Pre-Milling Interventions For Improving The Microbiological Quality Of Wheat

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Generally, wheat-based products have been considered as safe food produced for human consumption. The reason for this is because they are low moisture food and most of the finished products are thermally processed. However, raw flour can have potential hazards, which, if not properly managed, may have the potential to result in serious health consequences. Most of these hazards are related to microbial contamination, which can come during growth, harvest, transportation, and storage of wheat grain.

Development of pre-milling intervention strategies to improve the microbial quality of wheat and wheat-based products is an emerging concern for the food industry. When steam tempering conditions for hard and soft wheat were applied, the microbial population associated with the grain was significantly reduced compared to controls. Further reductions were observed when acid was applied as part of the intervention which is an added benefit since the addition of a tempering solution is required for both wheat classes since the desired milling moisture cannot be reached by applying steam alone. On average, the highest reductions for hard wheat were achieved for Aerobic Plate Count (APC), molds and *Enterobacteriaceae* showing an average reduction of up to 4.78, 4.26 and 3.64 log CFU/g, respectively. While for soft wheat the highest microbial reduction was achieved for *Enterobacteriaceae* (up to 4.34 log CFU/g), followed by APC (up to 4.0

log CFU/g). For both wheat classes studied, the increased bed depth and temperature increased the microbial reduction. While the inoculation studies showed that saturated steam was most effective against the non-pathogenic *E. coli* population, with the highest reduction of 3.43 log CFU/g. On the other hand, *E. faecium* reductions showed a different trend. Acid with steam achieved the highest reduction for all bed depths and temperatures. When the temperature was decreased to 80°C the efficacy of the steam decreased as well. Furthermore, the functional properties of the flour were evaluated and a few significant differences between the control and treated samples. However, the treatments did not substantially affect the functional properties of soft and hard wheat straight-grade flour when cookies and bread were made from them, respectively. Therefore, the use of steam, or a combination of steam and acid, may be an alternative intervention applied by the milling industry to reduce microbial population in hard and soft wheat.

To mom, dad, and Vigan.

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CHAPTER 1. LITERATURE REVIEW

1. INTRODUCTION

Wheat is the world's leading cereal grain produced, consumed, and traded today. The United States is one of the largest world's wheat-producing countries. Wheat production is divided into five major classes: hard red winter, hard red spring, soft red winter, white, and durum wheat (USDA-ERS). On the last report from USDA – Economic research service, 2020/21 production including all wheat classes US was 1,825.82 (million bushels) (USDA, 2021).

Due to foodborne illness and outbreaks that have been associated with contaminated wheat flour, awareness regarding the importance of the microbial quality of wheat has been raised (Magallanes López & Simsek, 2020). Wheat milled products – flour is a staple ingredient in many of the commercial products consumed by millions of people and is also an essential ingredient for consumers. Flour is classified as low-moisture food. For several decades, the low water activity (0.85) found in these products has contributed to the belief that, from a microbiological point of view, these foods are considered to be safe (Sabillón Galeas, 2014).

The microbiological quality of wheat grain is a major contributor to the quality and safety associated to the ingredients provided by the milling industry (Berghofer *et al.*, 2003). Several studies on the microbial quality of wheat have been carried out over the last two decades due to the increasing awareness of the potential pathogens associated with it (Eglezos, 2010a; Laca et al., 2006; Manthey et al., 2004; Myoda et al., 2019;

Sabillón & Bianchini, 2016). The surface of wheat kernels is where the microbial load is typically found. This contamination can be transferred between milled products during the dry milling process (Berghofer *et al.,* 2003), which will lead to reduced microbiological quality of wheat flour.

The wheat microflora is affected by a number of factors, including irrigation water, insect infestation, soil, environmental conditions, and animal feces (Laca *et al.,* 2006). Grain storage and growing conditions are some of the main preventive steps to prevent fungal growth (Thielecke & Nugent, 2018). This includes avoiding factors that cause crop stress such as drought stress, bird damage, insect damage, early harvest, avoiding kernel damage during harvesting and processing. Additionally, it is important to mention that the handling of grains during processing, transport, and storage can also affect grain hygiene (Labbe *et al.*, 2014).

While most flour-based foods undergo a microbial reduction step (e.g., baking) before intended consumption, homemade flour-based mixes or commercial ready-to-bake refrigerated/frozen dough products may pose a potential safety hazard to consumers' health if consumed without proper cooking (Sabillón Galeas, 2014). The increased incidence of foodborne disease outbreaks caused by flour-containing products has highlighted the importance of high microbiological quality of wheat grain at the start of the flour supply chain (Laca *et al.*, 2006).

If safety interventions are developed based on consumer eating behavior, foodborne illness and outbreaks (**Table 1-1**) associated with contaminated wheat flour can be prevented (Magallanes López & Simsek, 2020). Consumers tend to eat unbaked products such as raw homemade dough, ready-to-bake cookie dough, and other types of dough

Table 1-1. Outbreaks and recalls associated with wheat flour and wheat flour products.

Product (source)	Pathogen	Number of cases	Isolated from the product?	Year	Location	Reference
Wheat Flour						
Flour	*Salmonella* Typhimurium phage type 42	67	Yes	2008-2009	New Zealand	(McCallum *et al.*, 2013)
Flour (General Mills, Kansas City, MO	*E. coli* O121, *E. coli* O26	63	Yes	2015-2016	USA	(CDC, 2016a)
Flour (Ardent Mills, Saskatoon, SK)	*E. coli* O121	30	Yes	2016-2017	Canada	(PHAC, 2017)
Flour (Rogers Foods, BC)	*E. coli* O121	6	Yes	2017	Canada	(BCCDC, 2017)
Wheat flour product						
Frozen pot pies (USA)	*Salmonella* serotype I 4, 5, 12:i:-	396	Yes	2007	USA	(CDC, 2007)
Cake mix, raw–in ice cream (USA)	*Salmonella* Typhimurium	26	Yes	2005	USA	(G. Zhang *et al.*, 2007)
Prepackaged, refrigerated cookie dough (USA)	*E. coli* O157:H7	80	Yes	2009	USA	(Neil *et al.*, n.d.)
Dough mix, dry (USA)	*E. coli* O157:H7	13	Yes	2016	USA	(CDC, 2016b)
Flour	*E. coli* O26	21	Yes	2019	USA	(FDA, 2019)

available in the retail market. One study reported *E. coli* infection (Neil *et al.,* n.d.), which was associated with raw cookie dough. Patients reported having eaten the dough uncooked which led to the hospitalization of 35 people. This case was defined as a diarrheal illness. Another recent case, also related to raw wheat flour was reported by Harris and Yada (2019). This outbreak was associated with a dough mix that was contaminated with *E. coli* O157:H7 and a cake mix contaminated with *Salmonella.* This highlights the importance of reducing microbial contamination of wheat and wheat flour products, so future outbreaks may be prevented. Another point to consider is the consumers' education about the risks of eating uncooked dough (Neil *et al.,* n.d.) as it carries potential hazards for their health. (*Sabillón*, 2018)**.**

2. MICROBIAL PROFILE OF WHEAT

Wheat is exposed to various forms of contamination through the production chain, from harvesting to final product consumption. Many factors such as soil, water, insects, and animal feces can be sources of microbial contamination to wheat kernels (Bullerman & Bianchini, 2008).

The number of microorganisms, including pathogenic ones, can be influenced by several factors including meteorological conditions during the growing season, the storage conditions like moisture and temperature, as well as the activity of pests through the supply chain (L. D. N. Doyle & Buchanan, 2013; Sabillón & Bianchini, 2016). The exterior of kernels is where the microflora associated with the kernel is mostly distributed consisting of a variety of microorganisms including spoilage and pathogenic bacteria. Even though these microorganisms are located on the surface of the grain, some of them

may reach the inner endosperm through the supply chain. These include *Micrococcaceae, Enterobacteriaceae, Bacillaceae*, as well as yeasts and fungi, including *Aspergillus, Cladosporium, Fusarium*, and *Alternaria* (L. D. N. Doyle & Buchanan, 2013; Laca et al., 2006; Sabillón & Bianchini, 2016). Several studies have reported that yeast and mold counts in wheat grain range from 1.4 to 6.0 log CFU/g (Berghofer et al., 2003; Eglezos, 2010a; Manthey et al., 2004; Sabillón & Bianchini, 2016). Spoilage bacteria counts have been reported between 0.9 to 8.4 log CFU/g (Berghofer et al., 2003; Eglezos, 2010a; Manthey et al., 2004; Sabillón & Bianchini, 2016). From these values, it is evident that microbial contamination levels vary greatly, suggesting that variables associated with geographic location and harvesting years may play a role (Sabillón & Bianchini, 2016).

Some microbial surveys have detected also the presence of *E. coli* in wheat kernels (Berghofer et al., 2003; Eglezos, 2010a; Sabillón & Bianchini, 2016). Human pathogens such as *Salmonella, E. coli,* and *Shigella* have been reported as well. *Salmonella* has been isolated from wheat samples in Australia (Berghofer et al., 2003; Eglezos, 2010a).

Mold contamination is of interest due to potential contamination with mycotoxin-producing fungi, which can occur during the growth of the plant or post-harvest. Some of the mycotoxins of the highest concern associated with wheat are deoxynivalenol (DON), zearalenone, T-2 toxin, and ochratoxin (OTA) (Magan *et al.*, 2010). These mycotoxins are of concern to human health and therefore many have maximum levels permitted in foods around the world.

3. UNIT OPERATIONS ASSOCIATED WITH MILLING

Several unit operations are needed before wheat can be milled into flour. **Figure 1-1** shows the order of primary processing of wheat: from grain to flour. Upon receiving, wheat is stored under dry conditions in order to prevent fungal growth The flour milling process begins with the cleaning of wheat grains to separate and remove non-wheat material (Delcour & Hoseney, 2009). Wheat is stored under dry conditions in order to prevent fungal growth. After that, in preparation for milling, the original moisture content of the wheat grain is adjusted through the process known as the conditioning step (Delcour & Hoseney, 2009). In order to adjust the moisture content, water is added to the wheat in a precise quantity and distributed as evenly as possible through the grain mass. The amount of water added is calculated from the initial moisture content of the wheat.

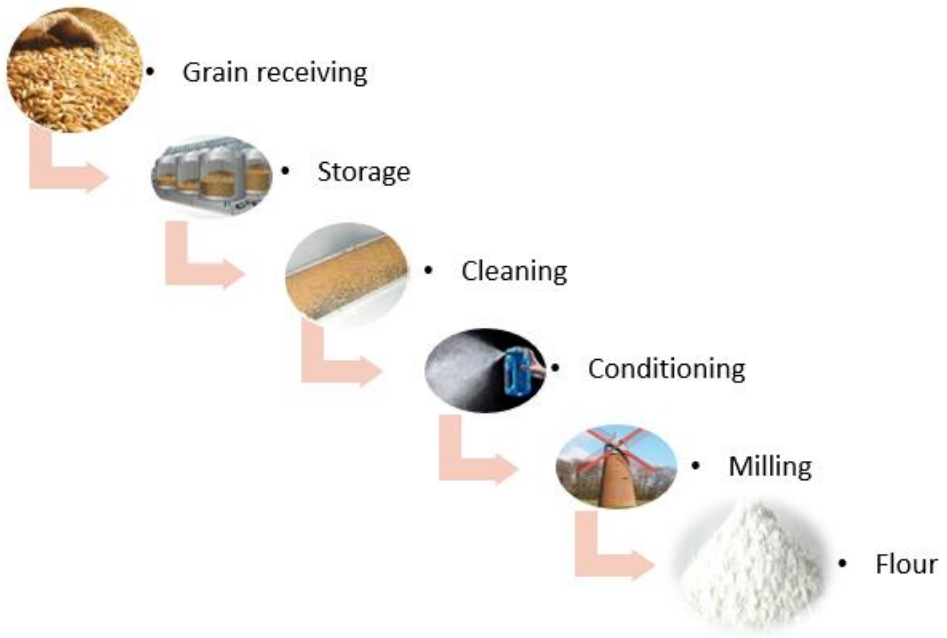

Figure 1-1. Flow chart including the main unit operations associated with the processing of wheat.

The objective of this step is to allow consistent separation of grain components (i.e., bran, germ, endosperm) during milling (Berghofer *et al.*, 2003; Rahim, 2010; Sabillón *et al.*, 2020). After adding the water, wheat is then allowed to rest for a specific period or until it reached the optimum moisture distribution which is considered suitable for milling (Posner & Hibbs, 2005). Based on the wheat class, the recommendation for moisture are as follows – 14.5-15.0% for soft wheat and 15.5-16.0% for hard wheat (Buhler, 2016). However, different pieces of literature have variations when it comes to the moisture content of these two wheat classes (±1.0%).

After this step, the wheat is milled using conventional milling equipment. During milling, grains are broken and undergo a sequence of reduction, grinding, and sifting operations to separate endosperm from outer grain layers (Berghofer *et al.*, 2003).

4. MICROBIAL PROFILE OF FLOUR

A study conducted by Mousavi *et al.*, (2018) showed that mycotoxin levels in the final product can be influenced by the unit operations steps included in the processing of different cereal-based products. More specifically, the milling operation showed a slight increase in the concentration of deoxynivalenol (DON) and total aflatoxin (TAF) in flour; while the levels of ochratoxin A (OTA) were reduced zearalenone (ZEN) were unchanged.

Wheat flour may also be contaminated, and some of the contaminants may include life-threatening microorganisms. This is the case because the wheat milling process used in the production of flour has no impact on the microbial inactivation step that may reduce the level of microorganisms contaminating the wheat grains. Most of these

microorganisms are transferred to the milled products (Sperber *et al.,* 2007), with the final flour still showing the presence of microorganisms (Richter *et al.,* 1993). Numerous studies have reported the presence of yeast, mold, spoilage bacteria (Berghofer et al., 2003; Eglezos, 2010a; Manthey et al., 2004; Richter et al., 1993; Sperber et al., 2007), and pathogenic bacteria (Ardent Mills, 2011).

Much like in wheat grain, yeast and mold are some of the common spoilage microorganisms found in flour. On a microbiological survey conducted by Richter *et al.,* (1993), more than 4,000 commercial wheat flour samples throughout the U.S. were evaluated indicating an average count of 2.1 to 2.9 log CFU/g for yeast and mold. More recent microbial surveys in North America by Manthey *et al.,* (2004) and Sperber *et al.,* (2007) reported similar counts for yeast and mold. The microbiological quality of wheat flour in Australia was evaluated by Berghofer *et al.,* (2003) and Eglezos (2010). They reported yeast and mold counts from 2.0 to 3.0 log CFU/g.

The North American surveys conducted by Richter *et al.,* (1993), Manthey *et al.,* (2004), and Sperber *et al.,* (2007) have shown varying aerobic bacterial counts in wheat flour from 3.8 to 7.0 log CFU/g. Berghofer *et al.,* (2003) also reported *Bacillus* spp., besides aerobic bacteria counts. The counts for *Bacillus* spp. were ranging from 2.0 to 5.0 log CFU/g. This group of researchers tested 71 flour samples, and 93% of the samples tested positive for *Bacillus cereus* (Berghofer *et al.,* 2003).

This review of the microbial profile of wheat and flour highlights the importance of the development of a method either before or after milling which would be a lethal step against pathogenic bacteria and other contaminants. This would ensure safer flour-based end-products and would prevent future illnesses and outbreaks associated with wheat-

based products.

5. PATHOGEN CONTROL IN WHEAT AND WHEAT FLOUR

Quite a few strategies have been studied for wheat grain and flour decontamination (**Table 1-2, 1-3,** and **1-4**). Some of the strategies include non-thermal technologies (Aron Maftei *et al.*, 2014; Butscher *et al.*, 2016; Du *et al.*, 2020; Selcuk *et al.*, 2008; Subedi *et al.*, 2020; Zahoranová *et al.*, 2016), thermal technologies (Chen, Guo, Xing, & Zhu, 2020; Hu *et al.*, 2016; Y. Jiao *et al.*, 2015; Liu *et al.*, 2018; Snelling *et al.*, 2020; Villa-Rojas *et al.*, 2017; Xu *et al.*, 2018), and grain tempering solutions (Chen, Guo, Xing, Sun, *et al.*, 2020; Dhillon *et al.*, 2007b; Ibanoglu, 2000; Sabillón *et al.*, 2016; Sabillón *et al.*, 2019).

5.1. Application of tempering solutions

In the industry, the wheat grain goes through the process called conditioning which is a process of adjusting its original moisture before milling. The aim of conditioning also known as tempering is the moisten the bran layers, and the starchy inner part of the grain, which would allow the inner part of the grain or endosperm to be reduced more easily into flour. Different sources of information indicate that moisture adjustment may vary depending on the grain hardness (Delcour & Hoseney, 2009). With regard to improving the microbiological quality of flour, there are tempering solutions that have been evaluated during conditioning to reduce microbial contamination on wheat.

Table 1-2. A review of decontamination strategies using tempering solutions for microbial load reduction of wheat.

Treatment type	Wheat type	Treatment description	Highest microbial load reduction	References
Tempering with ozonated water	Soft white wheat and hard red wheat grain	Tempering solutions with 1.5 and 11.5 ppm ozonated water.	With 11.5 ppm: up tp 2.0 log CFU[1]/g reduction for total bacteria and YMC[2]	(Ibanoglu, 2000)
Tempering with ozonated water	Durum and hard red spring wheat grain	Tempering solutions with 10 and 16 ppm ozonated water.	With 16 ppm: ≤0.5 log CFU/g reduction in YMC and APC[3]	(Dhillon *et al.*, 2007b)
Tempering with acid saline solutions	Hard red winter wheat grain	Tempering solutions: organic acids (acetic, citric, lactic, propionic; 1.0%, 2.5%, or 5.0%), NaCl (26% or 52%).	With 5.0% lactic acid and 52% NaCl: 4.3 log CFU/g reduction in APC; 4.7 log CFU/g reduction in Eb[4]	(Sabillón *et al.*, 2016)
Tempering with acid saline solutions	Soft red winter wheat grain	Tempering solutions: organic acids (acetic, lactic; 2.5% or 5.0%), NaCl (26.6%).	With 5.0% lactic acid and 26.6% NaCl: 3.2 log CFU/g reductions in APC; 4.5 log CFU/g reduction in Eb.	(Sabillón *et al.*, 2019)
Tempering with acidic saline solutions	Hard red winter and soft red winter wheat grain	Tempering solutions: organic acids (acetic, lactic; 2.5% or 5.0%), NaCl (26.6%)	With 5.0% lactic acid and 26.6% NaCl: 2.6 log CFU/g reduction in Salmonella spp., 2.4 log CFU/g reduction in E. coli O157:H7; 2.4 log CFU/g reduction in non-O157 STEC[5].	(Sabillón *et al.*, 2020)
Tempering with slightly acidic electrolyzed water (SAEW)	Hard white wheat grain	Tempering solutions: four concentrations of active chloride content of 10, 30, 50, and 70 mg/L.	With 70 mg/L: 0.93 log CFU/g reduction in TPC[6]; 0.78 log CFU/g reduction in YMC.	(Chen, *et al.* 2020)

5.1.1. Ozone

A study from Ibanglu (2000) showed the influence of tempering wheat with ozonated water on selected properties of wheat flour, as well as its use against the bacterial population in wheat. This study used 1.5 and 11.5 mg ozone/l and soft wheat samples were tempered to a moisture content of 15.0-15.5%, while hard wheat samples were adjusted to 16.0-16.5% moisture. This study showed a significant reduction in total bacteria, mold, and yeast counts after applying ozonated water on soft and hard wheat samples.

Dhillon *et al.*, (2007a) compared chlorinated water and ozonated water as an antimicrobial tempering solution. The tempering conditions for this study included ozonated water (10 and 16 ppm) and chlorinated water (700 ppm). Wheat grain was tempered to 17.0% moisture. The microbial quality of the wheat was evaluated by aerobic plate count (APC) and yeast and mold count (YMC). This study showed that there was a significant decrease in APC on hard red spring wheat samples when ozonated water was used for tempering or washing. And when the concentration of ozone was 16 ppm, both YMC and APC showed 1.0 to 2.0 log CFU/g reduction in durum wheat and hard wheat.

5.1.2. Acidic saline solutions

The application of organic acid and saline solutions for the reduction of microbial load on wheat has been studied by Sabillón *et al.* (2016). For this study, the researchers used different tempering solutions including acetic acid, lactic acid, citric acid, and propionic acid, at three different concentrations 1.0, 2.5, and 5.0% (v/v). The amount of tempering solution added to wheat was calculated in order to attain the moisture target for

the tempering step, depending on the type of wheat. This study showed that the initial microbial load was reduced by each acid solution. However, different acid concentrations were associated with different levels of microbial reduction. With the increase of acetic, lactic, and propionic acids to 5.0% concentration, the reduction resulted in up to 1.7, 2.3, and 3.8 log CFU/g in aerobic plate count (APC), *Enterobacteriaceae* (Eb), and mold counts, respectively (Sabillón *et al.*, 2016). This study also investigated the effect of saline solutions on microbial load, where a 26% NaCl tempering solution reduced the Eb population by 0.9 log CFU/g and the yeast and mold population by 1.4 and 1.0 log CFU/g, respectively. In later research, Sabillón *et al.*, (2019) studied the effect of the combination of both tempering solutions (acids, and saline) on the microbial quality of wheat. The combinations of organic acid and NaCl showed that lactic acid (5.0%) and NaCl (52%) were the most effective against APC, with an average reduction of 4.3 log CFU/g.

Another study evaluated the microbial reduction of wheat by tempering with saline organic acid solution at different seasonal temperatures (Sabillón *et al.*, 2020). Hard and soft red winter wheat samples were inoculated with pathogens to a 7 log CFU/g contamination. Test strains used for this research were as follows: five strains of *E. coli* O157:H7, six non-O157:H7 serotypes, and *Salmonella spp.* The seasonal temperatures selected for this study were 2.0, 10.8, and 24.2 (winter, spring/fall, and summer, respectively). After the inoculation process, wheat bags rested for 7 days for moisture adjustment and microbial adaptation. Bags were placed in the incubator at different testing temperatures. The microbial test was done at 0, 1, 3, 5, and 7 days after inoculation. Results showed that the survival rate of pathogenic microorganisms during

winter temperature (2.0°C) and spring/fall temperature (10.8°C) was higher than those associated with summer. The combination of NaCl (26.5%) and lactic acid (5.0%) was the most effective treatment against *Salmonella enterica, E. coli* O157:H7, and non-O157 STEC. This treatment showed an average reduction for *Salmonella enterica, E. coli* O157:H7, and non-O157 STEC was 1.8, 1.8, and 1.6 log CFU/g for soft wheat and 2.6, 2.4, and 2.4 log CFU/g for hard wheat, respectively. While other combinations of acids and NaCl (26.5%) gave lower log CFU/g reduction of *Salmonella enterica, E. coli* O157:H7, and non-O157 STEC. In general, the results from hard wheat showed a higher reduction in pathogenic load when compared to soft wheat. This might be due to the different moisture levels aimed during tempering with hard and soft wheat adjusted to 15.5 and 15.0%, respectively (Sabillón *et al.,* 2020).

5.2. Thermal technologies for pathogen control in wheat and wheat flour

5.2.1. Steam treatment

High temperatures, including steam, are considered a potential tool for decontamination of wheat. Hu *et al.,* (2016) studied the effect of superheated steam (SS) against microbial inactivation in a range of temperatures from 110 to 220°C, a processing time of 10 to 80 s, and velocities of 7.5 and 15.5 m^3/h. The superheated steam processing equipment had a closed chamber where 200 g of wheat grain samples were placed in a metal mesh tray and processed by exposure to superheated steam (upside and downside). This study showed that with the increase of the processing time, the reduction of bacteria was higher among organisms tested higher reductions value observed total bacteria, followed by mold, and *Bacillus* spp. Similar results were reported when the velocity was 15.0 m^3/h, total bacteria count was reduced up to 3.37 log CFU/g at 200°C for 80 s,

Table 1-3. A review of decontamination strategies using the thermal treatment for microbial load reduction of wheat.

Treatment type	Wheat type	Treatment description	Highest microbial load reduction	References
Pulsed light	Wheat grain (*Triticum aestivum* L)	Pulse duration: 0.3 ms; dose: 0.4 J/cm^2. Six treatments: 5, 10, 15, 20, 30, and 40 pulses.	With 40 pulses: 4.0 log CFU/g reduction in mold counts.	(Aron Maftei *et al.*, 2014)
Pulsed light through a fluidized bed	Wheat flour	Pulse duration: 0.3 μs, dose: 0.49 J/cm2. Six treatments: 2, 4, 8, 16, 32, and 64 pulses.	With 64 pulses: ~10.1% of *Saccharomyces cerevisiae* load was inactivated.	(Fine & Gervais, 2004)
Pulsed light-emitting diode (LED)	Unbleached wheat flour	Pulse duration: 10 ms, with varying doses depending on wavelength. Four different wavelengths: 275, 365, 395, and 455 nm.	With 395 nm: 2.48 log CFU/g reduction in *Salmonella* counts.	(Subedi *et al.*, 2020)
Pulsed LED	Unbleached wheat flour	Pulse duration: 10 ms, with 395 nm wavelength applied during 10, 30, or 60 min	With 60 min: 2.91 log CFU/g reduction in *Salmonella* counts.	(Du *et al.*, 2020)
Low-temperature plasma	Winter wheat grain	Power supply frequency: 0.1–83 kHz, voltage set at 8 kV. Treatment time: 3, 10, and 30 s	With 10 s treatment: 89.2% of the fungal load was reduced.	(Kordas *et al*. 2015)
Pulsed argon plasma	Wheat grain	Power supply frequency: 10 kHz, voltage: 8 kV. Treatment time: 5-3,6000 s.	With 3,600 s: 3 log CFU/g reduction in *Geobacillus stearothermophilus* endospore load.	(Butscher *et al.*, 2016)

whether wheat was tempered or not before the superheated steam treatment. Mold reduction was reported up to 3.03 log CFU/g with treatment time varying from 10-30. Hu *et al.,* (2016) reported a similar reduction as those obtained by Los *et al.,* (2018) for tepmpered and non-temepered wheat samples regarding *Bacillus* spp. which were 2.64 and 2.53 log log CFU/g, respectively.

The effect of vacuum steam treatment on hard red spring wheat was studied by Snelling *et al.,* (2020). Primary samples were preheated to 40 ± 4°C and then processed through the vacuum steam. Wheat samples were placed in a metal basket (1 kg) with a bed depth of 2.5 cm. The researchers used thermocouples to monitor the temperature in 15 s intervals. The temperature intervals used for this study were as follows: 65, 75, and 85°C and different pressures (mbar). They showed that vacuum steam was effective against *E.coli* O121 where a reduction of 2.46 log CFU/g was detected at 65°C treatment for 6.7 min. Increasing the time of the treatment would increase the log reduction of bacteria, similar to what Hu *et al.,* (2016) reported in their study with superheated steam. In this study *Salmonella* had a reduction of 2.56 log CFU/g at 65°C for 14.8 min (Snelling *et al.,* 2020).

The effect of superheated steam was also studied by Gou *et al.,* (2020). They studied the effect of superheated steam in microbial reduction and quality characteristics of wheat flour, and shelf-life stability of semi-dried whole wheat noodles. The steaming process of whole wheat flour took place at four different temperatures (155, 160, 170, and 190°C) and times (3, 5, 7, and 10 s). Bacterial Total Plate Count (TPC) was enumerated before and after treatment. The log CFU/g reduction of TPC at 155°C at 10 s treatment time was 1.82 log CFU/g. With the increased superheated steam exposure time

and temperature, the Total Plate Count gradually decreased. Gou *et al.*, (2020) reported that with an increased temperature to 190°C and time to 10 s, the TPC was reduced below the detection limit of the method. This relationship between temperature and time was also reported before (Hu *et al.,* 2016; Snelling *et al.*, n.d.), showing that the higher the exposure time and the temperature, the higher the microbial reduction achieved.

Steam is considered as a treatment that could be effective against the inactivation of pathogens during milling (Guo *et al.,* 2020; Hu *et al.,* 2016; Snelling *et al.,* 2020). By applying such a method at an industrial scale, it would help reduce the risk of possible outbreaks in the future from *Salmonella* and *E.coli*, as they are the pathogens of concern in the milling industry (Harris, L. J., & Yada, 2019).

5.2.2. Radio-frequency heating

Convectional thermal treatment in food processing is based on heat transfer by conduction and convection. Radio-frequency heating is an alternative convectional thermal treatment in which electromagnetic energy is transferred directly to the heated product (Altemimi *et al.,* 2019). Radio-frequency (RF) heating has been applied in the food industry as well, and it is considered a tool for the thermal inactivation of pathogens. This is because of the produced heat from the treatment, which increases the temperature and disrupts the living environment for pathogens (Y. Jiao *et al.,* n.d.).

The application of RF treatment on wheat flour has been studied by Villa-Rojas (2017a). In this research, a free-running RF system, with a power of 500 W and a frequency of 27.12 MHz studied the antimicrobial effect of RF heating on wheat flour with different water activities (0.25, 0.45m, and 0.65 a_w). Wheat flour was inoculated with *Enterococcus faecium* and *S. enterica* serovar Enteritidis PT 30. The exposure time

was 8.5-9 min in order to avoid overheating at a temperature of 75°C minimum. The log reduction achieved for these bacteria species was 3.5-7.0 CFU/g. Liu *et al.*, (2018) evaluated the suitability of *E. faecium* as a surrogate for *Salmonella* Enteritidis. They compared the time required to reduce the microbial population by 10-fold at a given temperature of 75, 80, and 85°C. This study showed that regardless of the treatment temperature, *E. faecium* was more resistant than *Salmonella* at any selected time.

In addition to studying RF heating treatment on wheat flour, Jiao *et al.*, (2015) examined the application of RF heating in wheat kernels. The goal of this research was to detect the hot and cold spots to improve the heat uniformity for microbial disinfection. However, they did not report any results regarding the antimicrobial effect of the treatment. This study did generate essential information on developing an RF treatment protocol to control microorganisms or insects in low-moisture granular products such as wheat kernels. Their results indicated that the temperature uniformity depends on the product size, shape, and location. This experiment was done in a pilot-scale free-running RF unit with a built-in hot air system and an inserted conveyor belt. Wheat kernels were placed inside a plastic container with small holes on the side and bottom for RF treatment.

Radio-frequency heating is a promising technology for the heat treatment of dry products in a short time (Villa-Rojas *et al.*, 2017), with the potential to extend the shelf-life of grain seeds, by inhibiting the growth of fungi (Jiao *et al.*, 2015). Some studies also consider RF technology as a method for inactivation of pathogens in wheat flour (Liu *et al.*, 2018; Villa-Rojas *et al.*, 2017). However, information is still lacking on the effect of

this technology on the functional properties of wheat flour – whether it has a positive or negative impact on it.

5.3. Non-thermal technologies for pathogen control in wheat

Some of the non-thermal technologies that have been studied include pulsed light (Aron Maftei *et al.,* 2014; Du *et al.,* 2020; Subedi *et al.,* 2020) and cold plasma (Butscher *et al.,* 2016; Kordas *et al.,* 2013; Los *et al.,* 2018; Selcuk *et al.,* 2008; Zahoranová *et al.,* 2016).

5.3.1. Pulsed light

Consumers want high-quality processed foods with minimal changes in nutritional and sensory characteristics. Generally, non-thermal methods are considered to keep food quality characteristics better than traditional thermal processing (Oms-Oliu *et al.,* n.d.). Non-thermal technologies are considered a superior decontamination alternative to thermal technologies because they can reduce microbial decontamination while preventing undesirable changes to food (Elmnasser *et al.,* 2007; Oms-Oliu *et al.,* n.d.). Oms-Oliu *et al.,* (2010) have been studying the effect of pulsed light in microbial inactivation on food processing equipment, food packaging materials, as well as on food surfaces of fruits and vegetables.

A pulsed light system has been considered as a decontamination strategy to achieve microbial load reduction in wheat, without changing the overall quality of grain. The inactivation of microorganisms during pulsed light treatment is explained by the photochemical and photothermal mechanisms (Rowan *et al.,* 1999). The photochemical mechanism consists of a temporary overheating of the cell which is due to the absorbed light which can cause protein denaturation, and water vaporization, this lead to cell

membrane disruption (Elmnasser *et al.*, 2007); while the photothermal effect is associate with the lethal action of pulsed light.

A study from Aron Maftei *et al.*, (2014) showed decontamination of wheat using pulsed light treatments. This study reported that this method was effective especially on mold inactivation; the greater the number of pulses applied, the greater was the mold inactivation. A 4.0 log CFU/g mold load reduction was achieved with 40 flashed of a dose of 0.4 J/cm^2. Aron Maftei *et al.*, (2014) also showed that pulse light treatment that had higher treatment times achieved greater microbial reductions.

Another study applied pulsed UV light treatment on flour. Fine and Gervais (2004) reported that 58 J/cm^2 were required to decrease the population of *Saccharomyces cerevisiae*. The researchers reported that the thermal effect of pulsed light dominated the UV effect on their treatments. The pulsed light treatment affected the color of the wheat flour by changing it, as the gluten proteins were oxidized and lost the gluten water holding capacity. However, researchers reported having an improvement in gluten viscoelastic properties.

Even though many researchers have shown pulsed light treatment as a way to achieve microbial load reduction, this method still has some drawbacks. According to Gómez-López *et al.*, (2007) and Elmnasser *et al.*, (2007), one of the challenges of a pulsed light system is the limited efficacy to control food heating. The color change of wheat flour reported by Fine and Gervais (2004) was detectable before microbial reduction was observed. Therefore this method is not seen as an adequate technology for cereals and grains due to their opaque nature (Oms-Oliu *et al.*, n.d.).

Table 1-4. A review of decontamination strategies using the non-thermal treatment for microbial load reduction of wheat.

Treatment type	Wheat type	Treatment description	Highest microbial load reduction	References
Superheated steam	Winter wheat grain (*T. aestivum* L.)	Steam temperatures: 110, 140, 170, and 200°C. Steam velocities: 7.5 and 15.0 m^3/hr; time: 10–80 s; and with or without tempering.	With 200°C, 15.0 m^3/hr, 80 s: 81.8% of *Bacillus* spp. counts were reduced.	(Hu *et al.*, 2016)
Vacuum steam	Hard red spring wheat grain	Three temperatures: 65, 75, and 85°C; time: 4 or 8 min in a closed system. Samples (1 kg) treated as a grain bed in a depth of 2.5 cm.	With 65°C for 8 min: 3.57 log CFU/g reduction of *E. coli* and 3.21 log CFU/g reduction of S*almonella.*	(Snelling *et al.*, 2020)
Radio frequency (RF) heating	Soft white wheat organic pastry flour (Eden Foods, MI)	0.5 kW, 27 MHz RF heating unit. Four electrode gaps (50, 60, 70, and 90 mm) and three surrounding materials (polyetherimide, polyethylene terephthalate, and polystyrene).	With 90 mm electrode gap using polystyrene cylinders: 7 log CFU/g reductions in *Salmonella.*	(Villa-Rojas *et al.*, 2017)
RF pasteurization	Soft winter wheat organic flour (Eden Foods, MI)	6 kW, 27.12 MHz RF heating unit. Electrode gap 35 mm. Aluminum test cells. Three temperatures (75, 80, and 85°C).	At 85°C, 33 min: 3.7 log CFU/g reductions *in Enterococcus faecium* and 5 log CFU/g reductions in *Salmonella.*	(Liu *et al.*, 2018)

5.3.2. Cold Plasma

The application of low-pressure cold plasma for decontamination of cereal grains has been reported for inactivation of indigenous microbial communities of grains (Kordas *et al.,* 2013; Selcuk *et al.,* 2008) and artificially contaminated cereal grains and seeds (Butscher *et al.,* 2016; Zahoranová *et al.,* 2016).

A study from Los *et al.* (2018) investigated the atmospheric cold plasma (ACP) as an alternative for decontamination of grain. In this study, both cereal grain decontamination and grain quality were investigated. The researchers inoculated wheat and barley with pathogens such as *E.coli, B. atrophaeus* (vegetative cells and endospores), and *P. verrucosum* (spores). The concentration of the inoculum was 8.0 log CFU/ml. *Bacillus atrophaeus* endospores showed the highest resistance with only 2.4 log CFU/g microbial reductions for 20 min treatment time. The maximum reduction achieved for wheat was 1.5 and 2.5 log CFU/g for bacteria and fungi, respectively.

The atmospheric cold plasma is considered a promising tool for effective decontamination of cereal grain and modulation of functional properties (Los *et al.,* 2018). Furthermore, Kordas *et al.* (2013) investigated the antifungal activity of low-temperature plasma on winter wheat grain. The fungi population was decreased by 89.2% with a 10 s optimum time. This treatment did not affect the germination ability of winter wheat. Zahoranová *et al.* (2016) conducted a study on the effect of cold atmospheric pressure plasma (CAPP) treatment on the inactivation of pathogenic and toxigenic microorganisms which are resent on the surface of wheat seeds (*Triticum aestivum*). Researchers reported the CAPP treatment led to a significant reduction of epiphytic bacteria, pathogenic and toxigenic filamentous fungi.

This method is considered advantageous when working in wet and dusty environments and continuous mode, making it easier for use in agricultural practice (Zahoranová *et al.,* 2016).

6. EFFECT OF DIFFERENT ANTIMICROBIAL TREATMENTS ON THE FLOUR FUNCTIONALITY

Wheat flour is one of the most popular ingredients in the kitchen and in the food processing industry. The pre-and post-milling interventions that may be applied to improve the microbial quality of the flour can affect the functional properties of this ingredient. Several methods have been studied for flour decontamination such as tempering solutions (Dhillon *et al.,* 2007a; Ibanoglu, 2000; Sabillón *et al.,* 2016; Sabillón *et al.,* 2019, 2020), non-thermal technologies (Aron Maftei *et al.,* 2014; Butscher *et al.,* 2016; Kordas *et al.,* 2013; Los *et al.,* 2018; Subedi *et al.,* 2020; Zahoranová *et al.,* 2016), and thermal technologies (S. Jiao *et al.,* 2015; Liu *et al.,* 2018; Villa-Rojas *et al.,* 2017).

Some of these studies, in addition to microbial reduction, showed how specific treatments would affect flour properties based on the treatment type, time, type of tempering solution, thermal or non-thermal treatment applied, etc.

A study by Ibanoglu (2000) showed the influence of tempering with ozonated water on hard and soft wheat flour. The wheat samples were tempered using 1.5 and 11.5 mg ozone/l. The application of ozonated water did not change the flour quality properties (Ibanoglu, 2000). The results of this study showed the α-amylase activity

of the final flours did not change after tempering with ozonated water. This study also evaluated chemical and physical changes associated with flour. Results showed no effect on sedimentation volumes. Even though ozone tends to decolorize some of the food components, Ibangolu's (2000) study did not show any changes regarding this flour property. Furthermore, the farinographs results also did not show any significant change due to the treatments for either soft or hard wheat samples.

Sabillón *et al.,* (2019) showed that the application of tempering solutions such as organic acid 2.5 and 5.0% (acetic and lactic acid) in combination with NaCl 26% can alter some of the properties of whole-grain and straight-grade flour. This study showed significant differences in the pasting properties of whole-grain flour. The treatment combinations of acetic acid 2.5 and 5.0 % + NaCl 26% and lactic acid 2.5% + NaCl 26% showed a reduction of peak viscosity, setback viscosity, and final viscosity when compared to the control samples. While lactic acid 5.0% + NaCl 26% did not show any significant difference from control. Since the application of acid will lower the pH of the flour, Sabillón *et al.,* (2019) reported that this could affect the time required to reach the peak viscosity. Similar trends regarding the pasting properties of the straight-grade flour were observed in the same study by Sabillón *et al.,* (2019). Furthermore, this study evaluated the Mixograph curves of the whole-grain flour, which showed no significant effect regarding the tempering solution used. However, for the straight-grade flour, the saline organic solutions did affect some of the Mixograph variables. The flour, which was tempered with 2.5% acetic acid, showed a shorter peak time compared to the control, while no significant increases in the mixing tolerance and the optimum mixing time were observed (Sabillón *et al.,*

2019). Even though some authors have reported that the dough development time and dough stability may be increased by the application of organic acid and NaCl, this study didn't show any significant increase in the mixing tolerance and optimum mixing time. Both whole-grain bread and straight-grade flour bread did not show any significant differences compared to the control bread.

The studies discussed here, indicate that the use of ozonated water as a tempering solution of wheat did not lead to any detrimental change in the chemical and physical properties of the flours evaluated (Ibanoglu, 2000). Similar conclusions were reported by Sabillón *et al.*, (2019) regarding the use of the saline organic acid solution as an antimicrobial tempering solution for hard wheat. According to the authors, this tempering solution did not show any significant changes in the functional properties of both types of flour (whole-grain and straight-grade flour), even though some individual variables were different when compared to control. Furthermore, the baking results showed that the bread firmness and volume were not affected by the treatments applied (Sabillón *et al.*, 2019).

Snelling *et al.*, (2020) studied the effect of vacuum steam on hard red spring wheat and flour functionality. The temperatures used for this study were 65, 70, 75, and 85°C for 4 and 8 min treatment time. The results showed that regardless of the treatment temperature or time, the milling yield did not change. While protein was reduced by 0.2% at temperatures ≥75°C, the authors indicated that this change would not affect the functionality of the flour since it was considered small. Flour color and starch analysis showed no differences when samples were compared to control samples. Generally, the water absorption was decreased from 1 to 2% from the

control samples, but the overall results from the farinograph were minor (Snelling *et al.,* n.d.). Furthermore, this study showed that with the increase of temperature to 75 and 85°C the wet gluten, peak viscosity, and loaf volume of the bread reduced significantly. Another study from Poudel and Rose (2018) did not observe any significant differences between samples treated with saturated steam for a time of up to 90 seconds.

Based on the studies reported here, whatever parameters are chosen for heat treatment, must be checked for their effect on functionality. The application of vacuum steam seems to be another tool that is effective for decontamination of wheat before milling; however, like with other thermal treatments, the functionality of the final flour must be evaluated.

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CHAPTER 2. THE EFFECT OF LACTIC ACID IN ASSOCIATION WITH STEAM IN REDUCING MICROORGANISMS IN HARD RED WINTER WHEAT

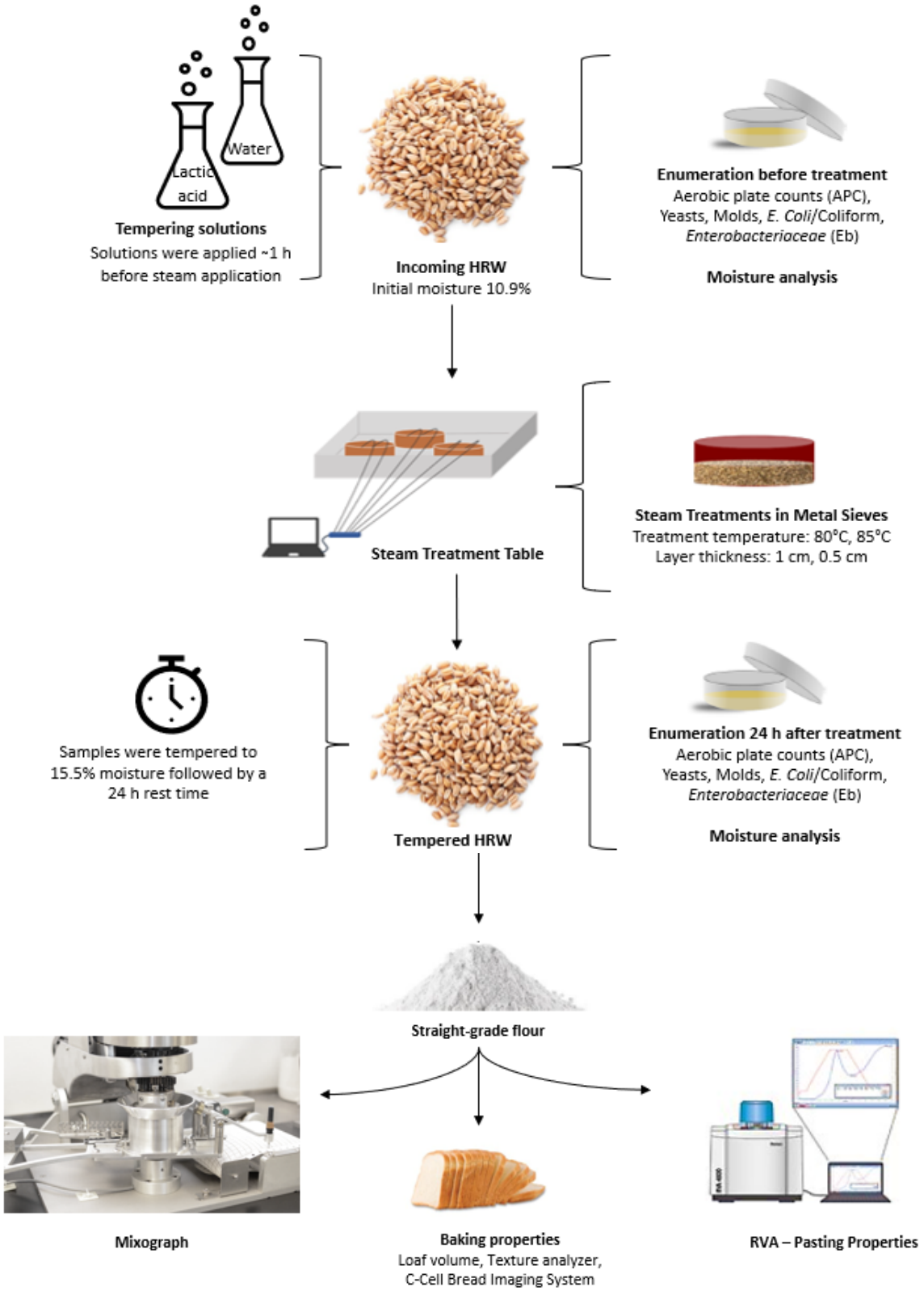
CHAPTER 2. THE EFFECT OF ORGANIC ACIDS IN ASSOCIATION WITH STEAM IN REDUCING MICROORGANISMS IN HARD RED WINTER WHEAT

ABSTRACT

Generally, wheat-based products have been considered as safe food for human consumption because they are a low moisture food and most of the finished products are thermally processed. Raw flour has been associated with microbial hazards, which, if not properly managed, may have the potential to result in serious health consequences. Previous studies have shown that tempering hard wheat with organic acids, as well as steam application, can have the potential to reduce the microbial load of wheat with minor changes on the functional properties of the flour. Thus, the objective of this study is (a) to study the effect of saturated steam by itself or in combination with lactic acid, to reduce the natural microbial load of hard red winter wheat, and (b) to evaluate the impact of these interventions on the functional properties of hard wheat flour. Hard red winter wheat samples were treated by adding acid to the tempering water, by steaming the kernels in replacement of the traditional steep, or by adding a combination of both. Samples were placed into sieves (bed depth 0.5 and 1.0 cm) for those treatments where steam was included and treated with steam in a steam table until grain temperature achieved 80 and 85°C. After treatment was applied, hard wheat was allowed to temper to 15.5% moisture for 24 h at room temperature (~24°C.and 60% RH) under aseptic conditions. Before and after tempering, wheat samples were tested for Aerobic Plate Count (APC), coliforms, *Enterobacteriaceae* (Eb), yeast, and mold. Selected treatments

were chosen for further evaluation of flour functionality. Wheat samples were milled into straight-grade flour using a pilot Bühler mill. The use of steam as a tempering intervention led to significant decrease in microbial population associated with the wheat grain, when compared to controls. Furthermore, the addition of acid prior to steaming increased the steam efficacy. Overall, the combination of acid and steam achieved the highest overall microbial reductions among the treatment combinations. More specifically, with the steam and acid treatment combination APC, Eb and coliforms were reduced by 4.0, 4.1 and 4.3 log CFU/g, on average, respectively. The tempering interventions using steam, and water or acid solution were used to adjust grain moisture, were chosen for further evaluation of straight-grade flour properties. When flour functionality was evaluated, the interventions applied resulted in differences for individual parameters associated with pasting properties. However, baking performance, including bread firmness and loaf volume, was not as affected by most of the treatments applied. Bread image analysis showed minimal changes when steam in association with acid was used as a tempering intervention. However, when water was added before steaming, the slice brightness, and number of cells were affected by the treatments. Even though the interventions suggested here provided promising application in the milling industry, further research is needed on different wheat classes and the impact of the treatment on sensory characteristics of the end-product.

VISUAL SUMMARY



1. INTRODUCTION

Wheat milled products, including flour, fall into the category of low-moisture foods. For several decades, the low water activity (0.85) found in these products has contributed to the belief that from a microbiological point of view, these foods were of no public health concern (Sabillón & Bianchini, 2016). The microbiological quality of wheat grain is a major contributor to the loss of the quality and safety of the ingredients provided by milled products and foods (Berghofer et al., 2003). Several studies on the microbial quality of wheat have been carried out over the last two decades due to the increasing awareness of its potential contamination with pathogens (Dhillon et al., 2007a; Eglezos, 2010a; Laca et al., 2006; Myoda et al., 2019; Sabillón et al., 2020). The wheat microflora is influenced by many factors, including irrigation water, insect infestation, soil, environmental conditions, and animal feces (Laca et al., 2006). The key preventive steps to avoid contamination are the growing conditions of the crops (Thielecke & Nugent, 2018). In addition, it is important to mention that the handling of grains during post-harvest, transport, processing, and storage also affects grain hygiene (Labbe et al., 2014).

To mitigate the risk of grain contamination it is important to establish realistic and efficient antimicrobial treatments that could be used by the industry for the processing and distribution of safer milled products. Since none of the operations used in the wheat dry-milling process require either chemical or thermal treatments, the effect of the operations on wheat microbial load reduction is insignificant (Sabillón et al., 2020). Recently, Sabillón et al., (2020) showed that decontamination of wheat by tempering hard wheat kernels with solutions containing a combination of organic acid and NaCl, regardless of tempering temperature, effectively reduced a load of pathogenic

microorganisms when compared to the traditional tempering process using water. The combination of NaCl 26.5%and lactic acid (5.0%) was the most effective treatment against Salmonella enterica, *E. coli* O157:H7, and non-O157 STEC. The average reduction for Salmonella enterica, *E. coli* O157:H7, and non-O157 STEC was 1.8, 1.8, and 1.6 log CFU/g for soft wheat and 2.6, 2.4, and 2.4 log CFU/g for hard wheat, respectively. While other combinations of acids and NaCl (26.5%) gave similar but less effective log CFU/g reduction of Salmonella enterica, *E. coli* O157:H7, and non-O157 STEC (Sabillón et al., 2020). Another study by Sabillón et al., (2016) showed that with the use of up to 5.0%, of acetic, lactic, and propionic acids, reductions of 1.7, 2.3, and 3.8 log CFU/g were achieved in aerobic plate count (APC), *Enterobacteriaceae* (Eb), and mold counts, respectively. While the combination of lactic acid (5.0%) and NaCl (52%) were the most effective against APC, with an average reduction of 4.3 log CFU/g.

Thermal treatment is another technology that may be applied to wheat kernels to achieve microbial reduction. Vacuum steam treatment is considered an effective pathogen inactivation method for the flour milling industry. A study from Snelling et al., (2020) treated hard wheat samples with steam under vacuum at 65, 70, 75, and 85°C for 4 and 8 min. Significant changes in dough and baked product functionality were observed at ≥70°C. This study showed that treatment time had no significant effect on flour functionality for those parameters evaluated. Another study has shown that the use of steam in wheat kernels is effective in reducing the lipolytic activity in flour; while not affecting its functional properties (Poudel & Rose, 2018). This study also showed that steaming time did not affect lipid oxidation in flour, starch and gluten properties. Effects of tempering with steam have been studied by Chen et al., (2020) where it showed that

yeast and mold counts (YMC) and mesophilic aerobic spores in flour were decreased by the use of temperature. Also, the authors suggested that steam tempering could shorten the tempering time and partially improve the functionality of the flour. Thus, the objective of this study was (a) to evaluate the effect of steam by itself or in combination with lactic acid, to reduce the natural microbial load of hard red winter wheat, and (b) to evaluate the impact of these interventions on the functional properties of hard wheat flour.

2. MATERIALS AND METHODS

2.1. Materials

Hard red winter wheat (HWR) was commercially available purchased for this research. Samples of HWR were received, mixed, and stored at room temperature before use. Lactic acid (85%) was obtained from Fisher Scientific™ (Pittsburgh, PA).

2.2. Experimental design

Figure 2-1 describes the experimental design used to evaluate the effect of steam, by itself or in combination with lactic acid, on the microbial load of wheat kernels. Steam treatment was applied to wheat directly kernels as the only source of water for moisture increase during tempering, and to wheat kernels that had been pre-tempered with water to ensure the desired final tempering moisture. Tempering with lactic acid solution was also evaluated, along with the combination of steam and lactic acid. The combination of mechanisms for microbial inactivation (acid and steam) was evaluated to observe if there was any synergetic effect between them while reducing the microbial load of hard wheat

(Figure 2-1). In the absence of an intervention, water was used as a control for the tempering process.

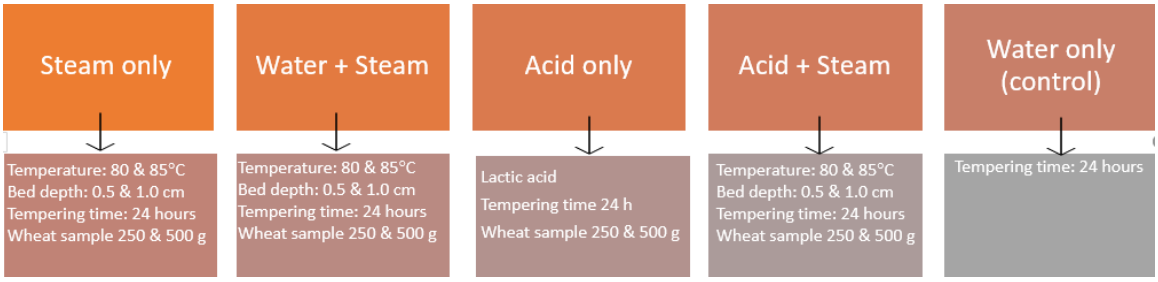


Figure 2-1: Experimental design to evaluate the effect of steam, by itself or in combination with lactic acid, on microbial load of hard wheat kernels

At first the effect of acid tempering (5% lactic acid) or steam treatment by itself (0.5 cm bed depth; 80 °C final temperature) and their combination (0.5 cm bed depth; 80 °C final temperature tempered with 5% lactic acid) were evaluated. And based on the results obtained, then the effect of temperature, bed depth, grain moisture and acid tempering were further studied as described in **Figure 2-1**.

2.3. Tempering with water

According to the experimental design (**Figure 2-1**), two-bed depths were evaluated. For the bed depth of 0.5 cm, a wheat sample of 250 g was required; while for the bed depth of 1.0 cm, the amount of wheat used was 500 g. The amount of distilled water required was based on the sample size and treatment temperature when samples undergo the steam treatment. However, temperature treatment was not taken into account for the control (untreated) samples; only sample size of 250 g was considered. Calculations were based on the initial moisture of the wheat (10.9%) and final weight of grain after tempering to 15.5% moisture. To allow the best distribution of the water in the wheat sample, the distilled water was applied to the kernels in a biosafety cabinet with the help

of an atomizer. The wheat samples were shaken every 15 min for 2 h of the tempering process and then allowed to temper for 24 h at room temperature.

2.4. Tempering with lactic acid

2.4.1. Preparation of tempering solution

A lactic acid solution was used to temper the wheat kernel. The amount of acid used during the tempering was 1.8 mL lactic acid (85%) per kilogram of tempered wheat. This acid application is equivalent to the one used by Sabillón et al., (2016) when 5.0% lactic acid solutions were used in their study. In this research, the amount of distilled water and lactic acid (85%) was reduced to achieve the desired acid treatment and final moisture upon tempering. Calculations were based on the initial moisture of the wheat (10.9%) and final weight of grain after tempering to 15.5% moisture. Taking into account the volume of acid needed, the remaining amount of distilled water required to achieve 15.0% moisture after tempering was also calculated for each size.

2.4.2. Application of tempering solution

According to the experimental design (**Figure 2-1**), two-bed depths were evaluated. For the bed depth of 0.5 cm, a wheat sample of 250 g was required; while for the bed depth of 1.0 cm, the amount of wheat used was 500 g. After the amount of lactic acid (85%) and distilled water required to achieve the acid treatment was determined, they were applied to hard wheat samples.

To allow the best distribution of the acid on the wheat sample, the distilled water and lactic acid (85%) were first mixed and then applied to the kernels in a biosafety cabinet

with the help of an atomizer. The wheat samples were shaken every 15 min for the first 2 h of the tempering process and then allowed to temper for 24 h at room temperature.

2.5. Tempering with steam

2.5.1. Direct application of steam to wheat kernels

Samples of hard red winter wheat were divided into 250 g subsamples to achieve a 0.5 cm bed depth, while 500 g subsamples were used for 1.0 cm bed depth when using a mesh sieve (standard 40 mesh sieves with 0.420 mm hole thickness) as a carrier for the samples. Two thermocouples (Omega TJ36-CPSS-116G-g, Omega, Norwalk, CT, USA) were placed in the center of the grain bed in each sieve. Three sieves were placed on a steam table that had been pre-heated to 100 ºC. The thermocouples were monitored using Omega Logging Software (Picto technology LTD, England, UK) which recorded the sample temperature every second. Sieves were placed on a steam table that was preheated up to 100ºC. After placing the sieves inside the steamer, grain temperature was monitored until it achieved 80 or 85ºC, according to the experimental design. When the target temperature was reached (80 or 85ºC), the wheat samples were taken out of the steam table. Right after the steaming process, the samples were placed into sterile plastic bags and immediately placed in an ice bath for ~5 min for cooling. Samples were allowed to temper at room temperature for 24 hours inside the plastic bags.

2.5.2. Steaming of pre-tempered wheat kernels

Hard wheat samples were also pre-tempered with water before steam was applied to ensure a final moisture content of 15.5% after tempering. With the direct application of steam to wheat kernels, the amount of water added could not be controlled. Therefore, pre-tempering with water was included to ensure that the final desired moisture was

achieved. Calculations for the amount of water to be added was done knowing the initial moisture of hard wheat kernels and the amount of moisture that each steam treatment (temperature and bed depth) adds, by conducting preliminary tests so that the final moisture after application of tempering solution and steam was at the desired level of 15.5%. Pre-tempered hard wheat was then steam as described under 2.5.1.

2.6. Tempering with steam and lactic acid

For this set of experiments, the amount of water required to be added to each sample as pre-tempering treatment was calculated as described under 2.5.2. Then the amount of acid for each sample size was calculated to achieve a concentration of 1.5 mL lactic acid (85%) per kilogram of tempered wheat. The lactic acid was combined with water required for pre-tempering and added prior to steaming (≤1 h before steaming). Once again, to allow for an even distribution of water and acid over the hard wheat samples, the mixture was applied in a biosafety cabinet with the help of an atomizer. The samples were then shaken every 5 min before the steaming process. After the pre-tempering with acid and water was completed, hard wheat kernels were steamed as described under 2.5.1.

2.7. Microbial analysis

To determine the effect of lactic acid and steam as antimicrobial interventions in wheat, the microbial load of the samples was determined in duplicate before and after each treatment. To perform the microbial analysis, 25 g of wheat samples and 225 mL of sterilized 0.1% peptone solution were placed into a sterile plastic bag. Wheat kernels were soaked for 5 min and then mixed with a stomacher blender (Stomacher 400, Seward Ltd, Bohemia, NY) for 120 sec. After mixing, serial dilutions were prepared using

sterilized 0.1% peptone solution, and microbiological tests were performed to include – Aerobic Plate Count (APC), *Enterobacteriaceae* (Eb), generic Escherichia coli (EC), Coliforms, Yeast, and Mold. For APC enumeration, dilutions were spread plated onto Standard Methods Agar (Remel, Thermo Fisher, Lenexa, KS) and incubated for 48 h at 35ºC. *Enterobacteriaceae* (Eb) was determined using Petrifilm™ (3M Microbiology, St. Paul, MN), with samples incubated at 37 ºC for 24 h. Coliforms and generic Escherichia coli (EC) were enumerated using EC Petrifilm™ (3M Microbiology, St. Paul, MN), with incubation at 37 ºC for 24 h (coliform count) or 48h (generic EC counts). Yeast and mold counts were determined using Dichloran Rose Bengal Chloramphenicol Agar (Oxoid, Basingstoke, Hampshire, UK). Samples were spread onto plates and incubated at 25ºC for 3 days (yeast) and 5 days (mold).

2.8. Moisture of tempered wheat

Except for the moisture of wheat samples that were directly steamed, all others by the end of the tempering process had their moisture adjusted to 15.5%. To verify the moisture content before treatment, after treatment, and before milling, sample moisture was determined using a forced-air oven method according to 44-15.02 (AACCI, 2009).

2.9. Experimental milling

The treatments with the highest overall microbial reduction (pre-tempering with acid and water followed by steaming) were selected to evaluate their impact on the functional properties of flour. Therefore, enough wheat was treated according to sections 2.5 and 2.6 to produce 800 g of wheat per replicate. Three replicates per treatment were evaluated. A Buhler experimental mill was used to obtain straight-grade flour following the AACC standard method 26-21.02 (AACCI, 2009). Milling room temperature and

relative humidity were kept at 22-24° and 60%, respectively, to ensure reproducibility of results. After the milling process, flour was mixed thoroughly for further analysis and stored in the refrigerator (4°C) until processing.

2.10. Functionality testing and flour properties

2.10.1. pH and acid content

The pH of the flour was measured according to AACC standard method 02-52.01 (AACCI, 2018). In short, 10 g of flour along with 100 mL of distilled water was placed in an Erlenmeyer flask and mixed for 15 min with a magnetic stirrer, followed by a 10 min rest. The supernatant liquid was decanted into a clean Erlenmeyer flask and the pH was determined with a calibrated Thermo Scientific Orion 2-star Benchtop pH meter. After measuring the pH, the acid content of the supernatant was determined by titrating with 0.1 N NaOH to a final pH of 7.0.

2.10.2. Mixing properties

Mixing properties of the straight-grade flour were evaluated using a Ten g Mixograph (National Manufacturing, Lincoln, NE, USA) following method 54.40.02 (AACCI, 2009). The Mixograph software allowed for the collection and reporting of data and results. The water absorption and the mixing time were determined and recorded using the results from the mixograph data, which were later applied when evaluating the baking properties of bread.

2.10.3. Pasting properties

The pasting properties of the flour were evaluated in duplicate by Rapid Visco Analyzer (RVA) (Model 4S, New Port Scientific; Warriewood, NSW, Australia)

following AACC standard method 76-21.01 (AACCI, 2009). In short, 3.5 g flour with moisture adjusted to 14% was mixed with 25 mL of distilled water and added to a test container. The sample was mixed by vigorously plunging the test container blade through the mixture for 30 s to avoid the formation of clumps during the analysis. Samples were analyzed using the Standard=1 test profile in the RVA software. Maximum viscosity, minimum viscosity after peak, final viscosity, and time to peak viscosity were recorded and collected from the RVA.

2.10.4. Baking properties

Baking properties for the straight-grade flour were determined in duplicate using AACCI Approved Methods 10-10.03 (AACCI, 2009). Bread volume was measured 1 h after cooling according to AACCI Approved Method 10-05.01 (AACCI, 2009). Using a bread-slicing guide and an electric knife, loaves were sliced to 12.5 mm. After that, the crumb structure was evaluated using a C-Cell Bread Imaging System (Calibre Control International, Warrington, U.K.). From the overall data collected by the C-Cell software, parameters such as slice area, height, brightness, number of cells, cell diameter, and average cell elongation were chosen for further statistical analysis. The texture of bread slices was evaluated according to AACCI Approved Method 74-10.02 (AACCI, 2009).

2.11. Statistical analysis

Statistical analysis of data from each microorganism group was conducted separately. For analysis of the first experiment comparing control, acid tempering, and steam treatments an analysis of variance (ANOVA) using treatment as the factor was performed (version 9.4, SAS Institute, Bray, NC USA). For analysis of different levels of tempering and steam treatment, a three-factor ANOVA with interactions was used with tempering

condition, wheat bed depth, and final temperature as the factors. When the ANOVA F-values were significant ($p<0.05$), Tukey's honestly significant difference was used to compute differences among treatments or factors. For comparison of experimental data with calculated values in the first experiment, a one-sample t-test was performed. PROC GLIMMIX procedure in SAS 9.4 was used to analyze the effect of the different treatments on the functional properties of the flour. When differences occurred, they were reported at the $\alpha=0.05$ significance level with Tukey-Kramer adjustment applied to obtain appropriate p-values. For the flour functionality parameters, a nested factorial treatment design was used to account for the fact that the control had no temperature or bed depth values associates with it. Tukey-Kramer and Dunnett adjustments were used to account for multiple comparisons, where appropriate.

3. RESULTS AND DISCUSSION

3.1. Effect of acid, steam and their combination in hard wheat tempering

3.1.1 Effect of tempering on grain moisture

All interventions applied to control microbial contamination resulted in moisture increase in the wheat kernels. However, different treatments contribute more or less to the final moisture of the grain. **Table 2-1** shows the results obtained for final moisture and moisture addition upon the tempering process.

When steam was used by itself to temper hard wheat, the final moisture of the grain was increased to 11.48%, but the range of 15.5% that is desirable for hard wheat milling was not achieved. However, when lactic acid was used or combined with steam for

tempering, the final moisture of wheat was close to the target moisture 15.5% (±0.5%). Therefore, based on the data provided here, it is necessary to add a tempering solution (lactic acid solution, or simply water) to the tempering with steam if the target milling moisture is to be achieved.

Table 2-1. Final moisture of hard wheat after tempering with acid, steam or their combination.

Treatment	**Temperature (◦C)**	**Bed Depth[2] (cm)**	**Moisture Addition[3] (%)**	**Final moisture[3] (%)**
Control (water)[1]	NA	0.5	5.32 ± 0.13	15.38 ± 0.52
Steam	80	0.5	1.36 ± 0.34[2]	11.48 ± 0.31
Acid	NA	0.5	5.37 ± 0.44	15.49 ± 0.51
Acid + Steam	80	0.5	5.78 ± 0.48	15.91 ± 0 .58

1 Sample size equivalent to 0.5 bed depth
[2] Bed depth represents the thickness of the wheat layer during steam treatment
[3] Values denote mean ± standard deviation

3.1.2 Reduction of natural microflora

Previous studies have reported that steam treatment can be an effective method for the inactivation of wheat natural microbiota (Hu et al., 2016; Wang et al., 2019). Additionally, the use of tempering solutions such as organic acids has also been reported as a tool for the reduction of microbial load on natural microflora on wheat (Sabillón et al., 2016; 2020). In this research, hard red winter wheat samples were treated with saturated steam (100°C) by itself and in combination with lactic acid. The efficacy of steam, lactic acid, and in combination against the natural microflora of hard wheat is presented in **Figure 2-2.** From the statistical analysis, different groups of microorganisms were affected differently by the interventions applied, with molds being the ones most reduced (up to 4.26 log CFU/g) followed by coliforms (up to 3.47 log CFU/g).

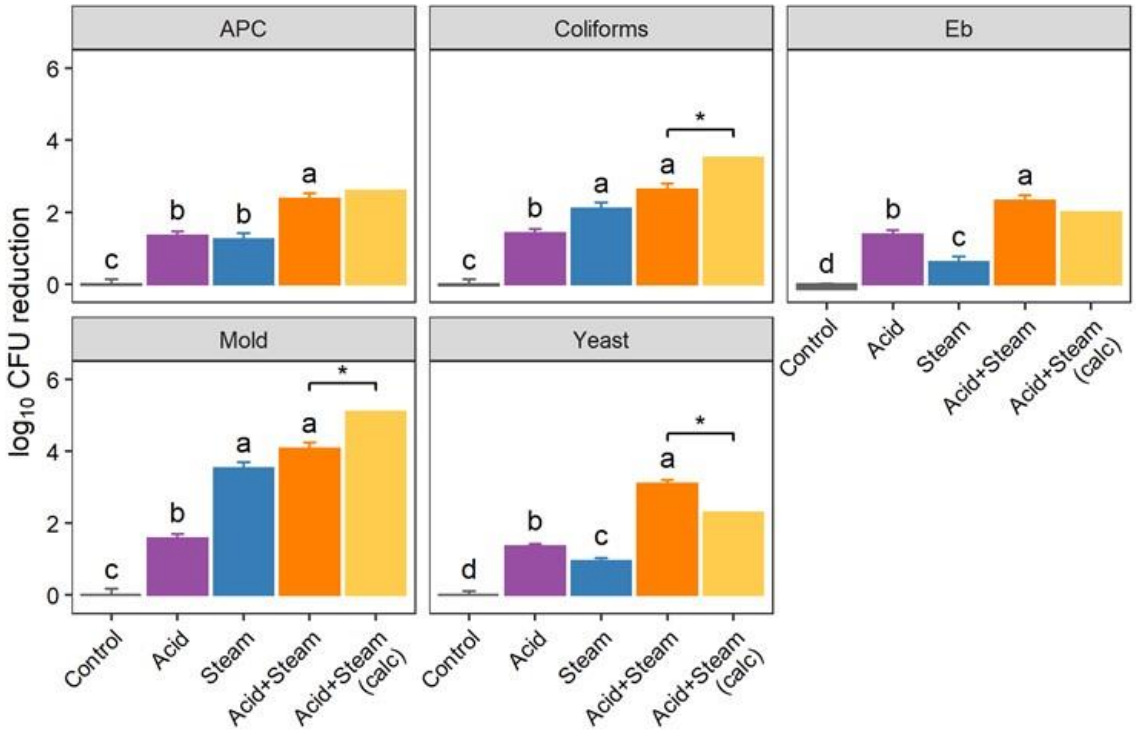


Figure 2-2. Effect of acid tempering (5% lactic acid) or steam treatment (0.5 cm bed depth; 80 °C final temperature) and their combination (0.5 cm bed depth; 80 °C final temperature tempered with 5% lactic acid) on natural microbiota reduction in hard wheat. Bars marked with different letters within panel are significantly different (Tukey's HSD $p<0.05$); *$p<0.05$ for experimental results compared with the calculated sum of acid and steam treatment.

When the effect of each intervention was evaluated, within microbial groups, the effect of steam was variable, showing results that were either similar (APC), superior (coliforms and mold), or inferior (Eb and yeast) to acid by itself. However, when acid and steam were combined, the results were always significantly superior then the effect of acid by itself. Statistical analysis also indicated that the effect of acid and steam combined is equivalent to the sum of the effects of the two interventions for APC and Enterobacteriaceae, but significantly lower than the sum for coliforms and molds.

In general, the results from these experiments indicated that a combination of acid and steam may be beneficial to the food industry in increasing the microbial safety of wheat-based products. Therefore, further variables were included in the experimental design to evaluate the efficacy of these interventions. The variables included were grain bed depth and final temperature. Also, from the moisture analysis it was evident that pre-tempering the grain prior to steam treatment would be required if ideal milling moisture was to be achieved at the end of the tempering interventions.

3.2 Effect of bed depth and temperature when tempering hard wheat with steam and acid

3.2.1. Moisture of tempered hard wheat

Table 2-2 shows the moisture data for hard wheat upon tempering when the expanded experimental design was followed, where bed depth and grain bed temperature were included as new variables. Additionally, for these experiments water was added to a set of samples, prior to the steam treatment, to aid with achieving the desired final grain moisture upon tempering.

In general, results showed that all interventions applied to control microbial contamination, once again, resulted in moisture increase in the wheat kernels. However, as noted earlier, those treatments where only steam was applied, the final tempered moisture (11.07% - 12.15%) was lower than desired; while the treatments where either water or an acid solution was applied prior to steaming led to grain tempered at the proper moisture levels of at least 15.3%.

Table 2-2. Final moisture of hard wheat after tempering with acid, steam or their combination when variables were expanded to include bed depth and grain temperature.

Treatment	Temperature (◦C)	Bed Depth[2] (cm)	Moisture Addition[3] (%)	Final moisture[3] (%)
Control (water)[1]	NA	0.5	5.32 ± 0.13	15.38 ± 0.52
Steam	80	0.5	1.36 ± 0.34[2]	11.48 ± 0.31
Steam	80	1.0	0.99 ± 0.68	11.11 ± 0.15
Steam	85	0.5	0.94 ± 0.68	11.07 ± 0.31
Steam	85	1.0	2.03 ± 0.59	12.15 ± 0.24
Acid	NA	0.5	5.37 ± 0.44	15.49 ± 0.51
Acid	NA	1.0	5.30 ± 0.27	15.32 ± 0.23
Water + Steam	80	0.5	5.64 ± 0.92	15.76 ± 0.02
Water + Steam	80	1.0	5.48 ± 0.31	15.60 ± 0.77
Water + Steam	85	0.5	5.20 ± 0.44	15.32 ± 0.35
Water + Steam	85	1.0	5.81 ± 0.16	15.93 ± 0.23
Acid + Steam	80	0.5	5.78 ± 0.48	15.91 ± 0 .58
Acid + Steam	80	1.0	5.77 ± 0.67	15.89 ± 0.79
Acid + Steam	85	0.5	5.82 ± 0.11	15.95 ± 0.17
Acid + Steam	85	1.0	5.99 ± 0.37	16.15 ± 0.12

1 Sample size equivalent to 0.5 cm
[2] Bed depth represents the thickness of the wheat layer during steam treatment
[3] Values denote mean ± standard deviation

Snelling et al., (2020) reported a slight decrease in moisture content when applying vacuum steam treatment on hard red spring wheat samples. Hu et al., (2016) reported moisture increase up to 16.0% using superheated steam.

3.2.2. Temperature profile of hard wheat during steam treatment

Figure 2-3 shows the time required to reach the target temperature for each specific bed depth. Based on the data presented in the chart there is a direct correlation between grain temperature, time, and bed depth.

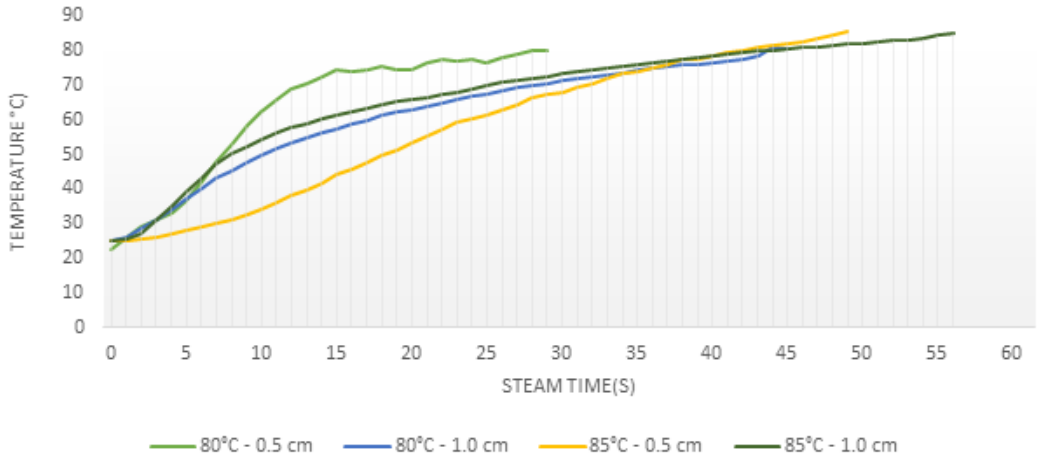


Figure 2 3. Temperature profiles of hard red winter wheat during steam application.

Increasing the bed depth from 0.5 to 1.0 cm at 80°C required the steaming time to be increased by 14 ± 2 sec. The same trend was observed for 85°C, with the increase in bed depth increasing the treatment time by 6 ± 4 sec. And as expected, the increase in target temperature from 80 to 85°C also led to an increase in treatment time for each of the bed depth tested. For example, for the bed depth of 0.5 cm, an increase in treatment time of 20 sec was observed when the final grain temperature was changed from 80°C to 85°C.

3.2.3. Reduction of natural microflora

Based on the results obtained from tempering hard wheat with steam, acid and their combination, the experimental design was expanded to include grain bed depth and final temperature. **Figure 2-4** shows the average log reduction achieved by these treatments when the tempering conditions, bed depth and final temperature were considered.

When the steam tempering conditions were considered within microbial groups, the addition of water to the steam treatment led to a significant reduction in steam

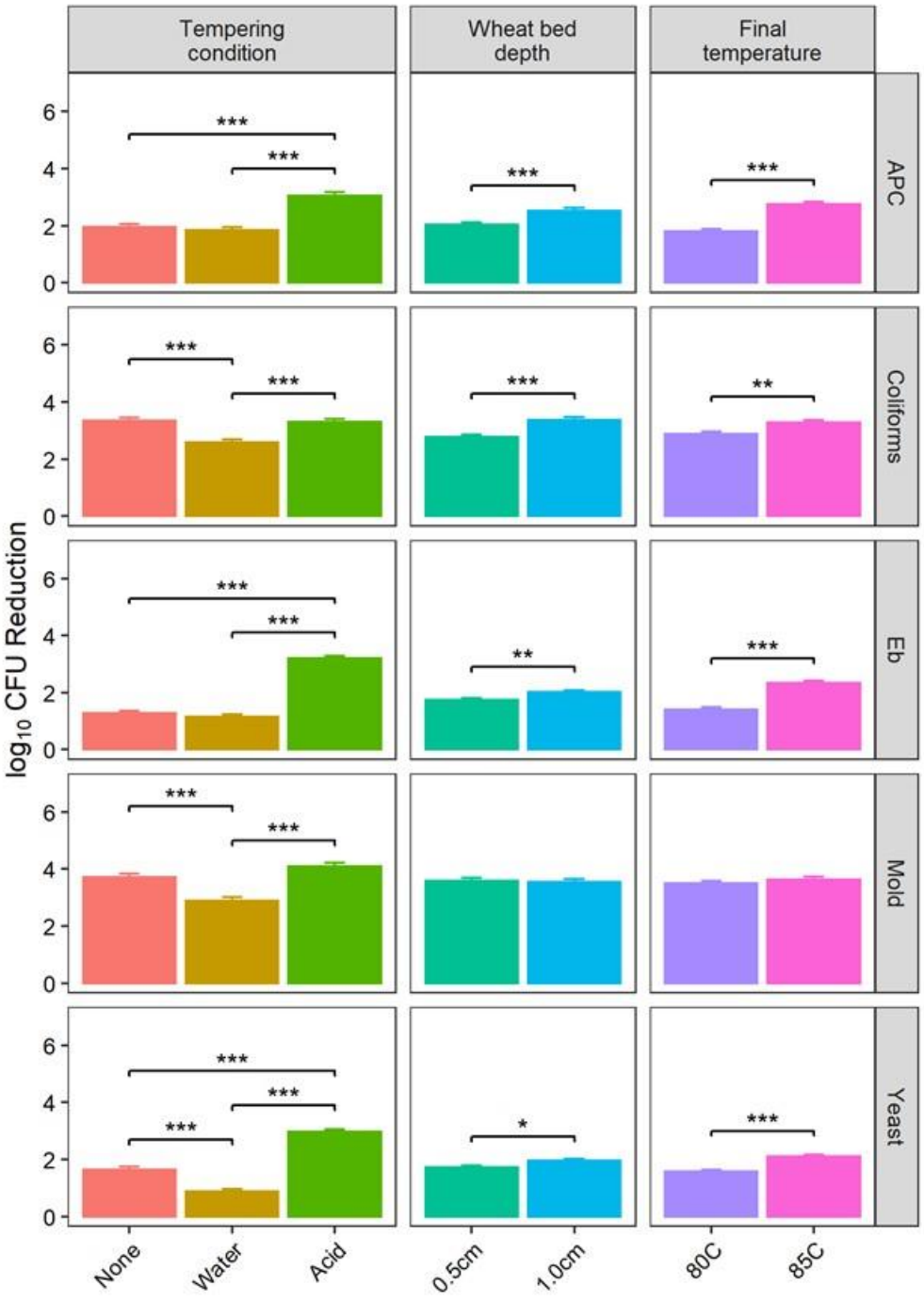


Figure 2 4. Effect of tempering and steam treatment variables (bed depth and temperature) on natural microbiota reduction in hard wheat *p<0.05, **p<0.01, ***p<0.001

efficacy for yeast, mold, and coliforms, but only at a significance level of $p<0.001$. It is worth mentioning that without the addition of water prior to steaming the wheat the final needed moisture for milling is not achieved. Therefore, for practical purposes, when using steam to reduce microbial contamination, efforts should be made to adjust the grain moisture prior to the intervention either by using water, or an acid solution. As a matter of fact, acid addition to the steam treatment in this research led to microbial reductions that were significantly higher for all microbial groups tested than those achieved by a pre-treatment with water. On average, the highest reductions were achieved for Eb (up to 3.64 log CFU/g) and molds (up to 4.26 log CFU/g), and APC showing an average reduction of up to 4.78 log CFU/g. Wang et al., (2019a) reported that superheated steam at 200°C for 180 s reduced 99.98% of bacteria (which is about 4 log CFU/g) present on barley; while Hu et al., (2016) reported a 3.03 log CFU/g reduction for APC when wheat kernels were treated at 110°C for 80 s using saturated steam. These results, when compared to those reported here, suggest the combination of acid with steam can be as effective as the application of higher treatment temperature and time.

Comparing the microbial reductions observed with the grain bed depth of 0.5 and 1.0 cm, for almost all microbial groups, the average reduction for 1.0 cm was higher than that achieved for 0.5 cm. This may be related to the amount of time required to heat up the grain, as the thicker the bed depth, the more grain needs to be treated and therefore more time is required. Indeed, when data from **Figure 2-3** is considered, an increase in bed depth from 0.5 to 1.0 cm at 80°C required the steaming time to be increased, on average, by 14 sec. This increased time for the thermal treatment is known to lead to a higher microbial reduction, as D-values for microorganisms is decreased with

temperature (Pankaj, 2015)(reference for D-value!). Similar results have been reported previously regarding the exposure time, where the concentration of microflora would decrease with the increase of processing time (10-80 s;110–200 °C) (Hu et al., 2016). However, the potential effect of this increased thermal treatment on the functional properties of the final wheat flour must be considered. Therefore, even though higher microbial reductions may be achieved with thicker bed depths and/or longer thermal treatments, the potential detrimental effect on grain functionality must be evaluated and a balance between these two effects must be achieved.

The last statistical comparisons made considered the final temperature of the grain bed. Except for molds, all other microorganisms experienced a higher microbial reduction at 85^{o}C, when compared to 80^{o}C. Thermal death of microorganisms is directly related to the temperature used, with higher temperatures leading to higher thermal death and lower D-values (Pankaj, 2015) (reference here again!) . In addition to the effect of the temperature itself, these results may also have a relationship with the time required to apply the treatments. **Figure 2-3** shows that for the bed depth of 0.5 cm, an increase in treatment time of 20 sec was observed when the final grain temperature was changed from 80^{o}C to 85^{o}C. This increase in time certainly contributes the higher microbial reductions observed at higher temperatures.

It is worth mentioning that the effect of bed depth and temperature was not observed on the log reduction of molds, most likely because even the less harsh conditions used (bed depth of 0.5 cm and 80^{o}C) were already enough to bring this microbial population to levels that were below the limit of detection of the method used for quantification. Average log reduction for molds was observed up to 3.57 log CFU/g.

When specific treatment combinations were considered, the combination of acid and steam at 85°C – 1.0 cm achieved the highest reductions for Eb and APC, at 3.79 and 3.64 log CFU/g, respectively. Similar results were reported by Sabillón et al., (2016) when tempering wheat with lactic acid 5.0% + NaCl 26 %, which led to reductions of 3.4 and 3.0 log CFU/g, for Eb and APC, respectively. The results from Eb and APC also indicate that even the mildest steam treatment (80°C – 0.5 cm) in combination with acid, led to microbial reductions but at lower levels (2.32 and 2.36 log CFU/g, respectively).

In summary, even though the addition of water prior to the steam treatment reduced its efficacy, for the reduction of some microbial groups, the desired final moisture of the tempered grain was only achieved when the wheat was pre-treated with water. Therefore, the addition of a tempering solution is required for proper tempering when steam is used. And this tempering solution could serve as the carrier for organic acids. Indeed, the results shown here indicate that the best microbial reductions were achieved with a combination of steam and acid for the temperatures/bed depths evaluated. To further define which tempering interventions would be practical and of value to the food industry, the functional properties of the final wheat flour must be evaluated.

3.3. Effect of tempering interventions on functional properties of straight-grade flour

Tempering treatments that showed the highest microbial reductions and final moisture suitable for milling (acid + steam and water +s team) were selected for further evaluation of flour properties and functionality. The pH and acid content of the different flours obtained are shown in **Table 2-3**. The pH of the flour where lactic acid was added

as part of the treatment showed a significantly reduced value compared to the control.

Table 2-3. pH and acidity values of straight-grade flour obtained from hard wheat pre-tempered with lactic acid or water and treated with steam.

Treatment	**pH**	**Titratable Acidity (ml 0.1*N* NaOH)**
Control (water)	6.79 ± 0.06a	1.69 ± 0.11a
Acid + Steam *85°C – 1.0 cm*	6.15 ± 0.13c[1, 2]	2.06 ± 0.4b
Acid + Steam *85°C – 0.5 cm*	6.08 ± 0.02c	2.16 ±0.6bc
Acid + Steam *80°C – 1.0 cm*	6.12 ± 0.01c	2.09 ± 0.03b
Acid + Steam *80°C – 0.5 cm*	6.04 ± 0.03c	2.25 ± 0.12c
Water + Steam *85°C – 1.0 cm*	6. 69 ± 0.04ab	1.80 ± 0.09a
Water + Steam *85°C – 0.5 cm*	6.72 ± 0.01ab	1.72 ± 0.05a
Water + Steam *80°C – 1.0 cm*	6.71± 0.11b	1.73 ± 0.03a
Water + Steam *80°C – 0.5 cm*	6.65 ± 0.12ab	1.83 ± 0.14a

[1]Mean values with the same letter in the same column are not significantly different from one another (P > 0.05).
[2]Values denote mean ± standard deviation

When the pH of the flour was lower, the acidity level measured increased. Similar results were observed by Sabillón et al., (2019) when tempering wheat with organic acids (acetic and lactic acid). These results showed that increasing the concentration of acetic acid from 2.5 to 5.0% would cause further pH reduction and would increase the acid content of the flour furthermore. Another study reported lower pH values as well when hard wheat was tempered with saline solutions (Sabillón et al., 2017).Each value of flour pH tempered with water and steam was lower than the control, but not significantly

different. Based on this we assume that maybe steam can affect the pH of the flour as well. However, no supporting reference was found for the assumption. A study reported results when wheat was tempered with water only and there were no significant changes in pH observed by the addition of water to wheat (Sabillón et al., 2017; Sabillón et al., 2020).

Pasting properties of straight-grade flour are also presented in **Table 2-4.** The Rapid Visco analyzer used to evaluate the flour measures the viscosity of samples during a given period of time (Gamel et al., 2012). After reaching the pasting temperature, the viscosity will increase due to the temperature increase in the equipment (Batey, 2007; Gamel et al., 2012). This defines the peak parameter. This parameter indicates the water-binding capacities of the starch granules or mixtures, it is associated with the final product quality and can be used to predict the bread firming behavior during the storage (Batey, 2007; Gamel et al., 2012; Sabillón et al., 2014). When evaluating the results obtained in general peak viscosity was no different from the Control all treatments at different temperatures and bed depths. Similar results were reported by Sabillón et al., (2017) where tempering of wheat with lactic acid, where no significant differences were observed from the control sample.

During the holding period at a constant temperature, breakdown viscosity takes place. When the starch granules reach the peak viscosity, the viscosity will decrease due to the physical destruction of swollen starch granules under mechanical shear stress and constant high temperature (Gamel et al., 2012; Sabillón et al., 2014). The combination of acid and steam, regardless of temperature, at bed depth 0.5 cm showed a higher breakdown viscosity when compared to control. Another study reported increased

breakdown viscosity when tempering wheat grains with steam (Chen et al., 2020). While the addition of organic acids, including lactic acid 5.0% did not show any difference when tempering wheat grains with acid solutions alone (Sabillón et al., 2014).

The final viscosity relates to the gelling process of the starch. It indicates the ability of the starch granules to form a viscous paste or gel after cooking and cooling (Gamel et al., 2012; Sabillón et al., 2014). All the treatments including steam when compared to the control did not show any significant differences. However, when the comparison was made for treatments including water + steam, the final viscosity for treatments 80°C - 0.5cm and 85°C - 1.0 cm were lower than the values obtained from the control. Usually, lower final viscosity can be linked to the longer steam exposure time, which explains the results observed for the treatment at 85°C. Similar results were reported by Chen et al., (2020) who observed a decreased final viscosity when the steam treatment time increased from 280 to 320 s. Another study showed no changes in the final viscosity of the flour due to the steaming (Poudel & Rose, 2018).

Table 2-4. Pasting properties of straight-grade flour obtained from hard wheat pre-tempered with lactic acid or water and treated with steam.

Treatment	**Peak (cP)**	**Trough (cP)**	**Breakdown (cP)**	**Final (cP)**	**Setback (cP)**	**Time to peak viscosity (min)**	**Pasting temperature (°C)**
Control (water)	2440.16 ± 168.3a[1, 2]	1420.83 ± 55.46a	987 ± 30.91bc	2536.16± 101.68a	1202 ± 75.40a	5.87 ±0.09cd	50.72 ± 0.25b
Acid + Steam 85°C – 1.0 cm	2447.66 ± 230.56a	1485.83 ± 126.25a	1024 ± 28.44ab	2607.66 ± 91.46a	1220.33 ± 78.26a	5.93 ± 0.03bc	51.98 ± 0.49a
Acid + Steam 85°C – 0.5 cm	2499.33 ± 243.88a	1394.33 ± 62.28a	1049.5 ± 18.00a	2636.5 ± 183.60a	1189.80 ± 15.09a	5.85 ± 0.04d	50.55 ± 0.30b
Acid + Steam 80°C – 1.0 cm	2349.33 ± 232.05ab	1216.33 ±108.04a	960 ± 25.23cd	2469.66 ± 188.35ab	1169.66 ± 77.23a	5.76 ± 0.04e	50.14 ± 0.04d
Acid + Steam 80°C – 0.5 cm	2279.16 ± 225.34b	1356.16 ± 150.94a	909.33 ± 9.39d	2453 ± 143.40ab	1200.83 ± 74.23a	5.91 ± 0.01bc	50.23 ± 0.06c
Water + Steam 85°C – 1.0 cm	2310.66 ± 199.53ab	1376 ± 138.69a	995.66 ± 56.01bc	2335 ± 121.48b	1046.83 ± 56.79bc	5.81 ± 0.04d	52.45 ± 0.89a
Water + Steam 85°C – 0.5 cm	2343.33 ± 237.98ab	1436.83 ± 158.04a	958.66 ± 37.62cd	2570.66 ± 164.69a	1025.83 ± 54.73c	5.78 ± 0.11de	52.72 ± 1.61a
Water + Steam 80°C – 1.0 cm	2374.33 ± 186.59ab	1376.33 ± 88.96a	977.83 ± 26.28bc	2603.66 ± 169.73a	1114.83 ± 86.16ab	6.13 ± 0.27ab	50.74 ± 0.44b
Water + Steam 80°C – 0.5 cm	2175.83 ± 252.29b	1249.83 ± 331.34a	968.5 ± 71.55bcd	2334.83 ± 235.15b	1220.83 ± 126.80a	6.29 ± 0.39a	50.74 ± 0.34b

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

[2]Values denote mean ± standard deviation

The setback viscosity, which is the last phase of the pasting curve represents a measure of the degree of retrogradation of the starch molecules during cooling (Gamel et al., 2012; Sabillón et al., 2014). None of the treatments showed any statistical difference, except water and steam combination at 85°C. regardless of the bed depth, which showed a lower setback viscosity than the control. This suggests that tempering wheat with water and steam might produce flour with less tendency to retrograde since the low setback viscosity indicate low rates of starch retrogradation (Gamel et al., 2012). However, another study evaluating the effect of steam did not show any significant difference in setback viscosity of the flour when steam was applied to the wheat kernels (Poudel & Rose, 2018). The time required to reach the peak viscosity is defined as the peak time. Acid and steam combination (80°C – 1.0 cm) showed lower peak time compared to the control. Similar results were observed for the whole grain flour when the wheat kernels were tempered with saline organic solution (Sabillón et al., 2014). Lower trough viscosity was observed when comparing acid and steam (80°C – 1.0 cm) with control, as well. Similar results were also reported when tempering wheat with steam (Chen, Guo, Xing, & Zhu, 2020). However, none of the other treatments did show any difference from the control. The pasting temperature suggests the beginning of the rise in viscosity (Sabillón et al., 2014). In general, among the treatments evaluated it seems that kernels treated at higher temperatures (i.e., 85°C) led to the production of flour with higher pasting temperatures. **Table 2-5** presents a summary of the bread firmness and loaf volume of bread made with straight-grade flour. The loaf volume of bread for all treatments was equal to the control, except for those made with flour from kernels treated with acid and steam at80°C – 1.0 cm and water and steam at 80°C - 0.5 cm which were significantly

lower compared to control. A study by Sabillón et al., (2017) applied acid saline solutions to wheat kernels. They did not report significant changes in loaf volume, neither on straight-grade flour or whole wheat flour.

When bread firmness of loaves made with straight-grade flour was evaluated, in general, no differences were observed between treatments and control. Application of lactic acid 5.0% on wheat kernels showed similar results regarding the bread firmness (Sabillón et al., 2017). The authors did not report any changes from the control samples when acid was applied as a tempering solution.

Table 2-5. Mean values and standard deviation for straight-grade bread loaf volume and firmness.

Bread analysis parameter	Treatments	Straight-grade bread
Loaf Volume (cc)	Control (water)	452.00 ± 10.55[1]a
	Acid + Steam 85°C-1.0 cm	492.83 ± 26.64ab
	Acid + Steam 85°C-0.5 cm	428.66 ± 31.80ab
	Acid + Steam 80°C-1.0 cm	415.50± 17.55b
	Acid + Steam 80°C-0.5 cm	444.83 ± 32.62ab
	Water + Steam 85°C-1.0 cm	429.51 ± 15.38ab
	Water + Steam 85°C-0.5 cm	458.50 ± 5.16a
	Water + Steam 80°C-1.0 cm	447.83 ± 13.28a
	Water + Steam 80°C-0.5 cm	410.50 ± 26.46b
Bread Firmness (N)	Control (water)	5.86 ± 0.70a
	Acid + Steam 85°C-1.0 cm	5.83 ± 0.88ab
	Acid + Steam 85°C-0.5 cm	5.97 ± 0.89ab
	Acid + Steam 80°C-1.0 cm	4.76 ± 0.75c
	Acid + Steam 80°C-0.5 cm	6.08 ± 0.55a
	Water + Steam 85°C-1.0 cm	5.67 ± 0.63abc
	Water + Steam 85°C-0.5 cm	6.14 ± 0.92a
	Water + Steam 80°C-1.0 cm	5.09 ± 0.58bc
	Water + Steam 80°C-0.5 cm	6.25 ± 0.54a

[1] Valued denote mean ± standard deviation

[2] Means followed by the same letter within the same column are not significantly different ($P > 0.05$)

Data associated with straight-grade bread image analysis as a result of different tempering treatments are shown in **Table 2-6**. In general, acid in combination with steam treatment did not affect slice area, slice height, slice brightness, number of cells, or average cell elongation when compared to control. The only parameter affected by the combination of acid and steam was the cell diameter, where observed values for treatment were larger than control. Similar results associated with bread image analysis were reported by Sabillón et al., (2017) where the authors reported no significant changes compared to control samples when wheat kernels were tempered with organic saline solutions. When wheat kernel had their moisture levels pre-adjusted before steam was applied, these bread slice height, cell diameter, and average cell elongation were affected by the treatments when compared to control values.

Figure 2 6. Mean values and standard deviation for parameters associated with straight-grade bread image analysis as result of different tempering treatments.

	Bread analysis Parameter					
T**reatment**	**Slice area (mm2)**	**Slice height (mm)**	**Slice Brightness**	**Number of Cells**	**Cell Diameter (mm)**	**Average Cell Elongation (mm)**
Control (water)	4229.5 ±156.80bc[1]	60.95 ± 1.73d[2]	154.16 ± 3.52bc	3253.5 ± 176.11a	1.61 ± 0.08c	1.58 ± 0.03bcd
Acid + Steam 85°C – 1.0 cm	4386.3 ± 78.73ab	64.99 ± 2.02d	158.88 ± 4.05abc	3277.6 ± 189.15a	1.68 ± 0.09bc	1.60 ± 0.08bcd
Acid + Steam 85°C – 0.5 cm	3968.2 ± 329.26c	60.91 ± 4.37d	159.34 ± 4.57a	3377.5 ± 504.4a	1.47 ± 0.10d	1.52 ± 0.02d
Acid + Steam 80°C – 1.0 cm	2349.33 ± 232.05abc	62.73 ± 3.70d	158.96 ± 3.48ab	3149.5 ± 112.12a	1.72 ± 0.11ab	1.56 ± 0.04cd
Acid + Steam 80°C – 0.5 cm	4269.8 ± 247.99abc	63.85 ± 2.09d	156.78 ± 2.88abc	3164.5 ± 231.36a	1.72 ± 0.16ab	1.55 ± 0.03cd
Water + Steam 85°C – 1.0 cm	4407.8 ± 178.22ab	81.45 ± 3.05b	156.61 ± 3.65abc	3172.9 ± 125.06a	1.73 ± 0.07ab	1.68 ± 0.02a
Water + Steam 85°C – 0.5 cm	4553.7 ± 92.94a	83.67 ± 1.20a	156.37 ± 2.13abc	3160.0 ± 122.33a	1.82 ± 0.09a	1.61 ± 0.03abc
Water + Steam 80°C – 1.0 cm	4430.3 ± 129.36ab	81.40 ± 2.93b	152.36 ± 2.70c	3102.9 ± 153.74a	1.73 ± 0.70ab	1.66 ± 0.04ab
Water + Steam 80°C – 0.5 cm	4018.8 ± 135.2c	76.67 ± 2.01c	159.3 ± 2.85a	2859.9 ± 152.31a	1.80 ± 0.11a	1.64 ± 0.06ab

[1]Valued denote mean ± standard deviation

[2] Means followed by the same letter within the same row are not significantly different (P > 0.05)

4. CONCLUSIONS

The tempering intervention evaluated here, which included steam, organic acid and their combination, resulted in an improvement of the microbiological quality of wheat. The use of steam by itself did not provide enough moisture for the tempering process, therefore additional water should be added to the treatment. According to the results reported here, this water could serve as a carrier for organic acids, leading to the combination of two microbial treatments – thermal (steam) and chemical (lactic acid). Indeed, results reported here have shown that the combination of acid and steam achieved the highest overall microbial reductions among the treatment combinations. More specifically, with the steam and acid treatment combination APC, Eb and coliforms were reduced by 4, 4.1 and 4.3 log CFU/g, on average, respectively.

When flour functionality was evaluated, the interventions applied resulted in differences for individual parameters associated with pasting properties. However, baking performance, including bread firmness and loaf volume was not as affected by most of the treatments applied.

Bread image analysis showed minimal changes when steam in association with acid was used as a tempering intervention. However, when water was added before steaming, the slice is, slice brightness, and number of cells were affected by the treatments.

Even though the interventions suggested here provided promising application in the milling industry, further research is needed on different wheat classes and the impact of the treatment on sensory characteristics of the end-product.

5. REFERENCES

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CHAPTER 3

THE EFFECT OF LACTIC ACID IN ASSOCIATION WITH STEAM IN REDUCING MICROORGANISMS IN SOFT WHEAT

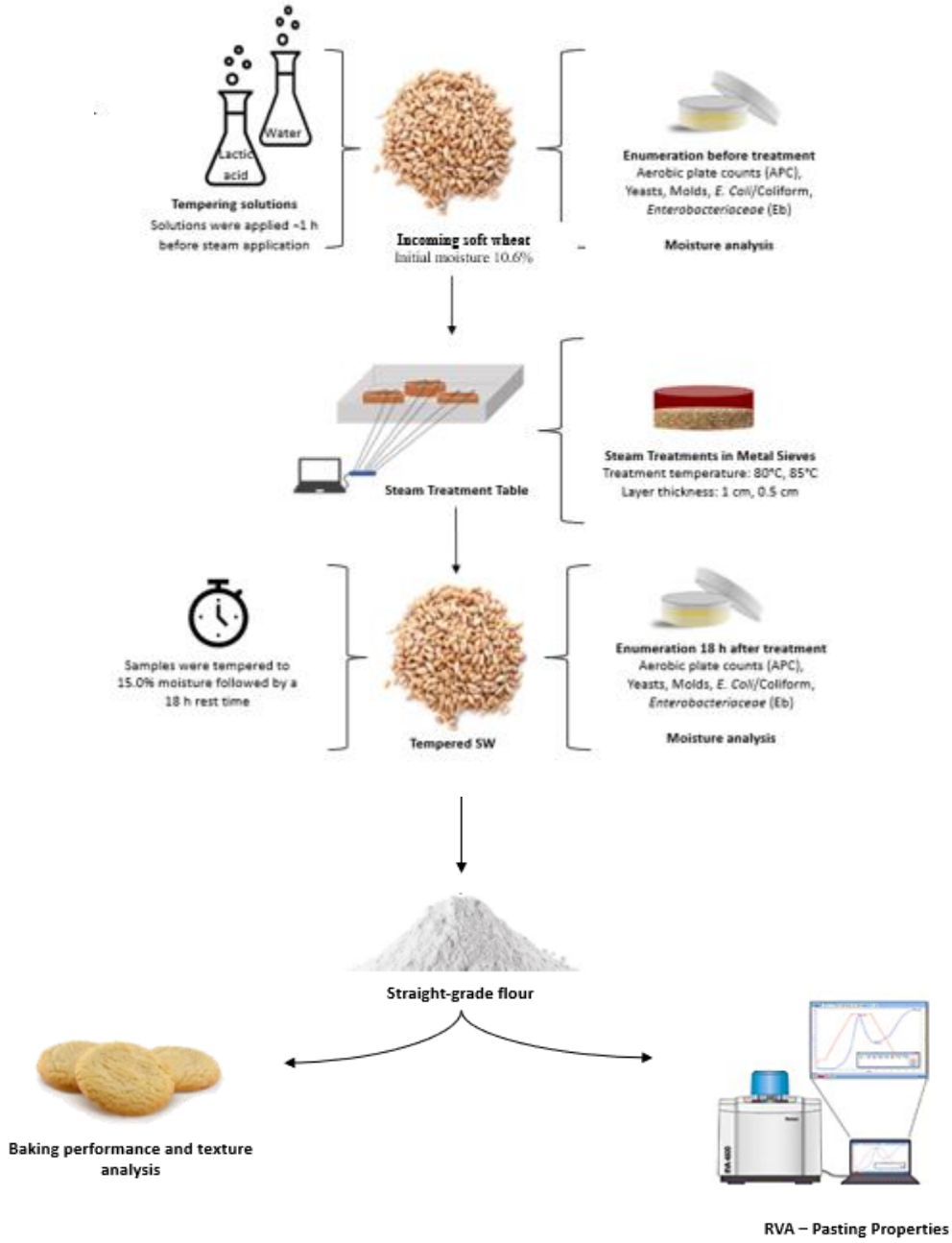
CHAPTER 3. THE EFFECT OF LACTIC ACID IN ASSOCIATION WITH STEAM IN REDUCING MICROORGANISMS IN SOFT WHEAT

ABSTRACT

Wheat-based products have been considered as safe food for human consumption because they are low moisture foods and most of the finished products are thermally processed. Within the food industry, steam is an effective method of microbial reduction for a variety of products. Previous studies have shown that tempering soft wheat with organic acids, as well as the steam application can have the potential to reduce the microbial load of wheat with minor changes on the functional properties of the flour. Thus, the objective of this study is (a) to study the effect of saturated steam by itself or in combination with lactic acid, to reduce the natural microbial load of soft wheat, and (b) to evaluate the impact of these interventions on the functional properties soft wheat flour. Soft wheat samples were treated by adding acid to the tempering water, by steaming the kernels in replacement of the traditional steep, or by adding a combination of both. Samples were placed into sieves (bed depth 0.5 and 1.0 cm) for those treatments where steam was included and treated with steam in a steam table until the grain temperature achieved 80 or 85°C. After treatment was applied, soft wheat was allowed to temper to 15.0% moisture for 18 h at room temperature (~24°C.and 60% RH) under aseptic conditions. Before and after tempering, wheat samples were tested for Aerobic Plate Count (APC), coliforms, *Enterobacteriaceae* (Eb), yeast, and mold. Selected treatments were chosen for further evaluation of flour functionality. Wheat samples were milled into straight-grade flour using a pilot Bühler mill. According to the results, tempering done

with steam alone or in combination with water for moisture adjustment, were equally effective in reducing the microbial flora of wheat grains in comparison to controls. However, the addition of acid furthermore increased the microbial reduction for all microorganism tested. The highest microbial reduction was achieved for Eb (up to 4.3 log CFU/g), followed by APC (up to 4 log CFU/g). At bed depth of 1.0 cm both temperatures tested (80 and 85°C) showed higher microbial reduction then at bed depth of 0.5 cm, which could be due to the longer exposure time to steam on a deeper bed. The tempering intervention using steam, and water or acid solution were used to adjust grain moisture, were chosen for further evaluation of straight-grade flour properties. Even though few significant differences were observed in the pasting properties of the straight-grade flour, the baking quality of cookies was not significantly affected by any treatment.

VISUAL SUMMARY



1. INTRODUCTION

One of the most important food commodities is cereal grains which represent up to 80% of the diet in some cultures (Maga, 1978; Olsson et al., 2000). And soft wheat is one of the major wheats grown in the United Kingdom, Europe, and Australia. Soft wheat flour use is not very common for bread making due to the resulting less appealing crumb structure and small bread loaves. This type of flour is mostly used in the manufacturing of cakes, biscuits, and cookies (Kent-Jones, et al.,).

Cereal grains are exposed to various contaminants including microorganisms during different stages of the production chain, such as harvesting, transportation, and storage (F.-Q. Li et al., 2002). The handling practices applied during these stages can affect wheat quality and safety. Traditional steps prior to wheat milling such as cleaning, conditioning, grinding, sieving, and purification may reduce some microbial contamination (M. Li et al., 2013). However, any remaining contamination may contaminate the flour and have a strong influence on the quality and safety of milling end products (Berghofer et al., 2003).

Foodborne diseases associated with products containing flour have been increasing (Sabillón et al., 2020) and this highlights the importance of developing methods that would provide consumers with flour that is safe and of high quality. In the past, several foodborne disease outbreaks have been associated with flour-based mixes that were contaminated with Salmonella spp. and Escherichia coli O157:H7 (McCallum et al., 2013; Neil et al., n.d.).

Yeast, mold, and a wide variety of bacteria are among the spoilage microorganisms reported on wheat grain. A range of 1.4 to 6.0 log CFU/g has been reported by several studies (Berghofer et al., 2003; Eglezos, 2010b; Manthey et al., 2004; Sabillón et al., 2020; Sabillón & Bianchini, 2016). In addition to spoilage bacteria, coliforms and *Enterobacteriaceae* are also a common group of microorganisms found in wheat kernels (Berghofer et al., 2003; Eglezos, 2010b; Sabillón & Bianchini, 2016).

Many studies have addressed the issue of the risk caused by pathogenic bacteria in wheat and the potential methods that could be used to improve the microbial quality and safety of wheat. Some of the methods include grain tempering solutions (Chen, Guo, Xing, Sun, et al., 2020; Dhillon et al., 2007; Sabillón et al., 2016, 2017; Sabillón et al., 2020), thermal technologies (Chen, Guo, Xing, & Zhu, 2020; Hu et al., 2016; Y. Jiao et al., 2015; Liu et al., 2018; Snelling et al., 2020; Villa-Rojas et al., 2017), and non-thermal technologies (Aron Maftei et al., 2014; Du et al., 2020; Subedi et al., 2020).

Tempering of hard and soft wheat with acidic saline solution has been studied on different occasions by Sabillón et al., (2016, 2019, 2020). A combination of lactic acid 5.0% and NaCl 52% resulted in a 4.0 and 4.7 log CFU/g reduction for aerobic plate counts (APC) and Enterobactericiae (Eb), respectively (Sabillón et al., 2016); while the combination of lactic acid 5.0% and NaCl 26.6% resulted in a 3.2 and 4.5 log CFU/g reduction for APC and Eb (Sabillón et al., 2019). Another study showed that this combination (lactic acid 5.0% and NaCl 26.6%) is effective in reducing Salmonella spp. (2.6 log CFU/g), *E. coli* O157:H7 (2.4 log CFU/g), and non-O157 STEC (2.4 log CFU/g) in soft wheat (Sabillón et al., 2020).

The effect of thermal treatment using vacuum steam was studied by Snelling et al., (2020) in hard red spring wheat. They showed a 3.57 log CFU/g reduction in *E. coli* and 3.21 log CFU/g reduction in Salmonella with the application of vacuum steam at 65°C for 8 min. Another study showed that using superheated steam at a temperature of 200°C for 18 s can reduce up to 81.8% of Bacillus spp. load. Thus, the objective of this study was: (a) to evaluate the effect of steam by itself or in combination with lactic acid, to reduce the natural microbial load of soft wheat, and (b) to evaluate the impact of these interventions on the functional properties of soft wheat flour.

2. MATERIALS AND METHODS

2.1. Materials

Soft wheat commercially available was purchased for this research. Samples of soft wheat were received, mixed, and stored at room temperature before use. Lactic acid (86%) was obtained from Fisher Scientific™ (Pittsburgh, PA).

2.2. Experimental design

Figure 3-1 describes the experimental design used to evaluate the effect of steam, by itself or in combination with lactic acid, on the microbial load of soft wheat kernels. Steam treatment was applied directly to wheat kernels as the only source of water for moisture increase during tempering, and also to wheat kernels that had been pre-tempered with water to ensure the desired final tempering moisture. Tempering with lactic acid solution was also evaluated, along with the combination of steam and lactic acid. The combination of mechanisms for microbial inactivation (acid and steam) was evaluated to observe if there was any synergetic effect between them while reducing the microbial

load of soft wheat **(Figure 3-1).** In the absence of an intervention, water was used as a control for the tempering process.

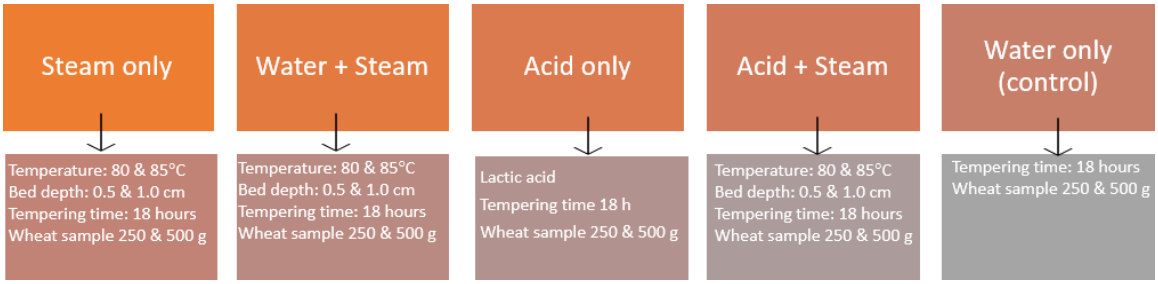


Figure 3-1. Experimental design to evaluate the effect of steam, by itself or in combination with lactic acid, on microbial load of soft wheat kernels.

At first the effect of steam treatment by itself (0.5 cm bed depth; 80°C final temperature) or acid tempering (5% lactic acid) and their combination (0.5 cm bed depth; 80°C final temperature tempered with 5% lactic acid) were evaluated. Based on the results obtained, then the effect of temperature, bed depth, grain moisture and acid tempering were further studied as described in **Figure 3-1.**

2.3. Tempering with water

According to the experimental design (**Figure 3-1**), two bed depths were evaluated. For the bed depth of 0.5 cm, a wheat sample of 250 g was required; while for the bed depth of 1.0 cm, the amount of wheat used was 500 g. The amount of distilled water required for tempering was based on the sample size and initial moisture of the kernels. However, temperature treatment was not taken into account for the control (untreated) samples; only sample size of 250 g was considered. Calculations were based on the initial moisture of the wheat (10.6%) and final weight of grain after tempering to 15.0% moisture. To allow the best distribution of the water in the wheat sample, the distilled

water was applied to the kernels in a biosafety cabinet with the help of an atomizer. The wheat samples were shaken every 15 min for the first 2 h of the tempering process and then allowed to temper for 18 h at room temperature.

2.4. Tempering with lactic acid

2.4.1. Preparation of tempering solution

A lactic acid solution was used to temper the soft wheat kernels. The amount of acid used during the tempering was 1.8 mL lactic acid (85%) per kilogram of tempered wheat. This acid application is equivalent to the one used by Sabillón et al., (2016) when 5.0% lactic acid solutions were used in their study. In this research, the calculated amount of distilled water and lactic acid (85%) was used to achieve the desired acid treatment and final moisture upon tempering. Calculations were based on the initial moisture of the wheat (10.6%) and final weight of grain after tempering to 15.0% moisture. Taking into account the volume of acid needed, the remaining amount of distilled water required to achieve 15.0% moisture after tempering was also calculated for each size.

2.4.2. Application of tempering solution

According to the experimental design (**Figure 3-1**), two sample sizes were evaluated: 250 and 500 g of soft wheat. After the amount of lactic acid (85%) and distilled water required to achieve the acid treatment was determined, they were applied to soft wheat samples.

To allow for an even distribution of the acid throughout the wheat sample, the distilled water and lactic acid (85%) were first mixed and then applied to the kernels in a biosafety cabinet with the help of an atomizer. The wheat samples were then shaken

every 15 min for the first 2 h of the tempering process and then allowed to temper for 18 h at room temperature.

2.5. Tempering with steam

2.5.1. Direct application of steam to wheat kernels

Samples of soft wheat were divided into 250 g subsamples to achieve a 0.5 cm bed depth, while 500 g subsamples were used for 1.0 cm bed depth when using a mesh sieve (standard 40 mesh sieves with 0.420 mm hole thickness) as a carrier for the samples. Two thermocouples (Omega TJ36-CPSS-116G-g, Omega, Norwalk, CT, USA) were placed in the center of the grain bed in each sieve. Three sieves were placed on a steam table that had been pre-heated to 100 ºC. The thermocouples were monitored using Omega Logging Software (Picto technology LTD, England, UK) which recorded the sample temperature every second. Grain temperature was monitored until it achieved 80 or 85ºC, according to the experimental design. When the target temperature was reached (80 or 85ºC), the wheat samples were taken out of the steam table. Right after the steaming process, samples were tempered into sterile plastic bags and immediately placed in an ice bath for ~5 min for cooling. Samples were allowed to temper at room temperature for 18 hours inside the plastic bags.

2.5.2. Steaming of pre-tempered wheat kernels

Soft wheat samples were also pre-tempered with water before steam was applied to ensure a final moisture content of 15.0% after tempering. With the direct application of steam to wheat kernels, the amount of water added could not be controlled. Therefore, pre-tempering with water was included to ensure that the final desired moisture was

achieved, Calculations for the amount of water to be added was done knowing the initial moisture of soft wheat kernels and the amount of moisture that each steam treatment (temperature and bed depth) adds, by conducting preliminary tests so that the final moisture after application of tempering solution and steam was at the desired level of 15.0%. Pre-tempered soft wheat was then steam as described under 2.5.1.

2.6. Tempering with steam and lactic acid

For this set of experiments, the amount of water required to be added to each sample as pre-tempering treatment was calculated as described under 2.5.2. Then the amount of acid for each sample size was calculated to achieve a concentration of 1.5 mL lactic acid (85%) per kilogram of tempered wheat. The lactic acid was combined with water required for pre-tempering and added prior to steaming (≤1 h before steaming). Once again, to allow for an even distribution of water and acid over the soft wheat samples, the mixture was applied in a biosafety cabinet with the help of an atomizer. The samples were then shaken every 5 min before the steaming process. After the pre-tempering with acid and water was completed, soft wheat kernels were steamed as described under 2.5.1.

2.7. Microbial analysis

To determine the effect of lactic acid and steam as antimicrobial interventions in wheat, the microbial load of the samples was determined in duplicate before and after each treatment. To perform the microbial analysis, 25 g of wheat samples and 225 mL of sterilized 0.1% peptone solution were placed into a sterile plastic bag. Wheat kernels were soaked for 5 min and then mixed using a stomacher blender (Stomacher 400, Seward Ltd, Bohemia, NY) for 120 sec. After mixing, serial dilutions were prepared

using sterilized 0.1% peptone solution, and microbiological tests were performed to include – Aerobic Plate Count (APC), *Enterobacteriaceae* (Eb), generic Escherichia coli (EC), Coliforms, Yeast, and Mold. For APC enumeration, dilutions were spread plated onto Standard Methods Agar (Remel, Thermo Fisher, Lenexa, KS) and incubated for 48 h at 35ºC. *Enterobacteriaceae* (Eb) was determined using Petrifilm™ (3M Microbiology, St. Paul, MN), with samples incubated at 37ºC for 24 h. Coliforms and generic Escherichia coli (EC) were enumerated using EC Petrifilm™ (3M Microbiology, St. Paul, MN), with incubation at 37ºC for 24 h (coliform count) or 48h (generic EC counts). Yeast and mold counts were determined using Dichloran Rose Bengal Chloramphenicol Agar (Oxoid, Basingstoke, Hampshire, UK). Samples were spread onto plates and incubated at 25ºC for 3 days (yeast) and 5 days (mold).

2.8. Moisture of tempered wheat

To verify the moisture content before tempering, after tempering, and before milling, sample moisture was determined using a forced-air oven method according to the 44-15.02 (AACCI, 2009).

2.9. Experimental milling

The treatments with the highest overall microbial reduction (pre-tempering with acid and water followed by steaming) were selected to evaluate their impact on the functional properties of flour. Therefore, enough wheat was treated according to sections 2.5 and 2.6 to produce 800 g of wheat per replicate. Three replicates per treatment were evaluated. A Buhler experimental mill was used to obtain straight-grade flour following the AACC standard method 26-32.01 (AACCI, 2009). Milling room temperature and

relative humidity were kept at 22-24° and 60%, respectively, to ensure reproducibility of results. After the milling process, flour was mixed thoroughly for further analysis and stored in the refrigerator (4°C) until processing.

2.10. Functionality testing and flour properties

2.10.1. pH and acid content

The pH of the flour was measured according to AACC standard method 02-52.01 (AACCI, 2018). In short, 10 g of flour along with 100 mL of distilled water was placed in an Erlenmeyer flask and mixed for 15 min with a magnetic stirrer, followed by a 10 min rest. The supernatant liquid was decanted into a clean Erlenmeyer flask and the pH was determined with a calibrated Thermo Scientific Orion 2-star Benchtop pH meter. After measuring the pH, the acid content of the supernatant was determined by titrating with 0.1 N NaOH to a final pH of 7.0.

2.10.2. Pasting properties

The pasting properties of the flour were evaluated in duplicate by Rapid Visco Analyzer (RVA) (Model 4S, New Port Scientific; Warriewood, NSW, Australia) following AACC standard method 76-21.01 (AACCI, 2009). In short, 3.5 g flour with moisture adjusted to 14% was mixed with 25 mL of distilled water and added to a test container. The sample was mixed by vigorously plunging the test container blade through the mixture for 30 s to avoid the formation of clumps during the analysis. Samples were analyzed using the Standard=1 test profile in the RVA software. Maximum viscosity, minimum viscosity after peak, final viscosity, and time to peak viscosity were recorded and collected from the RVA.

2.10.3. Baking properties

The baking quality of the soft wheat flour for the production of cookies was done according to AACC standard method 10-50.05 (AACC, 2009). After mixing all the ingredients, the cookie dough was rolled out to a thickness of 0.7 cm and was cut into 6 cm diameter pieces with a cookie-cutter mold. Cookies were baked on the oven (National Manufacturing Corporation; Lincoln, NE) at 205°C for 10 min. After cooling for 30 min, the average diameter (width) and thickness of six cookies was obtained and the spread factor (width/thickness ratio) was calculated. Baking experiments were replicated for each treatment that combined pre-tempering with water and acid, followed by steaming.

2.10.4. Texture analysis of cookies

The texture of baked cookies was conducted within 3h after baking using a texture analyzer (Model TA-TX2, Texture Technologies; Scarsdale, NY) equipped with a 25-kg load cell with a 3-point bending rig, following the American Institute of Baking (AIB) standard procedure for cookie hardness (AIB, 2020). The gap between the support beams was set to 40 mm to be half the diameter of the cookies. The support between the rig distance was kept constant throughout the analysis to ensure comparability of the results. Cookie hardness and flexibility were recorded.

2.11. Statistical analysis

Statistical analysis of data from each microorganism group were conducted separately. For analysis of the first experiment comparing control, acid tempering, and steam treatments an analysis of variance (ANOVA) using treatment as the factor was performed (version 9.4, SAS Institute, Bray, NC USA). For analysis of different levels of

tempering and steam treatment, a three-factor ANOVA with interactions was used with tempering condition, wheat bed depth, and final temperature as the factors. When the ANOVA F-values were significant ($p<0.05$), Tukey's honestly significant difference was used to compute differences among treatments or factors. For comparison of experimental data with calculated values in the first experiment, a one-sample t-test was performed. PROC GLIMMIX procedure in SAS 9.4 was used to analyze the effect of the different treatments on the functional properties of the flour. When differences occurred, they were reported at the $\alpha=0.05$ significance level with Tukey-Kramer adjustment applied to obtain appropriate p-values. For the flour functionality parameters, a nested factorial treatment design was used to account for the fact that the control had no temperature or bed depth values associates with it. Tukey-Kramer and Dunnett adjustments were used to account for multiple comparisons, where appropriate.

3. RESULTS AND DISCUSSION

3.1. Effect of acid, steam and their combination in hard wheat tempering

3.1.1. Effect of tempering on grain moisture

The target moisture for the soft wheat samples after tempering was 15.0%. Depending on the treatment type the moisture would vary, especially when wheat was treated by steam only. The reason for this is because the moisture addition can not be controlled when the samples undergo the steam process. **Table 3-1** highlight the differences between each treatment, where some contribute more or less to the final moisture of grain.

When wheat kernels were tempered with steam only, the final moisture of the grain was increased up to 12.39%, but the range of 15.0% that is desirable for soft wheat milling was not achieved However, when lactic acid was used or combined with steam for tempering, the final moisture of wheat was close to the target moisture 15.0% (±0.5%). Based on the data provided here, it is necessary to add a tempering solution (lactic acid solution, or simply water) to the tempering with steam if the target milling moisture is to be achieved.

Table 3-1. Treatment temperature and bed depth relationship with moisture addition to soft wheat during tempering.

Treatment	Temperature (◦C)	Bed Depth[3] (cm)	Moisture Addition (%)	Final moisture (%)
Control (water)[1]	NA	0.5	5.99 ± 0.06	14.92 ± 0.18
Steam	80	0.5	1.71 ± 0.37[2]	12.39 ± 0.26
Acid	NA	0.5	4.31 ± 0.21	14.98 ± 0.11
Acid + Steam	80	0.5	5.62 ± 0.65	14.96 ± 0.31

1 Sample size equivalent to 0.5 bed depth
[2] Values denote mean ± standard deviation
[3] Bed depth represents the thickness of the wheat layer during steam treatment
.

3.1.2. Reduction of natural microflora

Previous studies have reported that organic acid alone and thermal treatments such as steam were effective in reducing the microbial load of wheat (Hu et al., 2016; Sabillón et al., 2016). Therefore, this study used a combination of both, to see whether it would improve microbial reduction on soft wheat.

This research evaluated the effect of saturated steam (100°C), by itself and in combination with lactic acid, on the natural microflora of soft wheat and **Figure 3-2** presents the results obtained for the different treatments. Overall, all treatments applied

had a significant impact in reducing the natural microflora associated with soft wheat. Among the different microorganisms evaluated, the groups that were affected the most by the treatments were coliforms with reductions of up to 4.56 log CFU/g, followed by molds (up to 3.27 log CFU/g).

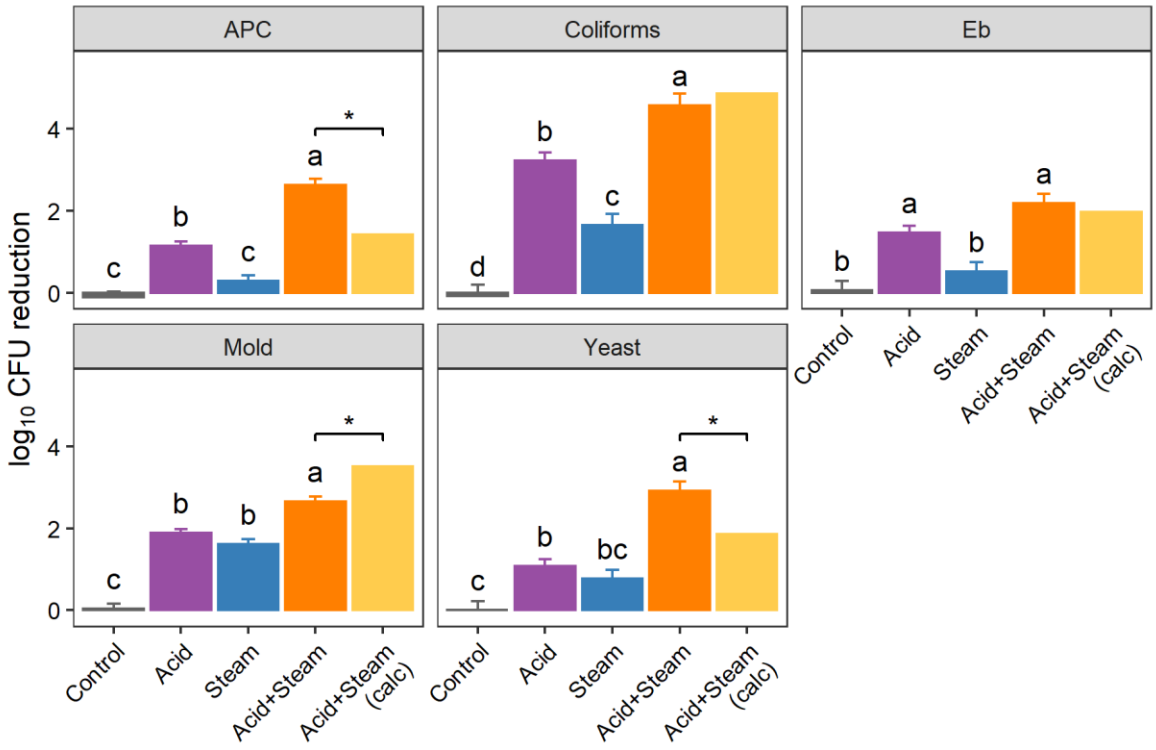


Figure 3-2. Effect of acid tempering (5% lactic acid) or steam treatment (0.5 cm bed depth; 80 °C final temperature) and their combination (0.5 cm bed depth; 80 °C final temperature tempered with 5% lactic acid) on natural microbiota reduction in soft wheat. Bars marked with different letters within panel are significantly different (Tukey's HSD $p<0.05$); *$p<0.05$ for experimental results compared with the calculated sum of acid and steam treatment.

Furthermore, the effect of each intervention was evaluated within microbial groups. These evaluations showed that the effect of steam was variable, showing results that were either inferior (APC, Eb, coliform) or similar (yeast and mold) to acid by itself. However, when acid and steam were combined, they showed superior effect for all microbial groups tested compared to acid by itself. Statistical analysis also indicated that

the effect of acid and steam combined is equivalent to the sum of the effect of the two interventions for coliforms and Eb, but significantly higher for APC and yeast, and lower for mold counts. A previous study by Sabillón et al., (2020) reported a 3.2 log CFU/g reduction in APC when tempering soft wheat with lactic acid 5.0% and NaCl 26.6%; while Eb was reduced by 4.5 log CFU/g. Additionally, a bacterial reduction of 3.03 log CFU/g was reported by Hu et al., (2016) when wheat kernels were treated at 110°C for 80 s using superheated steam. Another study reported similar results on the effect of superheated steam (Wang et al., 2019a). Compared to all those other studies, the interventions proposed here where acid and steam were combined, showed either superior or equivalent efficacy for the reduction of APC and Eb.

Generally, based on the data provided here, they indicate that a combination of acid and steam may be beneficial for the food industry by increasing the microbial safety of wheat-based products. Further studies were then conducted including other variables, such as bed depth and temperature, to evaluate their role on the efficacy of these interventions. Also, from the moisture analysis it was evident that pre-tempering with steam alone is not sufficient to achieve the required moisture for soft wheat milling. Therefore, moisture adjustments, with either water or an acid solution, were included in the experimental design.

3.2. Effect of bed depth and temperature when tempering soft wheat with steam and acid

3.2.1. Moisture of tempered soft wheat

Table 3-2 shows the moisture data collected for each treatment combination included in the experimental design, where bed depth and grain bed temperature were

included as new variables. Water was added to a set of treatments in the experimental design, so that the target milling moisture for soft wheat would be achieved based on the calculations that were made prior to adding water to the grain.

Table 3-2. Final moisture of soft wheat after tempering with acid, steam or their combination when variables were expanded to include bed depth and grain temperature.

Treatment	Temperature (◦C)	Bed Depth[3] (cm)	Moisture Addition (%)	Final moisture (%)
Control (water)[1]	NA	0.5	5.99 ± 0.06	14.92 ± 0.18
Steam	80	0.5	1.71 ± 0.37[2]	12.39 ± 0.26
Steam	80	1	1.12 ± 0.12	11.79 ± 0.14
Steam	85	0.5	1.30 ± 0.30	11.97 ± 0.31
Steam	85	1	1.68 ± 0.41	12.36 ± 0.14
Acid	NA	0.5	4.31 ± 0.21	14.98 ± 0.11
Acid	NA	1.0	4.72 ± 0.35	15.02 ± 0.17
Water + Steam	80	0.5	5.07 ± 0.49	15.24 ± 0.51
Water + Steam	80	1	4.85 ± 0.47	15.52 ± 0.14
Water + Steam	85	0.5	4.89 ± 0.45	15.16 ± 0.51
Water + Steam	85	1	5.28 ± 0.62	15.15 ± 0.25
Acid + Steam	80	0.5	5.62 ± 0.65	14.96 ± 0.31
Acid + Steam	80	1	6.06 ± 0.21	15.40 ± 0.41
Acid + Steam	85	0.5	6.81 ± 0.51	15.15 ± 0.23
Acid + Steam	85	1	6.17 ± 0.36	15.01 ± 0.41

1 Sample size equivalent to 0.5 cm bed depth
[2] Bed depth represents the thickness of the wheat layer during steam treatment
[3] Values denote mean ± standard deviation

Results provided on **table 3-2** showed that the moisture was increased for all treatment combinations, except for steam alone where the final moisture was between 11.79% to 12.39%, which is lower that the desired final grain moisture upon tempering

3.2.2. Temperature profile of hard wheat during steam treatment

Table 3-3 shows the time required to reach the target temperature for each specific bed depths. Based on the data presented in this table there is a direct correlation between grain temperature, time, and bed depth.

Table 3-3. The average time and temperature of soft wheat during steaming.

Temperature (°C)	Bed depth[1](cm)	Steam time (s)
85	1	42 ± 7[2]
85	0.5	24 ± 9
80	1	39 ± 8
80	0.5	19 ± 5

[1] Bed depth represents the thickness of the wheat layer during steam treatment
[2] Values denote mean ± standard deviation

Increasing the bed depth from 0.5 to 1.0 cm at 80°C, led to an increase in steam time 20 ± 7 sec. Similar trend was observed at 85°C, where the increase of bed depth from 0.5 to 1.0 cm increased the time needed to reach target temperature by 18 ± 8 sec. The higher the temperature, the longer it took to achieve the target grain temperature.

3.2.3. Reduction of natural microflora

The experimental design was expanded furthermore based on the results obtained from tempering soft wheat with steam, acid, and their combination, where it included grain bed depth and final temperature. **Figure 3-3** shows the effect of tempering and

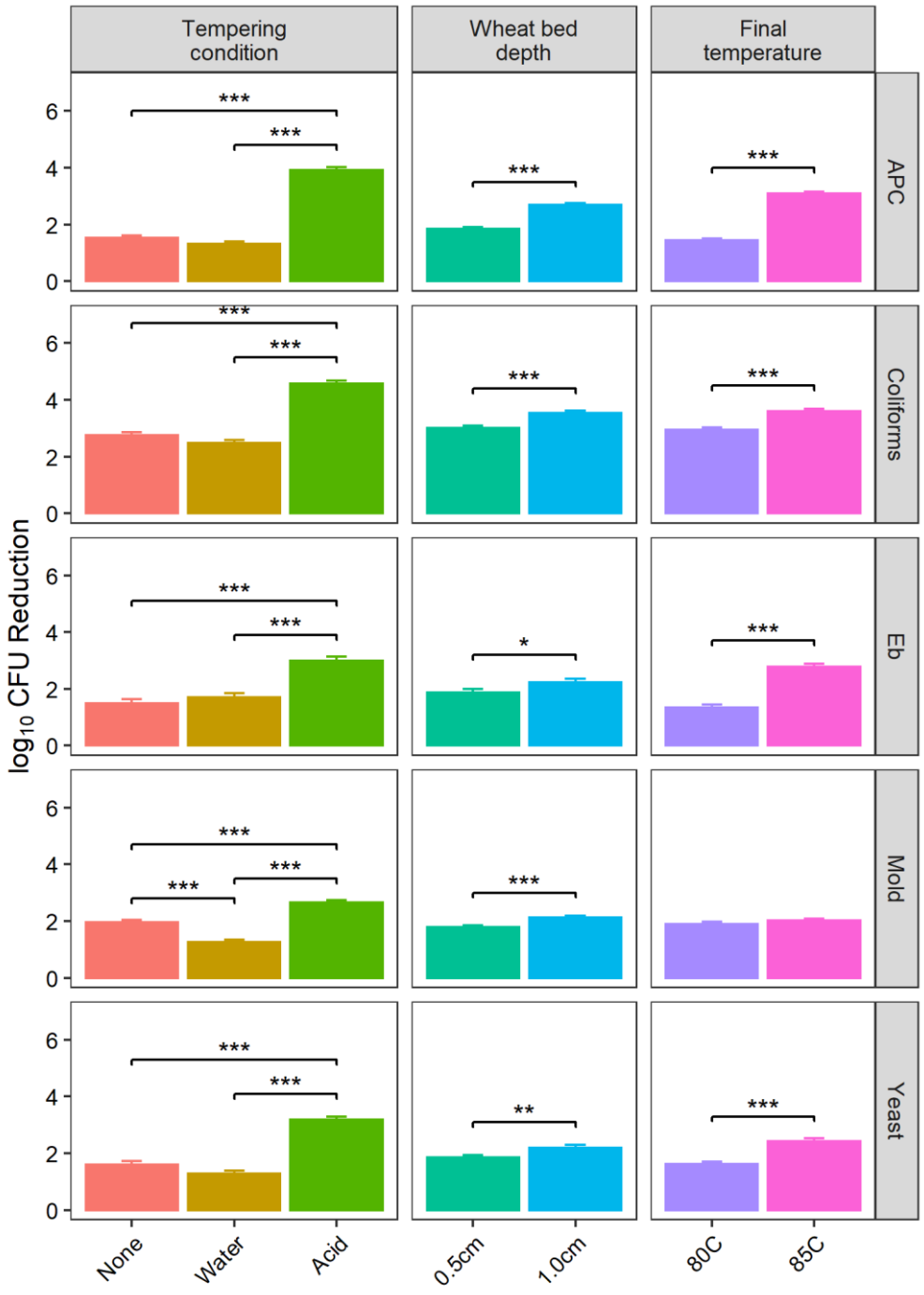


Figure 3-3. Effect of tempering and steam treatment variables (bed depth and temperature) on natural microbiota reduction in soft wheat.*p<0.05, **p<0.01, ***p<0.001

steam treatment variables such as bed depth and temperature, and the average log reduction achieved by these treatments when the tempering conditions, bed depth and final temperature were included in the experimental design.

When steam tempering conditions were considered within microbial groups, the addition of water to steam treatment did not show any significant difference for all the microbial groups, except for mold at a significance level $p<0.001$. The addition of water or acid prior to steaming is necessary so that the final moisture of wheat is achieved, as the steam alone does not reach the moisture for milling that is needed. Furthermore, the data provided here showed that acid addition to wheat prior to steam significantly increased the microbial reductions, when compared to those that were tempered with water only. The highest microbial reduction was achieved Eb (up to 4.3 log CFU/g), followed by APC (up to 4 log CFU/g). A study reported that using tempering solution of lactic acid 5.0% and NaCl a reduction of coliform up to 3.52 log CFU/g was observed (Sabillón et al., 2018). Ibanoglu (2000) reported a 1.0-2.0 log CFU/g reduction in yeast and mold count (YMC) using tempering solutions containing ozone, while tempering with organic acids and NaCl showed a reduction of 2.69 and 3.47 log CFU/g, for yeast and mold respectively (Sabillón et al., 2018). Another study where the application of superheated steam at 200°C for 20 seconds was evaluated, showed a reduction in fungal population from 3.03 log CFU/g to non-detectable levels. (Hu et al., 2016).

When comparing the microbial reductions with the grain bed depth of 0.5 and 1.0 cm, all microbial groups showed higher microbial reductions at 1.0 cm wheat bed depth. This may be related to the longer exposure time of wheat at a bed depth of 1.0 cm, since the time required to heat up the grain at 1.0 cm is longer than 0.5 cm. Increasing the bed

depth from 0.5 to 1.0 cm at 80°C, would increase the time up to 20 sec. However, increasing treatment time for the benefit of microbial reduction may lead to detrimental changes associated to the functional properties of the final wheat flour, which must be considered.

The last statistical comparisons made considered the final temperature of the grain bed. Except for mold, all other microbial groups tested, showed significantly higher microbial reduction when the treatment temperature was at 85°C, compared to 80°C. . In addition to the effect of the temperature itself, this may also be partially related to the time required to achieve them, since the time needed to heat up to 85°C was longer than to achieve 80°C.

In summary, the addition of water prior to steam treatment did not reduce its efficacy for the reduction of all microbial groups, expect for mold. Therefore, the addition of a tempering solution is required to achieve the optimum milling moisture. This study showed that if acid is used a tempering solution, it could help to further increase the microbial reduction of natural microflora in wheat. Indeed, the results presented here indicate that the best microbial reductions were achieved with a combination of steam and acid for the temperatures/bed depths evaluated. Further evaluations were made by testing functional properties of the final wheat flour to define which tempering interventions would be practical for the food industry.

3.3. Effect of tempering interventions on functional properties of straight-grade flour

Table 3-4 presents the pH and the acidity of the flour obtained from soft wheat flour pre-tempered with acid or water and then treated with steam. All treatment

combinations had lower pH than control (untreated). Adding acid prior to steaming further reduced the pH when compared to pre-tempering with water.

Table 3-4. pH and acidity values of straight-grade flour obtained from soft wheat pre-tempered with lactic acid or water and treated with steam.

Treatment	pH	Titratable Acidity (ml 0.1N NaOH)
Control (water)	6.94 ± 0.12a	1.57 ± 0.02a
Acid + Steam 85°C – 1.0 cm	6.76 ± 0.02bc[1, 2]	1.95 ± 0.01b
Acid + Steam 85°C – 0.5 cm	6.71 ± 0.02d	2.11 ± 0.08bc
Acid + Steam 80°C – 1.0 cm	6.32 ± 0.16f	2.15 ±0.13c
Acid + Steam 80°C – 0.5 cm	6.62 ± 0.10e	2.13 ± 0.05c
Water + Steam 85°C – 1.0 cm	6.77 ± 0.00b	1.96 ± 0.03b
Water + Steam 85°C – 0.5 cm	6.78 ± 0.07b	1.94 ±0.01b
Water + Steam 80°C – 1.0 cm	6.71 ± 0.07d	1.79 ± 0.11ab
Water + Steam 80°C – 0.5 cm	6.72 ± 0.07cd	1.75 ± 0.11ab

[1]Mean values with the same letter in the same column are not significantly different from one another (P > 0.05).
[2]Values denote mean ± standard deviation

Using acid as a part of the tempering intervention increased the acid content and decreased the pH for all the flour samples. However, the pH decrease observed here was different when compared to another study when soft wheat was tempered with lactic acid 5.0% and NaCl 26%, where the decrease in pH was higher than in this study (Sabillón Et al.,, 2018). Similar trends on pH and acidity were also observed in studies with wheat flour using tempering solution (lactic acid 5.0% and NaCl 26%) (Sabillón et al., 2017). A

higher acid content was measured in flours obtained from hard wheat when compared to those obtained from soft wheat (Sabillón Et al.,, 2018; Sabillón et al., 2017), These differences could be due to the endosperm characteristics in the two wheat classes (Posner & Hibbs, 2005).

The pasting properties of straight-grade soft wheat flours are summarized in **Table 3-5.** In general, the RVA pasting profiles showed significant changes for some of the parameters under conditions when treatments were applied compared to the control. The recorded values were lower for most of the RVA parameters listed in **Table 3-5** when compared to control. Similar trends were observed when soft wheat was tempered with lactic acid 5.0% and NaCl 26% (Sabillón et al., 2018). When thermal treatments are considered even though in this study significant differences were observed when steam was used as part of the tempering step. Poudel and Rose (2018) did not show any significant differences between samples treated with saturated steam up to 90 seconds and controls. More in line with the results reported here, other studies which used different steam treatment methods have shown differences in peak, trough, and final viscosities as a result of steaming (Hidalgo et al., 2008; Hu et al., 2017); In general, when steam was applied the peak viscosity, trough, breakdown, final viscosity, and set back were further from control, compared to when wheat was pre-tempered with water prior to steaming. Contrarily, time to peak viscosity and pasting temperature were more affected when soft wheat was pre-tempered with water prior to steaming.

Some studies which applied organic acid and NaCl to the flour have reported that the addition of these two may alter some of the viscoelastic properties of the flour such as peak, final viscosity, dough stability, and dough development stability, and dough

Table 3-5. Pasting properties of straight-grade flour obtained from soft wheat pre-tempered with lactic acid or water and treated with steam.

Treatment	Peak (cP)	Trough (cP)	Breakdown (cP)	Final (cP)	Setback (cP)	Time to peak viscosity (min)	Pasting temperature (°C)
Control (water)	2514.16 ± 147.65ab [1, 2]	1633 ± 100.49bc	671.33 ± 25.93ab	2940.5 ± 200.38a	1554.43 ± 148.17a	5.76 ± 0.11bc	52.59 ± 0.22a
Acid + Steam 85°C – 1.0 cm	1744 ± 115.97d	1256.33 ± 89.34e	517.33 ± 37.58d	2363.16 ± 174.97c	1180.5 ± 81.65d	5.69 ± 0.12c	52.48 ± 0.38a
Acid + Steam 85°C – 0.5 cm	2071.16 ±315.20c	2134.83 ± 451.85a	520.50 ± 5.73d	2634 ± 101.59b	1367.5 ± 112.66bc	5.76 ± 0.14c	50.29 ± 0.18bc
Acid + Steam 80°C – 1.0 cm	2692.5 ± 177.80a	1739.5 ± 128.31b	515.66 ± 5.00d	2816.33 ± 233.78ab	1483.33 ± 128.16ab	5.85 ± 0.63abc	50.06 ± 0.12r
Acid + Steam 80°C – 0.5 cm	1822 ±16.26d	1329.5 ± 80.72de	520.66 ± 5.62d	2626.33 ± 209.12b	1244.16 ± 70.62cd	5.72 ± 0.08c	50.69 ± 0.22b
Water + Steam 85°C – 1.0 cm	2598.83 ± 185.55ab	1705.16 ± 186.53bc	653.66 ± 35.48bc	2644.66 ± 255.36b	1343.33 ± 194.00c	6.17 ±0.03a	50.50 ± 0.30bc
Water + Steam 85°C – 0.5 cm	2437 ± 190.74b	1584 ± 73.51c	688.83 ± 58.11ab	2707 ± 204.57b	1361.33 ± 82.05bc	5.92 ±0.09a	51.78 ± 1.65ab
Water + Steam 80°C – 1.0 cm	2124.16 ± 106.16c	1404.16 ± 99.80d	693.16 ± 20.99a	2758.5 ± 136.60ab	1359.16 ± 71.68bc	5.87 ±0.05ab	50.6 ± 0.24b
Water + Steam 80°C – 0.5 cm	2124.16 ± 106.16c	1555.5 ± 100.94c	627.66 ± 15.57c	2745 ± 199.59ab	1350.83 ± 90.40bc	6.30 ± 0.27a	50.21 ± 0.15de

[1]Mean values with the same letter in the same column are not significantly different from one another (P > 0.05).
[2]Values denote mean ± standard deviation
saturated steam

development time (D'appolonia, 1972; Ganz, 1965; Wu et al., 2010). Pasting temperatures in this study were slightly lower when compared to another study that used saturated steam to treat wheat kernels for 90 s (Poudel & Rose, 2018).

Table 3-6 presents the baking quality of cookies made with soft wheat flour, which was obtained from kernels tempered with a combination of water or acid and saturated steam. The range values for cookie diameter were between 6.78 and 7.19 cm. Overall, the treatments at lower temperatures did not show any significant differences when compared to the control sample; even though values measured were lower than those with control samples. Treatments at higher temperatures showed significantly lower diameters than cookies made with the control flour. When soft wheat kernels were tempered with saline organic solutions Sabillón et al., (2018) reported higher diameter values ranging from 9.30 to 9.47 cm.

The thickness of cookies varied from 0.85 to 0.92 cm. No significant changes were observed when samples were pre-tempered with water prior to steaming. However, adding acid prior to steaming significantly reduced the thickness of the cookies. Similar values of thickness were observed in other studies when tempering soft wheat with organic saline solutions (Sabillón et al., 2018). The spread factor is a measure of cookie quality and it ranged from 7.85 to 8.36. The spread factor values reported here were lower when compared to another study where soft wheat samples were tempered with saline organic solution (Sabillón et al., 2018). In general, a higher spread ratio is desirable for better cookies (Mudgil et al., 2017).

Significant differences were observed in cookie hardness where most of the treatments showed lower values compared to the control sample. On the other hand, the flexibility of

the control sample showed lower values when treatments were applied. The flexibility of cookies also increased when organic saline solutions were applied in soft wheat (Sabillón et al., 2018). A few studies have reported that the amount of damaged starch in flour may influence the cookie characteristics such as diameter, hardness, and spread ratio (Barrera et al., 2007; Mudgil et al., 2017).

Table 3-6. Cookie quality characteristics from soft wheat flour tempered with water or acid in combination with steam.

	Cookie dimensional characteristics			Cookie textural characteristics	
Treatment	**Diameter (cm)**	**Thickness (cm)**	**Spread Factor[1]**	**Hardness (g)**	**Flexibility (mm)**
Control	7.19 ± 0.05a[2, 3]	0.92 ± 0.005a	7.99 ± 0.01c	2327.80 ± 18.40a	1.18 ± 0.005b
AS[4] 85°C -1.0cm	6.95 ± 0.11bc	0.86 ±0.01b	8.03 ± 0.30bc	2066.60 ± 101.75b	1.54 ± 0.21ab
AS 85°C - 0.5cm	6.78 ± 0.66abc	0.87 ± 0.02ab	7.85 ± 0.13c	2740.54 ± 189.76a	1.39 ± 0.14b
AS 80°C - 1.0cm	7.06 ± .011ab	0.84 ±0.01b	8.36 ± 0.11a	1563.62 ± 152.09cd	1.45 ± 0.06ab
AS 80°C – 0.5cm	7.05 ± 0.12ab	0.86 ± 0.01b	8.17 ± 0.18ab	1912.81 ± 299.59bc	1.39 ± 0.14b
WS[5] 85°C -1.0cm	6.99 ± 0.20abc	0.86 ± 0.08ab	8.12 ± 0.33b	2074.82 ± 181.91b	1.60 ± 0.12ab
WS 85°C - 0.5cm	6.95 ± 2.68c	0.87 ± 0.03ab	7.95 ± 0.05c	1507.05 ± 20.40d	1.66 ± 0.07a
WS 80°C - 1.0cm	7.15 ± 0.11ab	0.88 ± 0.03ab	8.10 ± 0.24b	1570.32 ± 173.44cd	1.61 ± 0.46ab
WS 80°C – 0.5cm	7.05 ± 0.21abc	0.87 ± 0.01ab	8.09 ± 0.36b	1977.35 ± 86.26b	1.39 ± 0.13b

[1]Spread factor = Diameter/Thickness

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

[3]Values denote mean ± standard deviation

[4]AS – Acid + Steam

[5]WS – Water + Steam

4. CONCLUSIONS

The application of saturated steam by itself and in combination with lactic acid, during tempering showed a significant reduction in the natural microbial load of soft wheat. Among the treatments tested, the use of acid in combination with steam provided the highest microbial reduction for all groups of microorganisms evaluated. No significant differences were observed where steam was applied alone or after pre-tempering with water, suggesting that if moisture adjustments for the milling process are needed then water can be added as needed without interfering with the effect of steam in the final microbial counts. When steam and acid by themselves were compared as a tempering intervention, the microbial reduction was for the most part similar for all treatments.

There were a few significant differences between the control and treated samples on flour pasting properties and baking quality of cookies. However, the treatments did not substantially affect the functional properties of soft wheat straight-grade flour. This suggests that the application of steam in combination with lactic acid during tempering can be a potential method used by the industry to reduce the risk of microbial contamination associated with soft wheat flour. Measures like this may contribute to reducing the risk of possible food outbreaks related to wheat-based products. Further research is now warranted to evaluate the sensory characteristics of baked goods made with flour produces using the same tempering interventions suggested here.

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CHAPTER 4. EFFICACY OF SATURATED STEAM AND LACTIC ACID ON THE REDUCTION OF SURROGATE ORGANISMS FOR PATHOGENS IN HARD RED WINTER WHEAT INOCULATED SAMPLES

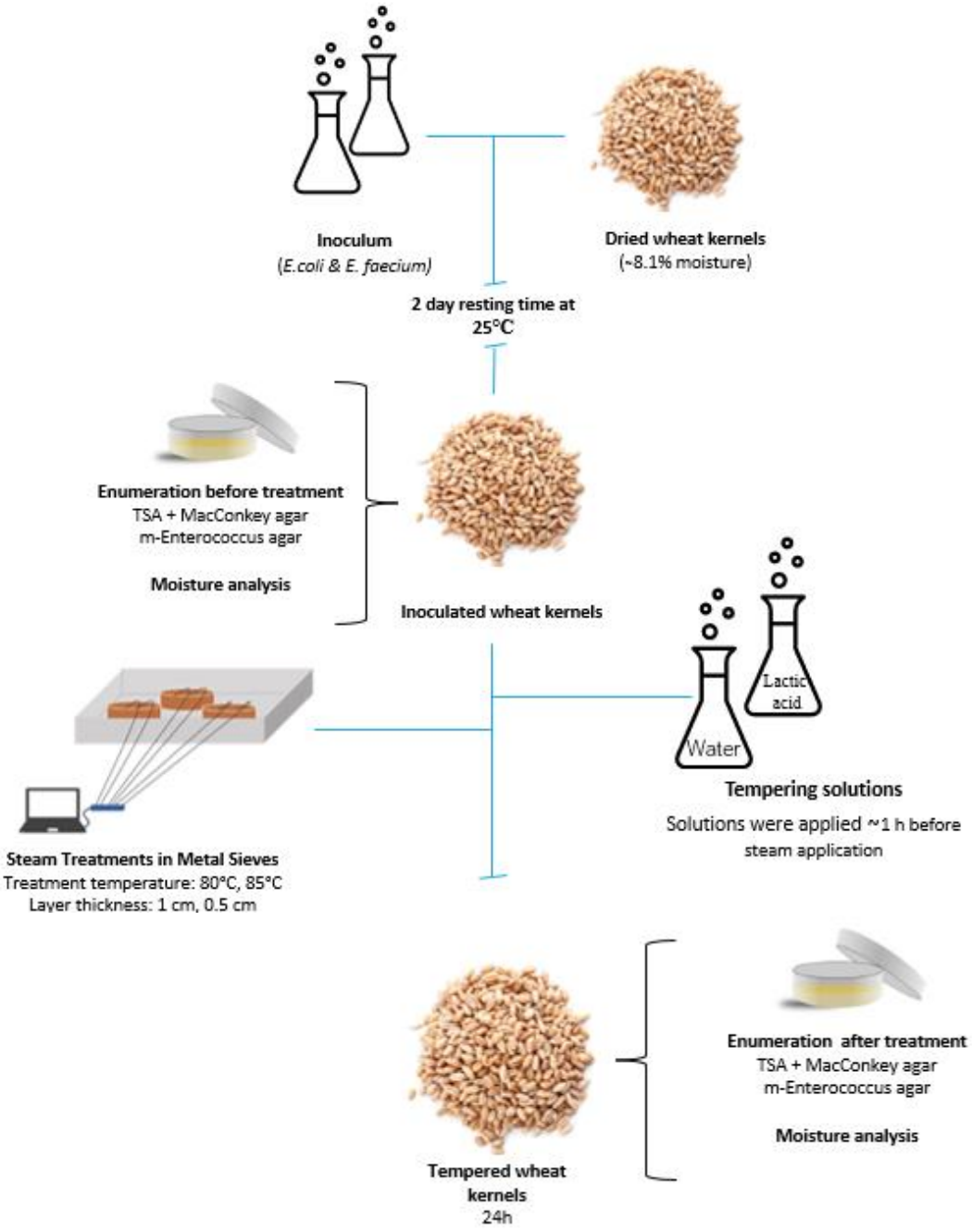
CHAPTER 4. THE EFFICACY OF SATURATED STEAM AND LACTIC ACID ON THE REDUCTION OF MICROBIAL LOAD IN HARD WHEAT INOCULATED SAMPLES

ABSTRACT

Due to outbreaks of *E. coli* and Salmonella which have been associated with wheat-based products, there is an essential need for developing a lethal step prior to milling to increase the safety of these products, potentially preventing future outbreaks from happening. Compounding this issue has been the increased number of ready-to-cook wheat-based products, which in some cases are consumed raw or uncooked. All these have the potential to risk the safety of the consumer's health. This study investigated the efficacy of saturated steam alone or in combination with tempering solutions (water or lactic acid) to reduce the populations of *E. coli* and *E. faecium* on hard wheat inoculated samples. Hard wheat samples were inoculated to a ~7.0 log CFU/g contamination level. Upon treatment, saturated steam significantly reduced the *E. coli* population, with the highest reduction of 3.43 log CFU/g being achieved for steam alone at 80°C – 1.0 cm, followed by three other treatments, which did not show any significant difference for 80°C – 1.0 cm. On the other hand, *E. faecium* reductions showed a different trend. Acid with steam achieved the highest reduction for all(?) bed depth and temperatures. When the temperature was decreased to 80°C the efficacy of the steam decreased as well. This suggests that at lower temperatures, for *E. faecium* the tempering of wheat with lactic acid is more efficient than the steam itself. The results included here for *E. coli* and *E. faecium* suggest that depending upon the microorganism of interest, different treatments can be applied to achieve the same results. Further research is now necessary to

determine if these surrogates are adequate to represent the inactivation of pathogenic *E. coli* and/or Salmonella in wheat kernels when thermal and no-thermal tempering interventions are applied.

VISUAL SUMMARY



1. INTRODUCTION

Wheat is exposed to various forms of contamination through the production chain. Soil, water, insects, and animal feces are a few of many factors that are a source of the contamination that wheat kernels are exposed to (Bullerman & Bianchini, 2008). Wheat is exposed to multiple sources of microbial contamination in the production chain, such as during harvest, storage, and transport. Microbial contamination includes enteric pathogens such as Salmonella and Escherichia coli, which have been associate with wheat milled products representing a food safety risk (Sabillón & Bianchini, 2016). Other factors influence the number of microorganisms in wheat, such as the meteorological conditions during the growing season, the storage conditions (i.e. moisture and temperature), as well as the presence of pets through the supply chain (M. P. Doyle & Buchanan, 2013; Sabillón & Bianchini, 2016).

Several microbiological surveys have detected the presence of pathogenic microorganisms, such as pathogenic *E. coli* on wheat kernels entering the milling system (Berghofer et al., 2003; Eglezos, 2010a). Other human pathogens such as Salmonella and Shigella have been also reported as well. For example, Salmonella has been isolated from wheat samples in Australia (Berghofer et al., 2003; Eglezos, 2010a). Wheat kernels may have contaminants on their surface. During the milling process which is involved in the production of flour, most of the microorganisms may be transferred to the final product. In the flour, microorganisms can survive during long periods of time depending upon storage conditions such as temperature and humidity (Laca et al., 2006; Sabillón et al., 2016).

Nowadays, there is an increased demand for ready-to-bake products such as cookie dough and pizza crust doughs, which sometimes may be consumed uncooked or partially cooked (Wales, 2011). If safety interventions are developed with these consumer eating behavior in mind, foodborne illness and outbreaks associated with wheat flour can be prevented (Magallanes López & Simsek, 2020). In the past, there have been reports of several outbreaks which were related to wheat flour-based products. One of them was associated with *E. coli* contamination of cookie dough (Neil et al., n.d.). In this outbreak, patients reported having eaten the dough uncooked, which resulted in the hospitalization of 35 individuals. This was the first reported Shiga toxin-producing *E. coli* (STEC) outbreak associated with consuming ready-to-bake cookie dough, even though the instructions on the package indicated the need to cook before eating. Another article reported an outbreak of STEC which was associated with raw flour as the source of the infections (Crowe et al., 2017). Despite the low moisture in flour, these are examples that flour and flour-based products can still be a carrier for foodborne pathogens. Another recent case, also related to raw wheat flour was reported by Harris and Yada (2019). This outbreak was related to dough mix contaminated with *E. coli* O157:H7 and a cake mix contaminated with Salmonella. A study in New Zealand reported an outbreak of Salmonella Typhimurium phage type 42 (STM42), which again was related to eating wheat-based uncooked baking mixture (McCallum et al., 2013).

In a survey regarding the eating behavior among consumers, 53% of 4,343 adults admitted that they eat homemade raw cookie dough (Byrd-Bredbenner et al., 2008). All the reported outbreaks and eating behavior among the consumers highlight the importance of the development of a lethal step against microorganisms present in wheat

microflora to ensure the safety of wheat-based products. Therefore, this study evaluates tempering interventions against indicator organisms. A non-pathogenic *E. coli* strain (ATCC 25922), which is commonly used as a quality control strain, was used as a surrogate for *E. coli* (STEC) (Sabillón et al., 2021), and *Enterococcus faecium* as a surrogate for Salmonella. Previous studies have shown that *E. faecium* has a higher inactivation temperature than Salmonella, which makes it an appropriate surrogate for this study (Bianchini et al., 2014). The main objective of this study was to evaluate the efficacy of saturated steam by itself and in combination with lactic acid during wheat tempering, prior to the milling process, to inactivate surrogates of pathogenic organisms in hard wheat samples.

2. MATERIALS AND METHODS

2.1. Materials

Commercially available hard red winter wheat (HWR) was used for this research. Bacterial strains used were non-pathogenic *E. coli* (ATCC 25922) and *E. faecium* (B-2354). The non-pathogenic *E. coli* strain was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Lactic acid (86%) was obtained from Fisher Scientific™ (Pittsburgh, PA).

2.2. Bacterial strains and inoculum preparation

A non-pathogenic *E. coli* strain (ATCC 25922) and *E. faecium* (B-2354) were chosen for this study. *E. faecium* was selected as a surrogate for Salmonella since in previous studies it has been suggested as an adequate surrogate for this pathogen during thermal processes (Bianchini et al., 2014; Jeong et al., 2011). Both cultures were stored at -80°C and were reactivated by transferring a small portion of the frozen broth into 9 mL Tryptic Soy Broth (TSB) using a sterile loop. For non-pathogenic *E. coli*, the 9 mL TSB tube was incubated following the method by Sabillón et al., (2021) at 37°C for 24 h. *E. faecium* was incubated at 32°C for 24 h (Bianchini et al., 2014). After the incubation process, bacterial cells were aseptically transferred into a 50 mL sterile conical tube and harvested by centrifugation at 4,000 x g / 4°C for 8 min (Sorval™ ST 16R Centrifuge, Thermo Fisher Scientific INL. Waltham, MA). The pellet was washed with 0.1% sterile peptone solution and then finally resuspended in 0.1% sterile peptone solution to conclude the preparation of the inoculum.

2.3. Inoculation of wheat kernels

The initial moisture of hard wheat samples was determined according to AACC approved method using a forced-air oven 44-15.02 (AACCI, 2009). Before inoculation, the wheat samples were dried overnight (~16h at 40°C) to 8.1 ± 0.5% moisture using a forced-air oven. This drying step was included because the inoculation step using a liquid inoculum would increase the moisture of the kernels. Subsamples of either 250 or 500 g of hard wheat were placed in sterile bags. Prepared bags were placed in a biosafety cabinet for inoculation. The amount of inoculum added was calculated to achieve 7.0 log CFU/g in each bag for both bacterial strains used. The inoculum was sprayed into the bags in order to ensure even moisture distribution onto the kernels. After that, the samples were allowed to rest for 2 days for the microbial adaption and moisture distribution. Bags were closed tightly and stored at 25°C (room temperature). On the first day of inoculation, samples were shaken every 12 minutes during the first 3 h, and then once a day during the resting period.

2.4. Experimental design

Figure 4-1 describes the experimental design used to evaluate the effect of steam, by itself or in combination with lactic acid, on the microbial load of inoculated hard wheat kernels. Steam treatment was applied to wheat directly kernels as the only source of water for moisture increase during tempering, and to wheat kernels that had been pre-tempered with water to ensure the desired final tempering moisture. Tempering with lactic acid solution was also evaluated, along with the combination of steam and lactic acid. The combination of mechanisms for microbial inactivation (acid and steam) was

evaluated to observe if there was any synergetic effect between them while reducing the microbial load of inoculated hard wheat **(Figure 4-1).** In the absence of an intervention, water was used as a control for the tempering process.

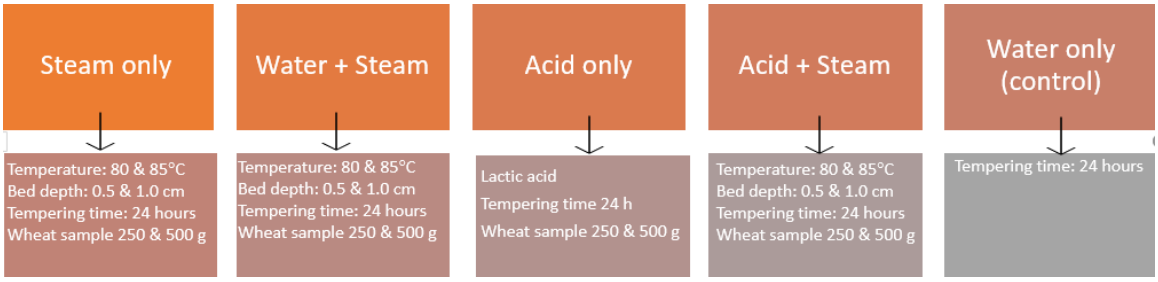


Figure 4-1. Experimental design to evaluate the effect of steam, by itself or in combination with lactic acid, on microbial load of inoculated hard wheat kernels

2.5. Tempering with water

According to the experimental design (**Figure 4-1**), two bed depths were evaluated. For the bed depth of 0.5 cm, a wheat sample of 250 g was required; while for the bed depth of 1.0 cm, the amount of wheat used was 500 g. When the inoculated samples were treated with water as part of the control, the amount of water added was calculated based on the initial moisture (after inoculation) of the wheat kernels and the target moisture (15.5%); To allow the best distribution of the water in the inoculated wheat sample, the distilled water was applied to the kernels in a biosafety cabinet with the help of an atomizer. The wheat samples were shaken every 15 min for 2 h of the tempering process and then allowed to temper for 24 h at room temperature.

2.6. Tempering with lactic acid

2.6.1. Preparation of tempering solutions

A set of samples were tempered by adding lactic acid to the process (**Figure 4-1**). The amount of acid used during the tempering was 1.8 mL lactic acid (85%) per kilogram of

tempered wheat. This acid application is equivalent to the one used by Sabillón et al., (2016) when 5.0% lactic acid solutions were used in their study. In this research, the calculated amount of distilled water and lactic acid (85%) then used to achieve the desired acid treatment and final moisture upon tempering. Calculations were based on the initial moisture of the wheat (10.6%) and the final weight of inoculated wheat after tempering to 15.5% moisture.

2.6.2. Application of tempering solution

According to the experimental design (**Figure 4-1**), two-bed depths were evaluated. For the bed depth of 0.5 cm, a wheat sample of 250 g was required; while for the bed depth of 1.0 cm, the amount of wheat used was 500 g. After the amount of lactic acid (85%) and distilled water required to achieve the acid treatment was determined, they were applied to inoculated hard wheat samples.

To allow the best distribution of the acid on the inoculated wheat sample, the distilled water and lactic acid (85%) were first mixed and then applied to the kernels in a biosafety cabinet with the help of an atomizer. The wheat samples were shaken every 15 min for the first 2 h of the tempering process and then allowed to temper for 24 h at room temperature.

2.7. Tempering with steam

2.7.1. Direct application of steam to wheat kernels

Samples of inoculated hard red winter wheat were divided into 250 g subsamples to achieve a 0.5 cm bed depth, while 500 g subsamples were used for 1.0 cm bed depth when using a mesh sieve (standard 40 mesh sieves with 0.420 mm hole thickness) as a

carrier for the samples. Two thermocouples (Omega TJ36-CPSS-116G-g, Omega, Norwalk, CT, USA) were placed in the center of the grain bed in each sieve. Three sieves were placed on a steam table that had been pre-heated to 100 ºC. The thermocouples were monitored using Omega Logging Software (Picto technology LTD, England, UK) which recorded the sample temperature every second. Sieves were placed on a steam table that was preheated up to 100ºC. After placing the sieves inside the steamer, grain temperature was monitored until it achieved 80 or 85ºC, according to the experimental design. When the target temperature was reached (80 or 85ºC), the inoculated wheat samples were taken out of the steam table. Right after the steaming process, the inoculated samples were placed into sterile plastic bags and immediately placed in an ice bath for ~5 min for cooling. Inoculated samples were allowed to temper at room temperature for 24 hours inside the plastic bags.

2.7.2. Steaming of pre-tempered wheat kernels

Inoculated hard wheat samples were also pre-tempered with water before steam was applied to ensure a final moisture content of 15.5% after tempering. With the direct application of steam to inoculated wheat kernels, the amount of water added could not be controlled. Therefore, pre-tempering with water was included to ensure that the final desired moisture was achieved, Calculations for the amount of water to be added was done knowing the initial moisture of hard wheat kernels and the amount of moisture that each steam treatment (temperature and bed depth) adds, by conducting preliminary tests so that the final moisture after application of tempering solution and steam was at the desired level of 15.5%. Pre-tempered hard wheat was then steam as described under 2.7.1.

2.8. Tempering with steam and lactic acid

For this set of experiments, the amount of water required to be added to each sample as pre-tempering treatment was calculated as described under 2.5.2. Then the amount of acid for each sample size was calculated to achieve a concentration of 1.5 mL lactic acid (85%) per kilogram of tempered wheat. The lactic acid was combined with water required for pre-tempering and added prior to steaming (≤1 h before steaming). Once again, to allow for an even distribution of water and acid over the hard wheat samples, the mixture was applied in a biosafety cabinet with the help of an atomizer. The samples were then shaken every 5 min before the steaming process. After the pre-tempering with acid and water was completed, hard wheat kernels were steamed as described under 2.7.1.

2.9. Microbial analysis

To determine the effect of the tempering interventions, inoculated wheat kernels were analyzed before and after treatments. Serial dilutions of the inoculated samples with *E. faecium* were prepared using phosphate-buffered saline (225 mL) and mixing it with 25 g of sample for 120 s. Further dilutions were prepared as needed. *E. faecium* samples were plated on m-Enterococcus agar (m-EA; Acumedia, Neogen Corporation) and incubated at 35 ± 2°C for 48 h. The m-EA is a selective medium that uses sodium azide to restrain the growth of gram-negative microorganisms, selecting for the organisms of interests.

To determine the number of microbial cells for non-pathogenic *E. coli*, 25 g of inoculated or treated samples were mixed with 225 mL of 0.1% sterile peptone solution for 120 s. Additional serial dilutions were prepared as needed. Dilutions were plated on

Tryptic Soy Agar (TSA) and incubated at 37°C for 3 h to allow the recovery of stressed, injured cells. After the cell-recovery period, TSA plates were overlaid with 9 mL MacConkey Agar. All the plates were incubated at 37°C for 48 h.

2.10. Moisture of tempered wheat

The moisture content of wheat samples was determined before inoculation, after inoculation, and after tempering using a forced-air oven, according to the method 44-15.02 (AACCI, 2009).

2.11. Statistical analysis

The PROC GLIMMIX procedure in SAS 9.4 was used to evaluate log reduction of the microorganisms of interest due to the tempering interventions. When differences among treatments occurred, they were reported at $\alpha=0.05$ significance level with Tukey-Kramer adjustment applied to obtain appropriate p-values. For analysis of the log reduction response, generalized linear models were used.

3. RESULTS AND DISCUSSION

3.1. Moisture management during grain inoculation

Hard wheat samples were dried overnight (~16 h at 40°C) to 8.1 ± 0.5% moisture. The reason for adding a drying step to the experimental design was to avoid excessive moisture in the wheat kernels due to the water which was added by the liquid inoculum. Because of the added drying step, upon inoculation the original moisture of wheat (10.6%) was restored after inoculation. The moisture of the samples was measured at three points: (2) before the inoculation, (2) 24 h after the inoculation and (3) 24 h after the treatment.

3.2. Moisture of tempered wheat

Moisture for each treatment is presented in **Table 4-1.** When inoculated wheat was pre-tempered with acid alone or with a combination of water or lactic acid and steam, the moisture of inoculated wheat was close to 15.5 ± 0.5%**.** However, for the intervention where steam was applied directly to kernels, the target milling moisture was not achieved due to the short time of steam exposure. Based on the data provided here, it is necessary the addition of a tempering solution to achieve the target milling moisture if steam is used as part of the tempering. Other studies have applied vacuum steam as a treatment for hard red spring winter wheat and reported a slight decrease in moisture content (Snelling et al., 2020). Since vacuum steam does not add up moisture to the kernels, that creates the possibility of combining vacuum steam treatment with antimicrobial tempering solutions without exceeding the moisture of tempered wheat. However, superheated steam may prevent limitations if multiple strategies are desired as it can increase the moisture of

Table 4-1. Treatment temperature and bed depth relationship with moisture addition to hard wheat inoculated samples during tempering.

Treatment	Temperature (◦C)	Bed Depth[3] (cm)[3]	Moisture Addition (%)	Final moisture (%)
Control (water)[1]	NA	NA	5.84 ± 0.62	15.48 ± 0.35
Steam	80	0.5	1.45 ± 0.11[2]	12.04 ± 0.54
Steam	80	1	1.68 ± 0.15	11.65 ± 0.06
Steam	85	0.5	1.84 ± 0.33	12.01 ± 0.64
Steam	85	1	1.56 ± 0.05	11.99 ± 0.15
Acid	NA	0.5	3.95 ± 0.57	15.05 ± 0.54
Acid	NA	1.0	4.02 ± 0.41	15.32 ± 0.11
Water + Steam	80	0.5	4.65 ± 0.42	15.68 ± 0.65
Water + Steam	80	1	5.01 ± 0.11	15.74 ± 0.02
Water + Steam	85	0.5	4.64 ± 0.22	15.02 ± 0.50
Water + Steam	85	1	5.99 ± 0.23	15.48 ± 0.32
Acid + Steam	80	0.5	4.98 ± 0.20	15.00 ± 0.60
Acid + Steam	80	1	5.54 ± 0.26	15.56 ± 0.45
Acid + Steam	85	0.5	5.96 ± 0.32	15.15 ± 0.18
Acid + Steam	85	1	6.05 ± 0.11	16.15 ± 0.32

1 Sample size equivalent to 0.5 & 1.0 bed depth
[2] Values denote mean ± standard deviation
[3] Bed depth represents the thickness of the wheat layer during steam treatment

the wheat kernels by itself. A study by Hu et al., (Hu et al., 2016) reported an increased moisture up to 16.0% using superheated steam. Moisture content is an important factor when it comes to the milling process of wheat and different wheat classes have different requirements for moisture levels.

3.3.Temperature profile of steam treatment

Figure 4-2 represents the time needed to achieve the target temperature for each treatment and bed depth. Based on this figure it can be noticed that when the bed depth increases from 0.5 to 1.0 cm, so does the time. Using 0.5 cm bed depth would have the advantage of saving time and energy if a similar approach would be used by industry. On

the other hand, if the time to achieve the target temperature is longer, the moisture of wheat kernels would increase due to the longer exposure time to steam.

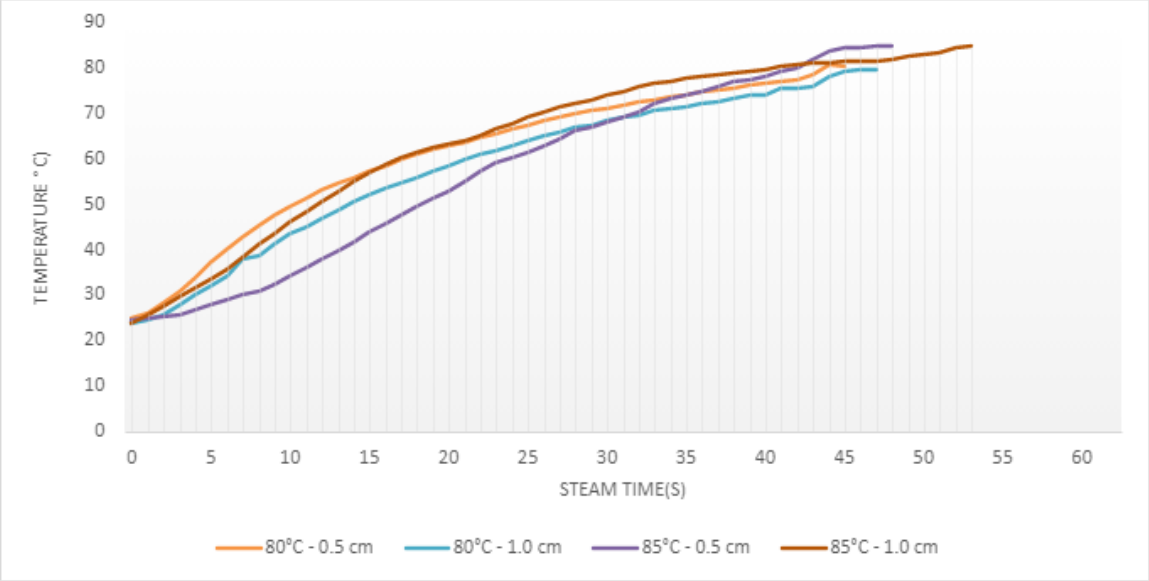


Figure 4-2. Temperature profiles of hard red winter wheat during steam application.

3.4. Reduction on surrogate organisms for pathogens in hard red winter wheat by tempering interventions

In the past, several studies have investigated different methods for the inactivation of wheat natural microflora, as well as targeted organisms in inoculated samples. These methods include the use of different tempering solutions, thermal and non-thermal treatments. Steam treatment and organic acids specifically have been reported to be effective methods when it comes to microbial intervention (Hu et al., 2016; Sabillón et al., 2016; Sabillón et al., 2019, 2020; Wang et al., 2019). In this, the efficacy of saturated steam by itself and in combination with lactic acid was studied as a pre-milling intervention step for hard wheat. The outcomes of the treatment combinations are

presented in **Figure 4-3.** When evaluating all charts for *E. coli* (**Figure 4-3** a, b, c, and d), steam by itself at bed depth 1.0 cm at 80°C achieved the highest log reduction 3.43 log CFU/g, followed by a reduction of 3.34 log CFU/g bed depth 0.5 cm at 85°C. However, for *E. coli* those reductions were not significantly different from values achieved with steam at other temperatures and bed depths, in the presence or absence of acid or water as a pre-tempering process. This suggests that no matter which temperature (85 or 80°C) is chosen for the treatment against non-pathogenic *E. coli* the results will be similar log reduction when steam is applied as part of the tempering process. When steam treatments were compared to tempering with acid only, the lowest log reduction was recorded in general, with the lowest value (1.58 log CFU/g) associated with bed depth 0.5 cm. A study by Snelling et al., (2020) described the effect of vacuum steam on inoculated hard red spring wheat samples with *E. coli* O121. The microbial reduction was recorded at 0, 2, 4, 6, and 8 min test times at 65°C. Similar to this study, they reported an increased log reduction when the processing time was increased with the highest log reductions achieved with 8 min (3.57 log CFU/g). It is worth mentioning that a study has reported that *E. coli* O157:H7 strains have higher survival ratios than the non-pathogenic strains (Yokoigawa et al., 1999), therefore direct comparisons under the conditions studied here would be ideal to ensure that the surrogates suggested in this study are appropriate to represent the pathogens of interest. Sabillón et al., (2020) investigated the effect of organic acids (acetic and lactic acid) 2.5 and 5.0% in combination with NaCl 26.5% against inoculated soft and hard wheat samples with non-O157 STEC and *E. coli* O157:H7. The highest microbial reduction overall was reported with the combination of lactic acid 5.0 % + NaCl 26.5%, with an average of 2.4 log CFU/g reduction for *E. coli*

O157:H7 and non-O157 STEC, while in this study the average reduction of non-pathogenic *E. coli* was 2.09 log CFU/g when tempering hard wheat with lactic acid. Once again direct comparison of pathogenic and non-pathogenic strains would be beneficial to evaluate their behavior under tempering with acids. Other studies have reported a reduction of pathogenic *E. coli* by tempering with acids in other wheat classes, for *E. coli* O157:H7 and non-O157:H7 STEC, respectively (Sabillón et al., 2020). Sabillón et al., (2021) studied the effect of high-pressure processing on sugar-cookie dough. For this study, they used the same strain used in this research (ATCC 25922) and reported a 2.0 log CFU/g reduction when treating the dough at 600 MPa for 6 min.

Figure 4-3 also presents the microbial reduction of *E. faecium* under the treatments tested. In general, reductions achieved for *E. faecium* were lower than those observed for *E. coli*, indicating higher resistance of *E. faecium* to the condition's testes. And unlike *E. coli*, the highest microbial reduction for *E. faecium* was achieved from the

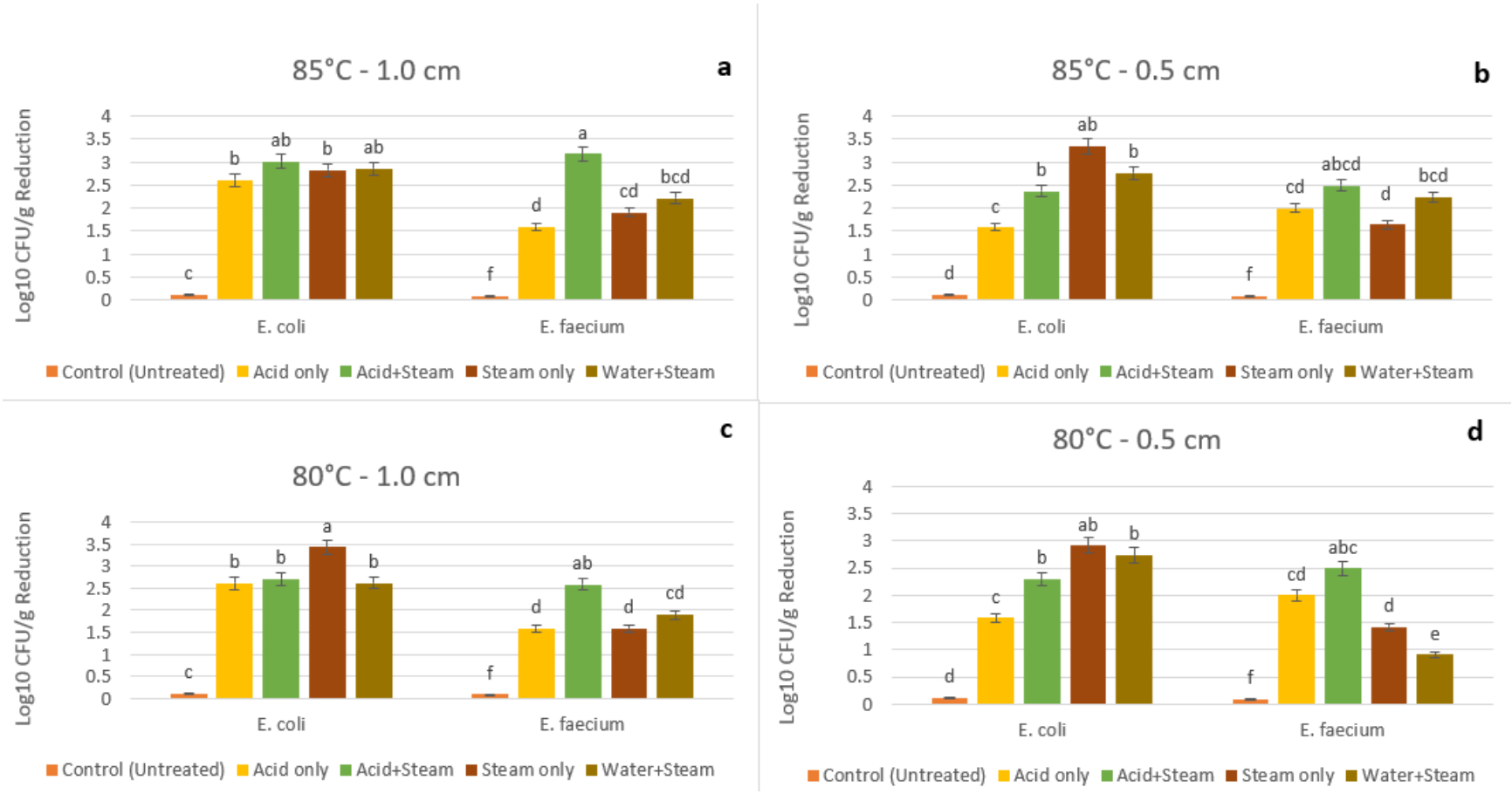


Figure 4-3. Effect of different treatment combinations on reduction of E. coli and E. faecium on inoculated hard red winter wheat. Legend: Error bard denotes ± standard deviation. Log reduction values within the same microbial group with the same letter indicate that there are no significant differences among the treatments ($P > 0.05$).

combination of lactic acid and steam (**Figure 4-3** a, b, c, and d) regardless of the temperature and bed depths evaluated. Among the treatments that involved steam, those where steam was applied by itself led to the lowed *E. facecium* reduction. When steam treatments were compared to acid alone for tempering, once again lactic acid led to lower *E. faecium* reduction, as observed with *E. coli*. As expected, for the most part, the addition of water prior to steaming did not show a significant difference when comparing to the of steam only. As far as evaluating steam as part of the tempering step acid Sabillón et al., (2020) reported a reduction of 2.6 log CFU/g of Salmonella when tempering hard wheat with lactic acid 5.0 % + NaCl 26.5%; for soft wheat using the same tempering solution the reduction was lower (~1.80 log CFU/g) compared to hard wheat. When evaluating steam as part of the tempering step a study using vacuum steam on hard red spring wheat reported a 3.21 log CFU/g reduction in Salmonella (Snelling et al., 2020). The treatment conditions to achieve such log reduction were 65°C for 8 minutes. However, none of these studies compared *E. faecium* and Salmonella on a single study, which makes comparisons difficult and shows the need for such studies to evaluate the efficacy of *E. faecium* as a surrogate for Salmonella during wheat tempering. The only study that evaluated both organisms simultaneously was the one conducted by Villa-Rojas et al., (2017) where microbial reduction was studied in wheat flour by radio-frequency heating. They used a free-running RF system type, with a power of 500 W and a frequency of 26.12 MHZ and studied the antimicrobial effect of RF heating of wheat flour with different water activities (0.25, 0.45m, and 0.65 a_w). Wheat flour was inoculated with *Enterococcus faecium* and Salmonella. The RF heating for 8.5 min at 0.25 a_w resulted in a 3.08 and 4.95 log CFU/g for Salmonella and *E. faecium*. The

minimum RF heating was 77.1°C and the maximum 99.9°C. Their study concluded that *E. faecium* was a feasible surrogate for Salmonella to validate the efficacy of RF treatment and that *E. faecium* was more resistant than Salmonella at any selected time. Studies are now needed to evaluate if the same would be observed when steam is used for wheat tempering. Non-thermal processes have also been evaluated to reduce Salmonella in wheat-based products. A study used pulsed light-emitting diode (LED) with a 395 m wavelength in unbleached wheat flour. A reduction of 2.48 log CFU/g in Salmonella counts was reported (Subedi et al., 2020). Du et al., (2020) applied pulsed LED as well on unbleached wheat flour, and with a 60 min treatment with 395 wavelengths, a 2.91 log CFU/g reduction in Salmonella counts was observed.

4. CONCLUSIONS

Steam treatment, by itself or in combination with lactic acid, is an effective way to reduce the natural microflora of wheat. This study revealed that saturated steam is an effective way to reduce non-pathogenic *E. coli* counts in hard wheat during tempering. Overall, there were no significant differences among the treatments that included steam in the reduction of *E. coli*. This suggests that either steam or a combination of steam with acid or water can give similar reductions. On the other hand, the differences in *E. faecium* reductions were more pronounced when it comes to different treatment combinations. Overall, lactic acid 5.0% alone or with steam was most effective against *E. faecium*. The highest reduction was noticed by the combination of acid and steam at 85° - 1.0 cm, however, the value was not statistically different from those reductions obtained at other temperature and bed depth combinations.

Further research is needed to test side by side the surrogate strains suggested here along with the pathogenic organisms of interest. This would allow to evaluate if they are adequate to represent the microbial reduction of the pathogen during tempering with steam.

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