Inhibition of Toxigenesis of Group II (Nonproteolytic) Clostridium botulinum Type B in Meat Products by Using a Reduced Level of Nitrite

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ABSTRACT

The effect of three different concentrations of sodium nitrite (0, 75, and 120 mg/kg) on growth and toxigenesis of group II (nonproteolytic) Clostridium botulinum type B was studied in Finnish wiener-type sausage, bologna-type sausage, and cooked ham. A low level of inoculum (2.0 log CFU/g) was used for wiener-type sausage, bologna-type sausage, and cooked ham. The products were formulated and processed under simulated commercial conditions and stored at 8°C for 5 weeks. C. botulinum counts were determined in five replicate samples of each nitrite concentration at 1, 3, and 5 weeks after thermal processing. All samples were positive for C. botulinum type B. The highest C. botulinum counts were detected in nitrite-free products. Toxigenesis was observed in nitrite-free products during storage, but products containing either 75 or 120 mg/kg nitrite remained nontoxic during the 5-week study period, suggesting that spores surviving the heat treatment were unable to germinate and develop into a toxic culture in the presence of nitrite. The results suggest that the safety of processed meat products with respect to group II C. botulinum type B can be maintained even with a reduced concentration (75 mg/kg) of sodium nitrite.

Foodborne botulism is a rare but severe disease that occurs after the ingestion of food containing preformed neurotoxin of Clostridium botulinum (20). The first documented foodborne botulism cases in the late 18th century were associated with consumption of meat and blood sausages (11). C. botulinum spores are commonly found in soil and in the intestinal tract of cattle and pigs; therefore, spores in dust, soil, or feces may contaminate meat during slaughter or meat processing (6, 7, 15, 16). Modern botulism cases related to meat are mainly caused by home-prepared meats, and outbreaks traced to commercial meat products are rare (26, 29, 31).

Because of nitrite’s antioxidant properties and ability to give an intense red color and the desired taste, this compound is widely used to cure meat (3). Nitrite also has antimicrobial activity, the most important aspect of which is the inhibition of growth of and toxin production by C. botulinum (28). Although the benefits of the curing process are well known, the use of nitrite in meat curing has raised public concern because nitrite can be a precursor of nitrosamines, many of which are known to be carcinogenic (28). However, studies have failed to show a consistent link between nitrite and cancer (1). High levels of nitrite cause methemoglobinemia (3), and epidemiological evidence indicates that nitrite may increase the risk of type I diabetes (8, 30). Because of health concerns, the concentration of nitrite permitted in meat products is regulated; the maximum concentration of added sodium nitrite in cured meat products in the European Union is 150 mg/kg (12). Although the approved levels of nitrite are deemed safe, consumers are constantly pushing for further reductions in the use of nitrite.

In Europe, foodborne botulism cases are often associated with meat products and are caused by group II (nonproteolytic) C. botulinum type B (15, 22, 26, 27, 31). Group II C. botulinum strains are psychrotrophic and thus able to grow at refrigerated temperatures. Although the spores of group II C. botulinum are less heat resistant than spores of group I (proteolytic) C. botulinum (25), group II spores may survive heat treatments used in production of meat products (19). The effect of nitrite on the growth and toxigenesis of group I C. botulinum in meat products has been widely studied (2, 5, 13, 17), but little information is available on the antibotulinal effect of nitrite on group II C. botulinum type B in nonfermented, vacuum-packaged meat products.

The aim of this study was to investigate the effect of various concentrations of nitrite on growth and toxigenesis of group II C. botulinum type B inoculated at various levels into three Finnish meat products to evaluate whether...
The three meat products studied were nitrite- and pH-reduced products in order to reduce nitrite concentrations can be used without sacrificing product safety.

**MATERIALS AND METHODS**

**Meat products.** The three meat products studied were wiener-type sausage, Finnish bologna-type cooked sausage, and cooked ham. The ingredients and NaCl concentrations of the products are described in Table 1. Three concentrations of nitrite (0, 75, and 120 mg/kg) were used in manufacture of all products. The wiener-type sausage and bologna-type sausage batters were prepared at the Department of Food Hygiene and Environmental Health (DFHEH; University of Helsinki, Helsinki, Finland), and cooked ham batter was manufactured at the Finnish Meat Research Institute (Hämeenlinna, Finland) and transported refrigerated to the DFHEH for further processing. Before inoculation, each batch of raw batter was sampled to test for the presence of spores of *C. botulinum* types A, B, E, and F.

**Inoculation with *C. botulinum* strains.** The *C. botulinum* spore inoculum consisted of equal numbers of three group II type B strains: Eklund 2B, Eklund 17B, and Hatthewy 706B. Before inoculation, the spore suspensions of each strain were enumerated as described by Doyle (10). A low level of inoculum (2.0 log CFU/g) was used for wiener-type sausage and bologna-type sausage, but both low (2.0 log CFU/g) and high (4.0 log CFU/g) levels of inoculum were used for cooked ham. The spore mixture was diluted in sterile distilled water and thoroughly mixed with the raw sausage or ham batter. After inoculation, the wiener-type sausage batter was stuffed into natural casings made from lamb intestine. A fiber-reinforced cellulose casing with inner barrier (75 mm in diameter; Nalo Top, Kalle GmbH, Wiesbaden, Germany) was used for finely chopped bologna-type sausage batter, and plastic casing (98 mm in diameter; Tripan WF 2000, Naturin GmbH & Co. KG, Weinheim, Germany) was use for the cooked ham batter.

**Thermal processing, packaging, and storage conditions.** The products were cooked (Vemag CS700, Vemag Maschinenbau GmbH, Verden, Germany) immediately after stuffing of the casings. The meat products were processed under simulated commercial conditions. The internal temperature of the cooking chamber and the products was monitored (DP-158, Envic, Turku, Finland) with a temperature probe (Ellab A/S, Roedovre, Denmark) during the heat treatment, and water cooling was initiated when the internal temperature of the products reached 72°C. The pasteurization times of the thermal processes, based on product internal temperatures and assuming a Tref of 85°C and a z-value of 7°C (21), were 0.04, 0.28, and 0.45 min for wiener-type sausage, bologna-type sausage, and cooked ham, respectively. After cooling, the bologna-type sausage and the cooked ham were sliced. After processing and cooling, all product samples were vacuum packed (Turbovac, HFE Vacuum Systems, ’s-Hertogenbosch, The Netherlands). Samples of each product were stored at 8°C for 5 weeks.

**Sampling procedures and *C. botulinum* MPN counts.** The most-probable-number (MPN) counts for *C. botulinum* were determined in five replicate samples for each nitrite concentration at 1, 3, and 5 weeks after thermal processing. A PCR assay for growth detection was used as described by Hielm et al. (14) with some modifications. Quantification was based on a five-tube MPN series. Ten-fold dilutions of samples were inoculated into tubes containing tryptone–peptone–glucose–yeast extract broth and incubated anaerobically at 30°C for 72 h, followed by similar overnight culture at 30°C. Cells from overnight cultures were washed and heated as described elsewhere (21) and used as a template for the PCR with primers specific to the *C. botulinum* neurotoxin type B gene (18). Each batch of raw batter was tested for the presence of *C. botulinum* types A, B, E, and F using parallel five-tube MPN series that were incubated at 30 and 37°C and subjected to multiplex PCR (18).

**Detection of botulinum neurotoxin.** The presence of botulinum neurotoxin in the samples was determined by the mouse bioassay (23) with approval from the State Provincial Office of Southern Finland (ESLH-2001-08351). All efforts were made to reduce the number of animals used, e.g., the presence of toxin was not determined on week 5 when all parallel samples tested positive for botulinum neurotoxin on week 3.

**Statistical analysis.** Statistical significance was determined by Student’s *t*-test and an analysis of variance using SPSS statistical software version 10.0 (SPSS Inc., Chicago, IL). The bacterial counts were log transformed before analysis.

**RESULTS AND DISCUSSION**

Each batch of raw batter was free of natural contamination with *C. botulinum* types A, B, E, and F. All inoculated samples studied were positive for *C. botulinum* type B regardless of the concentration of nitrite added (Table 2). Thus, the thermal process used in production of the meat products was insufficient to eliminate all inoculated *C. botulinum* spores. The highest counts were detected and significant growth of *C. botulinum* was observed only in nitrite-free products. In bologna-type sausages, a significant difference (*P* < 0.05) in *C. botulinum* counts was observed on week 5 between products with nitrite concentrations of 0 and 75 mg/kg. On weeks 3 and 5, the nitrite-free wiener-type sausages had significantly higher (*P* < 0.05) *C. botulinum* counts than did wiener-type sausages containing 75 or 120 mg/kg nitrite. In the cooked ham samples with low and high levels of inoculum, *C. botulinum* counts were significantly higher (*P* < 0.05) on week 3 in nitrite-free hams than in hams containing 75 or 120 mg/kg nitrite. In cooked ham with the high level of inoculum, a significant difference (*P* < 0.05) in *C. botulinum* counts was observed on week 5 between nitrite-free ham and ham with 75 mg/kg nitrite. In bologna-type sausages and in cooked hams with both levels of inoculum, no significant difference (*P* < 0.05) in *C.

**TABLE 1. Ingredients of meat products formulated for this study**

<table>
<thead>
<tr>
<th>Product</th>
<th>Ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wiener-type sausage</td>
<td>Pork (37%), beef (20%), pork rind, soy protein, NaCl (1.6%), spices, phosphate, ascorbate, sodium nitrite, water</td>
</tr>
<tr>
<td>Bologna-type sausage</td>
<td>Pork (30%), mechanically deboned poultry meat (13%), pork rind, potato flour, soy protein, NaCl (1.6%), spices, phosphate, ascorbate, sodium nitrite, water</td>
</tr>
<tr>
<td>Cooked ham</td>
<td>Ham (86%), NaCl (1.80%), phosphate, sodium ascorbate, sodium nitrite, water</td>
</tr>
</tbody>
</table>

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**References**

1. Allen, J. Food Prot., Vol. 75, No. 7 INHIBITION OF TOXIGENESIS OF GROUP II C. BOTULINUM BY NITRITE

2. Statistical analysis. Statistical significance was determined by Student’s *t*-test and an analysis of variance using SPSS statistical software version 10.0 (SPSS Inc., Chicago, IL). The bacterial counts were log transformed before analysis.

3. RESULTS AND DISCUSSION

4. Each batch of raw batter was free of natural contamination with *C. botulinum* types A, B, E, and F. All inoculated samples studied were positive for *C. botulinum* type B regardless of the concentration of nitrite added (Table 2). Thus, the thermal process used in production of the meat products was insufficient to eliminate all inoculated *C. botulinum* spores. The highest counts were detected and significant growth of *C. botulinum* was observed only in nitrite-free products. In bologna-type sausages, a significant difference (*P* < 0.05) in *C. botulinum* counts was observed on week 5 between products with nitrite concentrations of 0 and 75 mg/kg. On weeks 3 and 5, the nitrite-free wiener-type sausages had significantly higher (*P* < 0.05) *C. botulinum* counts than did wiener-type sausages containing 75 or 120 mg/kg nitrite. In the cooked ham samples with low and high levels of inoculum, *C. botulinum* counts were significantly higher (*P* < 0.05) on week 3 in nitrite-free hams than in hams containing 75 or 120 mg/kg nitrite. In cooked ham with the high level of inoculum, a significant difference (*P* < 0.05) in *C. botulinum* counts was observed on week 5 between nitrite-free ham and ham with 75 mg/kg nitrite. In bologna-type sausages and in cooked hams with both levels of inoculum, no significant difference (*P* < 0.05) in *C.
botulinum counts was observed between products with 75 or 120 mg/kg. On weeks 3 and 5, wiener-type sausages with 75 mg/kg nitrite had significantly lower \( (P < 0.05) \) *C. botulinum* counts than did wiener-type sausages containing 120 mg/kg nitrite.

All product types processed without nitrite had evidence of toxigenesis after 3 or 5 weeks of storage at 8°C, but none of the products containing either 75 or 120 mg/kg nitrite became toxic during this storage period (Table 2). In wiener-type sausage and in cooked ham with the low level of inoculum, toxic samples were found after 5 weeks of storage, whereas in bologna-type sausage and in cooked ham with the high level of inoculum, toxicity occurred after 3 weeks of storage. The typical shelf life of similar commercially available products in Finland is 18 to 25 days at 6°C. Thus, if spores of *C. botulinum* are present in raw materials or contaminate the product during processing, toxin formation in nitrite-free meat products during the shelf life is highly likely, particularly when recommended storage temperature conditions are violated. Toxin production by group II *C. botulinum* can occasionally occur with very weak growth or no detectable growth \( (4) \). In the present study, no toxin was observed in products containing 75 or 120 mg/kg nitrite, suggesting that spores that survived the heat treatment were unable to germinate and/or grow to produce toxin in the presence of nitrite.

A low prevalence of group I *C. botulinum* spores in meats has been described \( (27) \), but the occurrence of group II *C. botulinum* in meats remains largely unknown \( (19) \). Although group II *C. botulinum* counts are generally low \( (19) \), meat-related botulism cases reported, especially in Europe, indicate that group II *C. botulinum* should be regarded as a potential meatborne hazard \( (26, 27, 31) \). The growing international trend of consumers demand for fresh food with minimal heat processing and limited use of preservatives is posing new challenges for the food industry \( (24) \). Group II *C. botulinum* type B strains can grow and produce toxin at refrigeration temperatures \( (9) \); thus, refrigeration alone cannot be used to control *C. botulinum* growth and toxigenesis. In this study, all meat products containing either 75 or 120 mg/kg nitrite remained nontoxic during the 5-week storage period at 8°C, suggesting that product safety with respect to group II *C. botulinum* type B can be maintained even with a reduced concentration \( (75 \text{ mg/kg}) \) of sodium nitrite. Similarly, in previous studies on group I *C. botulinum*, only one wiener sample containing 100 mg/kg nitrite became toxic after 56 days of incubation at 28°C, whereas products containing 50 mg/kg nitrite or less began to show toxicity after 7 days of incubation \( (2, 17) \). However, several factors, including product type, NaCl concentration, heat treatment, storage time, and storage temperature, affect the growth of and toxin production by *C. botulinum*. Therefore, the nitrite concentration needed to provide product safety with respect to *C. botulinum* differs for each meat product. In conclusion, nitrite has a clear beneficial function in this type of meat product with a long shelf life because all nitrite-free products exhibited evidence of toxigenesis. Thus, the use of nitrite as a curing agent in wiener-type sausage, bologna-type sausage, and cooked ham is justified.

### ACKNOWLEDGMENTS

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### REFERENCES


