Nitrite Burn in Fermented Sausage

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A rare outbreak of anaerobic nitrite burn in fermented sausage afforded the isolation and characterization of the offending microorganism which was identified as a Staphylococcus sp. Unlike most staphylococci, this organism reduces nitrite as well as nitrate.

Most manufacturers of fermented sausage use nitrite in the cure to effect the production of the cured meat pigment. In relatively rare instances, some fermented sausage makers still rely on a microbial reduction of nitrate to nitrite for color development. This sole reliance on microbes can lead to a color defect wherein a green discoloration is observed (2). The defect results from excessive nitrite accumulation and subsequent oxidation of the cured meat pigment to a distinct green color.

Usually, the defect is observed on the surface of the sausage where micrococci are associated with the nitrate reduction (aerobic nitrite burn). In extremely rare instances, anaerobic nitrite burn occurs wherein the entire sausage or a central area (in cross-section) evidences the green discoloration. The organism associated with the anaerobic defect has not been described.

We had the opportunity to investigate a commercial outbreak of anaerobic nitrite burn, and isolated and characterized a Staphylococcus sp. as the organism responsible for the defect. The investigation of the defect and the characterization and comparison of the isolate with known strains form the foundation for this communication.

MATERIALS AND METHODS

Source of strains. Staphylococcus sp. strain N9A was a culture isolated from a fermented sausage product in this outbreak of nitrite burn. S. hyicus (7) and other Staphylococcus strains were acquired from departmental stocks. All strains were maintained by daily transfer in brain heart infusion broth (Difco).

Media and techniques. The laboratory-formulated sausage mixture described previously (3) was employed where indicated. In experiments concerned with the evolution of carbon dioxide from broth media, the basal medium consisted of tryptone (Difco), 10 g; yeast extract (Difco), 5 g; NaCl, 5 g; KH₂PO₄, 7.5 g; glucose, 3 g; and distilled water, 1 liter. For each determination, a control flask (basal medium) and one containing 1.0% potassium nitrate (test medium) were prepared. Two-hundred-and-fifty-milliliter amounts of the respective media were dispensed into 500-ml Erlenmeyer flasks, and after sterilization and inoculation the flasks were flushed with helium to create anaerobic conditions. After incubation (24 hr, 37 C), the flasks were connected to a series of collection bottles containing 0.1 N Ba(OH)₂ solution and flushed with helium. The resulting precipitates in the traps were combined and washed twice with CO₂-free distilled water, dried (24 hr, 100 C), and weighed.

The physiological tests employed in the characterization of the strains were described previously (4).

RESULTS

Investigation of the defect. This manufacturer used a nitrate cure for over 20 years without any color problems. No lactic acid-producing starter culture or chemical acidulant was employed. With explosive suddenness, all products manufactured by this producer turned green in the smokehouse in the terminal days of processing. Total discoloration as well as green cores were observed. In a 10-day manufacturing period, only an occasional stick of sausage did not evidence the defect.

The final pH of the product averaged 5.2, and the residual nitrite varied from 60 to 175 μg per g. A definitive metallic odor was associated with the product and in the manufacturing area of the plant. The replacement of the nitrate with 100 μg of nitrite per gram of product resulted in complete and permanent rectification of the defect.

A numerically predominating Staphylococcus sp. was observed in all products with the defect. This organism readily reduced nitrate to nitrite, and ultimately it reduced the nitrite. Viable counts of the organism ranged...
from $10^4$ to $10^8$ organisms/g of product. In the laboratory, the discoloration was readily reproduced in the sausage mixture described previously (3). KNO$_3$ (1%) was added to the experimental sausage mixture. Ultimately a culture was selected for detailed study, and it was designated *Staphylococcus* sp. N9A.

**Growth experiments in laboratory-prepared sausage.** Preliminary experiments indicated significant gas production when *Staphylococcus* N9A was inoculated in the laboratory-formulated sausage mix with high nitrate concentrations but not with nitrite (up to 200 µg per g), or with a low concentration of nitrate (0.1%). In the commercial outbreak, excessive gas production was not characteristic, and only occasionally were gas holes noted in the product.

Experiments were devised to test the effect of the acidulating agent on defect formation by *Staphylococcus* N9A. The laboratory sausage mixture was prepared with 1.0% KNO$_3$. The mixture was divided into two batches, and one batch was inoculated with *Pediococcus cerevisiae* (3), and to the other batch glucono-delta lactone was added for chemical acidulation (1). One hundred-gram amounts were inoculated with *Staphylococcus* N9A as indicated in Table 1. The levels of inocula were estimated by plate counts. The sausages were observed for 72 hr for color development and gas production. In this experiment, 50 µg of KNO$_3$ per g was added to give the cured meat color initially.

The results indicated that approximately $10^4$ cells/g of sausage mix of the defect-producing bacteria must be present initially if acidulation (either biological or chemical) is practiced. Additional experiments indicated that inocula as low as 10 organisms/g of sausage mixture could produce the defect in 48 hr if neither a biological nor a chemical acidulation procedure was incorporated in the system. In essence this is the so-called “wild fermentation”, and this was the practice of the manufacturer in this current outbreak.

Preliminary experiments indicated that gas was produced in the sausage mixtures. The gas was identified as CO$_2$ in Ba(OH)$_2$ traps. Moreover, this was the predominant gas produced in broth media containing nitrate. Greatly reduced levels of CO$_2$ were produced when nitrate was omitted from the media used for broth cultures and from the sausage mixture.

**Other defect-producing staphylococci.** In further experiments, a collection of 25 *Staphylococcus* strains was screened for gas production and color defect formation in the laboratory sausage mixture formulated with 1.0% KNO$_3$. The strains were also tested for gas production in a broth medium with and without added nitrate. The collection included 17 *S. epidermidis*, three *S. aureus*, and four *Staphylococcus* sp. strains (all reduced nitrate but not nitrite), and one strain of *S. hyicus* that reduced both nitrate and nitrite (6). Twenty-two strains failed to produce gas in the broth medium and discoloration in the sausage mixture. One strain, *S. hyicus*, elicited reactions similar to that of strain N9A (Table 1).

### Table 1. Effect of acidulant and inoculum level of defect-producing *Staphylococcus* on production of nitrite burn

| Inoculum (Staphylococcus N9A cells/g) | Acidulant          | Results               |
|-------------------------------------|--------------------|-----------------------|
|                                     |                    | 24 hr | 48 hr | 72 hr |
|                                     |                    | Color | Gas | Color | Gas | Color | Gas |
| $10^2$                              | *P. cerevisiae*    | Red   | -   | Red   | -   | Red   | -   |
| $10^3$                              | *P. cerevisiae*    | Red   | -   | Red   | -   | Red   | -   |
| $10^4$                              | *P. cerevisiae*    | Red   | -   | Red*  | -   | Red*  | +   |
| $10^5$                              | *P. cerevisiae*    | Red   | -   | Green | +   |        |     |
| $10^6$                              | Green              | Red   | -   | Red   | -   | Red   | -   |
| $10^7$                              | GDL                | Red   | -   | Red*  | -   | Red*  | -   |
| $10^8$                              | GDL                | Red   | -   | Red   | -   | Red   | -   |
| $10^9$                              | GDL                | Red   | -   | Red*  | -   | Green | -   |
| $10^{10}$                           | GDL                | Red   | -   | Green | +   |        |     |

* *Pediococcus cerevisiae*, starter culture employed in commercial fermentations; inoculum = $\sim 10^7$ organisms/g.

* Predominantly red color with greening noticeable.

* Concentration of glucono-delta lactone = 0.75%.
2). Gas production in broth cultures containing nitrate was noted with two other *Staphylococcus* strains. Although both strains (one, a coagulase-positive *S. aureus* strain 196E, the other a coagulase-negative *Staphylococcus* sp. strain 217) produced gas in the broth medium containing nitrate; neither evidenced defect formation in the sausage mixture (Table 2). The ability of strains 196E and 217 to produce gas in broth media with nitrate but not in the sausage mixture was not pursued, but it may be a quantitative effect.

**Physiological studies.** An attempt was made to quantitate the amount of CO₂ produced in the presence of nitrate in broth cultures under anaerobic conditions. The defect-producing strains essentially produce a mole of CO₂ for every mole of glucose fermented in the presence of nitrate, assuming complete glucose fermentation (Table 3). The non-defect-producing staphylococci do not evolve as much CO₂.

A detailed physiological study was undertaken to detect any character that correlated with the peculiar ability of these strains to reduce nitrate anaerobically (Table 4). As seen in the table, no correlation could be made, and it appears that nitrate and nitrite reduction are the only significant characters that relate these organisms.

Repeated intraperitoneal inoculation of *Staphylococcus* strain N9A into mice and rats failed to demonstrate any pathogenic potential. Moreover, this strain lacks both coagulase and nuclease activity.

**DISCUSSION**

Historically, nitrate was added to meat to effect the cured meat color development.

| Inoculum culture | KNO₃ (%) | Color | CO₂ evolved (mmole) |
|------------------|----------|-------|---------------------|
| *Staphylococcus* N9A | 0 | Red | 0.894 |
| *Staphylococcus* N9A | 1 | Green | 4.42 |
| *S. hyicus* | 0 | Red | 0.674 |
| *S. hyicus* | 1 | Green | 5.43 |
| *S. aureus* 196-E | 0 | Red | — |
| *S. aureus* 196-E | 1 | Red | — |
| *Staphylococcus* sp. 217 | 0 | Red | — |
| *Staphylococcus* sp. 217 | 1 | Red | — |

Later, nitrite was identified as being closer to the active component involved in the color-developing reaction. However, some producers still rely on the microbial reduction of nitrate, although most producers now add nitrite for color development. Products made only with nitrate in the cure are subject to undercure (lack of microbial reduction of nitrate) or "overcure" which we refer to as nitrite burn. Usually, nitrite burn (in fermented sausages made with a nitrate cure) is seen in the peripheral areas of the sausage where the nitrate-re-

| Inoculum culture | KNO₃ (%) | CO₂ evolved (mmole) | Net CO₂ (mmole) |
|------------------|----------|---------------------|-----------------|
| *Staphylococcus* N9A | 0 | 0.530 | — |
| *Staphylococcus* N9A | 1 | 2.25 | 1.72 |
| *S. hyicus* | 0 | 0.250 | — |
| *S. hyicus* | 1 | 2.11 | 1.86 |
| *S. aureus* 196-E | 0 | 0.520 | — |
| *S. aureus* 196-E | 1 | 0.959 | 0.439 |
| *Staphylococcus* sp. 217 | 0 | 0.561 | — |
| *Staphylococcus* sp. 217 | 1 | 1.37 | 0.810 |

* Cultures were grown anaerobically in 250 ml of broth containing 1.66 mmoles of glucose.

| Determinations | *Staphylococcus* N9A | *S. hyicus* | *S. aureus* 196-E |
|----------------|---------------------|-------------|-------------------|
| Coagulase | — | — | + |
| Deoxyribonuclease | — | + | + |
| Acetylmethylcarbinol | — | — | + |
| Nitrite reduction | + | + | — |
| Litmus milk (acid) | 1° | 3 | 3 |
| Hydrolysis: | Esculin | — | + |
| Gelatin | — | + | + |
| Fermentation: | Mannose | — | 1° | 1 |
| Galactose | 3 | 1 | 1 |
| Maltose | — | — | 1 |
| Inulin | — | 1 | — |
| Mannitol | — | — | 1 |
| Glycerol (aerobic) | — | — | 1 |
| Glycerol (anaerobic) | — | 6 | — |

* All three strains were catalase positive, reduced nitrate, hydrolyzed arginine, and did not hydrolyze starch.
* Numerals refer to days required to observe reaction.
* All fermented fructose, glucose, lactose, and sucrose. None fermented xylose, arabinose, dulcitol, inositol, or sorbitol.
ducing micrococci are capable of growth. Anaerobic nitrite burn is relatively rare, and in the few previous outbreaks of this defect the offending organism was never isolated. This was supposedly due to either its acid sensitivity (killed by acid produced subsequently by the lactic acid bacteria) or self-sterilization due to excessive nitrite and nitrous acid formation. In this study large numbers of the organism were present and isolation was not a problem.

Prevention of the defect is easily and readily attained. Replacement of the nitrate in the curing mixture with 100 μg of KNO₂ per g results in good color development without defect formation, even in the presence of the offending microorganism.

The reduction of nitrate under anaerobic conditions is a characteristic not commonly observed in the genus Staphylococcus (5). The CO₂ produced by the organisms examined in this study, when grown anaerobically with nitrate, probably reflects an altered pathway wherein the regeneration of oxidized diphosphopyridine nucleotide is coupled with nitrate reduction. This would then afford a pyruvate dismutation reaction and the generation of CO₂. Preliminary observations substantiate this supposition.

There does not appear to be any significant relationship between anaerobic nitrate reduction and any other physiological character we examined. Thus, it appears that this may be just an atypical Staphylococcus epidermidis with the peculiar ability to reduce nitrate under anaerobic conditions.

Characteristically, gas was observed in the sausage prepared in the laboratory. We believe that the apparent absence of gas formation in the sausage manufactured commercially could reflect a significantly slower growth response and a protracted fermentative period resulting in the potential for CO₂ loss by diffusion.

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