

# *Staphylococcus aureus* survival, staphylococcal enterotoxin production and shelf stability of country-cured hams manufactured under different processing procedures

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Received 17 September 2001; received in revised form 6 December 2001; accepted 6 December 2001

## Abstract

Fresh hams were inoculated with *Staphylococcus aureus*, cured, equalized, cold smoked or non-smoked, and aged. Initial *S. aureus* populations of 8.57 and 8.12 Log<sub>10</sub> CFU/cm<sup>2</sup> for salt and salt + NO<sub>2</sub> hams decreased to below the levels of detection after the fourth month of aging. *S. aureus* was detected following enrichment for 75% of the inoculated and 62% of the control hams at the end of the aging period. Staphylococcal enterotoxin was detected in 40% of the inoculated and 50% of the control hams following the aging period. The NaCl content of these hams with or without nitrite were 4.45/3.37% and *a<sub>w</sub>* values 0.94/0.91. Country-cured ham products obtained from retail stores in Kentucky were all negative for *S. aureus* enterotoxin. These results indicate that higher salt content and lower *a<sub>w</sub>* values on country-cured hams play an important role in controlling the growth and toxin production of *S. aureus*. © 2002 Elsevier Science Ltd. All rights reserved.

**Keywords:** Country-cured hams; *Staphylococcus aureus*; Staphylococcal enterotoxin

## 1. Introduction

*Staphylococcus aureus* is the predominant species involved in staphylococcal food poisoning outbreaks. Enterotoxins involved in food poisoning are produced by approximately one-third of the coagulase positive strains of *S. aureus* (Halping-Dohnalek & Marth, 1989). Occasionally strains of coagulase negative species have also been reported to produce enterotoxin (Nanu & Narayan, 1992). Enterotoxins have been associated with foodborne disease, with Staphylococcus enterotoxin A (SEA) and Staphylococcus enterotoxin D (SED) being the most frequently associated (Minor & Marth, 1972). In 1983 *S. aureus* with cold smoked ham as a vehicle, was involved in a foodborne outbreak on a river boat, where USDA attributed the “cold smoked” method of processing for allowing *S. aureus* to grow (CDC, 1983). In 1992 food poisoning caused by SEA occurred in Germany after consumption of dry-cured smoked ham (Uterman & Müller, 1992). Commonly, food containing the pre-formed enterotoxin is often normal in odour,

appearance, and taste (Bryan, 1976). Therefore, due to the history of *S. aureus* in country-cured products, the safety of the meat supply as well as consumer safety and to provide technical assistance to the country-cured ham industry. This study was designed to validate a six log reduction in *S. aureus* population and consequently toxin production by the use of different processing procedures. Shelf stability of country-cured ham was monitored by assessing water activity (*a<sub>w</sub>*), salt content (NaCl), pH and moisture protein ratio (MPR).

## 2. Material and methods

*S. aureus* (ATCC 12600, 27543, 25923, 6538) were obtained from the culture collection of the Food Science Section, Department of Animal Science, University of Kentucky. Each strain of *S. aureus* was transferred twice into Brain Heart Infusion Broth (BHI) (Difco, Detroit MI) and incubated at 35 °C. Cultures with 10<sup>7</sup>–10<sup>8</sup> cells/ml were combined on the day the hams were inoculated. Approximately 3 ml of the microbial cocktail was inoculated onto hams via spraying (calibrated 250 ml plastic spray bottle). Two areas were inoculated, the center section, and ham face.

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### 2.1. Inoculation, curing, equalization, smoking and aging

One hundred and sixteen fresh hams, between 8.5 and 10.5 kg, were obtained from a commercial processor and divided into two curing treatments groups. Cure mixture was either (salt cure mixture) 7.5 g salt (Aberger Flake Salt, Cargill Foods, Inc., Minneapolis, MN) and 2.5 g sugar (Pure Cane Sugar, Savannah Food Industrial, Inc. Savannah, GA.), or (salt + NO<sub>2</sub> cure mixture) 7.5 g salt, 2.5 g sugar and 0.28 g of nitrite (AC Legg Burminham, AL) per kg of ham. The cure mixture was rubbed over the entire surface of the ham.

Hams were inoculated with a cocktail of *S. aureus* and then one-half was cured with salt, the remaining hams cured with salt and nitrite. (Fig. 1).

After equalization, half of each treatment as cold smoked for 8 h using Alkar, 450 mini-smoker, (DEC International, Inc. Lodi, Wisconsin 53555) at ≤ 18 °C (cold smoke).

Three hams per treatment were analyzed for microbial reduction after curing, equalization, smoking, and after each month of aging. One un-inoculated control ham cured with salt and one control ham with salt and nitrite were used for chemical analysis (Table 1).

### 2.2. Enumeration

Swab rinse procedure (APHA, 1972) with phosphate buffer was used to swab the hams. A series of decimal dilutions in phosphate buffer were plated in duplicate

on pre-poured and dried plates of Baird Parker Agar (Difco), spread with a sterile bent rod and incubated at 35 °C for 24 h. Shiny, brown-black colonies with an opaque halo were counted as *S. aureus*.

Table 1  
Number of hams analyzed during country-cured ham processing

Processing time (days)	Treatments					
	Salt cure			Salt + NO <sub>2</sub>		
	Control <sup>a</sup>	NS <sup>b</sup>	Smoked	Control <sup>a</sup>	NS <sup>b</sup>	Smoked
0		3			3	
45 (Curing)		3			3	
66 (Equalization)		3	3		3	3
94 (Aging)	1	3	3	1	3	3
122	1	3	3	1	3	3
150	1	3	3	1	3	3
178	1	3	3	1	3	3
206	1	3	3	1	3	3
234	1	3	3	1	3	3
	3 <sup>c</sup>			3 <sup>c</sup>	3	3
Total hams	9	27	21	9	27	21
		114 + 2 <sup>d</sup> = 116				

<sup>a</sup> Control hams used for chemical analysis.

<sup>c</sup> Control hams used for microbial evaluation at the end of the aging period.

<sup>b</sup> NS non-smoked.

<sup>d</sup> Two control fresh hams, with neither cure nor inoculation used to evaluate *S. aureus*.

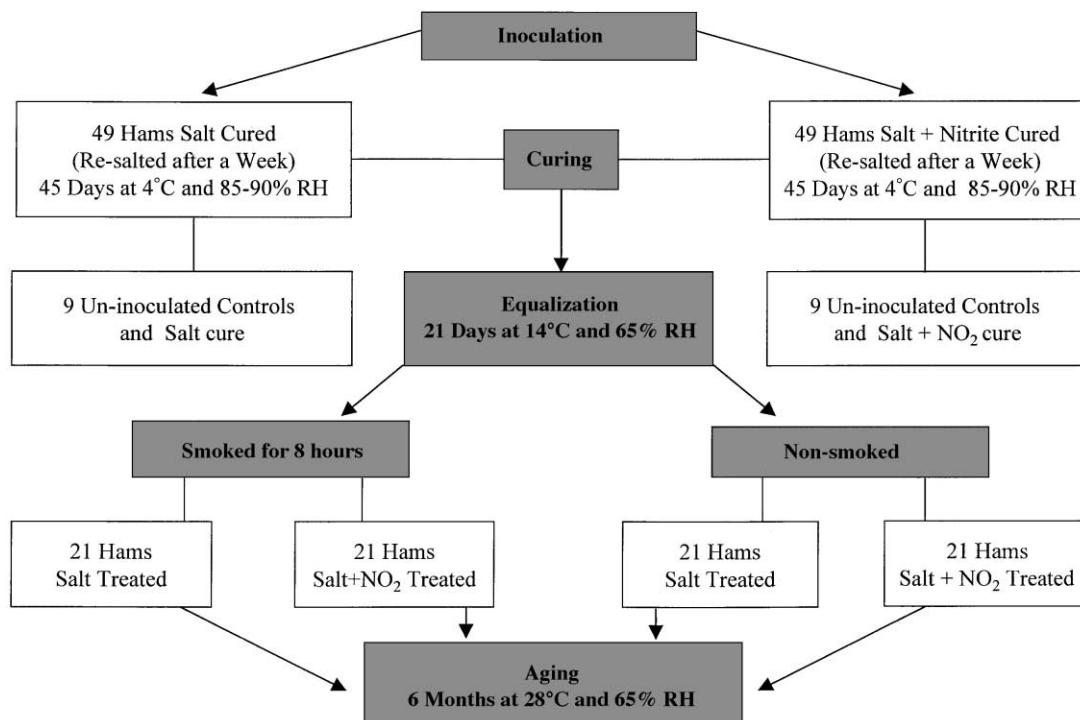


Fig. 1. Experimental design for inoculated country-cured ham.

### 2.3. Enrichment procedure and pathogen detection on inoculated and control hams

A 25 g sample from inoculated and control hams was stomached with 225 ml of tryptic soy broth (TSB) (Difco) with 10% sodium chloride and 1% of sodium pyruvate. Samples were incubated at 35 °C for 48 h. After incubation, samples were streaked onto Baird Parker Agar and incubated at 35 °C for 24 h (BAM, 1998).

### 2.4. Coagulase test

Suspect *S. aureus* were obtained from Baird Parker Agar and inoculated into BHI at 35 °C for 24 h. Then, a coagulase test was performed following Bacteriological Analytical Manual (BAM, 1998).

### 2.5. Detection of enterotoxin-production

ELISA test kit (TECRA), Staphylococcal enterotoxin (SET) visual immunoassay (Bioenterprice Pty Ltd (CAN 008 556 652), PO Box 20, Roseville, NSW 2069, Australia), was used to confirm enterotoxin production in the challenge (ATCC 12600, 27543, 25923, 6538) Staphylococci. A blend of surface and internal country-cured ham samples obtained during the aging process and on control hams at the end of the process were ground (Kitchen Aid- Food Processor. KSM90WH, Inc., St. Joseph, Michigan, USA) and also analyzed. Sample preparation followed protocol #6 and #5, respectively, in the kit instruction.

The enzyme-linked immunosorbent assay was performed following kit manual and read using a plate reader (HT 7000, HT-Soft 1.0. Filter 405 nm).

### 2.6. Chemical analysis

Slices of un-inoculated ham from salt cure and salt plus nitrite cure were ground using a Kitchen Aid-Food Processor (KSM90WH, Inc., St. Joseph, Michigan, USA). Ground samples were divided into well-labeled plastic bags for moisture, pH, protein, salt, and  $a_w$  analysis.

For moisture analysis, approximately 5 g of sample was placed on a dried pre-weighed aluminum pan and placed into a Programmable Lab Oven at 100–102 °C for 24 h or dried to constant weight. The sample was cooled in a desiccator and weighed. Percentage of weight loss was calculated and was described as% moisture content (AOAC, 1995).

For pH measurement, two 5 g samples from control un-inoculated hams were placed into 80 ml beakers and homogenized with 45 ml of distilled water using a Polytron homogenizer (PT10/35, Switzerland Kinematic, AG) until no lumps were observed. The pH of

the homogenates was measured using a potentiometric method (Accumet Basic AB15 pH meter with Silver/Silver Chloride reference, Fisher Scientific).

For protein content, a 100 g sample of frozen ground ham was freeze dried (Model 41 Sub Special. PePP. Division of the Virtis Co. Gardiner, New York) for 3 days at 150 °C. A 0.2 g sample in duplicate was evaluated using a Nitrogen analyzer apparatus (FP-2000, Leco Corporation, 3000 Lakeview Av., St. Joseph, MI 49085). Nitrogen content was determined by comparison to an EDTA standard (Calibration sample, Leco Corporation, 3000 Lakeview Av., St. Joseph, MI 49085). Nitrogen results were then multiplied by 6.25 factor to obtain the protein percentage of the samples. Protein and moisture content were used to calculate MPR, (formula: Moisture Protein Ratio (MPR) = % Moisture Content/%Protein).

Thirty-six samples, distributed into 5 g aliquots were analyzed for salt content according to AOAC (AOAC, 1995).

To evaluate water activity, duplicate ground ham samples were placed into plastic cups (40 mm) for  $a_w$  determination using a Benchtop System- $A_w$  meter (Rotronic Inc., Huntington, NY).

### 2.7. Commercial country-cured ham samples.

Sixty-four, vacuum-packed, country-cured samples were obtained from different retail stores in Kentucky. The samples consisted of vacuum packed country-cured slices, end slices, center-ends, wafer thins, ham chips, biscuit slices, pieces for seasoning and trimmings. Samples were ground and stored at 5 °C until analyzed for  $a_w$ , NaCl and *S. aureus* enterotoxin analysis.

### 2.8. Statistical analysis

Chemical and microbiological values were analyzed according to a complete block design. Correlation coefficients between microbial population reduction and chemical variables were determined. Bacterial counts were transformed into logarithms for statistical analysis. Differences were determined using Least Square Difference. Analysis was done using the General Linear Model procedure (SAS Institute Inc., Cary, NC, version 1998). Probability levels of  $P < 0.05$  were considered significant.

## 3. Results and discussion

*S. aureus* populations on hams prior to curing was  $< 10$  CFU/g. Previous studies on intact hams stored frozen prior to curing showed detectable levels of Staphylococci either on surface or core samples (Kemp, Obidoye, Langlois, Franklin, & Fox, 1980). The average

inoculum concentration of *S. aureus* population on inoculated country-cured hams was 8.57 and 8.12 Log<sub>10</sub> CFU/cm<sup>2</sup> for salt and salt+NO<sub>2</sub> cured hams respectively (Fig. 2). A 2-log reduction in the *S. aureus* population occurred following the curing process (45 days). After equalization (66 days) the population continued to decrease 2 and 4 logs for salt and salt+NO<sub>2</sub> cured hams, respectively. After 94 days (1 month aging), the *S. aureus* populations continued to decrease for salt cured, either smoked or non-smoked hams, and salt+NO<sub>2</sub> non-smoked hams but increased for the salt+NO<sub>2</sub> smoked hams. At 150 days (3 months aging), the salt cured hams, either smoked or non-smoked, had *S. aureus* counts below the level of detection. However, the salt+NO<sub>2</sub> smoked hams increased 3.6 logs and salt+NO<sub>2</sub> non-smoked hams decreased 0.40 log. It was not until the fourth month of aging that the overall *S. aureus* population decreased below the level of detection for all treatments (Fig. 2). There were significant differences in population reductions throughout the time period either for salt or salt + NO<sub>2</sub> and smoked or non-smoked hams ( $P < 0.01$ ), with the non-smoked ham group demonstrating a more rapid reduction than the smoked hams.

Regardless of treatment this study demonstrated that *S. aureus* was detected after the enrichment procedure on 75% of the inoculated hams and 50% of the control intact hams at the end of the aging period. These results can be explained by the presence of stressed cells as a result of the curing and aging process which provided adverse environmental conditions. Injured cells may lose metabolic, growth and toxin production capabilities or produce toxin in lesser quantities (Busta,

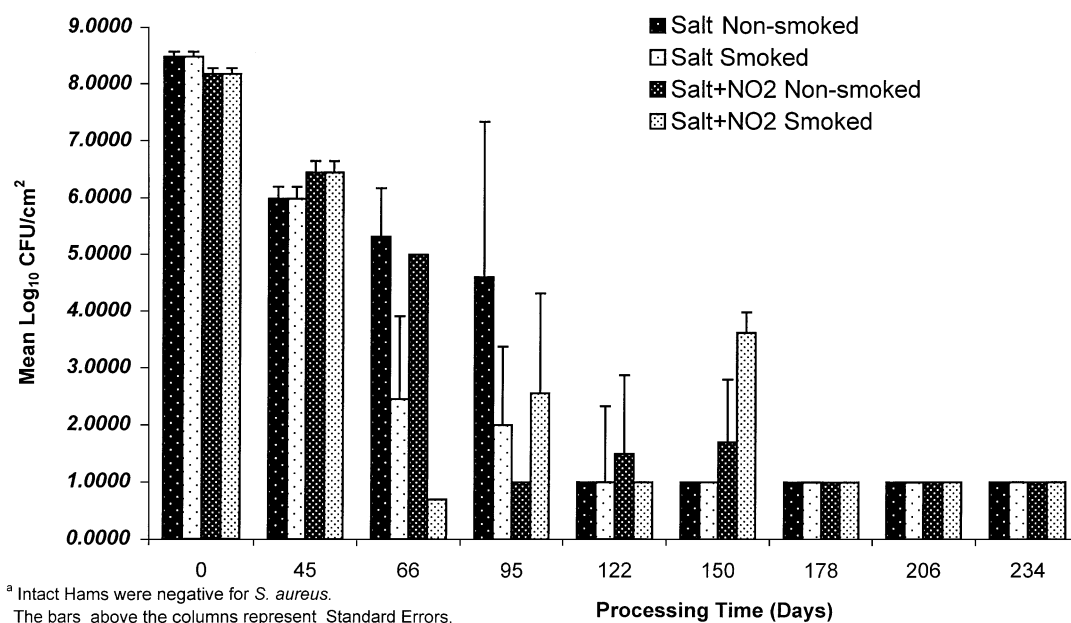
1976). These injured cells may not be able to initiate growth during the plating but could be recovered following enrichment procedure.

The chemical analysis for control hams demonstrated significant differences in the  $a_w$ , NaCl%, MPR and pH between the cure treatment ( $P < 0.05$ ; Table 2). It is well known that *S. aureus* may withstand the stress of desiccation remarkably well (Holley, 1985). *S. aureus* can grow from 6.7 to 47.8 °C at minimum  $a_w$  range of 0.83–0.86 and can produce enterotoxin from 10 to 46 °C at  $a_w$  above 0.88 (Lotter & Lesitner, 1978). The  $a_w$  values in this study had a mean of 0.94 for salt and 0.91 for salt+NO<sub>2</sub> treatment which is not considered a risk when compared with previous studies (Uterman & Müller, 1992). However, any potential problem would be overcome by drying hams at cooler temperatures after curing and then cold smoking at a temperature less than 18 °C as it has been described in this study. The mean salt concentrations were 4.45% for salt and 3.37% for salt+NO<sub>2</sub> showing significant differences in population reduction and NaCl content among treatments ( $P < 0.05$ ). Previous studies have shown NaCl content of 6.44%, much higher than the hams in this

Table 2  
Mean results of chemical analysis on country hams<sup>a</sup>

Treatments	$a_w$	NaCl(%)	MPR	pH
Salt Cure	0.94±0.01b	4.45±1.51b	2.76±0.71b	6.13±0.21b
Salt+NO <sub>2</sub> Cure	0.91±0.02c	3.37±0.33c	2.20±0.61c	6.27±0.39c

<sup>a</sup> Mean of six samples. Mean in the same columns with different letters differ ( $P < 0.05$ ) as per LSD procedure.



<sup>a</sup> Intact Hams were negative for *S. aureus*.  
The bars above the columns represent Standard Errors.

Fig. 2. *Staphylococcus aureus* evaluation on inoculated<sup>a</sup> country cured hams.

study (Lotter & Lesitner, 1978). In fact,  $a_w$  mean values (0.86) and salt content (6.44%) for the commercial hams analyzed in this study demonstrated more appropriate values comparing with the non-commercial study group, suggesting that lower  $a_w$  and higher salt content together play a intricate role in *S. aureus* inhibition and control in country-cured hams.

Losses in moisture are reflected by decreased MPR and in decreased proportion of  $a_w$  values. MPR is known as an indicator of shelf stability (Palumbo, Kissinger, Miller, Smith, & Zaika, 1979). MPR at first month of aging were 4.01/1 and 2.43/1 for salt and salt + NO<sub>2</sub> with a mean of 2.76/1 and 2.20/1 for the entire process. These MPR results were higher comparing with related products such as semi-dry smoked sausages which showed a MPR 1.25/1. Also, fully dry pepperoni with MPR of 1.60/1 (Palumbo et al., 1979). Therefore, higher MPR values in this study could be the product of lower salt concentration during the process and consequently promoting less water and moisture losses. There were no significant differences in *S. aureus* reduction and MPR or pH ( $P < 0.05$ ).

Enterotoxin production by *S. aureus* is a key to food intoxication caused by this microorganism, it has been frequently associated with high population numbers (10<sup>5</sup> organism/g) prior to the detection of enterotoxin (Barber & Deibel, 1972; Lee, Harmon, & Price, 1977; Pullen & Genigeorgis, 1977). Enterotoxin was detected in this study during the aging period on many of the hams tested. Forty percent of the hams (29 hams) were positive at the end of the aging period and three hams

from the six un-inoculated control hams were also positive for toxin (Table 3). None of the commercial hams analyzed in this study had positive toxin. Fang, Langlois, and Moody, (1997) did not detect toxin on vacuum-packaged dry-cured ham slices stored at 2 and 25 °C, results that agree with the commercial hams analyzed in this study.

Studies indicate that even under sub-optimal conditions, *S. aureus* can initiate, sustain growth and the cell can easily synthesize and secrete toxin (Lee, Silverman, & Munsey, 1981). Also, Enterotoxin SEA and SED are produced under less favorable conditions than Staphylococcus enterotoxin B (SEB), C (SEC), and E (SEE) (Ewald & Notermans, 1988). In agreement with these studies, organisms used to inoculate hams in this research contained SEA and SED genes.

Higher toxin positive samples, either on inoculated or control hams, may have several explanations. Pre-formed toxin may have already been present on the fresh ham prior to processing; however, we assumed that the hams were treated under conditions which would not allow the contamination, survival or proliferation of *S. aureus* and consequently enterotoxin production in the fresh product. *S. aureus* counts prior to curing detected no cultivable *S. aureus*. However, the country-cured commercial hams were negative for *S. aureus* enterotoxin, suggesting that the commercial hams were probably free of toxin and the further processing procedures were effective and efficient in controlling any possible enterotoxin producing *S. aureus* contamination.

Table 3  
*Staphylococcus aureus* enterotoxin evaluation on inoculated and un-inoculated control country-cured hams

Days of aging	Treatments				% Positive per month
	Non-smoke		Smoked		
	Salt cured	Salt + NO <sub>2</sub> Cure	Salt cured	Salt + NO <sub>2</sub> Cure	
<i>Inoculated hams</i>					
94	0/3	1/3	1/3	1/3	25%
122	0/3	1/3	2/3	3/3	50%
150	2/3	1/3	2/3	1/3	50%
178	2/3	0/3	0/3	0/3	16%
206	0/3	3/3	0/3	0/3	25%
234	1/3	3/3	2/3	3/3	75%
Total positives	5/18	9/18	7/18	8/18	
	<sup>a</sup> Total 29 toxin positives/72 hams			40.27%	
<i>Un-inoculated control hams</i>					
	Salt Cure	Salt + NO <sub>2</sub> Cure	Positive		
	2/3	1/3	3/6		
	<sup>b</sup> Total 3 toxin positive/6 hams		50%		

<sup>a</sup> Three inoculated hams were tested per treatment with a total of 72 hams after aging process.

<sup>b</sup> Three un-inoculated hams was tested per treatment with a total of six hams after aging process.

Since staphylococci is a normal flora in cured ham (Cassens, 1994), and even though enterotoxin production has been generally related with coagulase positive *S. aureus*, it is possible that other coagulase positive or non-coagulase positive staphylococci could be playing an important role in toxin production (Hirooka, Muller, Freitas, Vicente, Yashimoto, & Bergoll, 1988; Valle, Gomez-Lucia, Piriz, Goyache, Orden, & Vadillo, 1990). Strains of coagulase negative staphylococci also have been associated with toxin production (Bautista, Gaya, Medina, & Nuñez, 1988; Ewald & Notermans, 1988; Morita, Patterson, & Woodburn, 1979; Nanu & Narayan, 1992; Valle et al., 1990). These organisms were not isolated and identified in this study.

Many factors have been suggested to play important roles in enterotoxin production, including: *S. aureus* growth (Barber & Diekel, 1972; Lee et al., 1977; Pullen & Genigeorgis, 1977), presence and concentration of salts (McLean, Lilly, & Alford, 1968), pH and temperature (Uterman & Müller, 1992),  $a_w$  (Troller, 1986), processing exposure, microbial competition (Noletto & Bergoll, 1980) among others. However, none of the conditions alone has been productive in explaining toxin production and prevention. In this study, there was no correlation between the detection of enterotoxin and any of the chemical variables analyzed (data not shown).

Finally, *S. aureus* can survive in country-cured ham and remain as a dormant population. The fact that uninoculated control hams were also positive for toxin infers that other staphylococci strains may have been present with these competing organisms, which may mask the enterotoxigenic ones and possibly inhibit their growth but not enough to prevent enterotoxin production (Noletto & Bergoll, 1980). Even though cross-contamination of the samples could occur during the curing and aging process, *S. aureus* populations were undetected using plating procedures after 150 days. Nevertheless, negative *S. aureus* enterotoxin in commercial country-cured hams suggest that probably certain biological and/or chemical factor combinations such as lower  $a_w$  and higher salt content in the product and control of processing conditions should have a positive effect on toxin control. Therefore, additional research should be conducted to evaluate other staphylococci strains and types of toxins present when *S. aureus* is absent at early stages of the country-cured ham process to ensure that the product will be safe for human consumption.

### Acknowledgements

Special acknowledgment is made of the National Pork Producers Council for partial support of this research. Thanks to Katherine Akers for technical assistance and advice.

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