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Aflatoxin Production in Meats

II. Aged Dry Salamis and Aged Country Cured Hams[1]

LLOYD B. BULLERMAN,[2] PAUL A. HARTMAN, AND JOHN C. AYRES[3]

Department of Food Technology and Department of Bacteriology, Iowa State University, Ames, Iowa 50010

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Italian-type salamis contaminated with *Aspergillus flavus* were more likely to develop aflatoxins during aging than were smoked Hungarian-type salamis under the same conditions. Temperatures below 15 C and humidities of less than 75% were found to prevent aflatoxin development during the aging of salami. The aging of salami for 8 weeks and the presence of curing ingredients, especially pepper and sodium nitrite, tended to reduce the amounts of aflatoxins found. Aflatoxins were produced by *A. flavus* and *A. parasiticus* on 6- to 9-month-old country cured hams during aging when the temperature approached 30 C.

There is no direct evidence that aflatoxins can cause human disease. However, because of the carcinogenic properties of the aflatoxins and the large number of animal species and biological systems (including certain human cell cultures) known to be affected by aflatoxins, considerable interest has been generated in the presence and use of molds in the production of human food.

In addition to mold contamination of human food, a number of fungi are used in the manufacture of certain foods. Some of these organisms are added to the foods as inocula; in other instances, a characteristic flora develops by chance during an aging process. Certain cured and aged meats, especially country cured hams and European dry salamis, are examples of the latter. Country cured hams are produced in the southern United States, and European dry salamis are traditionally produced in many countries of Europe, including Hungary, Italy, Spain, Greece, Yugoslavia, Romania, and Czechoslovakia. Also, there is a limited production in the San Francisco-Oakland Bay area of the United States (1).

Country cured hams are dry-cured hams that may or may not be smoked, followed by aging for 6 months to 2 years. This aging, in many instances, is done in rooms or attics in which the temperature and humidity are not controlled, but allowed to fluctuate with the environmental conditions. Thus, summer temperatures of 30–35 C are not unusual. Heavy mold growth occurs particularly on the flesh side of the ham. The type of mold found is determined by the moisture content of the hams. Thus, during early stages of aging, penicillia predominate; as the available water of the ham decreases, aspergilli begin to grow. Eventually, as the water activity (available water) decreases to a lower level (0.65), *Aspergillus ruber* and other xerophilic molds predominate (1, 5; L. Leistner, J. C. Ayres, and D. A. Lillard, paper presented at the 4th Symp. World Ass. Vet. Food Hyg., Lincoln, Neb., July, 1965; D. A. Lillard and J. C. Ayres, paper presented at the 28th annual meeting of the Inst. Food Technol., Philadelphia, Pa., May, 1968).

Dry European type salamis are made from ground pork and beef combined in variable proportions with salt, spices, nitrate, and nitrite. Hungarian-type salami are smoked, whereas Italian-type salami are not. The Italian-type salami are hung in "greening" rooms for 4 to 5 days where excess moisture drips from the sausages and characteristic molds begin to grow. After smoking, or after growth has started, the salami are moved to rooms where they are aged for 30 to 60 days at temperatures of 10 to 16 C and a relative humidity of about 75%. During aging, profuse mold growth may develop. The predominant molds are either penicillia or penicillia and *Scopulariopsis*, but aspergilli may also be present (1). In a recent study in our laboratory (3), a strain of *A. flavus* isolated from an Italian-type salami was found capable of aflatoxin production. The present study was conducted to determine the levels of aflatoxins that could be produced by a known toxinogenic strain of *A. flavus* on salami

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[2] Present address: Product Research, Green Giant Company, Le Sueur, Minn. 56058.

[3] Present address: Division of Food Science, University of Georgia, Athens, Ga. 30601

TABLE 1. *Salt solutions used to obtain humidities within the desired humidity range for each temperature at which salamis were aged*

Desired humidity range	Calculated humidity obtained	Temp (C)	Saturated salt solution used	Ref.
%	%			
65–70	68.0	10	NH_4NO_3	(8)
	68.0	15	NH_4NO_3	(8)
	67.0	20	NH_4NO_3	(7)
76–80	76.3	10	NaCl	(7)
	77.2	15	$NaNO_3$	(7)
	75.8	20	NaCl	(7)
85–90	86.0	10	KBr	(7)
	86.7	15	KCl	(7)
	86.3	20	KCl	(7)

under simulated conditions of manufacture and the levels of aflatoxins that could be produced by known toxinogenic strains of *A. flavus* and *A. parasiticus* on country cured hams at different stages of aging.

MATERIALS AND METHODS

Two basic types of salamis, with three different treatments, were studied. The types of salami studied were: Italian type, not smoked; Hungarian type, inoculated before smoking; and Hungarian type, inoculated after smoking. The salami were made by the method of Ayres et al. (1). The same formula was used for both Italian- and Hungarian-type salami: 8 kg of lean pork, 2 kg of fresh side pork, 5 kg of lean beef, 350 g of sodium chloride, 75 g of ground white pepper, 22 g of garlic powder, 140 g of sucrose, 28 g of sodium nitrate, 3 g of sodium nitrite, and 10 g of monosodium glutamate. The meat was ground with the fine cutting blade of a sterilized grinder. The salts and spices were evenly mixed with the meat, and the mixture was kept at 2 to 3 C overnight before stuffiing into 4-cm diameter natural beef casings. The stuffed casing was tied off into ca. 8-cm lengths to give minature salamis weighing about 100 g each.

The salamis were inoculated with conidia of *A. flavus* NRRL A-1600, originally isolated from an Italian-type salami (3). Inocula were produced by growing the mold at 25 to 30 C for 10 days on thin-layers of potato-dextrose-agar in Roux bottles. The conidia were harvested and suspended in 100 ml of sterile 0.05% Tween 80 solution (Mann Research Laboratories, Inc., New York, N.Y.). Inoculum levels used were 10^2 and 10^6 spores per salami. The salamis were inoculated by dipping an individual salami for 30 sec in a spore suspension containing the desired number of spores per ml. The salami was removed from the inoculum and allowed to drain until no more liquid dripped from its surface; the liquid that drained from the salami was returned to the inoculum. When the salamis were inoculated in this fashion, the spore suspensions used for inoculum lost about 1 g of weight per salami dipped. The calculated number of spores per salami (10^2 and 10^6 spores/ml) was based on the assumption that each dipped and drained salami had absorbed an average of 1 ml of inoculum. One group of Hungarian-type salamis was inoculated before smoking and another group was inoculated after smoking. In both instances, the salamis were smoked for 1 hr a day for 8 consecutive days at tempeatures that never exceeded 32 C.

During the aging of the salamis, the temperature and humidity were controlled at 10, 15, and 20 C and 65 to 70, 75 to 80, and 85 to 90% humidity. Each inoculated salami was hung in a sterile 1-qt Mason jar (940 ml) equipped with a Mason lid modified by cutting a 1.5-cm hole off-center to accommodate a no. 1, one-hole rubber stopper fitted with a 5-cm length of glass tubing, plugged with cotton. A salami was hung on a small wire hook attached to the center of the lid and extending into the jar.

A 150- to 200-ml portion of a saturated salt solution, selected to give the desired humidities at the temperatures used, was placed in the bottom of each jar (7, 8). The humidities obtained from various saturated salt solutions at the temperatures used are shown in Table 1. As the salamis aged and lost moisture, the salt solutions became less saturated; this was compensated for by adding excess salt to the solution at the beginning of the experiment to maintain saturation. Additional salt was added to the solutions as needed during the experiment. The cotton-plugged glass tubing allowed sufficient gas exchange so the atmosphere within the jar was aerobic, but it was assumed that this arrangement would not permit such a large exchange of air that the humidity of the environment within each jar would be altered excessively.

The salamis were aged for 8 weeks, at which time they were stored at −18 C until they could be analyzed for aflatoxins. Another group of salamis, inoculated with 10^6 spores per salami and aged at 75 to 80% humidity at each temperature, was frozen each week for the first 4 weeks of the experiment, until aflatoxin analysis could be performed. This was done to determine whether aflatoxin levels were higher in the early stage of aging than at the end, at a humidity considered to be normal for a commercial product.

The amount of growth of the *Aspergillus* species was estimated visually. Random loopfuls of *Aspergillus*-like growth were streaked on potato-dextrose-agar plates. These cultures were grown and examined for (i) characteristics typical of *A. flavus* NRRL A-16100 and (ii) ability to produce aflatoxins on rice. The salami and rice cultures were extracted with chloroform and assayed for aflatoxins by visual comparison to aflatoxin standards by using thin-layer chromatography (4).

The effects of salami ingredients on growth and aflatoxin production by *A. flavus* NRRL A-16100 were evaluated. Glucose-ammonium-nitrate (GAN) broth, described by Brian et al. (2), was prepared by dissolving the following ingredients in 1 liter of distilled water: 50 g of glucose; 2.4 g of NH_4NO_3; 10 g of KH_2PO_4; 2 g of $MgSO_4 \cdot 7H_2O$; 1.3 ml of a solution consisting of 20 g of $ZnSO_4 \cdot 7H_2O$, 2 g of $CuSO_4 \cdot 5H_2O$, and 1 g of $Co(NO_3)_2 \cdot 6H_2O$ per liter; and 1.3 ml of a solution of 50 g of $CaCl_2$/liter. The GAN

broth was dispensed in 50-ml quantites into 250-ml Erlenmeyer flasks and autoclaved for 10 min at 121 C. Individual curing salts and spices were added to the hot broth in the same percentages as used in making the salamis. Other flasks contained GAN broth only or GAN broth plus all of the salts and spices in the same percentages as used individually. The percentages of salts and spices used were as follows: 2.2% NaCl; 0.4% ground white pepper; 0.14% garlic powder; 0.9% sucrose; 0.17% $NaNO_3$; 0.02% $NaNO_2$; and 0.07% monosodium glutamate. A mixture of two-thirds pork to one-third beef, with and without the combined curing ingredients, and casings alone were also studied. The samples were inoculated with *A. flavus* NRRL A-16100 and incubated at 25 C for 7 days. The meat cultures were extracted with chloroform and assayed for aflatoxins by visual comparison to aflatoxin standards by using thin-layer chromatography (4). The amount of growth of the *Aspergillus* species on the meat was estimated visually. The GAN broth cultures were filtered through Whatman no. 2v filter paper, by gravity filtration; the mycelial mats were collected, washed, dried, and weighed. The filtered GAN broth for each sample was extracted with two 50-ml portions of chloroform in a separatory funnel, and the extracts were combined. The extracts were concentrated and analyzed by thin-layer chromatography (4).

Portions of country cured hams, which had been experimentally produced and aged to 3, 6, 9, and 12 months (D. A. Lillard and J. C. Ayres, paper presented at the 28th annual meeting of the Institute of Food Technologists, Philadelphia, Pa., May 1968), were inoculated with 10^6 spores of either *A. flavus* NRRL 2999, *A. flavus* NRRL A-16100, or *A. parasiticus* CMI 15957. Other portions within each age group were kept as uninoculated controls. The ham pieces were wrapped in white butcher paper, and the aging was resumed at the following temperatures and times: 3-month hams at 10 C for 6 weeks; 6-month hams at 20 C for 4 weeks; 9- and 12-month hams at 30 C for 2 weeks. The different temperatures are representative of those that might occur during a normal aging time of 1 year. After the proper time had elapsed, the hams were frozen and stored at −18 C until analyzed for aflatoxin content.

The outer surface of each ham was sampled by removing the outer 1-cm layer of 100 g of the lean portion. This was the portion that most readily supported mold growth. These samples were cut into ca. 1-cm cubes, extracted with chloroform, and analyzed for aflatoxins (4).

RESULTS AND DISCUSSION

No aflatoxins were detected in any salamis aged at 10 C. Trace amounts of aflatoxins were found in Italian-type salamis aged at 15 C. Low levels of aflatoxins were detected in Italian-type salamis inoculated with 10^6 spores per salami and aged at 20 C under relative humidities above 75% (Table 2). Although temperature had a pronounced effect on aflatoxin production in aged salamis, the factors of humidity and type of salami produced equally striking effects. Also, there definitely appeared to be interactions between these three factors and their effects on aflatoxin production.

Increased reltive humidity during storage resulted in increased levels of aflatoxin production. The effect of smoking was equally striking. No aflatoxins were produced on Hungarian-type salamis at any temperature or humidity level when the salamis were inoculated before smoking. When salamis were inoculated after smoking, only those salamis aged at 20 C and at a relative humidity of 85 to 90% supported aflatoxin production.

No aflatoxins were produced during the first 4 weeks of aging on Hungarian-type salamis inoc-

TABLE 2. *Production of aflatoxins (μg B_1 + G_1/g) by A. flavus A-16100 on salamis inoculated with either 10^2 or 10^6 conidia/salami and aged for 8 weeks at 15 or 20 C at either of three humidities (65–70, 75–80, or 85–90%)*

Type of salami	Conditions											
	15 C						20 C					
	65–70%		75–80%		85–90%		65–70%		75–80%		85–90%	
	10^2	10^6	10^2	10^6	10^2	10^6	10^2	10^6	10^2	10^6	10^2	10^6
Italian	—[a]	—	—	0.02	—	0.06	0.04	1.11	0.68	0.68	1.2	1.8
Hungarian I[b]	—	—	—	—	—	—	—	—	—	—	1.0	1.0
Hungarian II[c]	—	—	—	—	—	—	—	—	—	—	—	—

[a] None detected.

[b] Inoculated after smoking.

[c] Inoculated before smoking. Smoking was done for 1 hr each day for 8 days. During smoking, the temperature never exceeded 32 C. Between smoking periods, the temperature was decreased to 20 to 25 C.

TABLE 3. *Production of aflatoxins by A. flavus NRRL A-16100 on Italian-type salamis inoculated with 10^6 conidia/salami and aged for several time periods at 20 C and 75 to 80% humidity*

Age of salami (weeks)	Aflatoxins $B_1 + G_1$ (μg/g)
1	0.3
2	0.9
3	2.8
4	2.4
8	0.7

TABLE 4. *Effect of salami ingredients on growth and aflatoxin production by A. flavus NRRL A-16100*

Ingredient or control	Mycelial weight (g) or growth	Aflatoxin[a] (μg/g)
Control[b]	0.74	38
Casings	—[c]	—
Pork + beef	+++[d]	—
Pork + beef + spices	+++	28
Garlic powder	1.10	87
Sucrose	0.95	55
Monosodium glutamate	0.98	32
NaCl	0.72	20
$NaNO_3$	0.79	21
White pepper	0.35	3
$NaNO_2$	0.16	0.19
Complete salt + spice mix	0.18	0.55

[a] Represents $B_1 + G_1$ and consisted of approximately 50% B_1 and 50% G_1.
[b] GAN broth only, no salami ingredients.
[c] None detected.
[d] Moderate to heavy growth.

ulated before or after smoking and aged at any of the temperatures and 75 to 80% humidity. Likewise, no aflatoxins were produced during the first 4 weeks of aging on Italian-type salamis aged at 10 and 15 C. In contrast to this, the levels of aflatoxins in Italian-type salamis aged at 20 C, increased during the first 4 weeks of aging, and declined by 8 weeks of aging (Table 3). These data suggest that, even though aflatoxins may be present during early stages of aging, the toxin levels may be lower in the finished product when long aging periods are used than when short aging periods are used. The low levels of aflatoxins in the finished product could be due to degradation of the toxins by *A. flavus* or by other microorganisms or by nonbiological oxidation.

Although it is significant that aflatoxins were not produced on many of the aged salamis under the conditions just described, it is equally significant that the amounts that were formed were very low. When *A. flavus* was reisolated from salami and grown on rice, levels of 330 to 480 μg of aflatoxin per g of rice were obtained. These are levels normally produced by this organism when grown on rice; thus, atoxinogenic mutants were not being selected during growth on the salamis. It was obvious that the substrate, though permitting growth, reduced aflatoxin production.

When the effects of individual salami ingredients on growth and toxin production by *A. flavus* were studied by using a defined medium, it was found that nitrite and pepper reduced growth and aflatoxin production substantially as compared to the other media (Table 4). The levels of aflatoxins found in media containing nitrite or pepper were of the same order of magnitude as the final levels of aflatoxins found in some aged salamis. The complete spice mix containing both nitrite and pepper reduced growth and toxin production in the same manner as did the two ingredients alone. The reduced amounts of aflatoxins produced in the presence of pepper agrees with the results of Frank (6), who found that *A. flavus* would grow on peppercorns but that no aflatoxins were produced. Casings did not support growth or toxin production, and the pork and beef mixture alone did not support mold growth because of bacterial overgrowth. Sodium chloride and sodium nitrate caused only slight reductions in toxin levels as compared to the control. Garlic powder and sucrose were found to stimulate growth and toxin production, and monosodium glutamate had no effect on toxin production.

The data indicate that low temperatures, low humidities, and smoking prevented growth and aflatoxin production by *A. flavus* contaminants on European-type salamis.

Aflatoxin production on country cured hams during aging was inconsistent (Table 5). No aflatoxins were produced on the 3-month old hams aged at 10 C. Only *A. flavus* NRRL 2999 produced aflatoxins on 6-month-old hams aged

TABLE 5. *Production of aflatoxins by A. flavus NRRL 2999, A. flavus NRRL A-16100, and A. parasiticus CMI 15957 on country cured hams inoculated with 10^6 conidia per ham and aged for various periods of time and at various temperatures*

Ham age (months)	Temp (C)	Aflatoxins $B_1 + G_1$ (μg/g)		
		NRRL 2999	NRRL A 16100	CMI 15957
3	10	—[a]	—	—
6	20	0.01	—	—
9	30	6.3	0.5	27
12	30	—	—	—

[a] None detected.

at 20 C with an average level of 0.01 μg/g of meat. All three molds produced aflatoxins on 9-month-old hams incubated at 30 C; *A. parasiticus* CMI 15957 produced the greatest amount. No aflatoxins were detected in 12-month-old hams aged at 30 C. With hams aged at 10 C, the temperature would be low enough to prevent mold growth. At 20 C, competition from other molds and the high salt concentration of the hams may put *A. flavus* and *A. parasiticus* at a competitive disadvantage, since their optimum temperature for growth is somewhat higher (28 to 30 C). The 12-month hams were fully aged, very dry hams. Even though the temperature of 30 C may have favored growth of *A. flavus* and *A. parasiticus*, the water activity was probably unfavorable for growth.

These data suggest that *A. flavus* and *A. parasiticus* could grow and produce aflatoxins on country cured hams during the aging process. The most likely time for this to occur would be when the aging temperature increased to 28 to 30 C but before the available water of the hams became too low to permit growth of the toxic molds. On the other hand, low temperatures (10 C) and low humidities (65 to 70%) during aging as well as high salt concentrations on the hams would prevent growth and aflatoxin production by *A. flavus* and *A. parasiticus*.

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