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UNDERSTANDING THE FACTORS AFFECTING MICROBIOLOGICAL QUALITY OF WHEAT MILLED PRODUCTS: FROM WHEAT FIELDS TO MILLING OPERATIONS

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UNDERSTANDING THE FACTORS AFFECTING MICROBIOLOGICAL QUALITY OF
WHEAT MILLED PRODUCTS: FROM WHEAT FIELDS TO MILLING OPERATIONS

by

Luis Eduardo Sabillón Galeas

A THESIS

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UNDERSTANDING THE FACTORS AFFECTING MICROBIOLOGICAL QUALITY OF WHEAT MILLED PRODUCTS: FROM WHEAT FIELDS TO MILLING OPERATIONS

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Although regarded as a low-risk commodity, wheat flour-based mixes have been implicated in several food safety incidents. The present thesis is a compilation of five scientific manuscripts on the effect of weather variation, milling steps and implementation of pre-milling interventions on the microbiological quality and safety of wheat and milled products. The first manuscript is a review of the microbiological quality and safety of wheat-based products. Despite the low water activity, wheat flour may harbor dormant but viable microorganisms, which could lead to safety concerns when flour is used to produce refrigerated dough products. The second manuscript illustrates the effect of climatic conditions on the microbiological quality of wheat, where wheat grown under higher relative humidity and higher maximum temperature may be more susceptible to lower microbiological quality. In the third manuscript, the effect of the milling steps on the microbial load of wheat milled products is reported. While the cleaning and tempering steps did not affect the initial microbial counts, the grinding process caused a redistribution and concentration of microbial contaminants into the bran and germ fractions. The fourth manuscript reports the effect of novel tempering solutions on reducing microbial load in wheat prior to milling. Results showed that tempering solutions containing organic acids and NaCl were capable of effectively reducing the microflora of wheat kernels when compared to the traditional tempering process. In the last manuscript, the combined antimicrobial effects of organic acid and NaCl during tempering and its impact on flour functionality were evaluated. The results indicated a synergistic effect between lactic acid and NaCl, which was very remarkable in reducing microbial counts. Moreover, the impact on mixing, pasting, and baking properties was minimal.

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

TABLE OF CONTENTS

Scientific Manuscript One: From Wheat Fields to Consumer's Tables: A Comprehensive Approach to the Microbiological Quality and Safety of Wheat-Based Products.....	1
Abstract	2
Introduction.....	3
Microbiological profile of wheat grain.....	5
Effect of the milling steps on microbial load and mycotoxins in milled fractions.....	10
Microbiological quality and safety of wheat flour.....	13
Effects of bakery and pasta processing on the microbiological quality and safety of flour-based foods.....	18
Potential safety risks associated to flour-based foods.....	21
Concluding remarks.....	24
References.....	26
Scientific Manuscript Two: Effects of weather variables on the microbiological quality of hard red winter wheat.....	39
Abstract.....	40
Introduction.....	41
Materials and methods.....	42
Results and discussion.....	45
References.....	57
Scientific Manuscript Three: Effect of the milling steps on the microbial load of wheat milled products from different wheat varieties.....	61
Abstract.....	62
Introduction.....	63
Materials and methods.....	64
Results and discussion.....	67
References.....	76

Scientific Manuscript Four: Effect of novel tempering solutions on reducing microbial load in wheat prior to milling.....	78
Abstract.....	79
Introduction.....	80
Materials and methods.....	81
Results and discussion.....	83
References.....	94
 Scientific Manuscript Five: Pre-milling interventions to reduce the microbial load of straight-grade and whole grain flour with minimal impact on flour functionality.....	98
Abstract.....	99
Introduction.....	100
Materials and methods.....	101
Results and discussion.....	106
References.....	130

LIST OF TABLES AND FIGURES

Scientific Manuscript Two

Figure 1. Winter wheat-producing districts in the State of Nebraska, United States.....	43
Table 1. Name, latitude, and longitude of study locations, and sowing, flowering and harvesting dates during both growing seasons at each location.....	46
Figure 2. 2011-2012 (a) and 2012-2013 (b) growing season precipitation levels (in) for Nebraska.....	48
Table 2. Average high and low temperature (T-Max, T-Min), relative humidity (RH), and total precipitation (Precip) distribution during the two growing seasons at each district.....	49
Table 3. Mean and standard deviation (SD) for microbial counts in winter wheat samples from the 2011-2012 growing season.....	50
Table 4. Mean and standard deviation (SD) for Deoxynivalenol, moisture content and water activity in winter wheat samples from the 2011-2012 growing season.....	51
Table 5. Mean and standard deviation (SD) for microbial loads in winter wheat samples from the 2012-2013 growing season.....	53
Table 6. Mean and standard deviation (SD) for Deoxynivalenol, moisture content and water activity in winter wheat samples from the 2012-2013 growing season.....	54
Table 7. Correlation coefficients for weather variables versus microbial loads, moisture content, and water activity for winter wheat samples.....	55

Scientific Manuscript Three

Table 1. Mean and standard deviation (SD) for different groups of microorganisms in wheat samples before and after cleaning (categorized by wheat variety).....	68
Table 2. Mean and standard deviation (SD) for different groups of microorganisms in wheat samples before and after tempering (categorized by wheat variety).....	70
Figure 1. Average water activity of wheat grain before and after conditioning and milling end products for all wheat varieties. Error bars denote \pm standard deviation.....	71
Figure 2. Comparison of the distribution of APC, Eb, yeasts, and molds among milled fractions and milling end-product (straight-grade flour) in the different wheat varieties. Error bars denote \pm standard deviation. No differences among varieties for each fraction and microorganisms were observed.....	73

Scientific Manuscript Four

Table 1. Range, mean and standard deviation (SD) for different groups of microorganisms in winter wheat samples.....84

Figure 1. Effect of organic acid and saline solutions on aerobic plate counts (APC) during wheat tempering. Error bars denote \pm standard deviation. One log reduction is equivalent to a 90% reduction in CFU per gram of wheat grain. Log reduction values with the same letter are not significantly different.....86

Figure 2. Effects of organic acids and saline solutions on *Enterobacteriaceae* (Eb) counts during wheat tempering. Error bars denote \pm standard deviation. One log reduction is equivalent to a 90% reduction in CFU per gram of wheat grain. Log reduction values with the same letter are not significantly different.....88

Figure 3. Effects of organic acids and saline solutions on yeast during wheat tempering. Error bars denote \pm standard deviation. One log reduction is equivalent to a 90% reduction in CFU per gram of wheat grain. Log reduction values with the same letter are not significantly different.....90

Figure 4. Effects of organic acids and saline solutions on mold counts during wheat tempering. Error bars denote \pm standard deviation. One log reduction is equivalent to a 90% reduction in CFU per gram of wheat grain. Log reduction values with the same letter are not significantly different.....91

Scientific Manuscript Five

Figure 1. Effect of the combined addition of organic acid and NaCl in tempering water on reducing aerobic plate counts (APC). Error bars denote \pm standard deviation. One log reduction is equivalent to a 90% reduction in CFU per gram of wheat grain. Log reduction values with the same letter are not significantly different.....107

Figure 2. Effect of the combined addition of organic acid and NaCl in tempering water on reducing *Enterobacteriaceae* counts (Eb). Error bars denote \pm standard deviation. One log reduction is equivalent to a 90% reduction in CFU per gram of wheat grain. Log reduction values with the same letter are not significantly different.....110

Figure 3. Effect of the combined addition of organic acid and NaCl in tempering water on reducing yeast counts. Error bars denote \pm standard deviation. One log reduction is equivalent to a 90% reduction in CFU per gram of wheat grain. Log reduction values with the same letter are not significantly different.....112

Figure 4. Effect of the combined addition of organic acid and NaCl in tempering water on reducing mold counts. Error bars denote \pm standard deviation. One log reduction is equivalent to a 90% reduction in CFU per gram of wheat grain. Log reduction values with the same letter are not significantly different.....114

Figure 5. Microbial load of different wheat milling end products during sixteen consecutive runs in a Buhler experimental mill. ^a APC, Aerobic plate counts; Eb, *Enterobacteriaceae*.....118

Table 1. Mean values and standard deviation for the microbial load of straight-grade and whole grain flour.....119

Table 2. Mean values and standard deviation for pH and acidity of straight-grade and whole grain flour.....120

Table 3. Mean values and standard deviation for dough and gluten properties of straight-grade and whole grain flour.....123

Table 4. Mean values and standard deviation for RVA pasting parameters of straight-grade and whole grain flour.....126

Table 5. Mean values and standard deviation for straight-grade and whole grain bread loaf volume and firmness.....127

Table 6. Mean values and standard deviation for straight-grade and whole grain bread image analysis.....128

FROM WHEAT FIELDS TO CONSUMER'S TABLES: A COMPREHENSIVE
APPROACH TO THE MICROBIOLOGICAL QUALITY AND SAFETY OF WHEAT-
BASED PRODUCTS *

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ABSTRACT

Throughout history, wheat-based foods have been considered among the safest of all foods produced for human consumption. In part, this claim reflects both the low risk profile of low moisture foods and the thermal processes used to produce the finished product. Nevertheless, raw flour contains a number of potential hazards, which, if not properly managed, may have the potential to result in serious public health consequences. These hazards are mostly microbiological in origin and arise mainly during production and distribution through the wheat supply chain. The physical processes carried out during milling have minimal impact on the level of contamination present on grain; therefore, the initial microbiological quality of wheat grain has a strong influence on the ultimate quality and safety of milling end products. While most flour-based foods are processed and consumed in forms that are less likely to be contaminated with pathogens, many refrigerated dough products possess a substantial safety hazard to consumer health, since they are more likely to be consumed raw or undercooked. The potential for exposure to pathogenic microorganisms present in raw flour by eating uncooked baking mixture has been demonstrated by several outbreaks. Such food safety incidents have led to heightened manufacturer and consumer awareness about safety related to flour-containing foods.

INTRODUCTION

Historically, wheat flour has symbolized a pure and wholesome product. Furthermore, as a low moisture food, it has not traditionally been a discussion point in terms of food safety (7). However, recent events have caused the food industry and consumers to reconsider the safety level offered by wheat milled products. Studies have indicated that bacterial contaminants may survive in a latent state for extended periods in wheat flour, despite the low moisture content, and emerge from dormancy when flour is added to environments that are more receptive to growth, such as batter or mixes (13, 22). The multistate outbreak of *Escherichia coli* O157:H7 infections in the United States in 2009 associated with consumption of ready-to-bake pre-packaged cookie dough represents the most striking recent example of the potential for harm caused by products containing raw flour (35). And it is far from an isolated event, since the safety of wheat flour-based products has been threatened over the years. Low levels of *Salmonella* contamination have been detected in wheat flour; and flour and flour-based mixes have been involved in foodborne *Salmonella* outbreaks (7, 27, 52, 64, 84, 90, 100, 101). Coliforms and generic *E. coli* species are commonly used as hygiene and safety indicators in many food industries to assess the microbiological safety of processing environments and foods. However, some species of enteric bacteria normally live and feed on dead organic matter (e.g., plants), thus these microorganisms are likely to be present in grain or milled products to some extent (104). Indeed, these microorganisms have been found in wheat flour (5, 7, 64, 84), where high levels may indicate unhygienic processing or handling and the potential presence of fecally-transmitted pathogens. Wheat flour is usually contaminated with *Bacillus* spores as a result of processing or post-processing contamination (83). *Bacillus* species have been associated with the spoilage of flour-containing foods (e.g., rope spoilage of bread) as well as food safety incidents (18, 45, 83, 89, 102). The presence of spore-forming pathogenic *Bacillus* species, such as *B. cereus*, in starchy foods like wheat-pasta is frequently the cause of several foodborne illnesses (18, 17, 50).

The safety of flour-containing foods has been compromised over the years, not only by pathogenic bacteria, but also by fungal contamination, especially those mycotoxin-producing molds. In addition to the negative changes in the chemical composition of flours, bread, and pastries, the production of mycotoxins is one of the several major consequences of mold growth (42). Mycotoxins are fungal secondary metabolites that have various acute and chronic effects on human health (33), therefore they represent another important risk associated with wheat product consumption. Mold growth and subsequent mycotoxin production usually occurs during grain ripening at the field level but also as a result of poor storage conditions (61, 71, 103). As wheat kernels are further processed into flour, fungal and mycotoxin contamination is redistributed, undestroyed, and concentrated in certain milling fractions; therefore, wheat flour may carry a significant mycological and mycotoxigenic load (12, 92). Mycotoxin-producing species of the genus *Aspergillus*, *Penicillium* and *Fusarium* have been found in wheat-based retail products, especially whole-grain flours (10, 26, 42, 47). Moreover, low but persistent levels of multiple mycotoxins in wheat-based products sold directly to the public have been reported in several countries (3, 26, 47, 69, 91, 99). Besides potentially pathogenic microorganisms and mycotoxins, wheat milling end products harbor a wide variety of spoilage microorganisms, including aerobic psychrophilic, mesophilic or thermophilic bacteria (e.g., spore-forming and lactic acid bacteria), yeasts, and molds (3, 7, 22, 44, 64, 84). Consequently, the use of raw flour (not heat-treated) in foods such as refrigerated dough products, pie pastry or pasta products could also be the source of accelerated spoilage.

The increased incidence of foodborne disease outbreaks caused by products containing flour has highlighted the importance of high microbiological quality of wheat grain at the beginning of the flour supply chain. During wheat production, including harvest, storage and transport, microbial contaminants are hosted and concentrated mainly on the surface of the grain and from there they reach the flour during milling (39). The microbiological quality of wheat grain is considered to be the major contributor to loss of quality and safety of milled products and

foods produced from this ingredient (7, 79). A comparison between the milling end products of high and low microbiological quality wheat showed that flour with higher microbial counts is usually obtained from wheat of inferior microbiological quality (7). Since wheat flour is an agricultural product, as such, it is exposed to naturally occurring microbiological threats, and therefore the assumption that flour and other milled products are microbiologically safe is risky for consumers. Nowadays, many flour-based products rely on the consumer to perform baking or cooking steps, which could be dangerous, since an alarming percentage of consumers often eat these products (e.g., refrigerated cookie or biscuit dough, frozen pizzas or pies) without fully cooking them as directed by the manufacturer (13). Therefore, it is important to understand how to mitigate the risk of microbial contamination in wheat flour, especially the one used in ready-to-cook or ready-to-bake products. In light of several food safety incidents implicating wheat flour as a potential carrier of pathogens, this article aims to provide an overview of the microbiological safety of the flour supply chain, from wheat grain to finished products, and to describe the effects of milling procedures and thermal processes on the microbial load and mycotoxins of wheat grain and finished products. Hence, to help evaluate the potential risk related to wheat-based products, within each section of this article, the microbial load has been categorized into three classes: spoilage microorganisms, pathogenic microorganisms, mycotoxin-producing molds and mycotoxins.

MICROBIOLOGICAL PROFILE OF WHEAT GRAIN

Throughout the growing season, wheat is exposed to multiple sources of microbial contamination, including soil, water, insects, and animal feces (15, 16, 39). Additionally, microbial contamination also occurs during harvesting and subsequent handling and storage (80, 103). Thus, throughout the wheat production chain, there is ample opportunity for molds to produce toxins and for microbes to colonize the wheat grain. The number and type of microorganisms present in wheat is influenced by several factors, including the meteorological

conditions prevailing during grain ripening and harvesting, harvesting and handling equipment, insect, bird and rodent activity during production and storage, and moisture control (64, 79, 103).

The microflora found in wheat grain is large and varied, and includes bacteria that mainly belongs to the families *Micrococcaceae*, *Pseudomonadaceae*, *Enterobacteriaceae*, *Lactobacillaceae* and *Bacillaceae*; yeasts, and molds that mostly belong to *Alternaria*, *Cladosporium*, *Fusarium*, *Helminthosporium*, *Aspergillus*, *Penicillium*, and *Eurotium* (39, 103). Within these families, pathogenic microorganisms (e.g. *Bacillus cereus*, *Clostridium botulinum*, *Clostridium perfringens*, *Escherichia coli*, *Salmonella*, *Staphylococcus aureus*), mycotoxin-producing molds, and spoilage microorganisms (e.g. *Bacillus* spp. and lactic acid bacteria) are found (7, 24, 32, 64, 103). These microorganisms are mostly distributed in the surface of the grain, although some species can occupy the inner part of the kernel (e.g. fungal hyphae), mainly thorough the germ or due to mechanical damage during harvesting (34, 39). Generally, wheat grain stored under proper conditions (e.g. temperature and humidity controlled) have water activity below the minimum needed for microbial growth; however, pathogenic and spoilage microorganisms may survive in a dormant state and be transferred to processed products where they become a problem (44, 103).

Spoilage microorganisms. Yeast and mold are among the common spoilage microorganisms found on wheat grain. In a two-year survey, Seiler (79) reported mold counts of 5.1 log colony forming units/gram (log CFU/g) in British wheat for the 1976 growing season and 6.0 log CFU/g for the 1977 growing season. Eyles et al. (24) found yeast-mold counts in the order of 4.2 log CFU/g in Australian wheat. In another Australian survey, Berghofer et al. (7) analyzed 58 wheat samples and found yeast and mold counts in the range of 2.0 to 5.0 log CFU/g. In a 2001 North American survey, the yeast-mold counts found in durum wheat samples as received from growers, farms bins, and elevators ranged from 1.4 to 5.0 log CFU/g, with an average of 3.3 log CFU/g (44). More recently, in another Australian wheat survey, Eglezos (22) reported mean counts of 3.7 log CFU/g and 2.7 log CFU/g for yeast and mold, respectively. Moreover, Peles et

al. (48) examined the microbiological status of Hungarian organic wheat grain, and found mean counts of 3.9 log CFU/g and 3.5 log CFU/g for yeast and mold, respectively. Riba et al. (63) reported that mean value counts of fungi ranged from 2.4 to 3.1 log CFU/g in Algerian wheat.

In addition to fungi, a wide variety of spoilage bacteria, including bacilli, lactic acid bacteria, and micrococci are also present in wheat grain. Eyles et al. (24) reported total aerobic counts of 4.9 log CFU/g in Australian wheat. The same levels of aerobic bacteria (4.9 log CFU/g) were observed in 54 U.S. wheat samples (65). Berghofer et al. (7) reported that the most frequent (modal) counts in the Australian wheat for aerobic mesophilic bacteria was 5.0 log CFU/g, with 55% of the samples analyzed containing counts greater than or equal to 4.0 log CFU/g, up to a maximum of 7.0 log CFU/g. In addition, the most frequent count for *Bacillus* spp., bacteria responsible for rope spoilage of bread, was 4.0 log CFU/g. In the same study, mesophilic aerobic spores and thermophilic aerobes were also commonly detected but at levels (1.0 log CFU/g) lower than other spoilage microorganisms. In the 2001 durum wheat survey in North America, the aerobic plate counts ranged from 0.9 to 8.4 log CFU/g (44). Peles et al. (48) also reported aerobic bacterial counts at a mean of 4.9 Log CFU/g in Hungarian organic wheat.

Pathogenic and fecal microorganisms. Enteric pathogens, such as *Salmonella* spp. and *Escherichia coli*, may be among the microflora of wheat grain creating a food safety risk in milled products. Likewise, the food poisoning spore-former *Bacillus cereus* may also be present in wheat grain. In 1992, *Salmonella* Havana was isolated from wheat grain in Queensland, Australia (22). More recently, Eglezos (22) reported a single isolation of *Salmonella* Give from 50 unscreened wheat samples. Similar occurrence was reported by Berghofer et al. (7), where only 2 environmental serovars of *Salmonella*, *S. Chester* and *S. Hvittingvoss*, were isolated from 2 milling samples out of 412 analyzed. Berghofer et al. (7) also reported that 47 out of 58 wheat samples evaluated tested positive for *B. cereus*. However, the majority of the positive samples contained less than 1 spore per gram. A low incidence of *B. cereus* in wheat was reported by Eglezos (22), since only 2 out of 50 samples analyzed showed the presence of this organism, at a

mean of 2.1 log CFU/g. Some species of enteric bacteria are associated with plant materials, and wheat is not the exception. In the same study, Eglezos (22) detected *E. coli* in one of the samples evaluated, at a mean of 0.6 log CFU/g. Eyles et al. (24) found coliforms counts at 1 most probable number/gram (MPN/g) in Australian wheat. However, Berghofer et al. (7) reported higher levels, 10 MPN/g, also in Australian wheat. *Escherichia coli* O157:H7 have not been isolated or detected in any of the other surveys carried out.

Mycotoxin-producing molds and mycotoxins. Fungal contamination is frequently divided into two groups, field fungi and storage fungi (49, 103). Field fungi invade grain before harvest when they have high moisture content (18 to 30%), whereas storage fungi infect grain post-harvest when they have lower moisture contents (14 to 16%). Field fungi consist primarily of species of *Alternaria*, *Cladosporium*, *Fusarium*, and *Helminthosporium*, while storage fungi include species of *Eurotium*, *Aspergillus*, *Penicillium*, and *Mucor*. Riba et al. (63) analyzed the mycobiota composition of 27 samples of freshly harvested field wheat grain collected from two regions in Algeria. Their results revealed the dominance of *Aspergillus* spp. followed by *Fusarium* spp., in both areas. A post-harvest survey by Birck et al. (105) found that *Aspergillus* (100%), *Fusarium* (80%) and *Penicillium* (60%) were the most frequently recovered genera from stored wheat samples during 180 days. Bensassi et al. (106) analyzed the mycobiota of freshly harvested wheat grain in Tunisia and found *Alternaria* as the dominant fungal species followed by *Fusarium* species. *Fusarium culmorum* and *Alternaria alternata* were among the most predominant isolated mycotoxigenic species. In another study, wheat samples were analyzed directly after harvest as well as after three months of storage (107). The most predominant species on wheat grain after harvest belonged to the genus *Alternaria*, *Cladosporium* and *Fusarium*, whereas after three months of storage the dominant species were those belonging to *Aspergillus* and *Penicillium*.

When wheat grain is colonized by these fungi there is a significant risk of contamination with mycotoxins and consequently, the safety of the grain can be greatly reduced. Mycotoxins

have multiple effects on eukaryotic cell functions; hence, it can cause significant harm to humans and animals when ingested (33). The mycotoxins of greatest significance on wheat grain include deoxynivalenol (DON) and zearalenone (ZEA) produced by *Fusarium graminearum* and *Fusarium culmorum*, respectively, during wheat flowering, and ochratoxin (OTA) produced by *Penicillium verrucosum* and *Aspergillus ochraceus* during wheat storage (51). Nishio et al. (53) studied the levels of *Fusarium graminearum* infection in wheat and observed that the amount of DON produced was directly related to the incidence of fungal growth. Among the mycotoxins produced by *Fusarium* spp., DON is the predominant and most economically important mycotoxin in small grain production, such as wheat (116). Several surveys have been conducted on the levels of mycotoxins in wheat worldwide (108, 109, 111, 112). Regulatory agencies such as the European Commission, have established maximum limits for these toxins to minimize the exposure and toxic effects in humans and animals (19, 20).

The microbial load and mycotoxin production in wheat grain depends on several factors, among them the climatic and geographical conditions. Seiler (79) demonstrated this by comparing the microbial load at the time of harvest of wheat grown under warm dry weather conditions (5.7 log CFU/g for total bacteria and 5.1 log CFU/g for molds) and wheat grown under unusually wet conditions (8.1 log CFU/g for total bacteria and 6.0 log CFU/g for molds). More recently, Manthey et al. (44) also found differences in the microbial load of durum wheat planted across the North Plains region in the United States as a result of precipitation levels during the growing season. Moreover, some studies have shown that moisture related variables, such as daily average relative humidity and total daily precipitation can be positively correlated with mold infection and mycotoxin production (114, 115). According to these studies, wheat grown under higher precipitation rates and humidity may be more susceptible to lower microbiological quality and higher mycotoxin content.

EFFECT OF THE MILLING STEPS ON MICROBIAL LOAD AND MYCOTOXINS IN MILLED FRACTIONS

The dry milling process of wheat involves three general operations: cleaning, tempering, and milling. None of these operations involve chemical or thermal treatments and thus do not significantly impact the safety of milled products. Nevertheless, the milling process causes a redistribution of contaminants and may lower the contamination levels in milled fractions intended for human consumption (flour and semolina).

Cleaning. Flour milling begins with removal of unwanted materials from wheat grain. Once harvested, wheat contains a significant percentage of non-wheat materials, including sticks, stems, stones, unsound wheat kernels, attached soil, insects and other kind of materials that may come from wheat fields (62). The cleaning process is carried out by a sequence of operations that removes these impurities based on shape, density, size, and magnetism (62). The foreign matter separated during the cleaning process may contribute to improve the safety of wheat grain prior to milling. In terms of microbiological quality, Manthey et al. (44) demonstrated that after cleaning dirty grain with a dockage tester and cyclone grain cleaner, on average, the microbial load was reduced in 1.0 log CFU/g for aerobic bacteria and similar reductions were also seen with yeast and molds counts. Seiler (79) also observed small reductions in counts of bacteria and mold in wheat when evaluating different dry scourers, aspirators and brushes. In contrast, Riba et al. (63) observed no differences in the fungal contamination between unclean wheat and wheat after cleaning, with fungal counts varying from 2.7 to 2.9 log CFU/g.

Likewise, physical separation and cleaning procedures may also reduce mycotoxin concentrations by removing contaminated and damaged kernels (9). According to Abbas et al. (1) the cleaning step can reduce deoxynivalenol concentrations from 5.5 to 19% from scab infected wheat. More recently, Lancova et al. (40) achieved an average reduction of 48% in deoxynivalenol content by sieving, scouring and polishing wheat grain. Pascale et al. (57) reported a 62% and 53% reduction of T-2 and HT-2, respectively, in wheat after cleaning.

Although these cleaning procedures may reduce microbial and mycotoxins contamination, the extent of the reduction is variable and there are no reports on the effectiveness of these cleaning steps for pathogen reduction. Therefore, kernels may still contain a substantial amount of contaminants (44).

Tempering. This process involves the addition of water to the clean wheat, primarily to enhance the efficiency of flour extraction (62). Water is added in precise amounts to reach the desired moisture level. To ensure even penetration of moisture into the kernels, wheat is held in large conditioning bins during specific holding periods (7, 62). This is a critical step in milling operations, since an improper handling can encourage microbial growth either in the wheat or on the milling equipment. Berghofer et al. (7) found higher mesophilic aerobic counts more frequently after wheat conditioning. In the same study, *E. coli* was detected after tempering in previously non-contaminated wheat. Since the holding periods are relatively short (6-18 hr) and the water activity of the grain during tempering is not enough to support bacterial growth (7, 62), the increase in contamination may be more likely to be due the use of poorly cleaned conditioning bins and equipment. Indeed, Berghofer et al. (7) during an inspection survey in Australian mills, detected build-up of grain residues in conditioning and storage bins, which may harbor substantial amounts of microbial contamination. Therefore, attention should be given to sanitary conditions and cleaning protocols of the equipment involved in the tempering process.

Milling. Once conditioned, wheat undergoes a series of reduction, grinding and sifting operations to separate the germ and the bran fractions from the endosperm (62). As in cleaning and tempering, milling processes do not destroy mycotoxins and microorganisms but rather redistributes and concentrates these contaminants in certain milled fractions, particularly in germ and bran (1, 57, 64). As wheat passes through break, coarse and fine reduction stages in the mill, the overall microbial load in the milled products is reduced when compared with that originally present on the conditioned grain. In an earlier study, Hesseltime (31) showed that the dry-milling process reduced bacterial counts in 1.3 log CFU/g from that originally present in wheat before

processing (5.3 log CFU/g); however, fungal counts were relatively unchanged. More recently, Berghofer et al. (7) reported that maximum aerobic bacterial count decreased from 7.0 to 5.0 log CFU/g, yeast counts from 6.0 to 3.0 log CFU/g, and mold counts from 6.0 to 4.0 log CFU/g after fine reduction. In the same study, the bran and germ fractions showed higher microbial counts than the incoming wheat, with aerobic bacterial count increasing from 6.0 to 7.0 log CFU/g in the bran portion. In fact, the dry milling process concentrates in excess of 90% of aerobic bacteria present on wheat into the bran and germ fractions (84).

The physical removal of microorganisms and other contaminants during the early break stages of the milling process seems to exert the greatest influence over the microbial levels of milled products, since only small reductions have been observed between the microbial load in the final reduction stages and the end product (e.g., flour) (7). When the microbial load of milling end products is compared, flour tends to have lower microbial loads than the initial wheat and other milled fractions (e.g., germ and bran) (7, 44, 64). This improvement in safety is achieved by the physical removal of the more heavily contaminated outer parts of the kernel (germ and bran fractions), that are first colonized by fungi and microorganisms (30, 39, 40, 44). The germ and bran fractions are less likely to be used for food production; however, whole grain and whole grain milled products consumption is growing at a remarkable rate due to their known health benefits (82), representing a unique food safety challenge for the milling and food industry.

Regarding mycotoxins, similar trends in redistributions of deoxynivalenol, aflatoxin, and zearalenone have been previously documented (1, 21, 56, 96). In general, the levels of mycotoxins are lower in the flour fraction than in the germ or bran fractions; however, the extent of the reduction may depend on the variety of wheat, the penetration degree of mycotoxin-producing molds, transfer of mycotoxins to the inner parts of the kernel, and flour extraction rate (60).

Therefore, even though the levels of microorganisms and mycotoxins are lower in milled fractions intended for human consumption (flour or semolina), these fractions can still retain

unsafe contaminants and represent a potential risk for consumers' health (7, 64). Furthermore, it is important to consider that the energy needed to break apart the wheat kernels can generate considerable amount of heat, hence moisture condensation is likely to occur in the break rolls, sifters and reduction rolls, which may lead to build-up of flour residues which supports bacterial growth (7). Thus, the final microbiological quality of wheat flour can be greatly influenced by these residues if they are not properly controlled.

MICROBIOLOGICAL QUALITY AND SAFETY OF WHEAT FLOUR

The cleaning and grinding procedures have minimal or no direct impact on the level of contamination present in wheat; therefore, the initial quality of the grain have a strong influence on the ultimate safety and quality of milling end products. Thus, the majority of microorganisms, including pathogenic and spoilage microorganisms, and mycotoxins originally present on the wheat might be expected to be present in the milled products (84).

Spoilage microorganisms. As in the original wheat grain, yeast and mold are among the common spoilage microorganisms found in flour. A survey by Seiler (79) revealed that the mold counts in flour (3.7 log CFU/g) were only about 10 times less than in the corresponding wheat (4.3 log CFU/g). Richter et al. (64) provided a comprehensive microbiological profile for more than 4,000 commercial wheat flours of four different types collected throughout the United States. On average, flour samples had counts of 2.1 log CFU/g and 2.9 log CFU/g for yeast and mold, respectively. More recently, Manthey et al. (44) evaluated the microbiological quality of 219 wheat flour samples produced from the 2001 durum wheat crop in the United States. The mean yeast-mold counts for these flour samples were 2.2 log CFU/g. A survey by Sperber et al. (84) analyzed more than 6,500 flour samples from the period 2003 through 2005, which represent the most recent microbiological profile of North American wheat flour (whole-wheat flour and durum flour). A mean yeast counts of 1.3 log CFU/g was observed for 6,573 flour samples analyzed, and a mean mold counts of 2.4 log CFU/g was observed for 6,869 flour samples. The

maximum count for both microorganisms was 5.9 log CFU/g. A survey by Berghofer et al. (7) in the late 90s, showed the microbiological status of freshly milled Australian wheat flour. The yeast and mold counts in the flour samples ranged from 2.0 to 3.0 log CFU/g, being 2.0 log CFU/g the most frequent count. Eglezos (22) reported higher fungal counts than the previous Australian survey. The mean count in flour samples were 3.0 log CFU/g and 2.8 log CFU/g for yeast and mold, respectively. A survey by Aydin et al. (5) found mean mold counts of 2.2 log CFU/g in 142 Turkish flour samples. Total mold counts obtained from retail wheat flour samples in Spain ranged from 1 to 3.2 log CFU/g (10). Weidenbörner et al. (98) reported total fungal counts of 3.2 and 3.3 log CFU/g in white and whole-grain German flour, respectively. Considerably higher levels of yeast and mold in the order of 3.7 to 5.3 log CFU/g, respectively, have been reported in Pakistani flour (88).

In addition to fungi, a wide variety of spoilage bacteria are also present in flour. Seiler (79) also reported that bacterial count in flour was, on average, 1.9 log CFU/g lower than the initial level present in the wheat (6.4 log CFU/g). In an earlier study, a mean aerobic bacterial count of 4.9 log CFU/g was observed in wheat before milling, while the flour had a mean aerobic bacterial count of 3.6 log CFU/g (65). In the survey carried out by Richter et al. (64), flour samples presented a mean count of 4.2 log CFU/g for aerobic bacteria. Moreover, Manthey et al. (44) reported aerobic bacterial counts in the order of 5.7 log CFU/g for durum wheat flour. A mean aerobic plate count of 3.7 log CFU/g with a maximum of 6.9 log CFU/g were reported in 6,598 flour samples analyzed in North America by Sperber et al. (84). In a recent Australian survey, Eglezos (22) reported higher aerobic bacterial counts than both the previous Australian survey and the North American survey, with a mean aerobic bacterial count of 4.2 log CFU/g. Besides yeast and mold, Berghofer et al. (7) also detected a wide range of other spoilage microorganisms in freshly milled flour. Aerobic mesophilic and thermophilic bacteria counts ranged from 1.0 to 7.0 log CFU/g and from 1.0 to 2.0 log CFU/g, respectively. In addition, *Bacillus* spp. and mesophilic aerobic spores' counts ranged from 2.0 to 5.0 log CFU/g and from

non-detectable to 3.0 log CFU/g, respectively. More recently, in a Turkish study, high levels of aerobic bacteria were found in 32 of 142 wheat flour samples analyzed (5). The aerobic bacterial counts in these flour samples ranged from 5.0 to 7.2 log CFU/g. In the same set of samples from Turkey, rope spore counts ranged from 30 to 4500 MPN/g in 62 flour samples (5).

Pathogenic and fecal microorganisms. Several microbiological surveys of wheat flour have also indicated the presence of pathogenic and fecal microorganisms, including *Escherichia coli*, *Salmonella* spp., *Bacillus cereus* and coliforms. A survey carried out during the period 1984 through 1991 in North America revealed that 1.05% of the 4,210 samples of wheat flour analyzed were contaminated with *Salmonella*, and 12.8% of the 3,350 samples of wheat flour analyzed were *E. coli* positive (64, 84). In contrast, the prevalence of *Salmonella* in 4,358 samples of wheat flour analyzed during the period 2003 through 2005 was significantly lower (0.14%) than the earlier reported incidence in 1984-1991 (84). The same study in 2003-2005 also reported mean counts of 0.7 log CFU/g for *E. coli* (petrifilm) in 2,921 samples analyzed, up to a maximum of 3.0 log CFU/g. Berghofer et al. (7) detected *E. coli* in 1 out of 72 flour sample analyzed at the level of 9 MPN/g, while in another Australian survey 2 out of 300 flour samples were *E. coli* positive (22). However, high levels of contamination with *E. coli*, 72 out of 142 flour samples contained more than 9 MNP/g, have been reported in Turkey (5). Victor et al. (55) found *E. coli* at levels of 3.8 and 4.1 log CFU/g in white and whole-grain flour, respectively, produced in Lesotho. A number of coliforms species are also part of the microbial flora found in wheat flour. In an earlier study, Spicher (85) reported mean coliform count of 2.0 log CFU/g in German flour. Richter et al. (64) reported mean coliform count of 1.2 MPN/g in 1,477 samples analyzed in the first comprehensive microbiological survey of North American wheat flour carried out in 1989. More recently, slightly higher levels of coliform (1.6 MPN/g) have been reported by another North American (84). Coliform counts reported in Australian flour varied from 1 to 1,000 MPN/g, being the most frequent count 1 MPN/g (7). In a recent study, coliform counts ranged from 3.0 to 4.0 log CFU/g in Pakistan flour (88). Victor et al. (55) also detected coliforms in

white and whole-grain flour produced in Lesotho, at levels of 3.4 and 3.7 log CFU/g, respectively.

Regarding sporeformers, Berghofer et al. (7) reported that 93% of 81 flour samples analyzed in Australia tested positive for *B. cereus*, although the most frequent count was 0.1 MPN/g. In the Turkish survey, only 6 out of 142 flour samples contained *B. cereus* at levels of more than 2.0 log CFU/g (5). *B. cereus* was not isolated in any of the flour samples analyzed in the 2006 Australian survey, and it is not mentioned in the North American surveys. Aydin et al. (5) was the only report to mention with an occurrence of *Clostridium perfringens*, in 14 out of 142 flour samples at levels above the detection limit of 2.0 log CFU/g.

Mycotoxin-producing molds and mycotoxins. The mycological profile of retail wheat flour is somewhat similar among reports found in the literature. *Aspergillus* and *Penicillium* species (storage fungi) have been recorded among the most prevalent ones in wheat flour by many authors. Riba et al. (63) evaluated the mycoflora of Algerian soft and durum wheat flour, especially for the presence of *Aspergillus* strains and its potential for ochratoxin A production. Results showed that fungi of the genus *Aspergillus* prevailed (95%) in soft wheat flour, while in durum flour *Penicillium* (43%) was as abundant as *Aspergillus* (57%). High frequency of ochratoxigenic species of *Aspergillus* was detected in all flour samples, being *A. candidus* the most predominant specie in both soft flour (47%) and durum flour (31%). Similar results were reported by Weidenböner et al. (98) in commercial samples of white and whole-grain flour in Germany. The mycobiota of whole-grain and white flour was dominated by *Aspergillus* spp. accounting for 84% and 77.3% of the isolations, respectively. *A. candidus* was also the most frequently encountered mold. From the 3,325 isolations, 93.3% belonged to 32 fungal species that are well known for their mycotoxin producing potential. Likewise, the most common genera isolated in 50 retail wheat flour samples from Saudi Arabia were *Aspergillus* (70%) and *Penicillium* (30%) (26). Cabañas et al. (10) have also shown that retail wheat flour in Spain have *Aspergillus* spp. and *Penicillium* spp. species as the predominant fungi. The most frequent

isolated species by this group were *A. flavus*, *A. candidus*, *A. versicolor*, and *Penicillium verrucosum*. However, a survey of the fungal and mycotoxin contamination in South African wheat products revealed that the major fungal contamination was *Fusarium* spp. (field fungi) and their related mycotoxins (47). Berghofer et al. (7) compared the fungal genera present on wheat before and after milling and found that field fungi such as *Fusarium*, *Alternaria*, and *Cladosporium* were usually detected in lower numbers in milling end products than on incoming wheat, while the storage fungi *Aspergillus* and *Penicillium* prevailed in all milled fractions. Therefore, mold genera entering the milling system are mostly comprised by storage fungi, which is consistent with the abovementioned mycological profile of retail wheat flour.

The majority of fungal species encountered on wheat flour are well known for their mycotoxin producing potential; therefore, improper conditions during wheat flour storage (e.g., high moisture levels and temperature) may promote spore germination, mold growth and mycotoxin production (98). However, mycotoxin contamination in wheat flour generally comes from infected kernels during the growing season or during storage. The incidence of several mycotoxins, including DON, ZEA, OTA and T-2 in commercial wheat flour has been reported in numerous studies. According to a study carried out in southwest Germany (74) in 1998, the incidence of DON for white flour (n=134) and whole-grain flour (n=77) were 78% and 66%, with contents ranging from 15 to 624 µg/Kg and from 15 to 1670 µg/Kg, respectively. Likewise, a follow-up study on the occurrence of DON in 1999 from the same German region showed again high frequency of occurrence of DON. Almost all (98%) 60 wheat flour samples, characterized by different ash contents, evaluated showed DON in concentrations ranging from 15 to 1379 µg/Kg (73). Also, it was noticed that the overall degree of toxin contamination tended to be lower with decreasing ash content. Abdulkadar et al. (2) analyzed 106 food products in Qatar and found 2 wheat flour samples contaminated by DON at levels ranging from 148.22 to 182.94 µg/Kg. In a survey of the occurrence of mycotoxins in wheat flour collected from the Serbian markets, results

showed that the most prevalent mycotoxin was DON (86.7%), with an average value of 325 µg/Kg (81).

Concerning the occurrence of ZEA and OTA, it has been shown that their levels are rarely high. A survey done by Kumagaia et al. (69) analyzed 50 retail wheat flour samples in Japan, where 28 samples were contaminated with OTA at levels ranging from 0.10 to 0.48 µg/kg. Nonetheless, Riba et al. (63) detected OTA in one Algerian flour sample at high concentration of 41.55 µg/Kg. Schollenberger et al. (73) encountered ZEA in German flour at levels ranging from 1 to 24 µg/Kg; while Skrbic et al. (81) reported an average value for this toxin of 4.6 µg/Kg in Serbian flour. A survey of cereal flour (including wheat flour) collected from local stores in Valencia, Spain revealed that 1 out of 21 samples tested positive for ZEA; however the sample presented levels below the maximum limit established by the European Commission (19, 20, 59). T-2 and HT-2 *Fusarium* toxins have also been detected in wheat flour sold in traditional markets. Kassim et al. (36) evaluated 15 wheat/wheat powder samples for the presence of T-2 and HT-2 toxins in South Korea, and found T-2 toxin in 3 samples with levels ranging from 65.2 to 431.0 µg/kg, while HT-2 was found at the high level of 355.3 µg/kg. According to these studies, for the most part consumers worldwide are exposed to low but persistent levels of multiple mycotoxins in commercial wheat flour. Nevertheless, samples of wheat flour that exceeded the maximum permitted level for most of these mycotoxins established by regulatory agencies were also reported.

EFFECTS OF BAKERY AND PASTA PROCESSING ON THE MICROBIOLOGICAL QUALITY AND SAFETY OF FLOUR-BASED FOODS

The methods commonly used to produce flour-based products generally involve the application of heat, and includes baking, frying, cooking, steaming and extrusion. However, nowadays there is an increased use of flour in a non-conventional manner to produce refrigerated and frozen products, which may not include a validated processing step to ensure safety. For

those processes that include a thermal treatment, the product achieves temperatures lethal to vegetative cells, effectively reducing the number of spoilage and pathogenic microorganisms that may be present. However, bacterial spores of the genus *Bacillus* or related genera are highly resistant to thermal destruction, thus they may survive and multiply during the cooling step. Furthermore, some mycotoxins have proven to be highly stable to heat, thereby also are not affected by the production processes. Therefore, regarding thermally processed products, in terms of food safety, both bacterial spores and mycotoxins represent a potential threat to consumers' health that must be considered.

Bakery processing. The main stages involved in the bread making process are fermentation, proofing, and baking. The effects of the baking fermentation step on mycotoxin concentration are rather contradictory, some studies have reported that fermentation of wheat flour dough reduced detectable levels of DON (40, 70, 75), while others showed an increase or no change in DON content (6, 38). During dough proofing, some authors reported an increased in DON levels (95). Regarding baking, DON content in some products like Egyptian flat bread have shown to be stable, withstanding baking temperatures (23); while the baking of regular bread provided reductions of 24-71% and a 35% reduction in biscuits and cookies (75). Kostelanska et al. (38) reported that DON content in the breadcrumbs barely decreases, whereas degradation of DON content take place mostly in the bread crust as it reaches the highest temperature. OTA have shown to be stable during bread making, with no apparent reduction (76, 95). However, Subirade (86) demonstrated that baking of biscuits resulted in the destruction or immobilization of about two-thirds of the toxin. Studies on heat stability have demonstrated that ZEA can also resist thermal treatments. Cano-Sancho et al. (11) did not find any reduction on ZEA during bread making, neither after fermentation nor after baking. Although some published studies indicate a slight reduction in some mycotoxins, the extent of the reduction in the final product may depend on fermentation and baking temperatures, baking times, loaf size, and the type of mycotoxin (95). In addition, it is important to consider that mycotoxin concentration in

the starting flour may be diluted when flour is mixed with other ingredients during processing; consequently, mycotoxins levels in finished products are usually lower than in the starting flour (76, 94).

Concerning the microbiological safety of baked products, spore-forming bacteria of the genus *Bacillus* or *Clostridium* are the major organisms responsible for the spoilage (ropiness) and poisoning of baked goods. Wheat and flour are usually the sources of heat resistant *Bacillus* spores (58, 67). Studies have shown that multiplication of vegetative cells of *Bacillus* spp. does not occur in the dough before baking (66), although some spores may germinate (25). Heat-resistant *Bacillus* spores can survive the baking process, mostly in the center of the crumb, where the temperature reaches up to 97–101°C for a few minutes (93). During prolonged cooling or improper storage, favorable conditions to spore germination may develop. When spore germination occurs, vegetative cells not only cause rope spoilage of bread but also cause food poisoning through the production of toxins.

Pasta processing. Unlike bakery processing, extrusion employs high heat, high pressure and mechanical shear simultaneously. It is also notable that the extrusion process for pasta products may lead to a reduction in some mycotoxins. Several studies have reported reduction in DON content in pasta or noodles ranging between 40% and 70% (54, 87). Although, Scudamore et al. (78) reported minimal effect on DON in wheat flour during simple extrusion. Processing semolina into spaghetti did not significantly alter DON concentrations (44). The addition of additives may also play a significant role in reduction of mycotoxins during extrusion as 62% reduction of DON was observed in the presence of sodium bisulfate (3). DON levels may also be reduced in pasta or noodles during cooking because of leaching into the boiling water, since DON is water-soluble (54, 87, 97). However, around 53% of DON content was retained in the noodles after cooking (54). Regarding ZEA, extrusion cooking of wheat flour at 120-140°C resulted in 73-83% reduction of ZEA content, the highest reduction was achieved when shear stress was applied (68). Both temperature and initial moisture content seems to play an important role in

reducing OTA content during extrusion cooking of wheat whole-meal, since 11-39% reduction rate of OTA was achieved at 30% moisture and 116-136°C, while only 8-33% reduction rate occurred at 17.5% moisture content (77).

The efficacy of extrusion in reducing microbial load when processing semolina into spaghetti has been evaluated (44). Semolina with a moisture content of 32% was extruded as spaghetti at 45°C and 25 rpm screw speed, and then the spaghetti was dried at 40°C. The starting semolina and the dried spaghetti were analyzed for microbial load. Reduction in aerobic mesophilic bacterial population ranged from 2.2 to 4.1 log CFU/g compared to semolina, whereas the yeast-mold population was reduced 0.1 to 1.7 log CFU/g from those for semolina. It is interesting that fungal populations were reduced in a lesser degree than the aerobic mesophilic bacterial flora, indicating that this group of microorganisms might be more resistant during spaghetti processing. The presence of these microorganisms in the finished product may lead to quality and safety problems.

POTENTIAL SAFETY RISKS ASSOCIATED TO FLOUR-BASED FOODS

Although historically regarded as a low-risk commodity for microbial contamination, raw flour and finished flour-based products have been implicated in foodborne disease outbreaks over the last few years. In baked or extruded goods, incidents where consumer health has been compromised are usually related to, but not entirely, germination of bacterial spores that have survived normal processing conditions. Whereas food-safety incidents associated with ready-to-bake type products appear to be related to the breach of cooking instructions and consumer eating habits. In addition to microbiologically related incidents, consumer health has also been threatened by mycotoxin contamination. Food safety incidents related to mycotoxin involving baked goods have been previously documented.

Processed and ready-to-eat products. Reports of food poisoning associated with spore-forming bacteria and involving extruded products have been documented. *Bacillus cereus* is

probably the leading cause of illnesses caused by foods of plant origin that have undergone heat treatments; however, infection with this organism is not commonly reported because of its usually mild symptoms (28). Nevertheless, a more severe form of the emetic type of foodborne illness associated with the consumption of spaghetti has been reported (43). More recently, Dierick et al. (18) described a fatal case of liver failure due to emetic toxin contamination in pasta salad. Moreover, bakery products have also been implicated in food safety incidents involving *Salmonella* spp., but due to transfers from production workers or food handlers rather than contamination of the product itself (37). Research has shown that, *Salmonella* spp. can survive for more than one year in dried pasta, which can represent a potential safety and public health hazard if the pasta is not properly cooked (117).

Mycotoxin contamination in finished bakery products has been implicated in foodborne gastroenteritis outbreaks in several countries. Bhat et al. (8) reported an outbreak of trichothecenes mycotoxicosis in India associated with consumption of bread made from mold damaged wheat. In China, foods made from wheat highly contaminated with DON and ZEA were linked to an outbreak that involved 130,000 people with gastrointestinal disorders (41). Moreover, several publications devoted solely to surveys of mycotoxins in wheat-based products, especially breakfast cereals, have reported low but persistent levels of multiple mycotoxins in these products sold directly to the public (46, 47, 69, 91, 99, 110).

Raw or ready-to-bake products. Within this product category are included: refrigerated cookie, pastry, and biscuit dough; frozen pizzas and pies; cake, brownie and many other bakery mixes. Unlike processed foods, these ready-to-bake type products have not undergone any validated kill step during processing to ensure that microbial food safety hazards are minimized. Therefore, these types of products represent a substantial safety challenge; since they rely on the consumers to perform the kill step. Moreover, a recent nationwide survey of 1,032 consumers in the United State revealed that many of them have eaten raw bakery goods or without fully cooking them as directed by the manufacture (13). The same survey indicated that 58% and 67%

of consumers, respectively, have tasted refrigerated store-bought cookie dough and raw homemade dough before they have been baked, and another 11% and 24% admitted to having eaten pizza dough and biscuit dough before it is fully cooked.

Bacterial contaminants cannot grow and multiply in dry flour because of the small amount of available water (7). Nevertheless, these microorganisms survive in a dormant state, retaining their viability and the potential to multiply if flour is incorporated to a more receptive environment for microbial growth, such as batter or mixes (13, 22). However, some pathogenic bacteria such as *Salmonella* spp. and *E. coli* do not need to grow to cause illness, since they require only a small number of cells (infective dose) to begin an infection (72). When such risky eating practices are associated with a potential microbial contamination of raw flour, ready-to-bake products can serve as a vehicle of serious and life threatening foodborne illness. Indeed, in recent years, wheat flour has been the “suspected agent” in several food safety incidents.

The first incident where consumer health was compromised due to a flour-based food occurred in Australia in 1952. Among the ingredients, flour was the suspected carrier agent of *Salmonella*, but the organism was not isolated (14). In 2005, 25 people in the United State became ill with salmonellosis due to cake batter ice cream. In this case, a dry cake mix was the suspected agent, but flour was not the ingredient of contamination (90). However, The Federal and Drug Administration issued a warning that dry cake mix should not be considered ready-to-eat unless has been treated to ensure safety. The first confirmed involvement of flour as a vehicle for transmission of pathogens occurred in 2008 in New Zealand (22). Sixty-six people became ill with salmonellosis, of which 8 were hospitalized. The investigation revealed that they had eaten uncooked flour, raw cake and had licked beaters after mixing batter. Nevertheless, *Salmonella* is not the only pathogen that has been related to an outbreak caused by a product containing flour. The last incident, in 2009, was caused by Shiga toxin-producing *Escherichia coli* (STEC) infection. This was the first reported STEC outbreak associated with consumption of ready-to-bake commercial pre-packaged cookie dough made with wheat flour, where 77 people in the

United States were sickened (35). The investigation did not conclusively implicate flour, but it remains the prime suspect for introducing the pathogen to the product. Over 3.6 million packages of cookie dough had to be withdrawn in this incident (35).

CONCLUDING REMARKS

Wheat from which flour is obtained is a raw agricultural commodity that is subject to naturally-occurring microbiological threats, therefore it is the primary contamination source for milled products. Physical processes carried out before and during milling influence the level of contamination present originally in the wheat. The milling process may reduce the concentration of mycotoxins and microorganisms in fractions commonly used for human consumption, but has the drawback of concentrating these contaminants into fractions that are becoming more widely consumed for their nutritional value, thus creating an emerging food safety challenge. Since none of the operations carried out during milling includes chemical or thermal treatments to ensure safety, milling end products should not be considered safe for consumption without further processing. Microbial contaminants that may have passed into flour during milling can survive in a dormant state, retaining their viability and the potential to multiply; therefore, foods containing raw flour (not heat-treated) should be considered as possible vehicles of infection. The thermal treatments applied to bakery goods or pasta products simultaneously reduces the microbial load and moisture content, thereby limiting the types of microorganisms that can grow and cause safety problems. However, minimal impact on mycotoxin levels occurs during such food processing operations; therefore, many wheat-based foods such as breads, pastas, and breakfast cereals, may contain at least trace amounts of mycotoxins.

Unlike thermally processed foods, refrigerated dough products can represent a substantial safety hazard since eating uncooked dough appears to be a popular practice among consumers. With increased use of flour in refrigerated and frozen products, even the low incidence of

pathogen contamination in wheat flour should not be ignored; instead, it should warrant the application of further treatments to make flour safer for human consumption. Relying only on consumers' education about the health risks associated with eating bites of raw dough may not guarantee the absence of food safety incidents. Therefore, even though label statements warn them against the danger of such risky eating practices, manufacturers should implement the use of heat-treated flour in ready-to-cook or ready-to-bake products to make them as safe as ready-to-eat products (35). Moreover, to ensure safety several control procedures should be used to reduce microbial load and mycotoxins in flour and flour-based products. These include the prevention of microbial establishment and growth within the mill and throughout the production chain through proper sanitation of production equipment, stringent temperature control during baking, testing of incoming wheat and the resulting flour, and hygienic packaging and shipping procedures.

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EFFECTS OF WEATHER VARIABLES ON THE MICROBIOLOGICAL QUALITY
OF HARD RED WINTER WHEAT *

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ABSTRACT

The meteorological conditions in farm fields during grain ripening have shown to have a strong influence on microbial development in wheat grain. In this study the effects of weather variables on the microbiological quality of wheat grain were evaluated over two consecutive seasons in the state of Nebraska, USA. Three hard red winter wheat cultivars were planted in three meteorologically contrasting regions to ensure developing seeds were exposed to different weather conditions. The natural microbial flora, mycotoxin, moisture content, and water activity of 54 freshly harvested wheat samples were analyzed and correlated with the weather conditions that prevailed from flowering to harvesting. In both growing seasons, localized areas of high rainfall occurred in the Southeastern district, while low precipitation rates were registered in the Panhandle district. There was considerable variation in the microbial load of the wheat harvested as the planting site moved from the western to the eastern part of the state. For the 2012 season, Aerobic plate count (APC), *Enterobacteriaceae* counts (Eb), yeast and mold counts, and internal mold infection (IMI) were lowest in grain samples collected from Panhandle and highest in grain from the South Central and Southeastern districts. For the 2013 season, there were no significant differences in the yeast counts found in grain collected from different districts, but levels of IMI and mold counts differed significantly between samples from the Southeastern district and the Panhandle district. Deoxynivalenol (DON) was detected in all districts, however, in very low concentrations. Among the climatic variables, relative humidity positively correlated with APC, Eb, yeasts, molds, and IMI. Molds counts were also positively correlated with mean minimum temperatures. A strong positive correlation was found between precipitation levels and the moisture content of the grain. In general, microbial load in wheat grain tended to decrease in those areas with lower relative humidity and lower maximum temperatures.

INTRODUCTION

The prevailing environmental conditions during the growing season not only determine how quickly or slowly the wheat plant develops but also the amount and type of microbial flora that might be present. Once the grain head emerges from the flag leaf sheath, it is exposed to multiple sources of contamination, including air, dust, water, insects, and animal feces; therefore, there is ample opportunity for microbes to colonize the developing grain. Thus, wheat grain may harbor a large and varied microflora that includes bacteria, yeasts, and molds (3, 5).

The availability of water is one of the most important factors driving the dynamics of these microbial populations. Therefore, weather may influence the rate of microbial colonization of grain. Under favorable conditions, bacteria begin to colonize the ripening grain, closely followed by yeasts and filamentous fungi (12). However, the colonization of grain by individual groups of microorganisms is highly variable and depends on the prevailing climatic conditions. Thus, the dominant species in warmer climates may differ from those in colder climates (12). Weather related variables, such as daily average relative humidity and total daily precipitation have been positively correlated with mold infection and mycotoxin production (7, 11, 14). The intensity of fungal diseases in wheat, such as *Fusarium* head blight (FHB) produced primarily by *Fusarium graminearum* Schwabe, is mainly determined by meteorological conditions (8, 19, 28). Indeed, particular weather conditions have been associated with some phases of the FHB disease cycle. For instance, maturation of perithecia in infested wheat is favored by prolonged periods of warm temperatures and high moisture or relative humidity (9, 10). In addition, the formation and density of ascospores on wheat spikes has been associated with high rain intensity and moderate warm temperatures (19). Besides the economic importance of FHB and other fungal diseases, colonization of the wheat spikes by fungal species also represents a food safety concern due to the production of mycotoxins. Moreover, statistical correlations between patterns of solar activity and microbiological quality of cereals have been reported (17). Weather patterns in the winter wheat-

producing regions in the High Plains of the United States have experienced cycles of drought and flooding, and extreme temperatures over the last decades. These fluctuations in environmental factors have likely made a significant impact on the amount and type of microflora associated with harvested wheat crops (24, 25).

There is a substantial amount of research that has associated specific environmental conditions in farm fields with fungal diseases development in wheat. However, much less effort have been directed towards evaluating the relationship between weather variables and overall microbiological quality of wheat grain. To fill this knowledge gap, the climatic conditions prevailing from flowering to harvesting of three winter wheat cultivars (*Triticum aestivum* L.) at three locations along a west-east transect of the state of Nebraska, USA, were evaluated to determine its impact on microbial load, mycotoxin, moisture content, and water activity of harvested wheat grain. In addition, the environmental variables most associated with annual fluctuation of microbial load in wheat were determined.

MATERIALS AND METHODS

Winter wheat variety trial locations. The University of Nebraska – Lincoln divides the state of Nebraska into five districts for the purpose of wheat variety testing (Fig. 1). The trials described in this study were conducted in the southeastern and western part of the state, since weather conditions change significantly from east to west. Three hard red winter wheat cultivars, two commercial lines (Overland and McGill) and one experimental line (NW07505), were planted in the Southeast district at Saunders County, South Central district at Clay Center County, and Panhandle district at Box Butte County, during two growing seasons, 2011-2012 and 2012-2013. Wheat cultivars were planted in a single day at each location, but planting dates varied among districts (Table 1). The sowing followed a randomized complete block design with three replications. Each plot was 15 ft x 35 ft with six rows. Plants were grown under rainfed conditions and the plots were well fertilized before sowing.

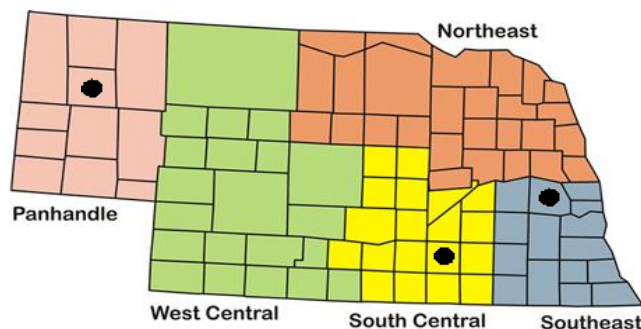


FIGURE 1. Winter wheat-producing districts in the State of Nebraska, United States. ● Study locations.

Sample collection. Wheat samples were collected over the 2011-2012 and 2012-2013 growing seasons. The Department of Agronomy and Horticulture at University of Nebraska-Lincoln, NE, USA, randomly collected samples (~2.0 kg) directly from the planting site during harvest, reflecting the condition of the grain at the point of origin. Wheat cultivars were harvested in a single day at each location, but harvesting dates varied among districts (Table 1). Three samples were collected for each wheat cultivar, with a total of 9 samples collected during harvest from each district. In total, 27 samples for each growing season were collected.

Meteorological data. Climate data throughout each growing season were obtained from the Automated Weather Data Network (AWDN) of the High Plains Regional Climate Center (HPRCC) for each location. Weather variables monitored included maximum and minimum daily air temperature, daily total precipitation, and daily relative humidity. In this study, only the weather conditions prevailing from flowering to harvesting were considered, since it is when the grain is exposed to the environment.

The flowering dates of the winter wheat cultivars were estimated based on the amount of heat energy that plants had received over the growing season, known as growing degree days (GDD) concept. The lower developmental threshold or base temperature and upper temperature threshold were set to 32°F and 86°F, respectively (16). The starting point for the accumulation of GDD was the seeding date. The basic equation used was:

$$\text{GDD} = \Sigma [(\text{daily maximum temperature} + \text{daily minimum temperature})/2] - \text{base temperature}$$

Because wheat does not grow significantly when temperatures are below freezing, GDD was considered to be zero if (daily maximum temperature + daily minimum temperature)/2 was less than 32°F. In addition, if (daily maximum temperature + daily minimum temperature)/2 was greater than 86°F, GDD was considered to be equal to 86°F, the upper temperature threshold.

Moisture and water activity analysis. The moisture content of all wheat samples was determined using the Approved Method 44-15A (AACC International 2002), and the water activity was measured using an Aqualab® S-3 TE water activity meter (Decagon, Pullman, Washington, USA).

Microbial and mycotoxin analysis. Microbial and mycotoxin analysis were conducted on all samples collected in both growing seasons. Wheat samples were analyzed for mesophilic aerobic bacteria (Aerobic Plate Count; APC), yeasts, molds, coliform, generic *E. coli*, and *Enterobacteriaceae* (Eb). In addition, samples were also examined for the presence of *Salmonella* spp. and *E. coli* O157:H7. Both APC and yeast/mold counts were determined by the spread plating technique according to standard methods described in the U.S. Food and Drug Administration's *Bacteriological Analytical Manual* (15, 26). The aerobic bacteria plates were incubated at 35 °C for 48 h, and the yeast/mold plates were incubated at 25 °C in the dark for 5 days. Coliform/*E. coli* and *Enterobacteriaceae* counts were determined according to AOAC official methods 991.14 and 2003.01, respectively, using Petrifilm™ (3M Microbiology, St. Paul, MN). For *Salmonella* spp., samples were inoculated in enrichment medium (Buffered Peptone Water) and incubated at 37 °C for 24 h. After incubation, 1 ml of sample was transferred to 9 ml of selective enrichment medium (Tetrathionate Broth, Acumedia) and incubated at 41 °C for 24 h. For *E. coli* O157:H7, samples were directly inoculated in selective enrichment medium (Modified Tryptone Soya Broth with Novobiocin, Acumedia) and incubated at 37 °C for 24 h. The presence or absence of *Salmonella* spp. and *E. coli* O157:H7 was determined using RapidChek™ (Romer

Labs) and Neogen Reveal™ (Neogen Corporation, Lansing, MI) lateral flow ELISA tests, respectively. Internal Mold Infection (IMI) and Internal *Fusarium* Infection (IFI) were determined by first randomly selecting 50 kernels from each sample, which were then surface disinfected and aseptically plated as described by Manthey et al. (13), with the exception that dichloran rose bengal chloramphenicol (DRBC) agar and czapek-dox iprodione dichloran (CZID) agar were used to determine IMI and IFI, respectively. Plates were incubated for 5 days, without light at room temperature. Deoxynivalenol (DON) concentration was determined using Neogen Veratox® 2/3 (Neogen Corporation, Lansing, MI) competitive direct ELISA test, following the USDA-GIPSA method for sample preparation and extraction (27).

Data analysis. Data was analyzed using the Statistical Analysis System SAS; (version 9.3, SAS Institute, Cary, NC, USA) where a general linear model of variance at the 5% level followed by Tukey's test was used to determine significant differences between cultivars, microbial load, and growing seasons. The correlation between weather variables and microbial load was determined based on Pearson's correlation coefficients, which were also obtained using SAS.

RESULTS AND DISCUSSION

There was a large variation in environmental conditions between the two growing seasons. Warmer than normal temperatures and low precipitation rates occurred during the 2011-2012 growing season, leading plants to develop at a greater rate and reach maturity 20-21 days earlier in 2012 as compared with 2013 (Tables 1 and 2). In contrast, the 2012-2013 growing season was dominated by moist and cool conditions, which resulted in a longer growing period and disease incidence above normal. The comparison between the precipitation levels between districts and growing seasons is illustrated in Figure 2. Despite weather differences between growing seasons, both years show localized areas of high rainfall in the Southeastern district, while lower precipitation rates were confined to the Panhandle district.

TABLE 1. Name, latitude, and longitude of study locations, and sowing, flowering and harvesting dates during both growing seasons at each location.

District	Farm Location			Season 2011-2012			Season 2012-2013		
	Location ^a	Longitude	Latitude	Sowing	Flowering	Harvesting	Sowing	Flowering	Harvesting
Southeast	Ithica	41.161	-96.414	Sept 26 th , 2011	May 17 th , 2012	June 28 th , 2012	Sept 25 th , 2012	June 18 th , 2013	July 18 th , 2013
South Central	Harvard	40.576	-98.134	Sept 23 th , 2011	May 17 th , 2012	June 22 th , 2012	Sept 19 th , 2012	June 14 th , 2013	July 09 th , 2013
Panhandle	Hemingford	42.249	-103.01	Sept 20 th , 2011	June 10 th , 2012	July 04 th , 2012	Sept 17 th , 2012	June 25 th , 2013	July 23 th , 2013

^a Ithica, NE (UNL ARDC) located in Saunders county; Harvard, NE (South Central Res. & Ext. Center) located in Clay Center county; Hemingford, NE (Cullan Farms) located in Box Butte county.

Phenological changes in wheat plants are governed mainly by temperature (29), thus plant growth and flowering depend on the accumulated temperature over the growing season (20). The accumulated GDD were used to estimate the date of the flowering stage of winter wheat cultivars according to the Zadoks scale (30). Previous studies that evaluated the amount of energy that wheat requires to progress through the different growth stages in the High Plains region have determine that from planting to flowering (Zadoks stage 69) approximately 3200 GDD are required (16). Therefore, the date of wheat flowering was estimated for each field by assuming that cultivars have the same base temperature for growth and development, and that growth response to temperature was the same among cultivars (Table 1).

The flowering date of winter wheat is mainly a function of spring temperatures but may also be affected by other environmental conditions (29). Earlier than usual flowering dates were observed in 2012 due to warmer temperatures and wetter conditions during spring and summer in the region (Table 1 and 2). In addition to differences observed on amount of rainfall amount between both growing seasons, the distribution of the rainfall throughout the growing season was also different between years. Higher precipitation levels from wheat flowering to harvesting were observed in the 2011-2012 season, with about 10 to 33 percent of the total rainfall occurring during this period (Table 2). In contrast, only 3 to 7 percent of the total rainfall occurred in the same period during the 2012-2013 season. However, lower mean temperatures and lower relative humidity from wheat flowering to harvesting were registered in the 2011-2012 season when compared with the 2012-2013 season.

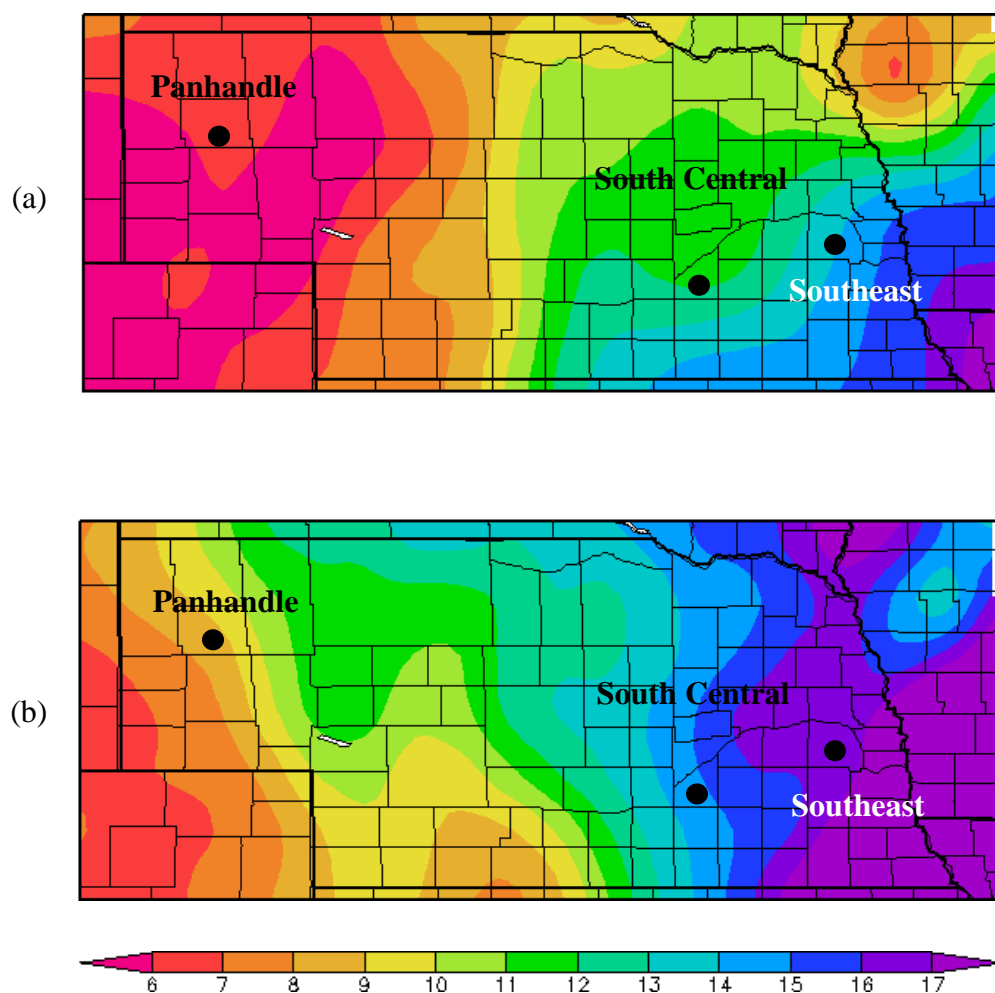


FIGURE 2. 2011-2012 (a) and 2012-2013 (b) growing season precipitation levels (in) for Nebraska. • Study locations.

TABLE 2. Average high and low temperature (T-Max, T-Min), relative humidity (RH), and total precipitation (Precip) distribution during the two growing seasons at each district.

Season	District	Throughout the Growing Season				From Flowering to Harvest			
		Precip (in)	T-Max (°F)	T-Min (°F)	RH (%)	Precip (in)	T-Max (°F)	T-Min (°F)	RH (%)
2011- 2012	Southeast	14.79	60.69	34.45	64.24	4.87	83.86	58.24	60.92
	South Central	12.87	60.99	33.19	65.75	3.95	83.45	56.71	61.51
	Panhandle	7.34	60.19	28.92	54.96	0.75	91.12	56.00	42.96
2012- 2013	Southeast	17.80	56.59	32.85	69.99	1.28	87.74	64.53	66.67
	South Central	16.50	56.61	31.37	69.42	0.53	88.42	63.34	62.93
	Panhandle	8.48	57.91	28.36	61.04	0.49	88.67	56.69	55.45

Table 3 summarizes the microbial analysis of winter wheat samples for the 2011-2012 growing season. According to the results, there was considerable variation in the microbial load of the wheat as the planting site moved from the western to the eastern part of the state. Mean TPC, Eb, yeasts, molds, and IMI were lowest in grain samples collected from Panhandle and highest in grain collected from the South Central and Southeastern districts. Coliform and *E. coli* were not detected in samples collected from the Panhandle district, but low levels were found in samples from the Southeastern and South Central districts. *Salmonella* spp. and *E. coli* O157:H7 were not detected in any of the samples analyzed. Levels of IFI did not differ among districts.

In general, there was no statistical difference in the microbial load found among cultivars in each location, indicating that microbiological quality is more dependent on weather conditions than wheat varieties. Local weather conditions prevailing from flowering to harvesting in South Central and Southeastern districts were characterized by high precipitation levels, high relative humidity and cooler temperatures. In contrast, post-flowering weather conditions in Panhandle districts were dominated by low precipitation rates and relative humidity, and warmer temperatures. Mean relative humidity during the post-flowering period in South Central and Southeastern districts was approximately 61% compared with 43% in Panhandle district (Table 2).

TABLE 3. Mean and standard deviation (SD) for microbial counts in winter wheat samples from the 2011-2012 growing season ^a

District	Cultivar	APC	Coliform/ <i>E. coli</i>	Eb	Yeasts	Molds	IMI	IFI
		Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
Southeast	Overland	5.64 (0.09) A ^b	ND (ND) B	4.31 (0.15) B	4.08 (0.02) B	3.33 (0.17) A ^b	100 (0.00) A	17.14 (2.86) A
	McGill	5.71 (0.10) A	0.78 (0.68) B	4.65 (0.09) B	4.07 (0.10) B	3.51 (0.26) A	100 (0.00) A	18.10 (5.95) A
	NW07505	5.69 (0.12) A	0.56 (0.98) B	4.65 (0.15) B	4.09 (0.14) B	3.50 (0.07) A	100 (0.00) A	15.98 (6.20) A
South Central	Overland	6.01 (0.02) A	2.46 (0.73) A	5.30 (0.03) A	4.63 (0.10) A	3.11 (0.11) A	98.67 (1.15) A	15.24 (6.60) A
	McGill	6.05 (0.11) A	1.80 (2.54) A	5.12 (0.34) A	4.65 (0.13) A	3.51 (0.17) A	100 (0.00) A	7.14 (2.02) A
	NW07505	5.89 (0.04) A	2.00 (1.74) A	4.84 (0.16) A	4.54 (0.05) A	3.40 (0.26) A	99.33 (1.15) A	12.38 (5.95) A
Panhandle	Overland	3.09 (0.65) B	ND (ND) B ^c	1.55 (0.21) C	2.62 (0.05) C	2.34 (0.07) B	48.00 (5.66) B	15.89 (2.27) A
	McGill	2.83 (0.56) B	ND (ND) B	1.51 (1.14) C	2.78 (0.16) C	2.33 (0.03) B	51.00 (18.38) B	12.50 (10.61) A
	NW07505	3.18 (0.53) B	ND (ND) B	2.14 (0.69) C	2.43 (0.14) C	2.13 (0.21) B	45.00 (5.66) B	11.25 (5.30) A

^a APC, Aerobic plate counts; Coliform/*E. coli*; Eb, *Enterobacteriaceae*, Yeasts, and Molds log CFU/g for counts. IMI, percentage of internal mold infection; IFI, percentage of internal *Fusarium* infection.

^b Different letters within the same column indicate differences based on $P < 0.05$.

^c ND, none detected.

Differences in the microbial load of wheat from different districts noticeably reflect the amount of precipitation and temperature that prevailed from flowering to harvesting (Figure 1 and Table 2). This pattern was also evident in the data presented by Seiler (25) where a steady decrease in bacterial and mold counts was observed as warm dry weather conditions prevailed during wheat ripening and a steep increase in microbial counts was observed in wheat grown under wetter conditions.

Table 4 summarizes the results for DON, moisture content, and water activity of grain samples collected during the 2011-2012 growing season. DON was found in all districts, however, in very low concentrations. Grain samples collected from the Southeastern and South Central districts contained higher moisture content than samples collected from Panhandle district. The high precipitation levels and high relative humidity from wheat flowering to harvest observed in the Southeastern and South Central districts in the 2011-2012 season may have contributed to the higher moisture content encountered in samples from these districts.

TABLE 4. Mean and standard deviation (SD) for Deoxynivalenol, moisture content and water activity in winter wheat samples from the 2011-2012 growing season

District	Cultivar	Deoxynivalenol ($\mu\text{g/g}$)	Moisture content (%)	Water activity
		Mean (SD)	Mean (SD)	Mean (SD)
Southeast	Overland	0.10 (0.09) A ^a	13.26 (0.39) A	0.63 (0.03) A
	McGill	0.05 (0.09) A	13.33 (0.24) A	0.63 (0.03) A
	NW07505	0.07 (0.12) A	13.38 (0.25) A	0.65 (0.01) A
South Central	Overland	0.03 (0.06) A	12.84 (0.29) A	0.58 (0.01) B
	McGill	ND (ND) A ^b	12.96 (0.25) A	0.60 (0.01) B
	NW07505	0.12 (0.20) A	13.35 (0.40) A	0.63 (0.04) B
Panhandle	Overland	0.13 (0.04) A	11.95 (0.09) B	0.60 (0.02) AB
	McGill	0.08 (0.11) A	11.81 (0.04) B	0.61 (0.02) AB
	NW07505	0.20 (0.07) A	12.27 (0.19) B	0.63 (0.00) AB

^a Different letters within the same column indicate differences based on $P < 0.05$.

^b ND, none detected.

Table 5 provides the results for the microbial analysis of winter wheat samples for the 2012-2013 growing season. Unlike the 2011-2012 growing season, the climate conditions that prevailed from flowering to harvesting in the 2012-2013 growing season were dominated by a very low precipitation levels, high relative humidity and warmer temperatures. As an example, mean relative humidity during post-flowering period in Panhandle district increased from 43% in 2012 to 55% in 2013 (Table 2). However, weather patterns were similar among districts for both growing season. In both seasons, the South Central and Southeastern districts were characterized by higher levels of precipitation, higher relative humidity and warmer temperatures than the registered in the Panhandle district.

The variability in microbial counts among districts in the 2012-2013 season was less pronounced compared with the 2011-2012 season. Coliform/*E. coli* and APC were found at highest concentrations in grain samples collected from Southeast district. *Salmonella* spp. and *E. coli* O157:H7 were not detected in any of samples analyzed. There were not significant differences in the yeast counts found in grain collected among districts.

As in the 2011-2012 season, for the 2012-2013 growing season there was no statistical differences in the microbial load among cultivars. In general, the microbial counts in the samples collected in the different districts in the 2012-2013 growing season were higher than the microbial counts found in the 2011-2012 growing season, except for IFI which showed higher values in the 2011-2012 season than in the 2012-2013 season.

TABLE 5. Mean and standard deviation (SD) for microbial loads in winter wheat samples from the 2012-2013 growing season ^a

	Cultivars	APC	Coliform/ <i>E. coli</i>	Eb	Yeasts	Molds	IMI	IFI
		Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
Southeast	Overland	6.14 (0.38) A ^b	3.84 (0.04) A	5.09 (0.72) A	4.79 (0.25) A	4.40 (0.04) A ^b	100 (0.00) A	9.00 (1.41) A
	McGill	6.00 (0.26) A	4.17 (0.30) A	4.89 (0.32) A	4.69 (0.05) A	4.20 (0.00) A	100 (0.00) A	7.00 (1.41) A
	NW07505	6.42 (0.15) A	4.02 (0.09) A	4.38 (0.13) A	4.76 (0.05) A	4.48 (0.03) A	100 (0.00) A	10.00 (2.83) A
South Central	Overland	4.60 (0.36) B	ND (ND) B ^c	2.87 (0.71) B	4.34 (0.02) A	3.67 (0.03) B	99.00 (1.41) A	6.00 (2.83) AB
	McGill	4.73 (0.31) B	ND (ND) B	3.43 (0.05) B	4.93 (0.58) A	3.59 (0.11) B	99.00 (1.41) A	6.00 (0.00) AB
	NW07505	5.04 (0.33) B	ND (ND) B	3.22 (0.28) B	4.59 (0.11) A	3.73 (0.16) B	100 (0.00) A	8.00 (5.66) AB
Panhandle	Overland	5.82 (0.13) AB	ND (ND) B	5.47 (0.21) A	5.16 (0.04) A	3.54 (0.01) C	85.00 (4.24) B	5.00 (1.41) B
	McGill	5.67 (1.58) AB	ND (ND) B	3.98 (0.45) A	4.76 (0.09) A	3.64 (0.01) C	71.00 (12.73) B	3.00 (1.41) B
	NW07505	5.05 (0.10) AB	ND (ND) B	4.45 (0.08) A	4.74 (0.01) A	3.46 (0.01) C	72.00 (5.66) B	3.00 (1.41) B

^a APC, Aerobic plate counts; Coliform/*E. coli*; Eb, *Enterobacteriaceae*, Yeasts, and Molds log CFU/g for counts. IMI, percentage of internal mold infection; IFI, percentage of internal *Fusarium* infection.

^b Different letters within the same column indicate differences based on $P < 0.05$.

^c ND, none detected.

Table 6 illustrates the results for DON, moisture content, and water activity of samples collected during the 2012-2013 growing season. DON was not detected in samples collected from Panhandle but was found in very low concentrations in the Southeastern and South Central districts. Grain samples collected from the South Central district contained higher moisture content than samples collected from the Panhandle district. Water activity was lowest in grain collected in the Panhandle district. In general, the moisture level of wheat harvested in 2012-2013 season was lower (Table 6) than samples from the 2011-2012 season (Table 4), the low precipitation levels during post-flowering periods in the 2012-2013 season may have contributed to the lower moisture content encountered in wheat harvest in this period.

TABLE 6. Mean and standard deviation (SD) for Deoxynivalenol, moisture content and water activity in winter wheat samples from the 2012-2013 growing season

District	Cultivars	Deoxynivalenol ($\mu\text{g/g}$)	Moisture content (%)	Water activity
		Mean (SD)	Mean (SD)	Mean (SD)
Southeast	Overland	0.35 (0.07) A ^a	11.23 (0.54) AB	0.60 (0.01) A
	McGill	0.10 (0.14) A	11.57 (0.24) AB	0.59 (0.03) A
	NW07505	1.10 (0.42) A	11.06 (0.32) AB	0.58 (0.00) A
South Central	Overland	0.15 (0.14) B	11.72 (0.34) A	0.59 (0.02) A
	McGill	ND (ND) B ^b	10.87 (0.86) A	0.59 (0.01) A
	NW07505	0.10 (0.14) B	11.55 (0.28) A	0.60 (0.00) A
Panhandle	Overland	ND (ND) B	10.39 (0.12) B	0.53 (0.00) B
	McGill	ND (ND) B	10.97 (0.91) B	0.55 (0.01) B
	NW07505	ND (ND) B	10.17 (0.06) B	0.53 (0.01) B

^a Different letters within the same column indicate differences based on $P < 0.05$.

^b ND, none detected.

Wheat varieties were combined for correlation analysis, since there was no differences between them. Correlation coefficients between weather variables and microbial counts, moisture content, and water activity are summarized in Table 7. A significant positive correlations between relative humidity and all microbial indicators was observed, except for coliform/*E. coli* and IFI. In general, the microbial load of winter wheat samples increased with an increase in the relative

humidity of the environment during flowering to harvest. A strong positive correlation was also found between precipitation levels and the moisture content of the grain. As the precipitation rates from flowering to harvesting increased, the final moisture content in the harvested wheat grain also increased. Mold counts were positively correlated with the mean minimum temperatures; therefore, mold counts increased with an increase in the mean minimum temperature recorded. Among all of the correlations, the strongest ones were observed between mold counts and relative humidity and IMI and relative humidity.

TABLE 7. Correlation coefficients for weather variables versus microbial loads, moisture content, and water activity for winter wheat samples.

Weather Variables ^a	Microbial Indicators ^a							Moisture content	Water Activity
	APC	Coliform/ <i>E. coli</i>	Eb	Yeast	Mold	IMI	IFI		
Precip	0.446	0.169	0.473	0.050	0.029	0.471	0.752	0.905* ^b	0.680
T-Max	-0.719	-0.299	-0.749	-0.438	-0.337	-0.739	-0.433	-0.716	-0.410
T-Min	0.338	0.500	0.197	0.441	0.762*	0.569	-0.295	-0.323	-0.021
RH	0.869*	0.591	0.802*	0.834*	0.926*	0.971*	-0.172	0.045	-0.003

^a Precip, precipitation; T-Max, highest air temperature; T-Min, Lowest air temperature; RH, relative humidity; APC, aerobic plate count; Eb, *Enterobacteriaceae*; IMI, internal mold infection; IFI, internal *Fusarium* infection.

^b * Significant at $P < 0.05$

As a raw agricultural commodity, wheat grain is vulnerable to microbial infection shortly after head emergence and remains susceptible until harvesting or until climate conditions are not favorable for microbial growth. Flowering has been considered by many authors as the key stage for mold infection in the field (6, 21), and previous studies have reported on the correlation between mold infection and weather conditions. For instance, FHB infection occurs by the penetration of infectious hyphae on the inner surfaces of the floret (4), and is favored by low temperatures and high humidity (22). Reis (23) and Panisson (18) reported that peaks in *Gibberella zeae* counts were associated with rain events and high relative humidity. These reports are in agreement with the positive correlation between mold counts and weather variables

described here, which implies that mold counts increases as minimum temperature and relative humidity increases. Because relative humidity has a strong positive correlation with IMI, the higher levels of relative humidity in the 2012-2013 season in the Panhandle may explain why samples from this area had a much higher incidence of IMI when compared with samples from the same region in the previous year.

In conclusion, the results of the present study indicated that average minimum temperature and relative humidity during post-flowering periods play an important role on the microbiological quality and safety of wheat grain. The microbial flora of wheat can therefore be greatly influenced by the weather conditions during this period and an annual variation in microbial load may be expected. In general, microbial load in wheat grain tended to be lower in those areas with lower relative humidity and lower minimum temperatures. Besides weather conditions, other variables such as type of soil, use of fertilizers or foliar chemicals to treat diseases may also affect the number and type of microorganisms present. This study contributes to a better understanding on how environmental conditions may affect the microbial load of wheat. In addition, provides to grain buyers and processors the post-flowering weather conditions as an additional parameter to consider when selecting for higher microbiological quality grain.

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EFFECT OF THE MILLING STEPS ON THE MICROBIAL LOAD OF WHEAT
MILLED PRODUCTS FROM DIFFERENT WHEAT VARIETIES *

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ABSTRACT

Microbial contaminants are hosted and concentrated mainly on the surface of the grain and from there they may reach flour during wheat milling. In this study, samples taken during the milling process of four wheat varieties were analyzed for mesophilic aerobic bacteria (APC), yeasts, molds, and *Enterobacteriaceae* (Eb), to evaluate the effect of cleaning, tempering and milling stages on microbial load. Based on the results, the removal of foreign materials using a Dockage tester did not affect the microbial load of wheat grain. The conditioning process under aseptic conditions did not cause significant changes in the microbial populations of wheat grain, since the water activity during this process does not reach the minimum needed for microbial growth. When wheat from all four varieties was conditioned with sterile water and milled, the microbial load was distributed into the different milled fractions. As the layers of the kernel were separated, surface contaminants were concentrated in the bran (APC increased on average by 0.66, yeast and mold by 0.88 and 0.74, respectively, and Eb by 0.45 log CFU/g); while counts remained the same in the wheat germ. The inner endosperm had a lower microbial load; since the fractions obtained from break rolls and reduction rolls (break flour and reduction flour) had counts substantially lower than the conditioned wheat. When both fractions were combined to produce flour, the average reduction obtained for APC was 1.54 log CFU/g, Eb counts was 1.88 log CFU/g, and for yeast and mold counts were 1.10 and 0.16 log CFU/g, respectively. Among the different microbial groups the least redistribution was observed with molds. When comparing microbial counts at any given fraction, there were no differences among varieties. Although the milling process removes much of the outer layers of the wheat kernel, removing the majority of the microflora, flour can still retain significant amounts of microbial contamination representing a potential risk for consumers' health.

1. INTRODUCTION

Throughout the production chain, including harvest, storage and transport, wheat is exposed to multiple sources of microbial contamination (14). Thus, wheat kernels usually host a large and varied microbial flora, including pathogenic and spoilage microorganisms (3, 8). Most of these microbial contaminants are located in the outer layers of the kernel, the pericarp; although some species may reach the inner part through mechanical damage that may be caused during harvesting (6, 7).

Wheat arriving in the mill usually contains foreign material, such as sticks, stones, adhered dust, wood, insects, or metal, which may contain a significant microbial load and represent a potential hazard for the milling operation. Therefore, flour milling begins with removal of these impurities through a sequence of operations based on physical characteristics (11). The wheat-cleaning system varies greatly among milling operations regarding the sequence, number, and complexity of the methods used. Over time, new grain cleaning technologies have been introduced such as infrared or ultraviolet optical sorters; however, traditional mechanical methods for removing impurities based on size, shape and density are the basis of most cleaning operations. The foreign matter separated during the cleaning process may contribute to improve the safety of wheat prior to milling by reducing microbial load and mycotoxin concentrations (4,8). However, the extent of the reduction in the microbial load may be variable since microorganisms are strongly adhered to the surface of the grain (7).

After the initial cleaning, the next step in wheat preparation for milling is conditioning, which involves the addition of water to bring the wheat to its optimum milling moisture. To ensure even penetration and distribution of moisture into the kernels, wheat is held in large conditioning bins during specific holding periods (3, 11). This adjustment in moisture content mellows the endosperm and toughens the bran, ensuring an easier separation later in the milling

process. Conditioning is a critical step in milling operations, since an improper handling and poor sanitary conditions can encourage microbial growth in the wheat kernels (3).

Once conditioned, wheat undergoes a series of reduction, grinding, sifting and purifying operations to separate the germ and the bran fractions from the endosperm (11). During this grinding process, the microorganisms are redistributed and concentrated in certain milled fractions, particularly in germ and bran (3, 13). Therefore, the majority of microorganisms originally present on the wheat kernels might be expected to be present in the milled products (15). Moreover, the physical separation of the grain components (bran, endosperm, and germ) and the subsequent reduction of the endosperm to flour generate heat and thus moisture condensation is likely to occur, which may lead to build-up of flour residues in the milling equipment, which supports bacterial growth that can also be a source of contamination to the product.

A thorough review of the scientific literature has shown the lack of reports that compare wheat varieties and their effect on microbial load distribution during milling. Therefore, the objectives of this study were (i) to evaluate the effect of cleaning and conditioning on the microbial load of wheat grain, (ii) to examine the influence of the milling stages on the microbiological quality of milled fractions, and (iii) to evaluate the effect of wheat variety on microbial load distribution during milling.

2. MATERIALS AND METHODS

2.1 *Wheat samples*

Four hard red winter wheat varieties, two commercial lines (Overland and McGill) and two experimental lines (NW07505 and NW03666) were obtained from the 2012 winter wheat trials in the state of Nebraska, USA. Trials were conducted in the South Central district at Clay Center County during the period September 23th, 2011 (Sowing) – June 22th, 2012 (Harvesting). Samples were provided by Husker Genetics, the University of Nebraska-Lincoln Foundation Seed Division.

2.2 *Sample cleaning*

The wheat samples (1100 g each) were cleaned using a dockage tester (CDT/B; Carter-Day International, Minneapolis, Minn., USA) to remove shrunken and broken kernels, sticks, stones, lighter particles like chaff and straw, and other foreign materials according to their particle size. Dockage tester was equipped with a no. 25 riddle and a no. 2 and no.8 sieves.

2.3 *Conditioning*

The initial moisture content of wheat samples was determined using a forced-air oven method 44-15A (AACCI 2002) and the water activity was measured using an Aqualab® S-3 TE water activity meter (Decagon, Pullman, Washington, USA). The amount of tempering water needed was calculated based on the initial moisture content of each wheat sample. A one-stage tempering procedure was used to achieve a good repeatability of milling results (Method 26-10.02, AACCI 2002). Three cleaned samples (1000 g) from each wheat variety were placed in sterile plastic bags and tempered to 15.5% moisture using sterile distilled water under aseptic conditions (Biosafety cabinet). Tempering water was applied to wheat using an atomizer to ensure even distribution. Samples were shaken vigorously during the first two hours after the addition of water and were left to stand for 24 hr under controlled conditions (23-24°C and 60% relative humidity).

2.4 *Experimental milling*

Nine hundred grams of each tempered wheat sample were milled using an Buhler experimental mill (Buhler, Minneapolis, MN) according to method 26-21.02 (AACCI 2002). Milled fractions (flour, bran, and shorts) were collected in sterile plastic bags, as they were coming out of the mill. Flour fractions were recombined in a tumbling mixer for 3 min to obtain straight-grade flour. The tumbling mixer consisted of a sterile cylindrical container, which was held at a 45° angle and rotated at 6.7 rpm on a circular axis. The water activity of each milled

fraction was determined using a forced-air oven method 44-15A (AACCI 2002) and an Aqualab® S-3 TE water activity meter (Decagon, Pullman, Washington, USA), respectively.

2.5 Microbiological analysis

Microbial analysis was performed in wheat samples collected directly from growers, after cleaning, after tempering, in each of the milled fractions, and in the final flour. Samples were analyzed for mesophilic aerobic bacteria (Aerobic Plate Counts, APC), yeasts, molds, and *Enterobacteriaceae* (Eb). An initial dilution was prepared from each sample by mixing 25 g sample into 225 ml 0.1% aqueous peptone solution. For APC, serial dilutions were spread plated on Standard Methods Agar (SMA, Acumedia) and incubated at 35 °C for 48 hr, according to the U.S. Food and Drug Administration's *Bacteriological Analytical Manual* (9). For yeast and mold counts, appropriate dilutions were spread plated onto Dichloran Rose Begal Chloramphenicol agar (DRBC, Acumedia) and incubated at 25 °C in the dark for 5 days, according to the U.S. Food and Drug Administration's *Bacteriological Analytical Manual* (16). *Enterobacteriaceae* counts were determined according to AOAC official method 2003.01, using Petrifilm™ (3M Microbiology, St. Paul, MN). Serial dilutions were inoculated onto Eb Petrifilm™ and incubated at 37 °C for 24 hr.

2.6 Statistical analysis

The microbiological counts obtained were subjected to analysis of variance at 95% level of confidence. To determine significant differences among samples, a generalized linear mixed model analysis of variance followed by a Tukey's test was used. Significance was defined as $P < 0.05$. The statistical software SAS version 9.3 (SAS Institute, Cary, NC, USA) was used to analyze the data.

3. RESULTS AND DISCUSSION

3.1 *Wheat cleaning*

The removal of unwanted materials from wheat grain prior to milling is the first step in any milling operation. In the milling industry, wheat is cleaned thorough a sequence of operations, including magnetic separators, separators, aspirators, disc separators, and scourers, which separate foreign matter and remove firmly attached soiling. Dockage tester removes impurities like stones, sticks and other coarse and fine materials by the action of reciprocating screens and aspiration. Target microorganisms were detected in all wheat samples at very similar levels before and after cleaning as shown in Table 1. Cleaning dirty grain with a dockage tester did not significantly affect the microbial counts in none of the wheat samples analyzed. Therefore, the removal of coarse and fine materials did not have any significant effect on reducing microbial counts in wheat grain.

However, the addition of dry scourers and brushes to the cleaning system has shown to cause a small reduction in microbial counts, since these operations scour off impurities and remove more firmly attached soiling on wheat grain. Manthey et al. (8) demonstrated that cleaning dirty grain with a dockage tester followed by a cyclone grain cleaner may reduce, on average, aerobic bacterial counts in 1.0 log CFU/g. Similar trends of overall reduction in yeast and mold counts were also observed. In another study, Seiler (14) also observed small reductions in counts of bacteria and mold in wheat when evaluating different dry scourers, aspirators and brushes. By contrast, Riba et al. (12) observed no differences in the fungal contamination between unclean durum wheat and durum wheat after cleaning, with fungal counts varying from 2.7 to 2.9 log CFU/g. Therefore, the extent of the reduction in microbial counts by the cleaning step is variable and may depend on the type and number of cleaning methods used.

Table 1. Mean and standard deviation (SD) for different groups of microorganisms in wheat samples before and after cleaning (categorized by wheat variety) ^a

Wheat variety	APC		Yeasts		Molds		Eb	
	Dirty ^b	Clean ^c	Dirty	Clean	Dirty	Clean	Dirty	Clean
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
Overland	6.01 (0.03) A ^d	6.16 (0.07) A	4.73 (0.10) A	4.69 (0.13) A	3.11 (0.11) A	3.25 (0.12) A	5.30 (0.03) A	5.02 (0.25) A
McGill	6.05 (0.11) A	5.93 (0.10) A	4.65 (0.13) A	4.27 (0.30) A	3.51 (0.17) A	3.30 (0.12) A	5.12 (0.34) A	5.05 (0.33) A
NW03666	6.32 (0.21) A	5.95 (0.13) A	4.73 (0.04) A	4.47 (0.09) A	3.50 (0.30) A	3.26 (0.21) A	5.25 (0.18) A	4.80 (0.39) A
NW07505	5.89 (0.05) A	6.00 (0.16) A	4.54 (0.05) A	4.75 (0.07) A	3.41 (0.26) A	3.41 (0.24) A	4.84 (0.16) A	4.95 (0.18) A
Average	6.04 (0.18) A	6.02 (0.14) A	4.66 (0.11) A	4.58 (0.23) A	3.36 (0.25) A	3.31 (0.17) A	5.11 (0.25) A	4.96 (0.24) A

^a APC (Aerobic plate counts), Yeasts, Molds, and Eb (*Enterobacteriaceae*) log CFU/g for counts.

^b Sample as collected

^c Sieved grain

^d Different letters within the same row and microorganism indicate differences based on $P < 0.05$.

3.2 Wheat conditioning

The availability of water is one of the most important factors determining the growth of microorganisms. Usually, wheat kernels have a water activity below the minimum needed (0.90) for microbial growth (Fig. 1); however, microorganisms may survive in states of minimal metabolic activity for long periods of time. The presence of nutrients or the increase in water availability may stimulate the exit from dormancy of microbial cells; therefore, the addition of water to condition the wheat kernels prior to milling may encourage microbial growth if not properly managed. As a result of the addition of water for tempering, the water activity of wheat kernels increase (Fig. 1); however, still remains at levels (0.73) below the minimum needed to support the growth of microorganisms.

Table 2 illustrates the mean counts for all target microorganisms before and after the conditioning process. After conditioning, the mean counts of target microbial populations decreased in most samples, although this change was not statistically relevant. However, in two wheat varieties (NW07505 and Overland), the amount of mold was reduced significantly after conditioning. In these samples, the mean mold counts decreased from 3.25 to 2.78 log CFU/g and from 3.41 and 2.88 log CFU/g for varieties Overland and NW07505, respectively. Moreover, the mean yeast count in NW07505 was reduced from 4.75 to 3.84 log CFU/g. A similar trend was also observed by Berghofer et al. (3), who reported that the populations of *Bacillus* spp., yeasts and molds were reduced after conditioning. In this later study, the percentage of wheat samples containing more than 3.0 log CFU/g of mold counts were reduced by up to 26% after conditioning.

Table 2. Mean and standard deviation (SD) for different groups of microorganisms in wheat samples before and after tempering (categorized by wheat variety) ^a

Wheat variety	APC		Yeasts		Molds		Eb	
	Before ^b	After ^c	Before	After	Before	After	Before	After
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
Overland	6.16 (0.07) A ^d	5.95 (0.08) A	4.69 (0.13) A	4.28 (0.19) A	3.25 (0.12) A	2.78 (0.03) B	5.01 (0.27) A	5.34 (0.16) A
McGill	5.93 (0.10) A	5.83 (0.14) A	4.27 (0.30) A	3.85 (0.23) A	3.30 (0.12) A	2.81 (0.11) A	5.05 (0.33) A	5.11 (0.19) A
NW03666	5.95 (0.13) A	6.03 (0.09) A	4.47 (0.09) A	3.88 (0.08) A	3.26 (0.21) A	2.78 (0.20) A	4.80 (0.39) A	4.90 (0.23) A
NW07505	6.00 (0.16) A	5.87 (0.23) A	4.75 (0.07) A	3.84 (0.19) B	3.41 (0.24) A	2.88 (0.15) B	4.95 (0.18) A	5.03 (0.15) A
Average	6.01 (0.10) A	5.92 (0.09) A	4.55 (0.22) A	3.96 (0.21) A	3.31 (0.07) A	2.81 (0.05) B	4.95 (0.11) A	5.10 (0.18) A

^a APC, Yeasts, Molds, and Eb log CFU/g for counts.

^b Clean wheat before being conditioned

^c Wheat after conditioning

^d Different letters within the same row and microorganism indicate differences based on $P < 0.05$.

The conditioning process under aseptic conditions did not cause significant changes in the microbial populations of wheat kernels prior to milling. As previously mentioned, the minimum water activity requirement for growth of most bacteria is 0.90; while yeasts and molds are able to grow at lower water activities, such as 0.80 and 0.72, respectively (10). The relatively short holding period used in these experiments (24 hr) and the low water activity of about 0.70-0.75 found on wheat grain after conditioning (Fig. 1), supports the findings that the tempering process by itself may not promote microbial growth. However, an inspection survey carried out by Berghofer et al. (3) in commercial mills found an increase in mesophilic aerobic counts after conditioning and the presence of microorganisms that were not previously detected in wheat grain. Nevertheless, the same survey detected build-up of grain residues in conditioning and storage bins used for the process, which may contain a significant microbial load. Therefore, the observed increases in microbial load were most likely due to transfer from poorly cleaned bins and equipment and not necessarily due to the tempering process itself.

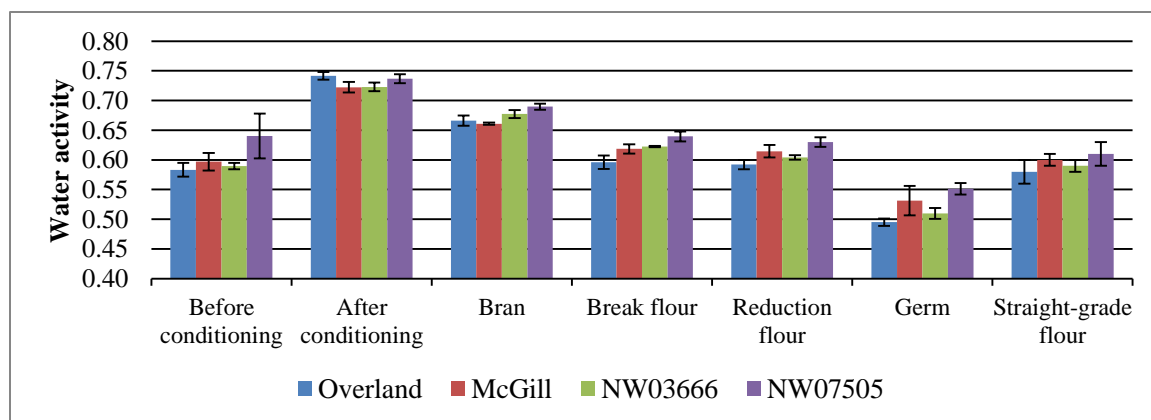


Figure 1. Average water activity of wheat grain before and after conditioning and milling end products for all wheat varieties. Error bars denote \pm standard deviation.

3.3 Wheat milling

Once conditioned, wheat passes through the various systems in the mill, allowing the removal of the endosperm from the bran and germ, and the reduction in particle size of the isolated endosperm. The milling process may differ among milling operations, depending on

process flow and the type of wheat used. However, all milling systems cause a physical separation of the grain components, thus the microorganisms originally present in the grain may be distributed across the milled fractions.

Figure 2 illustrates the distribution of APC, yeasts, molds and Eb counts among milled fractions, along with end product flour observed in the different wheat varieties. As a general trend, as the outer grain layers are separated by the corrugated rolls in the break system, microbial contaminants are concentrated in the bran and wheat germ fractions, with flour milled from the inner endosperm in the reduction system being the least contaminated end-product of the milling process (Fig. 2).

In the bran fraction, when compared to wheat kernels, APC increased by 0.7 log CFU/g, on average; while yeast and mold counts increased, on average, by 0.9 log CFU/g and 0.7 log CFU/g, respectively. The microbial counts in the germ fraction remained relatively unchanged when compared with that originally present on the conditioned grain. Berghofer et al. (3) reported that the bran and germ fractions showed higher microbial counts than the wheat entering the milling system, with APC increasing from 6.0 to 7.0 log CFU/g in the bran portion.

The flour obtained from the inner endosperm tends to have lower microbial counts, since most microorganisms are located in the outer layers of the grain (7, 8). In this study, mean counts were reduced significantly for all microorganisms in the flour fractions. Aerobic bacterial counts decreased, on average, by 1.7 log CFU/g in the reduction flour when compared to tempered wheat; while yeast and mold counts were reduced by 1.5 log CFU/g and 0.4 log CFU/g, respectively. The mean Eb counts in the reduction flour also decreased significantly when compared with tempered wheat (2.1 log CFU/g). Berghofer et al. (3) reported in the same study, that maximum aerobic bacterial count decreased from 7.0 to 5.0 log CFU/g, yeast counts from 6.0 to 3.0 log CFU/g, and mold counts from 6.0 to 4.0 log CFU/g after fine reduction.

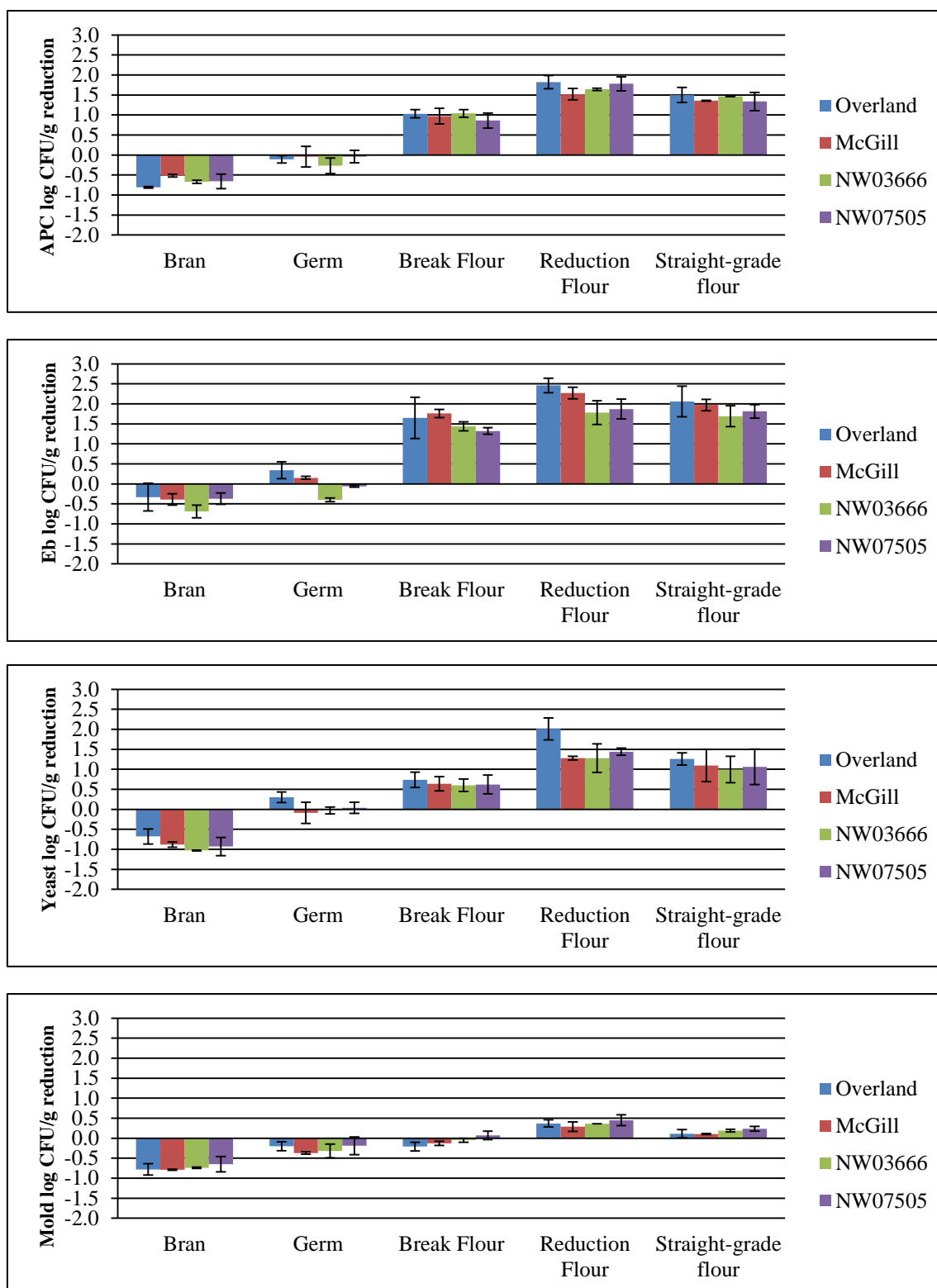


Figure 2. Comparison of the distribution of APC, Eb, yeasts, and molds among milled fractions and milling end-product (straight-grade flour) in the different wheat varieties. Error bars denote \pm standard deviation. No differences among varieties for each fraction and microorganism were observed.

During the milling process, there was a clear redistribution in the population of all microorganisms tested among the product fractions, which led to significant differences among the microbial loads of the different fractions for all wheat varieties tested. Among the different microbial groups the least redistributions were observed with molds (Fig. 2). Hesseltine (5) showed that the dry-milling process reduced bacterial counts in flour, but have minimal impact on fungal counts since they remained relatively unchanged when compared to that originally present on the conditioned wheat.

When both flour fractions were combined to produce straight-grade flour, the microbial load was significantly lower than that of conditioned wheat. A survey by Seiler (14) revealed that the mold count in flour (3.7 log CFU/g) was about 10 times lower than in the corresponding wheat (4.3 log CFU/g). Richter et al. (13) provided a comprehensive microbiological profile for more than 4,000 commercial wheat flour samples of four different types (hard red winter, spring, soft red winter, and durum) collected throughout the United States. On average, flour samples had counts of 2.1 log CFU/g and 2.9 log CFU/g for yeast and mold, respectively. These counts are lower than the ones obtained in the present study. When comparing microbial counts at any given fraction, there was no difference among varieties. Therefore, wheat varieties did not show any difference in the microbial distributions obtained.

In conclusion, this study contributes to a better understanding on how the general operations involved in the milling process affect the microbial load of the wheat milled products when compared with the wheat entering the system. Results indicated that removing foreign materials will not reduce microbial load in wheat grain. However, the incorporation of abrasive cleaning methods may cause a slight reduction in microbial counts as reported by some authors (3). The water activity encountered in wheat grain after tempering is not enough to support microbial growth, thus the tempering process by itself does not encourage microbial growth if proper sanitary conditions exist. The milling process causes a redistribution of the microbial load

into the different milled fractions, with the flour fractions showing lower microbial load when compared to conditioned wheat. However, although the milling process removes much of the outer layers of the wheat kernel, removing the majority of the microflora, flour can still retain unsafe contaminants representing a potential risk for consumers' health. The distribution of microorganisms, originally present in the kernels, during the milling process is not affected by wheat variety.

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EFFECT OF NOVEL TEMPERING SOLUTIONS ON REDUCING MICROBIAL
LOAD IN WHEAT PRIOR TO MILLING *

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ABSTRACT

The microbial flora of wheat kernels is the primary contamination source for milled products; therefore, reducing the microbial load is desirable before or during processing. In this study, the efficacy of adding organic acids and salt (NaCl) to tempering water to reduce microbial contamination in hard wheat prior to milling was evaluated. Hard red winter wheat (12.2% moisture) was tempered to 15.5% moisture by adding sterile distilled water (control) or tempering solutions containing organic acids (acetic, citric, lactic, or propionic; 1.0%, 2.5% or 5.0% v/v) or NaCl (26%, or 52% w/v) and holding for 24 hr at 23-24°C and 60% relative humidity. After tempering, the initial microbial load was reduced significantly by all the acid and salt treatments when compared to the control which was tempered with water. Tempering of wheat with organic acids at the 1.0% concentration level resulted, on average, in a significant reduction of 0.9 log CFU/g in the initial counts of Eb. The greatest reduction (2.7 log CFU/g) in the number of Eb when compared to incoming wheat was achieved by wheat tempered with lactic acid at 5.0%. Lactic and propionic acid at the 5.0% concentration level reduced APC by 2.0 and 1.7 log CFU/g reduction, respectively. The greatest reduction (4.7 log CFU/g) in the number of yeasts was achieved by lactic acid treatment at the 5.0% level, which resulted in counts below the limit of detection of the method. Similarly, the greatest reduction (3.8 log CFU/g) in mold counts was achieved by acetic, propionic, and lactic acid treatments at the 5.0% concentration level, which also resulted in counts below the limit of detection. Saline solutions were as effective as organic acid solutions at the concentration levels tested. The milling process of hard wheat tempered with organic acids and saline solutions may provide milled products with improved microbiological quality when compared with the traditional tempering process which uses water.

1. INTRODUCTION

As a raw agricultural commodity, wheat is subject to naturally-occurring microbiological threats; therefore, wheat kernels usually harbor substantial amounts of microbial contamination (7). Although the usual water activity of wheat grain and the storage practices normally used by the milling industry are not conducive to the growth of microorganisms, pathogenic and spoilage microorganisms may survive in a dormant state for long periods of time (13). Indeed, several studies have documented the presence of enteric pathogens, such as *Salmonella* spp., *Escherichia coli*, and the food poisoning spore-former *Bacillus cereus* (5, 13). Moreover, mycotoxin-producing molds, such as *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp. are commonly isolated from wheat grain (6, 25). In addition to pathogenic or toxigenic microorganisms, a wide variety of food spoilage bacteria are among the natural microbial flora of wheat kernels (5).

The milling steps, including cleaning, tempering and grinding have no direct impact on the level of contamination present in wheat. As wheat passes through the various systems in the mill, microbial contaminants are redistributed and concentrated in certain milled fractions, particularly in germ and bran (26, 33). Thus, the majority of microorganisms originally present on the wheat might be expected to be present in the milled products. Therefore, the microbial flora of the kernels is the primary contamination source for milled products; consequently, flour with higher microbial counts are usually obtained from wheat of inferior microbiological quality (5).

The increased incidence of foodborne disease outbreaks caused by products containing flour has highlighted the importance of high microbiological quality of wheat prior to milling. Since the microbial load is concentrated in the outer bran layer of the grain (19), some strategies to reduce microbial counts have been focused on pre-milling interventions. Chlorinated water (600 to 700 mg/L) has been widely used to disinfect wheat grain during tempering, for instance (9, 15). However, there has been some safety concerns about the use of chlorine since under

certain circumstances it may induce formation of potentially harmful by-products due to its reactivity with organic matter (4). Therefore, other methods have been evaluated to reduce the microbial load of wheat, rather than using chlorine-based sanitizers. Studies have focused on the application of ozonated water or acidified ozonated water as antimicrobial treatment for wheat during tempering (8, 9, 11, 17). Approaches evaluated include physical methods such as washing, soaking, and fluidization with ozonated water (10, 18).

Strategies other than chlorination and ozonation during tempering have not been employed to reduce microbial load in wheat prior to milling. Since, there is a growing interest in exploring and developing practical and effective antimicrobial treatments to improve safety and quality of wheat prior to milling, the objectives of this research were (i) to determine the efficacy of adding organic acids and NaCl to tempering water to reduce microbial load in hard wheat prior to milling, and (ii) to compare each organic acid and salt's ability to decrease microbial load in tempered wheat.

2. MATERIALS AND METHODS

2.1 Materials

Hard red winter wheat (McGill) samples were obtained from the 2013 growing season in the state of Nebraska, USA. Samples were provided by Husker Genetics, the University of Nebraska-Lincoln Foundation Seed Division. Lactic acid (86%) and citric acid granular were obtained from Mallinckrodt Pharmaceuticals. Propionic acid (99%) was obtained from Alfa Aesar Company. Glacial acetic acid was obtained from EM Science. Sodium chloride was obtained from Fisher Scientific.

2.2 Preparation of tempering solutions

A 100 ml stock solution for each organic acid (acetic, citric, lactic, and propionic) and salt (NaCl) at each desired concentration level was prepared. To prepare each organic acid solution, the respective organic acid and sterile distilled water were mixed in appropriate proportions to yield a solution wherein the final acid concentration was 1.0%, 2.5%, and 5.0% (v/v), respectively. For the saline solutions, concentrations used were 1.0% and 2.0% flour basis (% of flour weight on a 14% moisture basis). To prepare each saline solution, NaCl and sterile distilled water were mixed in appropriate proportions to yield a solution wherein the final salt concentration was 0.26 g/ml (26% w/v ~ 1% flour basis) or 0.52 g/ml (52% w/v ~ 2% flour basis). The solutions were always prepared fresh prior to tempering and under aseptic conditions.

2.3 Application of tempering solutions

The initial moisture content of the wheat samples was determined using a forced-air oven method 44-15A (AACCI 2012). The amount of tempering solution needed was calculated based on the initial moisture content of the wheat samples (12.2 %). Three samples (150 g) for each treatment were placed in sterile plastic bags and tempered to 15.5% moisture by adding sterile distilled water (control) or replacing the tempering water with solutions containing acid (acetic, citric, lactic, or propionic; 1.0%, 2.5% or 5.0% v/v) or NaCl (26%, or 52% w/v), under aseptic conditions (Biosafety cabinet). Tempering solutions were applied to wheat using an atomizer to ensure even distribution. Samples were shaken vigorously during the first two hours after the addition of tempering solutions and were left to stand for 24 hr under controlled conditions (23-24°C and 60% relative humidity).

2.4 Microbial analysis

To determine the microbial load of wheat before and after tempering under various experimental conditions, 25 g of wheat with 225 ml of phosphate-buffered saline solution was

placed in a stomacher bag and mixed for 90 seconds. Serial decimal dilutions were prepared with phosphate-buffered saline solution and analyzed for aerobic mesophilic bacteria (Aerobic Plate Counts (APC)), yeasts, molds, and *Enterobacteriaceae* (Eb). For APC, dilutions were surface plated in triplicate on Standard Methods Agar (SMA, Acumedia) and incubated at 35 °C for 48 hr, according to the U.S. Food and Drug Administration's *Bacteriological Analytical Manual* (21). For yeast and mold counts, dilutions were spread plated in triplicate on Dichloran Rose Begal Chloramphenicol agar (DRBC, Acumedia) and incubated at 25 °C in the dark for 5 days, according to the U.S. Food and Drug Administration's *Bacteriological Analytical Manual* (34). *Enterobacteriaceae* counts were determined according to AOAC official method 2003.01 (3), using Petrifilm™ (3M Microbiology, St. Paul, MN). Dilutions were inoculated in triplicate on Eb Petrifilm™ and incubated at 37 °C for 24 hr.

2.5 Statistical analysis

Data was analyzed using the statistical software SAS version 9.3 (SAS Institute, Cary, NC, USA). Differences on microbial counts caused by the tempering solutions were assessed at a 0.05 level of significance by analysis of variance (ANOVA), followed by a multiple comparison Tukey's test.

3. RESULTS AND DISCUSSION

The dry milling process of wheat involves three general operations: cleaning, tempering, and milling. Of all these operations, the tempering step has the greatest potential to minimize microbial safety hazards in the milling end products. Therefore, in this study the tempering water was used as a vehicle for delivery of antimicrobial agents, specifically organic acids and sodium chloride (NaCl).

A thorough review on the sodium and salt content in food products revealed that baked goods commonly contain 0.5-2.0% NaCl flour basis (16). Moreover, AACCI approved methods

10-10.02 and 10-13.03 suggest 1.5% NaCl flour basis in bread (1). Therefore, we restricted the NaCl treatments to 1.0% and 2.0% flour basis. Regarding organic acids, these substances have been accepted as GRAS (Generally Recognized As Safe) food additives, due to the absence of toxicological effects. In the United States, acetic acid (21 CFR 184.1005), citric acid (21 CFR 184.1033), lactic acid (21 CFR 184.1061), and propionic acid (21 CFR 184.1081) are approved as GRAS food additives. In accordance with the Code of Federal Regulations, these additives are used in food with no limitation other than current good manufacturing practices. Therefore, different concentration levels were evaluated to find the minimum but effective concentration to reduce the microbial counts during wheat tempering. The ability to reduce microbial counts among organic acid treatments was compared at equivalent concentrations (1.0%, 2.5%, and 5.0% v/v); while salt treatments, were at 1.0% and 2.0% flour basis, which are equivalent to 26% and 52% w/v, respectively.

Table 1 summarize the range and mean counts obtained for all microorganisms tested in the incoming wheat, as well as after tempering with water (control). Mean APC, yeasts, molds and Eb counts after tempering with water remained similar to those in the incoming wheat. The results in the previous manuscript demonstrated that the tempering process under aseptic conditions does not cause significant changes in the microbial populations of wheat kernels, which agrees with the findings of this study. Moreover, Dhillon et al. (11) found no significant changes in the microbial load of wheat after washing for 3 min with distilled water at 2L/kg.

Table 1. Range, mean and standard deviation (SD) for different groups of microorganisms in winter wheat samples ^a

Sample	APC ^b	Yeasts	Molds	Eb ^b
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
Incoming wheat	5.87 (0.04)	4.71 (0.03)	3.81 (0.02)	4.69 (0.03)
Control ^d	5.76 (0.12)	4.38 (0.05)	3.63 (0.04)	4.69 (0.03)

^a Microbial counts are presented in log₁₀ CFU/g

^b APC, aerobic plate counts; Eb, *Enterobacteriaceae* counts

^d Control, wheat tempered with sterile distilled water

The reductions of microorganisms by organic acids starts with the lowering of the pH in the environment and disruption of the permeability of the cytoplasmatic membrane, thus affecting metabolic functions by the accumulation of anions and protons in the cytoplasm and depletion of cellular energy (27). Figure 1 illustrates the effect of tempering solutions on the total aerobic mesophilic microflora. The initial microbial load was reduced significantly by all the acid and salt treatments compared to the control. Wheat samples tempered with 1.0% organic acid solution resulted, on average, in 1.0 log CFU/g reduction in the population of aerobic mesophilic bacteria. There was no difference in the reduction achieved among the citric acid levels evaluated; therefore, increasing the citric acid concentration beyond 1.0 % had no significant impact on reducing microbial counts in wheat grain.

Increasing the acid concentration from 1.0 % to 2.5% did not significantly increase the reduction in aerobic mesophilic bacteria. In fact, there were no significant differences in the reductions achieved by the different organic acids at the concentration levels of 1.0% or 2.5%. Likewise, increasing the acetic and citric acid concentration from 2.5% to 5.0% did not affect significantly the microbial counts. However, increasing lactic and propionic acid up to 5.0% significantly decreased the microbial counts, resulting in 2.0 and 1.7 log CFU/g reduction, respectively.

The antimicrobial action of sodium chloride is mainly due to the reduction of water activity in the substrate in which salt is added, thus causing an osmotic shock to the bacterial cell (32); however, studies have suggested other possible mechanisms of microbial inhibition, including alteration of pH, toxicity of sodium and chloride ions, limitation of oxygen solubility to the microbial cell and interference with substrate utilization in microorganisms (30, 31).

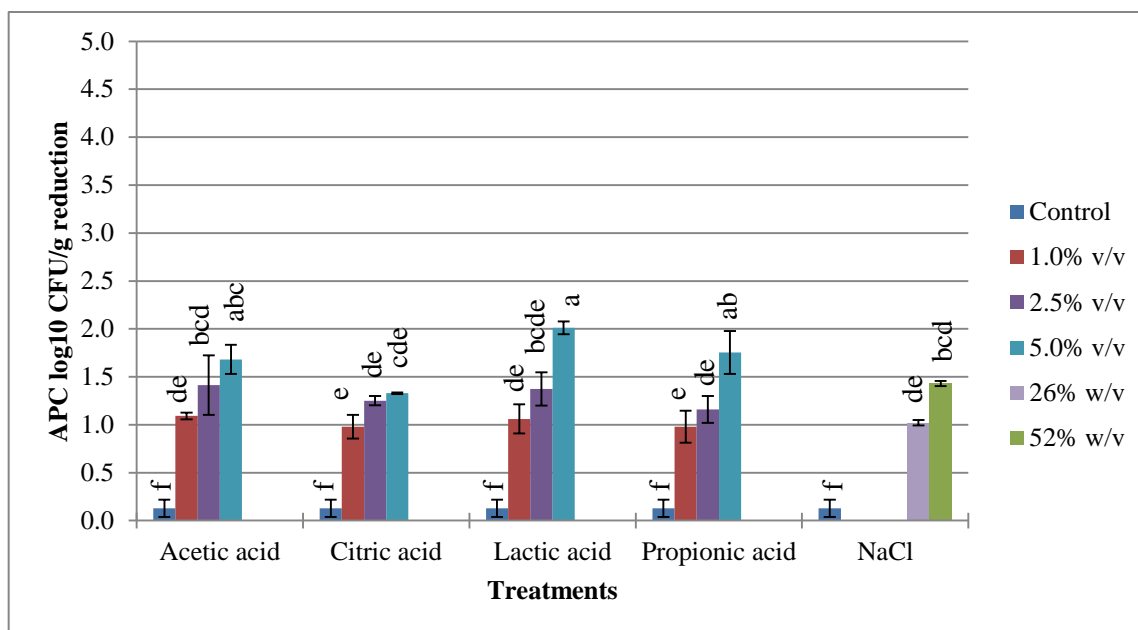


Figure 1. Effect of organic acid and saline solutions on aerobic plate counts (APC) during wheat tempering. Error bars denote \pm standard deviation. One log reduction is equivalent to a 90% reduction in CFU per gram of wheat grain. Log reduction values with the same letter are not significantly different ($P > 0.05$).

The tempering solution with 26% NaCl (~1% flour basis) reduced the mesophilic bacteria population by 1.0 log CFU/g, which was not significantly different to the reduction obtained by the organic acid solutions at the concentration levels of 1.0% and 2.5%. Increasing the NaCl concentration from 26% to 52% (~2% flour basis) slightly further reduced microbial counts, although the change was not statistically relevant. In addition, the reduction achieved by the 52% NaCl tempering solution was not significantly different from the one obtained by the acetic, citric, and propionic acid solutions at the 2.5% or 5.0% concentration levels. A saturated solution will contain 26.5g NaCl in 100 g of water (28). Significantly high amounts of NaCl are needed to cause bacteriostatic effects in foods; for instance, a 16.54% salt solution is needed to bring the water activity to 0.90, the minimum required for bacterial growth (28), which explains the results found here, since the concentration of NaCl used in the tempering water was higher than 16.54%, thus may induce a bactericidal effect on wheat grain.

Dhillon et al. (11) reported a 3.0 and 0.2 log CFU/g reduction in APC after washing wheat kernels for 3 min with acetic acid (1.0%) and ozonated water (16.5 mg/L) at 2L/Kg, respectively. Since an acidic pH may increase the dissolution of ozone in water, a powerful oxidizing agent, Dhillon et al. (11) also treated wheat kernels with a combination of ozonated water (20.5 mg/L) and acetic acid (1%) for 3 min. The addition of acetic acid enhanced the antimicrobial power of the ozonated solution, although the reduction in APC achieved by this treatment was not significantly different compared with acetic acid (1.0%) alone.

Ozone is widely used in the food industry as antimicrobial agent; therefore, the influence of ozone on the microbial load of wheat grain has been the subject of interest of many researchers. Ibanoglu (18) after washing wheat kernels with ozonated water (1.5 mg/L) for 30 min found a reduction of approximately 1 log CFU/g in aerobic mesophilic bacterial counts. Dhillon et al. (9) used a 2-step tempering procedure with resting time of 6 hours after each step, to temper durum wheat and hard red spring wheat using ozonated water at 10 and 16 mg/L. However, ozone treatments did not significantly affect microbial load (less than 0.5 log CFU/g reduction in APC) compared with distilled water tempering treatment. In a follow-up study, Dhillon et al. (10) developed a fluidized bed system for wheat disinfection. In this later study, the tempering solution that was a combination of acetic acid (0.5%) and ozonated water (26 mg/L) was the most effective against APC, with an average reduction of 1.6 log CFU/g.

The effects of tempering treatments on *Enterobacteriaceae* population are shown in Figure 2. Reduction trends of *Enterobacteriaceae* counts in wheat tempered with organic acids and saline solutions were similar to those observed for APC counts. Tempering of wheat grain with organic acids at the 1.0% concentration level resulted, on average, in a significant reduction of 0.9 log CFU/g when compared to the initial counts of *Enterobacteriaceae*. As in APC, there was no significant difference in the microbial reduction achieved by the organic acids at the 1.0% concentration level. Increasing the citric and lactic acid concentration from 1.0 % to 2.5%

significantly reduced the number of *Enterobacteriaceae* by 1.4 and 1.6 log CFU/g, respectively. In contrast, increasing the acetic and propionic acid concentration from 1.0 % to 2.5% did not significantly affect the population of *Enterobacteriaceae*. Nevertheless, there were no significant differences in the reductions achieved between organic acids at the 2.5% concentration level.

There were significant differences between the concentrations tested for citric acid and for lactic acid, where an increase in the concentration levels of these acids resulted in a greater reduction of *Enterobacteriaceae* counts. Akbas et al. (2) found that 5ml/L lactic acid and 5g/L citric acid solution reduced the numbers of *Enterobacteriaceae* in fresh-cut iceberg lettuce by 1.5 to 2.0 log CFU/g. The lowest *Enterobacteriaceae* counts were found in lactic acid-dipped lettuce. Likewise, in this study the greatest reduction (2.7 log CFU/g) in the number of *Enterobacteriaceae* when compared to the incoming wheat was achieved by lactic acid at 5.0%, although not statistically different than the reduction achieved (2.3 log CFU/g) by acetic acid at the same level. In fact, lactic acid exhibits a broad-spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria including *Enterobacteriaceae* (2).

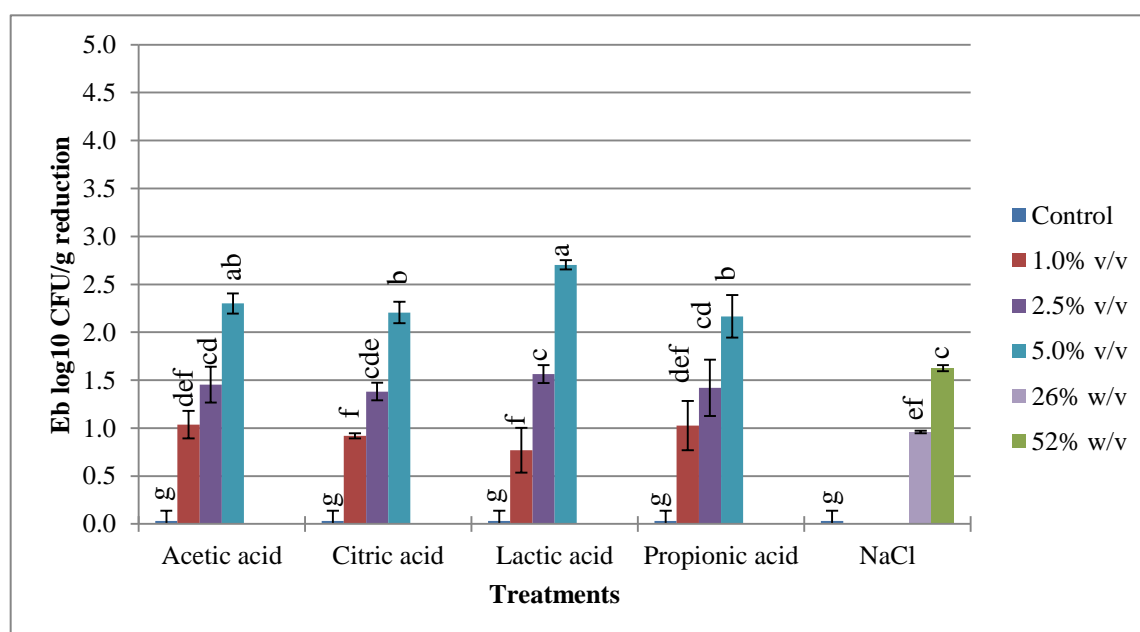


Figure 2. Effects of organic acids and saline solutions on *Enterobacteriaceae* (Eb) counts during wheat tempering. Error bars denote \pm standard deviation. One log reduction is equivalent to a 90% reduction in CFU per gram of wheat grain. Log reduction values with the same letter are not significantly different ($P > 0.05$).

The tempering solution with 26% NaCl (~1% flour basis) reduced the *Enterobacteriaceae* population by 0.9 log CFU/g, which was not significantly different than the reduction achieved by the organic acid solutions at the 1.0% concentration level (Fig. 2). Increasing the NaCl concentration from 26% to 52% (~2% flour basis) significantly reduced *Enterobacteriaceae* counts (1.7 log CFU/g) compared to control. In addition, the reduction achieved by the 52% NaCl tempering solution was not significantly different from the one obtained by the organic acids at the 2.5% concentration level.

Populations of yeasts and molds in wheat, as affected by tempering treatments, are represented in Figures 3 and 4, respectively. Similar reduction trends were observed in both microbial populations. In general, tempering wheat with organic acid and saline solutions resulted in a significant reduction in the initial population of yeasts and molds when compared to control. The greatest reduction (4.7 log CFU/g) in the number of yeasts was achieved by lactic acid treatment at the 5.0% level, which resulted in counts below the detection limit of the method for this group of microorganisms (Fig. 3). However, the reduction was not significantly different than the one achieved by acetic acid at the same concentration level. Similarly, the greatest reduction (3.8 log CFU/g) in mold counts was achieved by the acetic acid, propionic acid, and lactic acid treatments at the 5.0% concentration level, which also resulted in counts below the limit of detection (Fig. 4).

Lactate compounds have demonstrated strong antifungal activity against *Aspergillus* spp. (14), and acetic acid have shown to have a strong inhibition effect against many fungi (22). For any given concentration, the greatest reduction in yeast and mold counts was achieved by the acetic acid treatments. In fact, acetic acid was the most active chemical food preservative when compared with other chemical preservatives in inhibiting the growth of food-associated fungi (23).

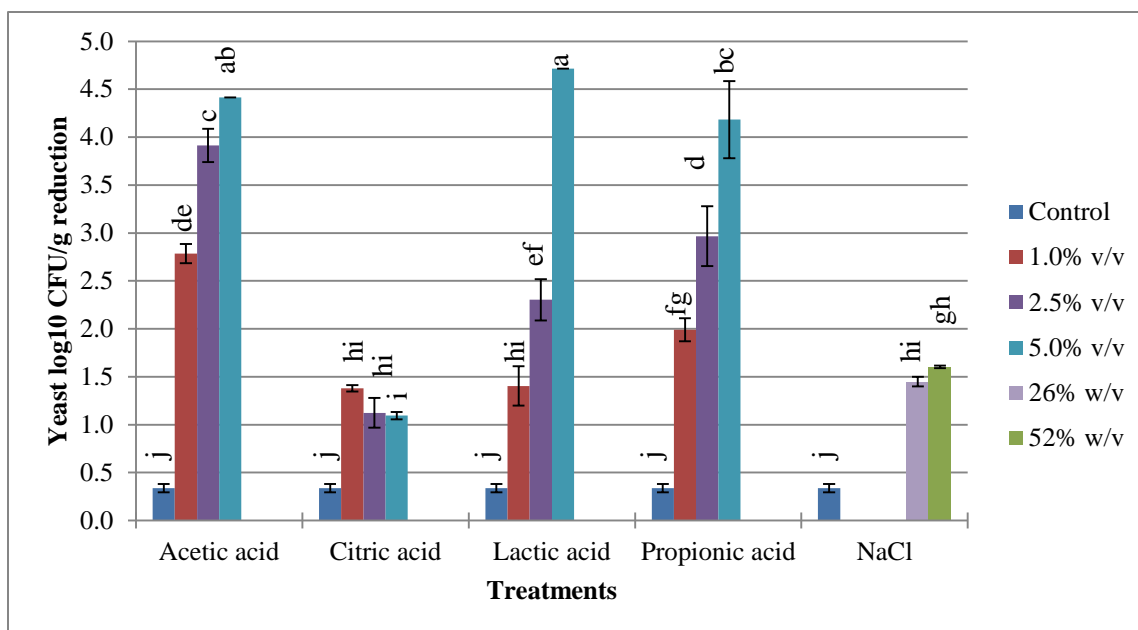


Figure 3. Effects of organic acids and saline solutions on yeast during wheat tempering. Error bars denote \pm standard deviation. One log reduction is equivalent to a 90% reduction in CFU per gram of wheat grain. Log reduction values with the same letter are not significantly different ($P > 0.05$).

Similar reduction levels were reported by Dhillon et al. (10), although using a fluidized bed system. Under the conditions tested by them, a combination of gaseous ozone (6 ppm), acetic acid (0.5%), and ozonated water (26 mg/L) was the most effective in reducing yeast and mold counts by 3.3 log CFU/g. In another study, Dhillon et al. (11) reported that a combination of acetic acid (1.0%) and ozonated water (20.5 mg/L) was the most effective treatment with yeast and mold reductions in the order of 4.1 log CFU/g.

In this study, there was significant difference between the concentrations evaluated for acetic acid, lactic acid, and propionic acid; therefore, increasing the concentration levels of these acids resulted in a greater reduction of yeast and mold counts (Fig. 3 and 4). Published reports show that effectiveness of acetic acid increases as concentration increases and pH decreases (24), which agrees with these findings. Doyle et al. (12) reported the antifungal activity of acetic acid against *Aspergillus* spp., *Penicillium* spp., *Rhizopus* spp., and some strains of *Saccharomyces*.

Lind et al. (20) studied the antifungal activity of propionic, acetic and lactic acid produced by propionibacteria, and reported that propionic acid, followed by acetic and lactic acid were the most potent antifungal acid.

Tempering wheat with citric acid was the least effective treatment for reducing the number of yeasts and molds. When comparing the reduction on yeast counts given by the citric acid treatments, there was no difference among concentration levels. The reduction in mold counts obtained from wheat after tempering with citric acid at 2.5% level were not different than those obtained with the traditional tempering process using water (Fig. 4). These results are in agreement with Pundir et al. (23) who found that citric acid was the least effective antimicrobial against food associated fungi isolated from bakery products and pickles. The tempering solution with 26% NaCl (~1% flour basis) reduced the yeast and mold populations by 1.4 and 1.0 log CFU/g, respectively. Increasing the NaCl concentration level from 26% to 52% did not significantly change the reduction in counts obtained for yeast and mold.

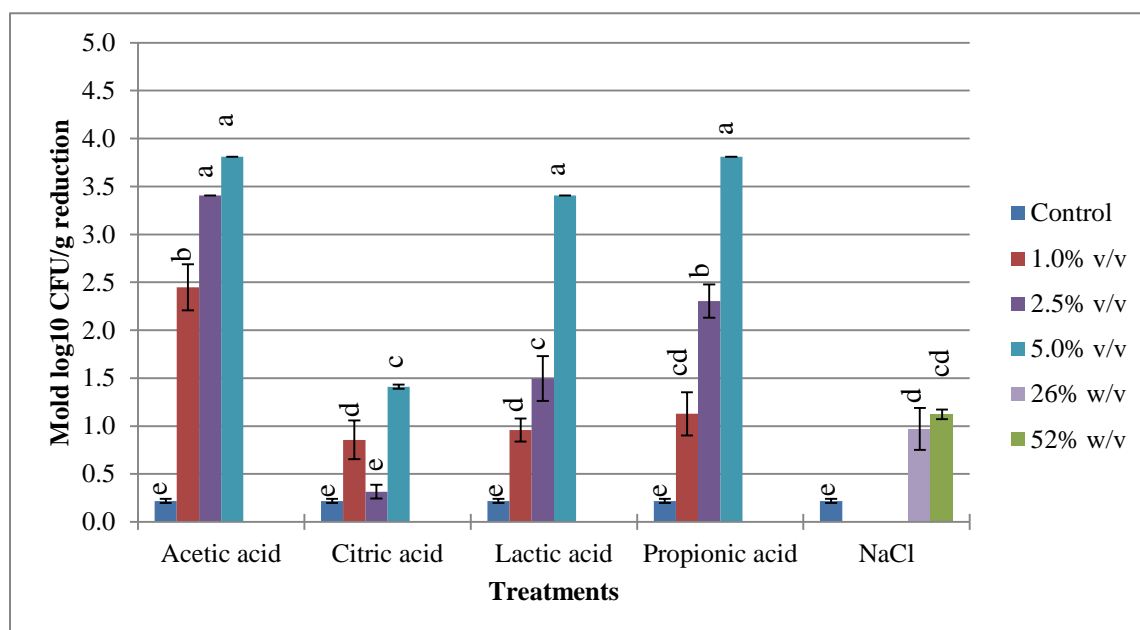


Figure 4. Effects of organic acids and saline solutions on mold counts during wheat tempering. Error bars denote \pm standard deviation. One log reduction is equivalent to a 90% reduction in CFU per gram of wheat grain. Log reduction values with the same letter are not significantly different ($P > 0.05$).

Several reports describe lower reduction in counts than the ones obtained in this study with the most effective tempering solutions. Dhillon et al. (11) reported a 0.5 log CFU/g reduction of yeast and mold counts in wheat after washing with ozonated water (16.5 mg/L). In the same study, 1.9 log CFU/g reduction in yeast and mold counts was achieved with chlorinated water (700 mg/L) washing. Similar results were shown by Ibanoglu (18), who found a slight reduction in yeast and mold counts (0.4 log CFU/g) after washing hard and soft wheat with ozonated water (1.5 mg/L) for 30 min. In an earlier study, Ibanoglu (17) also reported that tempering of wheat with ozonated water (11.5 mg/L) resulted in 1.0 log CFU/g reductions in yeast and mold counts. Reductions obtained with washing treatments may be the result of a combined antimicrobial effect of ozone and/or chloride and the removal of more firmly attached soiling from the wheat kernels.

When comparing the effect of ozonated water on the microbial load present in wheat and produce like fruits and vegetables, ozonated water may be less effective on wheat, since the rough surface and crevices of the kernels may partially shield microorganisms from the ozone (11). However, although the washing treatments used by some authors may increase the exposure of grain surfaces, resulting in uniform and quick contact of grain with antimicrobial agents, it has the drawback of increasing the moisture content thus an additional drying step may be needed to achieve an optimum milling moisture.

In conclusion, the results of this study revealed that organic acid and saline solutions were capable of effectively reducing the microflora of wheat kernels when compared with the traditional tempering process using water. Therefore, these solutions could be useful as surface sanitizers while the kernels reach the optimum milling moisture, thus reducing the risk of microbial contamination in the milled products. In general, lactic acid at 5.0% level was found to be the most effective treatment to reduce the numbers of APC, *Enterobacteriaceae*, yeasts and molds. Saline solutions were shown to be as effective as organic acid solutions at the lower

concentrations tested. However, further studies are needed to define the effects of these tempering solutions on the functional properties of flour, and to establish whether organic acids in combination with sodium chloride would result in greater microbial reduction.

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PRE-MILLING INTERVENTIONS TO REDUCE THE MICROBIAL LOAD OF
STRAIGHT-GRADE AND WHOLE GRAIN FLOUR WITH MINIMAL IMPACT ON
FLOUR FUNCTIONALITY *

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ABSTRACT

The initial microbial load of the grain is a safety concern for wheat-based products. The purpose of this study was to determine the efficacy of adding organic acids and NaCl to tempering water to reduce microbial load in wheat prior to milling, and to determine the impact that these treatments might have on the microbial quality and functional properties of straight-grade and whole grain flours. To accomplish this, wheat was tempered to 15.5% moisture under controlled (24h, 23-24°C, 60% RH), aseptic conditions by adding water (control) or tempering solutions containing acid (acetic or lactic; 2.5% and 5% v/v) and NaCl (~1% and ~2% flour basis (fb)). Wheat was analyzed before and after tempering for Aerobic Plate Counts (APC), yeasts, molds, and *Enterobacteriaceae* (Eb). The microbial load of the tempered wheat was significantly reduced by all organic acid-NaCl treatments. The combination of lactic acid (5%) and NaCl (~2%) was the most effective against APC and Eb, with an average reduction of 4.3 and 4.7 log CFU/g, respectively. After experimental milling, fractions were collected and recombined to obtain straight-grade or whole grain flour. All combinations of organic acid-NaCl were effective in reducing the microbial load of the final milled product. Even though some significant differences in mixing, pasting, and baking properties were observed, their impact on functionality was minimal. For example, whole grain flour from wheat tempered with acids at 5% required longer times (5.3 min) for optimum dough development compared to the control (4.5 min). Additionally, dough mixing tolerance in both types of flour was not affected, regardless the acid level. When bread made with any of the straight-grade flour samples, the cell structure characteristics of bread slides were uniform. Therefore, implementation of organic acids and NaCl in tempering water provides milled products with improved microbiological quality, without compromising functionality.

1. INTRODUCTION

The safety level offered by wheat flour-based products has been questioned over the last decade in light of several food safety incidents. Although regarded as a low-risk commodity for microbial contamination, flour-based mixes have been implicated in foodborne disease outbreaks caused by *Salmonella* and *Escherichia coli* O157:H7 infections (Gilbert et al. 2010; NZFSA, 2008; Neil, 2012). Furthermore, spore-forming *Bacillus* spp. and other spoilage microorganisms (e.g., lactic acid bacteria, yeasts and molds) are among the microbial flora of wheat flour, which have been associated with the spoilage of flour-containing foods (Sorokulova et al. 2003). Even though the usual water activity of wheat milled products are not conducive to microbial growth, microbial contaminants may survive at minimal metabolic activity levels representing a potential health hazard (Eglezos, 2010).

Throughout the wheat production chain there is an ample opportunity for microbes to colonize the surface of the grain; thus, wheat kernels usually harbor a large and varied microbial flora (Laca et al. 2006). Indeed, these microorganisms are considered to be the primary contamination source for milled products. The steps involved in the milling operations, including cleaning, tempering, and grinding, have shown to have a minimal impact on the microbial load of wheat grain (Seiler, 1986). Several studies have documented that the grinding process may cause a redistribution of microbial contaminants across the milled fractions, being the germ and the bran fractions where the microorganisms tend to be concentrated, although flour can still contain a significant amount of microbial contamination (Berghofer et al. 2003).

The sanitary conditions during the tempering and grinding operations may also play an important role in the ultimate safety and quality of milled products. Inspections surveys in commercial milling operations have detected the presence of encrusted grain dust in the tempering bins and build-up of flour residues within the mills, which may harbor a significant

microbial load, thus increasing the risk of microbial contamination during processing (Berghofer et al. 2003). Consequently, the final microbiological quality of wheat milled products can be greatly influenced by these residues if they are not properly controlled.

The susceptibility of flour components (e.g., gluten and starch) to deterioration have limited the number of treatments that may be applied, either to the wheat kernel or to the flour itself, to improve safety. However, some methods have been evaluated to reduce the microbial counts in wheat kernels prior to milling, including chlorination and ozonation of tempering water, and irradiation of wheat kernels (Ibanoglu, 2002; Dhillon et al. 2010). In the previous chapter, the value of adding antimicrobial agents such as organic acids and salt (NaCl) to tempering water as a mean of reducing microbial contamination in wheat grain prior to milling was studied. In this later study, the tempering solutions containing either lactic acid or acetic acid at 5.0% level were remarkably effective at reducing microbial contamination.

Since the use of organic acid and saline solutions during tempering have shown promising results in reducing the microbial load of wheat prior to milling, the objectives of this study were (i) to evaluate the effectiveness of combining organic acid and salt (NaCl) to reduce microbial load in hard wheat during tempering, (ii) to determine the impact that these treatments might have on the microbiological quality and functional properties of straight-grade and whole grain flour, and (iii) to evaluate if a consistent use of wheat tempered with these tempering solutions would reduce microbial establishment within the mill.

2. MATERIALS AND METHODS

2.1 Materials

Hard red winter wheat (McGill) samples were obtained from the 2013 growing season in the state of Nebraska, USA. Samples were provided by Husker Genetics, the University of Nebraska-Lincoln Foundation Seed Division. Food grade lactic acid (85%) and sodium chloride

were obtained from Fisher Scientific. Food grade glacial acetic acid was obtained from BDH Chemicals.

2.2 Preparation of tempering solutions

Individual organic acids and salt (NaCl) were combined in an aqueous solution to examine whether they showed any synergistic effects. Each organic acid (acetic or lactic, at a concentration of 2.5% and 5% v/v) was combined with NaCl (at a concentration of 26% w/v and 52% w/v) to yield a total of 8 different treatment combinations. To prepare each organic acid/salt solution, a 500 ml stock saline solution for each desired concentration was prepared by mixing NaCl and sterile distilled water in appropriate proportions to yield a solution wherein the final salt concentration was 0.26 g/ml (1% flour basis) or 0.52 g/ml (2% flour basis). Then, the respective organic acid and the stock saline solution were mixed in appropriate proportions to yield a solution (100 ml) wherein the final acid concentration was 2.5% and 5.0% (v/v) and the final salt concentration was approximately 1% and 2% (flour basis, fb), respectively. The solutions were always prepared fresh prior to tempering and under aseptic conditions.

2.3 Application of tempering solutions

The initial moisture content of the wheat samples was determined using a forced-air oven method 44-15A (AACCI 2002). The amount of tempering solution needed was calculated based on the initial moisture content of the wheat samples (12.2 %). Three samples (1000 g) for each treatment were placed in sterile plastic bags and tempered to 15.5% moisture by adding sterile distilled water (control) or tempering solutions containing acid (acetic or lactic; 2.5% and 5.0% v/v) and NaCl (~1% and ~2% fb), under aseptic conditions (Biosafety cabinet). Tempering solutions were applied to wheat using an atomizer to ensure even distribution. Samples were shaken vigorously during the first two hours after the addition of tempering solutions and were left to stand for 24 hr under controlled conditions (23-24°C and 60% relative humidity).

Moreover, to evaluate the effectiveness of these solutions in reducing microbial establishment within the mill, sixteen additional samples (1000 g) were tempered with a solution containing lactic acid (5.0%) and NaCl (~1% fb) under the same conditions previously described.

2.4 Experimental milling

The milling experiment was limited to those samples tempered with solutions containing a ~1% (fb) NaCl concentration. Nine hundred grams of each tempered wheat sample were milled using an Buhler experimental mill (Buhler, Minneapolis, MN) according to method 26-21.02 (AACCI 2002). Milled fractions (flour, bran, and shorts) were collected in sterile plastic bags and recombined in appropriate proportions in a tumbling mixer for 3 min to obtain straight-grade and whole grain flour, respectively. The tumbling mixer consisted of a sterile cylindrical container, which was held at a 45° angle and rotated at 6.7 rpm on a circular axis. To evaluate the effectiveness in reducing the microbial load within the mill, sixteen consecutive samples (tempered with lactic acid (5.0%) and NaCl (~1% fb)) were milled and the milled fractions were collected in sterile plastic bags every three runs.

2.5 Evaluation of flour properties and functionality

2.5.1 pH and acidity of flour

pH and total titratable acidity of straight-grade and whole grain flour were determined using the Thermo Scientific Orion 2-Star Benchtop pH meter. A 10g sample was blended with 90 ml distill water and the suspension was then titrated with a 0.1 mol/L NaOH to a final pH of 7.0. The total titratable acidity was expressed as the amount (ml) of NaOH used.

2.5.2 Dough and gluten properties

Dough and gluten properties of straight-grade and whole grain flour were evaluated in duplicate using a Ten g Mixograph (National Manufacturing, Lincoln, NE, USA) according to

AACCI approved method 54.40. (AACC International 2002). The optimum water absorption used during the experiment was estimated based on the equation $Y = 1.5X + 43.6$, in which X is flour protein content on a 14% moisture basis; however, for whole grain flour a +1.8% adjustment in the optimum water absorption was made (Bruinsma et al. 1978). Mixing time, peak height, and right of peak slope were calculated by the Mixograph software (National Manufacturing, Lincoln, NE, USA).

2.5.3 Pasting properties

Pasting properties of straight-grade and whole grain flour were evaluated by rapid viscosity analyzer (RVA). A 3.50 g flour sample (adjusted to 14% moisture basis) and 25 ml distilled water were mixed in an RVA canister by vigorously jogging the stirrer through the sample for 30 s to avoid formation of clumps during the analysis. The mixed samples were then analyzed by RVA (Model 4S, Newport Scientific, Warriewood, NSW, Australia), using Standard-1 profile, following AACCI Approved Method 76-21 (AACC International 2002). Peak viscosity, breakdown, final viscosity, setback, peak time and pasting temperature were recorded by the RVA software (Newport Scientific, Warriewood, NSW, Australia).

2.5.4 Baking properties

Baking quality of straight-grade and whole grain flour were determined using the AACCI approved methods 10-10B and 10-13A, respectively (AACC International 2002), except that the water absorption and mixing time were estimated from the Mixograph data (National Manufacturing, Lincoln, NE, USA; AACCI approved method 54.40). Addition of NaCl to the flour mix formulation was adjusted to maintain equivalent levels of salt and flour in the dough of the treatments and the control. Loaf volume was determined according to AACCI approved method 10-05 (AACC International 2002) after cooling for about 1 hr. Loaves were sliced, 12.5 mm thick per slice, using an electric knife and a bread slicing guide (Black & Decker

Corporation, Towson, MD USA). Bread slices were analyzed for firmness according to AACCI approved method 74-10A (AACC International 2002), and their images were analyzed using a C-Cell imaging system (Calibre Control International Ltd., UK) following the manufacturer's instructions.

2.6 Microbial analysis

Microbial analysis was performed in wheat samples before and after tempering, in each milled fraction, and in the straight-grade and whole grain flour. To determine the microbial load in each sample, 25 g of sample along with 225 ml of phosphate-buffered saline solution was placed in a stomacher bag and mixed for 90 seconds. Serial decimal dilutions were prepared with phosphate-buffered saline solution and analyzed for aerobic mesophilic bacteria (Aerobic Plate Counts (APC)), yeasts, molds, and *Enterobacteriaceae* (Eb). For APC, dilutions were surface plated in triplicate on Standard Methods Agar (SMA, Acumedia) and incubated at 35 °C for 48 hr, according to the U.S. Food and Drug Administration's *Bacteriological Analytical Manual* (Maturin et al. 1998). For yeast and mold counts, dilutions were spread plated in triplicate on Dichloran Rose Begal Chloramphenicol agar (DRBC, Acumedia) and incubated at 25 °C in the dark for 5 days, according to the U.S. Food and Drug Administration's *Bacteriological Analytical Manual* (Tournas et al. 1998). *Enterobacteriaceae* counts were determined according to AOAC official method 2003.01 (AOAC, 2013), using Petrifilm™ (3M Microbiology, St. Paul, MN). Dilutions were inoculated in triplicate on Eb Petrifilm™ and incubated at 37 °C for 24 hr. In addition to APC, yeasts, molds, and Eb, each milled fraction was also analyzed for coliform and generic *E. coli* according to the AOAC official method 991.14 (AOAC, 2013), using Petrifilm™ (3M Microbiology, St. Paul, MN). Dilutions were inoculated in triplicate on coliform Petrifilm™ and incubated at 37 °C for 24 hr.

2.7 Statistical analysis

Data was analyzed using the statistical software SAS version 9.3 (SAS Institute, Cary, NC, USA). To determine differences among treatments, the dough, pasting and baking properties of flour, as well as the microbiological counts obtained were assessed at a 0.05 level of significance by analysis of variance (ANOVA), followed by a multiple comparison Tukey's test.

3. RESULTS AND DISCUSSION

3.1 Combined antimicrobial effects

The previous manuscript showed that the addition of organic acids or NaCl to tempering water was effective in reducing the microbial load of wheat prior to milling. Because the mode of inhibition across the acid-antimicrobials is similar, but differs from salt, it was hypothesized that the interactive effects of two different mechanisms of microbial inhibition would be more effective in reducing microbial load. Therefore, in the present study the combination of organic acid (lactic or acetic) and NaCl was evaluated.

In general, the antimicrobial effect of organic acids, including acetic and lactic, have been attributed to the ability of its protonated (undissociated) form to freely permeate the cell membrane, thus acidifying the cytoplasm and affecting the proton gradient and chemical transport across the membrane (Ricke, 2003). However, the antimicrobial activity may vary among acids depending upon concentration, dissociation level, environmental pH, and temperature (Ricke, 2003). On the other hand, the antimicrobial mechanisms of sodium chloride differ from organic acids, and are mainly due to the reduction in water activity in the product and the toxicity of sodium and chloride ions, when present in excess inside the cytoplasm (Shelef et al. 2005). Moreover, injury of bacterial cells at high concentrations of NaCl could be due to extreme plasmolysis, resulting in indirect damage to the cell outer membrane (Smith et al. 1987).

The effects of tempering solutions containing a combination of organic acid and NaCl, on the total aerobic mesophilic microflora are shown in Figure 1. The mesophilic bacterial population present in wheat was reduced significantly by all organic acid-NaCl treatments. The combination of lactic acid (5.0%) and NaCl (~2.0%) was the most effective against APC with an average reduction of 4.3 log CFU/g. In contrast, tempering wheat with a combination of acetic acid-NaCl was, in general, the least effective treatments for reducing the number of mesophilic bacteria, with reductions ranging from 1.9 to 2.6 log CFU/g.

The previous manuscript demonstrated the ability of individual organic acids and NaCl to reduce the microbial load of wheat during tempering. In such a study, tempering solutions containing lactic acid at 2.5% and 5.0% (v/v) reduced aerobic plate counts by 1.4 and 2.0 log CFU/g, respectively. Similarly, solutions containing NaCl at the concentration levels of 1% and 2% (flour basis) produced a reduction in bacterial counts by 1.0 and 1.4 log CFU/g, respectively.

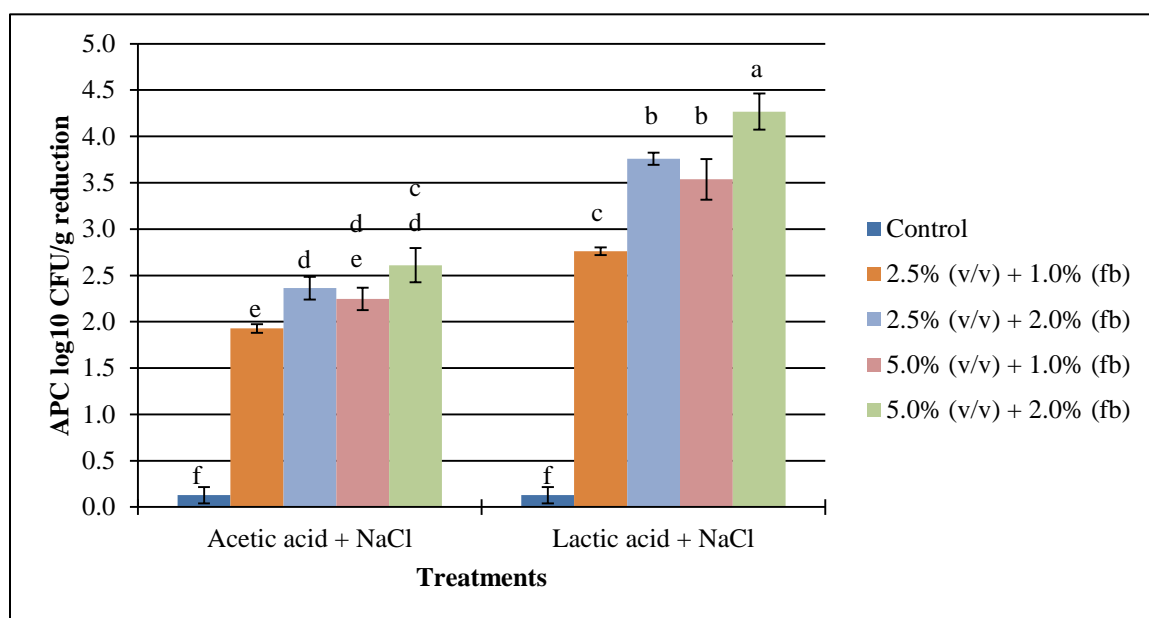


Figure 1. Effect of the combined addition of organic acid and NaCl in tempering water on reducing aerobic plate counts (APC). Error bars denote \pm standard deviation. One log reduction is equivalent to a 90% reduction in CFU per gram of wheat grain. Log reduction values with the same letter are not significantly different ($P > 0.05$).

In the present study, the combined effect of lactic acid and NaCl on reducing APC was significantly greater than the individual effect of the two antimicrobial compounds alone at any given concentration (Fig. 1). When different concentrations of NaCl (~ 1.0% and 2.0% fb) were added to the solution containing 5.0% lactic acid, an additional reduction in APC was observed compared to lactic acid alone. The observed additional reductions varied from 1.5 (5.0% lactic acid + ~1.0% NaCl) to 2.3 log CFU/g (5.0% lactic acid + ~2.0% NaCl). Interestingly, increasing the NaCl concentration at any given lactic acid level significantly further reduced the microbial counts. The reductions obtained by all the lactic acid-NaCl combination treatments were significantly greater than the algebraic sum of the reductions obtained by the individual antimicrobial agent reported in the previous manuscript (Sabillón, 2014), which suggests a synergistic interaction. A synergistic interaction occurs when the effect of two compound taken together is greater than the sum of their separate effect at the same concentration levels.

The previous study reported a reduction of 1.4 and 1.7 log CFU/g in APC using tempering solutions containing acetic acid at the concentration levels of 2.5% and 5.0%, respectively (Sabillón, 2014). Adding NaCl at 1.0% or 2.0% (fb) to the tempering solutions containing acetic acid slightly further reduced the mesophilic bacteria population compared with the previous study (Sabillón, 2014). However, increasing the acetic acid concentration from 2.5% to 5.0% at any given NaCl level did not affect significantly the microbial counts (Fig. 1). Unlike the combination lactic acid-NaCl, the reductions in APC obtained by any given combination of acetic acid-NaCl were not significantly different from the algebraic sum of the reductions achieved by their individual parts, which suggests at the most an additive interaction.

Possible mechanisms underlying the antimicrobial activity of acetic acid are similar to lactic acid, in which the undissociated form of the acid can also traverse the lipid membrane and release protons into the cytoplasm (Guldfeldt et al. 1998); however, the antimicrobial activity

vary with different acids what could explain the different results between acetic and lactic acid reported here.

Previous studies have shown lower levels of microbial reduction than in this experiment. For example, the effect of ozonated or chlorinated washing on microbial load in wheat at different concentration levels was investigated by Dhillon et al. (2009). At 16.5 mg/L ozone concentration, the washing treatment (2L/Kg) reduced APC by 0.2 log CFU/g. Ibanoglu (2002) reported a reduction of 1.0 in APC after washing wheat kernels with ozonated water (1.5 mg/L) for 30 min.

Figure 2 illustrates the effect of tempering treatments on *Enterobacteriaceae* population. The greatest reduction (4.7 log CFU/g) in Eb counts was achieved by lactic acid-NaCl treatments, with one of the combinations potentially being able to reduce Eb population even more since it resulted in counts below the limit of detection. As in APC, in general, the least effective treatments for reducing the number of Eb were the tempering solutions containing a combination of acetic acid and NaCl.

In the previous manuscript reduction levels in Eb counts by 1.6 and 2.7 log CFU/g using tempering solutions containing lactic acid at 2.5% and 5.0% level, respectively, were reported. Likewise, tempering solutions containing NaCl at the concentration levels of 1% and 2% (flour basis) reduced Eb counts by 1.5 and 2.3 log CFU/g, respectively. In the same study, reductions of 1.5 and 2.3 log CFU/g in Eb counts were achieved by tempering solutions containing acetic acid at the concentration levels of 2.5% and 5.0%, respectively.

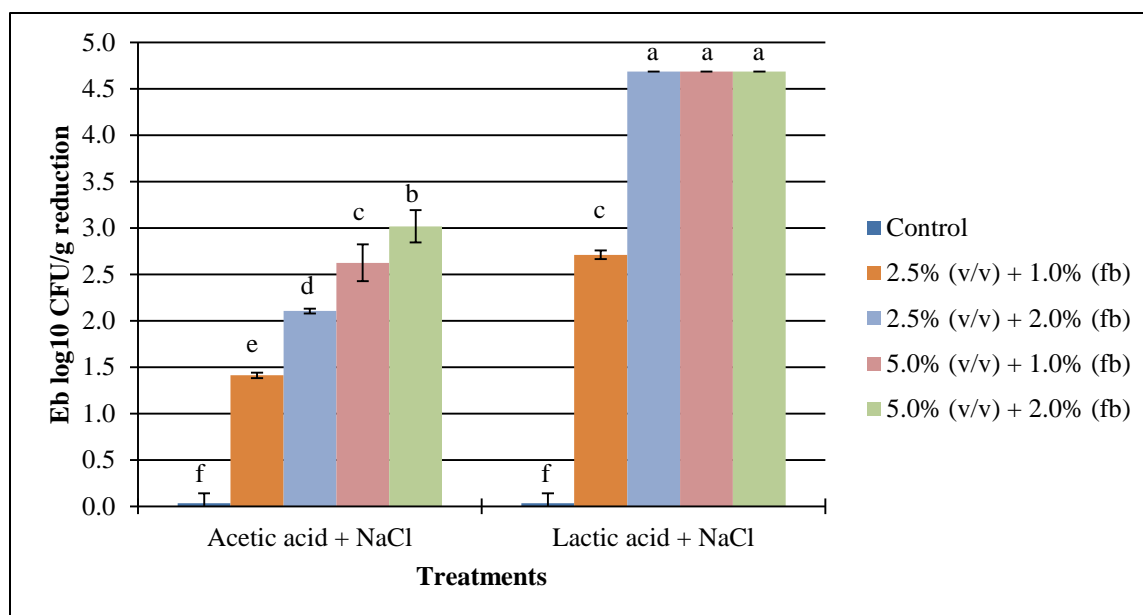


Figure 2. Effect of the combined addition of organic acid and NaCl in tempering water on reducing *Enterobacteriaceae* counts (Eb). Error bars denote \pm standard deviation. One log reduction is equivalent to a 90% reduction in CFU per gram of wheat grain. Log reduction values with the same letter are not significantly different ($P > 0.05$).

As observed with the reductions in APC, a similar synergistic effect between lactic acid and NaCl was observed for Eb counts. The addition of NaCl, regardless the concentration level, to the tempering solution containing lactic acid at 5.0% caused a significant and synergistic additional reduction in Eb counts when compared to lactic acid alone. However, this synergistic effect was not observed by the combination containing lactic acid (2.5%) and NaCl (~1.0%). Even though the Eb counts were significantly further reduced by the addition of NaCl, this reduction was not bigger than the additive effect of both antimicrobials. Likewise, the reductions in the number of Eb obtained by any given combination of acetic acid-NaCl were not significantly different from the algebraic sum of the reductions achieved by their individual parts. In fact, the addition of NaCl at 1.0% to the tempering solutions containing acetic acid it seems not to have an impact, since the reduction in Eb counts was very similar when compared to the reduction achieved by the acetic acid alone in the previous manuscript (Sabillón, 2014).

The permeabilizing nature of the protonated form of lactic acid not only reduces the intracellular pH and disrupt the transmembrane proton-motive force, but also has a potent outer membrane-disintegrating effect on Gram-negative bacterial cells by releasing lipopolysaccharide of the outer layer, thus increasing the permeability of the cell membrane (Alakomi et al. 2000). A synergistic antimicrobial effect against enteric pathogens was reported with a combination of lactic acid and copper. Ibrahim et al. (2008) found that the effectiveness of a 0.2% lactic acid treatment on inhibiting the growth of *Salmonella* spp. and *E. coli* O157:H7 on carrot juice was increased when combined with copper sulfate (50 ppm). When a combination of lactic acid (0.2%) and copper (40 ppm) was sprayed on the surface of previously inoculated lettuce and tomatoes with *E. coli* O157:H7, the population of *E. coli* was reduced significantly by 3.93 and 3.39 log CFU/g, respectively (Gyawali et al. 2011). The authors of these studies suggested that the destabilization of cell membrane caused by lactic acid and its permeabilizer properties facilitate the entrance of copper ions into the bacterial cells which cause a toxic effect, therefore producing a synergistic effect against enteric pathogens. The synergistic effect observed between lactic acid and NaCl in the present study may be explain by the disruptive action of the lactic acid in the outer cell membrane that may have contributed to a greater diffusion and accumulation of NaCl ions into the cytoplasm, thus causing a toxic effect.

The effect of tempering treatments on the populations of yeasts and molds are represented in Figures 3 and 4, respectively. The tempering solution containing either acetic or lactic acid at the 5.0% level, regardless the NaCl concentration, were the most effective in reducing the number of yeasts and molds originally present in wheat.

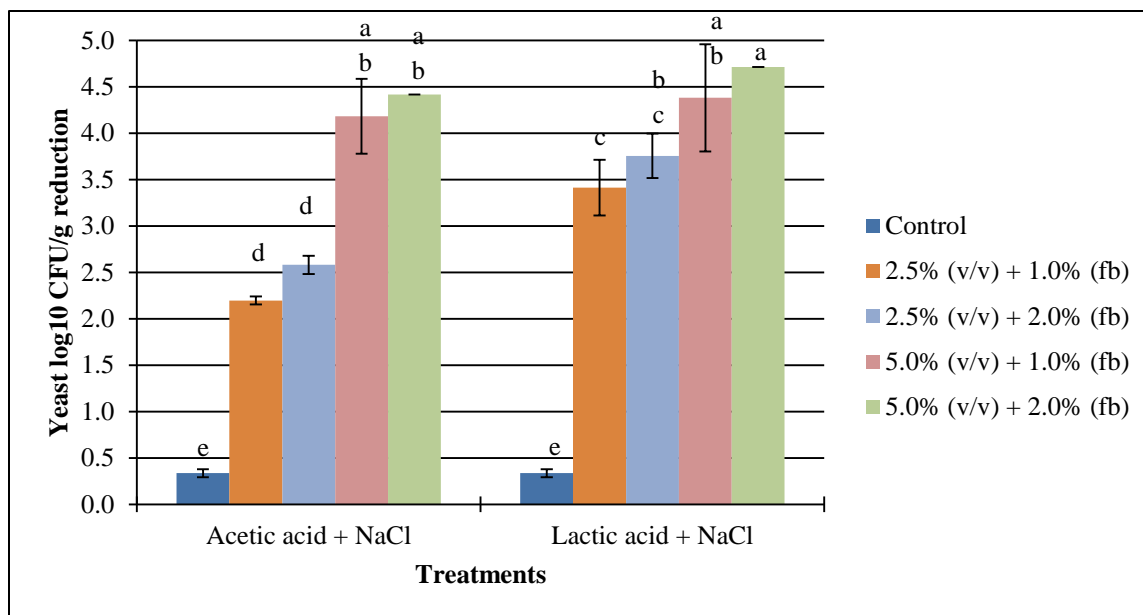


Figure 3. Effect of the combined addition of organic acid and NaCl in tempering water on reducing yeast counts. Error bars denote \pm standard deviation. One log reduction is equivalent to a 90% reduction in CFU per gram of wheat grain. Log reduction values with the same letter are not significantly different ($P > 0.05$).

The previous manuscript reported a reduction of 2.3 and 4.7 log CFU/g in yeast counts using tempering solutions containing lactic acid at the concentration levels of 2.5% and 5.0%, respectively (Sabillón, 2014). In addition, these tempering solutions also achieved a reduction in mold counts of 1.5 and 3.4 log CFU/g, respectively. Although, adding NaCl at 1.0% or 2.0% (fb) to the tempering solutions containing lactic acid at 2.5% further reduced the yeast and mold counts, compared with lactic acid alone (Sabillón, 2014), the reduction achieved was not greater than the sum of the individual effects, which suggests at the most an additive interaction.

In contrast, an antagonist effect of acetic acid and sodium chloride was observed for yeast and mold counts when NaCl, either at 1.0% or 2.0% (fb), was added to the solutions containing acetic acid at 2.5%, since the best reductions achieved for yeasts (~ 2.4 log CFU/g) and molds (~ 1.8 log CFU/g) with the combination of antimicrobials (acid/NaCl) was lower than the previously reported reduction of 3.9 and 3.4 log CFU/g, respectively, achieved by acetic acid alone (Sabillón, 2014).

Sodium chloride (NaCl) has been shown to work as a synergist and an antagonist under different conditions with other antimicrobial components, such as acetic acid. An antagonist effect of acetic acid and sodium chloride has been reported, in which *E. coli* O157:H7 and *Salmonella* were found to be protected by the hypertonicity from acetic acid (Chapman et al. 2009; Lee et al. 2010). Also, combinations of acetic acid and salt were less effective at reducing *E. coli* O157:H7 counts in cucumber puree than did treatment with acetic acid alone (Lee et al. 2010). A similar antagonistic effect observed for the combination of lactic acid and salt for *E. coli* and *E. coli* O157:H45 has been reported by Casey et al. (2002). According to the authors of these studies, several physiological responses of the microorganisms to a hypertonic and acidic environment could explain the protective effect observed. Bacteria may be able to maintain intracellular pH in the presence of NaCl by coupling the import of Na⁺ ions to the H⁺ export (Casey et al. 2002). Hypertonic solutions containing 10% sucrose or 3% NaCl were more protective than 0.5% NaCl. This suggests that an increase in osmotic pressure may induce a slightly plasmolysed physiological state, thus diffusion of protonated acetic acid into the cytoplasm may be slowed by a more rigid cell membrane (Chapman et al. 2009).

The reduction levels in microbial load obtained in the present study are similar to those reported by Dhillon et al. (2009), who studied the influence of combining ozone with acetic acid in tempering water, and found that washing wheat with a combination of acetic acid (1.0%) and ozonated water (20.5 mg/L) was remarkably effective against yeast and mold, with a reduction of 4.1 log CFU/g. Similar reduction levels were obtained in a follow-up study by Dhillon et al. (2010), although using a fluidized bed system. In this later study, washing wheat with an increased concentration of ozone (26 mg/L) in combination with acetic acid (0.5%) was effective in reducing the number of APC and yeasts/molds, with an average reduction of 3.3 log CFU/g.

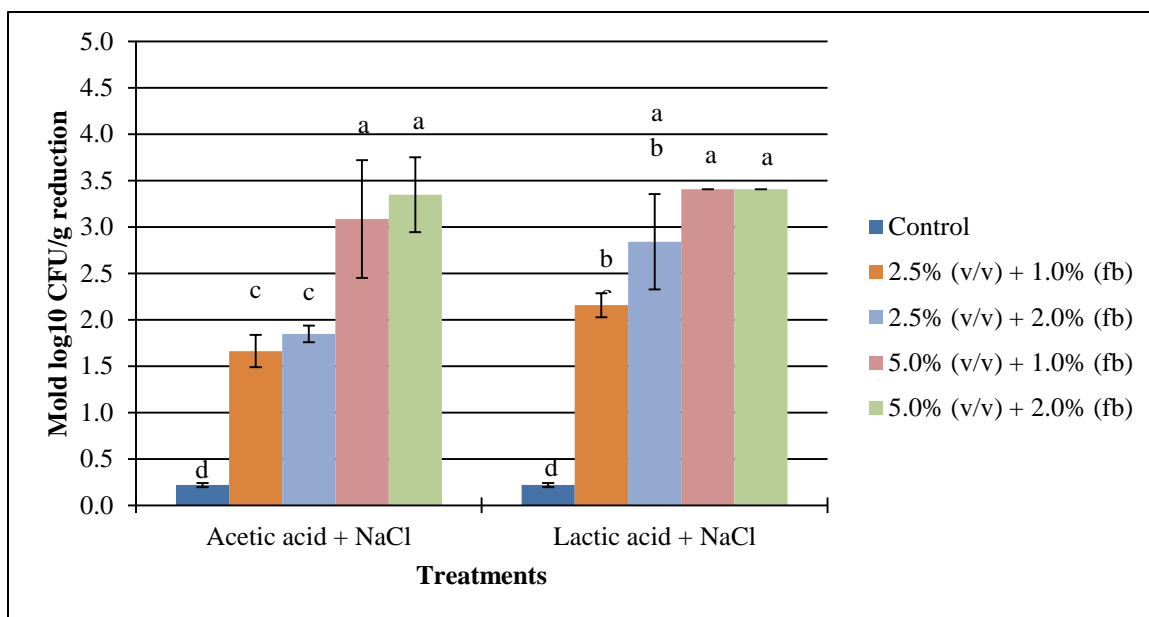


Figure 4. Effect of the combined addition of organic acid and NaCl in tempering water on reducing mold counts. Error bars denote \pm standard deviation. One log reduction is equivalent to a 90% reduction in CFU per gram of wheat grain. Log reduction values with the same letter are not significantly different ($P > 0.05$).

Therefore, combinations of lactic acid and acetic acid with NaCl resulted in different antimicrobial effects against different groups of microorganisms. The antimicrobial synergism observed against certain groups of the wheat microbial flora, for example with the combinations of lactic acid and NaCl could be explained by the permeabilizing and disrupting ability of lactic acid in the cell membrane, thus facilitating the entrance and accumulation of Na^+ and Cl^- ions into the cytoplasm, which eventually results in a cascade of events that is catastrophic to microbial cell viability. On the other hand, the antagonist or in some cases additive effect observed by the combination of acetic acid and NaCl, could be explained by an increase in membrane rigidity, thus slowing the diffusion of acetic acid into the cytoplasm.

3.2 Effect of tempering solutions on reducing microbial load within the mill

Once conditioned, wheat passes through the various systems in the mill to separate the endosperm from the outer grain layers and to reduce the particle size of the endosperm fractions (Posner et al. 1997). The initial process in wheat grinding is the break system, which is designed to remove the endosperm from the bran and germ by the action of corrugated rolls. The ground material is then separated by size and density by a set of sieves or purifiers. The large endosperm particles are reduced into fine particle of flour in the reduction system by the action of smooth rolls. The isolated bran and germ are further process in the millfeed system to remove any adhered endosperm and to purify and separate each fraction.

The milling process may vary among milling operations depending upon the type of wheat used and the end-use of the milled product. However, regardless of the variations in the milling system, wheat kernels experience a shearing action as well as a crushing action to break apart the bran and endosperm, and later in the process the isolated endosperm also experiences a crushing action to reduce the particle size. This grinding operation creates a considerable amount of heat; hence moisture condensation is likely to occur throughout the milling system, especially in the break and reduction rolls, which may lead to build-up of flour residues inside the milling equipment (Berghofer et al. 2003).

Wheat kernels usually harbor a large and varied microbial flora, which are usually not affected by the traditional cleaning and tempering procedures prior to milling (Berghofer et al. 2003). Therefore, wheat entering the milling system may contain significant amounts of microbial contaminant, which may become established in the build-up residues and in the surface of the milling equipment, thus increasing the risk of microbial contamination during milling. Berghofer et al. (2003) reported that 47 out of 58 wheat samples tested positive for *Bacillus cereus*; however, after milling a higher proportion of milled products (93% of flour and 94% of bran

samples) tested positive for *B. cereus*, thus indicating that *B. cereus* spores may be present in the milling equipment.

Figure 5 illustrates the postprocessing contamination levels of wheat milled products during sixteen consecutive runs in a Buhler experimental mill. The wheat entering the milling system was of a superior microbiological quality, since the tempering solution containing lactic acid (5.0%) and NaCl (~1.0%), which reduced the microbial population of wheat kernels to below the limit of detection used for this evaluation (data not shown). Since microbial contamination could not be detected in wheat after tempering, it was hypothesized that a consistent use of this wheat, over time, would reduce microbial establishment within the mill.

Target microorganisms that were not detected in wheat grain after tempering, were detected in all milled fractions after milling, which suggested a recontamination as wheat passed through the various systems in the mill (Fig. 5). Since the microbial load is concentrated in the surface of the grain, the mill sections where the bran and germ pass through may be expected to be the most contaminated sections of the mill. Indeed, as the wheat passed through the mill on the first run the fractions that were recontaminated the most were the germ and the bran fractions. For any given fraction, the least reduction in microbial counts was observed for APC and molds. After sixteen consecutive runs, the APC counts in the germ fraction were reduced from 5.2 to 2.9 log CFU/g. However, as a general trend, as wheat samples were milled in consecutive runs, the microbial load within the mill was reduced by the wheat samples as indicated by the microbial load of each milled fraction. After sixteen consecutive runs, the yeast and Eb counts were below the detection limit used for evaluation of these two groups of microorganisms in all milled fractions.

In a Buhler experimental mill, coarse bran particles comes off the top of the sieves of the last break stage, while the germ (shorts) comes off the top of the sieves of the last reduction stage (Posner et al. 2005). Among milled fractions, the germ fraction spends the highest amount of time in the milling system and also contains the highest amount of lipids (~11%), which could explain why this fraction suffered the most recontamination after the first run and in general required the most amount of runs to allow for a reduction in its microbial load.

Therefore, these results suggests that the use of wheat with a higher microbiological quality, over time, may contribute to reduce the microbial establishment within the mill, thus reducing the risk of microbial contamination or recontamination of the milled fractions during processing.

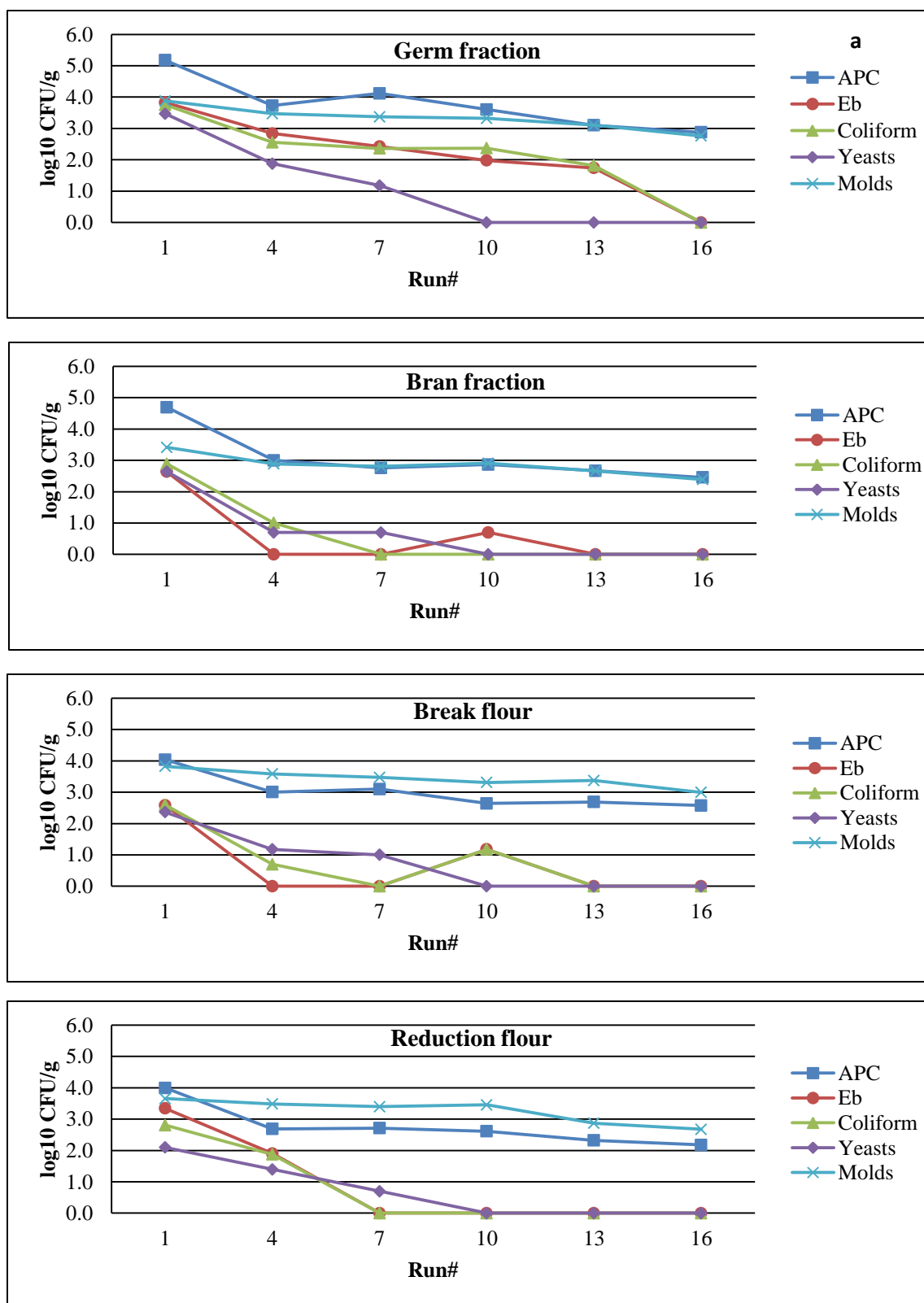


Figure 5. Microbial load of different wheat milling end products during sixteen consecutive runs in a Buhler experimental mill. ^a APC, Aerobic plate counts; Eb, *Enterobacteriaceae*.

3.3 Effect of tempering solutions on the microbiological quality of flour

The microbiological quality and properties of flour were examined on all tempering solutions containing ~1% NaCl. Although effective as antimicrobial treatment, the combinations using ~2% NaCl were not included for further analysis as they presented saturation issues (lack of ability to completely dissolve the salt content); making them not useful in larger scale from a practical standpoint.

Table 1 illustrates the microbial load of straight-grade and whole grain flour obtained from the different treatments. All combinations of organic acid-NaCl were effective in reducing the microbial load of the final milled product. In general, whole grain flour presented higher microbial counts than straight-grade flour. Since the microbial contaminants are concentrated in the bran, it was expected that the microbiological quality of whole grain flour would be lower than the flour obtained just from the inner endosperm. Tempering solutions containing organic acids at the 5.0% level were the most effective in reducing the number of yeasts, and molds in the whole grain flour.

Table 1. Mean values and standard deviation for the microbial load of straight-grade and whole grain flour ¹

Flour type	Treatments ²	Final Counts (Log CFU/g) ³			
		APC	Yeasts	Molds	Eb
Straight-grade flour	Control (water)	3.27 (0.03)a ⁴	2.80 (0.08)a	2.59 (0.03)a	2.37 (0.10)a
	Lactic acid 2.5%	2.62 (0.22)b	1.54 (0.13)b	2.07 (0.16)b	1.81 (0.18)bc
	Lactic acid 5.0%	2.28 (0.07)c	1.07 (0.19)c	1.51 (0.16)c	1.56 (0.27)bc
	Acetic acid 2.5%	2.59 (0.12)bc	1.37 (0.04)bc	1.95 (0.03)b	1.99 (0.21)b
	Acetic acid 5.0%	2.45 (0.06)bc	1.13 (0.10)c	1.34 (0.10)c	1.49 (0.13)c
Whole grain flour	Control (water)	4.39 (0.04)a	3.64 (0.10)a	3.00 (0.03)a	3.12 (0.03)a
	Lactic acid 2.5%	3.39 (0.12)b	1.96 (0.11)b	2.34 (0.10)b	2.64 (0.10)b
	Lactic acid 5.0%	3.26 (0.08)b	1.23 (0.16)c	1.73 (0.13)c	2.28 (0.15)c
	Acetic acid 2.5%	3.48 (0.16)b	1.91 (0.05)b	2.26 (0.01)b	2.64 (0.09)b
	Acetic acid 5.0%	3.32 (0.10)b	1.22 (0.03)c	1.88 (0.12)c	2.43 (0.10)bc

¹ Values denote mean±(standard deviation)

² The tempering treatments also contain ~1.0% NaCl, except the control

³ APC, Aerobic Plate Counts; Eb, *Enterobacteriaceae*

⁴ Means followed by the same letter, within the same column and type of flour, are not significantly different ($p > 0.05$).

3.4 Effect of tempering solutions on flour properties and functionality

3.4.1 pH and acidity of straight-grade and whole grain flour

Table 2 summarizes the pH and acidity of straight-grade and whole grain flour obtained from the different treatments. As a general trend, as the acid concentration increased from 2.5 to 5.0% the pH of the flour decreased and the total acid concentration (titratable acidity) increased, except for lactic acid treatments in straight-grade flour. The titratable acidity provides a good estimate of the total acid content, and based on the results the organic acids used in tempering solutions seem to be concentrated in the outer bran layer of the kernel, since the acid content in whole grain flour was higher than in straight-grade flour.

Table 2. Mean values and standard deviation for pH and acidity of straight-grade and whole grain flour ¹

Test	Treatments	Flour type	
		Straight-grade flour	Whole grain flour
Flour pH	Control (water)	5.74 (0.04)a ²	6.25 (0.02)a
	Lactic acid 2.5% + NaCl 1.0%	5.73 (0.02)a	5.79 (0.02)b
	Lactic acid 5.0% + NaCl 1.0%	5.58 (0.01)b	5.51 (0.05)c
	Acetic acid 2.5% + NaCl 1.0%	5.40 (0.02)c	5.74 (0.01)b
	Acetic acid 5.0% + NaCl 1.0%	5.28 (0.02)d	5.41 (0.02)d
Titratable Acidity (ml 0.1N NaOH)	Control (water)	1.50 (0.28)c	1.53 (0.07)e
	Lactic acid 2.5% + NaCl 1.0%	1.43 (0.06)c	2.90 (0.00)d
	Lactic acid 5.0% + NaCl 1.0%	1.60 (0.00)c	4.23 (0.15)b
	Acetic acid 2.5% + NaCl 1.0%	2.00 (0.00)b	3.16 (0.06)c
	Acetic acid 5.0% + NaCl 1.0%	2.28 (0.04)a	4.59 (0.02)a

¹ Values denote mean±(standard deviation)

² Means followed by the same letter, within the same column and test, are not significantly different ($p > 0.05$).

However, trace amounts of acid, especially acetic acid, seems to be able to traverse the outer layer and reach the inner endosperm, since the total acid content in the straight-grade flour obtained from wheat tempered with acetic acid was higher than the flour obtained from lactic acid treatments and the control.

Food labeling regulations is an important part that needs to be addressed when adding additives to foods or their processing. The Code of Federal Regulations 21CFR101.100 provide guidelines about the exemptions from food labeling requirements regarding processing aids and incidental additives. According to the code, incidental additives that are present in a food at insignificant levels and do not have any technical or functional effect in that food may be exempt from compliance with the food labeling requirements. According to 21CFR101.00, incidental additives are: “substances that are added to a food during processing, are converted into constituents normally present in the food, and do not significantly increase the amount of the constituents naturally found in the food”. Therefore, based on the total acid content found in the flour samples, straight-grade flour tempered with 2.5% or 5.0% lactic acid in combination with ~1.0% NaCl would not require labeling declaration; however, all the other treatments, especially the ones applied to produce whole grain flour might require labeling declaration, since the acids are retained by the outer layers of the grain (bran), it is added back to the flour when producing whole grain flour which leads to an increase in the amount of acid naturally present in the wheat kernel.

3.4.2 Dough and gluten properties

The mixing properties of straight-grade and whole grain flour are shown in Table 3. Whole grain flour from wheat tempered with acetic acid at 5.0% required longer time (5.3 min) for optimum dough development (peak time) compared to the control (4.5 min). In contrast, organic acid-NaCl treatments did not affect dough-development time for straight-grade flour. Galal et al. (1978) reported that a combination of six organic acids isolated from sourdough and NaCl (1.5%) increased dough-development time and dough stability of hard red winter wheat flour, although using a Farinograph. Similar results were also observed by Bakhoun et al. (1982),

who found that mixing time and stability were increased as acid and salt were added to hard red winter flour.

Straight-grade flour obtained from all treatments showed higher maximum force for mixing than the control flour (peak height), but that was not observed in whole grain flour. Additionally, dough mixing tolerance (right of peak slope) in both straight-grade and whole grain flour was not affected, regardless the acid level.

No significant differences were found in the water absorption between the different treatments in the straight-grade flour. However, whole grain flour obtained from wheat tempered with acetic acid at 5.0% showed a lower water absorption compared to the control flour, which may account for the longer time required to reach optimum dough development (peak time). Galal et al. (1978) found that the water absorption decreased significantly when organic acid and NaCl were added to the flour, regardless the level of NaCl (1.0 to 1.5%). The author argued that acids would create a repulsive force by increasing the number of positively charged sites in the protein structure thus causing the coiled part of the molecule to unfold, while NaCl would tighten the unfolded part of the protein by suppressing the repulsion force, thus reducing the water absorption.

Results presented here suggest that even though some significant differences in mixing properties were observed, their impact on functionality was minimal. Perhaps because organic acids seem to be retained by the bran (Table 2), slight variations, if any, in mixing properties are more likely to occur in whole grain flour than in straight-grade flour.

Table 3. Mean values and standard deviation for dough and gluten properties of straight-grade and whole grain flour ¹

Mixograph Parameter	Treatments	Flour Type	
		Straight-grade flour	Whole grain flour
Peak Time (min)	Control (water)	5.75 (0.32)a ²	4.48 (0.61)b
	Lactic acid 2.5% + NaCl 1.0%	5.58 (0.41)a	4.61 (0.24)ab
	Lactic acid 5.0% + NaCl 1.0%	5.29 (0.43)a	5.05 (0.35)ab
	Acetic acid 2.5% + NaCl 1.0%	5.10 (0.36)a	4.68 (0.26)ab
	Acetic acid 5.0% + NaCl 1.0%	5.23 (0.28)a	5.32 (0.73)a
Peak Height (%)	Control (water)	49.36 (0.87)b	45.33 (1.00)a
	Lactic acid 2.5% + NaCl 1.0%	51.55 (2.02)a	45.85 (1.23)a
	Lactic acid 5.0% + NaCl 1.0%	53.18 (0.48)a	45.56 (0.69)a
	Acetic acid 2.5% + NaCl 1.0%	52.31 (0.44)a	45.58 (0.99)a
	Acetic acid 5.0% + NaCl 1.0%	52.41 (0.69)a	45.95 (1.03)a
Right of Peak Slope (%/m)	Control (water)	-1.08 (0.51)a	-0.88 (0.47)a
	Lactic acid 2.5% + NaCl 1.0%	-0.52 (1.16)a	-1.42 (1.04)a
	Lactic acid 5.0% + NaCl 1.0%	-1.23 (0.59)a	-1.42 (0.75)a
	Acetic acid 2.5% + NaCl 1.0%	-0.43 (1.14)a	-1.05 (1.05)a
	Acetic acid 5.0% + NaCl 1.0%	-1.16 (0.64)a	-1.58 (0.65)a
Predicted Water Absorption (%)	Control (water)	57.14 (0.01)a	64.35 (0.18)a
	Lactic acid 2.5% + NaCl 1.0%	57.13 (0.01)a	64.13 (0.14)ab
	Lactic acid 5.0% + NaCl 1.0%	57.12 (0.03)a	64.13 (0.19)ab
	Acetic acid 2.5% + NaCl 1.0%	57.11 (0.06)a	64.16 (0.05)ab
	Acetic acid 5.0% + NaCl 1.0%	57.12 (0.02)a	64.10 (0.09)b

¹ Values denote mean±(standard deviation)

² Means followed by the same letter, within the same column and Mixograph parameter, are not significantly different ($p > 0.05$).

3.4.3 Pasting properties

Pasting properties of straight-grade and whole grain flour are presented in Table 4. The peak viscosity indicates the water-binding capacity of the starch granules or mixtures, and this parameter in the straight-grade flour obtained from wheat tempered with acetic acid at the 5.0% level did show a significant reduction in the peak when compared to the control flour. Moreover, the use of organic acids and NaCl, at any concentration level, did not significantly affect the peak viscosity of whole grain flour compared to the control flour. Published reports suggested a significant increase in the peak height of wheat starch with the inclusion of NaCl (1 to 3.5%) (Ganz, 1965; Medcalf et al. 1966; D'Appolonia, 1972). The authors of these studies argue that the presence of NaCl causes the starch granules to remain intact for a longer period of time before breaking apart. However, in the presence of high ascorbic acid concentrations, a decrease in peak height was observed in starch mixtures by D'Appolonia (1972), who attributed the change in the pasting characteristics to accelerated hydration of the starch. In the present study, in general, no such changes were observed possibly because the organic acids and NaCl were not directly applied to the flour itself and the final organic acid and NaCl concentration in the flour was not enough to cause a detrimental effect.

Once the starch granules reach peak viscosity, a progressive decrease in viscosity occurs as a result of the physical destruction of swollen starch granules under constant high temperature and mechanical shear stress. The breakdown viscosity (difference between “peak viscosity” and “trough viscosity”) represents the granules’ susceptibility to applied shear and heat. Based on results shown in Table 4, no appreciable differences were noted in the breakdown viscosity of the straight-grade and whole grain flour in any of the treatments compared to the control flour.

The final viscosity indicates the ability of the starch granules or mixtures to form a viscous paste or gel after cooking and cooling. In addition, it is largely determined by the

retrogradation tendency of the soluble amylose on cooling (Olkku et al. 1978). The final viscosity of the straight-grade flour obtained from wheat tempered with acetic acid at the 5.0% level was significantly lower than the one obtained with the control flour. Moreover, the final viscosity of the whole grain flour obtained from all the organic acid-NaCl treatments was also significantly lower than the control flour. Similar results were observed by Wu et al. (2010), although in nonwaxy rice flour. In their study, a significant decrease in final viscosity was noticed with the addition of 0.1 mM organic acid (acetic, lactic, malic and citric) when compared to control. Ohishi et al. (2007) also found that final viscosity and setback of rice flour were significantly decreased with addition of 0.05–0.2 M acetic acid.

The setback is a measure of the degree of retrogradation, or re-association, of the starch molecules during cooling. No statistical difference was observed in the level of retrogradation among the different straight-grade flour samples, including the control flour. However, as in the final viscosity, whole grain flour obtained from tempering treatments showed lower setback tendencies than the control flour, which suggest that tempering wheat with organic acids and NaCl may produce flour with less tendency to retrograde.

The peak time indicate the time required for a mixture to reach its peak viscosity. There was no statistically significant difference in the time required among straight-grade flour to reach such peak viscosity. However, less time compared to control was required for the whole grain flour obtained from the organic acid-NaCl treatments to reach the peak viscosity. As shown in Table 2, organic acids seem to be concentrated in the outer bran layer of the wheat kernel, thus reducing the pH of the whole grain flour-water mixture, which may cause starch granules to become fragile and break down relatively rapidly after acidification, thus reducing the time required to reach the peak viscosity. The pasting temperature, which indicates the onset of the rise in viscosity, did not differ among treatments for straight-grade or whole grain flour.

Table 4. Mean values and standard deviation for RVA pasting parameters of straight-grade and whole grain flour ¹

RVA Parameter	Treatments	Flour type	
		Straight-grade flour	Whole grain flour
Peak Viscosity (cp)	Control (water)	3459.33 (37.86)a ²	2232.66 (12.50)a
	Lactic acid 2.5% + NaCl 1.0%	3403.66 (73.66)ab	2077.33 (24.83)a
	Lactic acid 5.0% + NaCl 1.0%	3353.00 (59.23)ab	2011.66 (25.42)a
	Acetic acid 2.5% + NaCl 1.0%	3340.33 (24.03)ab	2266.33 (245.70)a
	Acetic acid 5.0% + NaCl 1.0%	3309.00 (59.30)b	2073.00 (26.23)a
Trough (cp)	Control (water)	1824.66 (60.19)a	1154.33 (27.30)a
	Lactic acid 2.5% + NaCl 1.0%	1868.00 (31.19)a	1043.00 (17.35)ab
	Lactic acid 5.0% + NaCl 1.0%	1833.67 (100.6)a	981.000 (37.51)b
	Acetic acid 2.5% + NaCl 1.0%	1895.00 (108.0)a	1094.67 (104.6)ab
	Acetic acid 5.0% + NaCl 1.0%	1740.00 (50.57)a	972.000 (53.67)b
Breakdown (cp)	Control (water)	1634.66 (29.87)a	1078.33 (17.90)a
	Lactic acid 2.5% + NaCl 1.0%	1535.66 (65.43)a	1034.33 (23.03)a
	Lactic acid 5.0% + NaCl 1.0%	1519.33 (99.03)a	1030.66 (18.58)a
	Acetic acid 2.5% + NaCl 1.0%	1445.33 (96.78)a	1171.66 (142.1)a
	Acetic acid 5.0% + NaCl 1.0%	1569.00 (84.18)a	1101.00 (28.58)a
Final Viscosity (cp)	Control (water)	2892.33 (39.17)a	2015.66 (19.30)a
	Lactic acid 2.5% + NaCl 1.0%	2859.33 (37.22)ab	1749.33 (4.160)b
	Lactic acid 5.0% + NaCl 1.0%	2858.66 (43.62)ab	1665.33 (39.26)b
	Acetic acid 2.5% + NaCl 1.0%	2849.33 (70.49)ab	1764.67 (147.5)b
	Acetic acid 5.0% + NaCl 1.0%	2730.66 (45.32)b	1672.66 (49.10)b
Setback (cp)	Control (water)	1067.66 (94.77)a	861.33 (8.620)a
	Lactic acid 2.5% + NaCl 1.0%	991.3300 (6.03)a	706.33 (13.80)b
	Lactic acid 5.0% + NaCl 1.0%	1025.00 (62.23)a	684.330 (4.93)b
	Acetic acid 2.5% + NaCl 1.0%	954.330 (42.22)a	770.00 (69.86)b
	Acetic acid 5.0% + NaCl 1.0%	990.660 (22.50)a	700.660 (6.51)b
Peak Time (min)	Control (water)	6.02 (0.04)a	6.04 (0.08)a
	Lactic acid 2.5% + NaCl 1.0%	6.04 (0.04)a	5.84 (0.04)b
	Lactic acid 5.0% + NaCl 1.0%	6.04 (0.08)a	5.88 (0.04)b
	Acetic acid 2.5% + NaCl 1.0%	6.06 (0.07)a	5.80 (0.07)b
	Acetic acid 5.0% + NaCl 1.0%	6.00 (0.07)a	5.82 (0.08)b
Pasting Temperature (°C)	Control (water)	52.11 (1.76)a	52.65 (4.12)a
	Lactic acid 2.5% + NaCl 1.0%	51.00 (1.43)a	51.51 (1.32)a
	Lactic acid 5.0% + NaCl 1.0%	50.43 (0.49)a	52.15 (0.52)a
	Acetic acid 2.5% + NaCl 1.0%	50.95 (0.85)a	50.83 (0.75)a
	Acetic acid 5.0% + NaCl 1.0%	52.08 (0.49)a	53.00 (2.30)a

¹ Values denote mean±(standard deviation)² Means followed by the same letter, within the same column and RVA parameter, are not significantly different (p > 0.05).

3.3.4 Baking quality

Table 5 summarizes the loaf volume and firmness of bread made from straight-grade and whole grain flour. In loaves made from straight-grade flour, no significant differences in volume and firmness were observed. Bread firmness was higher when whole grain flour obtained from wheat tempered with organic acid, either lactic or acetic, at the 2.5% level was used. However, as the acid concentration increased to 5.0% the firmness decreased, up to a point where it was not significantly different than the control bread. Bakhoun et al. (1982) using white flour observed that, in the presence of NaCl, loaf volume may increase with increasing acid concentration up to a certain level. However, this effect was not evident in this study for neither type of flour evaluated, since organic acids may not reach the inner endosperm in sufficient amounts to cause the same raising effect and whole grain flour contains a number of non-functional components (i.e., bran and germ) which also may interfere with bread volume (Table 2).

Table 5. Mean values and standard deviation for straight-grade and whole grain bread loaf volume and firmness ¹

Bread Analysis		Bread type	
Parameter	Treatments	Straight-grade bread	Whole grain bread
Loaf Volume (cc)	Control (water)	609.67 (34.27)a ²	358.67 (4.27)a
	Lactic acid 2.5% + NaCl 1.0%	612.00 (12.96)a	358.33 (5.89)a
	Lactic acid 5.0% + NaCl 1.0%	605.50 (20.53)a	367.83 (15.04)a
	Acetic acid 2.5% + NaCl 1.0%	626.17 (14.63)a	355.00 (12.70)a
	Acetic acid 5.0% + NaCl 1.0%	622.00 (24.08)a	367.50 (12.29)a
Bread Firmness (N)	Control (water)	0.79 (0.09)a	4.09 (0.75)b
	Lactic acid 2.5% + NaCl 1.0%	0.81 (0.07)a	4.76 (0.92)a
	Lactic acid 5.0% + NaCl 1.0%	0.79 (0.11)a	4.43 (0.72)ab
	Acetic acid 2.5% + NaCl 1.0%	0.73 (0.12)a	4.85 (0.40)a
	Acetic acid 5.0% + NaCl 1.0%	0.80 (0.09)a	3.91 (0.42)b

¹ Values denote mean±(standard deviation)

² Means followed by the same letter, within the same column and bread analysis parameter, are not significantly different ($p > 0.05$).

Table 6 illustrates the image analysis of bread slices made from straight-grade and whole grain flour. Some parameters, including slice area, height, brightness, and number of air cells,

presented some differences among the treatments, especially in bread made from whole grain flour.

Table 6. Mean values and standard deviation for straight-grade and whole grain bread image analysis ¹

Bread Analysis		Bread type	
Parameter	Treatments	Straight-grade bread	Whole grain bread
Slice Area (mm ²)	Control (water)	5381.50 (273.98)a ²	3553.16 (264.54)a
	Lactic acid 2.5% + NaCl 1.0%	5452.16 (118.55)a	3363.55 (231.86)b
	Lactic acid 5.0% + NaCl 1.0%	5362.00 (171.16)a	3453.72 (186.29)ab
	Acetic acid 2.5% + NaCl 1.0%	5477.11 (154.23)a	3438.16 (96.730)ab
	Acetic acid 5.0% + NaCl 1.0%	5402.16 (176.20)a	3562.77 (109.20)a
Slice Height (mm)	Control (water)	63.97 (1.43)a	65.46 (4.37)a
	Lactic acid 2.5% + NaCl 1.0%	64.69 (2.06)a	61.58 (4.98)b
	Lactic acid 5.0% + NaCl 1.0%	64.01 (1.47)a	63.25 (2.21)ab
	Acetic acid 2.5% + NaCl 1.0%	64.86 (1.95)a	64.92 (1.47)a
	Acetic acid 5.0% + NaCl 1.0%	64.72 (1.05)a	66.10 (1.79)a
Slice Brightness	Control (water)	165.27 (4.22)a	92.01 (3.71)a
	Lactic acid 2.5% + NaCl 1.0%	159.25 (6.75)b	89.43 (1.34)b
	Lactic acid 5.0% + NaCl 1.0%	156.92 (5.34)b	88.00 (1.88)b
	Acetic acid 2.5% + NaCl 1.0%	159.76 (2.95)b	89.53 (2.10)b
	Acetic acid 5.0% + NaCl 1.0%	160.52 (3.77)b	88.45 (2.08)b
Number of Cells	Control (water)	2881.11 (151.15)a	1913.55 (169.42)b
	Lactic acid 2.5% + NaCl 1.0%	2947.44 (176.01)a	1903.00 (125.00)b
	Lactic acid 5.0% + NaCl 1.0%	2990.83 (153.79)a	1966.50 (116.10)ab
	Acetic acid 2.5% + NaCl 1.0%	2916.05 (101.24)a	1956.72 (82.820)ab
	Acetic acid 5.0% + NaCl 1.0%	2981.55 (109.75)a	1984.11 (62.530)a
Cell Diameter (mm)	Control (water)	2.39 (0.19)a	2.39 (0.16)a
	Lactic acid 2.5% + NaCl 1.0%	2.35 (0.12)a	2.24 (0.11)b
	Lactic acid 5.0% + NaCl 1.0%	2.28 (0.15)a	2.18 (0.11)b
	Acetic acid 2.5% + NaCl 1.0%	2.34 (0.13)a	2.18 (0.13)b
	Acetic acid 5.0% + NaCl 1.0%	2.30 (0.10)a	2.14 (0.10)b
Average Cell Elongation (mm)	Control (water)	1.70 (0.03)a	1.75 (0.06)a
	Lactic acid 2.5% + NaCl 1.0%	1.71 (0.03)a	1.74 (0.06)a
	Lactic acid 5.0% + NaCl 1.0%	1.70 (0.04)a	1.79 (0.05)a
	Acetic acid 2.5% + NaCl 1.0%	1.70 (0.04)a	1.77 (0.05)a
	Acetic acid 5.0% + NaCl 1.0%	1.68 (0.03)a	1.78 (0.05)a

¹ Values denote mean±(standard deviation)

² Means followed by the same letter, within the same column and bread analysis parameter, are not significantly different ($p > 0.05$).

Differences in cell structure among the loaves made from straight-grade flour were not readily apparent; in fact, straight-grade flour provided, regardless of the treatment, bread slices with more cell uniformity than bread slices made from whole grain flour, including diameter, elongation and number of cells (Table 6).

Treated whole grain flour provided bread slices with smaller cell diameter than the control flour; however, the average cell elongation did not differ from the control. However, treated straight-grade and whole grain flour provided darker bread slices. Usually, blackness is related to the occurrence of air cells which may be a consequence of a higher loaf volume. However, in this study the loaf volume obtained with treated flour did not differ from control (Table 5).

In conclusion, the milling process of hard red winter wheat tempered with organic acids and saline solutions provided milled products with improved microbiological quality when compared with the traditional tempering process using water. Even though some significant differences in mixing, pasting and baking properties were observed, the impact of tempering solutions on functionality was minimal. Furthermore, a consistent use of wheat with high microbiological quality may contribute to reducing the microbial establishment and growth within the mill, thus reducing the risk of microbial contamination during milling.

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