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# Comparison of Tempering Treatment Technologies for Pathogen Reduction on Wheat Grain

## ABSTRACT

Outbreaks and recalls of wheat flour because of contamination with *Salmonella* or Shiga toxin-producing *Escherichia coli* (STEC) have raised concerns regarding food safety and generated interest in determining mitigation treatments that can be incorporated into existing unit operations. We examined four technologies that can be incorporated into the tempering step during flour milling. Wheat grain was inoculated with five-strain cocktails of *Salmonella* and STEC at 8 log CFU/g, and water activity was equilibrated, followed by a week of storage before tempering. Sterile tap water, lactic acid (LA at 5% and 20%), peracetic acid (PeraGrain 3000 and 6000 ppm), Neo-Temper (NT I and II), and hypochlorous acid (1200 and 2000 ppm) were applied at 4% (v/w) grain. With the exception of 5% LA and NT I for STEC, all technologies evaluated significantly reduced pathogen populations ($P < 0.05$) during tempering compared with the water control.

## INTRODUCTION

The bacterial foodborne pathogens Shiga toxin-producing *Escherichia coli* (STEC) and *Salmonella* have contributed to food safety concerns in flour and related products *(1–3)*. Flour is a low-water-activity food; previous studies have demonstrated while inhibiting the growth of bacteria that these pathogens could persist under desiccation stress on wheat grain or in wheat flour for lengthy periods *(8, 10)*.

Several mitigation strategies have been evaluated to reduce the risk of foodborne illnesses attributed to flour that include thermal and nonthermal treatment of flour and its raw commodity, wheat grain. Tempering is a step before flour milling during which moisture is added to allow separation of bran and endosperm. There has been increasing interest in using tempering solutions with antimicrobial effects to treat wheat grain. Such treatments can be easily implemented in current milling plant systems, are potentially more cost effective than thermal treatments, and require less validation if using chemicals generally recognized as safe *(4)*.

When water is used as a tempering solution, microbe levels—both inoculated pathogens and naturally occurring

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microbes—on wheat grain do not significantly change over time *(9, 13)*. To date, chlorinated water, lactic acid (LA), peracetic acid (PAA), and sodium bisulfite (SBS) have been investigated for use as tempering solutions. Although most applications reduced pathogen numbers on grain, they are difficult to compare directly because of differences in experimental design. For example, one common difference is how the inocula were cultivated. Wheat grain tempered using 800-ppm chlorinated water *(12)* and PAA *(9)* was inoculated with agar-grown cultures, whereas wheat grain tempered with 5% LA and 26% sodium chloride solution *(16)* and 5% SBS were inoculated with a broth-grown inoculum *(15)*. Other variations in experimental design that have been observed in these studies include use of different bacterial strains, different methods of sample preparation, different methods for inoculum cultivation and application of the inoculum, and different amounts of tempering solution applied.

Here, we examined four commercially available technologies that could be used as tempering solutions, each at two concentration levels. By keeping the experimental design the same, other than the wheat type used, the goals of this study were to (1) examine the efficacy of pathogen inactivation using these technologies as a tempering treatment and (2) directly compare these technologies to provide actionable data for the milling industry.

## MATERIALS AND METHODS

### Selection of bacterial strains

Five strains of *Salmonella* isolated from low-moisture-food outbreaks were chosen to create the inoculum. The cocktail consisted of *Salmonella* strains associated with low-moisture foods and low-moisture-food-associated outbreaks: *Salmonella* Enteritidis PT30 (ATCC BAA-1045, isolated from a 2001 almond outbreak in Canada), *Salmonella* Typhimurium (FSL S10-1766, isolated from the environment), *Salmonella* Montevideo (488275, isolated from black and red pepper), *Salmonella* Tennessee (K4643, isolated from peanut butter), and *Salmonella* Mbandaka (698538, isolated from tahini). In addition, five strains of STEC were chosen that included serotype O157, as well as other serotypes commonly associated with low-moisture-food outbreaks. This cocktail included *E. coli* O157:NM (LJ1723, isolated from soy nut butter), *E. coli* O157:H7 (SEA13B88, isolated from an apple cider outbreak), *E. coli* O157:H7 (LJH1357, isolated from cookie dough), *E. coli* O121:H19 (PNUSAE002568, a clinical isolate from a 2016 flour outbreak), and *E. coli* O26:H11 (LJH1728, isolated from a 2016 flour outbreak).

### Wheat grain

Both hard red spring wheat and soft red winter wheat were evaluated for the application of the different tempering treatments. For treatment using PeraGrain A PAA and Bio-SAIF hypochlorous acid (HClO), hard red spring wheat was used. Soft red winter wheat was used for testing with Purac FCC 88 LA and Neo-Temper (NT). Water was added to both hard and soft wheat when used as control for each tempering treatment. Hard red spring and soft red winter wheat were provided by the Mennel Milling Company (Fostoria, OH). Upon arrival, the wheat grain was stored under refrigerated temperature in sealed Mylar bags (Uline, Pleasant Prairie, WI) and moved to room temperature 2 days before the experiment.

### Inoculum preparation

The 10 strains used were previously selected for rifampicin (Thermo Fisher Scientific, Waltham, MA) resistance (80 μg $ml^{-1}$) *(14)* and stored at −80°C long term. When the inoculum was created, selected strains were streaked onto Luria Bertani agar (Thermo Fischer Scientific) with 80 μg $ml^{-1}$ rifampicin (LBr) from frozen stocks and incubated at 37°C for 20 h. After incubation, a single colony from every strain was selected and transferred to 5 ml of LBr broth each and incubated at 37°C for 24 h. Following this incubation period, 250 μl of LBr broth culture was spread evenly across LBr plates (one 100-mm plate per strain) and incubated at 37°C for 24 h to achieve confluent lawn growth across the plate. The inoculum cocktail was created by harvesting the lawn plate growth from each of the five strains using a sterile L-shaped spreader. The harvested cells were transferred to 2.5 ml of Butterfield's buffer. The cocktail was then vortexed in the buffer, applied directly to 750 g of wheat grain in a sterile Stomacher bag (Nasco Whirl-Pak, Madison, WI), and hand massaged thoroughly for several minutes. The grain was sampled in quadruplicate via 11-g samples to confirm a homogenous inoculation of ~8 log CFU/g (±0.5 log CFU/g standard deviation).

### Water activity equilibration

The inoculated grain was transferred to a water activity equilibration chamber *(7)* at ambient temperature and 50% relative humidity for 48 h *(12)*. After this equilibration period, the water activity decreased to ~0.55. The wheat grain was then sealed in Mylar bags and stored in a controlled chamber at 20°C and 45% relative humidity for 5 days before tempering.

### Tempering solution preparation

PeraGrain A PAA (EnviroTech/Colorado Chemical, Modesto, CA), Purac FCC 88 LA (Corbion, Lenexa, KS), NT (Agri-Neo, Toronto, ON, Canada), and Bio-SAIF HClO (BioIonix, Fitchburg, WI) tempering solutions were prepared at two concentrations each. PeraGrain solutions were prepared at 3000 and 6000 ppm, LA solutions were prepared at 5% and 20% concentrations, organic oxidizer NT solutions were prepared according to manufacturer directions in two levels (NT I and NT II), and HClO solutions were prepared at 1200 and 2000 ppm, with pH adjusted to pH 5.5–6 with

LA. The solutions were diluted using sterile tap water, with the exception of NT treatments, which were provided as a working concentration that did not require further dilution and were applied as directed by the manufacturer for 50-g samples. For the remaining treatments, after dilution, 2 ml of solution at the desired concentration was combined with 50 g of wheat grain, corresponding to 4% (v/w) moisture content. Additional sterile tap water was added to ensure the total volume of tempering solution introduced reached 2 ml.

### Wheat tempering

The inoculated wheat was separated into 50-g aliquots and transferred to 250-ml centrifuge bottles (Nalgene, Rochester, NY). The target moisture content was 16% after 18 h of tempering. The initial moisture content of the samples was ~12% ± 0.8%. This initial moisture content was the determining factor for the volume of tempering treatment introduced to the sample. Therefore, 2 ml (4% v/w) of tempering solutions was introduced per 50 g of wheat. For moisture content reference before and after tempering, a 500-g sample of wheat was tempered in a 1-liter centrifuge bottle (Nalgene). The initial 30 min of tempering consisted of continuously rotating the centrifuge bottles via a custom tempering machine, with ~24 rpm. After this stage, the wheat samples were stored at ambient temperature for the remainder of the tempering process. For each bacterial species, three biological replicates of each tempering treatment were performed.

### Bacterial enumeration

Before tempering, pathogen levels from each biological replicate were enumerated by diluting 11 g of wheat grain with Butterfield's buffer in a ratio of 1:10 and placing this in a masticator (Seward, Worthing, West Sussex, UK) for 60 s, followed by serial diluting with Butterfield's buffer in ratio of 1:10, plating onto LBr, and incubating at 37°C for 24 h. Colonies were enumerated with a Scan 300 colony counter (Interscience, Woburn, MA). All tempered wheat was sampled in 11-g aliquots in duplicate. Dey–Engley neutralizing broth (Neogen, Lansing, MI) was added at a ratio of 1:10 to neutralize the tempering solutions and homogenized in a masticator for 60 s. Samples were diluted as needed in Butterfield's buffer, plated in duplicate onto LBr, and incubated at 37°C for 24 h before using the Scan 300 plate counter to enumerate *Salmonella* and STEC.

### Data analysis

For microbial analysis, the count data in CFU per gram were first transformed to log CFU-per-gram reduction by logging the count after treatment over the initial count. Then, the log reductions resulting from different treatments were checked for normality assumption. To determine whether various treatments were effective against inoculated pathogen on grain, the treatments were compared with the water control using an individual student's *t*-test. The *t*-test was also used to compare whether pathogen reduction because of water during tempering was affected by wheat type. To test whether treatment efficacy was associated with bacterial species or wheat type and to test which tempering treatments were significantly different from one another, the Tukey honestly significant difference test was used post hoc, followed by two-way analysis of variance (ANOVA). All statistical analyses were done using R (version 4.3.1), and plots were created with ggplot2 *(17)*.

## RESULTS AND DISCUSSION

### Moisture content

The initial water activity for both types of grain was 0.44, the average moisture content of hard red spring wheat was 12% ± 0.5%, and the average moisture content of soft red winter wheat was 11.6% ± 0.5%. After storage, the moisture content of the reference wheat grain was measured before and after each tempering treatment. Before tempering, the moisture content of the grain was 12% ± 0.5% for both hard and soft wheat. After tempering treatment, upon adding 4% (v/w) water, the moisture content increased by 3.6%–3.7%. After tempering, the moisture content of the grain was ~15.6% ± 0.5%.

### Pathogen reduction

Average initial bacterial populations before tempering were 7.93 ± 0.27 log CFU/g for *Salmonella* and 7.41 ± 0.32 log CFU/g for STEC. The effect of tempering treatments on inactivation depended on pathogen species, as determined by a significant interaction effect ($P < 0.05$) with two-way ANOVA. Log reductions were significantly higher ($P < 0.05$) for *Salmonella* than those for STEC on grain treated with PeraGrain 3000 ppm, and reductions were significantly higher ($P < 0.05$) for STEC than those for *Salmonella* on grain treated with HClO 1200 ppm *(Table 1)*. Most significant differences ($P < 0.05$) were among the different tempering treatments, and no significant difference ($P > 0.05$) was observed between species for the cases that were not described here.

Tempering with 5% LA led to a 0.99 ± 0.16 log reduction of *Salmonella*, and increasing the concentration to 20% led to a log reduction of 1.71 ± 0.22, although this was not significantly different ($P > 0.05$) from 5% LA *(Fig. 1)*. STEC log reductions when grain was tempered with 5% LA were not significantly different from those when tempering with water *(Table 1)*, whereas increasing to 20% LA led to a significantly greater ($P < 0.05$) log reduction of STEC. The effect of increasing concentrations of LA on greater log reductions on wheat grain has been observed: increasing from 2.5% to 5% led to significantly greater reductions of *Salmonella* and STEC on both soft and hard wheat *(16)*. Tempering with varying concentrations of LA has been evaluated in multiple studies, with the highest concentration

**TABLE 1. Average log reduction attributable to each treatment during tempering[a]**

| Tempering treatment | *Salmonella* log ($N/N_0$) | STEC log ($N/N_0$) |
|---|---|---|
| Water | $0.32 \pm 0.29^{Aa}$ | $0.49 \pm 0.21^{Aa}$ |
| LA 5% | $0.99 \pm 0.16^{Ba}$ | $1.08 \pm 0.41^{ABa}$ |
| LA 20% | $1.71 \pm 0.22^{BCa}$ | $1.70 \pm 0.43^{Ba}$ |
| PeraGrain 3000 ppm | $2.62 \pm 0.26^{Da}$ | $2.07 \pm 0.22^{Cb}$ |
| PeraGrain 6000 ppm | $3.30 \pm 0.14^{Da}$ | $3.34 \pm 0.36^{Da}$ |
| NT I | $1.63 \pm 0.62^{BCa}$ | $1.34 \pm 0.59^{ABCa}$ |
| NT II | $2.56 \pm 0.46^{Da}$ | $1.98 \pm 0.68^{Ca}$ |
| HClO 1200 ppm | $0.98 \pm 0.13^{Ba}$ | $1.60 \pm 0.15B^{Cb}$ |
| HClO 2000 ppm | $1.83 \pm 0.25^{Ca}$ | $1.79 \pm 0.24B^{Ca}$ |

[a]Capital letters indicate significant difference between treatments. Lowercase letters indicate significant difference between species.

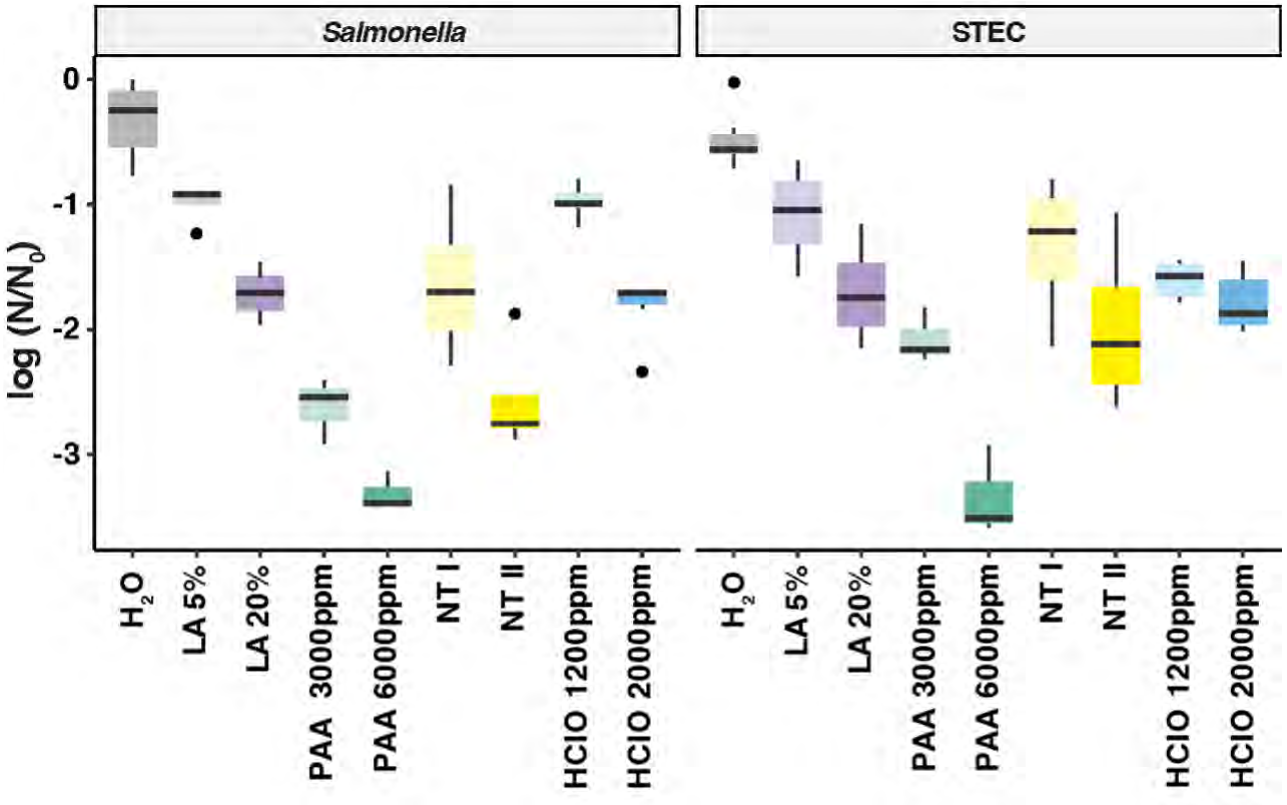


**FIGURE 1. Reduction of bacterial population (log N/N0) during different tempering treatments: water, LA 5% and 20%, PAA (PeraGrain) 3000 and 6000 ppm, NT I and NT II, and HClO 1200 and 2000 ppm. Boxplots show distribution of reductions for each bacterial species for each tempering treatment.**

reported at 10.9% *(9)*. The use of 5% LA is most commonly reported, with log reductions of 1.6–2.6 log CFU/g for *Salmonella* and STEC when tempered with 5% LA with 26.6% salt *(16)*. In the same study, reductions of *Salmonella*, *E. coli* O157:H7, and non-O157 STEC improved by 0.8, 0.6, and 0.8 log CFU/g, respectively, when tempering hard wheat compared with soft wheat, although the differences were attributed to differences in target moisture content and resting time for the two wheat types. Jung and Harris *(9)* reported a 1.23 and 1.09 log CFU/g reduction for *Salmonella* and STEC, respectively, when treated with 5.4% LA. In the same study, a 1.75– 2.14 log CFU/g reduction was observed for STEC when tempered with 10.4% LA. In another study, Rivera et al. *(15)* observed a 2.0 and 2.6 log CFU/g reduction for STEC treated with 5% and 10% LA, respectively. The reported ranges of reduction were different across studies, possibly because of variation in experimental design, such as wheat type *(16)*, which led to differences in tempering times and amount of solution added, inoculation level *(9)*, and preparation of the inoculum *(15)*.

Tempering with PeraGrain 3000 and 6000 ppm, as well as NT II treatment, achieved the greatest average log reduction, and these results were not significantly different from one another, with a 2.62 ± 0.26, 3.30 ± 0.14, and 2.56 ± 0.46 log CFU/g reduction of *Salmonella*, respectively *(Table 1)*. For STEC, PeraGrain 3000 ppm and NT II led to 2.07 ± 0.22 and 1.98 ± 0.68 log reductions, respectively, whereas the greatest log reduction ($P < 0.05$) was seen with PeraGrain 6000 ppm, with a log reduction of 3.34 ± 0.36 log CFU/g. Tempering with NT I resulted in significantly lower log reductions for both *Salmonella* and STEC than for NT II and either concentration of PeraGrain *(Table 1)*. For STEC, tempering with NT I was not significantly different from the water control ($P > 0.05$). PAA as a tempering treatment has been previously evaluated against STEC on wheat grain, although at lower concentrations than were evaluated here. Tempering with 100 and 500 ppm led to reductions of 0.73 and 1.21 log CFU/g, respectively, for STEC on wheat grain *(9)*.

Tempering with HClO led to a greater average log reduction for STEC than for *Salmonella* at 1200 ppm: 1.60 log and 0.98 log CFU/g, respectively. The log reductions were similar, ~1.8 log CFU/g, for both pathogens at 2000 ppm *(Table 1)*. Tempering with HClO 2000 ppm led to similar log reductions for STEC as for all other tempering treatments except PAA 6000 ppm. For *Salmonella*, HClO 2000 ppm had similar log reductions as NT I and 20% LA *(Table 1)*, ~2 log CFU/g. The use of HClO 70 ppm was previously only reported for efficacy in reducing aerobic microbe counts of wheat grain by 0.65 log CFU/g after tempering *(5)*. In comparison, when using 800-ppm chlorinated water, a 2-log reduction was found when treating *Salmonella*-inoculated wheat grain during tempering, and a 0.38–1.23 log CFU/g reduction was found for STEC on inoculated grain *(12)*. This may result from the use of different strains, because only some strains are consistent between the studies. The use of HClO resulted in a greater and more consistent reduction in levels of STEC at both 1200 and 2000 ppm. The reduction in *Salmonella* was lower for HClO when treated at 1200 ppm, and at 2000 ppm, the log reduction was similar to that of STEC.

**Comparison of tempering technologies**

Previous studies *(12, 15, 16)* have typically focused on evaluating the antimicrobial effects of a single type of tempering treatment, often at multiple concentrations. In many cases, increasing the concentration of the antimicrobial led to greater pathogen reduction *(15, 16)*. In other cases, the concentration did not produce significant differences in pathogen reduction *(12)*. The levels of each tempering treatment evaluated here were the recommended levels of use based on information from each manufacturer. We found that increasing the concentration of tempering technologies led to significantly higher log reductions for some treatments, including NT and HClO for *Salmonella* and PeraGrain for STEC *(Table 1)*. The tempering technologies evaluated here pose different types of stress to the microbes on the wheat grain, likely leading to inactivation. LA poses acid stress, HClO poses oxidative stress, and PAA and NT pose both oxidative and acidic stress, in which oxidative stress could lead to cellular damage and damage to the DNA *(6)*. All of these are nonspecific stressors, meaning they interact with all types of microbes that may be present on grain, not just foodborne pathogens.

Direct comparison of tempering technologies evaluated for wheat grain based on published data have not been possible because of differences in experimental design. For example, it has been shown that the inoculum preparation method leads to significantly different reductions of STEC and *Salmonella* during tempering *(9, 11)*. When inoculum was applied to the grain and then immediately tempered with 5% LA, the log reductions were significantly greater than when the inoculum was allowed to dry on the grain for 24 h before tempering *(9)*. Allowing the inoculum to adapt to the low-moisture conditions on the grain, which was done in the current study, is likely more representative of the conditions pathogens encounter when present on wheat grain.

One potential limitation of this study was that the wheat type used for testing LA and NT was different from the wheat type used for testing HClO and PeraGrain. Although a previous study suggested the difference in bacterial reduction on hard versus soft wheat mainly resulted from the difference in target moisture content and resting time *(16)*, we kept the moisture content and resting time the same in the current study. As expected, the *t*-test showed no significant difference ($P > 0.05$) in pathogen reduction between soft and hard wheat tempered with water. Further analysis using ANOVA showed pathogen reductions have no significant association ($P > 0.05$) with wheat type among all treatment replicates.

Future investigation of specific wheat types with suggested target moisture and tempering time will help to better understand each specific technology.

## CONCLUSIONS

Direct comparison of different commercially available tempering technologies shows that most treatments are effective at reducing *Salmonella* and STEC populations on wheat grain, although differences exist in the extent of reduction among the treatments. With the exception of 5% LA and NT I for STEC, all treatments led to reductions that were significantly greater than what is achieved with water alone. Use of a standardized inoculation and evaluation process will be helpful for additional comparisons of other tempering technologies. Future studies are needed to investigate the sensory and functional characteristics of flour milled from grain treated with the different tempering technologies, as well as to assess the potential impact on milling equipment. The data presented here will be useful for those in the milling industry considering use of an antimicrobial tempering technology.

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