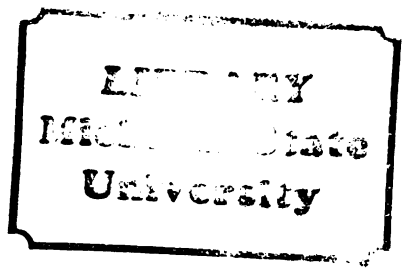



This is to certify that the

thesis entitled

Some Factors Influencing Production of Aflatoxins by Aspergillus flavus in Fermented Sausages

presented by

Valente Alvarez Barrera

has been accepted towards fulfillment of the requirements for

M.S. degree in Food Science

A. M. Pearson

Major professor

Date 6/12/81

# SOME FACTORS INFLUENCING PRODUCTION OF AFLATOXINS BY *Aspergillus flavus* IN FERMENTED SAUSAGES

By

Valente Alvarez-Barrera

A THESIS

Submitted to
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# ABSTRACT

## SOME FACTORS INFLUENCING PRODUCTION OF AFLATOXINS BY *Aspergillus flavus* IN FERMENTED SAUSAGES

By

Valente Alvarez-Barrera

Three types of sausages (smoked inoculated, unsmoked inoculated and smoked uninoculated control) were produced. They were stored at either 10 or 30°C at relative humidities of either 79% or 89% for either 3 or 6 weeks.

After 3 weeks storage at 10°C, no aflatoxins were detected in any of the samples, but 0.26 µg/kg of aflatoxin $B_1$ were detected in the unsmoked inoculated sausages held at 30°C and high relative humidity (89%). After 6 weeks storage, mold growth was present on all sausages. In the samples held at 10°C, however, aflatoxin $B_1$ was detected only on the unsmoked inoculated samples held at high relative humidity. On the other hand, after incubation at 30°C aflatoxins were found at higher levels (2.60-6.60 µg/kg) in all samples stored at either low or high relative humidities.

Although application of smoke retarded mold growth and aflatoxin formation, longer periods of time, high temperature and high relative humidity were demonstrated to be important determinants of aflatoxin production.

## ACKNOWLEDGEMENTS

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TABLE OF CONTENTS

# LIST OF TABLES

LIST OF FIGURES

# INTRODUCTION

Aflatoxins are a group of highly toxic secondary metabolites produced by some strains of Aspergillus flavus and by most, if not all, strains of Aspergillus parasiticus. Chemically, the aflatoxins are difuranocumarin derivatives, are slightly soluble in water, are soluble in solvents of intermediate polarity (including alcohols, chloroform, and acetone) and are insoluble in hexane. Through studies with experimental animals, strong evidence exists for involvement of aflatoxins in induction of cancer. Aflatoxin $B_1$ is the most important in terms of occurrence and toxicity.

In addition to mold contaminated agricultural commodities, aflatoxins have been found in milk and milk products, and in fresh, cured and smoked meat products. Most methods that have been used for aflatoxin extraction from meat products are based on methods used for extraction and determination of aflatoxins in crop products. The methods utilize extraction with suitable solvents, followed by purification, and a final separation and quantification of the aflatoxins, which is generally carried out on thin layer chromatography plates by visual comparison with aflatoxin standards.

Two general methods have been reported for extraction and identification of aflatoxins in cured meat products (Bullerman *et al.*, 1969; Strzelecki, 1973). These methods have been used by other workers for studying the presence of aflatoxins on ham, country cured ham, salami, bacon and fresh beef. However, they are not suitable for some meat products since interfering materials cause problems in spectrophotometric quantification of aflatoxins. Nevertheless, little is known about the growth and production of aflatoxins by *A. flavus* on fermented sausages.

Thus, the present study was designed to investigate the levels of aflatoxins produced by an aflatoxin producing strain of *A. flavus* grown on fermented sausages under different conditions of manufacture and storage. The variables investigated were the effects of temperature, relative humidity, smoking versus no smoking and the length of storage.

# REVIEW OF LITERATURE

## Occurrence of Aflatoxins in Feeds and/or Foods

Aflatoxins are produced by some strains of Aspergillus flavus and by most, if not all, strains of Aspergillus parasiticus (Diener et al., 1969; Bullerman, 1979). Studies on aflatoxin production in mold contaminated food and agricultural commodities began with the outbreak of Turkey X disease in England in 1960, which has been reviewed by Ciegler and Lillehoj (1968), Stoloff (1977), Wyllie and Morehouse (1978), and Bullerman (1979).

Aflatoxins have been found in a number of foods/feeds, such as peanuts and peanut products, cottonseed, corn, Brazil nuts, pistachio nuts, almonds, pecans, and walnuts (Stoloff, 1976). Other workers have studied the formation of aflatoxins in inoculated cheeses and in cured and smoked meat products (Lie and Marth, 1967; Van Walbeek et al., 1969; Oldham et al., 1971; Shih and Marth, 1972).

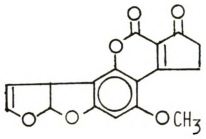
## Chemistry of Aflatoxins

Four compounds were originally isolated and were designated as aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$. The specific designation was due to their blue (B) and green (G) fluorescence under ultra violet light, respectively (Hartley et al.,

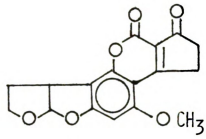
1963). Chemically, the aflatoxins are difuranocumarin derivatives and have been characterized by a number of researchers (Asao et al., 1963; Stoloff, 1977). In addition to these four aflatoxins, two others are of significance, $M_1$, $M_2$ which are hydroxylated derivatives of $B_1$ and $B_2$. They are excreted in the urine, feces and milk as metabolic products of $B_1$ and $B_2$ following their consumption by mammals (Frazier and Westhoff, 1978). Wyllie and Morehouse (1978) reported 12 related compounds, whereas Beuchat (1978) stated that there are 18 known aflatoxins. The chemical structures for the six aflatoxins of significance are shown in Fig. 1.

Beuchat (1978) pointed out that these compounds are slightly soluble in water, soluble in solvents of intermediate polarity, including alcohols, chloroform, and acetone, and are insoluble in hexane. Stoloff (1977) reported that aflatoxins are remarkably stable in normal food and feed systems, but are highly reactive at either end of the pH scale (pH $<3$ and pH $>10$) or on exposure to ultraviolet light in the presence of $O_2$.

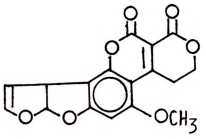
The site of unsaturation in the furan moiety and the lactone structure are the reactive portions of the aflatoxin molecule (Marth and Dyle, 1979). Several investigators have demonstrated the sensitivity of aflatoxins to oxidizing agents, particularly to alkaline oxidants, such as sodium hypochlorite (Fischbach et al., 1965; Trager et al., 1967;



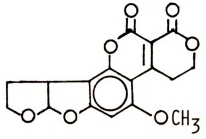

$B_1$



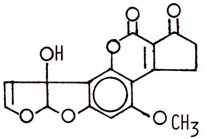

$B_2$



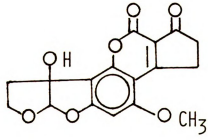

$G_1$




$G_2$




$M_1$




$M_2$

Figure 1. Structure of aflatoxins $B_1$, $B_2$, $G_1$, $G_2$, $M_1$ and $M_2$.

Nartarajan et al., 1975). Studies on the mechanism of detoxification with ammonia by Beckwith et al. (1975) showed that the phenolic acid exposed on opening of the lactone ring is particularly reactive. They also found that alcohol in acid media reacted with the vinyl ether of the difuran moiety, and resulted in hemiacetals that are very sensitive to oxidative change. This is typified by the formation of the water adduct, aflatoxin $B_{2a}$ (Beckwith et al., 1975).

## Presence of Aflatoxins in Meat Products

Ayres et al. (1967) tested ninety samples of cured and aged meats for characteristic fungal flora, representing all major types of cured and aged meats produced in the U.S. and Europe. They isolated 670 fungal strains (456 molds and 214 yeasts). The principal fungal flora of cured and aged meat were species of Penicillium, Aspergillus, Scopulariopsis, Pebaryomyces, Candida, and Torulopsis. They observed that these genera had in common a tolerance for low water activity. They showed that molds may be present in large numbers on the surface of certain cured and aged meats, especially on country cured hams and fermented sausages. They isolated strains of A. niger, A. ruber, A. wentii, A. flavus, P. frequentans, P. variable, and P. puberulum.

Bullerman and Ayres (1968) studied the potential hazards to human health constituted by the presence of these organisms on meat. He screened selected isolates from meat for the production of aflatoxins. Of the mold strains

tested, only the A. flavus isolates produced chloroform extractable fluorescent compounds, which coincided with aflatoxin standards on thin layer chromatographic plates. He demonstrated that A. flavus does indeed produce aflatoxins. These results suggest that given the proper circumstances, a potential health hazard could exist from molds growing on meat substrates.

Bullerman et al. (1969) attempted to determine the quantities of aflatoxins produced on fresh and cured meats during storage at various temperatures. Important factors investigated included the effects of type of meat, strain of mold, storage temperature, length of storage and number of mold spores present. Aflatoxins were produced on three different stored meat products, i.e., fresh beef, in which bacterial spoilage was delayed with antibiotics, ham and bacon. These products were stored at three different temperatures, 15, 20 and 30°C. When stored at 10°C, the products were spoiled by bacteria and yeast before detectable levels of aflatoxins were produced. At 20°C, high levels of aflatoxins (up to 630 ug/g of meat) were formed during storage. Below 30°C, both A. flavus and A. parasiticus produced more of aflatoxin $G_1$ than $B_1$. Above 30°C, however, A. flavus produced equal amounts of $B_1$ and $G_1$, whereas A. parasiticus continued to produce more $G_1$ than $B_1$.

Bullerman et al. (1969) also determined the levels of aflatoxins produced on salami by a known toxinogenic strain

of A. flavus under simulated conditions of manufacture. He also studied aflatoxin production by known toxinogenic strains of A. flavus and A. parasiticus on country cured hams at different stages of aging. During aging of Italian-type salami, A. flavus produced more aflatoxins than was the case for smoked Hungarian-type salami held under the same conditions. Temperatures below 15°C and humidities of less than 75% were required to prevent aflatoxin development during the aging of salami. A temperature of about 30°C was required for maximum production of aflatoxins. The presence of curing ingredients, especially pepper and sodium nitrite, tended to reduce the amounts of aflatoxins in these products.

Strzelecki et al. (1969) identified four strains of A. flavus from a heavily mold contaminated country cured ham. They concluded that the predominance of aspergilli on country cured hams was due to its low moisture content (approximately 30%). They grew molds on yeast extract containing 20% sucrose (YES), and extracted the aflatoxins with chloroform. A concentrate of the extract was resolved on thin-layer chromatograms of silica gel, and the aflatoxin concentration was determined visually by comparing with aflatoxin standards. They found that out of 10 different mold isolates, four of the five A. flavus isolates produced aflatoxins.

To determine the incidence of mycotoxic molds in domestic and imported cured and smoked meat, Bullerman (1977) analyzed 171 samples. Analyses were carried out for visible mold growth, isolation and classification of visible molds, ability of mold isolates to produce known mycotoxins and development of visible mold growth during simulated storage of each sample. A total of 393 isolates were obtained from domestic meat and 645 from imported meat. Of the total isolates, he found 68.1% were Penicillium, 2.4% Aspergillus, 4.9% Cladosporium, 5.8% Alternaria, 0.9% Fusarium and 17.9% were from various other genera. He concluded that among potentially toxigenic species of molds found on meat were P. eyclopium, P. viridicatum, A. flavus and A. glaucus. Five mycotoxins were identified on the meat, with aflatoxins being one of them.

Oldham et al. (1971) investigated the growth of A. flavus on certain perishible foods and the production of aflatoxins at normal refrigerator temperatures. They inoculated samples of cheddar cheese and luncheon meat with one strain of A. flavus. The products were held for 12 days at 4.4, 7.2, and 25°C. All samples, except those inoculated and incubated at 25°C, were negative for aflatoxin production. Therefore, they reported that the mold isolated did not produce detectable aflatoxins when kept at normal refrigeration temperatures for up to 12 days.

Mintzlaff et al. (1972) analyzed Penicillium culture isolated from mold-ripened sausages of European origin. They found that the cultures produced nine different mycotoxins, among which were aflatoxins $B_1$ and $G_1$. Some 88 isolates out of a total of 422 were found capable of toxin synthesis. Fourty four of these cultures produced penicillic acid, 17 ochratoxin A, 11 tremortin, 10 citrinin, and 6 patulin. However, toxins were not detected on sausages containing mold producing mycotoxins during storage up to 70 days. Although results indicated that consumption of sausage containing mold is not generally a health hazard, they recommended that manufacturers of these products could utilize pure cultures of molds known to be toxicologically safe for speeding up the aging process in meat.

Strzelecki (1973) followed the growth of aflatoxins in raw and country cured hams, as well as in salami. He reported that A. flavus will grow and produce aflatoxins in country cured hams, with trace amounts being present after 84 and 126 days at storage of 5 and 30.1°C, respectively. In salami, aflatoxins were found at levels of 104.85 and 213.4 µg/100 g after 13 and 78 days of storage, respectively. Recovery of added aflatoxins from meat products resulted in 16% recovery from raw ham after 6 weeks storage, 7% recovery from country cured ham after 126 days, and 19% in salami after 78 days.

In similar work, Murthay et al., (1975) studied the recovery of aflatoxin $B_1$ injected into frozen beef at different intervals of storage. On storing beef at -18°C for 20, 25, 32, 152 and 183 days, there was a diminution in the recovery of injected aflatoxin $B_1$. The author discussed the possibility that a portion of the aflatoxin was not recovered due to its combining with the meat constituents as a consequence of physicochemical changes occurring in the muscle during storage.

Strzelecki (1973) investigated the effect of the meat curing ingredients on aflatoxin production. He reported that aflatoxins were totally inhibited by potassium nitrite, although sodium nitrite caused a slight stimulation. The curing mixtures per se, which included 5.71% NaCl, 2.16% sucrose, 0.10% $KNO_3$ and 0.05% $NaNO_2$, resulted in somewhat more aflatoxin production. Meier and Marth (1977) studied the production of aflatoxins by mold grown in the presence of curing salts. They reported that the effects of individual curing salts or mixtures of curing salts on growth and toxin elaboration was substrate dependent. A combination of $NaNO_2$ and NaCl in yeast extract-sucrose (YES) broth depressed growth and/or aflatoxin production. However, biosynthesis of aflatoxin $B_1$ was enhanced by presence of 1 and 4% NaCl in YES broth. Similar results were obtained when working with sucrose-ammonium salts (SAS) broth. In work on sausages, they found that 100 and 200 ppm of $NaNO_2$

and NaCl, respectively, supported more mold growth and aflatoxin production than was the case for control sausages containing 3% NaCl and 100 ppm of $NaNO_2$. Addition of 2 and 3% NaCl without nitrite resulted in less aflatoxin production than that of the control sausage. Overall results suggested that high levels of 2 to 3% NaCl are inhibitory to aflatoxin production.

Van Walbeek et al. (1969) surveyed the influence of refrigeration on aflatoxin production by unidentified strains of A. flavus. They stated that aflatoxins were produced in significant concentrations under conditions simulating household refrigeration (7.5 to 10°C). The rate of aflatoxin production at 10°C was markedly influenced by preincubation culture at room temperature for 24 hours. The authors found that toxin production was higher in solid cultures than in liquid cultures.

Furtado et al. (1981) studied the effects of cooking and/or processing upon levels of aflatoxins $B_1$ and $B_2$ in meat from pigs fed a contaminated diet for 42 days. Fresh, fresh-cooked, cured-smoked (hams only) and cured-smoked-cooked products were analyzed. Some destruction of aflatoxins $B_1$ and $B_2$ occurred during cooking and/or processing. It was concluded that the maximum amount of inactivation did not exceed 41% of the total and was generally in the range of 15-30%. Thus, results suggested that aflatoxins are quite stable during cooking and/or processing.

Aflatoxin Carry Over into the Tissues of Animals Fed on Contaminated Feeds

Since studies have shown that ingested aflatoxins may be deposited in tissues as the original aflatoxin, or as one of its metabolites, the problem of transmission of aflatoxins through farm animals to human food has been studied by several investigators (Newberne et al., 1955; Burnside et al., 1957; Forgacs et al., 1958; Asplin and Carnaghan, 1961; Loosmore and Markson, 1961). Allcroft and Carnagham (1963) were the first to investigate the presence of aflatoxin residues from animals fed toxic material. Keyl and Booth (1971) determined the adverse effects of graded levels of aflatoxins in the rations of swine, beef cattle, dairy cattle and poultry. They found no adverse effects of aflatoxins at levels of 230, 300, and 450 ppm of aflatoxin.

Van Zytveld et al. (1970) studied the presence of aflatoxins in the livers and skeletal muscle of chickens, which had ingested a daily dose of aflatoxins over a six week period. Aflatoxins were detected as a result of aflatoxin ingestion. Mabee and Chipley (1973) investigated aflatoxin carry over in broiler chickens by administering low levels of $^{14}C$-labeled aflatoxins. The radioactivity was detected in the liver, heart, gizzard, breast and leg meat. Other similar studies have been reported by Platonow (1965) and Kratzer et al. (1969).

Allcroft and Carnagham (1962) reported that extracts of milk from cows fed aflatoxin contaminated rations and aflatoxins administered directly induced identical lesions. A relationship between aflatoxin intake and the concentration of aflatoxin M in milk has been reported by several investigators (Allcroft and Roberts, 1968; Keyl et al., 1968; Keyl and Booth, 1971; Polan et al., 1974).

The effect of aflatoxins on pigs has been studied by feeding them aflatoxin-contaminated diets (Krogh et al., 1973). It has been reported that the response of swine to aflatoxins depends on whether the aflatoxin-contaminated protein is fed separately or is incorporated directly into the total ration (Murthy et al., 1975). An appreciable amount of aflatoxin $B_1$ was found by Jacobson et al. (1978) in the tissues of pigs fed aflatoxin $B_1$. Furtado et al. (1979) assessed the possibility of aflatoxin carry-over into meat products from animals fed contaminated rations. They reported no evidence of gross pathological lesions, however, they detected residues of both aflatoxins $B_1$ and $B_2$.

These experiments demonstrated that ingested aflatoxins are deposited as the original aflatoxin, or as one of its metabolites. By using sensitive chemical methods, aflatoxin residues were found in the organs and muscle of swine and chickens, as well as in the organs and milk from cattle. Since a recent study reported that aflatoxin $B_1$ was detected in liver samples from patients with primary hepatocellular

carcinoma (Siraj et al., 1980), the transmission of aflatoxins through farm animals to human foods is an important problem.

## Methods for Aflatoxin Extraction and Identification

### General Extraction of Aflatoxins.

Sargent et al. (1961) were the first to extract aflatoxins using mold-contaminated peanut meal. They used exhaustive Soxhlet extraction of the sample with methanol, followed by further extraction with chloroform and defatting of the final extract with petroleum ether. Simultaneous defatting and extraction was used by Nesheim (1964), who blended the sample with aqueous methanol and hexane. Acetone was utilized as the solvent to extract aflatoxins from cottonseed, peanuts and other similar products by several other workers (Pons and Goldblatt, 1965; Pons et al., 1966; Stoloff et al., 1966).

Since the earlier methods yielded extracts having large amounts of interfering materials, Coomes and Sanders (1963) used liquid-liquid partition chromatography with methanol, water and petroleum ether as solvents. Pons and Goldblatt (1965) and Pons et al. (1966) used lead acetate to precipitate interfering substances from the extracts.

A more efficient purification system in which the aflatoxins were not destroyed or altered was utilized by DeIongh et al. (1962). The method used silica gel column chromatography to purify a crude extract that was sequentially

eluted with chloroform and methanol. Nesheim (1964) used partition column chromatography of an aqueous methanol extract of peanut meal on diatomaceous earth (celite) to separate aflatoxins. Then the aflatoxins were extracted with a chloroform:hexane mixture (1:1, v/v). Pons et al. (1966) used a similar method, however, they eluted interfering substances with diethyl ether and the aflatoxins with chloroform; methanol (97:3, v/v). Stoloff et al. (1966) utilized partition chromatography on a cellulose column, in which aflatoxins and the interfering substances were eluted with hexane and chloroform (1:1, v/v).

The first analytical methods for separation of aflatoxins used either paper chromatography or filter paper (Sargent et al., 1961; Coomes and Sanders, 1963). Introduction of silica gel (Kiesegel G) for the separation of several fluorescent spots of a purified aflatoxin extract from contaminated groundnut meal, was first accomplished by De Iongh et al. (1962). Later, TCL plates were adopted by other workers with variations in the solvent developing systems (Broadbent et al., 1963; Hartley et al., 1963). Although fluorescence of aflatoxins under UV light was widely utilized for detection and estimation (Pons and Goldblatt, 1969), until recently measurement had been by visual estimation. Nabney and Nesbitt (1965) improved the accuracy and precision of aflatoxin measurement by use of absorption spectrophotometry at 366 nm. Ayres and Sinnhuber (1966)

reported a more sensitive fluorodensitometric measurement of aflatoxin $B_1$ directly on silica gel coated plates using a recording densitometer equipped for fluorescence emission measurements. This basic system has been widely adopted.

Extraction and Identification of Aflatoxins in Meat Products. Methods for extraction, purification, determination and measurement of aflatoxins in meat were briefly mentioned by Murthay et al. (1975). These methods are modifications of those used in extraction and determination of aflatoxins from cereals and other food products (Bullerman et al., 1969; Strzelecki, 1973; Brown et al., 1973). Bullerman et al. (1969) made the first attempt to extract and analyze aflatoxins from meat. They extracted by blending a 100 g meat sample with chloroform. The water content of the meat (ca 50-60%) and the chloroform, which contained the aflatoxins, separated into two layers. They then decanted the chloroform layer and filtered it before making visual estimation of the aflatoxins. They then used this method for analyzing ham, bacon, salami and fresh beef.

A similar technique was utilized by Strzelecki (1973) who analyzed salami, raw ham and country cured ham for aflatoxins. He used 300 ml of methanol in distilled water (2:1) for extraction and a mixture of petroleum ether-hexane-benzene (2:1:2, v/v) for defatting the samples. In order to eliminate emulsion formation, 10 g of soidum chloride were added after filtering. Brown et al. (1973)

used a similar method but added 300 ml 5% NaCl solution. They did not need to defat the sample since they worked with raw tissues.

Furtado (1980) reported a suitable method for extraction and analysis of aflatoxins from the tissues of pigs and meat products. The method was a modification of the procedure of Trucksess and Stoloff (1979), which requires acetone and NaCl for aflatoxin extraction. After filtering, $(NH_4)_2SO_4$ and $Pb(OAC)_2$ solutions were added to insure better extraction of the aflatoxins.

Neither Bullerman (1969) nor Brown (1973) used liquid-liquid partitioning for removal of interfering lipids, carbohydrates and pigments from the primary extracts. However, Strzelecki (1973) defatted the samples by using a mixture of petroleum ether, hexane and benzene. The use of liquid-liquid partition systems was adopted and modified by several workers (Coomes and Sanders, 1963; DeIongh et al., 1964). Trucksess and Stoloff (1979) reported good results when using petroleum ether to remove the fat before extraction, cleanup, and quantitative determination of aflatoxins $B_1$ and $M_1$ from beef liver.

The use of chromatographic systems for partially purifying crude or primary extracts offer an efficient system, provided that aflatoxins are not destroyed or altered during the treatment. Therefore, different column systems were reviewed by Pons and Goldblatt (1969). A

aluminum oxide and anhydrous sodium sulfate column has been utilized by Strzelecki (1973). Silica gel and celite columns have been reported to provide good cleanup by other researchers (Bullerman et al., 1969; Brown et al., 1973; Trucksess and Stoloff, 1979).

Cleanup of aflatoxin extracts using TCL plates developed in one or two dimensions, and quantification of the aflatoxins by either visual estimation or densitometric analysis have been widely utilized in studies of mold contaminated feeds and other agricultural commodities (Nesheim, 1964; Coomes and Sanders, 1963; Pons and Goldblatt, 1965; Pons et al., 1966; Whitaker and Dickens, 1979). Modifications of these procedures have been adopted to purify and quantify aflatoxins in meat and meat products (Bullerman et al., 1969; Brown et al., 1973; Strzelecki, 1973; Trucksess and Stoloff, 1979; Furtado, 1980).

## EXPERIMENTAL

### Testing the Aflatoxin-Producing Ability of Two Different Strains of A. flavus

Two strains of A. flavus obtained from the culture collection at the Northern Regional Research Laboratory of the U.S. Department of Agriculture, Peoria, Ill., were examined for aflatoxin-producing ability. (1) NRRL-6549 A. flavus obtained from A. cieflar, Kulmback, Germany and isolated February, 1972 from raw ham; and (2) NRRL-6550 A. flavus obtained from B.G. Maxfield, Cullman, Alabama and isolated from the brain of a 14 day old broiler.

### Inoculum Production and Harvesting

Inocula from the two molds were produced by growing the molds at 28°C for 8 days on a thin layer of potato-dextrose-agar in Roux bottles. The conidia were harvested and suspended in 50 ml of sterile water, and gently agitated. After harvesting, the spore suspensions were transferred to a sterile bottle, enumerated with a hemocytometer and diluted to contain $1x10^6$ conidia/ml.

### Culture

Demineralized water was used to prepare the basal medium, which contained 2% yeast extract (Difco) and 20%

sucrose (YES). Flasks (500 ml) containing 50 ml of medium per flask were stoppered with foam plugs and autoclaved for 15 minutes at 20 psi. Media were inoculated with spores from 8 day old cultures of the two strains of A. flavus and incubated 8 days at 28°C as stationary cultures. Experiments were replicated five times each and the results are reported as averages.

Aflatoxin Extraction

Aflatoxins were extracted from cultures (mycelium medium) according to the techniques described by Buchanan et al. (1976). Chloroform (25 ml) was added to the culture, which was then agitated on a shaker (Model 6130, Eberbach Corp., Ann Arbor, Mich.) for 10 minutes. The liquid was then transferred to 125 ml separatory funnels and agitated vigorously. The chloroform layer was drawn off into a 500 ml round bottom flask. The extraction procedure was repeated twice with the three extracts from each replication being combined in the round bottom flask. Extracts were evaporated almost to dryness using a Rotovapor R (Buchi, Switzerland). The extracts were redissolved in chloroform to a volume of 0.5 ml. They were then stored at -16°C while holding for analysis.

## Identification, Quantification and Confirmatory Test for Aflatoxins

Aflatoxin assays were run by thin-layer chromatography using precoated 20x20 cm silica gel plates (Sil-G-HR-25, Brinkman Instruments Inc.). The plates were developed with chloroform-acetone (85:15, v/v) in the first direction and ether-methanol-water (95:4:1, v/v) for the second dimension. The aflatoxins were quantitated by densitometric analysis with a double beam scanning recording integrating Schoeffel SD 3000-3 spectrodensitometer.

After quantification, general confirmatory tests for aflatoxins were carried out according to the method of Przybylski (1975). This procedure is described later herein.

## Preparation of the Sausages

Three types of summer sausages were prepared. They were all prepared using the same formulation, but differed in the following ways: (a) lot 1-control-smoked, uninoculated; (b) lot 2-smoked and inoculated; and (c) lot 3-not smoked but inoculated. The formula was made using 9.5 kg of lean beef, 2.04 kg of lean pork, 2.04 kg of pork fat, 340.5 g of sodium chloride, 1.2 g of sodium nitrite, 2.4 g of sodium nitrate, 136.2 g of sucrose, 33.9 g of coarse black pepper, 12.6 g of coriander, 4.2 g of garlic powder, 4.2 g of allspice, 17.1 g of whole mustard seed, 17 g of starter culture (Lactacel Plus). The meat and fat were

ground separately in a Hobart meat grinder with a 4.8 mm plate. The spices, cure and starter culture were mixed with the meat and fat in a 80 lb capacity paddle mixer (Model Buffalo, John E. Smith's Sons Co., Buffalo, N.Y).

The mixture was stuffed into 4.5 cm diameter natural casings using an automatic stuffer (Model E-Z Pak water pressure sausage stuffer, E-Zuber Engineering and Sales Co., Minneapolis, Minnesota). After stuffing, the sausages were tied into 8-8.5 cm lengths to give small sausages weighing about 100 g each. Following the smoking and fermentation of the sausages for 13 hours, they were showered with hot water, tempered at room temperature, showered again with hot water, dried at room temperature and then chilled.

Inoculation of the Sausages

The sausages were inoculated with conidia of A. flavus NRRL-6549, which was previously shown to produce aflatoxins. Inocula were produced according to the method described by Bullerman et al. (1969). The mold was grown at 28°C for 10 days on thin-layers of potato-dextrose-agar in Roux bottles. The conidia were harvested and suspended in 2000 ml of sterile 0.05% Tween 80 solution. The inoculum level used was $10^6$ spores per sausage. The sausages were inoculated by dipping individually for 30 seconds in a spore suspension containing $10^6$ spores per ml. The calculated number of spores per sausage ($10^6$ spores/ml) was based on the assumption that each dipped sausage had adsorbed an average of

1 ml of inoculum.

The three lots of sausages were aged for 3 and 6 weeks at different conditions of smoking, temperature and humidity as shown in Table 1. This gave the following treatments: (1) 10°C and 75% relative humidity, (2) 10°C and 87% relative humidity, (3) 30°C and 79% relative humidity, and (4) 30°C and 89% relative humidity. About nine inoculated sausages were hung in fermentation pails similar to those described by Costilow and Uebersax (1978). The experiment employed twelve 5-gallon plastic pails equipped with lids modified by cutting 2.5 cm off-centered holes to accommodate #6 rubber stoppers. A small wire hook was attached to the center of each stopper so that a wire rack could be suspended in the pail to support the free hanging sausages. Close to the hole in the center of the lid, another 2.5 cm hole was made and was stoppered with a foam plug. The whole system is shown in Figure 2.

A saturated salt solution (900 ml), selected to give the desired humidities at the temperatures used, was placed in the bottom of each pail (Rockland, 1960). The saturated salt solutions and the relative humidities they provided are shown in Table 2. Each salt was added to the solutions to insure and maintain saturation, however, some sausages lost moisture as they aged, so salt was added when needed. The foam plugs provided sufficient gas exchange to get an aerobic atmosphere in the pails. As in previous studies

Table 1. Experimental conditions for holding sausages.

| Temperature ($^{o}$C) | 10 | 30 |
|---|---|---|
| Lot No. | Humidity | Humidity |
| | % | % |
| Lot 1 | 75 | 79 |
| Control[a,b] | 87 | 89 |
| Lot 2 | 75 | 79 |
| w/o smoke[c] | 87 | 89 |
| Lot 3 | 75 | 79 |
| with smoke[a,c] | 87 | 89 |

[a]Sausages prepared under regular conditions of processing.

[b]Sausages were not inoculated with A. flavus.

[c]Sausages inoculated with A. flavus NRRL-6549 at about $10^{6}$ spores/sausage.

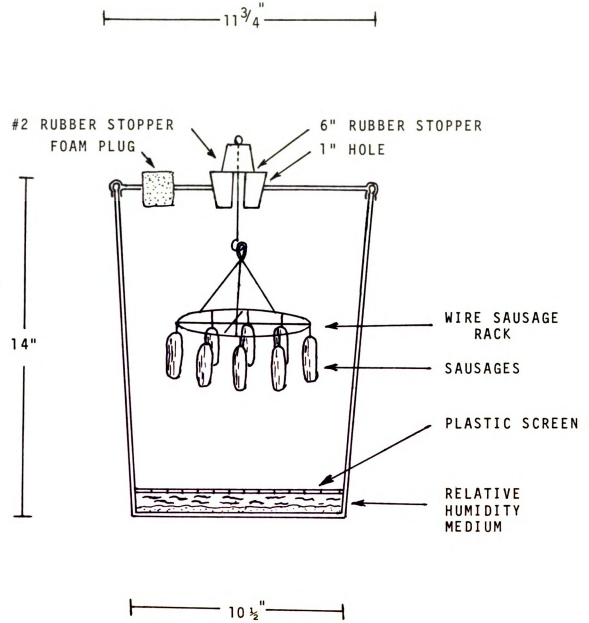



Figure 2. Design of controlled humidity 5 gallon plastic pail for aging the sausages.

Table 2. Salt solutions used to obtain desired relative humidities for each temperature at which sausages were aged.

| Temperature (°C) | Saturated Salt Solution Used | Humidity |
|---|---|---|
| | | % |
| 10 | NaCl | 75 |
| | KCl | 87 |
| 30 | $NH_4SO_4$ | 79 |
| | BaCl | 89 |

(Bullerman et al., 1969), it was assumed that this would not permit exchange of air to alter the humidity of the environment within each pail.

After holding the sausages for either 3 or 6 weeks, they were removed from the pails. They were placed in plastic bags, frozen and stored at -16°C until they were removed for analysis for aflatoxins.

Extraction of Aflatoxins from Sausages

Preliminary tests for extraction of aflatoxins from sausages were carried out using the methods of Bullerman et al. (1969) and Strzelecki (1973), which have been reported to be suitable for the analysis of aflatoxins in meat, such as ham, bacon, salami and some fresh meat. However, interfering materials from the added smoke, the spices, and perhaps from the high fat content of the sausages per se made the TLC manipulations very difficult. The plates showed an unresolved streak without spot differentiation. Thus, changes were made to eliminate the interfering materials from the extracts but they were time consuming and more complicated.

After considerable modification, extraction and quantification of the aflatoxins in the sausages were then carried out using a modification of the procedure described by Furtado et al. (1979, 1981), who reported a highly sensitive method for determination of aflatoxins in tissues.

The modified procedure began with 100 g of sausage, which was sliced and blended for two minutes in a Waring blender at moderate speed with 42 ml of saturated NaCl solution (40 g NaCl/100 ml $H_2O$) and 3 g of citric acid. Then 300 ml of acetone were added to the homogenate while washing the sides of the blender jar. An additional blending was then performed at moderate and high speeds. After blending, the material was filtered through fast filtering prefolded paper (Whatman 114 V) and the filtrate was collected in a 500 ml Erlenmeyer flask. After complete draining of the acetone from the residue on the filter paper, the flask was stoppered with a cork stopper and frozen at -16°C for 5 minutes. The precipitated fat was removed by filtering, using the same type of paper.

After filtration was completed, the meat residue was discarded. Then 150 ml of water, 7.5 g of $(NH_4)_2SO_4$ and 20 ml of $Pb(OAC)_2$ solution (200 g $Pb(OAC)_2 \cdot 3H_2O$ in 500 ml $H_2O$ containing 3 ml of acetic acid and made to a volume of one liter with $H_2O$) were added to the filtrate. The solution was stirred for ½ minute with a magnetic stirring device. Then 10 g of diatomaceous earth were added to the solution and stirring was continued for an additional ½ minute. The solution was allowed to stand for about 5 minutes before filtering through fast filtering folded paper. The final filtrate was collected in a 500 ml Erlenmeyer flask.

Purification of the Aflatoxin Extract

Liquid-Liquid Partition. The filtrate was transferred to a 500 ml separatory funnel. Then 100 ml of petroleum ether (35-60°C b.p.) were added. The separatory funnel was shaken vigorously for about 1 minute. The layers were allowed to separate, and the lower aqueous-acetone layer was drained into a second 500 ml separatory funnel. The petroleum ether layer was then discarded. Then 50 ml of chloroform were added to the aqueous-acetone solution and the separatory funnel was shaken vigorously as before. After the layers separated, the lower chloroform layer was collected in a 500 ml flask. Aflatoxin extraction from the aqueous-acetone layer was repeated one more time using 50 ml of chloroform:acetone (50:30, v/v). The aqueous layer remaining after the chloroform extraction was discarded. The chloroform-acetone extract was then evaporated to dryness in a Rotavapor R (Buchi, Switzerland), using a water bath setting at 35°C.

Silica Gel Column Chromatography. A 250x25 mm glass column (Fisher & Porter) was filled using about 50 ml of chloroform. A slurry of 10 g of silica gel 60 (70-230 mesh ASTM-EM Laboratories Inc.) in 60 ml of chloroform was added to the column and the chloroform was then drained to about 10 cm above the top of the silica gel. Another 3 cm layer of anhydrous $Na_2SO_4$ was added on top of silica gel. The excess of chloroform was drained by gravity to the top of

the upper $Na_2SO_4$ layer.

The aflatoxin extract was dissolved in about 5 ml of chloroform-hexane (1:1, v/v) and then transferred to the column with a disposable glass pipet. The sides of the flask were washed three more times with chloroform-acetone (1:1, v/v) and the washings were added to the column. After each addition of the chloroform extract, the column was drained to the top of the packing and the eluate was discarded. Interfering substances were eluted from the column in 100 ml of the ether-hexane (3:1). The eluate was then discarded.

The aflatoxins were eluted from silica gel column with 150 ml of chloroform-methanol (97:3, v/v). The eluate was collected in a 250 ml flask and evaporated to near dryness in a rotary evaporator as described earlier herein. The sample extract was dissolved in acetone and quantitatively transferred to 2 dram vials using a disposable glass pipet. The acetone solution was evaporated to dryness (N-Evap evaporator, Model 106, Organomation Assoc.) under a steam of nitrogen using a water bath setting of $35^{o}C$. Special care was taken to avoid overheating of the dry extract. The aflatoxin extract was then dissolved in a 100 µl of benzene-acetonitrile (9:1, v/v), and the vial was sealed with a teflon lines screw cap. The vial containing the aflatoxins was shaken vigorously for about 1 minute on a vortex shaker before removing the samples for analysis.

Thin Layer Chromatography. Precoated 20x20 cm silica gel plates (Sil-G-HR-25, Brinkman Instrument Inc.) were scored and spotted as shown in Figure 3. A 20 µl sample of aflatoxin extract was applied to the plate with a 25 µl syringe (Hamilton Co.). Standards of 0.5131, 0.1668, 0.540 and 0.1791 µg of aflatoxin $B_1$, $B_2$, $G_1$ and $G_2$, respectively, were spotted on TLC plates about 1 cm from the edge (Figure 3).

The plates were developed in the first direction with chloroform:acetone (85:15, v/v) in a sealed and unequilibrated tank. After the development in the first dimension was completed, the plates were removed from the tank and dried under a hood for about 2 minutes, the drying was finished by placing in a forced-draft oven for 1 minute and immediately developed in a second dimension with anhydrous ethyl ether-methanol-water (95:4:1, v/v). The plate was removed from the tank and dried as before to remove the ether. After drying the plate was analyzed visually and prepared for densitometric analysis.

Densitometric Analysis of Aflatoxins. Aflatoxins were quantified with a double beam scanning recording-integrating spectrodensimeter SD 3000-4 (Shoeffel Instruments). The plates were scored prior to spotting as shown in Figure 3 with a Schoeffer Scoring Device SDA 303, which provides 10 mm strips. Three aflatoxins standards were spotted within three strips parallel to the second dimension of development.

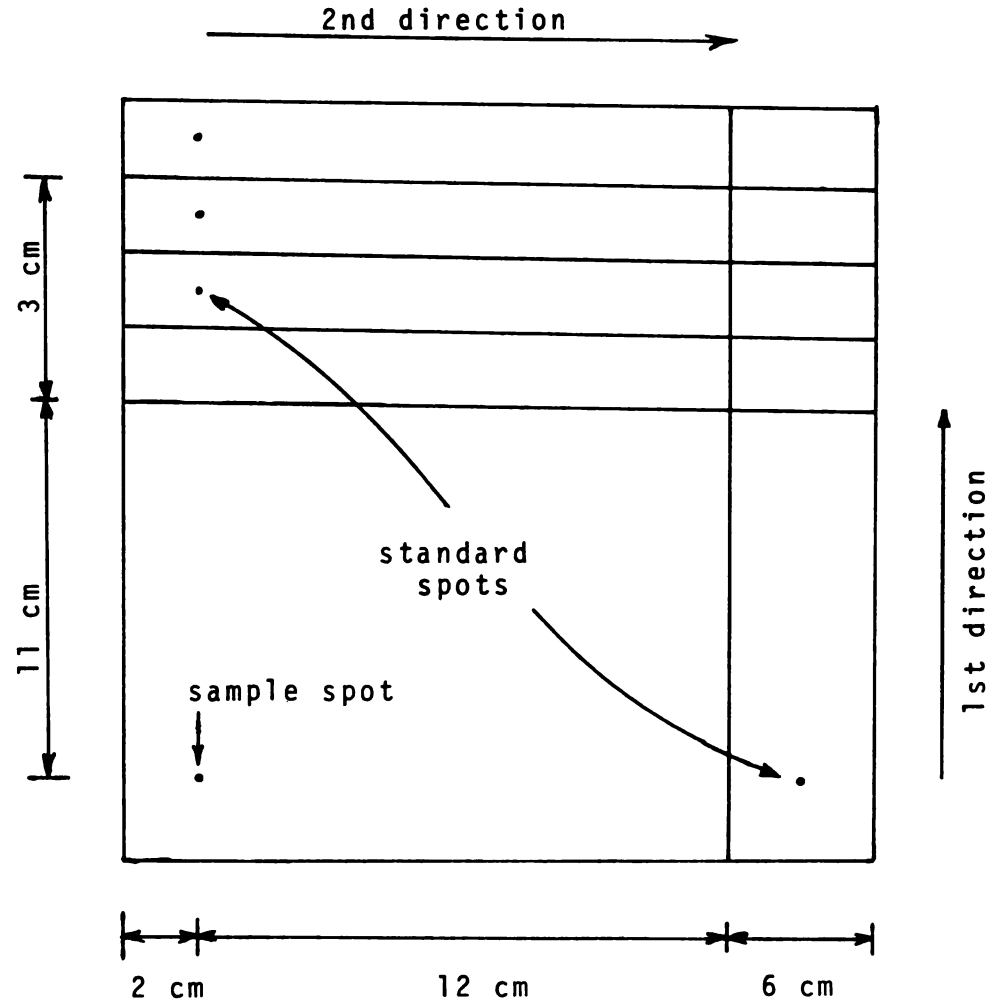



Figure 3. Spotting and scoring pattern for 2-dimensional TLC plate.

The average of the three readings of the aflatoxin standards was used to compare and calculate the concentration of the sample. For analysis of the standard, the scanning head was placed within each of the three strips and parallel scanning took place. For the sample spot, the plate was viewed under uv light and each two spots were localized within an imaginary strip identified through four pencil marks made on the silica gel plate. Then the plate was placed on the plate carrier in such a way as to be driven and scanned parallel to the direction of the imaginary lines.

Aflatoxin concentrations were calculated according to the following formula:

$$\mu g/Kg=(B \times Y \times S \times V)/(Z \times X \times W)$$

where:

B = Area of aflatoxin peak in the sample

Y = Concentration of aflatoxin standard in µg/ml

S = µl of the aflatoxin standard

V = Dilution of sample extract in µl

Z = Area of aflatoxin standard peak (Average of three replications)

X = µl of sample extract spotted on the plate

W = Grams of sample in the final extract

General Confirmatory Test for Aflatoxin. After development of the TLC plates and identification of the spots under uv light, the plate was sprayed with 25% sulfuric

acid. The plate was then dried in a chromatographic oven at 45°C under a stream of nitrogen. Fluorescence change of the aflatoxins from blue or green to yellow after the treatment with $H_2SO_4$ was used as additional confirmation for the presence of aflatoxin in the sample (Przybylski, 1975).

Preparation of Aflatoxin Reference Standards. Aflatoxin reference standards were prepared according to the AOAC method (1980) and contained 0.5 µg/ml of aflatoxin $B_1$ and $G_1$ and 0.1 µg/ml of aflatoxins $B_2$ and $B_2$. A solvent mixture of benzene:acetonitrile (98:2, v/v) was used as the solvent for all aflatoxins. The aflatoxin standard solutions were stored at -16°C.

Fat and Moisture Analysis

Moisture Content. The A.O.A.C. (1980) procedure for determining moisture was used. About 5 g of sample were accurately weighed to four decimal places into a previously dried aluminum dish. The sample plus the dish were then dried for 18-24 hours in an oven at 100-105°C. The dried sample was cooled in a desiccator and weighed to four decimal places. The loss in weight was calculated as percentage moisture.

Fat Content. The fat content was determined using the Goldfisch extraction method of the A.O.A.C. (1980). The sample used for moisture analysis was utilized. The aluminum dish containing the dried meat sample was carefully folded into a porous thimble and clipped into the Goldfisch

extractor. The fat was extracted with anhydrous diethylether for approximately 8 hours into a previously dried and tared beaker. The extract was then dried for 1 hour at 100°C in an air convection oven, cooled in a desiccator and weighed as before. The percent fat was calculated as grams of fat extracted per hundred grams of sausage.

Safety Procedures. All material, glassware and vials in contact with aflatoxins were soaked either with 5-6% NaOCl (household bleach) or with sulfuric acid-dichromate solution (120 g $Na_2Cr_2O_7$-$2H_2O$ 1600 ml conc. $H_2SO_4$ and diluted to a volume of 3 liters with water)to destroy any residual aflatoxins. Plastic disposable gloves were worn routinely during all work with aflatoxins. Respirator masks were worn when handling culture of A. flavus, when making the spore suspension, during sausage inoculation and when preparing sausages for aflatoxins analysis. After the holding experiment, all surfaces of the containers were treated by spraying them with a solution of disinfectant solution (3-5% Lysol) and then with NaOCl solution. All TLC plates used for aflatoxin analysis were thoroughly soaked in NaOC solution before discarding. The waste material, after treatment with ammonia, was collected in plastic bags and placed inside tightly closed containers and labeled properly until removed by the MSU Animal Waste Disposal Unit. The discarded solvents involved in the experiment were removed by the MSU Chemical and Biological Safety Unit. All work involving

aflatoxins was done under a fume hood, this included preparation of silica gel columns, spotting, development and drying of the TLC plates. Similarly, any work involving the use of toxic solvents, such as ammonia, chloroform, benzene, methanol and acetonitrile, was also carried out under the fume hood.

# RESULTS AND DISCUSSION

## Testing of Two Strains of A. flavus for Production of Aflatoxins

Preliminary work testing two different strains of A. flavus on suitable media showed that only one of the strains produced aflatoxins. The two molds tested included A. flavus-NRRL-6549 and A. flavus-NRRL-6550. Only NRRL-6549 produced measurable amounts of aflatoxin $B_1$. Results are in agreement with the conclusions of Heathcote and Hibbert (1978), who showed that many strains of A. flavus do not produce aflatoxins.

Using the basal YES medium containing 2% yeast extract and 20% sucrose, the fungus produced from 1.71 to 3.80 µg of aflatoxin per liter of media. Davis et al. (1969) reported that some other strains of A. flavus produced aflatoxins at levels of 6.2 and 6.4 mg/100 g of mycelia. The amount of aflatoxin $B_1$ produced by the mold in the preliminary test was adequate, since the purpose of this experiment was to find out if aflatoxins could be produced under simulated conditions of manufacture and storage of summer sausage.

Visual Estimation of Mold Growth

The three lots of sausages consisted of: (a) lot 1-control, smoked and uninoculated, (b) lot 2-unsmoked and inoculated, and (c) lot 3-smoked and inoculated. They were aged for either 3 or 6 weeks at either 75-79% or 87-89% relative humidity at temperatures of either 10 or 30°C. After incubation, the sausages were analyzed visually for mold growth.

Incubation for 3 Weeks. After incubation of the samples at 10°C and a relative humidity of 75%, only slight mold growth was found on the surface of the sausages. Mold growth was only noticeable on the uninoculated-smoked control sausages, which showed about 4 small colonies per sausage. At the same temperature but at high relative humidity (87%), slight growth was observed on both the smoked uninoculated control sausages and on the smoked inoculated ones. However, the unsmoked inoculated sausages contained about 5 large colonies per sausage, with the colonies being considerably larger than those of the smoked uninoculated control.

On holding the sausages at high temperature and low humidity (30°C, 79% r.h.), considerable difference in the amount of mold growth was evident. All sausages in all treatments were covered by heavy mold growth. However, visible differences were evident between colonies for the uninoculated smoked control and for those from the other

samples which were inoculated with A. flavus. The uninoculated smoked control sausages exhibited some yellow-green, some white and some brown colonies. On the other hand, both lots of inoculated sausages (smoked and unsmoked) were entirely covered by mold, which exhibited the characteristic yellow-green color of A. flavus colonies. Shrinkage was observed on the surface of all sausages, and was probably due to the loss of surface moisture. This resulted in some surface drying and wrinkling, but was not confirmed by moisture analysis of the entire sausage (Table 3), indicating that the apparent shrinkage was a surface phenomenon.

At optimum conditions of temperature and humidity for mold growth (30°C, 89% r.h.), very heavy mold growth was present on all samples. As previously observed, the uninoculated smoked control showed a mixture of colonies that exhibited green, yellow-green, brown, black and white colonies. Both inoculated lots (smoked and unsmoked) had a heavy mold covering on all exterior surfaces. Under these conditions, no differences could be observed between the mold growth for the unsmoked and smoked sausages. No shrinkage was apparent on any sample under these conditions of incubation.

Incubation for 6 Weeks. All sausages held for 6 weeks at 10°C and 75% relative humidity showed slightly more mold growth than those held for 3 weeks. The colonies were larger in all cases. Slight mold growth was evident on

Table 3. Production of aflatoxin $B_1$ by A. flavus NRRL-6549 on summer sausages, after 3 weeks of incubation at 10°C and 30°C. Each value is the average of three samples expressed in µg/kg.

| Temperature | 10°C | | 30°C | |
|---|---|---|---|---|
| Relative Humidity | 75% | 87% | 79% | 89% |
| Control[a] | ---[c] | ---[c] | ---[c] | ---[c] |
| Smoked[b] | ---[c] | ---[c] | ---[c] | ---[c] |
| Unsmoked[b] | ---[c] | ---[c] | ---[c] | 0.26 |

[a]Uninoculated smoked sausages.

[b]Sausages inoculated with A. flavus at $10^6$ spores/sausage.

[c]No aflatoxin was detected at >0.02 ppb.

both the smoked and unsmoked inoculated sausages, but more growth was apparent on the smoked uninoculated controls.

Increasing relative humidity and keeping temperature the same (87% r.h. and 10°C, respectively) caused only slight changes in mold growth. The number of colonies was almost the same for the smoked uninoculated control and the inoculated smoked sausages, but 60-75% of the surface of the unsmoked inoculated sausages was covered with mold in comparison to less than 20% in the case of inoculated smoked sausages. In all cases the color of all colonies was blue-green, which is characteristic of A. flavus. Although some shrinkage was observed on all sausages at 10°C and 75% relative humidity, at 10°C and 87% relative humidity no shrinkage was apparent on visual examination, probably because the high relative humidity prevented moisture losses.

At 30°C and 79% relative humidity, a great deal of surface shrinkage was evident for all treatments. The surface of the smoked uninoculated controls was completely covered with brown, green and white colonies. The brown colored mold appeared to exude a dark brown fluid on the surface of the sausages. The smoked inoculated sausages were completely covered with a brownish-gold mold, which also exuded a similar dark fluid. In contrast to the uninoculated smoked controls, the inoculated smoked sausages showed only the brownish-gold mold colonies. The unsmoked sausages were somewhat different than the smoked inoculated

ones, in that they were completely covered with a brownish-gold mold, but in addition, also contained some greenish white secondary contaminations on each sausage.

At 30°C and 89% relative humidity, which are the optimum conditions for the growth of A. flavus, very little surface shrinkage was apparent. This was confirmed by moisture analysis of the sausages (Table 4). The uninoculated smoked control was completely covered with a mixture of colonies. Brownish-gold mold with brown fluid soaked areas and several white and green colonies of secondary contaminations were observed on each sausage. Under these conditions, both the inoculated smoked and the inoculated unsmoked sausages were similar in appearance. Their surfaces were covered with brownish-gold growth accompanied by fluid soaked areas similar to those observed on the controls. As in the case of the uninoculated smoked control, but to a lesser degree, the inoculated smoked sausages and inoculated unsmoked sausages both exhibited several small white colonies of secondary contaminations. Under these conditions all the sausages were soft, watery and lacked firmness upon slicing.

## Factors Influencing Aflatoxin Production

Aflatoxin Production During 3 Weeks Storage. Results show that temperature and time are important factors influencing the ability of the mold to produce aflatoxins. After

Table 4. Moisture content in summer sausages incubated for 3 weeks at 10°C and 30°C. Each value is the average of three samples expressed as percent moisture.

| Temperature | 10°C | | 30°C | |
|---|---|---|---|---|
| Relative Humidity | 75% | 87% | 79% | 89% |
| Control[a] | 49.65 | 47.74 | 57.84 | 46.65 |
| Smoked[b] | 49.67 | 45.52 | 58.06 | 50.38 |
| Unsmoked[b] | 49.44 | 47.39 | 59.04 | 49.00 |

[a]Uninoculated smoked sausages.

[b]Sausages inoculated with A. flavus at $10^6$ spores/sausage.

incubation for 3 weeks at 10°C, no aflatoxins were detected in any of the three lots at either 75% or 87% relative humidity (Table 3). These results agree with those reported by Bullerman *et al.* (1969), who concluded that there is a relationship between time, temperature and aflatoxin production by *A. flavus* on fresh beef, bacon and ham. Results of the present study confirmed those of Oldham *et al.* (1971), who reported that *A. flavus* did not produce detectable aflatoxin in cheddar cheese or luncheon-meat when kept at refrigeration temperatures.

Data in Table 3 also show that even after the temperature was increased (30°C) and all samples showed visible mold growth, only the unsmoked inoculated lot held at high relative humidity (80%) contained any measurable amount (0.26 µg/kg) of aflatoxin $B_1$. The data suggested that the mold requires both time and favorable conditions of humidity to produce aflatoxins.

The data in Table 3 also indicate that the combination of low temperature (10°C) at either low relative humidity (75-79%) or high relative humidity (87-89%), and smoking retarded mold growth and aflatoxin production on the sausages. Changes in relative humidity also affected the moisture content in the samples as shown in Table 4. High relative humidities tended to increase the moisture content, with the sausages apparently taking up moisture from the environment.

Austwick and Ayerst (1963) found that the most important factors in growth and aflatoxin production by A. flavus are the moisture content of the products and the relative humidity in the environment surrounding the natural substrate. Results of this study (Table 3) show that time and temperature also are important factors for aflatoxin production, since neither visible mold growth nor aflatoxin production were detected during 3 weeks of storage at low temperature ($10^{o}C$). On the other hand, 0.26 µg/kg of aflatoxins were produced during storage at high temperature ($30^{o}C$) and high relative humidity (89%).

Aflatoxin Production During 6 Weeks Storage. After 6 weeks incubation at $10^{o}C$ and either 75 or 87% relative humidity, there were no detectable levels of aflatoxins in any of the samples except for the unsmoked inoculated sausages held at high relative humidity (89%). These sausages that had previously shown visible mycelial growth produced measurable levels of aflatoxin (1.40 µg/kg, Table 5). In the rest of sausages held under the same conditions, no aflatoxins were detected. The data indicate that for periods of up to 6 weeks duration, low temperature, low relative humidity, and smoking prevented growth mold and aflatoxin production (Table 5).

Data in Table 5 also demonstrate that aflatoxins were produced by A. flavus during 6 weeks of incubation. In the smoked inoculated sausages, the levels of aflatoxins were

Table 5. Production of aflatoxin $B_1$ by A. flavus NRRL-6549 on summer sausages after 6 weeks of incubation at 10°C and at 30°C. Each value is the average of three samples expressed in µg/kg.

| Temperature | 10°C | | 30°C | |
|---|---|---|---|---|
| Relative Humidity | 75% | 87% | 75% | 89% |
| Control[a] | ---[c] | ---[c] | ---[c] | ---[c] |
| Smoked[b] | ---[c] | ---[c] | 4.04 | 2.30 |
| Unsmoked[b] | ---[c] | 1.40 | 1.64 | 6.60 |

[a]Uninoculated smoked sausages.

[b]Sausages inoculated with A. flavus at $10^6$ spores/sausage.

[c]Aflatoxins not detectable at >0.02 ppb.

4.04 µg/kg and 2.30 µg/kg at 79 and 89% relative humidity, respectively. The unsmoked inoculated sausages contained 1.64 µg/kg at low relative humidity (75%) and 6.60 µg/kg at high relative humidity (89%). These results demonstrated that A. flavus can produce aflatoxins during 6 weeks storage at 30°C, even at low relative humidity and after smoking. The optimal conditions for aflatoxin production were shown to be 30°C and 89% relative humidity.

The results of this study agree with the report of Austwick and Ayerst (1963) showing that the relative humidity surrounding a natural substrate is one of the most important factors in growth and aflatoxin production. The visual estimation of growth, previously described, and the data in Tables 3 and 5 demonstrate that mold growth and aflatoxin production were only detected when relative humidity was 79% or more. Thus, results indicate that an increase in relative humidity during storage favored increased levels of aflatoxin production.

Increasing the hold time resulted in a noticeable change in the moisture content of the sausages. Comparing the results in Table 4 with those in Table 6 shows that all samples increased in moisture content when they were held in a relative humidity of 75% at 10°C for 6 weeks. Similar changes were also observed during storage for 6 weeks at relative humidities of 79 and 89% at 30°C.

Table 6. Moisture content in summer sausages incubated for 6 weeks at 10°C and at 30°C. Each value is the average of three samples expressed as percent.

| Temperature | 10°C | | 30°C | |
|---|---|---|---|---|
| Relative Humidity | 75% | 87% | 79% | 89% |
| Control[a] | 57.87 | 51.08 | 73.00 | 62.84 |
| Smoked[b] | 59.17 | 44.90 | 68.36 | 58.83 |
| Unsmoked[b] | 60.77 | 48.47 | 68.31 | 60.38 |

[a]Uninoculated smoked sausages.

[b]Sausages inoculated with A. flavus at $10^6$ spores/sausage.

## Other Factors Related to Aflatoxin Production

Effects of pH. The pH values are given in Tables 7 and 8 and showed that the samples stored at low temperature (10°C) did not change in pH at either 75 or 87% relative humidity. At high temperature (30°C), on the other hand, the pH readings obtained after 6 weeks were higher than those after 3 weeks, regardless of relative humidity (79 or 89%). On studying the effects of pH on mold growth and aflatoxin production, Davis et al. (1966) concluded that pH did not influence either aflatoxin production or growth, so long as the pH was higher than 4.0. Based on their results and in light of the fact that yeasts and molds are more acid-tolerant than other microorganisms (Frazier and Westhoff, 1978), it is probable that the absence of aflatoxins in the present study was due to either low temperature or low relative humidity instead of pH.

Effects of Smoking. Data in Tables 3 and 5 demonstrated that smoke inhibits mold growth and aflatoxin formation. Smoking of inoculated sausages delayed mold growth. Thus, aflatoxins were not detected or were present at lower levels than in the unsmoked inoculated sausages. When A. flavus did grow, however, aflatoxins were present in both smoked and unsmoked inoculated sausages. Growth of mold and aflatoxin production thus required favorable conditions of time, temperature and relative humidity.

Table 7. The pH of summer sausages incubated for 3 weeks at 10°C and 30°C. Each value is the average of three samples.

| Temperature | 10°C | | 30°C | |
|---|---|---|---|---|
| Relative Humidity | 75% | 87% | 79% | 89% |
| Control[a] | 4.8 | 4.8 | 5.3 | 5.0 |
| Smoked[b] | 4.8 | 4.9 | 5.1 | 6.3 |
| Unsmoked[b] | 4.8 | 4.9 | 6.9 | 6.7 |

[a]Uninoculated smoked sausages.

[b]Sausages inoculated with A. flavus at $10^6$ spores/sausage.

Table 8. The pH of summer sausages incubated for 6 weeks at 10°C and 30°C. Each value is the average of 3 samples.

| Temperature | 10°C | | 30°C | |
|---|---|---|---|---|
| Relative Humidity | 75% | 87% | 79% | 89% |
| Control[a] | 4.9 | 4.9 | 6.99 | 6.97 |
| Smoked[b] | 4.9 | 4.9 | 6.70 | 7.24 |
| Unsmoked[b] | 4.9 | 5.5 | 7.23 | 7.40 |

[a]Uninoculated smoked sausages.

[b]Sausages inoculated with A. flavus at $10^6$ spores/sausage.

Effect of Curing Ingredients. There is controversy regarding the influence of curing ingredients on aflatoxin production. Strzelecki (1973) reported that potassium nitrate inhibited aflatoxin production, but that sodium nitrate stimulated production. He further concluded that the complete curing mixture stimulated aflatoxin formation to the greatest extent. These data are opposite to the results of Bullerman et al. (1969), who reported that the curing salts inhibited aflatoxin production. In this study the effect of curing ingredients was not investigated since the purposes of the experiment were to determine the production of aflatoxins by a known strain of A. flavus on sausages produced under simulated conditions of manufacture. Nevertheless, results of this experiment suggest that the curing ingredients do not inhibit aflatoxin formation, since aflatoxins were produced whenever environmental conditions were favorable. Further work will be needed to clarify the effects of curing salts on production of aflatoxins.

# CONCLUSIONS

A preliminary screening test showed that Aspergillus flavus NRRL-6550 did not produce any aflatoxins but NRRL-6549 produced large amounts of aflatoxin $B_1$. Thus, the latter strain was used in all subsequent studies.

Aflatoxins were not produced during the first 3 weeks of storage at 10°C, on any of the sausages at either 75 or 87% relative humidity. The growth of mold on the smoked inoculated sausages and the unsmoked inoculated sausages was greatly enhanced by increasing the temperature (30°C). However, high relative humidity (87%) was also required in order to produce aflatoxin $B_1$ during 3 weeks of storage at 30°C.

In periods of up to 6 weeks, low temperature (10°C), low relative humidity (75%) and smoking retarded mold growth and aflatoxin production. At 10°C and high relative humidity (87%), only the unsmoked inoculated sausages produced detectable levels of aflatoxins (1.40 ug/kg). However, during 6 weeks storage at 30°C, aflatoxin $B_1$ was produced in amounts from 2.30 to 6.60 ug/kg, even at low relative humidity and after smoking.

The presence or absence of aflatoxins was due to the effects of temperature and relative humidity rather than pH.

Smoking inhibited mold growth and aflatoxin formation. When A. flavus did grow, however, aflatoxins were detected in both the smoked and unsmoked inoculated sausages regardless of whether or not they were smoked.

Results demonstrate that aflatoxins can be produced on summer sausages under favorable conditions for the growth of A. flavus, which include time, temperature and relative humidity. Although low levels of aflatoxins may occur in summer sausages under refrigeration temperatures ($10^{o}C$), the amount of mold growth found on most samples held at high temperatures ($30^{o}C$) and high relative humidity (89%) would make the sausages unacceptable for human consumption.

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