Evaluation of post-fermentation heating times and temperatures for controlling Shiga toxin-producing *Escherichia coli* cells in a non-dried, pepperoni-type sausage

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Abstract

Coarse ground meat was mixed with non-meat ingredients and starter culture (*Pediococcus acidilactici*) and then inoculated with an 8-strain cocktail of Shiga toxin-producing *Escherichia coli* (ca. 7.0 log CFU/g). Batter was fine ground, stuffed into fibrous casings, and fermented at 35.6°C and ca. 85% RH to a final target pH of ca. pH 4.6 or ca. pH 5.0. After fermentation, the pepperoni-like sausage were heated to target internal temperatures of 37.8°, 43.3°, 48.9°, and 54.4°C and held for 0.5 to 12.5 h. Regardless of the heating temperature, the endpoint pH in products fermented to a target pH of pH 4.6 and pH 5.0 was pH 4.56±0.13 (range of pH 4.20 to pH 4.86) and pH 4.96±0.12 (range of pH 4.70 to pH 5.21), respectively. Fermentation alone delivered ca. a 0.3- to 1.2-log CFU/g reduction in pathogen numbers. Fermentation to ca. pH 4.6 or ca. pH 5.0 followed by post-fermentation heating to 37.8° to 54.4°C and holding for 0.5 to 12.5 h generated total reductions of ca. 2.0 to 6.7 log CFU/g.

Introduction

Shiga toxin-producing cells of *Escherichia coli* (STEC) continue to pose a significant threat to public health as evidenced by their recovery from a variety of higher volume and higher risk foods, the observance of both large and small recalls of such foods due to the presence of regulated serotypes of these bacteria, and the frequency and documentation of severe illnesses attributed to pathogenic strains of these bacteria associated with consumption of contaminated and perhaps under-processed and/or improperly handled foods (CDC, 1995a; 1995b; 2010; USDA-FSIS, 2010). Although not the sole/primary vehicle of sporadic cases and outbreaks, raw and further processed beef was responsible for several illnesses and recalls over the past 35 years (Griffin *et al*., 2003; Kaspar *et al.,* 2010; Page, 2018). Several reports have been published, particularly since the early- to mid-1990's and likely in response to the much publicized salami outbreaks in the U.S. (CDC, 1995a) and Australia (CDC, 1995b), detailing the fate of serotype O157:H7 cells of STEC in a variety of both short- and long-term ripened cured-dried sausage and reporting on validated interventions and processes for their control (Balamurugan *et al*., 2017; Calicioglu *et al.,* 2002; Faith *et al*., 1997, 1998; Glass *et al.,* 2012; Heir *et al*., 2013; Holck *et al*., 2011; Hinkens *et al*., 1996; McLeod *et al*., 2016; Riordan *et al*., 1998; Rode *et al*., 2012). As detailed in our previous publication (Hinkens *et al*., 1996), although there are a variety of pepperoni types and sizes, three main categories dominate the market: i) small diameter (28-36 mm) for the deli case, ii) medium diameter (49-55 mm) for pizza topping, and iii) large diameter (60-80 mm) for sandwiches. As a result of the *Jack-in-the-Box* outbreak attributed to undercooked hamburger patties in the early 1990's in the U.S. (CDC 1993) and to some extent the salami outbreaks attributed to survival of STEC in salami products soon thereafter (CDC 1995a, 1995b), both serotype O157:H7 and subsequently the following serotypes of STEC, namely O26:H11, O45:H2, O103:H2, O111:H-, O121:H19, and O145:NM (aka *The Big Six*), are considered adulterants in raw/non-intact meats (USDA-FSIS, 2011). As such, producers are required by the United States Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS) to validate a 2- or 5-log reduction of these pathogens during manufacture of fermented meats (Reed, 1995a, 1995b). With the exception of a study by Glass *et al*. (2012) wherein strains of the six regulated non-O157:H7 serotypes of STEC were evaluated, most prior studies evaluated the fate of serotype O157:H7 strains of *E. coli* in dry-fermented-type sausage (Faith *et al.,* 1997, 1998; Hinkens *et al*., 1996; Riordan *et al*., 1998). Given the current regulatory posture that *The Big Six* strains of non-O157:H7 serotypes and strains of O157:H7 are considered adulterants in raw/non-intact meats (USDA-FSIS, 2011), further studies are warranted to validate the comparative fate of these additional STEC strains/serotypes of *E. coli* in fermented meats.

Numerous studies were conducted since the early 1990's to monitor viability of STEC in a variety of dry and semi-dry fermented sausage. In general, results to date established that fermentation alone is sufficient to deliver about a 1- to 2-log reduction of pathogen levels in products such as soudjouk, pepperoni, Genoa salami, and

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Norwegian dry-sausage (Calicioglu *et al*., 2002; Faith *et al*., 1997, 1998; Glass *et al*., 2012; Heir *et al*., 2013; Hinkens *et al*., 1996; Holck *et al.,* 2011; Nissen and Holck, 1998; Porto-Fett *et al*., 2008, 2010; Riordan *et al*., 1998; Rode *et al.,* 2012). In addition to the abovementioned peer-reviewed publications that quantify reductions in levels of STEC during fermentation and drying of fermented meats, in the mid-1990's the National Cattlemen's Beef Association (NCBA) commissioned a study to validate typical processing parameters used by industry for comparative and collective lethality during manufacture of dry and semi-dry fermented sausage. In general, the resulting NCBA Blue Ribbon Task Force Report (Nickelson *et al.,* 1996) validated several processes that achieved either a 2- or a 5-log reduction of *E. coli* O157:H7 and identified processes/steps in the manufacture of dry/fermented sausage *"…useful in evaluating greater (more severe) or lesser processes when evaluating a lower or higher risk…."* Processes/steps shown to be of higher risk included: i) high pH, ii) beef ingredient, iii) high initial coliform count – ingredient, and iv) low fermentation temperature. It is general knowledge that pH, salt, fermentation and drying temperatures and times, in combination with relative humidity, curing salts, and presence of secondary metabolites, may also appreciably affect viability of STEC in fermented sausage. That being said, most of the data published to date suggests that post-fermentation heating is the only effective and reliable method to achieve a 5-log reduction of STEC in certain dry-fermented sausage products such as pepperoni without adversely affecting product quality (Faith *et al.,* 1997, 1998; Glass *et al*., 2012; Heir *et al*., 2013; Hinkens *et al*., 1996; Holck *et al*., 2011; Riordan *et al*., 1998). Collectively, these data confirmed that traditional processes for pepperoni production were only sufficient to deliver about a 2-log reduction of *E. coli* O157:H7, and with the possible exception of a relatively recent paper (Glass *et al*., 2012), there has been little information published on the fate of non-O157:H7 cells of STEC in fermented sausage. For control of *E. coli* O157:H7, the two most widely accepted and practiced post-fermentation heating parameters are heating to internal temperatures of 62.8°C instantaneous or to 53.3°C and then holding for 60 min (Hinkens *et al*., 1996; Nickelson *et al*., 1996). Thus, the purpose of this study was to validate the lethality of post-fermentation heating times and temperatures for lethality towards STEC to provide manufacturers with additional processes/options for ensuring the safety of fermented sausage.

Materials and Methods

Bacterial strains

The eight rifampicin-resistant (Rif^R) strains of Shiga toxin-producing *Escherichia coli* [STEC-8; (i) USDA-FSIS 380-94 (meat isolate, serotype O157:H7), (ii) JB1-95 (clinical isolate, serotype O111:H-), (iii) CDC 96-3285 (human stool, serotype O45:H2), (iv) CDC 90-3128 (human stool, serotype O103:H2), (v) CDC 97-3068 (human stool, serotype O121:H19), serotype O121:H19, (vi) 83-75 (human stool, serotype O145:NM), (vii) H30 (infant with diarrhea, serotype O26:H11), and (viii) ATCC BAA-2326 (human stool, serotype O104:H4)] used in this study were confirmed, cultured, and maintained as described previously (Luchansky *et al.,* 2008).

Manufacture of a pepperoni-type sausage

The pepperoni-type sausage for this study was prepared essentially as described previously (Hinkens *et al*., 1996) using fresh pork and beef trimmings (75:25 pork:beef with 30% fat) obtained from a local butcher (Illg's Meats, Chalfont, PA, USA). The dry ingredients, starter culture, and casings were donated by a cooperating commercial sausage manufacturing company (John Morrell Food Group, Lisle, IL, USA). The batter was comprised of a dry spice mix (3.69%; Saratoga Food Specialties, Bolingbrook, IL, USA), cure salt (3.60%; 6.25% sodium nitrite), and a commercial starter culture (0.0188%; *Pediococcus acidilactici*; Saga 200; Kerry Ingredients & Flavors, Beloit, WI, USA). For each trial, the batter (ca. 7 kg) was inoculated with 160 ml of the STEC-8 cocktail to achieve an initial level of ca. 7.0 log CFU/g. Next, the inoculated batter was fine ground through a 3/8-inch plate (Model 4346; Hobart, Troy, OH, USA), and then stuffed into a 55-mm fibrous casing using a floor-type, hydraulic-driven piston stuffer (50 lb capacity; Model SC-50, Koch Equipment, Kansas City, MO, USA). The resulting chubs (ca. 290 g; 18 cm L × 5.5 cm D) were stapled/clipped (Max HR-PS II; Salco, East Syracuse, NY, USA) and then fermented at 35.6°C and 85% relative humidity (RH) in an environmental chamber (Model ES 2000 CDC-DW; Bahnson Environmental Specialties, Winston Salem, NC, USA) until an endpoint target pH of either ca. pH 4.6 or ca. pH 5.0 was achieved. After fermentation (ca. 8 to 12 h), the environmental chamber temperature was adjusted to 37.8°C, 43.3°C, 48.9°C, or 54.4°C and the chubs were heated for up to 12.5, 8, 4, and 4 h, respectively, at a RH of 95%. Note, the resulting chubs were not dried after the post-fermentation heating component of this study. A single trial consisted of a freshly-grown cocktail comprised of each of the 8 STEC strains inoculated into a fresh meat block that was subsequently fermented to a single endpoint pH (either ca. pH 4.6 or ca. pH 5.0) and then separately subjected to one of each of the 4 post-fermentation heating regimens. At least two trials (N=2), but up to 4 trials (N=4), were conducted for each endpoint pH in combination with one of each of the associated 4 heating regimens.

Microbiological and physical-chemical analyses

At each sampling interval, a 25-gram portion from each of 3 chubs was separately analyzed (n=3) essentially as described (Hinkens *et al*., 1996). The samples were macerated (Stomacher 400; Seward, Cincinnati, OH, USA) and then plated, with and without prior dilution in 0.1% peptone water, onto sorbitol-MacConkey (Difco, BD, Franklin Lakes, NJ, USA) plus rifampicin (100 µg/ml; Sigma Chemical Company, St. Louis, MO, USA) agar plates. When pathogen levels decreased to below the detection limit (≤0.47 log CFU/g) by direct plating, chubs testing negative for the pathogen by direct plating were enriched as previously described (Hinkens *et al*., 1996). In addition to enumerating surviving STEC-8, the pH of each sample was measured as described (Porto-Fett *et al*., 2008) using a model 6000P pH/temperature electrode and a model 5500 pH meter (Daigger, Vernon Hills, IL, USA). Water activity was measured using an electronic water activity meter (Decagon Aqualab Model Series 3; Decagon Devices, Pullman, WA, USA).

Statistical analyses

Means and standard deviations were calculated for each of the endpoint pH and for each of the post-fermentation heating regimens using triplicate sausage samples at each sampling interval. Data were analyzed using the Microsoft Excel 2013 software (Redmond, WA).

Results

Fermentation at 35.6°C and 85% RH to a comparatively lower (*i.e.,* pH 4.6) or a comparatively higher (*i.e*., pH 5.0) endpoint pH delivered a 1- to 2-log decrease in levels of STEC-8 inoculated into the pepperoni-type sausage evaluated in the present study (Figures 1-4). More specifically, the average pH of the batter was pH 5.77±0.30

(range of pH 5.28 to pH 6.60), whereas the average pH in products fermented to a target pH of ca. pH 4.6 and ca. pH 5.0 was pH 4.56±0.13 (range of pH 4.20 to pH 4.86) and pH 4.96±0.12 (range of pH 4.70 to pH 5.21), respectively. No changes in a_w were observed during fermentation; the average a_w of the batter was a_w 0.944±0.006 (range of a_w 0.934 to a_w 0.959), whereas after fermentation the average a_w was a_w 0.944±0.011 and a_w 0.940±0.008 (range of a_w 0.917 to a_w 0.964) in products fermented to a target pH of ca. pH 4.6 and ca. pH 5.0, respectively. Fermentation delivered reductions of ca. 0.9 to 1.1 and ca. 0.3 to 1.2 log CFU/g in products fermented to an endpoint pH of ca. pH 4.6 and pH 5.0, respectively.

Products were also subjected to a post-fermentation heating step for lethality towards STEC-8. In general, additional reductions of ca. 0.4 to 2.0 and 0.4 to 1.2 log CFU/g were observed during the come-up-time (CUT) after fermentation to pH 4.6 and pH 5.0, respectively, until achievement of post-fermentation heating temperatures of 43.3° to 54.4°C (Figures 1-4); no additional reductions were observed during the CUT for post-fermentation heating temperatures of 37.8°C. Lastly, for sausage fermented to pH 4.6, additional reductions of 0.1 to 1.5, 0.3 to 4.0, 0.2 to 2.3, and 1.9 to 3.7 log CFU/g in levels of STEC-8 were achieved during post-fermentation heating to 37.8°, 43.3°, 48.9°, and 54.4°C, respectively. Similarly, for sausage fermented to pH 5.0, additional reductions of ca. 0.2 to 1.1, 0.3 to 5.2, 1.2 to 3.2, and 2.4 to 4.4 log CFU/g were achieved during post-fermentation heating to 37.8°, 43.3°, 48.9°, and 54.4°C, respectively., After post-fermentation heating, an additional decrease in pH of the sausage was observed for all fermentation and heating conditions tested. More specifically, the pH of sausage fermented to ca. pH 4.6 and ca. pH 5.0 after post-fermentation heating decreased to pH 4.14 to pH 4.30 and pH 4.23 to pH 4.40, respectively. However, no appreciable changes in a_w were observed after post-fermentation heating for sausage fermented to either ca. pH 4.6 (a_w 0.944) or ca. pH 5.0 (a_w 0.940). Regardless of the target endpoint pH, the average a_w of sausage after fermentation and heating was a_w 0.945±0.007 (range from a_w 0.929 to a_w 0.953). In general, longer times at higher temperatures and lower pH levels delivered greater reductions of STEC-8; however, survivors were recovered by direct plating and/or by enrichment for all pH, time, and temperature conditions tested. Although post-fermentation heating to 48.9° and 54.4°C for longer than 2 h resulted in greater reductions in STEC-8 numbers, these treatments adversely affected the texture of the product. Perhaps due to the fat content of the product (ca. 30%), as well as the above mentioned extended post-fermentation heating times and higher temperatures,

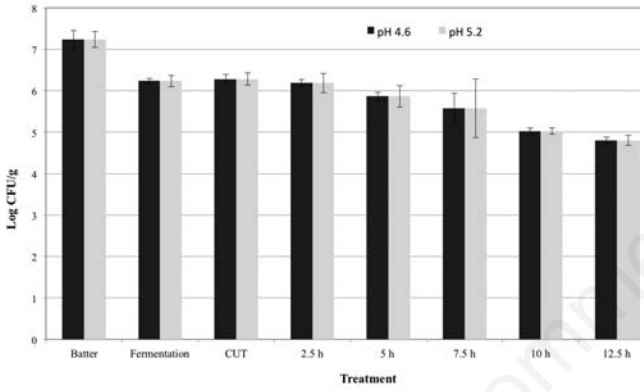

Figure 1. Inactivation of STEC in pepperoni-type sausage fermented to pH 4.6 or pH 5.0 with heating and holding at 37.8°C. Error bars represent the standard deviation of the mean (N=4, n=3).

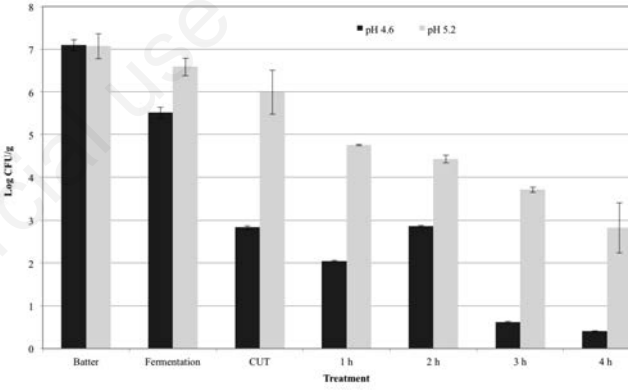

Figure 3. Inactivation of STEC in pepperoni-type sausage fermented to pH 4.6 or pH 5.2 with heating and holding at 48.9°C. Error bars represent the standard deviation of the mean (N=2, n=3).

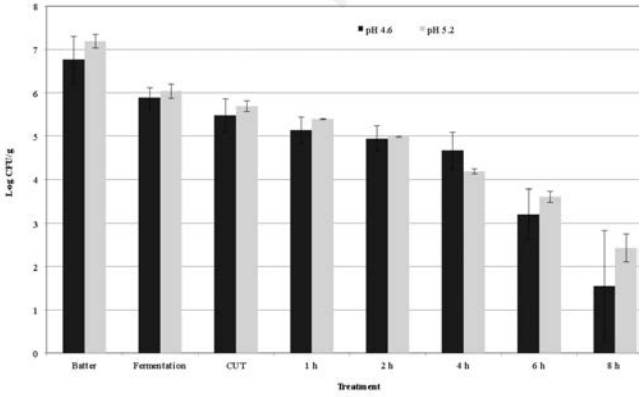

Figure 2. Inactivation of STEC in pepperoni-type sausage fermented to pH 4.6 or pH 5.0 with heating and holding at 43.3°C. Error bars represent the standard deviation of the mean (N=2, n=3).

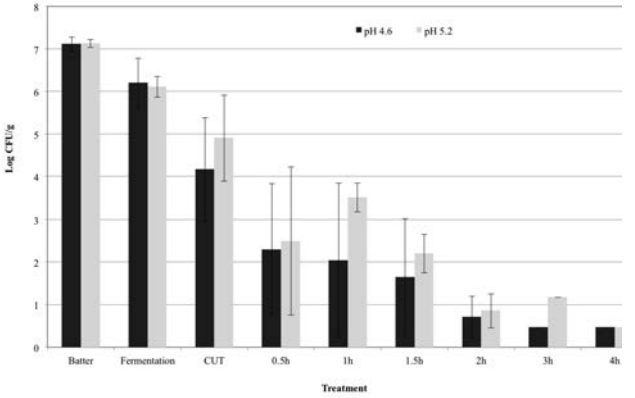

Figure 4. Inactivation of STEC in pepperoni-type sausage fermented to pH 4.6 or pH 5.2 with heating and holding at 54.4°C. Error bars represent the standard deviation of the mean (N=4, n=3).

liquified fat was observed throughout portions of some sausage chubs.

Discussion

Fermented sausage have been produced and consumed for centuries, and largely without untoward consequences until some 30 years ago with the emergence of acid-tolerant serotypes of *Escherichia coli* that produce Shiga toxins (Griffin *et al.*, 2003). In general, STEC are somewhat more tolerant of the lower pH and a_w of a traditional fermented sausage than most other foodborne pathogens; therefore, to achieve a 2- or 5-log reduction in levels of STEC as stipulated by USDA-FSIS (Reed, 1995a, 1995b) it may be necessary to develop additional ingredients or starter cultures to inhibit this pathogen or to validate post-fermentation interventions, including heat, high pressure, storage at ambient temperatures, freezing – thawing, and/or irradiation to deliver the required lethality (Heir *et al*., 2013; Holck *et al.*, 2011; Rode *et al.,* 2012). In so doing, every effort must also be made to preserve product quality and to manage costs. For these reasons, and based on information already published, the goal of the present study was to validate post-fermentation time/temperature heating regimens for lethality towards STEC.

Prior to the present study, the primary post-fermentative heating regimens for dry-fermented sausage that were validated and published in a peer-reviewed journal were: (i) holding the product at 53.3°C for up to 60 min, or (ii) heating the product to an internal instantaneous temperature of 62.8°C (Glass *et al*., 2012; Hinkens *et al*., 1996). Both of these heating regimens were selected primarily based on meeting the requirements for trichinae destruction (Hinkens *et al*., 1996). Additionally, the process of heating to an internal instantaneous temperature of 62.8°C approximated conditions established by USDA-FSIS for cooked and/or roast beef, and was subsequently approved as an option to control *E. coli* O157:H7 in fermented sausage (Hinkens *et al*., 1996). The process for heating to 53.3°C and holding for 60 min was selected because it was less damaging to the sensory attributes of pepperoni than heating to 62.8°C (Hinkens *et al.,* 1996). In related studies, (Heir *et al.,* 2013), salami and Norwegian *Morr* dry-fermented sausage were subjected to post-fermentation heating at 32°, 43°, 50°, 60°, or 65°C for 30 min to 6 days depending on the temperature; reductions of 3.5 to >5.5 log CFU/g were achieved with only minor untoward effects on product quality. According to these authors, the abovementioned time and temperature parameters were selected based on guidelines to achieve a 5-log reduction of STEC in ready-to-eat fermented sausage as published by the Health Protection Branch of the Health Canada Agency. In contrast, Graumann and Holley (2008) reported reductions of ca. 3.5 to 7.0 log CFU/g within 30 days of drying of a salami-type sausage via inclusion of 2 to 6% of non-deheated ground mustard to the batter with the cure ingredients.

The literature is replete with studies confirming that fermentation alone will only deliver a ≤2.0 log decrease in levels of STEC, and that post-fermentation heating is the only effective and reliable intervention to achieve a 5-log reduction of vegetative cells of most foodborne pathogens in fermented sausage while limiting untoward consequences on product quality (Faith *et al*., 1997, 1998; Glass *et al*., 2012; Hinkens *et al.,* 1996; Holck *et al*., 2011; Incze, 1998; Lindqvist and Lindblad, 2009; Riordan *et al*., 1998). In addition to the lowering of pH by the action of the starter culture, sodium nitrite, a common ingredient in fermented sausage, can also inhibit serotype O157:H7 strains of STEC (Morita *et al.,* 2004; Tsai and Chou, 1996). Collectively, inclusion of both intrinsic and extrinsic hurdles such as nitrite, starter cultures, or competitive flora, along with smoking and drying, can in large measure enhance product safety (Leistner, 2000; Bohnlein *et al*., 2016). That being said, the use of unrealistically high initial levels of the pathogen coupled with the uneven distribution in the meat of the pathogen, and the acid elaborated by the starter culture may explain, at least in part, the recovery of sporadic survivors of STEC even after post-fermentation heating of product as observed in the present study. More specifically, for sausage fermented to pH 4.6 and then heated, a ≥5-log CFU/g reduction was achieved in 8 h at 43.3°C, in >4 h at 48.9°C, and in 1 h at 54.4°C, whereas for sausage fermented to pH 5.0 and then heated, a ≥5-log CFU/g reduction was achieved in 8 h at 43.3°C, in 5 h at 48.9°C, and in 2 h at 54.4°C. Note, heating to 37.8°C delivered total reductions of 2.5 log CFU/g for chubs fermented to pH 4.6 and total reductions of 1.4 log CFU/g for chubs fermented to pH 5.0. Although a post-fermentation heating/drying step per se was not conducted in the present study, it is highly likely that further reductions in pathogen levels would be achieved following a typical drying regimen for a pepperoni-type sausage for a pepperoni-type sausage following a post-fermentation heating step.

Conclusions

Our results validated that fermentation to pH 4.6 or pH 5.0 delivered about a ≥5-log reduction in pathogen levels, but only after post-fermentation heating for 1 to 8 hours at 43.3° to 54.4°C. Further reductions likely achieved during subsequent drying may allow for lower temperatures and shorter times for post-fermentation heating and would be the primary objective of an interesting companion study. Regardless, the data herein provide manufacturers of dry-fermented sausage with several options to validate/achieve the required reduction of STEC while producing a high-quality and wholesome product. These data also confirm that processes previously validated as effective for serotype O157:H7 strains of *E. coli* will likely be as effective toward strains of the other six regulated serotypes of STEC and strains of serotype O104:H4 of *E. coli.*

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