VACUUM STEAM PASTEURIZATION OF HARD RED SPRING WHEAT

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MASTER OF SCIENCE

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ABSTRACT

Vacuum steam pasteurization (VSP) is a promising technology for pasteurization of low moisture foods. Recent outbreaks traced to wheat flour, a low moisture food, have created demand in the milling industry for a process that reduces pathogens in wheat while maintaining its functional properties. The objective of this study was to evaluate the efficacy of VSP on Hard Red Spring (HRS) wheat. First, HRS wheat samples were pasteurized at 65, 70, 75, and 85°C for 4 and 8 minutes. Significant changes in dough and baked product functionality were observed in treatments ≥70°C suggesting gluten denaturation occurred. After determining that 65°C processing conditions best preserved functionality, HRS wheat was inoculated with *Escherichia coli* O121, and processed at 65°C for 0, 2, 4, 6, and 8 min periods. The treatments achieved a maximum microbial reduction of ~3.5 log CFU/g. VSP shows potential as an effective pasteurization method for the flour milling industry.

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LIST OF ABBREVIATIONS

AAB…….…….Acetic Acid Bacteria

AACCI…….…….American Association of Cereal Chemists International

APC……...……Aerobic Plate Count

BHI……..……..Brain Heart Infusion

BU…….……..Braebender Unit

CDC……..……..Center for Disease Control

CFU…….Colony Forming Unit

DON…….Deoxynivalenol

EHEC……..…….Enterohemorrhagic *Escherichia coli*

FAO……..…….Food and Agriculture Organization

FDA……..Food and Drug Administration

FSMA…….….Food Safety Modernization Act

GMP……..……..Glutenin Macropolymer

HACCP……Hazard Analysis Critical Control Point

HPSEC…….High Performance Size Exclusion Chromatography

HRS…..……..…….Hard Red Spring

HUS……..……..Hemolytic Uremic Syndrome

LAB……..Lactic Acid Bacteria

MB…….……Moisture Basis

MTI……...……..Mixing Tolerance Index

NDSU……North Dakota State University

NIR……...……Near-Infrared

PPO……..……..Polyphenol Oxidase

RTE……..Ready To Eat

RVA……Rapid Visco Analyzer

SAS……..Statistical Analysis Software

STEC…….. Shiga toxin-producing *Escherichia coli*

VSP……...…….Vacuum Steam Pasteurization

WHO……..……World Health Organization

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1. INTRODUCTION

Historically, there has been little concern for the safety of wheat flour and its related food products as flour is low in moisture. Previously, it was not believed microbes could survive in such an environment; however it is now better understood that some microbes can survive in low moisture conditions for extended periods of time (Beuchat et al. 2013; Crowe et al. 2017; Sperber 2007). Many products made from wheat flour are intended to only be consumed after a cooking step has been implemented that would kill any pathogens, yet consumers may eat raw dough products including cookie dough, brownie or cake batter, or frozen dough despite warnings on food labels to avoid such practices. Recent outbreaks related to raw flour consumption, primarily caused by the *Salmonella* and *E. coli* pathogens, have raised concern about the safety of flour products (Harris and Yada 2018). Of particular interest was a multi-state an outbreak which occurred in 2016 in the United States, when shiga-toxin producing *Escherichia coli* O121 and O26 were linked to contaminated flour causing widespread illness (Crowe et al. 2017; Harris and Yada 2018)

Wheat kernels are exposed to many potential contaminants in the environment including animals, insects, soil, wind, and poor sanitation of harvesting equipment, and transportation and storage containers. Current wheat milling practices do not utilize techniques which actively aim to reduce microbial populations (Berghofer et al. 2003; Sperber 2007). Development of a process that can reduce pathogenic organisms while maintaining wheat quality is of great interest to wheat processors. Traditional pasteurization methods utilize high heat to kill pathogens and are undesirable in the flour industry as the high temperatures can reduce functionality of the grain. The gluten protein, imperative for the formation of dough and integral for quality of bread products, is denatured at high temperatures.

One potential method of processing for use in the milling industry is vacuum steam pasteurization (VSP). Steam is a desired medium for pathogenic reduction in the food industry as it has a high level of heat transfer. As its name implies, VSP utilizes steam application under the presence of vacuum, which creates lower temperatures of steam. The low temperatures in this method promise sustained quality of the wheat, while maintaining the effective heat transfer attained with steam allowing for higher reduction of pathogens. In previous research, VSP has been shown as an effective method to reduce pathogens in low moisture foods including almonds, sunflower seeds, quinoa, and peppercorns (Shah et. al, 2017). The method could be used on wheat kernels before the milling process to reduce pathogens that are commonly found on the outermost, bran layer, of the kernel.

2. LITERATURE REVIEW

2.1. Wheat

Wheat (*Triticum aestivum* L.) is the third most produced crop in the world, and is an important export commodity for the United States (Diekmann 2009). There are six recognized classes of wheat in the United States which are categorized by their growing season, hardness, and color. The six classes include: Hard Red Spring, Hard Red Winter, Soft Red Winter, Soft White, Hard White, and Durum wheat. The seed of the wheat plant is of primary interest, as it can be milled into flour. Wheat flour is a central component of many people's diets as an important ingredient in food products from around the world including breads, cakes, cookies, pastas, and noodles.

The wheat kernel, or caryopsis, is comprised of three main components: the bran, the germ, and the endosperm (Figure 1). A defining characteristic of the wheat kernel is the crease, a groove running the length of the kernel on the ventral side opposite the germ, which can be as deep as 60% to 70% of its thickness (Misailidis and Campbell 2013). The bran is the outer layer of the kernel, consisting primarily of insoluble fiber, and acts as hard layer of protection for the endosperm (Šramková et al. 2009). The bran is made of three primary layers, including the pericarp, testa (or seed coat), and aleurone layer (Parker et al. 2005). A majority of the vitamins and minerals in wheat are contained in the bran and are important for human health. Examples include iron, B and E vitamins, and thiamin (Onipe et al. 2015). The bran is removed from the endosperm when milling white, or refined flour, as opposed to whole wheat flour, which includes the bran portion. Before milling, the wheat is tempered with water, which acts as a plasticizer, making the bran tougher, easier to remove during milling, and less likely to

contaminate the flour. The bran byproduct can be used in other food products, or is sold as animal feed.

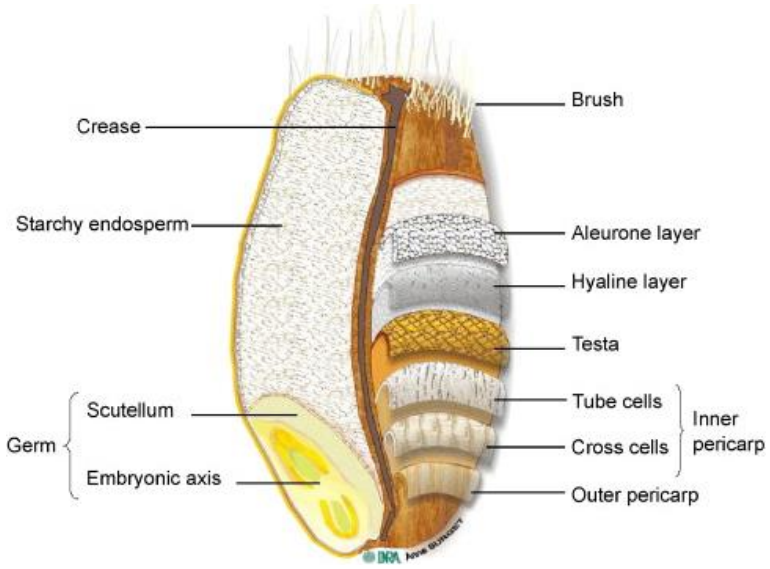

Figure 1. Diagram of wheat caryopsis.
Reprinted without changes from Saulnier et al. (2007)

The wheat germ comprises between 2-3% of the total caryopsis by weight, and is the embryo of the wheat seed, making it critical for germination (Brandolini and Hidalgo 2012). While the germ is primarily composed of lipids and proteins, some essential vitamins and minerals can also be included in its composition (Šramková et al. 2009). During milling of refined flour, the germ is removed.

The endosperm makes up the largest portion of the wheat kernel and is comprised of approximately 80% carbohydrate material by weight (Stevenson et al. 2012). The endosperm also contains important enzymes and proteins (Šramková et al. 2009). Two of these important proteins are glutenin and gliadin. Glutenin is responsible for giving strength to the dough; they are large aggregating proteins; with a molecular weight over 3 million units. In contrast, gliadin proteins do not aggregate, and are much smaller in size (molecular weight of 40,000 units). Gliadin is responsible for dough cohesion, which allows dough to remain extensible. In the

presence of water and mechanical energy in the form of mixing, these two storage proteins combine to form gluten.

2.1.1. Hard Red Spring Wheat

Hard Red Spring Wheat (HRS) is one of the six classes of wheat. There are three subclasses of HRS: Dark Northern Spring, Northern Spring, and Red Spring wheat, which are characterized by the percent of kernels that are dark, hard and vitreous (DHV). Dark Northern Spring contains >75% DHV kernels, while Northern Spring and Red Spring contain 25-74%, and <25% DHV kernels, respectively. HRS wheat is primarily used for bread baking, due to the flour's high protein content and strong gluten structure. It is often mixed with lower protein wheat to improve gluten strength in flour (U.S. Wheat Associates 2018). The crop is primarily grown in the Northern Plains of the United States including Minnesota, Montana, North Dakota, South Dakota, Idaho, Oregon, and Washington. In 2018, 16.0 million metric tons were produced in the United States. Compared to other classes of wheat, HRS generally has a higher water absorption which is desirable in the baking industry as more loaves of bread can be produced with increased shelf life and softness (U.S. Wheat Associates 2018).

2.2. Microbial Contamination of Wheat

A report released in 2014 by the Food and Agricultural Organization (FAO) and World Health Organization (WHO) ranked the cereals and grains category of highest concern from a microbiological food safety perspective in comparison with other low moisture foods (FAO and WHO 2014). The report utilized a sophisticated scoring system based on four criteria: international trade, burden of disease, food consumption, and food production. The cereals and grains category scored highly in all criteria compared to other categories which included dried protein products, spices and dried herbs, nuts and nut products, confections and snacks, dried

fruits and vegetables, and seeds for consumption. Most notable in the cereals and grains category was the high score in the international trade criteria, because of its large importance as a commodity of trade, and the consumption criteria as large portion sizes are associated with cereal and grain foods.

2.2.1. Wheat Kernel Microflora

Several studies have enumerated microbial populations of wheat kernels and wheat flour (Aydin et al. 2009; Berghofer et al. 2003; Bullerman and Bianchini 2009; Manthey et al. 2004; Richter et al. 1993; Sperber 2003; 2007). Results from these studies are difficult to summarize and compare because they lack uniformity in the microbial populations observed, methods for collection and enumeration, geographical region, and the environmental conditions crops were exposed to. A summary by Sabillón and Bianchini (2016) of recent research determining the microbiological profile on wheat kernels can be found in Figure A2.

In general, non-pathogenic organisms found on wheat include coliform populations, lactic acid bacteria (LAB), acetic acid bacteria (AAB), yeasts, and molds. Non-pathogenic organisms can cause spoilage of the wheat or resulting products. Some molds, while not themselves pathogenic, produce mycotoxins which can cause chronic effects on human health (Sabillón and Bianchini 2016). In wheat, the *Fusarium* genus produces a heat-stable mycotoxin known as deoxynivalenol (DON), which is widely monitored in the United States. Pathogenic organisms including *Bacillus cereus, Clostridium botulinum, Clostridium perfringens, Escherichia coli, Salmonella spp.,* and *Staphylococcus aureus* have been observed in wheat (Bullerman and Bianchini 2009; Manthey et al. 2004). Generally, there are low levels of detection of pathogenic organisms in wheat. Most microflora are found on the wheat bran, the outermost layer of the wheat kernel (Berghofer et al. 2003; Laca et al. 2006).

Wheat is an agricultural product and is thus subjected to many environmental conditions which are conducive to microbial contamination. Wheat may be contaminated from the soil, other crops, insects, birds, rodents, by harvesting equipment, or in storage/handling, and throughout milling/production (Bullerman and Bianchini 2009). Grain with higher microbial contamination has been correlated with lower quality parameters such as increased kernel damage, total defects, poorer U.S. grading numbers, and decreased test weight, vitreousness, and kernel weight (Berghofer et al. 2003; Manthey et al. 2004). This research suggests processors could use the assumption that higher quality grain contains less microbial contamination as a method of mitigating risk associated with food spoilage and safety.

2.2.2. Microflora During Wheat Processing

The low water activity (a_w <0.65) of wheat does not allow for microbial growth, however, cells can remain viable while dormant (Berghofer et al. 2003; Carter et al. 2015; Eglezos 2010). Storage conditions for wheat grains are carefully monitored to prevent microbial growth; kernel moisture is usually maintained between 8% and 12% (Berghofer et al. 2003; Sperber 2007). While the process of milling does not directly aim to reduce microorganisms, the microbiological profile of wheat changes throughout the cleaning, tempering, and milling steps (Berghofer et al. 2003; Sabillón and Bianchini 2016; Sperber 2007). Cleaning steps generally reduce microbial counts of the grain, while microbial populations tend to increase during tempering and during milling due to potential contamination by equipment.

Cleaning of grain before milling utilizes multiple techniques including sieving, scouring, metal detectors, aspiration, destoning, and disc separation to remove foreign materials based on shape, density, size, and magnetism (Sabillón and Bianchini 2016). Cleaning methods such as

dockage testers, cyclone grain cleaners, scourers, aspirators, and brushes were found to reduce aerobic bacteria by ~1 log CFU/g (Manthey et al. 2004; Seiler 1986).

Pearling is a method occasionally used prior to milling of cereal grains to remove the bran from the endosperm using abrasive forces. Though most commonly used for rice, Laca et al. (2006) studied its efficacy as a microbial reduction method for wheat. From the study, it was concluded that much of the microbial contamination is located in the pericarp layer of within the bran. As more bran was removed from the endosperm during pearling, microbes adhered to the bran's surface are also removed. When 4% of the kernel was removed from the outside of the grain, an ~87% decrease in mesophilic organisms was observed (Laca et al. 2006).

In traditional wheat milling, a tempering process is used to increase the moisture of the grain to between 12 and 17%. Water is applied to the kernels and is allowed to rest for 16-24 hours before milling to ensure equal distribution and absorption of moisture. While advantageous for bran removal, the high moisture conditions produced via tempering offer an opportunity for microbial growth before milling. Berghofer et al. (2003) studied the changes in microflora on wheat throughout the milling process in nine flour mills across Australia, and observed an alarming change of *E. coli* contamination after tempering. Prior to tempering, no *E. coli* was detected in the wheat sample, while after tempering, 14% of samples tested positive. This change was hypothesized to be a result of, "poorly cleaned conditioning bins and equipment" (Berghofer et al. 2003).

Most commercial milling operations utilize a series of break and reduction rolls to first remove bran particles from the outside of the kernel, and then crush the endosperm to produce a fine particle size. After milling, microbial populations are concentrated in the bran and germ fractions producing a flour with a higher microbiological purity than the initial wheat (Berghofer

et al. 2003; Manthey et al. 2004). Manthey et al. (2004) found an overall reduction of bacteria from 1.6 x 10^8 CFU/g to 5.0 x 10^5 CFU/g in the refined flour after cleaning and milling of the wheat. Bran is usually discarded as a byproduct of the milling process; however, recent trends towards increased whole wheat consumption may create an additional challenge for the milling industry (Sabillón and Bianchini 2016).

As the wheat moves through the milling system, many sifting and transport systems are used. Berghofer et al. (2003) observed higher microbial counts midstream in the milling process, which were indicative of equipment contamination. Buildup of residues in break rolls, reduction rolls, and sifters is likely due to warm conditions and moisture condensation in the mill, and could be a source of contamination (Berghofer et al. 2003).

2.2.3. Microflora in Wheat Flour

A summary by Sabillón and Bianchini (2016) of recent research determining the microbiological profile in wheat flour can be found in Figure A3. Similar to research completed studying microbiological profiles on wheat kernels, studies with wheat flour have shown drastic differences between region of the study, and even between mills from which the flour was sourced. A summary by Rose et al. (2012) shown in Table 1 shows this variation- especially between pathogenic organisms.

Table 1. Summary of microbiological profile of wheat grains from multiple regions.

Category	Origin[a]			
	Turkey (Aydin et al. 2009)	**Australia** (Berghofer et al. 2003)	**Australia** (Eglezos 2010)	**United States** (Richter et al. 1993)
Bacteria (log CFU/g)	4.14 (142)	NR	4.2 (100)	4.17 (1,354)
Mold (log CFU/g)	2.24 (142)	NR	2.80 (50)	2.90 (1,682)
Yeast (log CFU/g)	NR	NR	3.70 (50)	2.12 (1,648)
Escherichia coli (% positive)	51 (142)	1 (71)	2 (300)	12.8 (3,350)
Salmonella sp. (% positive)	ND (142)	NR	ND (150)	1.3 (3,040)
Bacillus cereus (% positive)	4.2 (142)	93 (71)	ND (350)	NR

[a] Values are means; samples sizes are provided in parentheses. NR= Not reported; ND= Not detected. Reprinted without changes from Rose et al. (2012).

A survey of flour samples collected in Germany between 2014 and 2017 indicated a frequent presence of shiga toxin producing *Escherichia coli* (STEC) (Mäde et al. 2017). Over the four year period, 39% of samples (n=98) evaluated using real-time PCR techniques showed presence of STEC. When subsamples were collected, the rate of detection increased significantly, which suggests testing procedures for future studies should utilize a defined subsampling technique. Results suggested certain flour mills were more prone to STEC detection, however statistical differences were not significant. As a part of the study, mills were visited regularly for inspection, and a trend between inferior hygienic conditions and detection of STEC was observed.

Most recently, Kindle et al. (2019) surveyed commercial flour samples randomly purchased from local stores in Switzerland. Of the 70 samples, 21 were wheat flour samples, with other flour samples containing alternative grains, or a mix of multiple grains. The 70 samples originated from 20 mills in three countries. *E. coli* O103:H2 was detected in 4.8% of the wheat flour samples using real-time *stx* PCR. These samples were milled in the same facility that 44.4% of contaminated samples originated from, suggesting poor hygienic conditions, or other persistent contamination sources. Similar to Mäde et al. (2017), detection was improved by

subsampling; in the majority of samples (55.6%), only one of the five subsamples tested positive. This suggests the need for development of a definitive subsampling methodology. In all flours surveyed, STEC was detected in nine (12.9%) samples, including spelt, rye, buckwheat, wheat and blends of flours.

2.2.4. *Escherichia coli* O121

E. coli O121 has recently been linked to illnesses caused by consumption of raw flour, (CDC 2016; Grant et al. 2011). It is a gram-negative bacillus that is enterohemorrhagic (EHEC) and produces signa toxins (STEC) (Grant et al. 2011). It is closely related to the well-known pathogen, *E. coli* O157:H7, and belongs to a group known as the 'Big Six', which includes the *E. coli* serovars O26, O45, O103, O111, O121, and O145. This group has been identified as causing 75% of non-O157 STEC illnesses (Grant et al. 2011).

E. coli adheres to and colonizes in the large intestine of humans, and produces Shiga toxins (*Stx*) that inhibit protein synthesis in the ribosome of the cell. The infectious dose of *E. coli* is low; as few as 10 cells can cause illness (Rose et al. 2012; Tuttle et al. 1999). Symptoms of the infection typically develop 3 to 4 days after ingestion and include diarrhea and severe abdominal cramps. Bloody diarrhea can also develop and can last as long as 4-10 days. In some cases, *E. coli* can cause hemorrhagic colitis and hemolytic uremic syndrome (HUS) (Grant et al. 2011; Montville et al. 2012; Sperandio and Nguyen 2012; Tuttle et al. 1999). Generally, between 3% and 9% of patients develop HUS, which causes red blood cells to rupture and induces kidney failure (Mele et al. 2014). Treatment for HUS typically requires blood transfusion. Antibiotics cannot be administered for treatment of *E. coli* infections, as they only encourage toxin production and place patients at higher risk of developing HUS (Sperandio and Nguyen 2012).

Portions of the population particularly vulnerable to infection include infants and elderly (Lund 2015).

2.3. Flour Food Safety

2.3.1. Flour Outbreaks and Recalls

Wheat flour was traditionally considered a low-risk food as it does not readily support microbial life due to its low water activity, and is usually only consumed after a cooking step has been implemented (Beuchat et al. 2013; Crowe et al. 2017; Sperber 2007). However, recent outbreaks related to raw flour consumption have raised concern about flour processing (Table 2).

Table 2. Outbreaks of foodborne illness associated with the consumption of flour and cereal grain products.

Product (source)	Pathogen	Year	Number of cases	Isolated from product?	Outbreak location(s)	Source
Wheat flour						
Flour (New Zealand)	*Salmonella* Typhimurium phage type 42	2008-2009	67	yes	New Zealand	McCallum et al., 2013
Flour (General Mills, Kansas City, MO)	*E. coli* 0121, *E. coli* 026	2015-2016	63	yes	USA (24 states)	CDC, 2015; Crowe et al., 2017; US FDA, 2017
Flour (Ardent Mills, Saskatoon, SK)	*E. coli* 0121	2016-2017	30	yes	Canada (6 provinces)	PHAC, 2017
Flour (Rogers Foods, BC)	*E. coli* 0121	2017	6	yes	Canada (1 province)	BCCDC, 2017; Beach, 2017; News Desk, 2017
Wheat flour product						
Cake mix, raw- in ice cream (USA)	*Salmonella* Typhimurium	2005	26	yes	USA (11 states)	Zhang et al., 2007
Frozen pot pies (flour could not be ruled out) (USA)	*Salmonella* serotype 1 4,5,12:i;-	2007	396	yes	USA (41 states)	CDC, 2008a; Mody et al., 2014
Prepackaged refrigerated cookie dough (USA)	*E. coli* 0157:H7	2009	80	yes	USA (31 states)	CDC, 2009; Neil et al., 2012
Dough mix, dry (USA)	*E. coli* 0157:H7	2016	13	yes	USA (9 states)	Gieraltowski et al., 2017
Cereal grain product						
Toasted oats cereal (USA)	*Salmonella* Agona	1998	400+	yes	USA (11 states)	CDC, 1998; Russo et al., 2013
Puffed rice or puffed wheat cereals (USA)	*Salmonella* Agona	2008	33	yes	USA (17 states)	CDC, 2008b; Hoffman et al., 2015; Russo et al., 2013
Puffed wheat cereal	*Salmonella* Mbandaka	2018	73	yes	USA (31 states)	CDC, 2018

Reprinted without changes from Harris and Yada (2018). Last updated 6/28/18.

Of particular interest was a multi-state an outbreak which occurred in 2016 in the USA, when shiga-toxin producing *Escherichia coli* O121 and O26 were linked to contaminated flour

(Crowe et al. 2017; Harris and Yada 2018). The outbreak occurred in 24 U.S. states and resulted in 63 reported infections, 17 of which were hospitalized, and one infection resulted in hemolytic uremic syndrome (HUS) (CDC 2016). Seventy-six percent of people interviewed after contracting the illness reported using flour in the previous week, 50% reported consuming raw dough or batter, and 3 children reported to have been in contact with the raw dough at restaurants. Investigators traced the source of the outbreak to a General Mills flour production facility. In the months following the outbreak, several recalls were issued effecting flour and boxed cake mix product lines.

A company may recall a food product to prevent outbreaks if internal testing reveals a potential risk to the public. Many recalls of wheat and wheat related products have occurred in recent years (Figure A1). One recall not listed in the table occurred on January 23, 2019, when General Mills issued a voluntary national recall of some of their 5lb Unbleached Flour product due to potential presence of *Salmonella*. While recalls often occur without causing consumer illness, companies who recall products face many severe consequences. Time and resources spent for a company to issue a recall and communicate with the public, as well as the collection and destruction of contaminated product can greatly impact the financial success of a company. Furthermore, the company's reputation is negatively affected, which can reduce its market share. Some companies may never fully recover from this loss of consumer trust.

The influx of flour related recalls have promoted an increase in advertisements and campaigns to teach consumers the importance of cooking flour completely before consumption, in addition to washing all hands and surfaces that come in contact with raw flour. Packaging on raw flour, refrigerated cookie dough, and frozen dough products currently display notices to

consumers to avoid consumption of raw dough or batter and to cook thoroughly before consumption.

2.3.2. Food Safety Compliance

The Food Safety Modernization Act (FSMA) was enacted into United States legislation in January, 2011. Aimed at holding food manufacturers to a higher standard throughout food processing procedures, the act gives the Food and Drug Administration (FDA), a stricter set of guidelines to ensure public safety (Taylor 2011). Focused primarily on the use of preventive controls, FSMA regulations require all food manufacturers to develop, implement, and record a series of procedures similar to Hazard Analysis Critical Control Point (HACCP) systems (Fowler 2013). HACCP builds on concepts of Good Manufacturing Practices (GMP). All food manufacturers are required to perform a full hazard analysis of their manufacturing systems and verify and implement techniques to prevent or minimize hazards (known as preventative controls). They must then develop monitoring systems to ensure hazards are reduced, and determine 'corrective actions' for processing when preventative controls are not achieved (Fowler 2013). All hazards identified in the hazard analysis step must have an associated preventative control measure. This poses a challenge for the flour milling industry, as pathogenic organisms present a hazard, and therefore require the industry to adopt preventative controls in their process.

Current milling practices do not utilize techniques which actively aim for microbial reduction (Berghofer et al. 2003; Sperber 2007). The FDA defines a processed food as, "Any food other than a raw agricultural commodity that has been subject to processing, such as canning, cooking, freezing, dehydrating, or milling" (Sperber 2007). In other food products, heat is commonly used as a method of direct inactivation for pathogens. However, in the milling

industry, processes involving heating of wheat or flour are generally avoided because of their negative effects on functional quality of the flour. While flour is considered a processed food, it is clear an effective kill-step process is not implemented during production. Development of a process that can actively reduce microbial populations while maintaining wheat quality is of interest to wheat processors.

Conversely, some individuals and organizations argue that further reduction of the microbiological profile of milled cereal grains provide no public health or food safety benefit because microbial populations observed on wheat grains are generally low, proper application of HACCP, GMP, sanitation practices, and regular monitoring are sufficient to mitigate risk. Microbiological testing of wheat can be extremely difficult, as incidences of many pathogens are quite low, and microorganisms are not equally distributed throughout the grain like they might be in liquid products such as milk. Sperber (2007) supports this claim with an example stating, "If a lot of food were contaminated at a level such that 1% of the analytical samples contained salmonellae, 300 samples would need to be tested to detect one positive sample at the 95% confidence level… such sampling plans are impractical". In addition, variability in technician skills and testing methods can result in differences of ±1 log in coliform counts, such as *E. coli* (Sperber 2007). These concerns and challenges are noteworthy for development of more safe flour production.

2.4. Vacuum Steam Pasteurization

Vacuum steam pasteurization (VSP) is a relatively novel processing technique which demonstrations promise in its ability to reduce microbial populations on low moisture foods, such as wheat, while preserving their quality. As its name implies, VSP utilizes a steam

application under the presence of vacuum. The theory of VSP can be better understood by referencing the saturated steam curve of water (Figure 2).

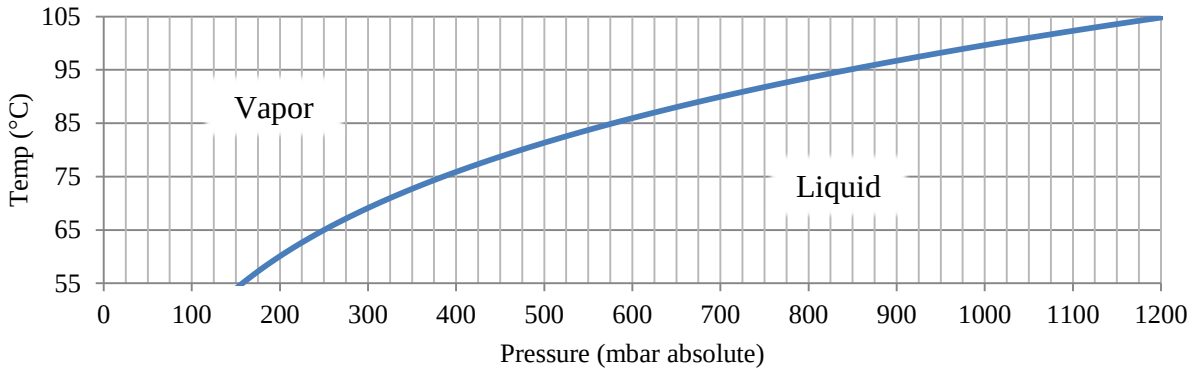

Figure 2. Saturated steam curve of water.

The line shown on the saturated steam curve of water indicates the boundary where water transitions between its liquid and vapor forms. As the pressure within a system decreases, the temperature at which water boils also decreases. At atmospheric pressure (1,000 millibar (mbar)) water boils at 100°C. Creating a vacuum in the processing chamber can lower the pressure below 1,000 mbar so that water below 100°C can remain in the vapor (or steam) form. For example, creating a vacuum to lower the pressure to approximately 400 mbar allows water molecules to remain steam at 75°C.

The vacuum within the chamber additionally helps to distribute steam throughout the chamber for even distribution of heat throughout the food product (Ivarsson 2011). Other studies involving processing techniques which apply both steam and vacuum, suggest a vacuum in the processing chamber allows the steam to penetrate porous regions of a food's surface where microbes may be hiding (Huang 2004; Morgan 1994; Morgan et al. 1996). Wheat bran has been characterized by its porous structure with pore sizes ranging from one micrometer to one nanometer (Jacobs et al. 2015). Morgan (1994) states that simple gas molecules are

approximately 10,000 times smaller than a bacterium, allowing gasses to easily enter pores where bacteria may be hiding. Another important role of the vacuum is to create a pressure gradient, which moves steam more quickly through the vessel to fill porous surfaces.

The technology works in four steps. First, the sample is preheated with warm, dry air to lessen the time required for steam pasteurization. Second, the product is inserted into the pasteurization vessel and a vacuum is applied. Next, steam treatment is applied under vacuum until target time/temperature parameters have been achieved. Lastly, the steam is turned off and a vacuum is applied to remove any remaining steam in the vessel and to help cool the product. Because the technology utilizes a closed chamber under which a vacuum is applied and must be opened and closed in order to rotate product, VSP is considered a batch, rather than continuous, system.

Steam is a desirable method of pasteurization because it uses moist heat, rather than dry heat, which is more effective for reducing pathogens (Anderson 2018; Grasso et al. 2014; Sperber 2007). The heat transfer observed with saturated steam is higher than that of other forms of steam, such as superheated steam or water, which results in in greater microbial reduction (Ivarsson 2011). Less moisture is applied to the product when processing with steam. Reduced moisture and lower temperature conditions promise less quality damage to the product, as lower heat can prevent protein denaturation. VSP could therefore be implemented into milling facilities as a preventative control and pathogen reduction while preserving the functional properties of the grain.

Shah et al. (2017) studied vacuum steam pasteurization of low moisture foods including whole flaxseed, quinoa, sunflower kernels, milled flaxseed and whole black peppercorns on commercially available pilot-scale VSP processing equipment. Samples were preheated to 40,

50, or 60°C and subjected to pasteurization temperatures of 75, 85, 95, and 105°C for 0.5, 1, 2, 3, 4, and 5 minute periods. The study concluded vacuum steam pasteurization was an effective method for reducing pathogens, *Salmonella* PT 30 and *E. coli* O157:H7 at temperatures as low as 75 °C and 85°C, depending on the product. Of the foods studied, sunflower kernels and flaxseed are most similar to wheat kernels. Flaxseed inoculated with 8 log CFU/g resulted in a >6 log reduction of *E. coli* O157:H7 after processing at 75°C for 1-5 minutes. In sunflower kernels, >6.4 log reduction of *E. coli* O157:H7 was observed at processing temperatures 85°C and above in as little as 30 seconds. These results show promise for VSP processing of wheat kernels.

To study the effects of VSP on product quality, Shah et al. (2018) studied the microbial and chemical shelf-life of whole and milled flaxseed after VSP processing. The samples were processed at 75, 90, and 105°C for 3 min, and 90°C for 9 min. Moisture content and water activity (a_w) initially increased after VSP processing, but decreased during storage (28 weeks) and were not significantly different ($p<0.05$) compared to untreated samples. The fatty acid profile, peroxide value and free fatty acid content of whole flaxseed were not affected by VSP processing during short pasteurization times and lower processing temperatures (75 and 90°C). Aerobic plate counts (APC), yeast, and mold counts were significantly reduced after pasteurization and throughout storage.

Newkirk et al. (2018) utilized a laboratory scale VSP system to determine the inactivation of *Salmonella enterica* and a surrogate, *Enterococcus faecium,* on whole black peppercorns and cumin seeds. A processing temperature of ~87°C was used, and results found 83 seconds of treatment were required to achieve a 5 log reduction of *Salmonella* on whole peppercorns, while only 73 seconds were required to achieve the same reduction in cumin seeds. The same group studied the effects of VSP on peppercorn and cumin seed quality for processing using both

sensory and analytical evaluations (Duncan et al. 2017). For the study, the samples were exposed to 82°C for 2 minutes. Sensory analysis found perceptible differences in odor of whole black peppercorns, which was supported with analytical evidence. In cumin seed, only slight differences were observed in color analysis.

Commercial VSP systems including Napasol®, Steripure®, Buhler®, and Log5® are used primarily for low moisture foods, and have all been validated according to the Almond Board of California for almond pasteurization. Use of VSP systems is most widely used in the nut pasteurization industry, but is also advertised for use with spices, dried fruits, powder blends, and other low moisture foods. Napasol® equipment is capable of processing up to 7,000 kg of product per hour in bulk containers (60 ft^3) that move through a series of processing steps along a conveyor system. Further advancement of commercial systems will result in increased demand of these processes. In the food-processing industry, steam is considered an energy-efficient source of heating, and VSP has been declared a processing treatment which allows, more precise control of temperature, no drying step, and homogenous treatment (Huang et al. 2013; Ivarsson 2011).

2.5. Alternative Pasteurization Methods

2.5.1. Wheat Kernels

Other pasteurization methods for a variety of low moisture foods have been studied and include baking, roasting, extrusion, fumigation, irradiation, radio frequency heating, and cold atmospheric plasma (Anderson 2018). Several microbial reduction techniques have been proposed specifically for wheat products. One strategy for pasteurization of wheat is to implement a kill step on whole kernels before milling (Sabillón et al. 2016). This would remove microorganisms located on the outside of the kernel before the kernel is opened and the

endosperm is exposed. In the milling industry, this is a desired method to prevent microbes from entering the milling facility where biofilms or other contamination may collect. Many proposed processes utilize a step already implemented in the milling process: tempering.

In some cases, chlorine is added to tempering water (600-700 ppm) with the intention of reducing microbial growth (Dhillon et al. 2007). Studies have proposed the addition of ozone or acidified ozone to tempering water to replace chlorine due to health concerns about residues left on the grain (Dhillon et al. 2007; Dhillon et al. 2009; İbanoğlu 2001; Spanoghe et al. 2016). Ibanoglu (2002) observed a ~1 log reduction using an ozone wash for yeasts, molds, and bacteria, while observing no significant differences in wheat quality, except for a small increase in dough extensibility. Dhillon (2010) utilized a wheat treatment with gaseous ozone, acetic acid, and ozonized water and found a 1.7 log reduction in bacteria with a 3.3 log reduction for yeast and mold counts. Use of organic acid and saline solutions in tempering water have also been studied and found that a combination of 5% lactic acid and 52% NaCl reduced APC by 4.3 log CFU/g (Sabillón et al. 2016). Potential residues remaining on flour or increased wear on milling equipment are some of the concerns for utilizing tempering water treatments.

2.5.2. Wheat Flour

An alternative strategy for wheat pasteurization is to implement a kill step after the milling process, on the wheat flour just before packaging (Hesseltine 1968). This strategy would control for additional contamination which might have occurred from equipment or human sources in the milling process. While each of the following studies found varying levels of success, all pose unique challenges for implementation on a commercial scale.

Cold plasma treatment has been studied for application on wheat flour, though no change in bacterial or mold counts were observed (Bahrami et al. 2016). This was hypothesized by the

researchers to be a result of the low moisture and low treatment levels, and that perhaps different treatment levels might have had an alternative effect. Treatment with cold plasma resulted in significant acceleration in lipid oxidation of the flour.

Irradiation techniques are traditionally used in wheat on the whole kernel to eliminate insects during storage, though some studies have evaluated its effects on flour (Rose et al. 2012). Studies using radiation in flour displayed a decrease in dough quality parameters, especially dough development time, and were not acceptable in consumer sensory panels (Köksel et al. 1998; Zaied et al. 1996). Hanis et al. (1988) utilized 1 kGy of radiation and observed a 2 log reduction of microorganisms, while treatments of 10kGy and higher completely eliminated bacteria. Though an effective method of microbial reduction, detrimental effects on quality and potential rejection by consumers because of a fear of 'irradiation' of foods limit its viability in the industry.

Other forms of radiation, perhaps more acceptable by consumers have been studied. Ultraviolet radiation alone did not result in a microbial reduction in wheat flour, however in combination with ozone treatment, a ~2 log reduction was observed (László et al. 2008). Pulsed electric fields were studied on rye flour and observed a 0.6 log reduction of APC with the application of field strengths greater than 20kV/cm (Keith et al. 1998). Microwave radiation has not been studied in wheat flours, perhaps because heating is uneven (Rose et al. 2012).

On a commercial scale, multiple solutions for wheat flour pasteurization are available. These include a verified kill step in which the produced flour can be considered a ready-to-eat (RTE) food. Buhler® offers an "ebeam" technology utilizing low energy electron beams for reduction of microbes in flour. Honeyville's TempSure® is a dry heat process which is applied after the product has been packaged. SimplySafe™ by Bay State Milling is a heat process used

for many other grains, but has not yet been validated for wheat products. Ardent Mills' SafeGuard® is a proprietary process that offers solutions for large and small scale applications and promises maintenance of acceptable gluten functionality. Another heat-treatment option for flour, Safesteril, is offered by Seimer's Milling Company. Safesteril offers a steam treatment followed by a flash cooler to quickly reduce temperature after treatment.

2.6. Effects of Heat Treatment on Wheat Quality

The effect of heat treatment on wheat quality has been studied for many years, with some publications dating as far back as the late 1920's. Traditionally, studies evaluated the efficacy of heating wheat kernels for the purposes of drying (Ghaly and Taylor 1982), or reduction of infestation by insects before storage of the grain (Dermott and Evans 1978; Ghaly and Van Der Touw 1982). More recent studies focus on improvement of functional properties of the wheat in dough and batter systems for product improvement (Chiqurupati and Pulverenti 1994; Gelinas et al. 2001; Nakamura et al. 2008; Ozawa et al. 2009; Ozawa and Seguchi 2006).

A fluidized-bed with air temperatures of 60, 70, 80, 90, and 100°C was designed by Dermott and Evans (1978) to study the disinfestation of various insect species in wheat. Though the air temperatures were much higher, after exposure for 12 minutes or less with temperatures up to 80°C, the grain surface temperature never exceeded 65°C. Quality tests concluded the treatment had no adverse effects on baking quality when heating with 80°C air for up to 30 minutes. Heating at 90°C for 15 min affected Extensograph and Viscograph results, but no adverse effects were observed in baking quality until heating at 100°C for longer than 5 minutes. Flour yield, calculated during milling, was unaffected by all treatments.

Ghaly and Taylor (1982) studied the effects of heating two wheat varieties for the purposes of drying. They exposed the grain at two moisture levels to temperatures of 60, 80, 100,

and 120°C for times ranging between 5 and 120 min using a fluidized bed for dry heat application. It was concluded that 60°C treatments did not cause significant differences for baking, germination or turbidity tests. They did, however, see significant differences in any sample heated at 80°C or higher. Additionally, it was concluded that temperature of the treatment, rather than the time of treatment, was the determining factor in the results. Grain with a lower moisture content (12%) showed less damage than grain with higher (14%) moisture content. Significant differences between varieties of wheat were also observed.

Investigating the impact of heat on both starch and protein fractions in flour, Ozawa et al. (2009) found heat had no significant effect on starch fractions, and that changes in flour properties could be attributed to alterations in gluten proteins. To perform the study, flour was heated with dry heat at 120°C for 120 min, and amylograph testing showed significant differences compared to the control when the entire flour sample was analyzed. However, after visual inspection of starch granules under a microscope, the starch did not appear to have been changed by processing. This suggested the changes in amylograph tests were not due to the starch, but gluten fraction of the flour. To investigate, the researchers separated the gluten fraction from both the heated and control flour samples, and re-ran the test. Amylograph results were un-changed between control and heat-treated flours. This suggested the increased viscosity observed by amylograph analysis in the entire flour sample could be attributed to the gluten fraction, which was altered at high temperatures (Ozawa et al. 2009).

Alternatively, heat-treating flour has been used as a technique to improve functional qualities in flour systems. Upreti et al. (2013) filed a patent for a process, which first dehydrates, then heats flour up to 260-350°F for between 2 and 56 minutes, which serves to reduce adhesiveness, stickiness, and/or increase dough strength. Similarly, Ozawa and Seguchi (2006)

investigated the effect of heat treated wheat flour on pancakes and found heat treatment improved springiness. Wheat flour is sometimes subjected to chlorine treatment to improve batter expansion and viscous properties in cake batters. After heat treating flour at 120°C for between 1 and 5 hours, Seguchi (1990) noted flour properties were similar to chlorinated wheat.

Schofield et al. (1983) studied the baking performance of gluten after heating. They washed gluten from flour, and heated it with a water bath between 55-75°C for 10 minutes, then freeze dried the glutens, and re-milled the freeze-dried portion. A majority of the functionality of the gluten was destroyed after heating above 75°C. They observed a decrease in gluten functionality within 2-3 minutes of heating, with no further loss in baking quality with prolonged heating at the same temperature. Additionally, they stated chemical changes occur in the glutenin protein fraction between 55 and 75°C, and above 75°C, gliadin proteins are affected. It was hypothesized that heating promotes the unfolding of the protein structure by, "facilitating sulfhydryl/disulfide interchange between exposed groups, and the protein is then 'locked' into the denatured state on cooling due to this disulfide bond rearrangement."

It is widely accepted that moist heat, rather than dry heat, is a more effective for microbial reduction, however, the use of moist instead of dry heat can also affect wheat quality. Sudha et al. (2016) compared the effects of quality between moist and dry heat in an attempt to improve the functionality and immunogenicity of whole wheat flour. The moist treatment consisted of steaming flour for 30 minutes, then drying it at 60°C for 2 hours, while the dry heat treatment consisted of heating at 100°C for 2 hours. Moist heat treatment reduced water absorption from 75% to 56%, and increased peak viscosity from 467 to 778 BU. A decrease in elasticity was observed in moist treated flours, and had a significantly lower loaf volume during

baking. The study concluded moist heat treatments had a greater influence on protein in flour than dry heat treatments.

While many studies characterize the effects of heat treatments on wheat quality and functionality, few recent studies have been published that utilize heat as a technique for microbial reduction while studying the effects on wheat quality.

3. OBJECTIVES AND NEEDS STATEMENT

3.1. Needs Statement

Wheat flour is an agricultural product, which does not undergo a kill step that would effectively reduce microbial populations during the milling process. While flour is not intended to be consumed as a RTE food, some products such as raw cookie dough, frozen doughs, or cake batters are often tasted by consumers before baking. Additionally, failure to properly wash hands and surfaces after contact with raw dough can lead to cross-contamination of other foods. An increase in outbreaks and recalls associated with wheat flour and wheat flour products, as well as pressure from new legislative regulations have presented the milling industry with a new challenge. Traditional pasteurization methods utilize high temperatures to reduce microbial populations; however, high temperature treatment can greatly reduce the functional properties of wheat flour. Development of a process that can actively reduce microbial populations while maintaining wheat quality is of great interest to wheat processors. VSP utilizes reduced temperature and moisture conditions, and shows potential as a processing technique that could be implemented in commercial milling facilities.

3.2. Research Objectives

The objective of this research is to investigate the efficacy of vacuum steam pasteurization on HRS wheat before milling. The following list outlines the major objectives of this research.

Objective 1: To determine VSP processing conditions (time and temperature) which preserve overall quality of hard red spring wheat.

Objective 2: To determine the overall microbial reduction of *E. coli* O121 that can be achieved under the same VSP conditions that preserve quality in hard red spring wheat.

4. MATERIALS AND METHODS

4.1. VSP Equipment Design

The VSP equipment used for this study was located in the Van Es building on the North Dakota State University campus in Fargo, ND. It was originally used at the Food Refrigeration and Process Engineering Research Centre at the University of Bristol, UK as a Steam Decontamination Rig. It was comprised of 4 main components: vacuum pump, condensing chamber, pasteurization vessel, and steam generator, which are shown in order from left to right in Figure 3.

Figure 3. VSP unit comprised of 4 main parts.

The steam generator (Wagner Power Steamer Model 705, Wagner Spray Tech Corp., Minneapolis, MN) was connected to a 1 cm diameter port in the base of the pasteurization vessel with plastic tubing insulated with a foam sleeve. A manually adjustable ball valve was used to control steam supply to the unit. To measure the temperature within the vessel, three Type T Wire Probe thermocouples (Sealed Teflon Tip, T-37X-T, ThermoWorks, American Fork, UT, USA) were used. Each thermocouple connected to a HOBO Thermocouple Data Logger (#UX100-014M, Onset Computer Corp, Bourne, MA, USA). The thermocouples were set to record the temperature every 15 seconds once started. The thermocouples were inserted through

a 1 cm port in the baseplate of the pasteurization vessel and sealed with a rubber bung and parafilm.

An 80 AV 3 Busch vacuum pump (Busch Manufacturing, LLC, Virginia Beach, VA, USA), connected to the baseplate of the pasteurization vessel with rubber tubing, and a right angle, lever operated, stainless steel vacuum valve (PV16MKS Isolation Valve, Edwards Vacuum LLC, USA) was used to pull a vacuum in the chamber. To measure the vacuum, a 910 DualTrans MicroPirani/Absolute Piezo Vacuum Transducer pressure gauge (MKS Instruments, Andover, MA, USA) was used. All pressure values were recorded in millibar (mbar) absolute, rather than gauge pressure.

To cool the air and prevent vapor from entering the pump, a condensing chamber was used between the VSP vessel and the pump. Depending on the projected length of the run based on target temperature, either a small (1 ft. diameter) or large (2 ft. diameter) chamber was used to hold more or less ice, as required by processing. The chambers were made of clear plastic and spherical in shape with two ports opposite each other. Ice sitting atop a false bottom filled the chamber, which was emptied and re-filled between each treatment.

The pasteurization vessel consisted of a metal baseplate and a large glass bell. The baseplate had a diameter of approximately 15 inches with four ports for air removal, thermocouple access, steam addition, and to monitor pressure. The glass bell, with a rubber strip along its bottom, sat on top of the baseplate to form a seal. The bell was approximately 12 inches wide and 18 inches tall.

Inside the vessel, a system was optimized for pasteurization of 1 kg batches of wheat. First, a copper piping system was designed which would evenly distribute steam throughout the bell-shaped vessel. A malleable copper pipe was fitted to the steam port in the metal baseplate of

the VSP system. The copper pipe directed steam to the top of the vessel. At the top, several branches were created to further disperse the steam. Though the steam almost instantly fills the entire vessel, thereby reaching the sample from all directions, this reduced the bias of the hottest steam contacting only one side of the sample. For sample containment, a metal autoclaving basket 10 inches in diameter and 6 inches tall, was used and placed on a plastic stand. To line the basket, window screen mesh was shaped to fit the basket dimensions. While processing, 1 kg of wheat was placed in the basket lining. The entire system, before the glass bell has been placed to form the vessel, is shown in Figure 4.

Figure 4. Pasteurization vessel arrangement in VSP.

4.2. Wheat Quality

4.2.1. Materials

Multiple varieties of HRS samples were obtained for a final wheat blend containing 29.5% Linkert, 29.5 % Glenn, 15.2% SY Soren, 9.8% Elgin-ND, 9.5% ND VitPro, and 6.5% SY Ingmar varieties. All samples were from the 2017 crop year. The mixture was homogenized on an American Process Systems homogenizer (Gurnee, IL, Type FPB-005). The wheat was cleaned for processing and milling on a Carter Day Dockage Tester with a number eight riddle.

Samples were divided into cotton bags in 500g aliquots and placed in cold storage (4°C) inside a plastic tote until processing.

4.2.2. VSP Procedure

The day before processing on the vacuum steam pasteurizer, samples were removed from cold storage and allowed to equilibrate to room temperature. Just before processing, samples were preheated from room temperature to 40±4°C with forced, dry air in a GCA/Precision Scientific Oven (American Sterilizer Company, Erie, PA, USA). The 1 kg sample took approximately 35 minutes to pre-heat. No sample preheated for longer than 50 minutes.

Preheated samples were removed from the oven and immediately placed on the vacuum steam pasteurization unit. Three wire-type T-37X-T Type T waterproof thermocouples (Thermoworks, American Fork, UT, USA) were placed in the bed of wheat (Figure 5). Two thermocouples were placed together in the center of the sample to verify internal sample temperature. The first thermocouple placed in the center of the grain was used as the reference thermocouple for temperature data collected throughout processing. A third thermocouple was placed further from the center to record temperature variations throughout the wheat bed. HOBO Thermocouple Data Loggers (ONSET, Bourne, MA, USA) were used with each thermocouple to record processing temperatures.

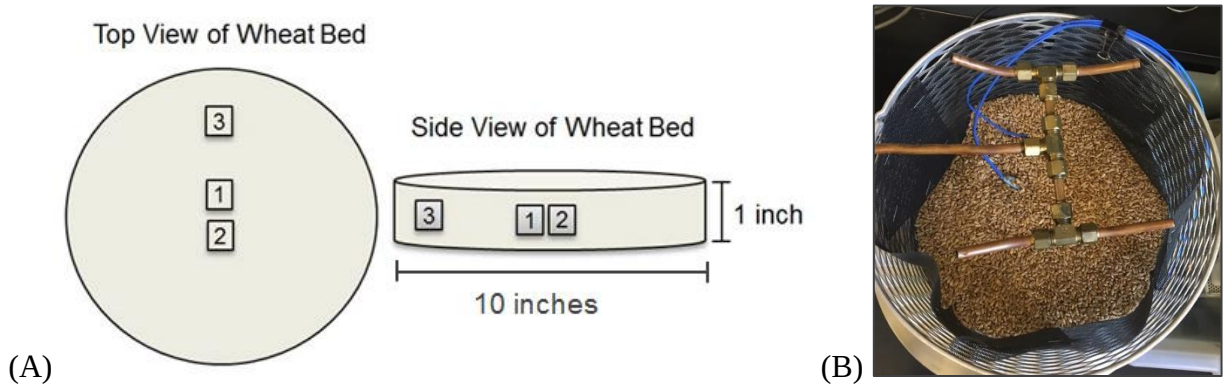

Figure 5. Diagram detailing thermocouple placement within wheat sample during processing. (A) Numbers 1, 2, and 3 indicate the three thermocouples (B) Blue thermocouples can be observed.

While the sample was being obtained from the oven, placed in the vessel, and thermocouples were arranged, the steamer was allowed to pre-heat for 5 minutes. Before heating, the steamer was filled with distilled water to the same volume using a marked fill line ¾ high on the outside of the steam vessel.

Table 3 shows the processing parameters which were met for each of the sample treatments. Because a single target pressure cannot be maintained, a pressure range was established using protocol contained in the VSP operations guide.

Table 3. Processing parameters for vacuum steam pasteurization in wheat quality study

Target Temperature (°C)	Target Pressure (mbar absolute)	Pressure Range (mbar absolute)		Treatment Time (min)
		Low	High	
65	250	230	275	4, 8
70	312	287	341	4, 8
75	386	356	421	4, 8
85	578	536	627	4, 8

To start the processing run, a vacuum was applied until the pressure reached 200 mbar absolute or lower. Then, the steam valve was fully opened. At the same time, the vacuum was partially closed- allowing the pressure in the vessel to remain between 770 mbar and 840 mbar absolute. As the temperature inside the wheat bed reached the desired treatment, the vacuum

valve would be slowly opened to create a stronger vacuum within the chamber. This was a delicate process because the more vacuum that is applied to the system, the more heat from the system is removed, and the cooler the wheat sample becomes. The balance between steam addition and vacuum application took several minutes to reach equilibrium. Once the target temperature was reached, the timer to record treatment time (4 or 8 minutes) would begin. The target pressure range was reached in the following 30 seconds or less. The run was considered valid so long as the temperature stayed within ±3°C of treatment target temperature for the entirety of the treatment time. If the system was losing heat too rapidly, steam was added and the pressure was allowed to extend above target range, so long as it was returned to target conditions within 30 seconds. Treatment time refers only to the amount of time the sample was subjected to target temperature processing conditions. Total processing time for the samples varied because the amount of time required to heat the sample to the target temperature varied.

Once the processing time was completed, the steam was turned off, and the vacuum left completely open to lower the pressure in the vessel between 125-180 mbar absolute. The grain was immediately double bagged in sealed plastic bags, and analyzed for moisture and test weight observations (GAC 2100b, Dickey John Corporation, Auburn, IL, USA). If necessary, samples were left to dry overnight to 14% moisture, approximately 20 hours, and analyzed for moisture and protein analysis (Infratec 1241 Grain Analyzer, Foss Analytical, Denmark) before being placed in cold storage to store for milling.

4.2.3. Milling

Samples were tempered to 16% moisture 18-24 hours before milling. Twenty minutes before milling, samples were tempered to 16.5% moisture. Samples were milled randomly over the course of three consecutive days on a Bühler MLU-202 mill (Bühler Industries, Uzwil,

Switzerland) according to AACCI Approved Method 26-21.02 (AACCI 2009). Milling conditions were maintained at 68% relative humidity and 22±1°C. Milling extraction was calculated by dividing the weight straight grade flour produced from the weight of the initial grain sample, and is expressed as a percent. A profile of the particle size of the flour can be found in Figure A4.

4.2.4. Flour Analysis

After milling, the flour sample was allowed to rest for 10 days before analysis. Proximate analysis of the flour was determined. Moisture content was analyzed using the AACCI Approved Air Oven Method 44-15.02 (AACCI 2009). Ash was analyzed using AACCI Method 08-01.0 and results were adjusted for 14% moisture basis (AACCI 2009). For protein analysis, the Dumas method (LECO FP628, St. Joseph, MI, USA) was used for nitrogen content, and protein was calculated based on a 14% moisture basis as detailed in AACCI method 46-30.01 (AACCI 2009). Flour color was analyzed using the CIE 1976 L*a*b* scale on a Minolta Chroma meter (CR-410, Konica Minolta, Ramsey, NJ, USA) with the Granular-Materials Attachment CR-A50.

To study starch pasting properties, Rapid Visco Analysis (RVA) was completed as detailed in AACCI Method 76-21.01 using a Perten RVA 4500 machine (Perten Instruments, Springfield, IL, USA) (AACCI 2009). For functional characteristics of the protein, wet gluten and gluten index were analyzed on a Glutomatic and Gluten Index Centrifuge (Perten Instruments, Springfield, IL, USA Model GM 2200, and CF 2015, respectively) according to AACCI Method 38-12.02 (AACCI 2009). Starch damage and total starch procedures followed AACCI Methods 76-31.01 and 76-13.01, respectively, utilizing kits purchased from Megazyme (Megazyme International Ltd., Bray, Ireland) (AACCI 2009).

Farinograph analysis was used to study water absorption, peak time, and mixing stability of the dough according to AACCI Method 54.21.02 (AACCI 2009) with a 50 g bowl attachment. Tests were conducted on the Brabender Farinograph E- software version 4.0.3 (C. W. Brabender, Duisburg, Germany).

Apparent enzymatic activity for α-amylase, xylanase, and polyphenol oxidase were studied. Megazyme kits (Megazyme International Ireland Ltd., Bray, Ireland) were utilized for these analyses. Polypheonl oxidase (PPO) analysis was performed using whole kernel samples, and procedures were followed as detailed in AACCI Method 22-85.01 (AACCI 2009; Anderson and Morris 2001). Absorbance was read at 475 nm in cuvettes with a spectrophotometer (DR/4000 U, Hach, Loveland, CO, USA).

Whole wheat flour samples were ground from kernels using Udy Cyclone Sample Mill (UDY Corporation, Fort Collins, CO, USA) for xylanase and α-amylase activity testing. Xylanase activity testing utilized the T-XAX Megazyme kit, with modifications as described by Courtin et al. (2005). Xylanase was extracted from the flour using sodium acetate buffer under refrigeration, then centrifuged at 3500 RPM for 20 minutes. AZCL-AX tablets were incubated with the samples for 17 hours, then filtered, and read for absorbance at 595nm using a microplate spectrophotometer (Mulitskan Ascent, Thermo Electron Corportation, Beverly, MA, USA). Standards were prepared with xylanase from *Aspergillus niger* at 250, 500, 750, 1,000, 5,000, 10,000, and 20,000 times dilutions (from 260mU/mL). The standards were plotted for absorbance and concentration, and the resulting linear trend line formula was used to calculate sample concentrations in units of mU/mL.

AACCI Method 22-05.01 was used for α-amylase measurement (AACCI 2009). Sodium maleate buffer was used to extract α-amylase in a heating block (Reacti-Therm III, Thermo

Scientific, Waltham, MA, USA) with amylazyme tablets from the Megazyme kit. After the reaction was stopped with Trizma base solution, absorbance was measured at 590 nm in cuvettes with a spectrophotometer (DR/4000 U, Hach, Loveland, CO, USA). The α-amylase activity was calculated as follows:

$$\text{Units/g} = ((122 \times \text{absorbance} + 4.3) / \text{sample weight}) \times 2 \times (0.001)$$

4.2.5. Baking Analysis

Bread was baked using a straight dough method and two-hour fermentation, according to AACCI Method 10-10.03. Samples were baked over the course of two consecutive days. During baking, absorption, mixing time, fermentation height, oven height, and weight were recorded. Evaluations for appearance, color, and crumb of bread followed AACCI Method 10-12.01. Additional analysis for bread texture was analyzed using a texture analyzer (TA-XT2i, Texture Technologies Corp, Scarsdale, NY, USA), using a cylindrical, 1 inch diameter, 35 mm tall, acrylic probe.

4.3. Microbial Reduction Experiment

4.3.1. Homogenous Testing

Before the experiment was run, it was important to verify that inoculation procedures were adequate to uniformly distribute the *E. coli* 0121 inoculum on the entire wheat sample. To test the procedure, 600 g of HRS wheat was inoculated, and randomly sampled eight times. Samples were then diluted with Butterfield's buffer, homogenized for 90 seconds with a stomacher (Masticator, IUL Instruments, Spain), further diluted to appropriate levels, and spread plated in duplicate on HiCrome ECC Selective agar plates (Sigma-Aldrich, St. Louis, MO, USA). The plates incubated at 37°C for 24 hours, and were counted. The procedures proved sufficient for homogenous inoculation.

4.3.2. a_w Equilibration

A concentrated liquid inoculum was used to inoculate the wheat sample before VSP processing. Applying the liquid inoculum increased the water activity (a_w) of the wheat sample. It is known that changes in water activity can alter the heat resistance of bacteria during processing (Archer et al. 1998), thus it was desired to adjust the wheat sample to its original water activity to test native conditions. To do this, the a_w of the wheat sample before inoculation was measured using an Aqualab 4TE a_w meter (Aqualab, Pullman, WA). After inoculation, the entire wheat sample was placed in a sterilized stainless steel tray (12in x 9 in). In a plastic weight boat, 25 g of Lithium chloride anhydrous 99% -20 mesh (Alfa Aesar, Ward Hill, MA) was added with water until the salt was just saturated. Lithium chloride acts as a desiccant in air systems. The tray containing the wheat sample and the weight boat with the salt solution were placed together in a sealed container (Coleman cooler 24in x 16 in, Coleman Company, Inc., Wichita, KS, USA). After 24 hours, the a_w equilibrated to the initial level, and the salt solution was removed from the container. The samples were processed 48 hours after inoculation.

4.3.3. Inoculation

For the microbial reduction study, *Escherichia coli* O121:H19 (TB0475) was obtained from the collection at Michigan State University (East Lansing, MI, USA). The strain was stored at -80°C in Brain Heart Infusion (BHI) broth with 15% glycerol. First, the culture was streaked on BHI agar (Criterion, Hardy Diagnostics, Santa Maria, CA, USA) and incubated at 37°C for 24 hours. One isolated colony from the agar was then transferred to 5mL BHI broth and incubated for 20 hours at 37°C. After incubation, 250µL of the BHI broth was dispensed onto 14 BHI agar plates, (100 mm x 15 mm), respectively. After spread plating with sterile hockey sticks, (Fisher Scientific, Waltham, MA) the plates were incubated for 24 hours at 37°C. The lawn of bacteria

on the surface of the plates was collected using a sterile hockey stick spreader, and suspended in 3 mL sterilized, distilled water creating a liquid suspension inoculum. This allowed enough bacterium to achieve ~8 log CFU/g on the inoculated wheat sample. The liquid inoculum and 600 g of wheat sample were poured into a large Whirl-pak bag (Nasco, Fort Atkinson, WI, USA) and shaken/massaged vigorously for 5 minutes. Prior to inoculation, the HRS wheat sample from the freezer was allowed to equilibrate to room temperature, and the a_w of the wheat before inoculation was recorded.

From the inoculated grain, 4 samples, 11 g each, were randomly sampled, diluted with Butterfield's buffer, homogenized for 90 seconds with a stomacher, spread plated at the appropriate dilution in duplicate on HiCrome ECC Selective agar plates. Plates were incubated at 37°C for 24 hours, and counted. These counts were used to verify homogenous inoculation for each replicate. The remaining inoculated grain was left in a sealed container with Lithium chloride salts, as described in section 4.3.2 above.

After the wheat sample's a_w was equilibrated, the sample was divided into 25 g portions and placed in 4in x 6.5 in muslin drawstring bags (Uline, Pleasant Prairie, WI, USA). Three bags containing 25 g of inoculated grain were used in each processing run, representing biological replicates. Each processing parameter was repeated in triplicate, representing technical replicates, for a total of 9 data points for each processing parameter.

4.3.4. VSP Procedure

VSP processing for the microbial analysis determination was largely similar to the procedure for wheat quality analysis, described in section 4.2.2 above. However, microbial reduction experiments were only performed at 65°C target temperature for 0, 2, 4, 6, and 8

minute treatment times. The target pressure to be maintained in the VSP unit was 250 mbar absolute, with the pressure range to be maintained between 230-275 mbar absolute.

It was desired to study the microbial reduction of *E. coli* O121 at the same processing conditions created during the wheat quality experiment. To simulate the 1 kg sample size processed for wheat quality analysis, 925 g of un-inoculated hard red spring wheat was added to a metal autoclave basket lined with window screen and pre-heated from room temperature to 40±3°C (approximately 35 minutes). After pre-heating, the basket was removed from the oven, and 3 cotton bags containing 25 g of inoculated seed were buried within the bed of grain (Figure 6).

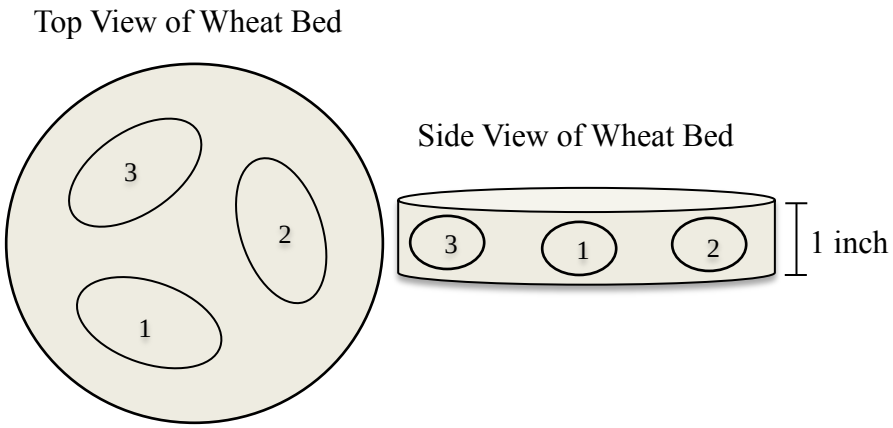

Figure 6. Diagram detailing inoculated wheat sample bag placement within wheat sample.

Methods describing inoculation procedures for the wheat are described in section 4.3.3. Three thermocouples with data loggers were utilized to monitor temperature conditions at 15 second intervals within the bed of wheat and were arranged in the same placement as the wheat quality experiment (Figure 5).

After the glass bell was placed over the sample on the baseplate of the pasteurization vessel to create a sealed vessel, an initial vacuum was created. Steam, which had preheated for exactly 5 minutes before operation, was allowed to fill the chamber between 770 and 840 mbar

absolute. When the temperature reached a target of 65°C, the timer began for 2, 4, 6, or 8 minutes. Throughout the processing time, the pressure within the vessel was required to remain within the target pressure range, unless the temperature of the sample was at risk to drop below target temperature (65±3°C), in which case steam addition was allowed so long as target processing conditions were achieved again in 30 seconds. For samples with a treatment time of 0 minutes, the VSP was allowed to heat until thermocouples in the bed of wheat read 65°C, after which the sample was immediately removed from the vessel. This allowed for analysis of microbial reduction that resulted from the "Come-up" process alone. Once the processing time was completed, a vacuum below 180 mbar absolute was created to cool the sample and remove excess moisture.

4.3.5. Plate Counts

After VSP processing, the muslin bags containing the inoculated grain were immediately removed from the processing vessel, contents were transferred into a sterile 13in x 7 in Whirl-pak bags (Nasco, Fort Atkinson, WI, USA), diluted with Butterfields buffer, and homogenized in a stomacher (Masticator, IUL Instruments, Spain) for 90 seconds. In addition to samples processed for 0, 2, 4, 6, and 8 minutes after reaching the target processing temperature of 65°C, a sample of inoculated wheat that was not processed at all, but had undergone a_w equilibration was plated as a control for initial log CFU/g counts. Serial dilutions were manually spread plated in duplicate on HiCrome ECC Selective agar plates (Sigma-Aldrich, St. Louis, MO, USA). Plates were incubated at 37°C for 24 hours, and counted on a Q-Count reader model 350 (Advanced Instruments Inc., Norwood, MA).

4.4. Statistical Analysis

4.4.1. Wheat Quality

All VSP processing conditions were repeated in triplicate. Four control samples were analyzed, and were all averaged for mean data presented. For optimal VSP operation, all similar temperature treatments were performed in increasing temperature. For example, all 65°C treatments processed first, with the replicates and processing times completed in a randomized order. The 70°C, 75°C, and 85°C treatments were processed in the same manner, in increasing order. Completely Randomized Design (CRD) was used. Mean separation and least significant difference tests were utilized to indicate significant differences (p<0.05) between treatments using one-way Analysis of Variance (ANOVA) in SAS for Windows, Version 9.4 (TS Level IM4). A two-way ANOVA was also performed to analyze the interaction of temperature and time in treatments. Control samples were not included for two-way ANOVA, so error values from the one-way ANOVA were utilized to more accurately calculate F-values. Correlation analysis was performed using the 'CORR' procedure.

To better study non-linear trends in data, Logistic Power (Sigmoid model) and Farazdahi-Harris models (Yield Spacing model) were calculated in Curve Expert Professional (Version 2.6.3). Equations for the models are as follows:

$$Logistic\ power: y = \frac{a}{[1 + \left(\frac{x}{b}\right)^{c}]}$$

$$Farazgdahi - Harris: y = \frac{1}{(a + b * x^{c})}$$

4.4.2. Microbial Reduction

In total, three biological and three technical replicates were processed and plated in duplicate. Duplicate plate counts were log transformed and averaged in Microsoft Excel.

Completely Randomized Block Design (CRBD) was used. One replicate of each treatment was completed on different processing days. Mean separation and least significant difference tests were utilized to indicate significant differences ($p<0.05$) between treatments using one-way Analysis of Variance (ANOVA) in SAS for Windows, Version 9.4 (TS Level IM4).

5. RESULTS AND DISCUSSION

5.1. Preliminary Experiments

Commercial vacuum steam pasteurization equipment often utilizes a pre-heating step before VSP processing to reduce overall processing time and preserve product quality by reducing exposure to moisture from steam. In preliminary trials optimizing the laboratory-scale system to process 1 kg samples of wheat, it was observed that the time to raise the temperature of the grain from room temperature to our desired temperature range was too long; over 40 minutes for one sample. The ice in the condensing chamber used to precipitate water in air before reaching the pump would melt during the long processing times. Without enough ice, steam would directly enter the vacuum pump, potentially causing damage. Thus, it was desired to use a preheating step in this experiment to raise the temperature of the grain before reaching the VSP system to reduce VSP processing time for more optimal processing using the laboratory scale system. However, before implementing the preheat processing, it was important to verify that the preheating step itself did not result in a loss of quality or functionality of the grain.

To ensure the preheating processing stem would not cause quality loss, a preliminary experiment was performed. To run the experiment, 1 kg samples of grain were separately placed in metal autoclave baskets lined with window screen meshing. A GCA/Precision Scientific Oven (American Sterilizer Company, Erie, PA, USA) was used to preheat samples until they reached 40°C. After reaching 40°C, basket of wheat (1 kg) was removed at 15 minute intervals between 0 and 60 minutes. The pre-heated wheat samples were then milled and farinograph and RVA analysis was performed. Farinograph and RVA quality tests were selected as they would allow a basic check that both the starch and gluten functionality would not be changed. Results from the preliminary experiment validating that the 40°C preheat step did not alter quality of the wheat are

displayed in Figure A2 and A3. No practical changes in farinograph results were observed. Absorption values ranged from 66.6 to 67.6, and stability time ranged between 7.4 and 9.3 min for all samples (Table A1). This indicated gluten proteins were not affected by the preheating step. Likewise, no practical changes were observed in RVA analysis (Table A2). Peak viscosity for all samples ranged from 1,869 to 1,951 cP, and final viscosities ranged between 2098 and 1295 cP, indicating the starch fraction of the wheat was not affected during the preheating step. These results indicated that the pre-heat treatment of 40°C was acceptable for use before VSP treatment to reduce processing time for up to 60 minutes of preheating.

5.2. VSP Processing

The 1 kg sample took approximately 35 minutes to pre-heat using dry forced air. No sample preheated for longer than 50 minutes. As expected, the processing time in the VSP unit varied based on the target temperature and treatment time (4 or 8 minutes). The amount of time required to increase the temperature of the grain from 40°C to the target temperature (65°C, 70°C, 75°C, or 85°C) is referenced as 'Come-up-Time', though it is also sometimes referred to as the 'lag' time (Table 4). The average 'Come-up-Time' for 65°C, 70°C, 75°C, and 85°C treatments was 9.71, 11.67, 15.04, and 21.04 min, respectively. Increasing the target temperature of the treatment by 10°C required an additional ~5 min of processing time. The 'Total Run Time' expresses the entire processing time when the grain was in the VSP system. It combines the 'Come-up-Time' preheating time, plus the target treatment time of 4 or 8 minutes. For all samples, Total Run Time ranged between 11.50 minutes and 29.25 minutes (Table 4)

For all treatments, variation in processing time between replicates (n=3) deviated by between 0.50 and 3.04 minutes. This variation is generally quite minor, and was expected as processing conditions changed slightly throughout the day, and across multiple days of

processing. For example, atmospheric pressure varied between days and with weather patterns, which may have affected the vacuum pump and the time required to lower the pressure in the processing chamber. Additionally, the temperature of the room where processing occurred changed, which may have affected the system's ability to retain heat. Before the grain was placed in the VSP system it was preheated with dry air to ~40°C. For all samples, the lowest and highest temperature of the wheat placed in the system was 35.87°C and 42.48°C, respectively. Slight variations in the temperature of the grain (±4°C) when placed in the VSP system could account for some variability.

As the treated flour was being analyzed for quality, it was observed that one of the sample replicates treated at 75°C for 4 minutes was a continual outlier showing increased gluten degradation. It was hypothesized that an error in processing resulted in this discrepancy. However, replicate treatments at 75°C for 4 min had the second lowest standard deviation in processing time compared to all other processing conditions, and processing data collected by the thermocouples in the system did not indicate deviations in the process. The two thermocouples placed in the center interior of the bed of grain were used to indicate the temperature of the wheat during processing and reached 75°C temperatures similar to other samples. A third thermocouple placed closer to the outer rim of the bed of wheat measured the gradient of temperature throughout the bed of wheat. The third thermocouple recorded temperatures of ~80°C for 1.5 minutes during processing. This was similar to the temperature variation observed for all other treatments, when the outside edges of the bed of grain were sometimes observed to be ~5°C higher than the internal temperature for 1-4 minutes during the 'Come-up-Time' process. Regardless, it is still possible that the wheat was subjected to higher temperatures than indicated by the thermocouples, and thus sustained greater loss in quality.

Table 4. Processing times and temperatures from VSP processing for quality testing.

Treatment	Pre-heat Grain Temperature [a] (°C)	Come-up-Time [b] (min)	Total Run Time [c] (min)
65°C- 4 min			
Mean	38.34	10.17	14.17
Min	36.40	7.50	11.50
Max	39.80	12.50	16.50
SD	1.75	2.52	2.52
65°C- 8 min			
Mean	39.76	9.25	17.25
Min	37.09	8.25	16.25
Max	42.20	10.00	18.00
SD	2.56	0.90	0.90
70°C- 4 min			
Mean	37.96	13.25	17.25
Min	36.95	12.75	16.75
Max	38.58	13.75	17.75
SD	0.88	0.50	0.50
70°C- 8 min			
Mean	37.72	11.33	19.33
Min	35.87	9.25	17.25
Max	38.90	12.50	20.50
SD	1.62	1.81	1.81
75°C- 4 min			
Mean	37.83	15.17	19.17
Min	36.95	14.25	18.25
Max	39.06	15.75	19.75
SD	1.10	0.80	0.80
75°C- 8 min			
Mean	40.11	15.17	23.00
Min	38.13	13.00	21.00
Max	42.48	18.25	26.50
SD	2.20	3.04	3.04
85°C- 4 min			
Mean	38.97	22.33	26.33
Min	36.25	21.50	25.50
Max	41.17	23.25	27.25
SD	2.50	0.88	0.88
85°C- 8 min			
Mean	39.37	19.75	27.75
Min	39.05	19.00	27.00
Max	39.81	21.25	29.25
SD	0.40	1.30	1.30

[a] Temperature of grain entering VSP system

[b] Time required for grain to heat from 40±4°C to target temperature (60°C, 70°C, 75°C, 85°C)

[c] Entire processing time of grain

5.3. Wheat Quality

5.3.1. Kernel Quality

Immediately after VSP treatment, the kernels were analyzed to determine the effect of VSP on the kernel size and moisture. Grain in the control samples averaged 62.38 lb/bu, while grain treated at temperatures above 70°C had lower test weights of 59.2 to 60.47 lb/bu, (Table 5). The decrease in test weight indicates an increase in kernel size, as kernels absorbed moisture during VSP processing. Kernel moisture increased significantly (p<0.05) for all treated samples from the control. Test weight and kernel moisture showed a negative correlation (p<0.001) (Table A14).

Table 5. Characteristics of kernels immediately after VSP treatment

Treatment	Test Weight (lb/bu)	Moisture (%)
Control	62.38a	13.73b
65°C- 4 min	60.80bc	15.07a
65°C- 8 min	61.00ab	14.97ab
70°C- 4 min	59.90bcd	15.60a
70°C- 8 min	59.57cd	16.07a
75°C- 4 min	59.93bcd	15.70a
75°C- 8 min	60.47bcd	15.23a
85°C- 4 min	59.20d	16.20a
85°C- 8 min	59.23d	16.13a

Mean values in a column followed by the same letter are not significantly different (p<0.05).

After kernel analysis, the samples were left at room temperature to dry to 14% moisture before placement in a cooler prior to milling, so the effect of the VSP treatment as a tempering method was not studied. Milling extraction, also known as milling yield, was the main parameter used to determine the milling quality of the grain. It was calculated as the amount of flour produced from the initial grain sample. Milling yield did not change significantly (p<0.05) between treatments, averaging 68.72%. The lowest average extraction rate was 68.72%, and the

highest was 69.29%. In a similar study evaluating the effect of heating on wheat quality, no significant difference in milling yield was observed (Dermott and Evans 1978).

5.3.2. Flour Analysis

After milling the kernels into flour, the composition of the flours was determined. Moisture of the flour, approximately 12.6%, was not significantly different between samples (p<0.05), except for 70°C treatments that had a slightly higher moisture of approximately 13.1% (Table 6). VSP processing of 70°C treatments was performed several weeks after 65°C, 75°C, and 85°C treatments, and was thus milled at a later date, which likely explains this small difference. Samples treated at 65°C, 75°C, and 85°C were milled in late May, while 70°C treatments were milled in mid-June. During this time, changes in weather including temperature and humidity occurred in Fargo, ND, and likely affected conditions in the milling room.

Ash is the measurement of the inorganic material in a sample. The majority of inorganic material in the wheat kernel is concentrated in the bran layer of the kernel, thus ash is used as an indicator of bran contamination, and overall milling performance. In refined, or white flour, low ash values are typically preferred. Differences in ash values between the control and heat treated samples were not significantly different (p<0.05), except for samples treated at 65˚C for 8 min, which were 0.1 % higher (Table 6). No significant differences (p<0.05) in ash content were observed at the same treatment temperature for different treatment times of 4 or 8 minutes.

Flour protein content is an important parameter for the buying and selling of flour as it indicates it potential baking properties. Protein content within a wheat kernel is dependent on the class and variety of the wheat, as well as the growing conditions experienced by the plant. Protein content in the flour reduced significantly (p<0.05) in samples that received VSP treatment at temperatures ≥70°C. Later discussion of other tests analyzing gluten protein

functionality reveal a similar trend with significant changes occurring at temperatures ≥70°C. Despite the statistical significance in reduction of protein content, the overall decrease in approximately 0.2% protein is unlikely to affect the functionality of the flour.

Starch, the largest component in flour, plays an important role in the quality of flour and baked good products. Total starch analysis showed no significant differences (p<0.05) between treatments, and ranged between 74.3% and 78.2% (Table 6). Starch damage plays an important role in the quality of flours produced from hard wheat varieties, as it can increase water absorption and decrease bread quality (Barrera et al. 2007). Analysis of starch damage revealed no significant differences (p<0.05) from the control for all treatments except 70°C treatments, which showed lower levels of starch damage. It is likely this difference was not caused by processing, but rather the later milling date for 70°C treatments as previously discussed.

Table 6. Proximate composition of flour after milling of VSP treated kernels.

Treatment	Moisture	Ash[b]	Protein[c]	Total Starch	Starch Damage
	----------(%)----------				
Control	12.81abc	0.44b	14.01ab	75.9a	7.7ab
65°C- 4 min	12.64c	0.51ab	14.03ab	77.0a	8.3a
65°C- 8 min	12.44c	0.54a	14.04a	76.7a	7.8ab
70°C- 4 min	13.12a	0.46ab	13.76c	76.1a	7.5b
70°C- 8 min	13.08ab	0.48ab	13.88bc	74.5a	7.5b
75°C- 4 min	12.67bc	0.44b	13.88bc	77.4a	7.9ab
75°C- 8 min	12.57c	0.45b	13.80c	76.1a	8.0ab
85°C- 4 min	12.80abc	0.47ab	13.80c	78.2a	8.2a
85°C- 8 min	12.51c	0.49ab	13.80c	74.3a	8.0ab

Mean values in a column followed by the same letter are not significantly different (p<0.05).
[b,c] Adjusted to 14% moisture basis

Analysis of flour color was performed using the CIE L*a*b* scale. L* values indicate the lightness of a sample on a scale of 0-100. Low values indicate a dark sample, while values closer to 100 are lighter in color. No significant difference (p<0.05) was observed between treatments for L* values. The redness or greenness of a sample is indicated by the a* values (red is positive, green is negative). No significant changes (p<0.05) in a* were observed, except for 70°C

treatments, which may be explained by the increase in apparent α- amylase activity (Table 8). Enzymatic activity can affect the color of the flour, and higher α- amylase activity in wheat flour has been associated with changes in color (Lorenz and Valvano 1981). It should be noted that visual inspection of the flours did not reveal observable differences in greenness. The blueness or yellowness of a sample is indicated by the b* values (yellow is positive, blue is negative). All VSP treated flour had significantly (p<0.05) lower b* values from the control, indicating decreased yellow color of the flour, however visual inspection of the flours did not reveal observable differences in yellowness.

Table 7. Color of refined flour expressed on CIE L*a*b* scale.

Treatment	Color		
	L*	a*	b*
Control	90.46a	-0.91bc	9.13a
65°C- 4 min	90.63a	-0.94bc	8.87bc
65°C- 8 min	90.65a	-0.93bc	8.90bc
70°C- 4 min	90.63a	-0.88ab	8.38e
70°C- 8 min	90.51a	-0.85a	8.51de
75°C- 4 min	90.69a	-0.93bc	8.79bc
75°C- 8 min	90.73a	-0.95c	8.70cd
85°C- 4 min	90.78a	-0.94c	8.68cd
85°C- 8 min	90.57a	-0.92bc	8.73bc

Mean values in a column followed by the same letter are not significantly different (p<0.05).

Apparent PPO, α-amylase, and xylanase activity was measured as heating conditions experienced during VSP processing could have promoted enzymatic activity, or denatured the enzymes. The PPO enzyme has been associated with darkening and overall discoloration of wheat flour and their resulting products, so its activity is undesirable (Fuerst et al. 2006). Levels of PPO were extremely low, and were not found to be significantly (p<0.05) different among treatments (Table 8). PPO activity is highly dependent on growing location (Park et al. 1997), and because PPO levels are low in the control sample, it is unclear whether VSP treatment

results in a decrease in PPO activity. However, it can be determined that VSP treatment did not increase apparent PPO activity.

Amylase is an enzyme that hydrolyses starch. High α-amylase activity is generally undesired as too much hydrolyzed starch can result in sticky dough and poor baking characteristics such as low bread loaf volume. Apparent α-amylase activity decreased significantly ($p<0.05$) from the control for all treatments except VSP temperature treatment of 70°C (Table 8). It is likely that differences in the environmental conditions during milling –such as increased temperature and humidity – of the 70°C treatments at a later date resulted in a higher α-amylase activity. No significant difference was observed in apparent α-amylase activity between temperatures of 65°C, 75°C, and 85°C, or with varying treatment times at each temperature.

Xylanase is an enzyme that can hydrolyze arabinoxylans, which are non-starch polysaccharides present in flour. They can be either water extractable, or water un-extractable (Simsek and Ohm 2009). Water extractable arabinoxylans play an important role in stabilizing the gas cells formed when producing baked goods, so xylanases are commonly added to bread formulations to increase water extractable arabinoxylan content, thus improving dough stability, loaf volume, crumb structure, and overall shelf life (Shah et al. 2006). However, in refrigerated doughs, xylanase activity is highly detrimental to product quality as the xylanase hydrolyzes the arabinoxylans thus decreasing its water holding capacity. As water is released from the hydrolyzed arabinoxylans, a phenomenon known as syruping occurs. Thus, the reduction of xylanase in flour is desired for all refrigerated doughs. Analysis of xylanase activity in these samples showed xylanase levels generally decreased significantly ($p<0.05$) as temperature of the treatments increased, with the exception of the 70°C treatment, which saw a higher reduction in

xylanase activity than 75°C treatment but lower than 85°C treatment (Table 8). As with other minor discrepancies observed in quality analysis, the deviation at 70°C treatments is likely due to changes in milling conditions.

Table 8. Apparent enzyme activity in whole wheat flour.

Treatment	PPO ($\Delta A475$/min*g flour)	α- Amylase (mU/g)	Xylanase (mU/g)
Control	0.025a	0.323a	0.892a
65°C- 4 min	0.032a	0.144b	0.555bc
65°C- 8 min	0.036a	0.137b	0.652ab
70°C- 4 min	0.035a	0.301a	0.311cd
70°C- 8 min	0.028a	0.288a	0.262cd
75°C- 4 min	0.034a	0.137b	0.460bc
75°C- 8 min	0.035a	0.133b	0.414bc
85°C- 4 min	0.034a	0.133b	0.042d
85°C- 8 min	0.030a	0.138b	0.035d

Mean values in a column followed by the same letter are not significantly different ($p<0.05$).

5.3.3. Starch, Gluten, and Dough

5.3.3.1. Pasting Properties

Rapid Visco Analysis (RVA) is a tool used to study the pasting properties of starch. The instrument measures the resistance of flour and water slurry to the constant stirring action of a paddle. The instrument heats and cools the sample using a water bath to induce gelatinization (heating) and gelation (cooling) of the sample. Upon initial heating, starch granules will begin to swell as they absorb water in the system. The swelling of the granules increases the viscosity, and the temperature at which the viscosity rapidly increases is referred to as the pasting temperature. Swelling of the starch granules, and increased viscosity, will continue until the curve peaks at a point known as the peak viscosity. The time of the peak viscosity is known as the peak time. After this point, the granules can become damaged by the stirring action and begin to align themselves in the direction of stirring. This causes a decrease in viscosity, and is known as the breakdown. Next, the system begins to cool and viscosity will increase again as a rigid

network of amylose forms around the outside of the starch granules. The increase in viscosity is referred to as the setback, and the final viscosity marks the viscosity of the sample at the end of the test.

RVA analysis revealed very few significant or practical differences ($p<0.05$) between the control and samples heat treated at 75°C and below (Table 9). The average peak viscosity below 85°C was 2,576 cP, and values ranged between 2,407 cP and 2,709cP. The average peak viscosity for 75°C treatments was significantly ($p<0.05$) higher than other treatments below 75°C, which may be explained by variation in processing in one of the replicates for this treatment showed considerably lower gluten functionality, and is hypothesized to have resulted from deviations in VSP processing. At 85°C treatments, for both 4 and 8 minute treatment times, peak viscosity, breakdown, final viscosity, and setback all increased significantly ($p<0.05$) from the control and other treatments.

Typically, changes in the pasting profile are attributed to starch, however it is possible that gluten protein fractions present in the flour also change the viscosity properties during testing. A study by Ozawa et al. (2009) wheat flour was heated at 120°C for 120 min, and amylograph analysis revealed significant increase in the peak viscosity of the treated flour when compared to the control. To investigate the cause of the increase in peak viscosity, they studied the starch granules under a microscope, where no difference in starch granule structure was observed. They then separated the gluten fraction from the flours and ran amylograph testing on the starch fraction alone. Results showed when the gluten fraction was removed from the flour, amylograph profiles were unchanged between heated and control flours. This led the researchers to conclude that changes in the gluten structure resulted in the increased peak viscosity of the amylograph test. It is possible that significant denaturation of the gluten protein at 85°C, as

observed in wet gluten, farinograph, and baking analyses in this study, was the cause of increased peak viscosity, breakdown, final viscosity and setback observed in RVA analysis. More research should be completed to verify changes in the starch were minimal.

Table 9. Pasting properties of flour as measured by Rapid Visco Analysis.

Treatment	Peak Viscosity	Breakdown	Final Viscosity	Setback	Peak Time	Pasting Temp
	----------(cP)----------				(min)	(°C)
Control	2407d	792e	2542e	926d	6.48ab	69.5bc
65°C- 4 min	2521cd	833de	2648de	960cd	6.51a	69.2c
65°C- 8 min	2591cb	861cde	2715cd	985c	6.51a	69.4bc
70°C- 4 min	2668cb	842de	2792cd	966cd	6.51a	69.9ab
70°C- 8 min	2517cd	792e	2648ed	922d	6.48ab	69.8b
75°C- 4 min	2709b	908bcd	2848bc	1047b	6.43ab	69.7bc
75°C- 8 min	2617cb	967abc	2694cde	1043b	6.33ab	69.5bc
85°C- 4 min	2899a	1011ab	3037a	1149a	6.30b	69.9ab
85°C- 8 min	2895a	1042a	2959ab	1106a	6.32b	70.4a

Mean values in a column followed by the same letter are not significantly different (p<0.05).

5.3.3.2. Wet Gluten

Wet gluten is a test used to evaluate gluten in a flour sample. Wet gluten represents the quantity, or amount of gluten in the sample, while the gluten index indicates the quality of the gluten present in the sample. At 65°C, there was no significant difference (p<0.05) observed in wet gluten or gluten index from the control sample. At 70°C the wet gluten content decreased by approximately 5%, though statistical analysis does not show significant differences (p<0.05). However, the quality of the gluten remains similar as indicated by the gluten index. Wet gluten analysis for one of the replicates (n=3) treated at 75°C for 4 minutes could not be completed as gluten could not be obtained from the sample, so the average wet gluten and gluten index was significantly lower (p<0.05) due to the '0' entered in the data set. Wet gluten and gluten index increased for samples treated at 75°C for 8 minutes as gluten could be isolated from all replicates. Though not statistically significant (p<0.05), the quality of the gluten as indicated by the gluten index decreased by approximately 15-30% between time treatments of 4 and 8 minutes. At 85°C no gluten could be obtained from the flour, indicating complete denaturation of

the protein. These results suggest denaturation of the gluten protein occurs above 65°C, with complete denaturation realized at 85°C treatments.

Table 10. Wet Gluten and Gluten Index analysis of flour.

Treatment	Wet Gluten (%)	Gluten Index (%)
Control	33.5a	94.3a
65°C- 4 min	32.7a	95.4a
65°C- 8 min	31.7a	96.0a
70°C- 4 min	25.6a	98.8a
70°C- 8 min	26.9a	96.3a
75°C- 4 min	17.1b	64.6a
75°C- 8 min	29.4a	80.7a
85°C- 4 min	0.0c	0.0b
85°C- 8 min	0.0c	0.0b

Mean values in a column followed by the same letter are not significantly different (p<0.05).

Regression analysis aids in the demonstration of the rapid reduction in gluten quantity and quality observed at temperatures greater than 70°C and 75°C (Figure 7).

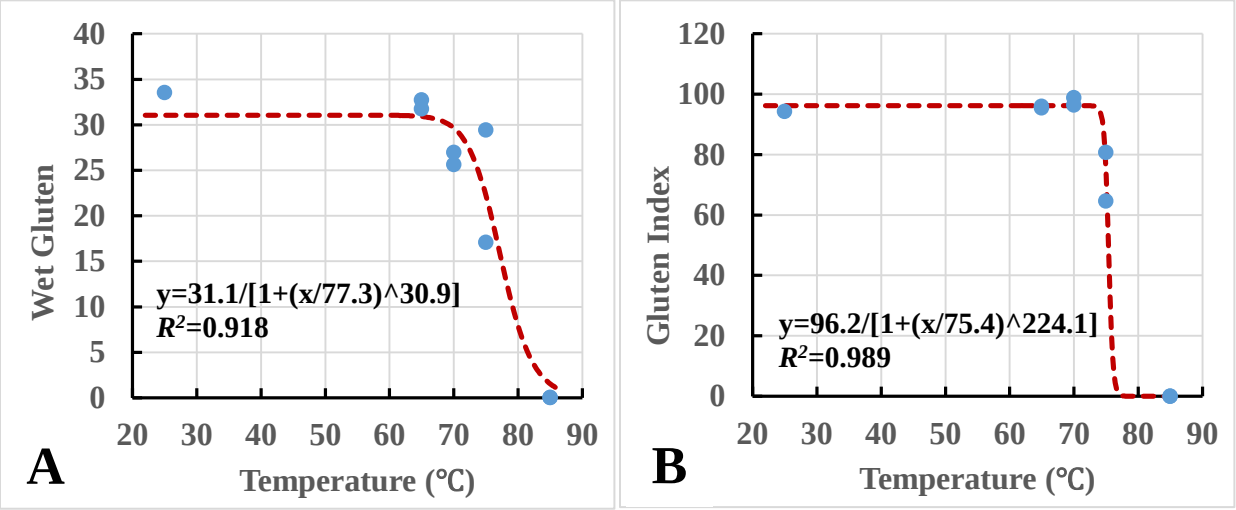

Figure 7. Graphs demonstrating negative sigmoidal relationship between both (A) Wet Gluten and (B) Gluten Index analyzes with increasing VSP temperature treatment
Data is fitted using the logistic power model: y=a/[1+(x/b)^c]

5.3.3.3. Farinograph

Farinograph analysis is used to understand the quality of dough produced from a flour sample and can be used to understand the gluten functionality. The test works by measuring the

resistance of dough to the mixing action of two paddles on a curve. Multiple parameters including absorption, stability, peak time, and mixing tolerance index (MTI) can be obtained from the curve, and are given in Table 11. Absorption is the amount of water the flour needs to form optimum dough consistency, and often, higher water absorption is desired for industrial uses. Analysis of VSP treated samples showed an overall trend of decreasing absorption ($p<0.05$) of 1-2% from the control at treatment temperatures up to 75°C. However, at 85°C treatments, the trend reverses and water absorption of the flour increases to values similar to the control, though overall changes are minor.

Table 11. Dough quality measured by farinograph.

Treatment	Absorption (14% MB[a])	Stability (min)	Peak Time (min)	MTI[b] (BU)
Control	63.4a	28.2ab	9.2a	8.0c
65°C- 4 min	62.1b	35.5ab	6.5b	10.0c
65°C- 8 min	61.5bc	39.1a	5.3b	8.3c
70°C- 4 min	60.9c	5.2c	2.3c	35.3b
70°C- 8 min	61.1c	4.7c	2.6c	32.7b
75°C- 4 min	61.3c	3.9c	2.2c	43.0b
75°C- 8 min	61.3c	5.0c	2.3c	39.7b
85°C- 4 min	62.1b	1.2c	1.5c	77.7a
85°C- 8 min	63.0a	1.2c	1.5c	78.3a

Mean values in a column followed by the same letter are not significantly different ($p<0.05$).
[a] MB= Moisture Basis
[b] MTI=Mixing Tolerance Index

Stability time, or the amount of time the dough remains at a desired consistency, is another important parameter for industrial uses as dough should remain tolerant to over-mixing. Typically, longer dough stability is desired and indicates a stronger gluten network. The control flour sample had a stability time of 28.2 minutes, indicating a strong wheat sample (Table 11). Interestingly, a slight increase in stability at 65°C of ~5-10 minutes was observed, though the change was not significantly different ($p<0.05$). At VSP temperatures of 70°C and higher, the

stability time decreased significantly ($p<0.05$) with an average reduction greater than 20 minutes. Stability time of samples treated at 85°C was only 1.2 minutes (Table 11).

Peak time indicates the amount of time it takes the dough sample to reach its peak viscosity, before the dough begins to break down and soften. Peak time of the control averaged 9.2 min, and decreased for 65°C treatments to 6.5 and 5.5 minutes for 4 and 8 minutes, respectively. VSP treatments above 70°C saw a significant ($p<0.05$) reduction in peak time from both the control and 65°C treatments of 2.6 min peak time or less. The peak time showed a negative linear correlation ($p<0.001$) with VSP temperature (Table A15).

The MTI is used to inform how much the dough will soften during mixing. It is typically desired to have low MTI for industrial uses, indicating less softening. The MTI of the VSP treated flours increased significantly ($p<0.05$) as treatment temperature increased above 65°C (Table 11). The control flour had an MTI of 8.0 BU. At 65°C, the MTI was not significantly different, averaging 9.2 BU between 4 and 8 minute treatment times, however, at 70°C treatments, a rapid increase ($p<0.05$) in MTI is observed. At 85°C the MTI increases significantly again ($p<0.05$). Results from all parameters of farinograph analysis suggest a significant decrease in dough quality is observed at temperature treatments of 70°C and higher, which further supported results observed in wet gluten analysis. MTI was negatively linearly correlated ($p<0.001$) with both wet gluten and gluten index analyzes (Table A15). Additionally, treatment time of 4 or 8 minutes within the same treatment temperature does not significantly ($p<0.05$) change dough quality.

5.3.4. Baking Analysis

Functional properties of the gluten and dough using wet gluten and farinograph analyses both indicated denaturation of the gluten protein occurred at processing temperatures ≥70°C.

However, the final indicator for end use quality of the flour was determined by bread baking analysis. Water absorption during baking was similar to farinograph water absorption. Absorption decreased significantly ($p<0.05$) from the control by 1-2% at 65°C, 70°C, and 75°C, and increasing again at 85°C, returning to levels of 70 and 71%, similar to the control ($p<0.05$) (Table 12).

Oven rise of the breads indicates the increased height of the breads during baking. Though much of the bread height can be attributed to gas retention from fermentation during proofing, bread will continue to rise during the baking process as water in the dough heats and turns to steam, which expands and pushes dough upward as it leaves the system. Strong gluten in the bread dough will allow dough to expand as the water expands, and is strong enough to remain cohesive and not break during the expansion. This allows for the formation of gaseous cell structures in the dough and larger loaf volume. The control sample averaged a 3.90 cm rise in the oven, similar ($p<0.05$) to the oven rise observed for 65°C treatments (Table 12). VSP treatment of 70°C-75°C observed a significantly reduced ($p<0.05$) oven rise of approximately 1.0cm, while 85°C treated samples collapsed slightly, by 0.30 and 0.10 cm in the oven. These results suggest gluten proteins were altered at VSP treatment temperatures of 70°C and above, compromising its ability to hold the expanding gas.

Loaf volume indicates the size of the loaf after baking. The control sample had a loaf volume of 1,011 cm^3. Volume of the bread loaf decreased significantly ($p<0.05$) as VSP temperature increased (Table 12). Treatment of 65°C for 4 minutes yielded a loaf volume similar to the control, while treatment for 8 minutes showed a significant ($p<0.05$) reduction in loaf volume at 880 cm^3. Samples VSP treated at 75°C and 85°C had just over one half and one third of the loaf volume compared to the control. Correspondingly, at the same temperatures where a

decrease in loaf volume was observed, the specific volume increased because the bread became more compact and dense.

Table 12. Bread baking quality.

Treatment	Absorption (%)	Oven Rise (cm)	Loaf Volume (cm^3)	Specific Volume (cm^3)	Firmness (mN)
Control	71a	3.90a	1011a	13.4c	1393b
65°C- 4 min	70ab	3.40a	922ab	14.7c	1620b
65°C- 8 min	70b	3.10a	880b	15.4c	1700b
70°C- 4 min	68d	1.00b	620c	22.8b	2933b
70°C- 8 min	68cd	1.20b	646c	21.9b	2115b
75°C- 4 min	69bc	0.30bc	555c	25.0b	3989b
75°C- 8 min	69bc	1.10b	648c	21.7b	3074b
85°C- 4 min	70b	-0.30c	338d	43.2a	14150a
85°C- 8 min	71a	-0.10c	353d	40.3a	16558a

Mean values in a column followed by the same letter are not significantly different ($p<0.05$).

Texture analysis of the slices of bread after baking revealed a significant ($p<0.05$) increase in firmness from flours treated at 85°C for both 4 and 8 min time treatments (Table 12). This increased firmness results from the dense crumb structure, as indicated by the increasing specific volume, and as can observed in Figure 9. The breads became more firm with increasing temperature treatment and decreasing loaf volume, and though statistical analysis did not reveal significant differences between the control, 65°C, 70°C, and 75°C treatments, it likely that sensory analysis of the breads would reveal a significant difference.

Images of baked bread samples can be observed in Figure 8 and 9. The breads were scored by an experienced baker for outward appearance as well as internal crumb structure using a scale of 1 to 10 while referencing a scoring guide. The results of this scoring are observed in Table A3; however, it is easily observed in Figure 8 and 9 that the overall quality and presence of desirable bread attributes such as high loaf volume, even coloring of the crust, and cell structure of the crumb decrease as temperature of the VSP treatment increases.

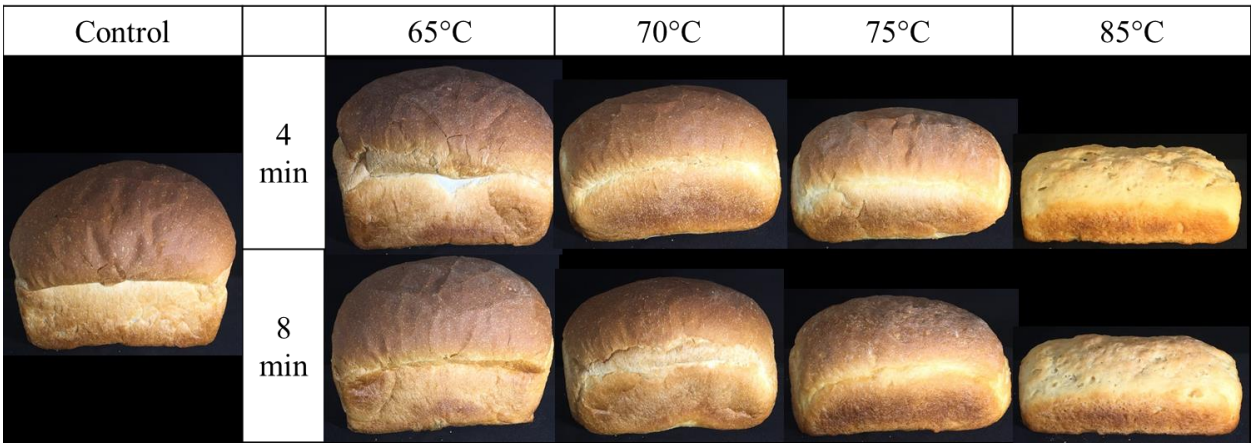

Figure 8. Pictures of sample bread loaves demonstrating bread baking quality.

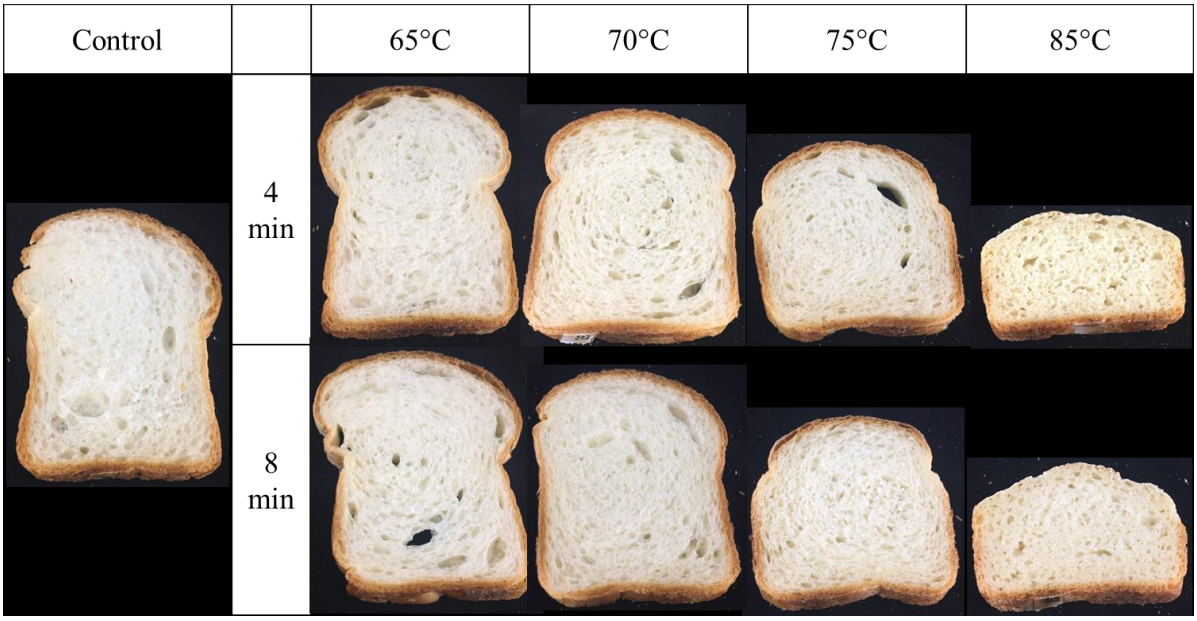

Figure 9. Pictures of sample bread slices demonstrating bread baking quality.

5.3.5. Discussion of Denaturation of Gluten with Heating

Gluten is responsible for the viscoelastic properties of dough. It is comprised of two proteins: gliadin and glutenin, which combine in when water is added and mixing occurs. Proper water addition is necessary to completely hydrate the proteins, as hydration promotes unfolding of the protein structure, thus allowing both intra-molecular and inter-molecular bonds to form. The mixing action is particularly important for proper development of the gluten as it promotes

formation of disulphide bonds that are important for strengthening of the dough via crosslinking and further act to stabilize hydrogen, ionic, and hydrophobic bonds (Wieser 2007).

Gliadin is associated with the viscous properties of the gluten and its ability to stretch great lengths while remaining a cohesive mass. Glutenin is associated with the elastic properties of the gluten, allowing for its resistance to extension and overall strength. In regards to the overall quality of a wheat flour's ability to form a dough and desired baked product, glutenin is generally considered to be of greater importance (Weegels et al. 1996). Of particular significance are high molecular weight glutenins, which are a component of the glutenin macropolymer (GMP). High molecular weight glutenins are large enough to entangle themselves across large spaces to form a vast network of disulphide bonds creating an "elastic backbone" for other subunits to bind (Sivam et al. 2010). Weegels et al. (1997) studied the importance of compositional changes in the GMP during mixing and identified an initial depolymerizatoin of the GMP, followed by a re-polymerization during dough resting. This change in GMP composition, brought about by an interchange of disulphide bonds during mixing is important for the overall strength of the dough.

In our experiments observing farinograph, wet gluten, and bread baking testing, a significant reduction in gluten quality was observed for treatment temperatures ≥70°C. Similar findings of gluten denaturation during heating were found by Schofield et al. (1983). Their experiments after heating treating isolated wet gluten samples indicated significant changes to the glutenin fraction of the gluten occurred at temperatures between 55 and 75°C. The group speculates that increased temperatures, promote unfolding of the 3D structure of the proteins, after which disulfide/sulfhydryl interchange reactions 'lock' unfolded proteins in their denatured state (Schofield et al. 1983). They further hypothesize that glutenin may begin conformational

changes at 50°C; however gliadin requires temperatures greater than 70°C to induce change. The heat sensitivity of the glutenin protein below 75°C, as demonstrated by Schofield et al. (1983) coupled with glutenin's demonstrated importance in dough quality (Weegels et al. 1997; Weegels et al. 1996) present a unique challenge for heat pasteurization methods in wheat products.

Multiple tests analyzing quality of VSP treated samples in this study indicated very few significant differences were observed between treatment time of 4 or 8 minutes at the same temperature. Two-way ANOVA revealed treatment time was never a significant factor ($p<0.05$) for any of the wheat quality parameters studied (Table A4-A11). Schofield et al. (1983) observed a similar result and hypothesized loss of gluten functionality occurred within 2 or 3 minutes, after which prolonged heating at the same temperature would not cause further functionality loss. This observation could be of importance when considering microbial reduction as prolonged heat treatment may not cause additional damage to the quality of the grain, but may allow further reduction of microbial populations. Processors of wheat products could potentially adopt a 'low temperature-long time' process for reduction of pathogens while maintaining gluten functionality.

An important distinction between this study and many of the other studies evaluating the effect of heating on gluten is the application of heat before or after the gluten formation. In this VSP study, heat was applied to whole kernels, when gliadin and glutenin proteins had not interacted to form a gluten complex. In contrast, the application of heat on wet gluten or isolated gluten portions of the flour (Schofield et al. 1983; Weegels et al. 1994a; Weegels et al. 1994b) occurred after gliadin and glutenin interaction forming the gluten matrix, which may affect the arrangement of bonds or mechanism of disassociation of the gluten network.

5.4. Microbial Reduction

After analysis of all quality parameters, it was determined that wheat was of an acceptable quality when processed at 65°C, and that the treatment time, of 4 or 8 minutes did not significantly affect quality. Thus, microbial reduction studies were completed at 65°C for 0, 2, 4, 6, and 8 minute periods. The pathogen *E. coli* O121 was used in this study as it has been associated with outbreaks from flour products in the United States in recent years (Harris and Yada 2018). Log reduction is a term commonly used in the food industry to indicate the difference between initial and final counts after a process, as a way of determining the success, or effectiveness of a process. For many food products, 5 log CFU/g reduction is the targeted standard when trying to reduce microorganisms in a system, and constitutes a 100,000-fold reduction of the bacteria; however an official standard for the milling industry has not yet been identified.

Figure 10 shows the relationship between increasing processing temperature and microbial reduction in wheat sample. The 'Come-up-Time', or amount of time it took the wheat sample to reach the target temperature of 65±3°C, was 9.5 minutes. This was very comparable to the average 'Come-up-Time' observed during quality analysis, 9.7 min; indicating samples underwent similar VSP processing conditions. The untreated, inoculated grain began with a microbial load of 7.98 ± 0.07 CFU/g. During the 'Come-up-Time' when the wheat increased in temperature from 40°C to 65°C, the *E. coli* O121 population reduced by 2.46 ± 0.34 CFU/g wheat. *E. coli* O121 populations continued to decrease with increased processing time, with log reductions of 2.99 ± 0.22 log CFU/g, 2.95 ± 0.26 log CFU/g , and 3.47 ± 0.28 log CFU/g for 2 min, 4 min, and 6 min treatment times, respectively. The highest overall average reduction in *E.*

coli O121 in wheat was 3.57 ± 0.33 log CFU/g, and was achieved after 65°C treatment for 8 minutes (Figure 10).

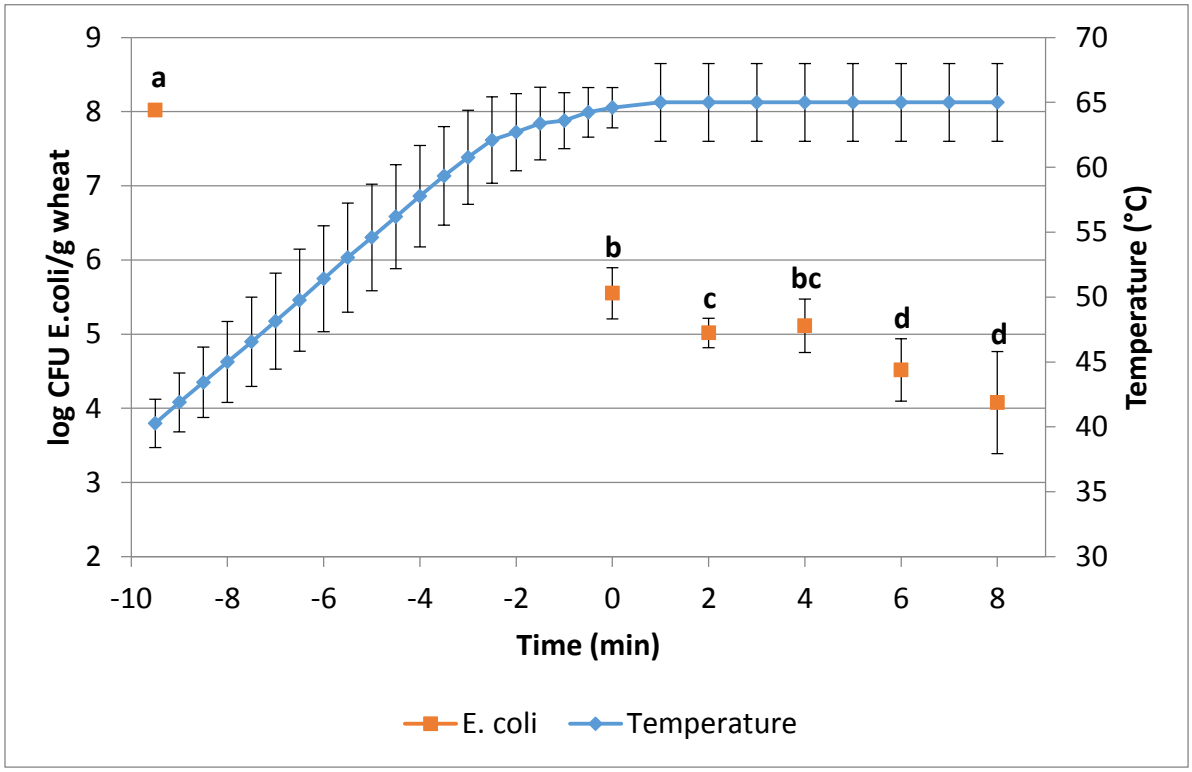

Figure 10. Microbial reduction of *E. coli* O121 shown with temperature profile of wheat bed. Data points are averages of technical replications, with error bars representing standard deviation *E. coli* populations with the same letter are not significantly different ($p<0.05$)

Comparison of this microbial reduction study to studies of other wheat products is difficult because of differences in microbial populations, inoculation procedures, and the kinds and types of heat treatments studied including the use of dry heat vs, steam, pressure, treatment times, and treatment temperatures. Discrepancies in any of these parameters can significantly change results. Variations in log reduction between 10 strains of *E. coli* 0121, all receiving a heat treatment of 60°C in a broth for 5 minutes, was more than 3 log CFU/mL in a study by Liu et al. (2015). The group reported log reductions in cell counts between ~2.5 log CFU/mL and ~5.5 log CFU/mL. Likewise, the group observed a similar range of variation when all 10 *E. coli* 0121 strains received a pressure treatment at 600MPa for 3 minutes, and should be a point of

consideration when interpreting and comparing results from this VSP wheat study as both temperature and pressure were used during processing in the VSP system.

Similar to this VSP research, Hu et al. (2016) studied microbial reduction on wheat kernels before milling. They applied saturated steam at multiple temperatures (110, 140, 170, and 200°C) and for 0-80 seconds on both tempered and non-tempered grain. They measured the reduction in total bacteria, *Bacillus* spp., and mold concentrations on un-inoculated grain. Temperatures of the wheat grain were not reported, though testing was performed on 200g of wheat spread in a thin layer for each replicate, with steam application at a higher velocity, so it may be assumed temperatures increased quickly. Maximum reduction in total bacteria counts wheat were observed after treatment with saturated steam at 200°C for 80 seconds on tempered grain, when microbial populations reduced to 1.95 ± 0.19 log CFU/g from 5.32 ± 0.15 log CFU/g for a total reduction of 3.37 log CFU/g. Most of the microbial reduction occurred within the first 40 seconds of processing. Mold populations were completely reduced below the limit of detection (1 log CFU/g), and *Bacillus* spp. populations were reduced at most from 2.63 ± 0.11 log CFU/g to 1.65 ± 0.16 log CFU/g at 200°C for 80 seconds. The study reported a higher reduction in microbial populations in tempered wheat, and thus suggests a processing step should occur after tempering but before milling.

The majority of published research relating to the microbial safety of wheat flour focuses on inoculation and treatment of the wheat flour, rather than on the kernels of grain before milling. However, treatment temperatures in wheat flour studies are more similar to those investigated in this VSP research than those studied on whole kernels by Hu et al. (2016), and have thus been summarized for comparison.

A 1957 study found complete reduction of *E. coli* in flour spread in a thin layer after treatment in an oven heated to 130°C for 45 minutes (Wiseblatt 1967). Wheat flour placed inside plastic bags and heat treated with a water bath achieved a 5 log reduction of *E. coli* 0157:H7 could be achieved when heating at a minimum of 70°C for 5 minutes (Greene 2012). At treatment of 65°C for 5 minutes, a log reduction of ~3.5 log CFU/g flour was achieved, comparable to the microbial reduction of 3.57 log CFU/g observed in this VSP study. Similarly, Forghani et al. (2018) achieved a 3.32 log CFU/g reduction, on average, of EHEC in flour after heat treatment at 65°C for 60 minutes in PCR tubes placed in a digital dry bath.

6. CONCLUSIONS

VSP processing shows promise in its ability to maintain quality of the functional characteristics flour produced from treated HRS wheat kernels, while achieving significant reduction in microbial populations. Using laboratory scale VSP equipment, it was determined acceptable quality of HRS wheat can be achieved at VSP processing temperatures of 65°C. Treatment temperatures exceeding 70°C inhibit the functionality of dough as there is apparent denaturation of the gluten protein. Most notable of all tests for quality of the flour were the wet gluten, farinograph, and baking analyzes, which suggest gluten denaturation begins at 70°C, with nearly complete denature of the gluten protein occurring during VSP treatment of 85°C. Changes in the starch portion of the flour as indicated by RVA analysis were not significant for product quality. VSP treatment was effective in reducing the apparent enzyme activity of PPO and xylanase. Treatment time of 4 or 8 minutes at the same temperature had no significant effect (p<0.05) on the quality of the wheat, indicating temperature was the most important parameter during processing.

Implementation of the VSP technology in industry will likely require that treatments maintain wheat functionality; thus, microbial reduction studies were only performed at 65°C when quality was maintained. Log reductions in *E. coli* 0121 populations ranged between 2.46 ± 0.34 CFU/g and 3.57 ± 0.33 log CFU/g wheat for 0 minute and 8 minute VSP treatments, respectively. Longer treatment times significantly (p<0.05) increased observed microbial reductions. VSP shows potential as an effective pasteurization method for the flour milling industry.

6.1. Future Work

Functional evaluation of the dough and baked bread products indicated alterations in the gluten protein as VSP heat treatment increased, and further analysis of protein chemistry using high performance size exclusion chromatography (HPSEC) could be completed to better investigate these changes. Additionally, this study did not consider alterations to the flavor of the wheat flour, and sensory analysis should be completed for final products that may be consumed using this flour. This study focused only on HRS wheat, however soft wheat classes should also be studied as they are more commonly associated with batter and dough systems including cookies, cakes, and brownies that may be more likely to be commonly consumed raw.

The *E. coli* 0121 pathogen was the only pathogen studied in this experiment, however *Salmonella* is another pathogen commonly associated with wheat product recalls and outbreaks, and should be studied using VSP treatment on wheat kernels. It was observed that treatment time had very few effects on the quality of the grain, however longer treatment times allowed for higher microbial reduction. Longer treatment times of >10 minutes may reveal acceptable quality while achieving >5 log CFU/g reductions in pathogenic organisms. Ultimately, this work should be performed on industrial equipment and considerations for the implementation of this equipment into a milling industry should be deliberated.

Additional work understanding the change in microbial populations during storage of the grain before milling, as well as location of the microbes on the kernels of grain would be helpful to better understand challenges in improving the food safety of the flour milling industry.

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APPENDIX

Date of recall mm/dd/yyyy (Reference)	Recall class	Product	Recalling firm	Reason given	Brand	Sub-recalls	Illnesses
06/14/2018 (18)	I	Puffed wheat cereal (23-oz and 15.3-oz boxes)	Kellogg Company (Battle Creek, MI)	*Salmonella* contamination; linked to outbreak	Kellogg's Honey Smacks		73
06/19/2017 (17)	II	Flour (20-kg bags), milled by Ardent Mills	Distributors: Flour Barrel (Guelph, ON); Hillcrest Home Baking (Floradale, ON)	Possible *E. coli* O121 contamination	Robin Hood - Super Keynote Strong Bakers Flour; Italian Style Flour		none reported
06/16/2017 (16)	II	Cookie dough, pie and tart shells	Various Canadian manufacturers	Possible *E. coli* O121 contamination	In-Dey-Go Fundraising Inc. (cookie dough); Apple Valley, Mildmay Cheese Haus		none reported
06/15/2017 (15)	II	All-purpose flour (bulk packs - various sizes)	Distributors: Country Bulk Inc. (Waterloo, ON) & Country Pantry Bulk Foods (Heidelberg, ON)	Possible *E. coli* O121 contamination	Robin Hood		none reported
06/15/2017 (14)	II	All-purpose flour (20-kg)	Ardent Mills (Brampton, ON)	Possible *E. coli* O121 contamination	Robin Hood - Baker's Hood	yes	none reported
06/07/2017 (12, 13)	II	All-purpose flour (10-kg bags)	Rogers Foods Ltd. (Armstrong, BC, Canada)	*E. coli* O121 contamination	Rogers		yes, 6 reported Feb–Apr 2017
05/31/2017 (11)	II	Durum wheat flour and wheat bran blends (20-lb bags)	Smucker Foods of Canada Corp. (Markham, ON)	Possible *E. coli* O121 contamination	Select Golden Temple, Swad, Maya		none reported
04/25/2017 (9, 10* recall list update to June 2, 2017)	II	Pie and tart shells (assorted sizes)	Harlan Bakeries (Edmonton, AB)	Possible *E. coli* O121 contamination	No name, Great Value, Apple Valley, Western Family		none reported
04/12/2017 (8)	II	Various flour and flour products	Ardent Mills (Saskatoon, SK, Canada)	*E. coli* O121 contamination; linked to outbreak	Brodie, Creative Baker, Golden Temple, Robin Hood	yes	yes, 30 reported in total: Nov 2016 – Apr 2017
04/04/2017 (7)	II	All-purpose flour (10-kg bags)	Smucker Foods of Canada Corp. (Markham, ON)	*E. coli* O121 contamination	Robin Hood	yes	yes, 1 reported March 2017
07/11/2016 (6)	II	Cake mix (2 flavors)	General Mills (Minneapolis, MN)	Made with affected flour recalled by General Mills	Betty Crocker Delights Super Moist		none reported
07/09/2016 (5)	II	Pancake mix (28-oz carton, 3.5-lb bag)	Continental Mills (Tukwila, WA)	Made with affected flour recalled by General Mills	Krusteaz (Blueberry)		none reported
07/01/2016 07/25/2016 (3, 4)	I	[expanded flour recalls]	General Mills (Minneapolis, MN)	*E. coli* O121 and/or O26 isolated from product; linked to multi-state outbreak	Gold Medal, Gold Medal Wondra, Signature Kitchens	yes	yes, 63 reported in total: Dec 2015 – Sep 2016
05/31/2016 (2)	I	All-purpose, quick-mixing, self-rising, or unbleached flour (13.5-oz carton; 2-, 4.25-, 5- and 10-lb bags)	General Mills (Minneapolis, MN)	*E. coli* O121 and/or O26 isolated from product; linked to multi-state outbreak	Gold Medal, Gold Medal Wondra, Signature Kitchens		yes, 38 reported Dec 2015 – May 2016
05/19/2009 (1)	I	Refrigerated cookie and brownie dough products	Nestlé USA (Solon, OH)	*E. coli* O157:H7 contamination; linked to outbreak	Toll House		yes, 80 reported

Figure A1. Recalls of wheat flour and wheat products, 2009 to present
Reprinted without changes from (Harris and Yada 2018).

TABLE I
Microbiological Profile of Wheat Grains in Different Geographic Regions[a]

	Geographic Origin						
Microbial Load	**Australia**	**Australia**	**Australia**	**Great Britain**	**United States**	**Hungary**	**Algeria**
Spoilage MOs							
Aerobic mesophilic bacteria[b]	4.9	5.0 (58)	NR	NR	6.4 (178)	4.9	NR
Bacillus spp.[b]	NR	4.0 (58)	NR	NR	NR	NR	NR
Yeast[b]	4.2[c]	3.0 (58)	3.7 (50)	NR	3.3 (178)[c]	3.9	NR
Mold[b]		3.0 (58)	2.7 (50)	5.1–6.0		3.5	2.4–3.1 (27)
Pathogenic and fecal MOs							
Escherichia coli[d]	NR	NR	2 (50)	NR	NR	NR	NR
Coliforms[d]	ND	93 (58)	NR	NR	NR	NR	NR
Salmonella spp.[d]	NR	0.5 (412)	2 (50)	NR	NR	NR	NR
Bacillus cereus[d]	NR	81.0 (58)	4 (50)	NR	NR	NR	NR
Reference	Eyles et al. (1989)	Berghofer et al. (2003)	Eglezos (2010)	Seiler (1986)	Manthey et al. (2004)	Peles et al. (2012)	Riba et al. (2008)

[a] Values are means; values separated by a dash are ranges. Sample sizes are provided in parentheses. MOs = microorganisms; NR = not reported; and ND = not detected.
[b] Expressed in log colony forming units per gram (log CFU/g).
[c] Combined yeast and mold counts.
[d] Values are percentages of positive samples.

Figure A2. Microbiological profile of wheat grains in different geographic regions
Reprinted without changes from (Sabillón and Bianchini 2016)

TABLE II
Microbiological Profile of Wheat Flour in Different Geographic Regions[a]

Microbial Load	Geographic Origin							
	Turkey	Australia	Australia	Great Britain	United States	United States	United States	Pakistan
Spoilage MOs								
Aerobic mesophilic bacteria[b]	5.0–7.2 (142)	1.0–7.0 (71)	4.2 (100)	4.5	4.2 (1,354)	5.7 (219)	3.8 (6,598)	NR
Bacillus spp.[b]	NR	2.0–5.0 (71)	NR	NR	NR	NR	NR	NR
Yeast[b]	NR	2.0–3.0 (71)	3.0 (50)	NR	2.1 (1,648)	2.2 (219)[c]	1.3 (6,573)	3.7 (150)
Mold[b]	2.2 (142)	2.0–3.0 (71)	2.8 (50)	3.7	2.9 (1,682)		2.4 (6,869)	5.3 (150)
Pathogenic and fecal MOs								
Escherichia coli[d]	50.7 (142)	1.4 (71)	0.7 (300)	NR	12.8 (3,350)	NR	0.7 (2,921)[b]	NR
Coliforms[e]	NR	1.0–1,000 (71)	NR	NR	1.2 (1,477)	NR	1.6 (3,688)	3.0–4.0 (150)
Salmonella spp.[d]	NR	NR	ND (150)	NR	1.3 (3,040)	NR	0.14 (4,358)	NR
Bacillus cereus[d]	4.2 (142)	93 (71)	ND (350)	NR	NR	NR	NR	NR
Clostridium perfringens[d]	9.9 (142)	NR	NR	NR	NR	NR	NR	NR
Reference	Aydin et al. (2009)	Berghofer et al. (2003)	Eglezos (2010)	Seiler (1986)	Richter et al. (1993)	Manthey et al. (2004)	Sperber (2007)	Batool et al. (2012)

[a] Values are means; values separated by a dash are ranges. Sample sizes are provided in parentheses. MOs = microorganisms, and NR = not reported.
[b] Expressed in log colony forming units per gram (log CFU/g).
[c] Combined yeast and mold counts.
[d] Values are percentages of positive samples.
[e] Expressed as most probable number per gram (MPN/g).

Figure A3. Microbiological profile of wheat flour in different geographic regions
Reprinted without changes from (Sabillón and Bianchini 2016)

Table A1. Farinograph analysis for preliminary preheating experiment

Treatment	Absorption (14% MB)	Stability (min)	Peak Time (min)
Control	67.1	7.9	7.7
40°C- 0 min	67.0	9.2	8.3
40°C- 15 min	67.2	8.7	8.3
40°C- 30 min	67.6	8.2	7.5
40°C- 45 min	67.3	7.4	7.0
40°C- 60 min	66.6	9.3	7.7

Table A2. RVA analysis for preliminary preheating experiment

Treatment	Peak Viscosity	Breakdown	Final Viscosity	Setback	Peak Time	Pasting Temp
	----------(cP)----------				(min)	(°C)
Control	1951	661	2158	868	6.3	69.3
40°C- 0 min	1989	586	2198	795	6.5	70.2
40°C- 15 min	1968	573	2172	777	6.4	70.2
40°C- 30 min	1928	599	2139	810	6.4	70.3
40°C- 45 min	1859	588	2035	764	6.4	70.3
40°C- 60 min	1896	543	2100	747	6.5	70.3

Table A3. Quality scoring of baked bread

Treatment [a]	Symmetry	Crust Color	Crumb Color
Control	8.8a	10.0a	8.0a
65°C- 4 min	7.0b	10.0a	7.0b
65°C- 8 min	6.3b	10.0a	7.0b
70°C- 4 min	2.7c	10.0a	6.3cd
70°C- 8 min	3.0c	10.0a	6.7bc
75°C- 4 min	2.7c	9.0b	6.0d
75°C- 8 min	3.3c	9.7a	6.7bc
85°C- 4 min	0.5d	0.5c	1.0e
85°C- 8 min	0.5d	0.5c	1.0e

Mean values in a column followed by the same letter are not significantly different ($p<0.05$).
[a] Score based on 10 point scale. 1=undesirable/10= desirable

Table A4. Analysis of variance for kernel characteristics of treated kernels.

Parameter	Source	DF	Mean Square	F Value	Pr > F
Test weight	Temp	3	3.07	4.50	0.02*
	Time	1	0.07	0.10	0.75
	Temp*Time	3	0.19	0.29	0.84
	Error	19	0.68	-	-
Moisture	Temp	3	1.46	2.42	0.10
	Time	1	0.01	0.02	0.90
	Temp*Time	3	0.22	0.37	0.78
	Error	19	0.61	-	-
Thousand Kernel Weight	Temp	3	0.12	0.51	0.68
	Time	1	0.40	1.65	0.21
	Temp*Time	3	0.11	0.44	0.73
	Error	19	0.24	-	-
Milling extraction	Temp	3	0.39	0.39	0.76
	Time	1	0.45	0.44	0.52
	Temp*Time	3	0.69	0.68	0.58
	Error	19	1.02	-	-

DF=Degrees of Freedom; MS=Mean Square; *=significance at $p<0.05$

Table A5. Analysis of variance for composition of treated flours.

Parameter	Source	DF	Mean Square	F Value	Pr > F
Moisture	Temp	3	0.38	6.41	<0.005*
	Time	1	0.15	2.54	0.13
	Temp*Time	3	0.02	0.28	0.84
	Error	19	0.06	-	-
Ash	Temp	3	0.01	2.79	0.07
	Time	1	0.00	1.08	0.31
	Temp*Time	3	0.00	0.05	0.98
	Error	19	0.00	-	-
Protein	Temp	3	0.07	8.35	<0.001*
	Time	1	0.00	0.06	0.81
	Temp*Time	3	0.01	1.23	0.33
	Error	19	0.01	-	-
Starch damage	Temp	3	0.47	2.86	0.06
	Time	1	0.13	0.79	0.38
	Temp*Time	3	0.13	0.82	0.50
	Error	19	0.16	-	-
Total starch	Temp	3	2.99	0.39	0.76
	Time	1	18.17	2.35	0.14
	Temp*Time	3	3.35	0.43	0.73
	Error	19	7.72	-	-

DF=Degrees of Freedom; MS=Mean Square; *=significance at $p<0.05$

Table A6. Analysis of variance for color analysis of treated flours.

Parameter	Source	DF	Mean Square	F Value	Pr > F
L*	Temp	3	0.02	0.59	0.63
	Time	1	0.03	0.70	0.41
	Temp*Time	3	0.02	0.55	0.65
	Error	19	0.04	-	-
a*	Temp	3	0.01	5.42	0.01*
	Time	1	0.00	0.79	0.39
	Temp*Time	3	0.00	0.45	0.72
	Error	19	0.00	-	-
b*	Temp	3	0.20	14.55	<0.0001*
	Time	1	0.01	0.38	0.54
	Temp*Time	3	0.01	0.96	0.43
	Error	19	0.01	-	-

DF=Degrees of Freedom; MS=Mean Square; *=significance at $p<0.05$

Table A7. Analysis of variance for apparent enzymatic activity of treated flours.

Parameter	Source	DF	Mean Square	F Value	Pr > F
Polyphenol Oxidase	Temp	3	0.00	0.30	0.82
	Time	1	0.00	0.28	0.60
	Temp*Time	3	0.00	0.83	0.49
	Error	19	0.00	-	-
Alpha Amylase	Temp	3	0.04	37.59	<0.0001*
	Time	1	0.00	0.12	0.73
	Temp*Time	3	0.00	0.09	0.97
	Error	19	0.00	-	-
Xylanase	Temp	3	0.35	9.26	<0.001*
	Time	1	0.00	0.00	0.99
	Temp*Time	3	0.01	0.19	0.91
	Error	19	0.04	-	-

DF=Degrees of Freedom; MS=Mean Square; *=significance at $p<0.05$

Table A8. Analysis of variance for pasting profile of treated flours.

	Source	DF	Mean Square	F Value	Pr > F
Peak viscosity	Temp	3	140542.37	14.75	<0.0001*
	Time	1	11726.26	1.23	0.28
	Temp*Time	3	14183.57	1.49	0.25
	Error	19	9531.15	-	-
Holding strength	Temp	3	31853.07	4.56	0.01*
	Time	1	22326.00	3.20	0.09
	Temp*Time	3	10417.64	1.49	0.25
	Error	19	6986.97	-	-
Breakdown	Temp	3	53542.07	13.51	<0.0001*
	Time	1	1691.76	0.43	0.52
	Temp*Time	3	3263.79	0.82	0.50
	Error	19	3964.49	-	-
Final viscosity	Temp	3	120672.54	14.19	<0.0001*
	Time	1	35766.76	4.21	0.05
	Temp*Time	3	15638.32	1.84	0.17
	Error	19	8505.77	-	-
Setback	Temp	3	40278.79	34.88	<0.0001*
	Time	1	1576.26	1.37	0.26
	Temp*Time	3	1677.62	1.45	0.26
	Error	19	1154.69	-	-
Peak time	Temp	3	0.05	4.43	0.02*
	Time	1	0.00	0.38	0.54
	Temp*Time	3	0.00	0.35	0.79
	Error	19	0.01	-	-
Pasting temp	Temp	3	0.85	7.25	0.002*
	Time	1	0.07	0.62	0.44
	Temp*Time	3	0.17	1.49	0.25
	Error	19	0.12	-	-

DF=Degrees of Freedom; MS=Mean Square; *=significance at $p<0.05$

Table A9. Analysis of variance for wet gluten analysis of treated flours.

Parameter	Source	DF	Mean Square	F Value	Pr > F
Gluten index	Temp	3	12555.61	29.08	<0.0001*
	Time	1	75.92	0.18	0.68
	Temp*Time	3	108.55	0.25	0.86
	Error	19	431.72	-	-
Wet gluten	Temp	3	1197.30	47.20	<0.0001*
	Time	1	60.46	2.38	0.14
	Temp*Time	3	57.64	2.27	0.11
	Error	19	25.37	-	-

DF=Degrees of Freedom; MS=Mean Square; *=significance at $p<0.05$

Table A10. Analysis of variance for farinograph analysis of treated flours.

Parameter	Source	DF	Mean Square	F Value	Pr > F
Water Absorption	Temp	3	2.81	14.78	<0.0001*
	Time	1	0.08	0.43	0.52
	Temp*Time	3	0.58	3.04	0.05
	Error	19	0.19	-	-
Peak time	Temp	3	23.25	18.78	<0.0001*
	Time	1	0.33	0.26	0.61
	Temp*Time	3	0.64	0.52	0.68
	Error	19	1.24	-	-
Stability	Temp	3	1423.24	9.88	<0.0005*
	Time	1	381.60	2.65	0.12
	Temp*Time	3	79.28	0.55	0.65
	Error	19	144.04	-	-
MTI	Temp	3	4861.82	41.99	<0.0001*
	Time	1	18.38	0.16	0.69
	Temp*Time	3	4.60	0.04	0.99
	Error	19	115.79	-	-

DF=Degrees of Freedom; MS=Mean Square; *=significance at $p<0.05$

Table A11. Analysis of variance for baking characteristics of treated flours.

Parameter	Source	DF	Mean Square	F Value	Pr > F
Oven Rise	Temp	3	12.70	40.56	<0.0001*
	Time	1	0.40	1.28	0.27
	Temp*Time	3	0.29	0.92	0.45
	Error	19	0.31	-	-
Loaf Volume	Temp	3	309648.76	86.89	<0.0001*
	Time	1	3278.34	0.92	0.35
	Temp*Time	3	4603.34	1.29	0.31
	Error	19	3563.82	-	-
Specific Volume	Temp	3	777.09	64.97	<0.0001*
	Time	1	15.62	1.31	0.27
	Temp*Time	3	5.39	0.45	0.72
	Error	19	11.96	-	-
Symmetry Score	Temp	3	38.94	99.77	<0.0001*
	Time	1	0.04	0.11	0.75
	Temp*Time	3	0.49	1.25	0.32
	Error	19	0.39	-	-
Crust Color Score	Temp	3	129.71	924.17	<0.0001*
	Time	1	0.17	1.19	0.29
	Temp*Time	3	0.17	1.19	0.34
	Error	19	0.14	-	-
Crumb Texture Score	Temp	3	51.49	489.12	<0.0001*
	Time	1	0.17	1.58	0.22
	Temp*Time	3	0.17	1.58	0.23
	Error	19	0.11	-	-
Crumb Color Score	Temp	3	47.71	453.23	<0.0001*
	Time	1	0.38	3.56	0.07
	Temp*Time	3	0.15	1.45	0.26
	Error	19	0.11	-	-
Texture	Temp	3	248579622.10	64.40	<0.0001*
	Time	1	214137.00	0.06	0.82
	Temp*Time	3	3585578.60	0.93	0.45
	Error	19	3859877.30	-	-

DF=Degrees of Freedom; MS=Mean Square; *=significance at $p<0.05$

Table A12. Correlations between VSP treatment temperatures, kernel properties, functionality of gluten, and baking analyses

	VSP Temp	Kernel Moisture	Gluten Index	Wet Gluten	Peak Time	Stability Time	MTI	Oven Rise	Loaf Volume
Test Weight	**-0.919*** **	**-0.995*** **	0.619^{NS}	0.753*	**0.935*** **	0.749*	-0.824**	**0.903*** **	**0.912*** **
Kernel Moisture	**0.923*** **	-	-0.578^{NS}	-0.713*	**-0.92*** **	-0.688*	0.777*	-0.87**	-0.876**
TKW	-0.698*	-0.517^{NS}	0.531^{NS}	0.574^{NS}	0.535^{NS}	0.455^{NS}	-0.522^{NS}	0.521^{NS}	0.563^{NS}
Milling Ext.	-0.492^{NS}	-0.246^{NS}	0.396^{NS}	0.3^{NS}	0.31^{NS}	0.158^{NS}	-0.368^{NS}	0.287^{NS}	0.327^{NS}
Xylanase	-0.869**	**-0.937*** **	0.731*	0.823**	0.887**	0.765*	**-0.901*** **	0.889**	**0.935*** **
Peak Viscosity	0.811**	0.759*	-0.895**	**-0.938*** **	-0.78*	-0.779*	0.923***	-0.854**	**-0.92*** **
Breakdown	0.714*	0.553^{NS}	-0.883**	-0.824**	-0.657^{NS}	-0.617^{NS}	0.857**	-0.729*	-0.797*
Final Viscosity	0.786*	0.76*	-0.878**	**-0.937*** **	-0.756*	-0.781*	0.897**	-0.844**	**-0.905*** **
Setback	0.708*	0.578^{NS}	**-0.913*** **	-0.873**	-0.646^{NS}	-0.653^{NS}	0.865**	-0.753*	-0.816**
Gluten Index	-0.587^{NS}	-0.578^{NS}	-	**0.967*** **	0.548^{NS}	0.684*	**-0.911*** **	0.719*	0.822**
Wet Gluten	-0.666^{NS}	-0.713*	**0.967*** **	-	0.673*	0.806**	**-0.952*** **	0.829**	**0.909*** **
Water Absorb.	-0.424^{NS}	-0.398NS	-0.408^{NS}	-0.253^{NS}	0.484^{NS}	0.065^{NS}	0.099^{NS}	0.247^{NS}	0.115^{NS}
Peak Time	**-0.912*** **	**-0.92*** **	0.548^{NS}	0.673*	-	0.782*	-0.809**	**0.944*** **	**0.912*** **
Stability	-0.583^{NS}	-0.688*	0.684*	0.806**	0.782*	-	-0.877**	**0.923*** **	0.898**
MTI	0.742*	0.777*	**-0.911*** **	**-0.952*** **	-0.809**	-0.877**	-	**-0.926*** **	**-0.974*** **
Oven Rise	-0.819**	-0.87**	0.719*	0.829**	**0.944*** **	**0.923*** **	**-0.926*** **	-	**0.98*** **
Loaf Volume	-0.831**	-0.876**	0.822**	**0.909*** **	**0.912*** **	0.898**	**-0.974*** **	**0.98*** **	-
Specific Volume	0.716*	0.761*	**-0.947*** **	**-0.981*** **	-0.744*	-0.822**	**0.982*** **	-0.871**	**-0.945*** **
Firmness	0.598^{NS}	0.608^{NS}	**-0.979*** **	**-0.962*** **	-0.565^{NS}	-0.686*	**0.915*** **	-0.711*	-0.828**

***p<0.001, ** p<0.01, *p<0.05, ^{NS} Not significant
MTI=Mixing Tolerance Index

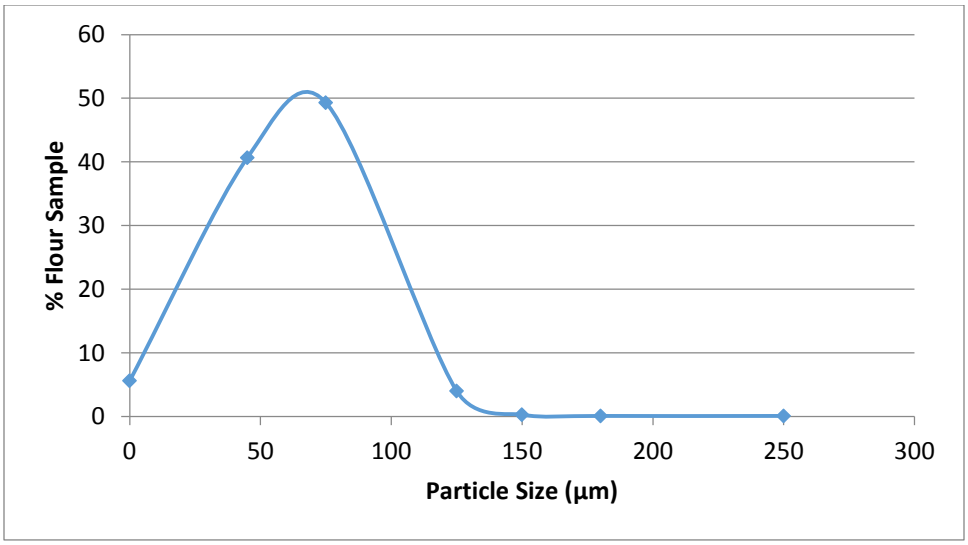

Figure A4. Particle size profile of flour from Buhler mill used in this study
Particle size analyzed using sieve sizes: 250, 180, 150, 125, 75, and 45 µm.