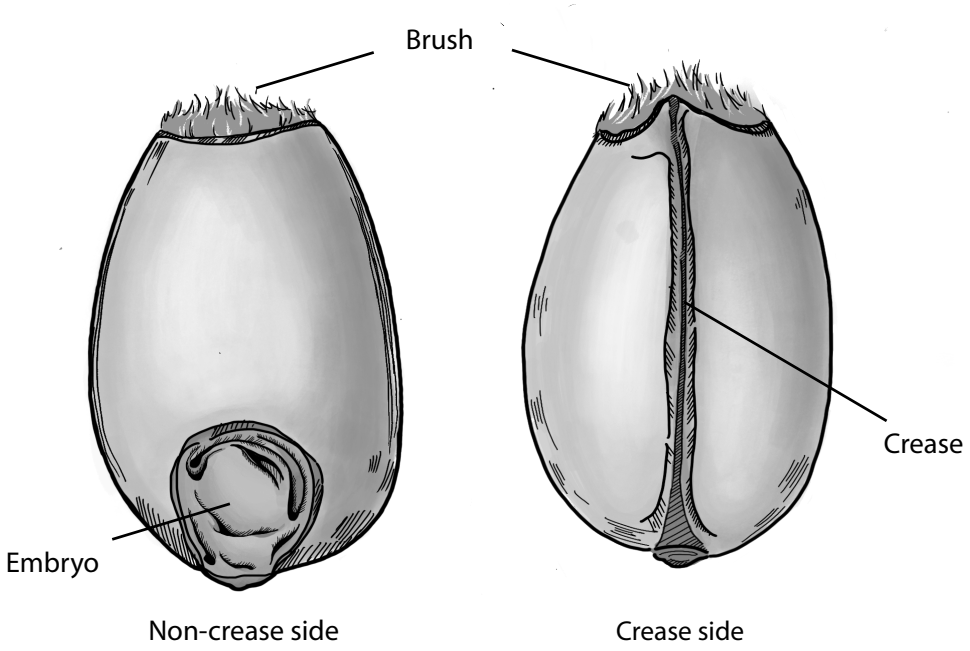

Figure 3-1 Dorsal and ventral views of wheat grain.

3.3 Method

Wheat grain. A commercial blend of soft red winter (SRW) wheat grain was kindly donated by Mennel Milling Company (Fostoria, Ohio). The grain was received in September 2020 and stored at 4°C in Whirl-Pak bags (Nasco Whirl-Pak, Madison, WI). The day before inoculation, one kilogram of wheat was brought to room temperature, and the moisture content of uninoculated wheat was measured using a handheld moisture meter (MINIGAC1SG4, Dickey-John; Minneapolis, MN), average moisture content of wheat was 12.5 ± 0.1%. The water activity of wheat was measured by AQUALAB 4TE water activity meter (METER, Pullman, WA), the average water activity was 0.50 ± 0.02.

Bacterial strains. Four strains of *Salmonella* and six strains of STEC were used. *Salmonella enterica* serovars included Enteritidis PT30 (ATCC BAA-1045, isolated from

almond, obtained from ATCC), Agona (FSL S9-0322, isolated from cereal), Tennessee (FSL R6-0494, clinical isolate), and Montevideo (FSL R8-3881, clinical isolate). The three *Salmonella* strains with the prefix "FSL" were obtained from the Food Safety Laboratory at Cornell University. STEC strains included *E. coli* O121:H19 (PNUSAE002568, flour outbreak, obtained from Michigan Department of Health and Human Services), *E. coli* O157:H7 (RMID 0509952, isolated from radish sprout), *E. coli* O26:H11 (TW16501, isolated from sprouts outbreak), *E. coli* O45 (TW07947, clinical isolate), *E. coli* O111 (TW07926, clinical isolate), *E. coli* O145 (TW09153, clinical isolate). All STEC strains except for the O121:H19 were obtained from the TS Whittam STEC Center at Michigan State University. STEC O157:H7 and STEC O103 strains described above were selected for resistance to 80 µg/ mL rifampicin (Thermo Fisher Scientific, Waltham, MA) using the method described in our previous study (*37*).

Inoculum preparation and inoculation of wheat grain. Each strain was streaked to Luria-Bertani (LB) agar from freezer stocks (Thermo Fisher), and plates were incubated at 37°C for 24 hours. A single colony was transferred to 5mL of LB broth, incubated at 37°C for 20 hours. Then 250µL of culture was evenly spread onto LB agar (in 100mm petri plates (Thermo Fisher) and grown for 24 hours at 37°C to achieve confluent lawn growth. Lawn grown cells were removed using a sterile L-shaped spreader (Thermo Fisher) and suspended in 2.5mL of sterile deionized water. For rifampicin resistant strains, all culture media contained 80 µg/ mL rifampicin.

To achieve ~6 log CFU/g target inoculum level on grains, one plate of lawn culture was scraped with a sterile L shaped spreader and suspended in 2.5 mL of room temperature sterile DI water to inoculate 1 kg of soft wheat for each strain, per replicate. The inoculum suspension was added directly to uninoculated wheat and massaged manually by hand for 3 minutes. After

mixing, the grains were transferred to a sterile stainless-steel tray and mixed for another 3 minutes with a sterile scoop. Four 25 g samples of inoculated wheat were assessed for the homogeneity; if the standard deviation of four samples was smaller than 0.5 log CFU/g, the inoculum was considered homogeneously distributed.

The inoculated wheat was transported in Whirl-pak bags (Nasco) to the water activity equilibration chamber described in (*23*). The relative humidity in the chamber was set at 55%, and inoculated wheat grain remained in the chamber for 48 hours at ambient temperature. The water activity of grain was measured to ensure it was 0.5±0.02 before removing from the equilibration chamber.

Tempering. Three biological replicates were tested, each with two technical replicates. For each technical replicate of each strain, the tempering process was conducted as described below. After water activity equilibration, seven sterile 250mL centrifuge bottles were each filled with 50 g of inoculated wheat (Nalgene, Rochester, NY), one bottle for each time point examined (t=0.5, 3.5, 6.5, 9.5, 12.5, 15.5, and 16 hours). Sterile deionized water was added to each bottle at the tempering times of t=0 and t=15.5 hours. The amount of water added was calculated following AACC standard method 26-95 (American Association of Cereal Chemists (AACC), accessed 2.3.22), targeting a final moisture content of 15%. A 1000 mL centrifuge bottle (Nalgene) containing 500 g of uninoculated wheat was used as the moisture content reference, under the assumption that the bottles with inoculated wheat was similar on moisture content changes throughout the tempering process. The tempering process is summarized as follows: water was added at t= 0 hours, targeting 14.5% moisture content, and after water addition, the bottles of inoculated wheat and reference wheat were placed in a rotating device (~23 rpm) for 30 mins at ambient temperature. At t= 0.5 hour, the rotation was stopped, grain

from one bottle was collected for enumeration, while the rest of the bottles remained still in the rotating device for the next 15 hours, and grain was collected from one bottle every three hours (t= 3.5, 6.5, 9.5, 12.5, 15.5) for enumeration of bacteria. At t=15.5 hours, the moisture content of the reference wheat was measured, and tempering water was added to target 15% moisture content, followed by continuous rotation at ambient temperature for half an hour. At t= 16 hours, the grain in the final bottle was sampled and the target microorganism enumerated. Of each enumeration point, the water activity of wheat was also measured. Throughout the experiment, the samples were maintained at ambient temperature of ~20°C.

Bacterial enumeration. Differential media were used for enumeration. Butterfield's buffer was added to 25 g of wheat that was randomly collected from 50g of grain of each time point, to 225mL of Butterfield's buffer to achieve a 1 to 10 initial dilution, and homogenized (Seward 400, Worthing, West Sussex, UK) for 120 seconds. Serial dilutions in Butterfield's buffer were plated onto modified trypticase soy agar (TSA) (BD, Franklin Lakes, NJ) supplemented with 0.1% ferric ammonium citrate (Thermo Fisher) and 0.006% sodium thiosulfate (Thermo Fisher) for *Salmonella*. Sorbitol MacConkey agar (BD) for STEC O157:H7, and MacConkey agar (BD) for non-O157 STEC. Native aerobes were quantified by plating onto Plate Count agar (Neogen, Lansing, MI), and coliforms were quantified using Violet Red Bile (VRB) agar (BD). The two rifampicin resistant strains were enumerated on MacConkey, or sorbitol MacConkey supplemented with 80 µg/ mL of rifampicin.

Strains transformed with pFCcGi plasmid to express mCherry. pFCcGi was a gift from Sophie Helaine & David Holden (Addgene plasmid # 59324; http://n2t.net/addgene:59324; RRID: Addgene_59324) (*19*). *E. coli* containing pFCcGi was streaked to LB agar with 100µg/ml ampicillin and incubated for 24 hours at 37°C. A single colony was transferred to 50 mL LB

broth with 100µg/ml ampicillin at 37°C for 16 hours with shaking, and centrifuged at 6000 x g for 15 minutes at 4 °C. The plasmid was then extracted using the QIAGEN Plasmid Midi Kit (QIAGEN, Hilden, Germany) as directed. Plasmid size was verified by electrophoresis on a 2.0% agarose gel (E-Gel Power Snap Electrophoresis Device (Invitrogen, Waltham, WA) with 1kB ladder (Invitrogen).

To make each recipient strain competent, the following method was adopted from previous studies (*13*, *44*). *Salmonella* PT30 and STEC O121:H19 were streaked for isolation onto LB agar and incubated at 37°C overnight. A single colony was transferred to 50mL of LB broth and grown to OD_{600} =0.6 at 37°C with shaking. After reaching the desired OD value, the culture was first heat shocked for 15 minutes in 42 °C water bath, then incubated in an ice bath for 10 minutes. The culture was then centrifuged at 4000 x *g* for 15 minutes at 4 °C. The cell pellet was washed three times with 1ml of 1mM MOPS (Thermo Fisher) + 20% glycerol (VWR, Radnor, PA) by centrifuging at 4000 x *g* for 30 seconds and suspended in 100µL 1mM MOPS + 20% glycerol. About 1 mg of the sample was electroporated at 2.5 kV using a MicroPulser Electroporator (Bio-Rad, Hercules, CA), then incubated in 3 mL of LB for 90 minutes at 37°C with shaking. Thereafter, 250 µL of culture was plated on LB agar with 100µg/ml ampicillin and incubated for 24 hours at 37°C. Transformed pink colonies that constitutively expressed mCherry were selected for confirmation of successful transformation. The selected colony was grown in 50ml of LB broth with 100µg/ml ampicillin and incubated at 37°C for 24 hours. After incubation, 800µL of culture was mixed with 75% glycerol and frozen at -80°C freezer stock for future use. The remaining culture was used for plasmid extraction and confirmation of plasmid size as described above.

Preparation and tempering for confocal microscopy. *Salmonella* PT30 and STEC O121:H19 with pFCcGi were streaked from freezer stock onto LB agar + 100µg/ml ampicillin and incubated at 37°C for 24 hours. A single colony was transferred to 5mL of LB + 100µg/ml ampicillin and incubated at 37°C for 24 hours, then, 50 µL of this culture was spread onto a 50mm petri plate of LB agar with 100µg/ml ampicillin and incubated at 37°C for 24 hours. The next day, the culture was harvested with a L-shape sterile spreader (Thermo Fisher) to 500 µL of DI water as described previously, and 75 grams of wheat was inoculated with the cell suspension. After massaging, the wheat was allowed to air-dry in a biosafety cabinet for 2 hours. The mean water activity was 0.51±0.02 before tempering. For tempering, 50 g of wheat was transferred to a bottle and tempered as described in 2.4. About 15 to 20 grains were randomly selected before and immediately after tempering (t=0 and 16) and fixed with 4% paraformaldehyde (Sigma) dissolved in 10X PBS for 1 5 minutes. After fixation, grains were washed three times with 1X PBS, and the grains were dried in the hood for 30 minutes. After fixation, 6 grains from each sampling point were randomly selected and stored in a Nuc Lab-Tek chamber cover glass system (Thermo Fisher) for imaging.

Confocal microscopy. Samples were imaged at the Center for Advanced Microscopy of Michigan State University using confocal laser scanning microscopy (Nikon Instruments A1 Confocal Laser Microscope). Z-series images were collected for the depth to sample to cover the whole crease depth of the kernel, with a 10x objective, after which the max intensity image of the z-series was recorded. Using Nikon NIS-Elements Advanced Research Software, multiple images were taken to view the whole kernel, as shown in Figure 3-2. Further analysis was conducted with Fiji version 2.5.0 (*56*). To remove the noise created by oversampling during z-series, four randomly selected uninoculated kernels were first analyzed to determine the

threshold, using the Shanbhag algorithm (*58*) embedded in Fiji. In analyzing the inoculated kernels, only the intensities greater than the threshold was identified in Figure 3-2B, with these areas calculated as a percentage by the software. To analyze if certain areas of wheat grains could potentially harbor more bacteria, wheat grains were divided into four areas. Three of these areas are on the crease (ventral) side of the kernel, as shown in Figure 3-2C, with the brush of the kernel on top, a rectangle with a width of 300μm to be the "crease" the area on the left of the crease as "left", and on the right of the crease as "right". The fourth area was the non-crease (dorsal) side of the kernel.

Statistical analysis. To determine if the changes in bacterial population and the changes in water activity during tempering were significant. The t-test used to determine if changes were significantly different from zero reduction with 95% confidence interval, the sample size of the test was equal. A two-sample student's t-test was used to analyze the change in bacterial population before and after water activity equilibration. To determine if the changes in bacterial population and the changes in water activity during tempering were significant. Analysis of variance (ANOVA) was used to determine if the mean fluorescence intensity of wheat grain at each area measured was significantly different.

All statistical analyses were done with R (version 4.0.3, www.r-project.org), and plots were made with ggplot2 version 3.3.3 (*26*).

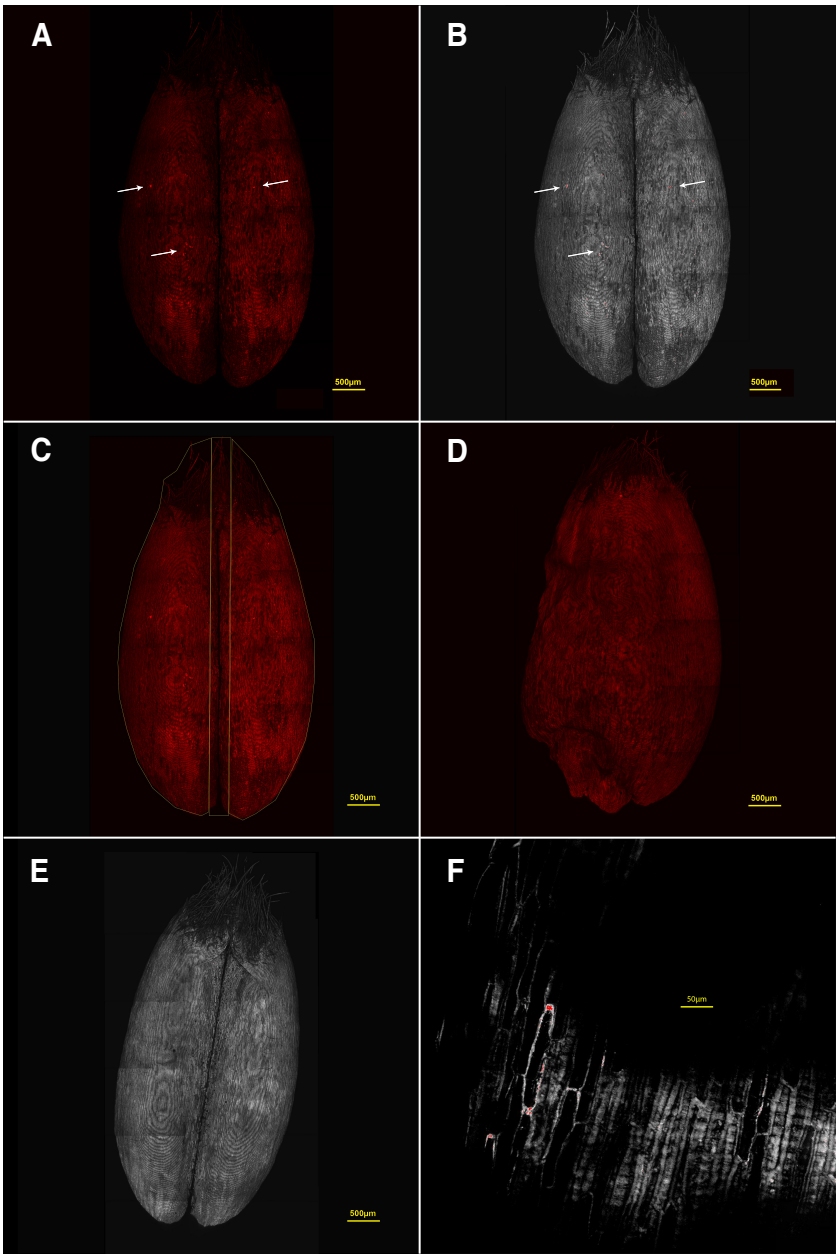

Figure 3-2 Kernel images taken with confocal microscopy, (A) maximum intensity image of crease side of an inoculated kernel, arrows pointing to strong intensity signal. (B) Image of the kernel after applying threshold. (C) Area defined with application Fiji; the crease side is defined by rectangle with width equals to 300 µm. (D) Maximum intensity image of non-crease side of an inoculated kernel. (E) Image of a uninoculated kernel after applying threshold. (F) Image of kernel surface after applying threshold, obtained with 40X objective.

3.4 Results

Bacterial population changes due to water activity equilibration. After inoculation, the average numbers of pathogens on wheat grain were 6.5±0.2log CFU/g of *Salmonella* strains,

and 6.4±0.3 log CFU/g of STEC, with these two populations statistically similar ($p>0.05$). After the 48-hour water activity equilibration period, the average *Salmonella* population reduction was 0.1±0.3 log CFU/g, while the mean decrease of population of STEC was 0.9±0.5 CFU/g, which was significantly ($p<0.05$) greater than that of *Salmonella*. The population of *Salmonella* on wheat grain before and after equilibration was not significantly ($p>0.05$) different, but the population of STEC on wheat grain before and after water activity equilibration was significantly($p<0.05$) lower.

Change in water activity during tempering. During the 16 hours of tempering, the average water activity (Figure 3-3) of wheat inoculated with *Salmonella* increased from 0.52±0.02 to 0.79±0.02, 0.51±0.01 to 0.79±0.02 for wheat inoculated with STEC strains, and 0.51±0.01 to 0.80±0.02 for uninoculated wheat grain. The change in water activity between every time point sampled was not significantly different among wheat inoculated with either pathogens or the uninoculated wheat ($p>0.05$), indicating that the pathogen inoculated onto wheat grains did not impact the change in water activity observed during wheat tempering. Therefore, water activity for all tempered wheat samples was assessed together to determine whether water activity changes significant at each time point.

The water activity of wheat grain increased at the two water addition points. During the first half hour when the larger volume of tempering water was added, a significant ($p<0.05$) increase in water activity by 0.32 ± 0.04 was observed. Water activity changes between the time intervals of 0.5 to 3.5 hours and 3.5 to 6.5 hours were -0.09 ± 0.04 ($p<0.05$) and -0.03 ± 0.02, respectively. The water activity of wheat grain remained stable after t= 6.5 hours before more water was added, as the change in water activity between 4 samplings points (t=6.5 to 9.5 hours, t=9.5 to 12.5 hours, and t=12.5 to 15.5 hours) were not significantly ($p>0.05$) different, the mean change

during these three intervals was -0.00±0.01. The second water addition significantly ($p<0.05$) increased the water activity by 0.09±0.02 during the last half hour of tempering.

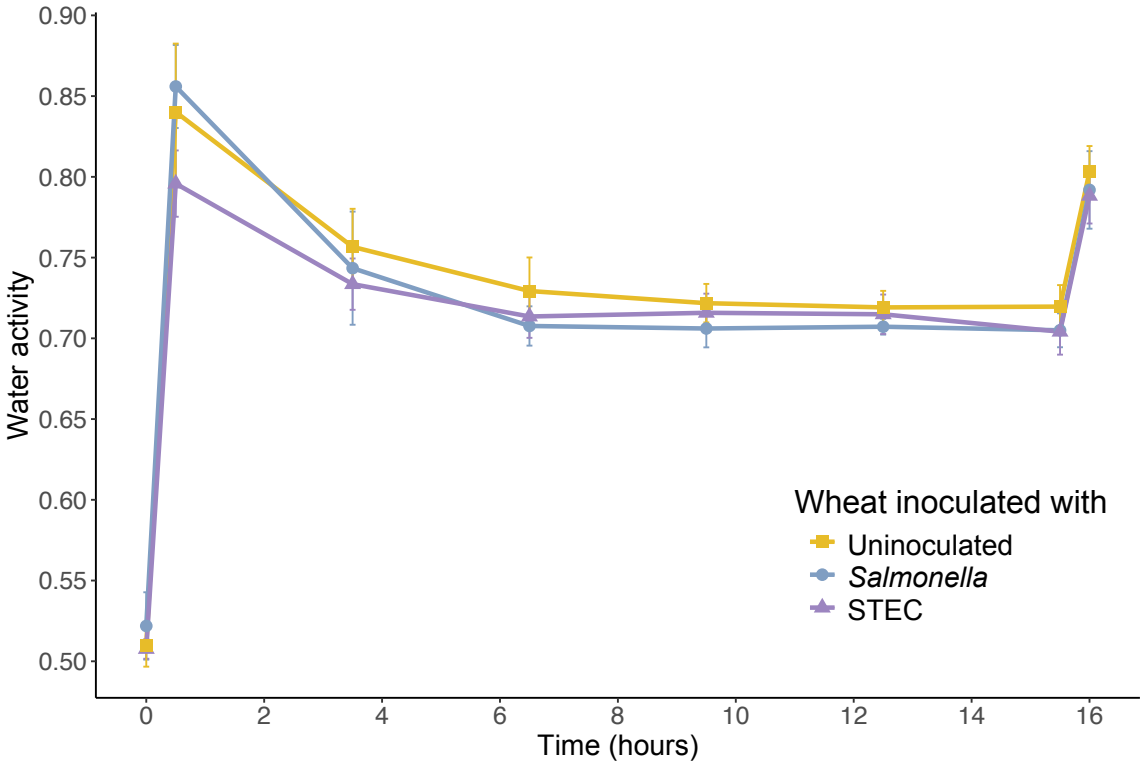

Figure 3-3 Changes in water activity during SRW tempering. Each data point represents the average of 3 replicates with error bar representing the standard deviation.

Changes in bacterial population during tempering. The mean populations for each strain on wheat grain over time during tempering are shown in Figure 3-4. While the starting populations were significantly different for the *Salmonella* and the STEC strains, the overall change in population during tempering were not significant ($p>0.05$). To conduct further analysis, the bacteria are grouped as: *Salmonella*, STEC, and native microbes (mesophiles and coliforms). Statistical analysis was conducted, and no significant differences ($p>0.05$) were found among strains in each group.

As described in section 3.2, adding water during tempering increases the water activity, and we wanted to determine if this increase in water activity led to an increase in bacterial

numbers. One-way ANOVA was performed to determine if the change in bacterial density during the first half hour (t=0 to t=0.5), and the overall change in density were significant ($p<0.05$) for the three groups of microbes. In the first half hour, the population changes of native microbes (native mesophiles and native coliform) and *Salmonella* strains were slight but statistically significant (Figure 3-5). On average, the population of native microbes increased by 0.3±0.1 log CFU/g, while the mean *Salmonella* population change was 0.0±0.1 log CFU/g, and the population of STEC change was 0.2±0.2 CFU/g during the first half hour.

During the whole 16 hours of tempering, the change in the native microbes and STEC population was significantly different ($p<0.05$). On average, the *Salmonella* population changed -0.2±0.2 CFU/g, and the STEC population changed -0.2±0.3 CFU/g, and the native microbe population change was 0.1±0.1 CFU/g.

Distribution of fluorescent signal during tempering. For wheat grain inoculated with *Salmonella* PT30 + pFCcGi and STEC O121:H19 + pFCcGi, the population density after inoculation was 6.5±0.5 log CFU/g for both *Salmonella* and STEC. The mean kernel areas sampled were 14.4±1.9 mm^2 for the non-crease side of the kernel, 1.8±0.02 mm^2 for the crease area, 6.6±1.2 mm^2, for left side of the crease, and 6.4±1.2 mm^2 for the right side of the crease, respectively. Based on the Shanbhag algorithm with uninoculated wheat, the threshold intensity for inoculated wheat was 3588 for the crease side and 3492 for the non-crease side. The percentage of the areas on inoculated wheat that had intensities greater than the thresholds defined are shown in figure 3-6, with the majority of data falling within a narrow range close to zero; the normality of the result was checked with the Shapiro test which found the data were found to be not ($p<0.05$) normally distributed for both STEC and *Salmonella*. A non-parametric Kruskal-Wallis rank sum test was run to determine if there is a significant difference in the

percentage of area with intensity greater than threshold at the different locations designated on the kernel. There were no significant differences ($p>0.05$) in the percentage of intensity greater than threshold among the 4 areas examined. Additionally, there was no significant ($p>0.05$) difference in the percentage of intensity greater than threshold between *Salmonella* and STEC.

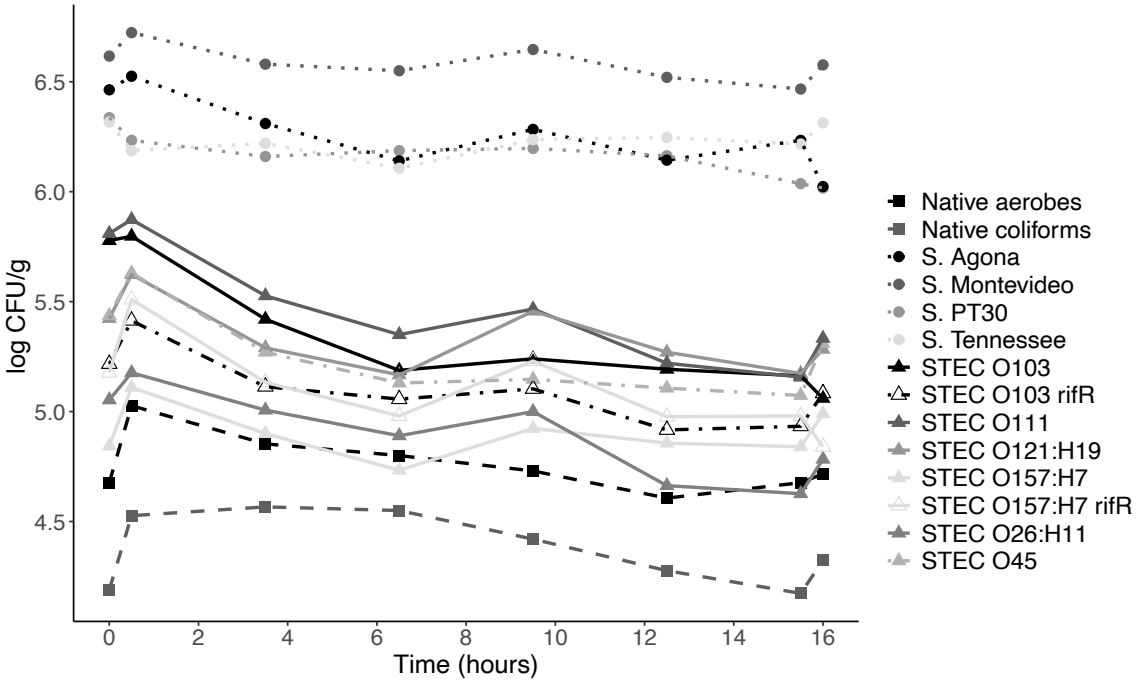

Figure 3-4 Changes in bacterial population during SRW tempering. Each data point represents the average of 3 replicates.

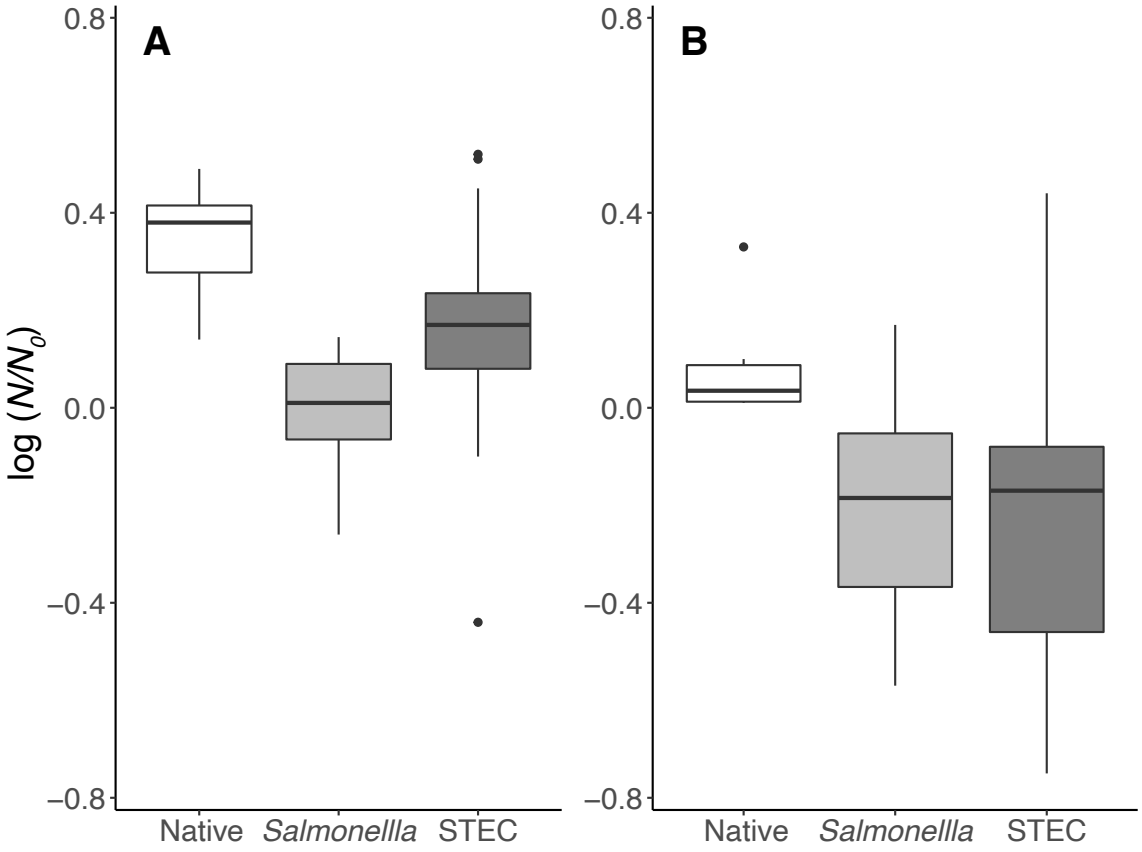

Figure 3-5 Changes in bacterial population of wheat inoculated with different microbe types (native microbe, Salmonella, and STEC) from time points (A) t=0 to t=0.5 hours, (B) t=0 to t=16 hours.

3.5 Discussion

Water activity increases significantly during tempering, but did not lead to changes in bacterial populations. The mean population changes during tempering were less than a half a log CFU/g for native mesophiles, native coliforms, *Salmonella* and STEC. The most significant increase in water activity occurred after the first water addition point, where a greater quantity of tempering water was added, which raised the water activity of wheat grains to about 0.85, which is still too low to support the growth of bacteria. Here we followed the AACC standard method to conduct the lab-scale tempering; in the milling industry, the second water addition is rarely performed, and the tempering time varies depending on many parameters: the type of wheat

tempered, initial moisture content of the wheat, as well as environmental factors such as the facility temperature and humidity. A recent study (*31*) of tempering inoculated hard wheat grains found an average change of -0.43 log CFU/g in the *Salmonella* cocktail population and -0.45 to –0.80 CFU/g in the STEC cocktail population over 12 hours of tempering. We found a similar level of reduction in *Salmonella*, and a relatively lower reduction in STEC population during tempering, which could be due to variation in the response of different STEC strains.

Sabillon et al. (2021) examined wheat samples from a mill in the United States and found no difference in aerobic plate counts (5.5 log CFU/g) for tempered wheat and wheat after cleaning. These results from commercial milling processes are similar to our findings with lab scale tempering. Even if the environment was not favorable for pathogen growth, the population remained relatively stable, which indicates that contamination on kernels either from the field or harvesting and storage needs to be effectively reduced to minimize food safety concerns.

Using tempering solutions to disinfect microbial contamination on wheat grains. Recent publications (*49*, *50*) suggested the risk of cross contamination between contaminated kernels to milling equipment and vice versa. Wheat inoculated with nonpathogenic *E. coli* was passed through a laboratory scale roller mill with two breaks, one sizing, and three reduction steps. Greater contamination was found in the non-flour portion compared to the flour portion, indicating that the debranning process greatly reduces the microbial load on wheat. However, continuous milling of contaminated wheat eventually led to an increasing count of *E. coli* in flour. The experiment was immediately followed by milling of uninoculated wheat and found even though the *E. coli* recovered for all fractions of milling dropped significantly ($p<0.05$) after the first run, the *E. coli* remained quantifiable after 20 batches of uninoculated wheat for both

flour and non-flour fractions. This finding indicates the necessity of milling equipment cleaning to prevent cross-contamination.

The milling process is a dry process after tempering. To reduce microbial contamination before wheat is milled, several kill steps before milling have been suggested. Addition of modified tempering solution to disinfect wheat grains could be one cost-effective way to reduce pathogen density by simply replacing water with a solution that has a disinfectant effect. Recent studies demonstrate that solutions containing chlorine, organic acids, or salts, can reduce pathogen populations on grain during tempering. Using 800ppm chlorinated water as a tempering aid resulted in reduction in *Salmonella* and STEC ranging from 0.3 to 2.4 log, depending upon the strain (*37*). Using 5% lactic acid and 26.6% NaCl as tempering aid resulted in a 2.4 log reduction in STEC and a 2.6 log reduction in *Salmonella* (*54*). And 5% and 10% of sodium bisulfate solution led to 2 log and 2.6 log reductions in STEC, respectively (*48*). A recent study (*31*) found the using 5.4% lactic acid led to a 1.79 log CFU/g reduction of *Salmonella*, and 0.45- 0.8 log CFU/g reduction in STEC. Increasing the lactic acid concentration to 10.9%, lead to 1.87-2.03 log CFU/g reduction in STEC. The study also explored the possibility of peracetic acid as tempering solution and reported reductions of STEC of 1.21 log CFU/g when exposed to 500ppm of peracetic acid.

Microscopic analysis shows minimal pathogen migration on wheat grain during tempering. The likelihood of contaminants being on the outer layer, or bran, is high, as it's the direct contact surface of wheat grains to environment. Due to differences in milling technology, research questions remain, for example, the percentage of microorganisms that could be removed when the bran is peeled away from the endosperm. Additionally, whether bacterial contaminants

can be spread from the bran to the endosperm during debranning as well as the rate at which this may occur.

Some of the above questions could potentially be answered by observing a fluorescently tagged pathogens under a microscope, a procedure we used in this study to evaluate if the distribution of pathogens present on the wheat kernels changed during the tempering process. When measuring the intensity of different areas of a kernel, the areas were defined manually; therefore, there existed potential for minor error in setting these areas. While changes in the distribution of pathogen cells on the surface of wheat kernels was not evident, we did identify some limitations of this technique. The wheat kernel is a relatively large and deep for confocal measurement. The two planes (crease side and non-crease side) that were measured with z-series in this study were representative, as the unobserved side has a similar texture to the "left", "right", and "back" sides. Other methods could be evaluated to examine the bran of the kernel in an efficient manner. One way could be the removal of the bran from the endosperm; in common milling processes, rolls are used to separate the endosperm from the bran with minimal damage to the bran layer. Imaging only the bran layer could greatly accelerate the sampling process, and looking at a relatively thin sample, using a higher objective to observe microorganisms would provide higher quality images. As discussed in recent publications, food samples usually need to be sectioned into thinner pieces in order to obtain high quality images (*18*, *30*).

The brush of the grain varies from long or short depending on the variety of wheat, and due to its natural shape and texture, the brush is more likely to catch undesirable particles (Posner and Hibbs, 2005). Under confocal microscopy observation, (*31*) found a higher concentration of fluorescence on brush hairs of wheat grains inoculated with GFP-labeled *Salmonella*. During milling, the miller aims to separate the bran from the endosperm. However,

due to the natural shape of the grains and the presence of the crease, the majority of commercially available flour contains bran. Therefore, some small particles of bran and brush hairs go downstream with the endosperm and could create food safety concerns if pathogens are attached. In our current study, even though we could see the brush hairs in the confocal images, the z-series distance was not calculated, brush hairs from both the crease, and the non-crease sides could be over or under sampled. The length and density of the brush hairs can vary, therefore making it difficult to quantify pathogens on the brush. Also, cells could stack up in the z-plane due to the uneven surface of wheat grain and lead to under-sampling when quantifying fluorescent intensity. Based on the observations above, a modified method would be useful to answer more questions regarding pathogen behavior on wheat kernels.

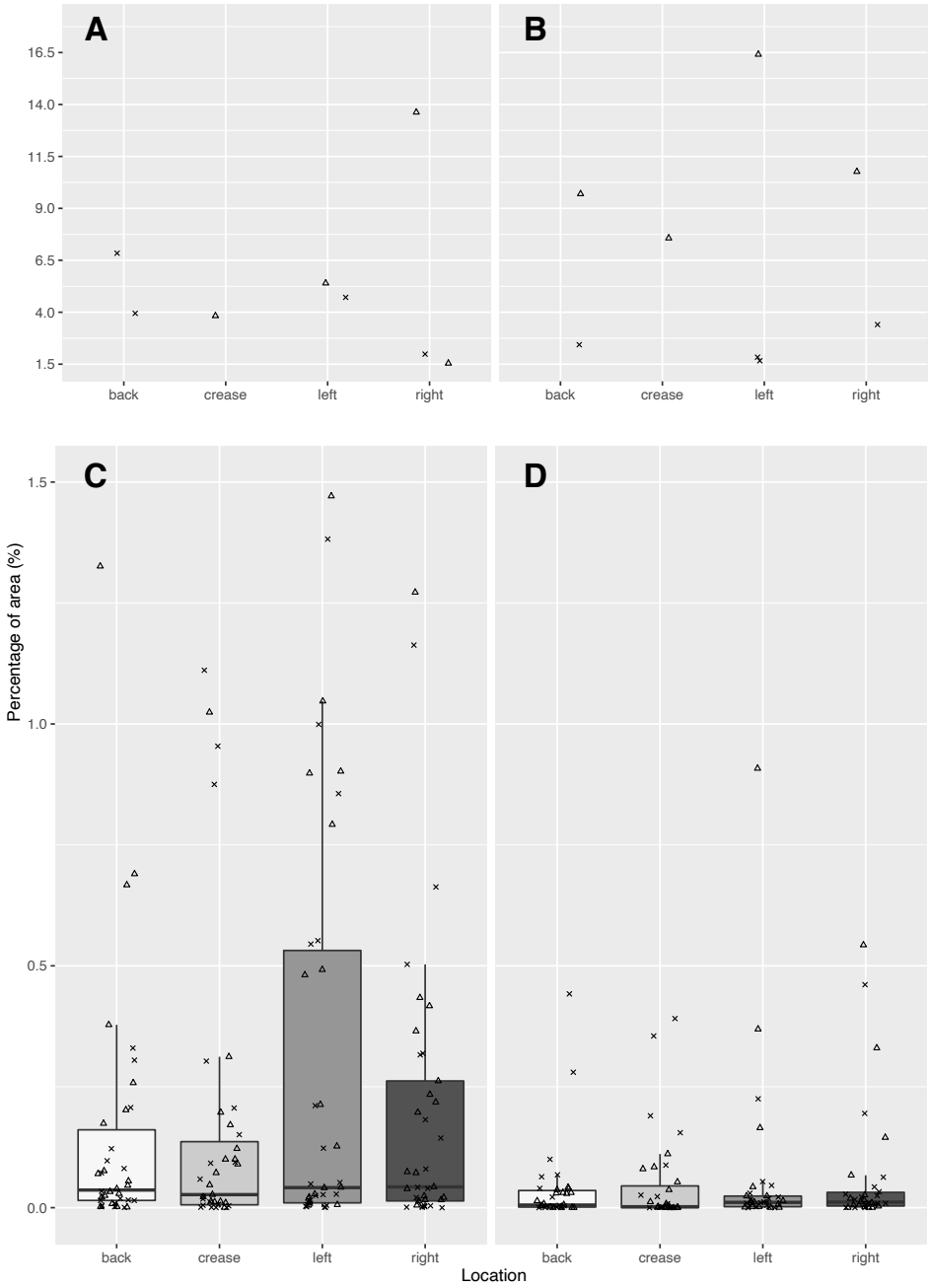

Figure 3-6 Percentage of area with intensity greater than threshold of wheat grains (A) inoculated with STEC O121:H19, 0-1.5%. (B) inoculated with Salmonella PT30, 0-1.5%. (C) inoculated with STEC O121:H19, 1.5-16.5%. (D) inoculated with Salmonella PT30, 1.5-16.5%. Each data point represents 1 technical replicate of percentage of intensity greater than threshold.

3.6 Conclusion

The process of tempering wheat grain prior to milling involves the addition of water. Here we show that while the water activity of wheat grain increases significantly ($p<0.05$) during tempering, it does not lead to significant changes ($p>0.05$) in the numbers of naturally occurring mesophiles and coliforms or inoculated *Salmonella* and STEC. Examination of STEC and *Salmonella* expressing mCherry inoculated onto the surface of wheat kernels showed no evidence of change in pathogen distribution on the wheat grain during tempering.

Acknowledgements

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4 IMPACTS OF CHLORINATED WATER ON PATHOGEN INACTIVATION DURING WHEAT TEMPERING AND RESULTING FLOUR QUALITY (*37*)

4.1 Abstract

Outbreaks of enteric pathogens linked to wheat flour have led the wheat milling industry to seek solutions addressing this food safety concern. Chlorinated water at 400 to 700 ppm has been used in the flour milling industry as a tempering aid to control growth of yeast and mold in tempering bins. However, the effectiveness of chlorinated water for inactivating enteric pathogens on wheat kernels was unknown. Five strains of Shiga toxin–producing Escherichia coli and two strains of Salmonella were inoculated onto hard red spring wheat at 7 log CFU/g and stored at room temperature for 1 month. Inoculated wheat was tempered with four concentrations (0, 400, 800, and 1,200 ppm) of chlorinated water (pH 6.5). The reduction due to chlorine was determined by calculating change in microbial loads at each chlorine level by using the response at 0 ppm as a reference. Uninoculated wheat tempered with chlorinated water was used to measure flour quality parameters. Changes in pathogen population over 18 h ranged from 2.35 to 0.30 log CFU/g with 800 ppm of chlorinated water and were not significantly different from changes at 400 and 1,200 ppm. Significant ($P < 0.05$) differences in the extent of reduction were observed among strains. However, the effect of chlorinated water at reducing native microbes on wheat kernels was minimal, with an average reduction of 0.39 log CFU/g for all concentrations. No significant ($P > 0.05$) changes occurred in flour quality and gluten functionality or during bread making for grains tempered at 400 and 800 ppm of chlorinated water. There were small but significant ($P < 0.05$) changes in flour protein content, final viscosity, and water absorption when tempered with 1,200 ppm of chlorinated water. The data showed that the level of chlorinated water currently used in industry for tempering could reduce

enteric pathogen numbers by 1.22 log CFU/g for Shiga toxin–producing Escherichia coli and 2.29 log CFU/g for Salmonella, with no significant effects on flour quality and gluten functionality.

4.2 Introduction

Low water activity (aw) restricts the growth of pathogens; thus, low aw foods were rarely considered a food safety concern until the international Salmonella outbreak associated with almonds in 2001 (*10*). In recent years, an increasing number of outbreaks associated with low aw foods have raised interest in methods to inactivate pathogens on these foods. Notably, wheat flour, one of the most consumed food ingredients, was associated with multiple outbreaks in the United States. In 2016, 63 people were infected with Shiga toxin–producing Escherichia coli (STEC) O121 and STEC O26 across 24 states (*8*). In 2019, an outbreak of STEC O26 was linked to wheat flour (*9*), and in 2021, an outbreak of STEC O121 was traced back to cake mix (*7*). The U.S. FDA has also issued several recalls (*65*, *66*) for potential contamination of *Salmonella* in wheat flour.

STEC can survive for extended periods in wheat flour (*21*, *22*). Before wheat grains are milled into flour, there are several points where contamination with enteric pathogens could occur. Preharvest contamination can come from seeds, soil, water, insects, as well as bird and rodent feces, and could be highly affected by environmental conditions, such as weather changes and other natural disasters; cross- contamination caused by equipment and improper handling are also the main contributors to microbial contamination of wheat grains (*42*). A survey of wheat grain collected from rail cars highlights the potential for contamination with STEC or *Salmonella* in the field or during harvest, where 5,176 wheat samples were tested for STEC and *Salmonella* spp.; 0.44% were positive for STEC, and 1.23% were positive for *Salmonella* (*45*). After harvest,

wheat is commonly transported to a storage facility and could be stored in wheat bins or grain elevators for months prior to arrival at the mill. The potential for long-term survival of STEC and *Salmonella* on wheat grain during storage was demonstrated in a recent study where STEC and *Salmonella* inoculated onto wheat grain was detected at > 2 log CFU/g for up to 44 weeks and 52 weeks, respectively (*36*). Upon arrival of wheat grain at a milling facility, grains go through a cleaning process that removes dirt and physical hazards. On average, 1-log CFU/g reduction of total aerobic microbes was observed during this process for durum wheat, with an initial aerobic count on grains at about 8.2 log CFU/g (*43*).

Tempering is the process that involves adding water to wheat grains to ease the bran removal process during milling. Chlorinated water (400 to 700 ppm) made from dissolving calcium or sodium hypochlorite in water has been used in the durum wheat milling industry as a tempering aid to reduce microbes, specifically mold, yeast, actinomycetes, and bacteria, such as Bacillus on grains (*35*). It was estimated that 85% of wheat mills in the U.S. use chlorine in various forms in their tempering water (*63*). Although use of chlorine as tempering aid is common in the milling industry, the efficacy of using chlorinated water as a tempering aid to reduce enteric pathogen load on wheat grains was not determined, possibly due to enteric pathogens being less of a flour safety concern in the past.

As tempering is a preexisting step in flour milling, potential control measures that can be incorporated into this step are desirable to avoid costs of an additional unit operation. Recent studies have focused on evaluating other tempering water additives that could reduce microbial load on grain. Proposed tempering aids include combinations of organic acids with salts, in which 5% lactic acid with NaCl (26.6%, w/v) was found to be most effective, with a 2.6-log CFU/g reduction of Salmonella and 2.4-log CFU/g reduction of both E. coli O157:H7 and non-

O157 STEC on hard wheat (*54*). led to a 2-log CFU/g reduction in E. coli O121 and O26 with 0.5% SBS, while increasing the SBS concentration to 1.5%, a greater than 4.0-log CFU/g reduction was observed. Even though the composition, dough, and bread-making properties of the resulting flour were comparable to the control, using 1.25 and 1.5% of SBS in tempering water resulted in acidified flours with pH 4.51 to 4.60 . A recent study (*48*) used 5 and 10% acidic water, both lactic acid and SBS, to temper wheat grains, which resulted in 2.0- and 2.6-log CFU/g reduction of STEC O121 and O26, respectively. Other tempering aids have been evaluated for the ability to reduce the overall microbial load on wheat, not specifically examining the inactivation of enteric foodborne pathogens. These methods include using slightly acidic electrolyzed water (*12*) as a tempering aid which led to a 0.65 log CFU/g reduction in total plate count. Tempering with 16 ppm ozonated water led to less than 0.5 log CFU/g reduction in aerobic plate counts (*15*).

As the use of chlorine as a tempering aid specifically to inactivate enteric pathogens has not been evaluated, the objectives of this study were (i) to determine the effectiveness of using different concentrations of chlorinat- ed water as a tempering aid to reduce pathogen loads on wheat grain inoculated with two strains of Salmonella and five strains of STEC and (ii) to evaluate the quality and gluten functionality of flour that had been milled from wheat kernels tempered at different concentrations of chlorinated water at 400, 800, and 1,200 ppm and control samples tempered with 0 ppm of chlorinated water.

4.3 Material and Methods

Wheat grain. An equal blend of hard red spring (HRS) wheat varieties, including ND Frohberg, MN Torgey, and Dapps harvested at Casselton, ND, in August 2020, sourced from the North Dakota State University Casselton Research Center, were used for this experiment.

Samples were placed in grain bins on the research farm at harvest, removed in late December, placed in 1- bushel (25.4-kg) bags, and stored at 58C until use. Grains were homogenized with a homogenizer (FPB-005, American Process Systems, Gurnee, IL) and cleaned on a dockage tester (Carter Day International, Minneapolis, MN) with a no. 8 riddle. Average moisture content of the blended wheat was 11.8% 6 0.3%, with an average aw of 0.49 6 0.01. The moisture content of the wheat was measured by a handheld moisture meter (MINIGAC1SG4, Dickey-John, Minneapolis, MN). The aw of wheat kernels was measured by using an AQUALAB 4TE aw meter (METER Group, Pullman, WA).

Bacterial strains and preparation. The selected seven pathogens (listed in Table 4) include two strains of Salmonella enterica isolated from low-moisture food outbreaks, Salmonella enterica Enteritidis PT30 sourced from the almond outbreak in 2001, and Salmonella enterica Agona isolated from cereal. Five serotypes of STEC, including one O157:H7 and four non-O157 STEC from the "big six" were selected, with STEC O121:H19 sourced from the multistate flour outbreak in 2016. All selected strains were assessed individually to determine efficacy of chlorinated water as a tempering aid to reduce the pathogen population.

The pathogens used in this experiment were first selected for resistance to rifampicin (Thermo Fisher Scientific, Waltham, MA) at 80 μg/mL. The selected strains were streaked to Luria-Bertani (LB) agar (Thermo Fisher Scientific) and grown for 18 h at 378C. A single colony was transferred to 50 mL of LB with 4 μg/mL rifampicin and incubated at 378C for 24 h with shaking. Next, 100 μL of overnight culture with 4 μg/mL rifampicin was transferred to 50 mL of LB with 40 μg/mL rifampicin and incubated at 378C for 24 h with shaking, and 100 μL of the overnight culture was then transferred to 50 mL of LB with 80 μg/mL rifampicin and incubated at 378C for 24 h with shaking. After confirming growth by streaking the overnight culture onto

LB agar with 80 µg/mL rifampicin, freezer stocks of the rifampicin-resistant strains were prepared by mixing 800 µL of the LB broth culture with 80 µg/mL rifampicin mentioned previously and 200 µL of 70% glycerol (Thermo Fisher Scientific) and stored at 808C. By using rifampicin-resistant strains, the chance of picking up native microbes from wheat kernels was minimized.

Table 4-1 Strains used to determine efficacy of chlorinated water as a tempering aid.

Species and serotype	Strain	Isolation source	Outbreak
Salmonella enterica Enteritidis	ATCC BAA-1045	Almonds	2001, Canada
Salmonella enterica Agona	FSL S9-0322	Dry cereal	
Escherichia coli O157:H7	TW14359		Spinach, 2006, United States
Escherichia coli O26:H11	TW16501	Human clinical	Sprouts, 2012 United States
Escherichia coli O121:H19	PNUSAE002568	Human clinical	Wheat flour, 2016, United States
Escherichia coli O103	TW08101	Human clinical	
Escherichia coli O111	TW07926	Human clinical	

Inoculation of wheat grain. The bacterial freezer stocks were streaked to LB agar with rifampicin (80 µg/mL) for isolation and incubated at 37°C for 20 hours. Then, a single colony was selected and grown in LB broth with rifampicin (80 µg/mL) at 37°C for 16 hours, and 250 µL of the broth culture was spread on LB agar with rifampicin (80 µg/mL) and incubated for 24 h to obtain a lawn growth over the 100-mm plate. To achieve an initial target inoculum level of ~6 log CFU/g, one plate of lawn culture was collected with a sterile spreader and mixed in 2.5 mL of sterile water. The mixture was then transferred to a Whirl-Pak bag (Nasco, Madison, WI) containing 1 kg of hard red wheat grain that was removed from 58C cold storage and left at

ambient temperature (~228C) overnight and massaged by hand for 3 min. After mixing, the kernels were transferred to a sterile stainless steel tray and mixed again with sterile scoop for 3 min. The homogeneity of inoculation was tested by randomly selecting three 11-g samples from the tray and plating on differential media with rifampicin (80 μg/mL) after serial dilution. If the standard deviation was less than 0.5 log CFU/g, the wheat was assumed to be inoculated homogenously as described in (*60*).

After initial experiments, we noted that the pathogen loads on wheat inoculated with STEC strains dropped significantly after 1 month of storage. STEC O157:H7, O103, and O111 were inoculated onto wheat grain at a higher level, and 1.5 plates of lawn cultures were collected with a sterile spreader in 2.5 mL of sterile deionized (DI) water to inoculate 1 kg of HRS wheat samples with same protocol described previously to achieve 7 log CFU/g.

Water activity equilibration. The average aw of wheat increased from 0.49 6 0.01 to 0.73 6 0.03 after inoculation. To lower the aw to the same level as before inoculation, the inoculated wheat grains were placed in an aw equilibration chamber set to target relative humidity of 55% for 48 h at ambient (~228C) temperature. The equilibration chamber included a central control system that received signals from relative humidity sensors inside the chamber. The system controlled the desiccation column, hydration column, solenoid valves, and air pumps that moved humidity in or out of the chamber. The average a_w of inoculated wheat after equilibration was 0.50 ± 0.01.

Wheat storage. After aw equilibration, the inoculated wheat kernels were transferred to a resealable Mylar bag (8 by 10 in. [203 by 254 mm]; Uline, Pleasant Prairie, WI), and the extra air in the headspace was pushed out manually and stored at ambient temperature (~228C) for 1 month before tempering. Wheat used to test the native microbes did not go through the

inoculation process but was removed from 58C cold storage and stored in Mylar bags at ambient temperature 1 month before the experiment.

Preparation of chlorinated water. Chlorinated water was prepared by dissolving calcium hypochlorite (Sigma-Aldrich Corp., Milwaukee, WI) in sterilized DI water. The pH was adjusted to pH 6.5 with 25% citric acid(w/v) (Sigma-Aldrich Corp.) using a Fisherbrand Accumet AB250 Benchtop pH/ISE meter (Thermo Fisher Scientific) at room temperature to obtain a 1200ppm chlorine solution. The 1200ppm chlorinated water was further diluted to 400ppm and 800ppm with sterilized DI water. The unchlorinated water (0ppm chlorine) used in this study was sterilized DI water. The total active chlorine of the chlorinated water was verified after dilution using a chlorine test kit (HACH, CN-21P; Loveland, CO).

Tempering. For every biological replicate of each strain studied, 50 g of inoculated wheat were placed into four sterile 250- mL centrifuge bottles (Nalgene, Rochester, NY), one for each time point examined. Another 500 g of uninoculated wheat grains was placed into a 1,000-mL centrifuge bottle (Nalgene) as a reference to measure changes in moisture content during tempering. The moisture content of the wheat was measured prior to tempering. To achieve a moisture content of 16%, water was added following the process described in the American Association of Cereal Chemists (AACC) Approved Methods of Analysis (1; see reference 1 for all AACC methods). Per AACC Method 26-95, chlorinated water was added at different concentrations (400, 800, and 1,200 ppm) or DI water for the 0-ppm treatment. The bottles containing wheat kernels were then shaken vigorously for 90 s at t 1⁄4 0 h to distribute water evenly on kernels. Bottles with kernels were held at room temperature for 17 h. At t 1⁄4 17.5 h, the moisture content of the wheat kernels was measured to calculate the volume of water needed to achieve a final moisture content of 16.5%. After the freshly prepared chlorinated water or DI

water was added to the sample, the bottles were shaken for 90 s and held at room temperature for 0.5 h. The bacterial populations were measured at t ¼ 0, 0.5, 17.5, and 18 h after the initial addition of the tempering solution.

Bacterial enumeration. D/E neutralizing broth (Neogen, Lansing, MI) was added to 25 g of sample that was randomly collected from 50 g of tempered wheat at each time point and homogenized with a Stomacher 400 (Seward, Worthing, West Sussex, UK) for 120 s. After serial dilution with Butterfield dilution buffer, strains were plated in duplicate onto differential media. For Salmonella strains, Trypticase soy agar (TSA; Neogen) with 80 μg/mL rifampicin supplemented with ferric ammonium citrate and sodium thiosulfate was used. Non-O157 STEC was plated onto MacConkey agar (Neogen) with rifampicin (80 μg/ mL) and E. coli O157:H7 onto sorbitol MacConkey agar (Neogen) with rifampicin (80 μg/mL). The plates were incubated at 378C for 24 h. Black colonies from modified TSA plates indicative of Salmonella, dark pink colonies on MacConkey indicative of E. coli, or translucent colonies on sorbitol MacConkey indicative of STEC O157:H7 were enumerated. To determine the native microbial population, plate count agar (Neogen) was used to quantify the total aerobes.

Wheat and flour quality. For flour quality testing, uninoculated wheat samples were tempered with the different levels of chlorinated water as described in the previous sections. Wheat samples were tempered to 16.5% moisture content 18 to 24 h before milling on a Bühler MLU-202 Mill (Bühler; Uzwil, Switzerland) following AACC 26-21.02. After milling, extraction yield was first calculated by dividing the weight of flour produced by the weight of initial grain sample, and various tests were performed on flour to determine the quality parameters. The moisture, ash, and protein content were determined by AACC 44- 12.02, AACC 8-01.01, and AACC 39-10.01, respectively. Flour color was analyzed with a CIE 1976 L*a*b*

lab scale colorimeter (CR-410 Chroma Meter, Konica Minolta, Tokyo, Japan) according to AACC 14-50.01. The pasting properties of the flour was determined by AACC 76-21.01 by using the Rapid Visco Analyzer (RVA; RVA 4500, Thermocline software version 3.16.347, Perkin Elmer, Waltham, MA). The rheological behavior of flour was determined by Farinograph-E (Brabender, Duisburg, Germany) with software version 4.0.4, following AACC 54.21.02. The falling number and wet gluten were determined by using AACC 56-81.03 and AACC 38-12.02.

The baking tests were performed for pan bread by using AACC 10-09.01 and 10-12.01. Residual chloride was measured by using a Fisherbrand Accumet AB250 Benchtop pH/ISE meter (Thermo Fisher Scientific) equipped with a Fisherbrand Accumet chloride combination electrode (Thermo Fisher Scientific).

Data analysis. For the microbial analysis, two factors were designated: the time of sampling (t = 0, 0.5, 17.5, and 18 h) and chlorinated water concentration (0, 400, 800, and 1,200 ppm). The initial count of inoculated wheat grains after 1 month of storage before tempering was also analyzed and recorded. Duplicate plate counts for each biological replication were conducted; three biological replications for each strain were performed on different dates. The microbial results were first checked for normality, and the changes in microbial load due to 400, 800, and 1,200 ppm of chlorinated water treatments were calculated by using the response at 0 ppm as a reference. One-sample t test was conducted for each strain to determine whether the changes in microbial population due to different chlorinated treatments were significantly different from the microbial population changes due to unchlorinated water (no difference from tempering with unchlorinated water). Analysis of variance (ANOVA) was conducted to determine if there was a significant difference in pathogen loads due to chlorine concentrations,

strains, or the interaction effect of both variables. The Tukey test was then used to determine if there was a significant difference among strains at the same chlorine concentration or among different chlorinated water levels for the same strains. The flour quality data was analyzed by using ANOVA and the Tukey test to determine if quality parameters were significantly different for wheat tempered with different levels of chlorinated water. All statistical analyses were done with R (version 4.0.3, www.r-project.org), and plots were created with ggplot2 version 3.3.3(*26*).

4.4 Results

Pathogen loads on wheat grain after 1-month storage. Immediately after inoculation, the levels of Salmonella and STEC on wheat grains were not significantly different ($P<0.05$), with an average of 6.76 ± 0.28 log CFU/g. The pathogen loads on inoculated wheat grains after 1 month of storage were significantly different ($P< 0.05$) between Salmonella and STEC. On average, the pathogen levels after 1 month of storage were 5.11 ± 0.37 log CFU/g for STEC and 6.45 ± 0.16 log CFU/g for *Salmonella*. For uninoculated wheat grain, the aerobic plate count of native microbes after 1 month of storage was 5.29 6 0.23 log CFU/g.

Changes in microbial loads on wheat tempered with unchlorinated water. Over 18 h of tempering with unchlorinated water, we observed an average increase of 0.45 ± 0.38 log CFU/g of native microbes (Fig. 4-1). For enteric pathogens, the average change in microbial load was not significantly different ($P <0.05$). The average log reduction was 0.32 6 0.42 CFU/g for the five strains of STEC and 0.30 6 0.38 CFU/g for the two strains of Salmonella. The results show that without active chlorine, water added to the grains followed by mixing can lead to changes in microbial load on kernels during tempering, and the change varied by strain, which ranged from 0.66 ± 0.28 to 0.20 ± 0.02 log CFU/g for STEC O26:H11 and STEC O103, respectively. Therefore, to focus only on the effect of chlorine on inactivating native microbes

and enteric pathogens, results for 400, 800, and 1,200 ppm were calculated in reference to changes seen with unchlorinated treatment for further analysis.

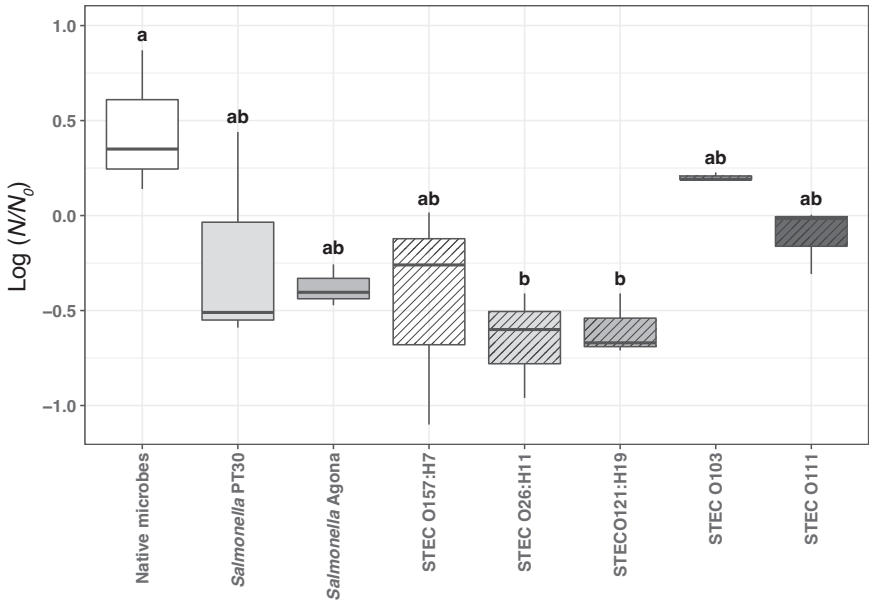

Figure 4-1 Change in overall population of microbes on wheat grain tempered with unchlorinated water for 18h. Different letters indicate mean values that are significantly different ($p<0.05$) and were the result of 3 replicates ($n=3$).

Changes in microbial loads on wheat tempered with different chlorine levels. The one-sample t test indicated that the reduction of pathogens was significant ($P< 0.05$) when tempered with 400 ppm of chlorinated water except for STEC O157:H7 and STEC O121:H19 ($P >0.05$). For these two strains, the reduction due to 400 ppm of chlorinated water was not significantly greater than the reduction with unchlorinated water. Using chlorinated water at 800 and 1,200 ppm during tempering, the overall reduction observed for all tested pathogens on grains was significant ($P< 0.05$). The reduction of native microbes was not significant for any of the three chlorine concentrations used.

The change in pathogen population on wheat when tempered with different chlorine levels of 400, 800, and 1,200 ppm was not significantly different ($P<0.05$) among the strains

(Table A1). Increasing the chlorine level did not result in a significantly greater log reduction of pathogens or native aerobes. The response to chlorinated water was significantly different among strains (P <0.05), and the log reductions observed varied by strain (Fig 4-2).

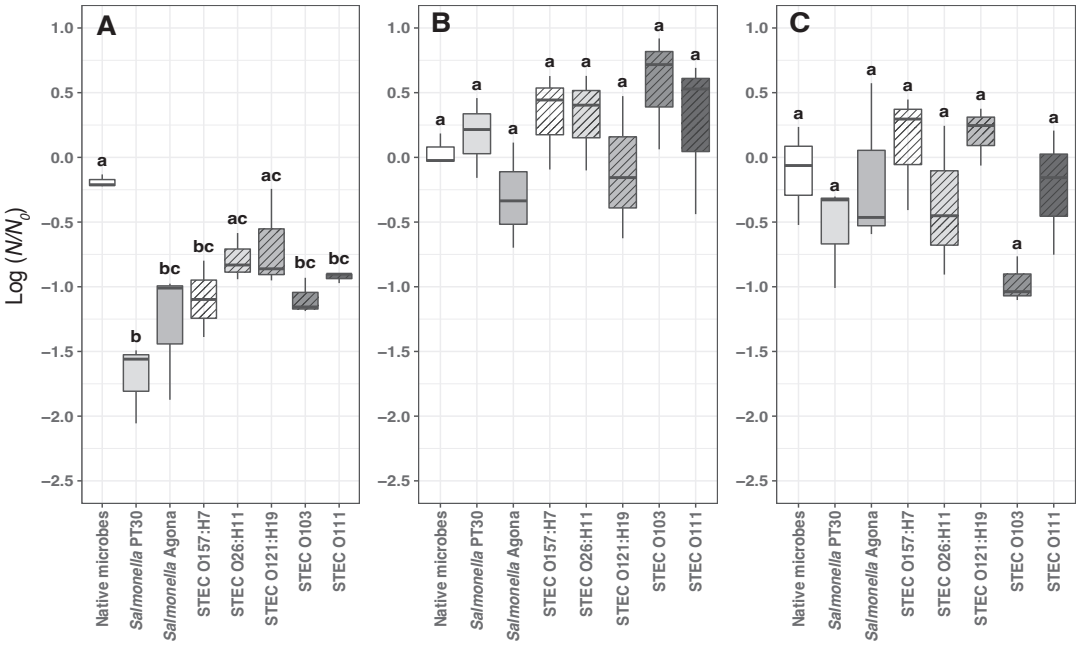

Figure 4-2 Change in microbial load of each strain at different chlorine level, (A) change in microbial load on wheat tempered with 400ppm chlorinated water for 18h, (B) change in microbial load on wheat tempered with 800ppm chlorinated water for 18h (C) change in microbial load on wheat tempered with 1200ppm chlorinated water for 18h. Different letters within each panel indicate mean values that are significantly different (p<0.05) and were the result of 3 replicates (n=3).

The reduction observed for the two strains of Salmonella was similar, with average reduction for all chlorine concentrations of 2.09 ± 0.39 log CFU/g and was significantly different (P <0.05) from STEC and native microbes. For the five strains of STEC, changes in population of four strains of non-O157 STEC were similar to each other (P> 0.05). The average change in non-O157 STEC on wheat grains tempered by different levels of chlorinated water was 1.23 ± 0.38 log CFU/g. However, the observed change in the STEC O157:H7 population due to

chlorine was 0.38 ± 0.24 log CFU/g, which was minimal and not significantly (P> 0.05) different from the change in native microbe population, which was 0.44 ± 0.35 log CFU/g.

Changes in microbial loads on wheat at each time interval during tempering with 800ppm chlorinated water. The process of tempering involves the addition of water at two different time points to increase the moisture content of the wheat grain. The most significant change in moisture content happened during the first half hour of tempering, when a larger amount of water is added to wheat grain. Here, we aimed to increase the moisture content of wheat grains by approximately 4% from 11.8% ± 0.3% to 15.8% ± 0.2%. The most significant change in pathogen load also occurred in the first half hour after the addition of chlorinated water. For wheat grain tempered with 800 ppm of chlorinated water (Fig 4-3), changes in microbial loads during the first half hour were 0.31 ± 0.01 log CFU/g for native microbes, 1.22 ± 0.39 CFU/g for STEC, and 1.54 ± 0.47 log CFU/g for Salmonella, and there was a significant difference ($P < 0.05$) among strains. In contrast, there was no significant ($P>0.05$) change in microbial loads among different strains for the next 17 h from t = 0.5 to t =17.5 h, with minimal changes in population for native microbes, Salmonella, and STEC at 0.15 ± 0.22, 0.09 ± 0.61, and 0.23 ± 0.61 log CFU/g, respectively. Chlorinated water was added in the final half hour of tempering, and the moisture content of wheat increased by about 0.6 to 16.3% ± 0.2%. From t = 17.5 to 18 h, changes in microbial population were similar ($P>0.05$) for all microorganisms tested; the change in population was 0.32 ± 0.23 log CFU/g for native microbes, 0.01 ± 0.45 log CFU/g for STEC, and -0.26 ± 0.58 log CFU/g for *Salmonella.* Similar trends for wheat tempered with 400ppm (Figure A-1) and 1200ppm (Figure A-2) chlorinated water were observed during each time interval.

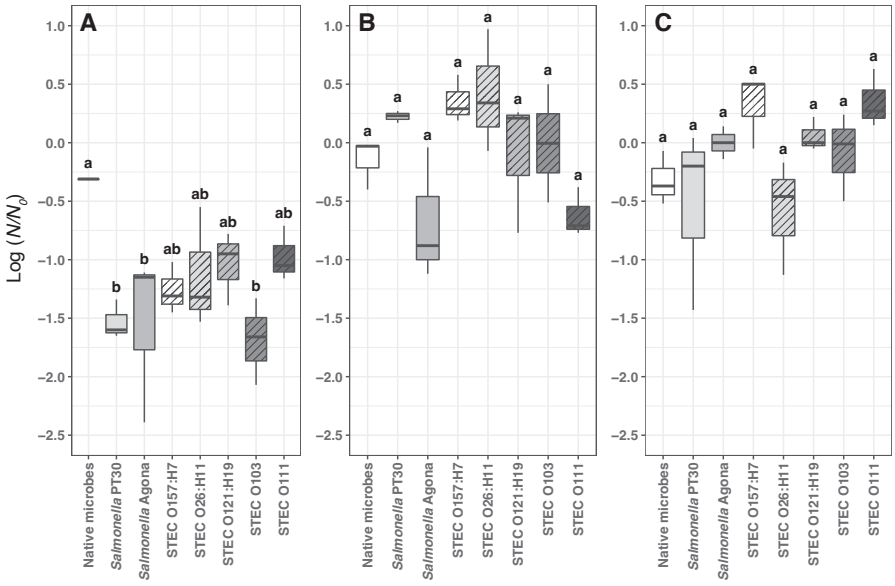

Figure 4-3 Change in microbial load of selected strains during different time intervals tempered by 800ppm chlorinated water, (A) changes from t=0 to t=0.5 hours, (B) changes from t=0.5 to t=17.5 hours, (C) changes from t=17.5 to t=18 hours. Different letters within each panel indicate mean values that are significantly different ($p<0.05$) and were the result of 3 replicates (n=3).

Quality parameters of flour milled from wheat tempered with chlorinated water.

The percentage of flour extracted from wheat tempered with different levels of chlorinated water was not significantly different ($p>0.05$). Flour composition and quality parameters (Table 5) of resulting flour tempered with different concentrations of chlorinated water were evaluated. Increasing the concentration of chlorine did not significantly change the moisture or ash content. The protein content was significantly($p<0.05$) different from the control when 1200ppm chlorine was used for tempering but using 400ppm and 800ppm chlorinated water did not significantly alter the protein content after tempering. Flour color was measured using CIE *L*a*b** scale, and no significant differences ($p>0.05$) were observed for *L*, a*,* or *b** values for different concentrations of chlorine, therefore there were no significant changes in flour brightness, red/green scale, or blue/yellow scale.

Falling number (Table 5) was measured for each treatment to determine the alpha-amylase activity, and no significant ($p>0.05$) differences from the control were observed for wheat tempered with chlorinated water. Wet gluten was measured to determine the amount of gluten-forming protein in flour, which directly impacts the dough mixing and baking properties of flour, and there were no significant ($p>0.05$) differences observed between treatments and control sample.

Rapid Visco Analyzer (RVA) measures the viscosity of flour and water mixture over a given period to determine the pasting ability, and the alpha-amylase activity. There was a significant ($p<0.05$) difference observed in final viscosity for wheat that had been tempered with 1200ppm chlorine compared to the unchlorinated control (Table A2).

Farinograph (Table 6) measures the torque produced during dough mixing, which estimates the water absorption of flour, and the stability of dough during mixing indicated by mixing tolerance index (MTI). The dough forming ability is a main quality indicator especially for hard wheat, and there was a significant ($p<0.05$) difference in absorption at 14% with flour tempered with 1200ppm chlorinated water when compared to control. There were no significant ($p>0.05$) differences observed for the other parameters measured by the Farinograph.

Residual chloride in wheat and milled products were measured (Table 7.) There was no significant change ($p>0.05$) in chloride level in flour tempered with different concentrations of chlorine.

Baking evaluation. HRS wheat is mainly milled to produce bread flour, therefore a bread baking test (Table A3) was conducted to determine if tempering with chlorinated water would have negatively impacted the gluten functionality for baking products. Bread loaves were evaluated by water absorption, oven rise, loaf volume, and specific volume. Control loaves made

from flour milled from wheat tempered with DI water had an average water absorption of 67.57%, oven rise of 5.56 cm, loaf volume of 957.5 cm^3 and specific volume of 6.91 cm^3 /g (Table A3). There were no significant ($p>0.05$) differences observed for the loaves made from flour milled from wheat tempered with chlorine, therefore increasing the chlorine level of tempering water did not alter gluten protein functionality of HRS wheat.

Table 4-2 Milling extraction and flour quality of wheat tempered with chlorinated water.

Chlorine Treatment	Flour Extraction %	Moist %	Protein % (14% MB[1])	Ash % (14% MB)	Falling Number sec.	Wet Gluten %, 14%MB	Gluten Index	Color L*	Color a*	Color b*
0 ppm	71.04a[2]	13.25a	14.46a	0.54a	377.33a	35.95a	96.58a	90.42a	-0.54a	8.24a
400 ppm	69.92a	13.26a	14.23a	0.53a	378.67a	35.55a	95.68a	89.82a	-0.51a	8.16a
800 ppm	69.91a	13.20a	14.02ab	0.53a	394.00a	34.93a	95.25a	90.03a	-0.59a	8.34a
1200 ppm	69.23a	13.18a	13.72b	0.52a	397.67a	34.78a	94.44a	90.05a	-0.65a	8.47a

[1.] MB = moisture basis
[2.] Different letters within each column indicate values that are significantly different ($p<0.05$) and were the result of 3 replicates ($n=3$)

Table 4-3 Dough quality of flour from wheat tempered with chlorinated water.

Chlorine Treatment	Absorption % (14% MB[1])	Peak Time min.	Stability min.	MTI FU[2]	FQN[3] mm
0 ppm	64.1a[4]	6.9a	9.5a	31.8a	130.5a
400 ppm	64.0a	7.1a	10.0a	29.3a	133.3a
800 ppm	63.3a	6.9a	9.4a	32.3a	129.3a
1200 ppm	61.9a	6.4a	8.8a	34.0a	118.5a

[1.] MB = Moisture basis
[2.] FU = Farinograph units
[3.] FQN = Farinograph quality number
[4.] Different letters within each column indicate values that are significantly different ($p<0.05$) and were the result of 3 replicates (n=3)

Table 4-4 Chloride levels in wheat and mill products of wheat tempered with chlorinated water.

Chlorine Treatment	Wheat ppm	Bran ppm	Shorts ppm	Flour ppm
0 ppm	0.18a[1]	152.33a	163.33a	103.33a
400 ppm	0.31b	154.00a	172.67a	109.67a
800 ppm	0.49c	155.33a	181.00a	108.00a
1200 ppm	0.65bc	153.67a	169.33a	111.00a

[1.] Different letters within each column indicate values that are significantly different ($p<0.05$) and were the result of 3 replicates (n=3)

4.5 Discussion

Differences in survival between *Salmonella* and STEC on wheat grain during storage. The significant difference in survival of *Salmonella* and STEC on wheat grain during the one-month storage period prior to tempering has been observed on wheat grain, wheat flour,

and other low moisture foods. Lauer et al. (*36*) reported significantly higher *D*-values for *Salmonella* compared to STEC on HRS wheat grain, with *D*-values of 24.1±1.6 weeks, and 12.4±1.0 weeks, respectively. Forghani et al. reported that *Salmonella* in wheat flour stored at room temperature had significantly higher first decimal reduction times compared to that of STEC O121 (*22*). Studies on other low moisture foods also found a greater reduction of STEC than *Salmonella* during storage at ambient temperature. For raw peanuts and pecan kernels stored at 22°C for 28 days, the rate of decline was 0.22 log CFU/g/30days for *Salmonella* and 0.37 log CFU/g/30days for STEC O157:H7 on peanuts, and 0.15 log CFU/g/30days for *Salmonella* and 0.34 log CFU/g/30 days for STEC O157:H7 on pecans (*4*). Kimber et al. (*33*) reported a 0.20 log CFU/g/month and 0.60 log CFU/g/month reduction on almonds, and 0.15 log CFU/g/month and 0.35 log CFU/g/month reduction on pistachios for *Salmonella* and STEC O157:H7, respectively, in a year-long assessment of survival at 24°C. While pecans, peanuts, and pistachios have different macronutrient profiles, they are all low water activity foods that require surviving pathogens to adapt to desiccation stress.

In contrast to the findings above, Sabillón et al. (*54*) reported greater reduction of *Salmonella* compared to STEC O157 on hard wheat stored at 24.2°C for 7 days. They observed 0.79, 0.38, and 0.51 log CFU/g reduction of *Salmonella*, STEC O157, and STEC non-O157, respectively, on hard wheat and 0.92, 0.83, 0.74 log CFU/g reduction of *Salmonella*, STEC O157 and non-O157 STEC on soft wheat after 7 days. One of the main differences was the inoculation and equilibration process, where the grain was inoculated with liquid grown cultures, whereas studies done on other low moisture foods described above, as well as the study presented here, employed a dry inoculum preparation method.

Allowing the inoculated pathogens to adapt to the low water activity environment of the grain may better mimic the adaptations that occur when contaminated grain is stored prior to milling. Methods used for inoculation and equilibration of water activity could impact pathogen survival during storage, and later performance during tempering. The variability and repeatability of *Salmonella* inoculation methods have been studied on wheat flour, showing that inoculums prepared from cells grown on an agar surface resulted in a more stable, higher population after water activity equilibration compared to those prepared from cells grown in liquid medium (*28*). In an evaluation of *Salmonella* survival on almonds performed by Uesugi et al., they found the inoculum preparation method impacted the initial sensitivity to desiccation, where cells grown in liquid medium exhibited a 3.7 log CFU/g reduction while cells grown on an agar medium had a 1.7 log CFU/g reduction on almonds after 24 hours of drying (*64*).The two studies above both inoculated *Salmonella* PT30 on low moisture food with different inoculum growth conditions, and significant differences in survivability were observed. Similar studies to evaluate if STEC would exhibit the same survival differences on low moisture food dependent on inoculum growth conditions have not been conducted.

A standard method for inoculation and water activity equilibration of wheat grain has not been proposed, and a range of methods have been used in recent studies (*47*, *48*, *54*, *55*). Variation in methodology can impact the ability to compare effectiveness across studies, and a recent study modeled factors that influence pathogen survival on low water activity foods based on published literature shows that survival of both *Salmonella* and *E. coli* were significantly influenced by temperature, water activity, and inoculum preparation (*19*). Many studies on wheat grains placed more emphasis on moisture content of wheat grains throughout the process, as it is a crucial parameter in the wheat milling industry. While a previous study showed that both water

activity and moisture content could serve as predictors of *Salmonella* heat resistance on low moisture food, similar information on the behavior of STEC strains, as well as the response to other inactivation kinetics remain unknown (*23*). The data we report here is the first evaluation of tempering aid efficacy for pathogens on wheat after 1 month storage following inoculation. This should be considered for evaluation of tempering aids, as desiccation stress on enteric pathogens posed by a low water activity environment has the potential to induce tolerance to other stressors such as acid or chlorine stress (*25*).

Impact of different tempering treatments on native microbe species. While the efficacy of different tempering treatments has been reported for wheat grains, many report effects on total microbes, yeasts, or molds, while few evaluated inactivation of enteric pathogens. In our experiments, the minimal reduction of native microbes suggests the native microbes present on wheat grains are relatively more resistant to chlorine treatment than enteric pathogens. The reduction seen in aerobic plate count of native microbes agrees with a previous study by Dhillon et al. (*14*) who observed a 0.4 log CFU/g reduction in aerobic plate counts on durum wheat grain after washing with 700ppm chlorinated water. Andrews et al. (*2*) observed a 20% inactivation of fungal spores on cereal grains when 4000ppm chlorinated wash water was directly applied to barley grain for 2 mins. *Bacillus* species were able to survive exposure to chlorine concentrations over 100ppm (*35*), and *Bacillus* is the most common bacteria present according to microbiota survey of stored wheat grains (*61*). The reduction of native aerobes by chlorinated water was similar to that observed from ozonated water treatment and slightly acidic electrolyzed water (*12*, *14*), with 0.65 log CFU/g reduction when wheat was tempered with 70mg/L of slightly acidic electrolyzed water, and 0.2 log CFU/g reduction when tempered with 16.5 mg/L ozonated water. Sabillón et al. found that using acid and saline solutions for

tempering resulted in about 1 to 2 log CFU/g reduction in aerobic microbes, and solutions with higher concentrations of organic acid resulted in a greater reduction (*55*). The combination of 5% lactic acid and 52% NaCl had the greatest reduction in aerobic microbes, with a reduction of 4.3 log CFU/g.

Impact of different tempering aid on enteric pathogens. Reduction of STEC and *Salmonella* on wheat grain with tempering aids has only been studied in the past few years. The most effective organic acid and saline combination against enteric pathogens was determined to be 5% lactic acid with 26.6%NaCl, which reduced *Salmonella enterica*, STEC O157:H7, non-O157 STEC on hard wheat by 2.6, 2.4, and 2.4 log CFU/g on hard wheat, and by 1.8, 1.8, 1.6 log CFU/g on soft wheat (*54*), respectively. The reduction is about half a log higher for *Salmonella* and roughly a log higher for non-O157 STEC compared to reduction due to 800ppm chlorinated water. Noticeably, the observed reduction in STEC O157:H7 was about 2 log CFU/g lower in this current study. This could be due to the differences in inoculation and moisture equilibration methods, as well as strain differences.

Two studies using SBS as a tempering aid to inactivate STEC O26 and O121 have been reported, with differing results. Tempering hard wheat with 0.5% SBS led to a 2 log CFU/g reduction of STEC O121 and O26 and tempering with 1.5% SBS led to 4 log CFU/g reduction, though the 1.5% SBS solution resulted in acidic flour with pH 4.51 to 4.60 (*47*). The wheat was autoclaved before inoculation, and only 30-minute equilibration time was used before tempering, in which the physiochemical properties of wheat grain were likely to be altered and influenced how the inoculum behaved on wheat grain. A second study compared 5 and 10% SBS as well as lactic acid as a tempering aid on non-sterilized wheat grain (*48*). The log reduction of STEC O121 and O26 on hard wheat following tempering with 5% SBS or lactic acid was determined to

be 2 log CFU/g on average, and 2.6 log CFU/g for 10% SBS or lactic acid. When accounting for the 1.1 log CFU/g reduction in pathogen load when tempered with water alone, tempering with 5% SBS achieved comparable results to the reduction we observed for STEC O121 and O26 when tempered with 800ppm chlorinated water, which was 1.5 log CFU/g and 1.3 log CFU/g, respectively.

Impact of different tempering methods on flour quality. For all the parameters measured in this study on flour quality and gluten functionality, no significant differences ($p>0.05$) were observed when comparing the unchlorinated water control to tempering with 400ppm or 800ppm chlorine, indicating the current use of 400-700ppm chlorinated water in the milling industry does not have a negative impact on flour quality or gluten functionality. When increasing the chlorine concentration to 1200ppm, protein content, final viscosity, and absorption of produced flour were significantly different from the control. Generally, the protein content of flour has a positive relationship with absorption, which determines the amount of water needed to develop a consistent dough. Higher chlorine concentration of 1200ppm also led to a significant ($p<0.05$) difference in final viscosity compared to the control, final viscosity is used to measure ability of starch in flour to form viscous paste after cooking and cooling. Finnie et al. (*20*) compared soft wheat pancake flour with and without commercial chlorination and found viscosity significantly different between the two. Viscosity is a main parameter to examine for soft wheat flour, which has a higher starch content and is used for products where starch quality is of high importance. Studies on impact of chlorination were mainly done on flour using chlorine gas. The flour is treated until a pH of 4.5-5.2 is reached depending on the degree of chemical modification desired for the requirements of specific end-product (*20*). There is limited research on how use of

chlorinated water as a tempering aid could impact the grain quality or the quality of resulting flour.

Sabillon et al. (*52*) studied the impact of using acid and saline in combination on flour quality, with the most effective combination against pathogens (*54*) of 5% lactic acid with 26.6% NaCl, resulting in flour pH significantly lower than the control. The RVA test showed no significant impact of viscosity, and no significant impact was observed for cookie quality test. Assessment of flour quality when using organic acid and saline water in combination as a tempering aid was performed only on soft wheat flour, its impact on hard wheat flour requires further study.

Rivera et al. (*47*) found that hard wheat tempered with SBS also led to a significantly more acidic flour compared to the control, and it had negative impact on flour composition on moisture, fat, starch content, and color appearance. The falling number and RVA also indicated the reduction in pasting strength of flour, and lowered retrogradation property. The breadmaking test produced a comparable bread volume, but texture properties were also negatively impacted.

Using acid with saline as tempering water and using SBS as tempering water face the same hurdle of resulting flour to be significantly more acidic than the untreated flour, which may lead to compromising flour quality in the end, especially for bread-making flour. The pre-existing method in mills using chlorinated water as a tempering aid, has proven to have antimicrobial activity against enteric pathogens on wheat in this study, and resulted in similar levels of reduction compared to other tempering aids. This study provides further proof that current chlorine levels used as tempering aid have minimal impact on flour quality and baking, which had already been recognized for decades in practice. Differences in survival among *Salmonella* and STEC were observed during 1 month of storage. Potential changes in desiccation stress

tolerance over storage time could alter how enteric pathogens respond to subsequent hurdles, impacting the ability to compare data from the current study with other studies.

While *Salmonella* had greater survival during the 1 month storage, we observed a significantly greater reduction of *Salmonella* when tempered with chlorinated water relative to STEC. A standardized inoculation procedure will be important in determining the most effective tempering aid to inactivate pathogens on wheat grain. Using chlorinated water at the current use level (400-700ppm) as a tempering aid could reduce enteric pathogens on wheat without compromising the flour quality or gluten functionality of the final product. However, the efficacy of chlorinated water on inactivating native microbes pre-existing on wheat grains was minimal.

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5 INACTIVATION OF *SALMONELLA* AND SHIGA-TOXIN PRODUCING *ESCHERICHIA COLI* ON SOFT WHEAT KERNELS USING VACUUM STEAM PASTEURIZATION

5.1 Abstract

Wheat, as the raw material for flour milling, can be contaminated with enteric pathogens, and therefore lead to outbreaks linked to flour. In previous lab-scale studies, vacuum steam treatment was able to reduce *Salmonella* and STEC loads on soft wheat kernels while maintaining flour quality and gluten functionality. This study used a newly designed lab-scale vacuum steam pasteurizer to determine the efficacy of using VSP to inactivate multiple strains *Salmonella* and STEC on soft wheat, modeling the non-isothermal time-temperature history during treatment and the reduction of microbial population. The result demonstrated vacuum steam treatment could effectively disinfect wheat grains inoculated with enteric pathogens. In this study, *Salmonella* strains were less thermally resistant than STEC serovars, the $D_{75°C}$ of *Salmonella* strains were 2.8 and 3.2 min, the $D_{75°C}$ of STEC ranged from 3.1 to 4.6 min. *E. faecium* had a $D_{75°C}$ of 3.3 min, which indicates that it could be used as surrogate for larger scale vacuum steam testing in the future but was not conservative compared to some STEC strains.

5.2 Introduction

The increasing occurrence of foodborne outbreaks (*7*, *9*) traced to flour has raised concerns regarding the safety of this low moisture food (LMF). Studies have shown that *Salmonella,* and Shiga-toxin producing *Escherichia coli* (STEC) are able to survive on both wheat grains ((*36*)and flour (*21*, *24*) for many months. While moisture plays an important role in the thermal inactivation of pathogens (*69*), milling is a dry process after tempering. Therefore, the inactivation of pathogens on wheat grains prior to milling may be the most effective treatment. Several microbial inactivation methods have been evaluated for microbes on flour and wheat grain (*12*, *16*, *37*, *48*, *54*, *59*, *68*). Even though thermal processing leads to a greater log reduction

of bacterial density than non-thermal processing, the resulting flour quality and functionality are the determining factors as to whether a specific treatment would be feasible.

López and Simsek (*40*) summarized technologies used on wheat, or flour, for the inactivation of microorganisms. These include the addition of tempering water to solutions with a disinfectant effect and other non-thermal technologies. For example, applying pulsed light to flour, led to a 2.91 log reduction in *Salmonella* after 60 minutes at 395nm (*16*). Different forms of plasma were also evaluated for their efficacy against yeast and mold on wheat grains. Thermal technology usually led to higher reductions of bacterial populations, some examples, including radio frequency pasteurization, led to a 7 log CFU/g reduction in *Salmonella* PT30 population inoculated on wheat flour, after heating 16 g of sample at 75°C for 9 min, surrounding with a polystyrene material to make an uniform electromagnetic field (*68*). Later, studies using radiofrequency pasteurization found a 3.7 log reduction in *Enterococcus faecium*, a common surrogate used in LMF thermal experiments, and 5 log CFU/g reduction in *Salmonella*, in wheat flour heated in aluminum test cells after 33 min at 85°C (*38*). The results showed that the surround materials are important to inactivation efficacy under similar inactivation method.

There are two thermal methods studied to inactivate microorganisms on wheat grains: superheated steam (*29*) and vacuum steam pasteurization (VSP) (*59*, *60*). Superheated steam led to a 2-log reduction when treated at 200°C for 15 m^3/hour (*29*) for mesophilic aerobic bacteria. While the high temperature of steam can compromise end-use product quality, steam could be maintained at a lower temperature under vacuum. Shah et al. (*57*) tested the efficacy of VSP to inactivate *Salmonella* PT30, *E. coli* O157:H7, and *E. faecium* on flaxseed, quinoa, sunflower kernels, and whole peppercorns. Overall, VSP could effectively reduce pathogens greater than 5 log CFU/g on flaxseed, sunflower seeds, and peppercorn when treated at 75°C for 1 min. In a

previous study (*60*), treating hard red wheat grain at 65°C for 8 minutes led to a 3.2 and 3.6 log CFU/g reduction in *Salmonella* PT30 and STEC O121:H19, respectively. In the same study, the bread baking test showed the volumes of baked bread decreased significantly when the wheat was treated at greater or equal to 75°C. Simsek et al. (*59*) conducted a similar study using soft red wheat, where cake and cookie quality were not impacted significantly when grain was treated at 65, 75, and 85°C for 8 mins. Protein was also denatured when treated at 85°C for 4 mins, which led to significant loss in bread volumes. However, soft wheat grains are usually milled to cake flour, where gluten plays a less significant role. Therefore, we believe that soft wheat could be processed with VSP at 75°C, while hard wheat needs to be processed at a lower temperature of 65°C to maintain flour functionality.

The objectives of this study were to: 1. assess the efficacy of a newly built vacuum steam pasteurizer to inactivate *Salmonella* and STEC inoculated on soft red wheat grains, 2. estimate the D-values when treating wheat at different temperatures, and 3. determine if *Enterococcus faecium* could be a feasible surrogate for *Salmonella* and STEC in similar studies.

5.3 Materials and Methods

Wheat grain. A commercial blend of soft red winter (SRW) wheat grain was kindly donated by Mennel Milling Company (Fostoria, Ohio). The grain was received in September 2020 and stored at 4°C in Whirl-Pak bags (Nasco Whirl-Pak, Madison, WI). The day before inoculation, one kilogram of wheat was brought to room temperature, and the moisture content of uninoculated wheat was measured using a handheld moisture meter (MINIGAC1SG4, Dickey-John; Minneapolis, MN); average moisture content of wheat was 12.5 ± 0.1%. The water activity of wheat was measured by AQUALAB 4TE water activity meter (METER, Pullman, WA), and the average water activity was 0.50 ± 0.02.

Bacterial strains and preparation. The bacterial strains used in this study include two strains of *Salmonella*, *Salmonella* enterica Enteritidis PT30 (ATCC BAA-1045, isolated from almonds, 2001 Canada outbreak), *Salmonella* enterica Agona (FSL S9-0322, isolated from dry cereal), four strains of STEC, *Escherichia coli* O157:NM (LJH1723, isolated from soy nut butter outbreak), *Escherichia coli* O26:H11 (TW16501, human clinical isolate, 2012 sprout outbreaks, US), *Escherichia coli* O121:H19 (PNUSAE002568, human clinical isolate, 2016 wheat flour outbreak, US), *Escherichia coli* O103 (TW08101, human clinical), E*scherichia coli* O111 (TW07926, human clinical), and *Enterococcus faecium* (ATCC BAA836) which was used to evaluate as a potential surrogate efficacy in this study. The bacterial strains selected for rifampicin resistance in LB contain 80 μg mL^{-1} (LBr), as described in our previous study(*37*). The freezer stock of *E. faecium* was prepared using tryptic soy agar (Neogen, Lansing, MI) supplemented with 0.6% yeast extract (RPI, Mount Prospect, IL) and glycerol (Thermo Fisher Scientific).

Inoculation of wheat grains. For each biological replicate, selected strains were streaked on LBr agar and incubated at 37°C for 20 hours, and a single colony was selected and transferred to LBr broth and incubated at 37°C for 24 hours. Each 250 μL of overnight culture was evenly spread to five LBr agar plates (100mm) and incubated for 24 hours at 37°C to achieve confluent lawn growth. The bacteria were harvested from the lawn culture with a sterile spreader and transferred to a beaker containing 2.5mL of sterile water and mixed by a sterile loop by constant stirring manually. The mixture was then transferred to a Whirl-Pak bag (Nasco Whirl-Pak, Madison, WI) containing 850 g of wheat grains and massaged by hand for 3 minutes to achieve ~8 log CFU/g initial pathogen density. After mixing, the wheat grains were transferred to a

sterile stainless-steel tray and mixed again with a sterile scoop for 3 min before water activity equilibration.

Water activity adjustment and sample preparation. Inoculated wheat grains were transferred to a_w equilibration chamber previously described (*23*) and allowed the a_w to be lowered for 24 hours at a relative humidity of 45%. The average final water activity was 0.49±0.01. After a_w adjustment, wheat grains were transferred to organza bags (4 x 6", ULINE, Pleasant Prairie, WI) by a sterile scoop, and each bag contained 25 grams of sample.

Design of pilot-scale vacuum steam pasteurizer. A custom pilot-scale vacuum steam pasteurization unit was developed for this study (Figure 5-1). The apparatus was composed of a 39 L aluminum non-electric steam sterilizer (All American Model 1941X, Manitowoc, WI) as the primary holding vessel for the product, a 6kW steam generator (Steam Planet, Cleveland, Ohio) fitted to a 14 L aluminum non-electric steam sterilizer (All American 1915X, Manitowoc, WI) to regulate the steam inlet pressure, and a vacuum pump (Cole-Parmer, Vernon Hills, IL). The container was wrapped in constant-wattage electrical heat tape (1440 W capacity, 8 cm wide by 240 cm long, McMaster Carr, Elmhurst, IL) to evenly heat the vessel. Product and vessel temperatures were measured by two digital temperature sensors (DS18B20, Adafruit Inc, New York, NY), and the pressure of the vessel was measured using a calibrated pressure transducer (PX3229, IFM, Malvern, PA). Recording of the temperature and vacuum pressure and control of the heat, steam, and vacuum pump of the apparatus was done by a custom programmable logic controller made on a Raspberry Pi model 3B microcontroller (Raspberry Pi Foundation, Cambridge, England, UK).

Vacuum steam pasteurization. Before treatment, the steam chamber was preheated by turning on the steam generator to allow the steam to build up in the 14L sterilizer. The electrical heat tape was also turned on to evenly heat the 39L treatment chamber.

Before each experiment, 1.9 kg of wheat grain used as filler was preheated in a wire mesh basket (10 x 6 x 6", Marlin Steel Wire, Baltimore, MD) lined with aluminum window screen mesh (Phifer, Tuscaloosa, AL) and placed inside the vacuum steam pasteurization unit and heated to 45˚C. Filler wheat was replaced every 5 to 6 experiments. The moisture content of the new batch of filler wheat was measured before treatment, after preheating to 45˚C, and at the treatment temperature (75 ˚C, t=0 mins). Inside the treatment chamber, the wire basket that contained the treatment sample was lifted by another wired basket of the same size to avoid water accumulation from steam during treatment.

After the filler wheat was heated to 45 ˚C, the filler was removed from the treatment chamber. For each treatment temperature and time, six bags of inoculated wheat grain of three technical replicates of two of the nine strains studied were buried in the filler wheat. Three treatment temperatures (65, 70, 75˚C) were measured. At treatment temperatures of 65 and 70 ˚C, two time points were measured at 0 min and 4 min. At a treatment temperature of 75 ˚C, 5 time points were measured at 0, 2, 4, 6, and 8 min.

After each treatment, samples were collected from organza bags and transferred to Whirl-Pak bags for bacterial enumeration. During treatment, pressure in the treatment chamber was maintained between -150 to -190 mbar.

Bacterial enumeration. The collected samples were diluted in Butterfield's buffer and homogenized by stomaching (Seward 400, Worthing, West Sussex, UK) for 1 min. Appropriate serial dilutions were performed using Butterfield's dilution buffer, wheat inoculated with

Enterococcus faecium was plated to TSA supplemented with 0.6% yeast extract (rifampicin resistance 80 μg mL^{-1}), wheat inoculated with pathogens was plated to LBr agar (80 μg mL^{-1}).

Modeling the inactivation rate. The differential form of the log-linear primary model is:

$$\frac{d(logN)}{dt} = -\frac{1}{D} \quad (1)$$

The Bigelow secondary model is used to describe how D changes with temperature T:

$$D(t) = D_r 10^{(\frac{T_r - T}{z})} \quad (2)$$

where T_r is a reference temperature that lies within the experimental temperature range. Substituting Eq. 2 into Eq. 1, and integrating both sides give the final model for non-isothermal conditions:

$$logN = logN_0 - \left(\frac{1}{D_r}\right) \int_0^t 10^{\left(\frac{T(t) - T_r}{z}\right)} dt \quad (3)$$

D_r, z, and $log\ N_0$ were estimated simultaneously by ordinary least squares non-linear regression using MATLAB, where $-(\frac{1}{D_r})$ is the slope, and $\int_0^t 10^{\left(\frac{T(t) - T_r}{z}\right)}$ is the time-temperature history for a given non-isothermal VSP experiment. T(t) is the dynamic temperature in the center among the wheat kernels. T_r is the optimal reference temperature that minimizes the error in the D_r parameter. For comparison, all D values were computed at 75 °C using Eq. 2. All data and the entire time-temperature histories were used in Equation (3).

Statistical Analysis. The data were analyzed using R (version 4.0.3, www.r-project.org), and plots were made with ggplot2 version 3.3.3 (*26*). The model was created and estimated using MATLAB (R2022a, The MathWorks Inc., Natick, MA).

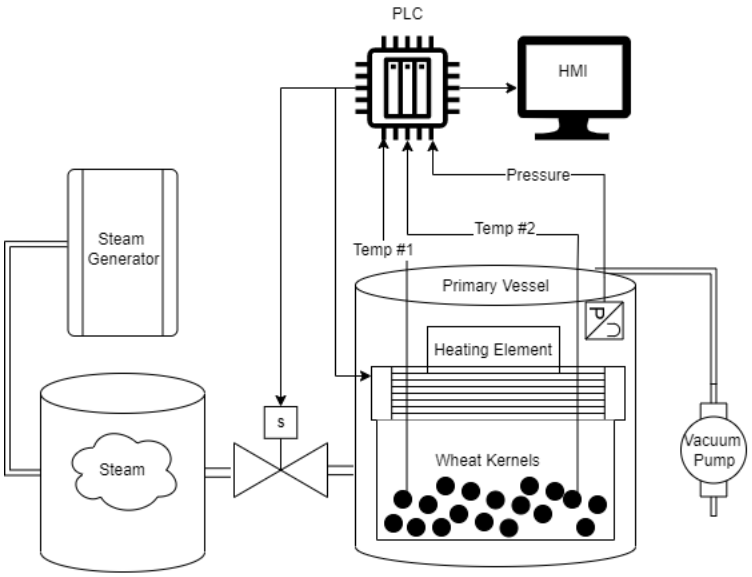

Figure 5-1 Picture of the custom-built vacuum steam unit.

5.4 Results

Efficacy of VSP for pathogen inactivation on wheat grain. The populations of STEC and *Salmonella* on wheat grain after water activity equilibration were significantly different ($p<0.05$), with a mean value of 7.8± 0.3 log CFU/g for *Salmonella* and 6.9±0.4 log CFU/g for STEC. STEC populations decreased an average of 1.0±0.3 log CFU/g after water activity equilibration, which was significantly ($p<0.05$) greater than that for *Salmonella,* with the change of -0.2±0.1 log CFU/g. No significant differences in reduction were observed among strains within the two species. This result was similar to previous findings where the reduction in STEC populations was significantly greater than *Salmonella* on LMF after water activity equilibration (*36*, *37*). Differences in starting bacterial populations could lead to the bacterial population reaching the limit of detection in a shorter time, in this case, 1 log CFU/g.

Figure 5-2 shows the time-temperature history of the log reduction model of the whole treatment (including come-up time) for each isolate examined. Table 1 shows the parameters estimated by the model. When comparing the 95% confidence intervals, we found, in general,

that STEC survives better than *Salmonella* when treated with VSP. Based on the 95% CI, the D-value of *Enterococcus faecium* was significantly greater than both *Salmonella* isolates examined, while not significantly different from *E. coli* O121:H19 and *E. coli* O103 but was significantly lower than *E. coli* O157:NM and *E. coli* O26:H11.

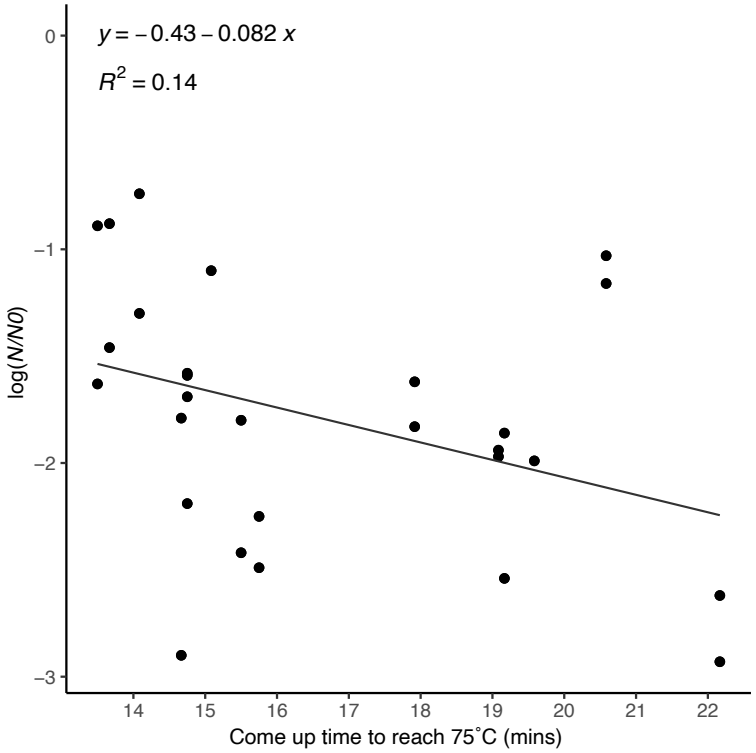

Figure 5-2 Come up time for filler wheat to reach 75 ˚C from 45˚C, and the pathogen population reduction during come up time. Each point represents one biological replicate of one stain studied.

In this study, we found the STEC generally had a higher D-value than the *Salmonella* (Table 2). Most previous LMF-related studies focused on the thermal inactivation of *Salmonella* Enteritidis PT30, as it is the primary pathogen of concern in other LMFs. However, given the increasing number of LMF outbreaks linked to STEC, thermal inactivation studies should also include STEC in addition to *Salmonella*. Even within the same species, the response to thermal stress could be different. When Ban and Kang (1) tested the efficacy of superheated steam to

inactivate enteric pathogens inoculated on almonds and pistachios, their *E. coli* O157:H7 strain had a greater D-value than *Salmonella* Typhimurium but a lower D-value than *S.* Enteritidis PT30.

In a previous VSP study on hard, soft wheat grain (*59*), the $D_{75^{\circ}C}$ for *Salmonella* PT30 and *E. coli* O121:H19 was 3.8 min and 3.6 min, respectively. In the present work, the $D_{75^{\circ}C}$ for *Salmonella* PT30 and *E. coli* O121:H19 was 2.8 mins and 3.1 mins, respectively. The $D_{75^{\circ}C}$ value was reduced by about 1.0 min for *Salmonella*, and 0.5 min for *E. coli* O121:H19, which could possibly be due to the enhanced efficacy of the new VSP unit design. The *E. coli* O121:H19 strain differed between these two studies, while the *E. coli* O121:H19 used in the current study was an isolate from a 2016 flour outbreak, suggesting that even within the same serovar, individual isolates may show different behavior.

As shown in Figure 5-3, the come-up time to bring the temperature from 45 ˚C to 75 ˚C varies from 13.5 to 22.2 minutes. Changes in bacterial population during the come-up time were measured and ranged from -0.74 to -2.93 log CFU/g. Microbial inactivation increased with come-up time. However, the low R^2 value indicates that using a linear regression model to calculate isothermal reduction was inappropriate. Moreover, similar come-up times could result in different reductions, potentially due to different heat-resistance capacities of the strains. Therefore, to determine the thermal inactivation rate of pathogens on LMFs treated with VSP, changes in both temperature and time changes need to be considered.

E. coli O157: NM isolated from soy nut butter had the highest D-value at 75 ˚C. In comparison, at 65 ˚C and 70 ˚C, the D-value is lower than some other strains. This is mainly due to the large z-value estimated by the model. The 95% confidence interval of the z-value also indicated that the data collected were too scattered. We included all data that were greater than

the detection limit. However, we found the *E. coli* O157:NM strain reached the limit of detection sooner when treated at 75°C since the starting populations of *E. coli*, in general, are lower than *Salmonella*, as observed in Figure 5-2A. With more data points collected above the limit of detection, the model is more likely to give a stronger estimation of D_r, z-value, and log N_0. To improve this, more data could be collected for wheat grains treated for longer time at lower temperatures (for example at 65°C) or treating wheat at 75°C with shorter time intervals before the microbial counts reach the limit of detection.

Table 5-1 Summary table of estimated parameters of the model, and D-value of each strain at temperature= 65, 70, and 75 ˚C.

Strains	**T_r** ˚C	**D_r [95% CI]** mins	**$D_{75\,°C}$** mins	**RMSE** CFU/g	**Z [95% CI]** ˚C	**$D_{70\,°C}$** mins	**$D_{65\,°C}$** mins
S. PT30	71.3	3.6 [3.4, 3,9]	2.8	0.61	32.3 [25.6, 38.8]	4.0	5.7
S. Agona	71.0	4.3 [3.9, 4.7]	3.2	0.61	30.6 [23.1, 39.2]	4.6	6.7
E. faecium	70.5	4.7 [4.3, 5.1]	3.3	0.68	30.1 [21.0, 39.1]	4.9	7.1
E. coli O26:H11	68.0	6.3 [5.5, 8.8]	4.3	0.75	42.5 [21.1, 63.8]	5.7	7.4
E. coli O157:NM	66.0	6.4 [5.9, 7.0]	4.6	0.47	61.1 [35.6, 86.5]	5.5	6.7
E. coli O121:H19	69.5	4.4 [4.0, 4.8]	3.1	0.59	36.6 [26.9, 46.4]	4.3	5.9
E. coli O103	72.0	5.0 [4.4, 5.6]	3.7	0.61	22.9 [16.1, 29.6]	6.1	10.1

***Enterococcus faecium* as surrogate.** These findings support *E. faecium* as a reasonable surrogate for larger scale testing in VSP. With inconsistent come-up time as a major limiting factor the custom lab-scale VSP, testing with a commercial grade pilot scale VSP is necessary to re-evaluate the process time for commercial application. The results in Table 1 show that *E. faecium* could be a conservative surrogate for testing VSP at a treatment temperature of 65°C. However, at 70°C and 75°C, even though *E. faecium* had a D-value greater than the two *Salmonella* strains tested, this microorganism is not as conservative a surrogate for STEC.

Increase in grain moisture content indicates potential for VSP to replace or accelerate the tempering process. The filler wheat was brought to ambient temperature one day before the experiment, and the average moisture content before treatment was 13.6±0.2% for three biological replicates. After the wheat was preheated to 45°C, the moisture content increased to 14.6±0.2%, and further increased to 16.4±0.2% after the temperature reached 75°C. The final moisture content of 16.5% after treatment was within the desired range for the final moisture content of hard wheat after tempering but higher than the desired range for the final moisture content of soft wheat, between 14.5 to 15%. Further studies should examine the variability in grain moisture content changes after VSP treatment and conduct analyses to determine if the water has migrated into the bran layer.

Future improvements for vacuum steam pasteurization. During vacuum steam pasteurization, moisture was added via steam to the wheat grain. A previous study that freeze-dried viable *Salmonella* PT30 cells and conditioned the cells to different moisture contents found the moisture content of bacterial cells is a key intrinsic factor in overcoming bacterial thermal resistance (*70*). In real life applications, the research found temperature, moisture, and process humidity impacted the lethality of *Salmonella* inoculated on pistachios exposed to hot-air heating (*5*). A similar finding in almond kernels suggested that increasing the moisture content of the product could achieve the desired reduction in a shorter time and at a lower temperature (*67*). The VSP is a treatment involving all three parameters of temperature, moisture, and process humidity, and the reduction found could also be influenced by these confounding elements. For example, during treatment, the moisture content of wheat kernels increased, presumably leading to a greater reduction. Therefore, future work relating to VSP should consider installing a

relative humidity sensor to determine the impact of moisture on microbial inactivation in this system.

5.5 Conclusion

Vacuum steam pasteurization provided a satisfactory result in inactivating *Salmonella* and STEC inoculated soft wheat grains. The D-value accounts for the time-temperature history during treatment, including the variation in come up time. The $D_{75°C}$ of *Salmonella* strains were 2.8 and 3.2 min, and the $D_{75°C}$ of STEC ranged from 3.1 to 4.6 min. *E. faecium* was found to be an adequate surrogate for the thermal inactivation of *Salmonella* and STEC on wheat grain. Further research should examine the impact of VSP treatment at 75°C based on the D-values estimated on wheat quality and flour functionality in final products.

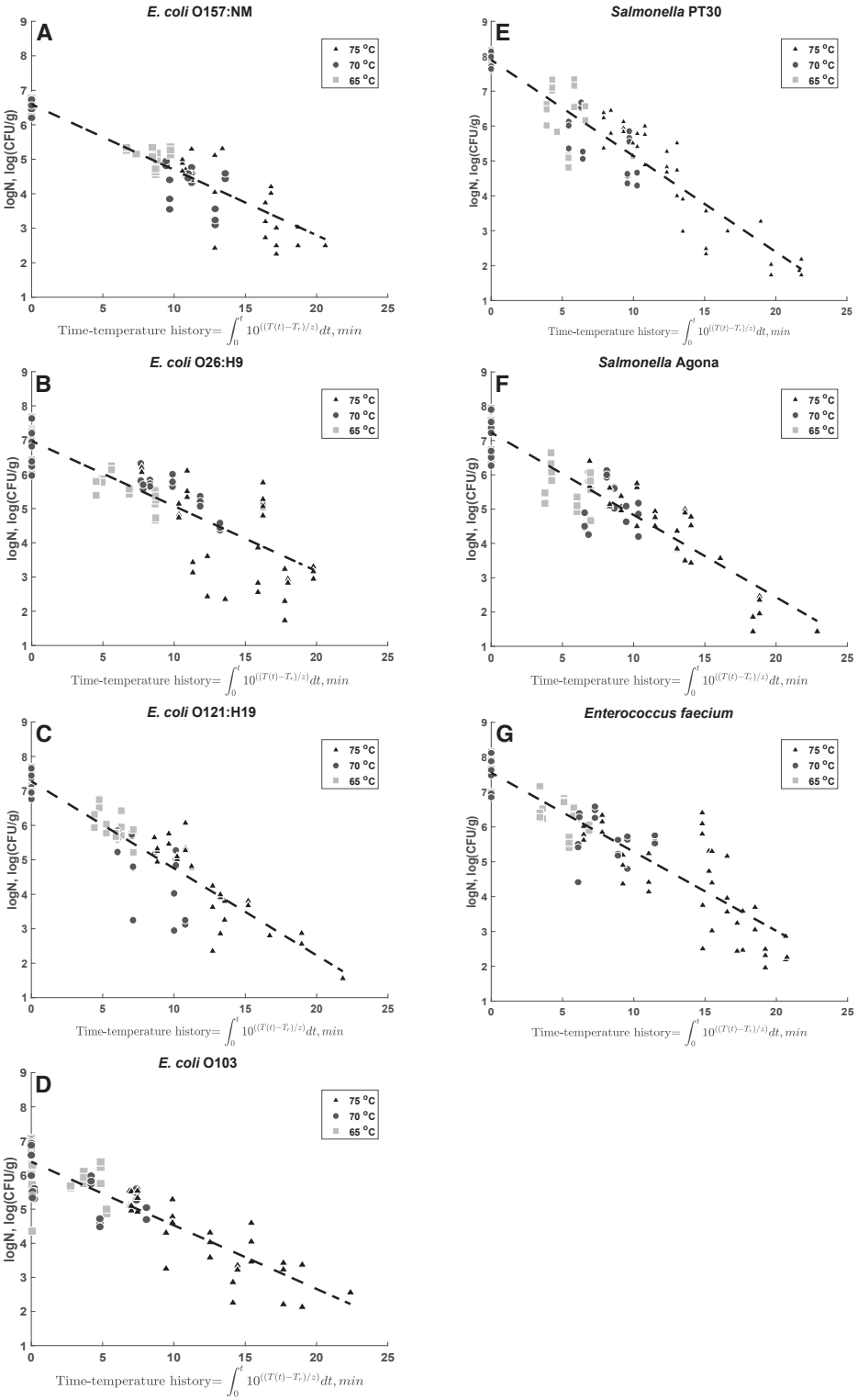

Figure 5-3 Log reduction at time-temperature history with estimated slope -(1/Dr) of strain, (A) E. coli O157:NM (B) E. coli O26:H9 (C) E. coli O121: H19 (D) E. coli O103 (E) Salmonella PT30 (F) Salmonella Agona (G) Enterococcus faecium. Each data point represents one technical replicate of one biological replicate for each strain.

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APPENDIX

Table A-1 Overall reduction due to different chlorine concentration. Different letters across each row indicate values that are significantly different ($p<0.05$) and were results of 3 replicates ($n=3$).

	Change in cell density (log CFU/g)		
Strains	400ppm	800ppm	1200ppm
Native microbes	-0.26±0.23a	-0.79±0.35a	-0.29±0.26a
Salmonella PT30	-2.08±0.48a	-2.35±0.23a	-2.13±0.23a
Salmonella Agona	-1.75±0.63a	-2.23±0.22a	-2.00±0.48a
STEC O157:H7	-0.32±0.42a	-0.31±0.07a	-0.52±0.10a
STEC O26:H11	-0.85±0.22a	-1.31±0.38a	-0.91±0.28a
STEC O121:H9	-0.96±0.45a	-1.49±0.39a	-0.98±0.17a
STEC O103	-1.50±0.32a	-1.39±0.22a	-1.72±0.31a
STEC O111	-0.90±0.27a	-1.25±0.08a	-1.56±0.27a

Table A-2 Pasting properties of flour from wheat tempered with chlorine.

Chlorine Treatment	Peak Viscosity cP	Hot Paste Viscosity cP	Breakdown cP	Final Viscosity cP	Setback cP	Peak Time min.	Pasting Temp °C
0 ppm	2078.2a[1]	1638.7a	439.5a	2382.8a	744.2a	6.63a	68.6a
400 ppm	2076.3a	1664.0a	412.3a	2405.8a	741.8a	6.63a	68.5a
800 ppm	2102.5a	1666.8a	435.7a	2439.5a	772.7a	6.59a	68.8a
1200 ppm	2103.8a	1700.5a	403.3a	2482.3b	781.8a	6.70a	69.4a

[1] Different letters within each column indicate values that are significantly different ($p<0.05$) and were result of 3 replicates (n=3)

Table A-3 End product quality of flour from wheat tempered with chlorine.

Chlorine Treatment	Absorption %, (14% MB[1])	Dough Handling 1 to 10[2]	Fermentation Height cm	Oven Rise cm	Loaf Volume cc	Specific Volume cc/g	Grain and Texture 1 to 10[2]	Crumb Color 1 to 10[2]
0 ppm	67.57a[3]	9.0a	7.38a	5.56a	957.5a	6.91a	7.0a	7.5a
400 ppm	67.40a	9.0a	7.35a	5.55a	933.3a	6.71a	7.0a	8.0a
800 ppm	67.62a	9.0a	7.33a	5.24a	898.3a	6.51a	7.0a	8.0a
1200 ppm	67.62a	9.0a	7.47a	5.56a	923.3a	6.67a	7.0a	7.8a

[1]MB = Moisture basis
[2]scores based on expert subjective evaluation where 1 is very poor and 10 is excellent
[3]Different letters within each column indicate values that are significantly different ($p<0.05$) and were result of 3 replicates (n=3)

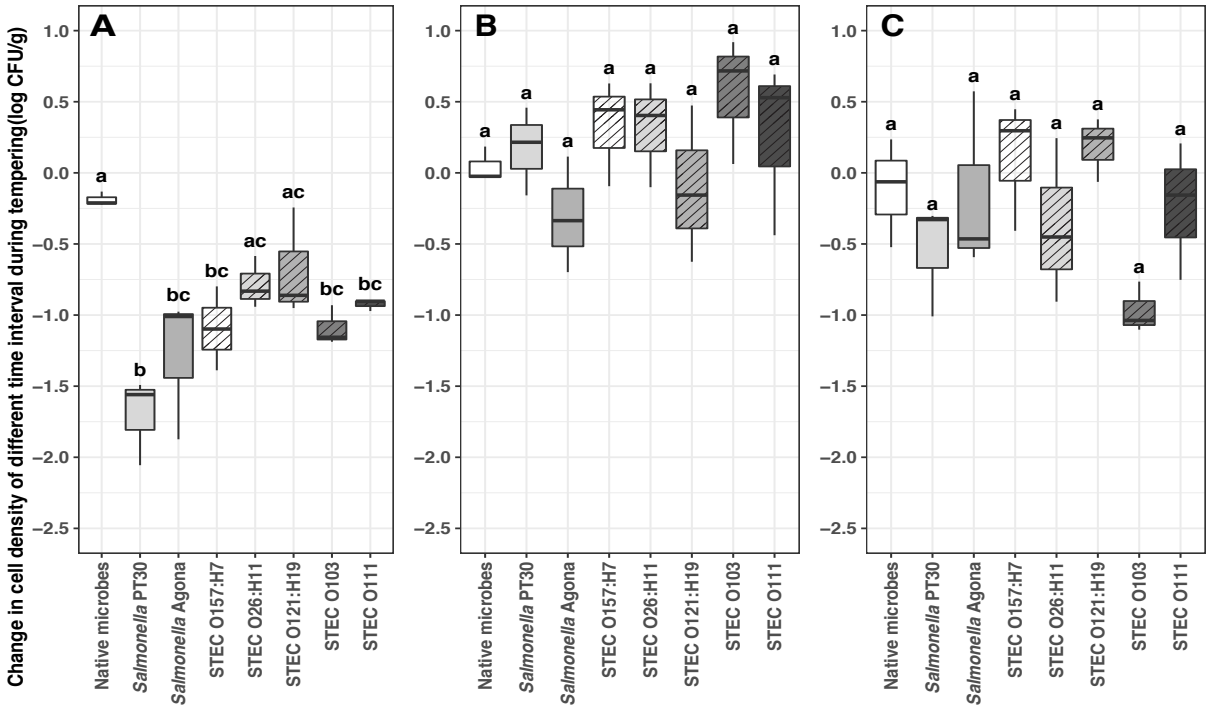

Figure A-1 Cell density changes of selected strains during different time intervals tempered by 400ppm chlorinated water. (A) changes from t=0 to t=0.5 hours, (B) changes from t=0.5 to t=17.5 hours, (C) changes from t=17.5 to t=18 hours. Different letters within each panel indicate values that are significantly different ($p<0.05$) and were result of 3 replicates (n=3).

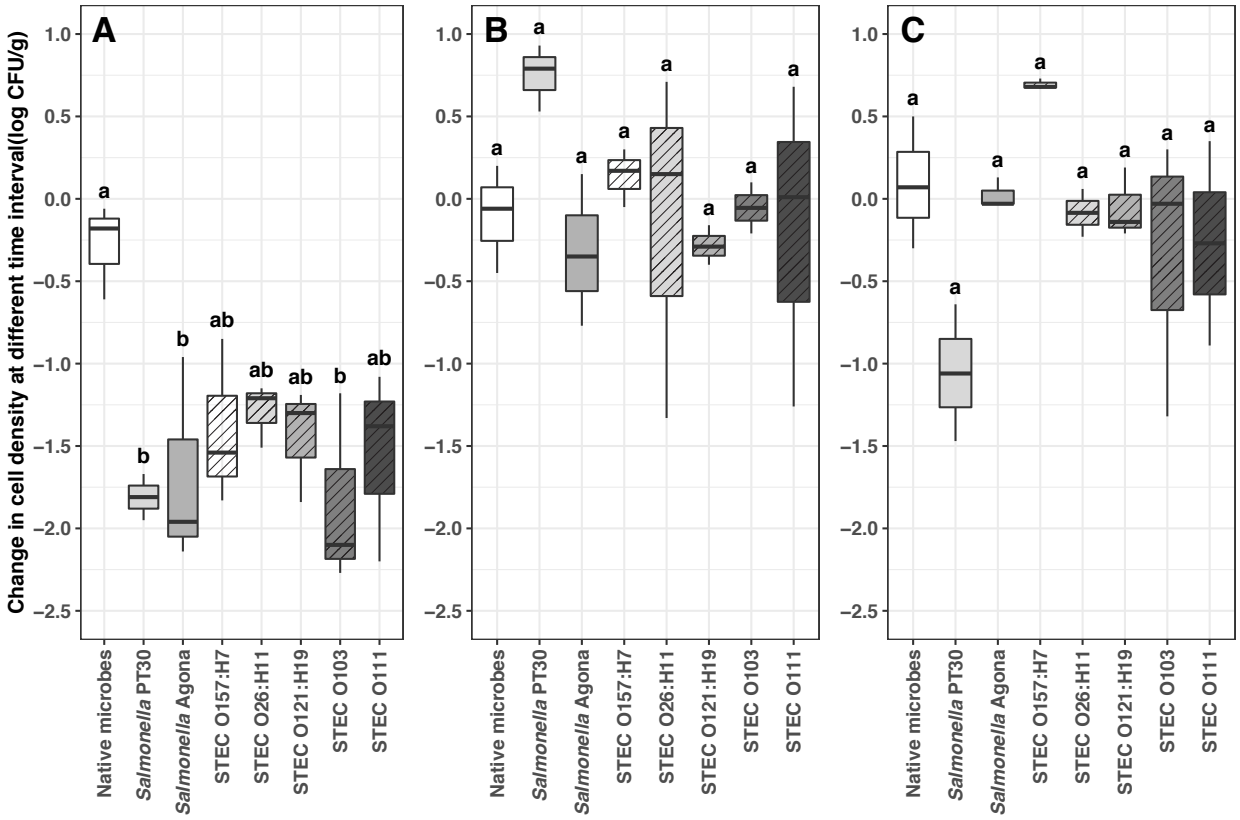

Figure A-2 Cell density changes of selected strains during different time intervals tempered by 800ppm chlorinated water, (A) changes from t=0 to t=0.5 hours, (B) changes from t=0.5 to t=17.5 hours, (C) changes from t=17.5 to t=18 hours. Different letters within each panel indicate values that are significantly different ($p<0.05$) and were result of 3 replicates (n=3).