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THE INHIBITION OF CLOSTRIDIUM BOTULINUM TYPE B AND E IN SALAMI SAUSAGE

By

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NORDAL, JOHN and ROAR GUDDING: *The inhibition of Clostridium botulinum type B and E in salami sausage.* Acta vet. scand. 1975, *16*, 537—548. — Vegetative cells and spores of Clostridium botulinum type B and E were inoculated into salami sausages with and without the preservatives sodium nitrite and sodium benzoate. The growth and toxin production of Clostridium botulinum type B and E were inhibited in this type of salami sausages, even without any addition of preservatives. The use of a starter culture with pH-lowering components has both technological and hygienic advantages.

Clostridium botulinum; salami sausages; nitrite; benzoic acid; starter culture.

The potential risk of the formation of nitrosamines from nitrite and amines in different food products has led to a change in the attitudes towards nitrite as a food additive. In Norway, the use of nitrite in meat products has been restricted to products where there may be a possibility for toxin production by Clostridium botulinum.

Salami sausage, which is produced by the fermentation and smoking of uncooked meat, may be added 200 mg nitrite per kg in order to prevent growth of Clostridium botulinum. In addition, the nitrite produces a more desirable red colour of the meat, improves taste and aroma, and may also have a positive effect on the quality of this type of sausage by the growth inhibition of putrefactive microflora (*Leistner et al.* 1973).

The use of starter cultures in the production of salami sausage, and other types of fermented sausage seems to have some technological advantages. However, the effect on hygienically hazardous agents like Clostridium botulinum has not been critically evaluated.

The preservative effect of benzoic acid is generally accepted in the food industry (*Souci & Mergenthaler* 1958). High concentrations of sodium benzoate (5000 mg/kg) also gave an inhibition of Clostridium botulinum type E in a laboratory medium, but the practical consequences of this observation have not been investigated further (*Gudding & Nordal* 1976).

The present study was carried out in order to gain information about the effect of food additives, such as nitrite and benzoic acid, on the growth and toxin production of Clostridium botulinum type B and E in salami sausage, and on the organoleptical quality of this product using these food additives. The effects of starter cultures in preventing growth of Clostridium botulinum were also subject to investigation.

MATERIALS AND METHODS

The salami sausages were produced in a meat factory according to the usual formula of the manufacturer. The premix was divided into 12 batches to which were added sodium nitrite, sodium benzoate and the starter culture, Fermatin®, as described in Table 1. Fermatin® was added in the prescribed amount (7.5 g per kg). This starter culture contains freeze-dried micrococci,

Table 1. The content of sodium nitrite, sodium benzoate and the starter culture Fermatin®, in the premix batches.

	Batch number					
Batches with Fermatin® (7.5 g per kg)	1	2	3	4	5	6
Batches without Fermatin®	7	8	9	10	11	12
Sodium nitrite mg per kg	200	200	200	0	0	0
Sodium benzoate mg per kg	0	1000	5000	0	1000	5000

different sugars and glucono-delta-lactone. Sodium chloride was added to the premix to a concentration of 4 %. The sausages had a diameter of about 60 mm, and the length was approx. 25 cm.

The sausages were immediately brought to the laboratory where bacteria and spores of Clostridium botulinum type B (strain Beans, *Skulberg* 1964) and Clostridium botulinum type E (NVH* 3013) were injected into the sausages. One day old cul-

* The type culture collection of the Department of Microbiology and Immunology, Veterinary College of Norway.

tures of Clostridium botulinum type B and type E grown in Robertson's broth (*Robertson* 1915—1916) at 37°C and 30°C, respectively, were used as vegetative cell inocula. The spore suspensions were prepared as described by *Tjaberg et al.* (1969). Amounts of 0.1 ml broth and spore suspension of each bacteria were injected into the same sausages at 15 cm intervals. The total number of bacteria and spores inoculated were as follows: $1 \cdot 10^6$ and $2 \cdot 10^5$ bacteria and spores of Clostridium botulinum type B, respectively, and $1 \cdot 10^5$ and $6 \cdot 10^5$ bacteria and spores of Clostridium botulinum type E, respectively. One control sausage, without any inoculate, was included for every 2 sausages with bacteria and spores of clostridia, for testing of organoleptic properties.

The salami sausages were placed in a climate room at 20°C and stored for 40 days. During the first 2—4 days the sausages were smoked for 12 hrs. at a temperature of 18—20°C. The smoke was produced by burning beech-wood. The relative humidity of the climate room was initially 92 % and decreased to 80 % after 40 days.

After 4, 8, 18, 25 and 60 days, 3 sausages of each batch were taken out for analysis.

Bacteriological examinations

The numbers of micrococci and lactobacilli were determined by a standard plate count method. Blood agar (7 % defibrinated blood) and MRS-agar (*de Man et al.* 1960) were used as media. The plates were incubated at 30°C for 48 hrs.

For the enumeration of Clostridium botulinum type B and type E samples of about 10 g were taken out from the area of the sausage where the clostridia were inoculated. The samples were diluted with saline (1:5), homogenized, and 0.1 ml of the homogenate was spread on the surface of blood agar plates (3 % agar). The plates were incubated anaerobically for 48 hrs. at 37°C (type B) and 30°C (type E).

Toxicological examinations

The homogenized samples were centrifuged at 3000 r.p.m. for 20 min., and the supernatants were used for toxicological titrations. One ml, or less, of each sample was injected intraperitoneally into albino mice, of both sexes, weighing about 20 g. Each dilution was tested on 2 mice and the dose killing both mice is given as the minimum lethal dose per g (MLD per g).

The water activity of the samples was recorded on a Durotherm a_w-Wert-Messer® (*Rödel & Leistner* 1971). A method described by *Kvåle & Dalhoff* (1963) was also used for the analysis of water activity.

The nitrite concentrations of the samples were determined using a spectrophotometrical method (*Follet & Ratcliff* 1963).

Organoleptic evaluation was performed by a test panel consisting of 10 persons of both sexes. The tests were arranged in a semi-dark room to exclude the influence of the visual judgement. The tastes of the sausages were classified according to a scale ranging from 1 to 6, the latter being the best quality. The ranking of the sausages was calculated from the average of the data given by each member of the test panel.

RESULTS

In all sausages, the number of micrococci was found to be in the range of 10^4—10^6 per g. The total counts of micrococci generally declined towards the end of the storage period. The lactobacilli counts were generally in the range of 10^6—10^8 per g. However, in all samples with 5000 mg sodium benzoate per kg the numbers of lactobacilli were 1—2 log. units lower than in the corresponding sausages without sodium benzoate, or with 1000 mg sodium benzoate per kg.

The pH values were generally lower in sausages with Fermatin® (Fig. 1). The differences in the pH values were most evident at the beginning of the experimental period. A decline in the pH values during the fermentation period could also be observed in most samples. This tendency could not be demonstrated in sausages with 5000 mg sodium benzoate added per kg.

The nitrite analyses performed 3—4 hrs. after production showed a reduction in the sodium nitrite concentration from 200 mg per kg initially to 137 and 152 mg sodium nitrite per kg in the samples with and without Fermatin®, respectively. On the 4th day, the nitrite residues were 2.4 and 25 mg nitrite per kg in sausages with and without Fermatin®, respectively.

The water activity was found to be 0.97 on the day of production and it decreased gradually to a_w 0.89.

Botulinal toxin was demonstrated in sausages inoculated with a culture of Clostridium botulinum type B (vegetative cells). The toxin concentrations were found to be in the range of 5—10 MLD

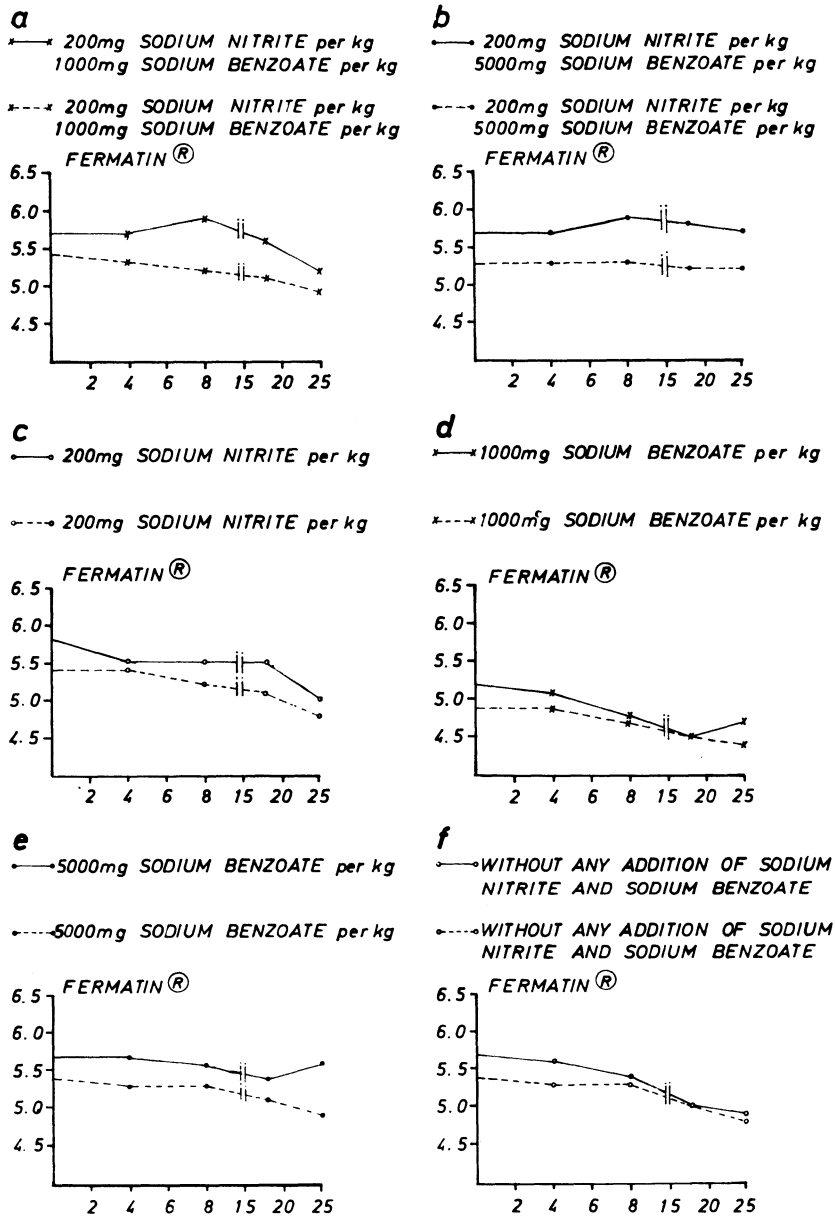

Figure 1. The pH values of sausages with and without the addition of different preservatives during the first 25 days of the experimental period.

per g, and these concentrations were demonstrated on one, or more, occasions in all sausages inoculated with vegetative cells of Clostridium botulinum type B. The demonstration of toxin was randomly distributed in sausages with and without different preservatives. No increase in toxin concentrations during the storage period could be observed. In sausages inoculated with spores of Clostridium botulinum type B, botulinal toxin was not demonstrated. Neither the inoculation of vegetative cells or spores of Clostridium botulinum type E caused detectable concentrations of botulinal toxin in the sausages.

The level of bacteria and spores of Clostridium botulinum type B and E found in the sausages corresponded to the inoculated numbers of vegetative cells and spores. A slight increase (up to 1 log.) in the number of Clostridium botulinum type B could be demonstrated following the inoculation of vegetative cells of this bacteria. This increase was found in most samples and there was no significant correlation with the content of certain preservatives in the sausages.

No change in the numbers of Clostridium botulinum was found during the storage of sausages injected with spore suspensions of Clostridium botulinum type B and E. The numbers of Clostridium botulinum type E seemed, however, to decline very slightly in sausages into which vegetative cells had been inoculated.

Sausages containing Fermatin® generally had a more solid consistency, and the colour of the cut surface was more homogenous, especially at the beginning of the storage period.

Table 2. The ranking of the salami sausages by the organoleptic examinations.

Ranking	Sausage	Score	Ranking	Sausage	Score
1	with N, B_1 and F	4.1	7	with N, B_2 and F	3.0
2	with F	3.8	8	with B_1	2.9
3	with N and F	3.4	9	with N and B_1	2.9
4	with B_1 and F	3.3	10	with no addition	2.8
5	with B_2 and F	3.1	11	with N	2.7
6	with N and B_2	3.1	12	with B_2	2.6

N: Sodium nitrite (200 mg per kg)
B_1: Sodium benzoate (1000 mg per kg)
B_2: Sodium benzoate (5000 mg per kg)
F: Fermatin® (7.5 g per kg)

The effect of nitrite on pigment formation could be observed in all sausages to which nitrite had been added. However, this effect was more distinct in sausages with starter cultures than in those without.

In the organoleptic evaluation, the test panel generally preferred sausages produced with Fermatin® (Table 2). The quality of sausages with 5000 mg sodium benzoate per kg was not found to be acceptable, as the colour, taste and consistency differed in a negative way from the rest of the sausages.

DISCUSSION

The production of salami sausages is a complex process, and several factors contribute to give the desired product. The fermentation also seems to create an environment which may be unfavourable for potentially dangerous microorganisms.

The numbers of micrococci and lactobacilli correspond to the results of *Tjaberg & Skjelkvåle* (1974) who studied the development of the fermentation flora very thoroughly. In this experiment, sodium nitrite and sodium benzoate in concentrations of 200 mg per kg and 1000 mg per kg, respectively, did not seem to influence the growth of the micrococci and lactobacilli. However, the growth of lactobacilli was significantly reduced in all samples containing 5000 mg sodium benzoate per kg, indicating that the lactobacilli are more sensitive to sodium benzoate than the micrococci. These results are in accordance with the findings of *Nordal* (unpublished) who studied the effects of benzoic acid on micrococci and lactobacilli in laboratory media.

The effects of high concentrations of sodium benzoate (5000 mg per kg) can be seen from the figures showing the pH values of the sausages. However, neither the addition of sodium nitrite (200 mg per kg) nor sodium benzoate (1000 mg per kg) influenced the fermentation process significantly as the pH decrease was similar to that in sausages with and without sodium nitrite and sodium benzoate.

A rapid lowering of the pH values is important from a hygienic point of view as the growth of contaminant microflora and possible pathogenic or toxinogenic microorganisms is depressed. Consequently, the use of a starter culture together with pH-lowering components such as glucono-delta-lactone seems to be favourable for the prevention of growth of hazardous micro-

organisms. Fermatin® also contains sugars which are favourable substrates for the fermentation process.

The decrease in the nitrite concentrations is due to several factors (*Hill et al.* 1973). The relatively high temperature during the fermentation is one reason for the nitrite depletion (*Nordin* 1969). The significant difference in nitrite residues in salami with and without Fermatin® may be due to the difference in pH values, as the nitrite depletion rate is more rapid at a low pH (*Nordin*). However, an increase in the activity of the micro-organisms may also be an explanation for this observation.

Botulinal toxin was demonstrated only in salami sausages inoculated with vegetative cells of Clostridium botulinum type B. It is reasonable to believe that the botulinal toxin type B originates from the inoculate which was a 1 day old broth culture with the bacteria. No increase in the toxin concentrations could be observed, and toxin was demonstrated in sausages with and without preservatives. These facts support the assumption that vegetative cells of Clostridium botulinum type B do not produce toxin in salami sausages of this type, or that the toxin production is insignificant. The germination of spores of Clostridium botulinum type B and E, and the growth and toxin production of Clostridium botulinum type E seem to be effectively inhibited in salami sausages.

In this experiment, no toxin production could be demonstrated, even in sausages without preservatives, indicating that the addition of chemical preservatives may not be necessary to prevent toxin formation by Clostridium botulinum.

The probability that vegetative cells of Clostridium botulinum will be found in salami sausages is small. As the inhibition of germination of Clostridium botulinum spores is effective, there is no practical risk for botulism following the consumption of salami sausages of the composition used in these experiments.

Different preservative principles acting together are responsible for the inhibition of germination and growth of Clostridium botulinum type B and E. The water activity of the salami was initially 0.97, equivalent to about 5 % NaCl by weight. As 4 % NaCl was added to the premix, this substance is the most important factor for the low initial water activity. According to *Ohye & Christian* (1966), the lowest a_w values permitting growth of Clostridium botulinum type B and E are 0.94 and 0.965, respectively. During germination and spore out growth the require-

ment for a high relative humidity is more important and the a_w values should be about 0.96 for type B and 0.97 for type E, respectively, when NaCl is used as a_w -lowering agent (*Baird-Parker & Freame* 1967). These results were obtained in experiments performed when other environmental factors were optimal.

The optimum temperatures for toxin production by Clostridium botulinum type B and E are 35°C and 25°C, respectively (*Ohye & Scott* 1953; 1957). The temperature during the smoking and storage of the salami sausages was maximum 20°C, and this temperature difference may also contribute to prevent the growth and toxin production by Clostridium botulinum type B. The pH values of the salami sausages were in the range 4.7—5.5. Clostridium botulinum may grow at a pH as low as 4.5 if other environmental factors are optimal (*Riemann* 1973). Neither the water activity of the salami nor the low pH may, by itself, give a complete inhibition of the growth of Clostridium botulinum type B and E. However, these 2 preservative factors together are responsible for the inhibition of Clostridium botulinum. The use of starter cultures, together with pH-lowering components which have technological advantages, also increases the hygienic security as the pH is lowered more effectively in the initial phase of the process.

No conclusion can be drawn concerning the anti-botulinal effect of benzoic acid, as the added clostridia were effectively inhibited by other preservative factors. However, the use of high concentrations of benzoic acid (5000 mg per kg) cannot be recommended for technological reasons, as the normal microflora of the products is inhibited.

Neither could the inhibitory effect of nitrite towards Clostridium botulinum be studied conclusively. However, the inhibition of Clostridium botulinum by nitrite in laboratory media is unquestionable, and the nitrite in the concentrations added may represent an extra security against the production of botulinal toxin.

Both the preservative effect, and the possible formation of nitrosamines, are dependent on the basal concentrations of nitrite (*Greenberg 1972, Christiansen et al.* 1973). However, the insignificant concentrations of nitrite found by control analyses of meat products give no information as to the amount of nitrite added to the product initially.

The organoleptic examinations showed that nitrite has a

desirable effect on the appearance of the sausages, as the colour of the cut surface was more homogenous. However, this effect may be obtained by the addition of lower concentrations of nitrite (*Nilsson* 1973).

The use of nitrite has also some technological advantages as the food-spoiling microflora may be inhibited (*Leistner et al.* 1973). This effect has not been studied in this investigation. It is, however, reasonable that the use of a starter culture with pH-lowering components will provide sufficient security for the quality of the products.

Even if the addition of nitrite is not necessary from a hygienic point of view, the use of nitrite in the meat industry may have technological benefits which should be evaluated against the toxicological risk.

CONCLUSIONS

1. The growth and toxin production of Clostridium botulinum type B and E are inhibited in salami sausage with an initial water activity of 0.97 and a pH of about 5.7.
2. The hygienic security may be increased by the use of a starter culture, containing pH-lowering components giving decreased initial pH values.
3. The use of sodium benzoate, in a concentration of 5000 mg per kg, has technological disadvantages as the fermentation flora is inhibited.

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SAMMENDRAG

Hemming av Clostridium botulinum type B og E i salamipølse.

Vegetative celler og sporer av Clostridium botulinum type B and E ble inokulert i salamipølse med og uten natriumnitritt og natriumbenzoat. Vekst og toksinproduksjon av Clostridium botulinum type B and E ble hemmet i de undersøkte salamipølsene både med og uten tilsetning av konserveringsmidler. Bruk av startkultur sammen med pH-senkende stoffer viste seg å ha både teknologiske og hygieniske fordeler.

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