Research Note

Effect of Salt and Sodium Nitrite on Growth and Enterotoxin Production of *Staphylococcus aureus* during the Production of Air-Dried Fresh Pork Sausage†‡

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ABSTRACT

*Staphylococcus aureus* contamination and enterotoxin production is a potential food safety hazard during the drying step of production of air-dried fresh country sausage. The growth characteristics and enterotoxin production of *S. aureus* during the drying step of this product with and without added sodium nitrite were evaluated. Three strains of *S. aureus* were grown to stationary phase and inoculated (10⁵ CFU/g) into sausage ingredients. Fresh pork sausages were stuffed into natural casings and allowed to dry for 10 days at 21°C with 60% relative humidity (RH). In control sausage (1.76% [wt/wt] salt) with no *S. aureus*, aerobic plate counts increased by 5.5 log/g during the 10-day drying period, and coliforms increased by 4.8 log/g. The addition of sodium nitrite (154 ppm of nitrite, 2.24% [wt/wt] salt) or increased salt (3.64%, wt/wt) to sausage limited the growth of coliform bacteria (*P* < 0.05). *S. aureus* numbers increased approximately 2 log units during the drying step, regardless of additional salt or nitrite. Additional salt or nitrite had no effect on *S. aureus* growth (*P* > 0.05). Staphylococcal enterotoxin (SE) was not detected in air-dried fresh sausages at any time. Our results suggest that drying of fresh pork sausage under similar parameters listed in this study does not support SE production.

*Staphylococcus aureus* has been responsible for food poisoning incidents in many types of food, including ready-to-eat (RTE) salads, ham, and sausage. With proper conditions, *S. aureus* can grow in food and produce enterotoxins. Staphylococcal enterotoxins (SEs) are heat stable and can cause foodborne intoxication, even following cooking of the food. According to the Centers for Disease Control and Prevention, there were 495 cases of *S. aureus* food-borne outbreaks in 2004, with the majority of staphylococcal food intoxications linked to meats and prepared foods (4).

There are nine SEs (SEA through SEJ). SEs are heat stable and can withstand heat at 121°C for 30 min (2). SEA and TED are the most common enterotoxins implicated in staphylococcal food poisoning (1, 5, 10). Enterotoxin production has been reported across a wide temperature range, from 10 to 48°C; pH 4 to 10; water activity, 0.87 to 0.99; and NaCl content, 1.7 to 17%. Optimum conditions for SE production are 37°C, pH 6 to 7, water activity 0.98, and 3.7% NaCl (9). As little as 20 ng of SE per g of food can result in foodborne illness in humans (13). However, depending on the type of food, enterotoxin may not always be formed, even though staphylococcal counts reach levels of more than 10⁷ CFU/g (16). Current immunoassays now have SE detection limits well below the <20 ng/g of food required for foodborne illness (12).

During growth of *S. aureus*, enterotoxins are produced in low amounts at exponential stage, but production increases in late exponential and stationary phases. Thus, significant growth of *S. aureus* has to take place in the food before toxic levels of enterotoxin are formed. In a range of inoculated foods, enterotoxin was not detected in any sample with less than 10⁷ *S. aureus* CFU/g. Bergdoll (3) reported that as few as 10⁵ CFU/g *S. aureus* were required for SE production. However, although many food samples with greater than 10⁷ *S. aureus* CFU/g were positive for enterotoxin (0.1 mg of enterotoxin per 100 g), several samples with counts of 10⁹ to 10¹⁰ CFU/g of *S. aureus* were negative for enterotoxin (16).

Air-dried fresh pork sausage is a popular regional meat product. It is fully cooked by the consumer and eaten as a breakfast side item. This sausage is commonly referred to as “dry” sausage; however, it does not achieve the adequate pH and water activity reduction characteristic of a semidry or dry fermented–style sausage. Traditionally, this sausage was made at home, and the finished product was allowed to dry outdoors for 5 to 7 days during the fall and winter months. As the process was commercialized, this drying step was moved into the processing facility, where the product is held at 16 to 24°C for 7 to 10 days. It is during this drying period that a potential hazard of *S. au-*
_reus_ may be introduced during processing and may grow and produce heat-stable enterotoxin. This sausage is produced by grinding lean pork trimmings and mixing with spices that commonly include salt, sage, black pepper, and red pepper flakes. Some sausage formulations may include sodium nitrite. The sausage is then stuffed into natural casings and allowed to dry. The finished dry sausage is commonly stored under refrigeration and is either vacuum packaged or wrapped in butcher paper. To our knowledge, there have been no reports of foodborne illness associated with consumption of this product.

The production of this style of sausage is a significant portion of the value-added meats sold in the southeast. Hazard analysis critical control point validation is a crucial measure for all meat processing operations under federal or state inspection. All meat processors must provide science-based data to validate that all hazards identified in a process are being adequately controlled. No research has been conducted on air-dried fresh pork sausage to determine the safety of its common production methods. Therefore, the objectives of this study were (i) to determine growth and enterotoxin production of _S. aureus_ during maturation of air-dried fresh pork sausage and (ii) to determine the impact of adding 154 ppm of sodium nitrite and increased salt (1.76 or 3.64%) on _S. aureus_ growth and enterotoxin production during maturation of air-dried fresh pork sausage. Salt is commonly added to the product in this concentration range, and nitrite addition is optional.

**MATERIALS AND METHODS**

**Bacterial strains.** Three enterotoxigenic _S. aureus_ strains were purchased from ATCC (American Type Culture Collection, Manassas, Va.). Strain ATCC 27154 (enterotoxin types A and D) was isolated from sliced turkey, strain ATCC 23235 (enterotoxin type D) was isolated from turkey salad, and ATCC 13567 (enterotoxin type A) was isolated from coconut cream pie involved in food poisoning. Each strain was activated in nutrient broth (Difco, Becton Dickinson, Sparks, Md.) for 20 h at 37°C. After activation, each strain was stored in 30% glycerol at −80°C. Cultures were activated prior to use by overnight growth in tryptic soy broth (TSB; Difco, Becton Dickinson) at 37°C at least twice before each experiment.

**Bacterial cocktail preparation.** Stationary-phase cells (10⁶ CFU/ml) were prepared in TSB at 37°C for 20 h. Ten milliliters of each strain was mixed in a 50-ml sterile centrifuge tube (Fisher Scientific, Fair Lawn, N.J.) and centrifuged at 11,950 × g for 2 min at room temperature. The supernatant was discarded, and 30 ml of 0.1% sterile peptone (Fisher) water was added and vortexed. The population of _S. aureus_ was 10⁶ CFU/ml, as confirmed by plating onto tryptic soy agar. This cocktail was serially diluted with 9 ml of 0.1% sterile peptone water to 10⁶ CFU/ml.

**Air-dried fresh pork sausage.** Air-dried sausage was prepared according to a standard formulation. The average composition of sausage was 1,000 g of ground pork (purchased locally), 17.6 g of table salt, 4.41 g of sage, 2.20 g of red pepper flake, and 6.61 g of black pepper. All of the ingredients were mixed with an electric hand-stand mixer (model 106742, GE Electronic Inc., Wal-Mart, Bentonville, Ark.). After mixing sausage ingredients, 10 ml of _S. aureus_ cocktail (10⁶ CFU/ml) was added and mixed for 5 min. Preliminary experiments with mixing time confirmed that the _S. aureus_ cocktail was dispersed throughout the batter in this time. The mixed ingredients were then stuffed into 30-mm natural small intestine hog casings (Natural Best, Chicago, Ill.) with a hand-cranked stuffer. Control and three different treatments of sausage were manufactured: (i) control, 1.76% salt, no _S. aureus_, no sodium nitrite; (ii) 1.76% salt, _S. aureus_, no sodium nitrite; (iii) 2.24% salt, _S. aureus_, 154 ppm of sodium nitrite; and (iv) 3.64% salt, _S. aureus_, no sodium nitrite. Sausages were dried at 21°C with 60% relative humidity (RH) for 10 days in an incubator. The entire experiment was replicated three times.

**Proximate analysis.** Water activity (Aquatic Lab, Pullman, Wash.) and pH (Orion model 250A, Orion Research Inc., Boston, Mass.) were measured in triplicate in dried sausage. Fat and salt content of finished dried sausages were determined by Soxhlet extraction and the Volhard method, respectively, by a commercial test laboratory (Microbac Laboratories, Inc., Wilson, N.C.).

**Microbiological analysis.** Samples of raw sausage were assayed before stuffing and prior to drying. Sausages were sampled daily during drying for microbiological analysis. Samples were plated for total aerobic bacteria, coliforms, and _Staphylococcus_ spp. with Petrifilms (3M Microbiology Products, St. Paul, Minn.) with 9-ml serial dilutions in 0.1% sterile peptone. For each sample, 25 g of sausage was aseptically taken from the 3- to 5-mm outer layer (aerobic bacteria, coliforms, and _Staphylococcus_ spp.), or separate 25-g cross-sectional samples (aerobic bacteria and coliforms) were taken and placed with 225 ml of 0.1% sterile peptone solution into a Whirl-Pak filter bag (Nasco, Fort Atkinson, Wis.). The outer 3- to 5-mm layer of sausages was sampled specifically for _Staphylococcus_ spp., because previous research with fermented sausages indicated the highest numbers of _staphylococci_ in these areas (1, 19). The contents were padded with a stomacher (Circulator 400, Seward, UK) for 2 min at room temperature. Petrifilm plates were incubated at 37°C for 24 to 48 h before counting. Numbers of CFU per gram were counted on duplicate Petrifilm plates having 15 to 150 colonies.

**SE.** An immunoassay for in vitro detection of SE in food samples was used to determine if SEs were present in sausages. The immunoassay (TECRA, Bioenterprises Pty, Ltd., Roseville, New South Wales, Australia) was equipped with polyclonal antisera against SE types A to E (SEA to SEE). SE detection in 10 g of the 3- to 5-mm outer layer of sausages from two samples from each treatment during each experimental replication (total of six analyses per treatment) was performed after 0, 2, 4, 6, 8, and 10 days of drying according to manufacturer’s instructions. The SE detection limit of the immunoassay was reported as 1 ng/g of sample.

**Statistical analysis.** The experiment was replicated three times. Data were subjected to the Statistical Analysis System (version 9.1, SAS Institute, Cary, N.C.). Analysis of variance of CFU per gram was then conducted (PROC GLM procedure in SAS) with least-square means used to determine significant differences (P < 0.05).

**RESULTS AND DISCUSSION**

The fat content of dried sausages was 17.3 ± 1.2 g of fat per 100 g of sausage (wt/wt). Salt contents of dried sausages were 1.76 ± 0.021 g/100 g (wt/wt), 2.24 ± 0.071 g/100 g (wt/wt), and 3.64 ± 0.170 g/100 g (wt/wt) for control (control formulation), sausage with added sodium nitrite, and sausage with increased salt, respectively. Initial pH and water activity were 5.99 ± 0.07 (23°C) and 0.98
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FIGURE 1. Growth of total aerobic bacteria from surface samples of air-dried country sausage during 10 days at 21°C and 60% RH. Bars represent standard error of the mean. Data points are the means from triplicate experiments.

FIGURE 2. Growth of coliforms from surface samples of air-dried country sausage during 10 days at 21°C and 60% RH. Bars represent standard error of the mean. Data points are the means from triplicate experiments.

FIGURE 3. Growth of S. aureus from surface samples of air-dried country sausage during 10 days at 21°C and 60% RH. Bars represent standard error of the mean. Data points are the means from triplicate experiments.

± 0.003 (23°C), respectively. Sausage final pH was 5.57 ± 0.007, and final water activity was 0.97 ± 0.003 (25°C). Generally, the average composition of commercial products will vary, with fat 20 to 40%, water activity 0.96 to 0.92, and pH 5.4 to 6.0. The CFU per gram of control sausage batter prior to drying was 10^2 ± 0.17 CFU/g of aerobic bacteria and 5 ± 0.75 CFU/g of coliform bacteria. No colonies were detected on Staph Express plates. After inoculation of S. aureus cocktail, aerobic and Staph Express plate counts were 10^4 ± 0.12 CFU/g (initial time point) after stuffing.

Raw uncooked sausage is a meat product that can provide a suitable environment for the proliferation of meat spoilage and foodborne bacteria, including S. aureus (6). During maturation and drying, the population of microorganisms increased on all four sausage treatments. Aerobic plate counts increased from 2.2 to 7.7 log on control sausage and from 3 to 7 log on other sausage treatments (Fig. 1). Coliform counts were less than 5 CFU/g for the four different sausages initially. After 10 days’ storage at 21°C and 60% RH, coliforms increased by 4 log for control sausages and control formulations with S. aureus sausages, whereas increases of 1 log were observed for sausage with 154 ppm of sodium nitrite and for those with the addition of 2 times higher salt (Fig. 2). Gram-positive bacteria are better equipped to cope with osmotic stress than gram-negative bacteria, and this may explain why coliform counts decreased with higher salt addition (14). No differences were detected between coliform and total aerobic bacteria counts from cross-sectional or outer 5-mm-layer sausage samples (P > 0.05).

If red-violet colonies are observed on Staph Express Petrifilm plates, these colonies may be considered a confirmed S. aureus (3M Microbiology Products) (11). When other than red-violet colonies are present, a DNase-reactive disk can be used to differentiate S. aureus from other colonies and to provide a confirmed count of S. aureus after a 3-h incubation at 35°C. However, only red-violet colonies were observed on Staph Express Petrifilm plates from sausages, confirming S. aureus counts.

No colonies were observed on Staph Express Petrifilm at any time points on control sausages with no added S. aureus. Initially, S. aureus counts were 4 log/g for the three sausage treatments with added S. aureus. After 10 days’ storage at 21°C with 60% RH, S. aureus increased by 3.1 log for sausages with higher salt, 2.2 log for control sausage formulations, and 2.5 log for sausages with added 154 ppm of sodium nitrite. The sausage formulation with increased salt (3.64%) resulted in the highest population of S. aureus during storage (Fig. 3). However, there were no significant differences between control formulation sausages, sausages with added sodium nitrite, and sausages with increased salt (P > 0.05). Between core (data not shown) and surface samples, aerobic plate count, coliform, and S. aureus counts were similar (P > 0.05). However, surface sampling from
the outer layer of sausages was important for detection of SE because previous studies have indicated that staphylococcal growth and enterotoxin production in sausage were localized in the outer layer of sausage where oxygen tension was highest (1).

On express Petrifilm plates, Wijnker et al. (21) reported a constant death rate of approximately 0.11 log CFU/day for S. aureus at five different brine concentrations (0.90, 0.87, 0.85, 0.83, and 0.75 water activity) in natural sheep casings stored at 20°C for 30 days. In contrast, the growth of S. aureus in bologna-type sausage was unaffected by salt concentrations of 2, 2.8, or 3.5% at 12, 15, or 18°C for 21 days (15). Previous research has also demonstrated that sodium nitrite does not inhibit S. aureus. Bacon with 0 to 120 ppm of NaNO₂ and 0.26% potassium sorbate did not inhibit the growth of S. aureus (5 log/g) at 27°C for 14 days (17).

SEs were not detected during the 10-day storage period for the four different sausage treatments. The important food safety implication of S. aureus growth is whether or not enterotoxin is produced. An increase in S. aureus numbers by 2 to 3 log during sausage maturation is not desirable in any food product, but the absence of enterotoxin from any sausages indicated that the raw sausages may be safe if properly cooked (71). Any sausages indicated that the raw sausages may be safe in any food product, but the absence of enterotoxin from any sausages indicated that the raw sausages may be safe if properly cooked (71). Any sausages indicated that the raw sausages may be safe in any food product, but the absence of enterotoxin from any sausages indicated that the raw sausages may be safe if properly cooked (71). Any sausages indicated that the raw sausages may be safe in any food product, but the absence of enterotoxin from any sausages indicated that the raw sausages may be safe if properly cooked (71). Any sausages indicated that the raw sausages may be safe in any food product, but the absence of enterotoxin from any sausages indicated that the raw sausages may be safe if properly cooked (71).

Environmental drying and temperature very likely played a critical role in the lack of SE formation, because the optimum temperature for SE production is 37 to 45°C (13, 20). Previous studies with fermented sausages demonstrated that aerobic conditions (e.g., outer surface layer of sausage) and fermentation temperatures (e.g., >37°C) promoted SE production (1). S. aureus is also a poor competitor, and background microflora may also aid in prevention of optimal conditions for SE production (7). Because S. aureus cells are likely to be present in raw meat, initial numbers of this pathogen are certainly critical, and the increases in coliform bacteria also suggest that enteric pathogens (e.g., Salmonella) can also be a concern. Quality ingredients, hygienic preparation, and proper temperature during drying and postdrying play critical roles in maintaining the traditional safety record of this product.

The addition of more salt, sodium nitrate, or a combination of these components may inhibit the growth of spoilage or pathogenic bacteria and SE production in air-dried meat. From experiments, adding 154 ppm of sodium nitrite or doubling the higher salt was effective in the inhibition of growth of coliform bacteria. However, higher salt (3.64%) may contribute to an optimum salt environment for S. aureus because 3.7% salt is the optimum salt concentration for S. aureus growth (9). Ingham et al. (8) mentioned that relatively high salt (=6% water phase salt) could support S. aureus growth on moderately dried RTE meat products.

Large numbers of staphylococci can be present in foods, but if the food is heated or boiled, the staphylococci will be killed; any enterotoxin produced will not be inactivated. Bergdoll (3) concluded that at least 10⁵ CFU/g of food was required to produce enterotoxin at concentrations that will cause foodborne illness. When assaying 94 brain heart infusion broth samples of S. aureus, enterotoxin was detected in all brain heart infusion broths with a positive optical density reading (10⁵ CFU/ml) (18). However, studies with a variety of foods also demonstrated that high numbers of S. aureus did not necessarily equate to SE production (16). These results are consistent with our study in that outgrowth of S. aureus in a food does not necessarily mean that enterotoxin will be produced. Our results suggest that drying of fresh pork sausage under similar parameters listed in this study does not support SE production.

The addition of sodium nitrite or increased salt to air-dried fresh pork sausage did not effectively prevent growth of S. aureus. However no enterotoxin was detected. Although increased salt did not inhibit growth of S. aureus, coliform growth was inhibited. These results suggest that nitrite addition enhances microbial quality of air-dried fresh pork sausage by limiting the growth of coliform bacteria and that this product is not at high risk for staphylococcal food poisoning, because enterotoxin was not detected. Meat processors may use this information to validate the process of manufacturing air-dried fresh pork sausage.

REFERENCES


