EFFECT OF BHA, BHT, TBHQ AND PG ON GROWTH AND TOXIGENESIS OF *ASPERGILLUS PARASITICUS* AND *ASPERGILLUS FLAVUS*

by

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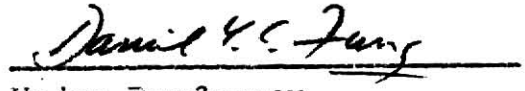

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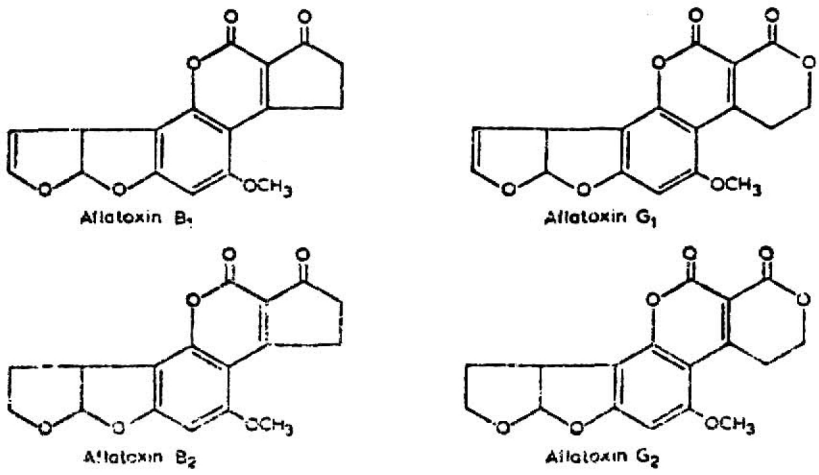
INTRODUCTION

In 1960 more than 100,000 young turkeys on poultry farms in the south and east of England died of a new disease that was termed "Turkey X disease". Systematic examination of the various feeds involved revealed the common factor to be a peanut meal of Brazilian origin. Through various identification procedures, scientists discovered that the disease was originated from a fungus called Aspergillus flavus, thus the toxic substance was named "Aflatoxin". Several reports have indicated that not only A. flavus but also A. parasiticus are able to produce aflatoxin. Aflatoxins are a group of acutely toxic and highly carcinogenic mold metabolites. The four major aflatoxins are named B_1, B_2, G_1, and G_2 since under UV light, B_1 and B_2 toxins emit blue color while G_1 and G_2 emit green color. Aflatoxin B_1 is among the most potent chemical carcinogens known. The toxins have similar structures and form a unique group of highly oxygenated, naturally occurring heterocyclic compounds (Goldblatt, 1969; Wogan, 1969). The structures of aflatoxins B_1, B_2, G_1, and G_2 are:



Aspergillus flavus appears to compete best in the microbiological world when the water activity is relatively low (0.84-0.86) and the temperature relatively high (25-40°C). When conditions are favorable, profuse growth and substantial aflatoxin production can be evident within 48 hours (Christensen et al., 1973). For aflatoxin production by A. flavus, glucose, sucrose and fructose are preferred carbon sources, and casamino acids are the preferred nitrogen source. The most striking effect of a minor element on aflatoxin production is that of zinc (Jarvis, 1971).

From numbers of surveys that have carried out, corn is the dietary staple in which aflatoxin is most likely to be encountered. Brazil nuts and postachio nuts are the dietary tidbits bearing the greatest risk of aflatoxin contamination. Cottonseed and copra are the oilseeds most likely to be found contaminated (Stoloff, 1977).

The pathological effects of aflatoxin were also reviewed by Stoloff (1977). The clinical signs of chronic aflatoxicosis in the avian and mammalian species studied are typically: lack of appetite, loss of weight, and general unthriftiness--hardly the basis for a specific diagnosis. Pathologic observations of low level intoxication reveal generalized jaundice and cirrhosis of the liver with bile duct cell proliferation and periportal fibrosis; acute poisoning results in jaundice of the mucous membrane, widespread hemorrhages, and fatty accumulation in the liver. The liver pathology is the most characteristic and easily identifiable effect in most species. Elevation of serum alkaline phosphatase activity is a good

indicator of liver malfunction associated with aflatoxicosis. Impairment of the immune system has been noted. The susceptibility of animals to both chronic and acute aflatoxicosis is variable between species and between strains of the same species. In general, susceptibility decreases with age, most probably related to development of the mixed function oxidase system of enzymes in the liver, and can be affected by the composition of the diet. The one recorded outbreak of acute aflatoxicosis in man, (Krishnamachari et al., 1975a and b) from consumption of corn contaminted with more than 6 mg/kg aflatoxin B_1, provides a clinical pattern similar to that seen in animals. Incidents such as this can be expected from consumption of badly molded foods under duress. The more widespread exposure of man to aflatoxins is at subacute levels. One of the most active and potentially fruitful areas of current research with aflatoxins is concerned with their metabolic fate and mode of action using both in vivo and in vitro studies with animals that show different overt responses to an aflatoxin challenge. Various reports have shown that aflatoxin contamination level and incidence of liver cancer have a positive correlation.

Aflatoxicosis in animals is caused by the ingestion of aflatoxin-contaminated foodstuffs, and this contamination may be controlled either by preventing molding or by detoxification of the contaminated material. Detoxification should only be considered as a last resort, since the simplest and most practical way of reducing the contamination of foodstuffs by aflatoxins and related metabolites is to prevent the growth

of those mold species which produce them (Goldblatt, 1971; Heathcote and Hibbert, 1978). Moisture and temperature are two important parameters to control aflatoxin production. Extraction with polar solvents such as alcohols and acetones can essentially achieve complete removal of aflatoxins. Heat is relatively ineffective for destruction of aflatoxin although normal roasting, as of peanuts for the preparation of peanut butter, results in considerable destruction of aflatoxin. Oxidizing agents readily destroy aflatoxin and treatment with hydrogen peroxide may be useful. Treatment of defatted oilseed meals with ammonia can reduce aflatoxin content to very low or nondetectable levels with only moderate damage to nutritive quality.

With the high degree of uncertainly in the risk involved, the approach to regulatory control of aflatoxin in food and feeds has varied from one country to another, depending on the importance of the susceptible commodity to the economy, whether a country is a producer or an importer of the commodity , and sometimes on political considerations between producer and consumer. The general tendency has been highly conservative ; in the United States, regulated tolerance for consumer peanut products are 15 ppb for totals (B_1, B_2, G_1 and G_2) and administrative guidelines for all other foods or feeds are 20 ppb totals (Food and Drug Administration, 1974).

Antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ), and propyl gallate (PG) commonly used to prevent rancidity in lipids and lipid-containing products also have

been cleared for use and listed as products "Generally recognized as safe" (GRAS) by the Food and Drug Administration in the U.S. In general, these antioxidants may be added to products at no more than 0.02% based on the fat content of the food. In meat and poultry products, the level of each individual antioxidant is restricted to 0.01%, but two or more may be added if the total does not exceed 0.02% based on the weight of the fat (Sims and Fioriti, 1980). Antioxidants are evaluated basically upon the benefits and risks that result from their addition to a food products. Food additives that have dual or multiple functions are more beneficial and more economical. In addition to their antioxidant properties, BHA, BHT, TBHQ, and PG are found to have antimicrobial activity against bacteria, molds, viruses, and protozoa (Branen, et al., 1980).

The objectives of this study were: 1) To determine the effects of BHA, BHT, TBHQ, and PG alone or in combination on growth, sporulation, and aflatoxin production by A. flavus and A. parasiticus on solid medium, 2) To evaluate the efficiency of these antioxidants in preventing the production of aflatoxin in salami.

LITERATURE REVIEW

Antimicrobial Activities of Antioxidants

Antioxidants are commonly used to prevent rancidity in lipids and lipid-containing products. They were also found to have antimicrobial activities. Ward and Ward (1967) were the first group to test the antimicrobial effectiveness of butylated hydroxytoluene (BHT) against Salmonella senftenberg 775W in commercial fish meal. Only slight inhibition of the organism was found at a concentration of 0.1% BHT in brilliant green agar.

Chang and Branen (1975) found that 250 ppm of butylated hydroxyanisole (BHA) prevented all growth and the production of aflatoxins B_1, B_2, G_1, and G_2 by Aspergillus parasiticus in glucose-salts-amino acid broth. They also reported that 150 ppm BHA inactivated growth of Staphylococcus aureus and 400 ppm BHA inhibited Escherichia coli in nutrient broth. This antimicrobial pattern is quite similar to that reported for another group of phenolic compounds--parabens. Both the BHA and parabens show the greatest antimicrobial activity toward molds and Gram-positive bacteria and are less effective against Gram-negative bacteria.

The antimicrobial effect of BHA, BHT, and propyl gallate (PG) against Bacillus subtilis, Saccharomyces cerevisiae, Aspergillus flavus and A. fumigatus was measured by the disc diffusion method and plate count method (Sandaran, 1976). BHA was effective in completely inhibiting growth of all the

test organisms at a concentration of 2500 ppm, while BHT and PG had no inhibitory effect. Concentration of BHA at 750-2000 ppm delayed the germination of A. flavus by 6-12 days and the appearance was different from normal colonies. Sankaran (1976) suggested that BHA may aid in delaying the germination and growth of spoilage microorganisms at lower concentrations and inhibiting them at higher concentrations. Its activity against sporulation of toxigenic fungi like A. flavus and A. fumigatus is of particular significance in cured meat and fish.

Fung et al. (1977) tested BHA and BHT against 6 toxigenic and 6 nontoxigenic strains of A. flavus by using a method similar to antibiotic susceptibility test. BHT was found not to be inhibitory to A. flavus growth, sporulation, pigmentation, or toxigenesis at any of the levels tested. BHA was found to inhibit A. flavus growth at 0.005-0.02 g per plate of solid media (60 or 100 mm in diameter). The production of aflatoxins B_2, G_1, and G_2 was completely inhibited by 0.01 g BHA in a 60 -mm-diameter plate of solid medium. Pigment formation which is directly related to toxigenesis was inhibited by BHA. Heating of BHA did not reduce the inhibitory effect of the compound on A. flavus when BHA was baked for 10 minutes at 200°C. Their study also indicated that the presence of BHA exerted an "environmental effect" rather than a genetic effect on growth and toxigenesis of A. flavus. Sporulation of the cultures had no direct relationship with toxigenesis in the presence of BHA.

Beuchat and Jones (1978) found that colony formation by heated and unheated conidia of A. flavus was totally prevented by 100 ppm BHA whereas BHT and PG had no effect.

Foudin et al. (1978) found antimicrobial activity of BHA and BHT against aflatoxin production in YES (yeast extract with sucrose) medium. BHT did not appreciably affect growth or aflatoxin production by A. parasiticus NRRL 2999 at concentrations of 25-150 ppm. BHA was fungistatic, and inhibition of growth was proportional to the concentration of the antioxidant. The amounts of all four aflatoxins decreased in a similar manner due to the depression of growth. Aflatoxin B_1 decreased from 67 µg/ml in the control to 0.3 µg/ml at 150 ppm added BHA and G_1 decreased from 91 µg/ml to 0.06 µg/ml. The effectiveness of ethoxyquin on growth and aflatoxin production by A. parasiticus and A. flavus in YES medium was also reported by Foudin et al.(1978). The effect of ethoxyquin at 100 ppm on 15 isolates of A. parasiticus and A. flavus, including some altered in aflatoxin-producing capability by mutation, was examined. Among wild-type isolates of A. parasiticus, G_1 decreased 90% and G_2 was completely inhibited; B_1 increased 67-1100% and B_2 increased 100-300%. Toxin synthesis was decreased 66-95% among wild-types and mutants of A. flavus and A. parasiticus producing only B toxins. The antioxidant ethoxyquin had an unique capability of selectively altering aflatoxin systhesis in A. parasiticus strains which produce both B and G type toxins. Large increases in the amounts of B aflatoxins with corresponding decreases in G

toxins may be due to specific inhibition of the conversion of B_1 to G_1 by ethoxyquin. They concluded that the use of ethoxyquin as a feed additive may aggravate potential mycotoxin hazards in contaminated feed-stuffs.

Beggs et al. (1978) reported synergistic antimicrobial activity of amphotericin B, an antifungal antibiotic, and antioxidants against certain opportunistic yeast pathogens. In the presence of 0.6 µg/ml of BHA - a subinhibitory level - either fungistatic or weakly fungistatic levels of amphotericin B became highly lethal to the Candida albicans and C. parapsilosis. The same synergistic effect was reported with propyl gallate and nordihydroguaiaretic acid against Candida spp. Torulopsis glabrata was not sensitive to this synergistic activity. The authors concluded that the antioxidants produced two effects: (1) autoxidation of amphotericin B, a polyene macrolide with 7 conjugated double bonds, thus stabilizing its activity, (2) antimicrobial activity against certain species.

The antifungal properties of BHA against eight species of Aspergillus, Penicillium and Geotrichum in synthetic media and food products was determined by Ahmad (1979). He reported that 200 ppm BHA in Glucose Salt Broth (GSB) caused 100% inhibition of all test molds. 200 ppm BHA gave total inhibition of growth of A. flavus or P. expansum in applesauce and Potato Dextrose Agar (PDA) but at least 400 ppm BHA were required to inhibit fungal growth in processed cheese spread. BHA was effective in GSB over a wide range of pH values and

incubation temperature against A. flavus at a concentration of 200 ppm. The presence of lipid in food decreased the inhibitory effect of BHA. BHA was found not to have synergistic antimicrobial activity with sorbate against A. flavus.

Shih and Harris (1977) tested the antimicrobial effects of BHA, PG, nordihydroquaiaretic acid (NDGA), and a combination of BHA-PG against E. coli and S. aureus in trypticase soy broth, PG and NDGA had a lethal effect against E. coli at the 400 ppm level, but BHA and a 50/50 combination with PG were not as effective in killing E. coli. BHA and combined PG-BHA at level of 400 ppm were required to totally inactivate S. aureus, while 50 ppm NDGA were highly inhibitory to S. aureus. NDGA had the strongest antimicrobial activity. BHA and a BHA-PG combination were more effective against S.aureus than against E. coli. Their results were in accord with the concept that phenols have more antimicrobial activity against Gram-positive than against Gram-negative cells.

Erickson and Tompkin (1977) reported the effect of tertiary butylhydroquinone (TBHQ) against growth of various types of microorganisms in plate count agar. Concentration of TBHQ at 0.003% (w/w) was found to totally inhibit S. aureus growth, whereas 0.01% TBHQ was effective against E. coli and soy flake aerobic sporeforming bacteria. A level of 0.1% TBHQ was needed to affect P. aeruginosa and fresh meat bacteria.

Ayaz et al. (1978) studied the effect of BHA and BHT on three enterotoxigenic strains of S. aureus in Brain Heart Infusion. Complete inhibition of S. aureus growth occurred in BHI with 200 ppm BHA or 150 ppm BHT and 150 ppm BHA or 100

ppm BHT inhibited enterotoxin formation in the same organism. The combination of BHA and BHT provided more effective inhibition than when the antioxidants are used alone. Inhibition of S. aureus growth by BHA or BHT was substantial at pH 7.0 and with 2% NaCl. Stern et al. (1979) reported that trypticase soy broth containing 100 ppm BHA with 5 or 7% NaCl at pH 5.0, 6.0, or 7.0 had a strong bactericidal effect against S. aureus.

Inhibition of Salmonella typhimurium and S. aureus in trypticase soy broth by BHA and propylparaben alone and in combination was studied by Pierson et al. (1980). A concentration of 200 ppm of BHA was bactericidal to S. aureus while up to 400 ppm of BHA was only restrictive to the growth of S. typhimurium. BHA is more effective than propylparaben in inhibiting growth of S. aureus and S. typhimurium in TSB. Combination of BHA and propylparaben showed no additional inhibition against S. aureus in comparison to BHA alone.

Robach and Stateler (1980) tested the effect of potassium sorbate alone and combined with BHA, TBHQ, or ethylenediamine tetraacetic acid (EDTA) on growth of two strains of S. aureus in trypticase soy broth at 37°C. The combination of sorbate and TBHQ or BHA resulted in a synergistic inhibition of growth of both strains of S. aureus, but 25 ppm of TBHQ alone inhibited growth of strain S-6. They also found that EDTA did not potentiate sorbate's activity against growth of strain S-6 but was synergistic with sorbate against growth of strain 12600.

Lahellec et al. (1981) evaluated the growth effect of potassium sorbate in combination with BHA, BHT, TBHQ, and PG in different pH media against four toxigenic strains of S. aureus. They found that potassium sorbate (1, 3, and 5%) in combination with BHA, BHT, PG (50 and 100 ppm) exerted greater bactericidal and bacteriostatec effects on S. aureus strains at pH 5 than at pH 7. 50 ppm of TBHQ was able to inhibit growth of S. aureus strains. They concluded that TBHQ had strongest inhibitory effect to S. aureus compared with BHA, BHT and PG. S. aureus S-6 was more resistant than 196, 326, and 137 in the presence of sorbate and antioxidants and shake cultures of S. aureus grew better than static cultures in the presence of sorbate and BHA.

Davidson et al. (1981) tested the antimicrobial activity of BHA, TBHQ, and potassium sorbate, alone and in combination, against S. aureus and S. typhimurium in trypticase soy broth. Salmonella typhimurium was totally inhibited by 150-200 ppm BHA, while S. aureus was 95% inhibited by 150 ppm BHA. Tertiary butylhydroquinone had dramatically different effects on the two bacteria. Salmonella typhimurium was able to grow even at 400 ppm TBHQ, whereas S. aureus was totally inhibited by as little as 25 ppm TBHQ. Sorbate-BHA and sorbate -TBHQ combinations were effective in synergistically delaying growth initiation and total growth of S. aureus. Combinations of potassium sorbate and BHA were also synergistic in suppressing final growth of S. typhimurium.

The effect of potassium sorbate alone and in combination

with BHA, TBHQ or PG on the growth of Salmonella senftenberg and S. aureus S-6 was studied by Poerschke (1981). Combination of sorbate with BHA, TBHQ and PG resulted in synergistic inhibition of growth of S. senftenberg, whereas 0.2% sorbate and 25 ppm TBHQ resulted in total inhibition of S. aureus S-6 at 12 hours. He also studied the effect of sorbate/antioxidant combinations in a food system - fresh gound beef. He found the sorbate/antioxidant combinations suppressed final growth of both S. senftenberg and S. aureus in fresh ground beef, but the antimicrobial effectiveness was markedly diminished, compared to media studies. The author attributed the diminished effect to autoxidation of the ground beef lipid content and used of antioxidant to prevent the autoxidation.

Growth of Vibrio parahaemolyticus 04:K11 was inhibited in TSB + 2.5% NaCl by 50 ppm BHA, while 400 ppm BHA was required to inhibit growth in crab meat homogenate (Robach et al., 1977). The higher level of BHA required for inhibition in the crab meat homogenates was thought to be due to partial inactivation of the antioxidant properties by the presence of oxidized crab meat lipids; therefore, the antimicrobial activity of BHA may depend on the lipid content and degree of lipid oxidation in the food product.

Vardaman et al. (1978) studied the in vitro and in vivo effect of BHT on Mycoplasma synoviae isolates. All of the cultures were totally inhibited by 10 ppm BHT in Mycoplasma medium in vitro for 7 days. BHT added at 100, 200 and 400 ppm in the feed did not have any significant in vivo effect on the

Mycoplasma synoviae (Ms) serological responses or show any chemoprophylactic effect on birds infected with the organisms.

Three strains of Clostridium perfringens were completely inhibited by 150 ppm BHA in fluid thioglycolate medium at 37°C (Klindworth et al., 1979). With 100 and 200 ppm BHA, the C. perfrigens cells were killed within 10 min after addition of the BHA to the culture in a buffer solution. These results indicated that BHA was bactericidal rather than bacteriostatic. BHA was also found to have a synergistic inhibitory effect when used in conjuction with nitrite, sorbic acid, or parabens. The authors reported that in the presence of a lipid and surfactant, the antimicrobial activity of BHA against C. perfringens was greatly reduced. The reduction was thought to be due to the reaction between BHA and a lipid molecule which "used up" the BHA molecule, making it no longer effective as an antioxidant. The same reaction could also result in destruction of the antimicrobial activity. Similarly, the hydrophobic nature of BHA and its solubility in lipophilic compounds might result in it being localized within the lipid portion of the medium, rendering it unavailable to act against those microorganisms which grow in the aqueous phase of the medium. They concluded that the age and physiological state of the culture, temperature, pH of the medium, and the presence of lipids can effect the extent of inhibition of C. perfringens by BHA.

Robach and Pierson (1979) studied the inhibitory effect of BHA, BHT, and PG on outgrowth and toxin formation of three

proteolytic strains of Clostridium botulinum in prereduced thiotone yeast-extract glucose medium (TYG) at 37°C. They found that C. botulinum 10755A was inhibited by 50 ppm BHA in TYG medium, whereas strains 62A and 613B were inhibited by 25ppm BHA. The antimicrobial effect of BHA was thought to be due to the antioxidant inhibition or competition with the electron donor systems within the cells, thereby inhibiting. BHT at 200 ppm inhibited outgrowth of the spores for the entire incubation period, but PG at 200 ppm had no inhibition of growth or toxin production. Since BHT is less effective than BHA as an antioxidant and as an antimicrobial agent, a hypothesis was proposed that the antimicrobial effectiveness of an antioxidant is related to the antioxidative capability of the compound.

Davidson and Branen (1980a) studied the antimicrobial activity of BHA against two psychrotrophic Pseudomonas species. In trypticase soy broth 100 ppm BHA delayed the growth of P. fluorescens at 22°C and totally inhibited growth at 7°C. In contrast, P. fragi was more resistant, and even in the presence of 400 ppm BHA at 7 and 22°C. Davidson and Branen (1980b) found that BHA caused leakage of intracellular proteins and altered the percentages of major fatty acids in P.fluorescens and P. fragi. These changes may have attributed to the antimicrobial properties of antioxidants.

Recently, Gailani (1981) studied the antimicrobial properties of BHA, BHT, TBHQ, and PG, alone or in combinations, on 16 Gram-negative and 8 Gram-positive bacteria in laboratory

media. He found that antioxidants inhibited Gram-positive bacteria more than Gram-negative bacteria and the inhibitory effects were bactericidal rather than bacteriostatic. The TBHQ-PG and TBHQ-BHT combinations had synergistic antimicrobial effects. Gailani also reported that the antioxidants at 100, 200, or 400 ppm significantly reduced psychrotrophs, coliforms , and fecal coliforms counts in ground pork after 4 weeks of storage at $4^{\circ}C$, but not after 1, 2 or 3 weeks.

The antiviral activity of antioxidants was first described by Snipes et al. (1975). They found that lipid-containing viruses such as Ø6 (a bacteriophage) and herpes simplex were 50% inactivated by 10^{-4} to 10^{-5} M BHT. Non-lipid-containing viruses such as poliovirus were not inactivated.

Wanda et al. (1976) found bacteriophage Ø6 to be totally inactivated by 30 µM BHT or 100 µM BHA. The action of BHT was enhanced by the presence of calcium, barium, and strontium. They also determined that in the presence of BHT, the phage lost its ability to attach to the host cells.

Kim et al. (1978) reported BHT inhibition of the lipid-containing human and murine cytomegaloviruses (CMV), and Semliki Forest Virus (SFV). Vaccinia virus, which also contains lipid, was not inhibited. The authors theorized that BHT react with the lipid-containing viral envelope, causing adsorption by the host cell to be disrupted.

Surak et al. (1976a) showed that 20 ppm BHA inhibited 50% growth of the protozoan Tetrahymena pyriformis. DNA,

RNA, and protein systhesis was inhibited 50% by 20 ppm BHA within 10 min after addition of BHA. They reported that BHA had no effect on the cell membrane. Surak et al. (1976b) reported later that BHT did disrupt the cell membrane and result in death of cells.

Safety Consideration

Butylated hydroxyanisole was first approved by the Food and Drug Administration (FDA) in 1948, while butylated hydroxytoluene was cleared in the U.S. in 1954, (Sims and Fioriti, 1980). Because of the widespread usuage of BHA and BHT, it is essential that the safety of these antioxidants be evluated critically.

Johnson (1971) proposed that the safety of food additives can be studied in three ways: (a) acute toxicity tests to determine the LD_{50}; (b) longer term toxicity tests with smaller intakes, usually with histopathological examination of the animals; and (c) study of the metabolites of the additives and of their biochemical effects. He also reported that BHA and BHT have toxic effects at extremely high doses, both having an LD_{50} of ca. 2000 mg/kg in most animal. According to his review, this would appear to be of little consequence, however, since it had been estimated that man undoubtedly consumes less than 0.1 mg/kg/day of BHA and BHT. The author mentioned that in metabolism of BHA in men, 27 to 77% of the BHA appeared in the urine as glucuronides in the

first 24 hours, less than 1% as free BHA, and little ethereal sulfate. Although the metabolism of BHT was considered slower than that of BHA, BHT was completely excreted by man within 14 days with no accumulation. Johnson concluded that at the levels of use fixed by food legislation there is no evidence of any danger to the consumer arising out of the use of BHA and BHT.

Branen (1975) reviewed the biochemical effects of subacute levels of BHA and BHT. It is estimated by Johnson (1971) that man consumes ca. 0.1 mg/kg body wt daily of these antioxidants. At levels 500 times this level (50 mg/kg/day), both BHA and BHT appear to be free of any obviously injurious effects. However, at larger doses (500 mg/kg/day), both BHA and BHT result in certain pathological, enzyme, lipid alterations in both rodents and monkeys, and BHT, in some cases, has been reported to have certain teratogenic and carcinogenic effects upon rodents. These alterations appear to differ markedly between rodents and monkeys, apparently as a result of differences which exist in the metabolism and excretion of BHA and BHT by these two species. However, in both animal species, the alterations appear to be physiological responses which are reversible upon removal of BHA and BHT from the diet. Long term chronic ingestion of BHA and BHT may be beneficial in sparing vitamin E and in modifying the acute toxicity of a number of mutagenic and carcinogenic chemicals.

During the early 1940s, propyl gallate (PG) and several

other alkyl gallates were allowed for use in food in U.S., (Sims and Fioriti, 1980). Orten et al. (1948) studied the toxicity of PG in rats, guinea pigs and dogs, They reported the LD_{50} of PG administered to rats orally as 3800 mg/kg. Death from this dose occurs usually within an hour and is due to cardiorespiratory failure. Heating PG at 425° to 450°F for one hour has no effect on the LD_{50} or on the toxic manifestations. The feeding of "sub-acute levels", 1.17 and 2.34 percent, of PG to rats resulted in inhibition of growth and a mild anemia probably due to the bitterness of the diet with a resulting low food intake. Death occurred within four weeks in about 40 percent of the animals fed the higher level. The only consistent pathological change found is tubular damage and the presence of albuminous casts in the kidney. The prolonged feeding of varying amounts of PG(0.00117%, 0.0117%, and 0.117%) for as long as two years to rats or for 14 to 15 months to guinea pigs and dogs had no detectable effect on gross appearance, growth or body weight, reproduction (guinea pig), hemoglobin, erythrocyte, or leucocyte levels in blood (rats), renal function as reflected by the qualitative composition of the urine (dogs), or histological appearance of the liver, kidney, spleen, stomach, gonads, lungs and heart (rats and guinea pigs). They concluded that no toxicity was observed following the ingestion of amounts of PG equivalent to at least 100 times that which would be consumed if all the dietary fat were treated with effective amounts of this antioxidant.

Tertiary butylhydroquinone (TBHQ) was cleared for food use in the U.S. in 1972 (Sims and Ficriti, 1980). The safety and biochemical behavior of TBHQ was evaluated in acute studied with rats and dogs (Astill, et al., 1975). They found the acute oral LD_{50} of TBHQ as a 5 or 10% solution in corn oil in rats was between 700-1000 mg/kg, and the intraperitoneal LD_{50} was between 300-400 mg/kg. Humans eliminated 0.002 g/kg single doses (high fat vehicle) almost completely in the urine in 2-3 days, with 0.1% unchanged, 73-88% as the 4-O-sulfate, and 15-22% as the O-glucuronide. Monotertiarybutylhydroquinone residues in most tissues of long term animals were below lower detection limits or negligible. Monotertiarybutylhydroquinone did not induce liver microsomal mixed function oxidases in short and long term rat and dog feedings. The feeding studies and comparative biochemical studies showed monotertiary-butylhydroquinone to be safe for its intended use.

From the above mentioned information it is concluded that the four antioxidants (BHA, BHT, TBHQ. and PG) under legislation are safe for human consumption in regular diet.

Effect of Chemical Agents and Food Components on Aflatoxin Production

Sorbic acid and sorboyl palmitate have been widely used as effective antifungal agents in various commercial foods. Groll and Luck (1970) tested the effect of sorbic acid and sorboyl palmitate on aflatoxin formation in commercial white bread. Sliced bread, with 0.13% sorbic acid or sorboyl

palmitate were sprayed with a spore suspension of A. flavus and incubated for 11 days under optimal conditions. In comparison with the control, they found the shelf-life of bread containing sorbic acid was extended by 100% and bread with sorboyl palmitate was extended by 180%. Aflatoxin production was also strongly inhibited by the two preservatives. Wallhausser and Luck (1970) investigated the influence of sorbic acid on the growth of A. flavus in orange drink, cheese, tomato pulp and wheat bread. Two aflatoxin producing strains of A. flavus were used to inoculate spores into tested food samples. They found that the growth of A. flavus in orange drink was completely inhibited by 0.0625% sorbic acid when stored in the light at 22°C for 9 or 21 days. Semi-hard cheese painted with 20% aqueous solution of potassium sorbate showed some sparse and retarded growth after 2 months storage at 27°C and 95% relative humidity. Commercial tomato pulp in the presence of 0.1% potassium sorbate remained free from growth for 2 months. Flour with 0.5% sorboyl palmitate was used by them to bake bread. The bread were packaged in plastics bags and contaminate with 1 ml of spore suspension (1000 spores/ml), no growth was observed after 14 days. All control samples without sorbic acid were heavily contaminated with A. flavus.

Effects of preservatives on aflatoxin formation by A. flavus were reported by Elbereir et al. (1975). Spore of aflatoxin-forming A. flavus strains were inoculated onto agar plates containing preservatives p-Hydroxybenzoic acid

esters, benzoic acid, sorbic acid and potassium sorbate at concentrations of 0-2000 ppm, and inoculated plates were incubated at 30°C for 3 days, then held for 5 months at room temperature. They found that all the preservatives inhibited both growth and aflatoxin formation; toxin formation was significantly reduced at levels at which no detectable inhibition of growth was observed. p-Hydroxybenzoic acid showed the strongest inhibitory activity, followed by sorbic acid, benzoic acid and potassium sorbate.

Bullerman (1974) tested the effectiveness of cinnamon against growth and aflatoxin production in bread, yeast-extract sucrose(YES) broth, and glucose-ammonium nitrate (GAN) broth by two toxinogenic strains of A. parasiticus. He found that the growth of A. parasiticus was very scant on raisin bread containing 1% cinnamon, whereas growth was abundant on rye, wholewheat and white bread which did not contain cinnamon. He also found the inhibitory effect of cinnamon on both strains in YES broth, the effect was especially pronounced with strain NRRL 2999. Increasing concentrations of ground cinnamon in YES broth resulted in detection of decreasing levels of aflatoxins. At the lowest level of cinnamon 0.02%, reduction in aflatoxin production ranged from 21-25%, while at the 2% level of cinnamon, the toxin production was reduced by 97-99%. The effects of an alcoholic extract of cinnamon on growth and aflatoxin production by strain NRRL 2999 in GAN broth were similar to those obtained with ground cinnamon in YES broth. At the 0.2% level of cinnamon extract, aflatoxin production was

inhibited by 98-99% and at the 1 to 2% level, aflatoxin production was reduced by more than 99%. Cinnamon is known as one of the more effective bacteriostatic spices and is also effective against yeasts. Bullerman concluded that cinnamon is an effective inhibitor of aflatoxin production even though mycelial growth may be permitted, whereas the primary function of the cinnamon in foods may not be preservative in nature, it has preservative properties, which result in built-in safety systems.

The effects of cinnamon oil, clove oil, cinnamic aldehyde and eugenol on growth and aflatoxin production by A. parasiticus were studied by using yeast extract sucrose broth as the substrate (Bullerman et al. 1977). All four substances inhibited mold growth and subsequent toxin production. Cinnamon and clove oils were inhibitory at 200-250 ppm, cinnamic aldehyde at 150 ppm and eugenol at 125 ppm. Since cinnamic aldehyde and eugenol are the respective major components of cinnamon oil and clove oils. They concluded that these are the major active antifungal ingredients of these two essential oils. The inhibitory effect of these substances was judged to be inhibition of growth rather than of toxin production. When growth occurred after a delay, aflatoxin production occurred when the cultures reached secondary metabolism. Given sufficient time, cultures which were inhibited initially , but which subsequently grew, produced toxin levels equivalent to control cultures. Levels of the oils above 250 ppm of cinnamic aldehyde and eugenol above 200 ppm completely

inhibited mold growth, or permitted only a small amount of growth that never reached secondary metabolism and never produced aflatoxin during the time of study.

Aspergillus parasiticus NRRL 2999 inoculated into meat mixtures with curing salts and into yeast extract sucrose (YES) and sucrose-ammonium salts (SAS) broth with and without curing salts had significantly affected growth and aflatoxin production (Meier and Marth, 1977). They found that the effect of individual curing salts or curing salt mixtures on growth and toxin elaboration by the Aspergillus was substrate dependent. When YES broth contained 100 ppm of $NaNO_2$, 2% NaCl, or 1 or 2% NaCl plus 200 ppm of $NaNO_2$ or $NaNO_3$, growth and aflatoxin production was depressed. Biosynthesis of aflatoxin B_1 was enhanced by 1 and 4% NaCl in YES broth. The SAS broth containing only NaCl or NaCl combined with nitrite or nitrate yield less aflatoxin than the control broth, while the growth and amount of aflatoxin production was increased in SAS broth with 200 ppm $NaNO_3$ alone. Sausages containing 100 or 200 ppm $NaNO_2$ and NaCl supported more mold growth and aflatoxin production than control sausage with 3% NaCl and 100 ppm of $NaNO_2$. Addition of 2 or 3% NaCl and no nitrite to sausage resulted in less aflatoxin than in control sausage. These experiments clearly demonstrate that mixtures of curing salts do not protect meat products from development of aflatoxin if toxigenic aspergilli are present and can grow.

Hitokoto et al. (1980) studied the inhibitory effect of 29 commercial powdered spices on the growth and aflatoxin

production of A. flavus by using low salt broth as the culture medium. Of the 29 samples tested, cloves, star anise seeds, allspice, and horseradish caused complete inhibition of the growth of A. flavus. Thyme inhibited the growth of A. flavus and showed 86-100% inhibition of aflatoxin production. Dill seeds, turmeric, marjoram, basil leaves, anise seeds, cumin fruits and coriander seeds showed partial inhibition of the growth and toxin production of A. flavus. They recovered the essential oil extract from powdered spices. The essential oil fraction of cloves contained 92% eugenol, star anise seeds contained 89% anethol, and thyme contained 54% thymol. At 250 µg/ml eugenol completely inhibited the growth of A. flavus, while at a concentration of 125 µg/ml eugenol was enough to cause total inhibition of aflatoxin production. Anethol caused complete inhibition on growth and aflatoxin production at a concentration of 2 mg/ml, whereas 400 µg/ml of thymol completely inhibited growth and aflatoxin production of A. flavus. They attributed the inhibitory effects of cloves, star anise seeds, and thyme to the major component in the essential oils of the powdered samples.

The effect of sucrose supplementation on aflatoxin production by A. parasiticus 2999 in soybeans and cashew fruit juice was investigated by Obidoa and Onyeneke (1980). They found the aflatoxin production showed a dual increase with increasing sucrose (0-20 g) and soybean (0.1-2.0 g) concentrations which "probably indicates the limited availability of suitable carbon souces for aflatoxin

synthesis in soy-bean". Two to five percent (w/v) sucrose supplementation was optimal for maximal toxin production in cashew juice. Above this range aflatoxin production dropped steeply, they related this phenomenon to the increased toxicity of cashew juice medium since fruit juices generally contain free osmotically active carbohydrates like fructose, glucose, and sucrose. Cooked soybeans supported higher yields of toxin than raw which is in direct contrast with data obtained from cashew fruit juice. Ripe cashew juice supported a greater quantity of toxin production than the unripe. Increases in aflatoxin production with cooking could be due to heat hydrolysis of complex carbon sources to readily assimilated compounds suitable for aflatoxin synthesis. They also found more fluorescent metabolites in cashew fruit medium than in soybeans. The new fluorescent metabolites produced are thought to be the potential of fungi to produce different amounts and types of toxins in different substrates and environments. The authors concluded that sucrose supplementation either enhances or suppresses the aflatoxigenic potential of A. parasiticus 2999 depending on the medium.

Arseculeratne et al. (1976) used smoked coconut and smoked broth to study the effect of the smoking process on aflatoxin accumulation by A. parasiticus NRRL 2999. Using a smoking process under laboratory conditions with fresh coconut and potato dextrose broth; aflatoxin accumulation was found to be significantly or totally inhibited in both smoked coconut and broth. Unsmoked coconut with 15% moisture content

or above permitted significant aflatoxin accumulation, while smoked coconut containing 40% moisture totally inhibited aflatoxin accumulation. They also found that light smoking slightly stimulated the growth of A. parasiticus. They concluded that the inhibitory effect of smoke is more marked on toxin accumulation than on mycelial growth; moisture level is critical for the fungistatic efficiency of a given degree of smoking. The antimicrobial effects of smoking are drying, heating and deposition of antimicrobial substances on foods during processing.

Buchanan and Ayres (1976) studied the effect of sodium acetate and propionate on growth and aflatoxin production by A. parasiticus NRRL 2999 in AMY medium (glucose + mineral salts + 2% yeast extract). At pH 4.5, sodium acetate (1.0g/100ml) completely inhibited growth and prevented aflatoxin production. At 0.6 and 0.8 g sodium acetate/100ml, growth was partially inhibited and aflatoxin production decreased by 70% and 99%. Propionic acid was more inhibitory than acetic acid against A. parasiticus since 0.2g/100ml completely inhibited mold growth and aflatoxin production. Propionic acid seemed to offer greater potential as a means of controlling aflatoxin. The authors suggested that surface application of acetic acid and propionic acid by spraying, dipping or flotation prior to storage may be a potential means of preventing the growth and aflatoxin production by A. flavus and A. parasiticus in the product.

The effects of caffeine and theophylline on growth and

aflatoxin B_1 production by A. parasiticus NRRL 2999 were studied in AMY medium at pH 4.5 by Buchanan and Fletcher (1978). Caffeine concentrations of 0.5, 1.0, and 2.0 mg/ml decreased aflatoxin production by 86, 96, and 100%, respectively. Theophylline concentrations of 2.0, 4.0, and 8.0 mg/ml were tested, but only the highest concentration was inhibitory, decreasing aflatoxin production by 54%. These data indicated that caffeine plays a significant role in preventing the production of aflatoxin during the storage of caffeine-containing commodities. Caffeine and theophylline are a source of naturally occurring methylxanthines. Methylxanthines are noted for their ability to inhibit the activity of phosphodiesterase, which catalyzes the hydrolysis of cyclic AMP. The preferential inhibition of aflatoxin production by caffeine suggests that aflatoxin biosynthesis is influenced by cyclic AMP concentration within the cell. The authors attribute the different inhibitory responses between caffeine and theophylline to theophylline being less effective as a phosphodiesterase inhibitor.

Wellford et al. (1978) evaluated the inhibitory action of honey on fungal growth, sporulation and aflatoxin production. They inoculated A. flavus NRRL 5862 and A. parasiticus NRRL 2999 into unprocessed honey. Growth and subsequent sporulation of fungi were seen only in media containing less than 60% honey. Growth and sporulation occurred in 40% honey after 2 days. Neither species of Aspergillus produced aflatoxins in 10% honey. They concluded that not only pure honey but

also diluted honey was capable of inhibiting toxin production or possibly neutralizing it.

Aflatoxins in Meats

Molds often grow on meats, especially cured meats during storage or aging. Some of these molds have toxigenic potential. A strain of A. flavus (NRRL A16,100) is isolated as a contaminant from an Italian type salami was found capable of producing aflatoxins (Bullerman and Ayres, 1968). This was the first report of the isolation of a toxigenic mold strain from cured meats.

The production of aflatoxins in various kinds of meats was observed by Bullerman et al. (1969a). They inoculated A. flavus and A. parasiticus into beef, bacon and ham to determine the quantities of aflatoxins production during storage at various temperatures. They found that no aflatoxins were detected in any of ten meat samples stored at 10°C. Meat stored at 10°C was spoiled by bacteria and yeast before detectable levels of aflatoxins were produced. High levels of aflatoxins were formed in meats stored at 20°C. Mold growth at 30°C was as abundant or greater than mold growth at 20°C, but the production of aflatoxin was less at 30°C than at 20°C. They also found that A. flavus and A. parasiticus developed higher levels of aflatoxin G_1 than B_1 in meat stored below 30°C, but at 30°C and above A. flavus produced equal amounts of G_1 and B_1, whereas A. parasiticus continued to produce more G_1 than B_1. The same authors inoculated

A. flavus NRRL A16,C O in European type salami and studied aflatoxin occurrence in salami during aging at different temperature and relative humidities. Bullerman et al. (1969b) indicated that Italian-type salami contaminated with A. flavus was more likely to develop aflatoxin during aging than was smoked Hungarian-type salami under the same conditions. A temperature below 15°C and a relative humidity of less than 75% was found to prevent aflatoxin development during the aging of salami. Salami ingredients were found to affect growth and aflatoxin production by Aspergillus. Pepper and sodium nitrite reduced growth and aflatoxin production by Aspergillus in glucose ammonium-nitrate broth.

Strzelecki et al. (1969) isolated 10 fungal species from a moldy country cured ham. Four fungi were identified as toxigenic strains of A. flavus. The amount of aflatoxin B_1 and B_2 production by these 4 strains in YES medium are from 49 to 85 µg/50ml of medium. Aflatoxins G_1 and G_2 were not detected. Biological toxicity of aflatoxin data were determined by using chicken embryo and duckling tests. Administration of 0.025 µg extract resulted in death of the chicken embryo and bile duct cell proliferation of 1-day-old ducklings. There were typical biological reactions caused by aflatoxin B_1.

Sutic et al. (1972) also investigated mold contamination in country cured hams. They used a swab method to sample 356 country cured hams from 11 ham processors in the State of Georgia. Of 562 mold isolates from 356 country cured hams,

403 were Penicillium, 123 were Aspergillus, and 36 were other mold isolates. The number of aspergilli isolated depended on the age of the hams and on the amount of moisture in the storage room. All aspergilli were more abundant on the surface of hams aged for 12 months or longer than on 1-to-3-month-old hams, whereas under moist conditions these hams yielded more pinicillia. Of 121 aspergilli isolates, only two strains were able to produce aflatoxin. Only aflatoxin B_1 was produced in detectable amounts by either strain. Biological assay of extracts failed to develop toxicity. They concluded that A. flavus isolated from country cured hams produced aflatoxin B_1 only when inoculated on experimental substrates.

Oldham et al. (1971) studied the production of aflatoxin in pre-packed luncheon meat at refrigerator temperatures. Salami, New England loaf, and bologna inoculated with A. flavus ATCC 15517 were incubated for 12 days at 4.4, 7.2 or 25°C. They found that all samples were negative for aflatoxin production except those incubated at 25°C. This indicated the mold would not produce detectable aflatoxin in the luncheon meat when kept at refrigeration temperatures (0-10°C) for 12 days.

Occurrence of aflatoxigenic molds on "Dry Cracower Sausage" was studied by Strzelecki and Badura (1972). They isolated 36 strains of mold from meat samples of ten different meat plants in Poland. Aspergillus and Penicillium are predominant isolates. They found 4 strains of A. flavus

were toxinogenic and produced 2.3 to 3.1 mg/100ml of medium of aflatoxins (B+G) in YES medium. Although A. flavus isolates produced small amounts of aflatoxin in laboratory medium, no toxin was found in any of the examined sausage samples. However, the occurrence of toxinogenic strains of A. flavus in foods is indicative of a potential hazard for human health.

Strzelecki (1973) studied the behavior of aflatoxins in raw ham, country cured ham and Italian type salami. Aspergillus flavus can grow and produce aflatoxins in country cured ham (traces after 84 days and 126 days of storage at 5 and 30.1°C) and in salami (104.85 and 213.40 µg /100g after 13 and 78 days of storage at 37, 31 and 10°C). Some chemicals like p-aminobenzoic acid, potassium sulfite, and potassium fluoride inhibited aflatoxin production in YES medium, while sodium nitrite and curing mixture stimulated aflatoxin production. These data were contradictory to the results of Bullerman et al. (1969), and they contributed the discrepancy to the use of different media between the investigations.

Hadlok et al. (1976) analyzed the mycoflora of 207 mold-contaminated meat products during a period of about 10 years (1965-1974) in the Federal Republic of Germany. A total of 425 strains were isolated; the great majority of isolates belonged to Penicillium (56.2%) and Aspergillus (33.9%). Aflatoxin, sterigmatocystin, penicillic acid, cyclopiazonic acid, ochratoxin and tremortin may found in dry sausages, cured ham, cooked meat products and raw meat.

MATERIALS AND METHODS

Effect of Antioxidants on Growth and Aflatoxin Production on Solid Medium

Organisms Tested

Three toxigenic strains of Aspergillus parasiticus (NRRL 2999, NRRL 4123, NRRL 5835) and three nontoxigenic strains of Aspergillus flavus (NRRL 5917, NRRL 5918, NRRL 5521) obtained from the Northern Regional Research Laboratory, Peoria, IL. were used in this study. Stock cultures were maintained on Potato Dextrose Agar (Difco). The cultures were stored at $7^{\circ}C$ and transferred every three months to maintain viability. Spore suspensions were made by washing the slants of 7 day-old culture with 3 ml of sterile distilled water with 2 drops of Tween 80 added. A sterile loop was used to gently dislodge the spores. The suspension was diluted to 10^{-4} in sterile distilled water to make a spore suspension. The spore suspension had about 10^5 to 10^6 spores/ml as determined by the plate count method.

Solid Medium Used

The fluorescent agar of Hara et al. (1974) was used. In this study, $HgCl_2$ was excluded from the medium because it has inhibitory effect on growth of A. flavus and A. parasiticus. The solution was adjusted to pH 5.5 before the addition of the agar. The sterile medium was poured into 100 mm diameter glass petri dishes and incubated overnight before use, each plate contained 15 ml of medium.

Antioxidants Tested

Antioxidants; butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ); and propyl gallate (PG) were obtained from Eastman Chemical Products Inc., Tennessee. A stock solution of each antioxidant was prepared by dissolving 10 g or 1 g of the antioxidant in 10 ml of 95% ethyl alcohol. Appropriate amounts were then transferred from the stock solution onto sterile blank disks by use of a micropipet. The disks were dried overnight before use.

Growth Inhibition Studies on Solid Medium

The procedure of Fung et al. (1977) was used in this study. Spore suspensions were swabbed on the fluorescent agar and then the disk, with desired amount of antioxidant, was placed in the center of the plate. Small amounts (0.0002-0.02 g per plate) of BHA, BHT, TBHQ and PG alone or in combination were used to challenge the test organisms. By this method the desired amounts of antioxidant, even in minute quantities can be accurately applied. The plates were incubated at room temperature (23 ± 1°C) for 5 days. Growth and sporulation on the plate were observed under a dissecting microscope. The diameters of inhibition zones, when present, were measured in cm. The plates were then placed under ultraviolet light (365 nm) to detect fluorescence in the agar. This is a presumptive test for the production of aflatoxin.

Quantification of Aflatoxins

Since fluorescence of the agar under UV light is a presumptive test of the presence of aflatoxin, the thin layer chromatography procedure of Pons et al. (1968) was used to identify and quantify the four primary aflatoxins (B_1, B_2, G_1, G_2). The following extraction procedures were used: 1) Crush entire culture and agar and put them into a 500 ml Erlenmeyer flask and cover with a layer of solid glass beads, add 150 ml of extraction solvent (750:150:8::acetone:distilled water:glacial acetic acid) and shake vigorously (speed 6) for 45 minutes in a mechanical shaker; 2) filter the extract through a folded 18.5 cm circle of Whatman No.4, to collect 125 ml filtrate, add 20 ml lead acetate solution (200 g lead acetate in 1 liter distilled water) and 25 ml distilled water; 3) add several boiling chips and boil liquid over a steam bath until volume is reduced to approximately 125 ml; 4) cool liquid and dilute it to 200 ml with distilled water; 5) add 4-5 g Filter-Aid (Celite), mix, and filter through a folded 18.5 cm circle of Whatman No.4; 6) to 160 ml filtrate, add 50 ml $CHCl_3$, shake vigorously in separatory funnel for 1 minute, let layer separate; 7) drain $CHCl_3$ (lower layer containing extracted aflatoxin) into 2" column of anhydrous sodium sulfate in an extraction tube, collect filtrate; 8) add 50 ml of fresh $CHCl_3$ and repeat steps 6 and 7; 9) wash sodium sulfate tube with 20 ml $CHCl_3$; 10) combine all extracts; 11) evaporate extracts to near dryness over a steam bath; 12) dissolve extract in 3 ml $CHCl_3$ and transfer liquid to vials;

13) store vials at 4°C until thin layer chromatography analysis. Plastic gloves were used at all times during extractions to protect the hands. Excess filtrates as well as discarded cultures were detoxified by treatment with Chlorox solution (800 ppm hypochlorite) for 24 hours.

The following thin layer chromatography procedure was used to identify and quantify aflatoxins. Stored vials with extracts were evaporated completely and reconstituted to 3 ml with 98:2::Benzene:Acetonitrile. Pre-coated thin-layer chromatography (TLC) sheets from Eastman Chemical Products Inc. were activated at 100°C for 30 minutes, this procedure can prevent moisture absorption by the sheet. Samples and standards were then spotted on the TLC sheets using a micropipet, approximately 2 cm from the bottom edge. Standards (5 ml) of known concentrations were spotted for comparison. Samples were applied or diluted until intensity of color matched that of standard. The TLC sheets were then developed by using Eastman Chromagram Developing Apparatus, sheets were put in a glass tank containing 88:12:1.5::$CHCl_3$:$CO(CH_3)_2$:distilled water solution (Stubblefield et al., 1969) for 30 minutes in complete darkness. Remove the plate and air dry in the dark for 15 minutes. The sheets were then observed for spots of fluorescence under UV light (365 nm) at R_f values comparable to the standard. Visual estimation was used in this study. Standards (B_1, B_2, G_1, G_2) were obtained from Sigma Chemical Company. The relative intensities of the standards and samples were compared and

amounts of aflatoxins in the extracts were calculated by using the following formula:

$$\text{Amount of aflatoxin in } \mu g/ml \text{ or } \mu g/g = \frac{(Vs)\ (Cs)\ (SD)}{(X)\ \ (W)}$$

Vs: microliters of standard for which the color intensity of the standard equals that of the sample.

Cs: concentration of aflatoxin in standard, micrograms per ml, B_1(5 µg/ml), B_2(1.5 µg/ml), G_1(5 µg/ml), G_2(1.5 µg/ml).

SD: volume to which sample extracts are diluted for thin layer chromatography analysis (ml).

X: volume of sample extract spotted, in microliters.

W: sample "weight" in "ml" or in gram.

Effect of Antioxidants on Salami

Salami was chosen as a meat model system to study the efficacy of antioxidants in preventing the production of aflatoxin.

Preparation of Cooked Beef Salami

The processing of cooked beef salami was done at the Department of Animal Sciences and Industry, Kansas State University, Manhattan, Kansas. The ingredients of salami is:

10 lbs 25/75 beef trim (25 % fat)
3 lbs water
100 grams salt
40 grams sugar
23 grams Heller salami seasoning
11 grams Prague powder (contains 6.25% sodium nitrite) dissolved in 1/2 cup warm water

2 grams sodium erythorbate

100 ppm antioxidant (BHA or BHT or BHT or TBHQ or PG or combination of BHA and TBHQ)

The following procedures were used for manufacturing salami: 1) grind beef trim through 1/2 inch plate and then through 1/8 inch plate; 2) add salt to ground beef and mix in a mixer; 3) add water and mix for 1 minute; 4) add sugar and Heller seasoning and mix for 30 seconds; 5) add Prague powder and mix for 30 seconds; 6) add sodium erythorbate and mix for 15 seconds; 7) add antioxidant and mix for 20 seconds; 8) stuff in shirred fibrous casing that has had one end tied with cord; 9) tie stuffed casing with cord; 10) hang on smoke stick and put into smokehouse to cook until internal temperature of salami reaches 150-155°F; 11) remove from smokehouse and shower; 12) chill to 38°F. Six batches of salami were made, the control batch contained no antioxidants and others contained different antioxidants; BHT, PG, BHA and TBHQ all singly or BHA plus TBHQ.

Growth Inhibition Studies in Salami

Salami samples were sliced with an alcohol flamed spatula, weighted in a sterile petri dish. One milliliter of Aspergillus spore suspension was applied onto sliced salami. Plates were incubated at room temperature (23 ± 1°C) for 5 days or refrigerator temperature (7°C) for 8 weeks and then room temperature for 5 days. After appropriate incubation time, samples were frozen for further analysis. Mold growth was estimated visually. Since the growth of species of

Aspergillus was readily distinguishable by characteristic color and appearance, distinction was made between the growth of the aspergilli and that of other microorganisms. The moldy salami samples were placed in a 400 ml steriled Stomacher Bag (Dynatech Lab, Inc., Alexandria, Va.) and 125 ml of extraction solution added. Samples were then mixed in a Stomacher Lab-Blender 400 (Model No. BA6021, A.J. Seward, London, Eng.) for 1 minute. Meat slurries were transferred into 500 ml Erlenmeyer flasks. The quantificative analysis of aflatoxin in the meat slurry (placed in 500 ml Erlenmeyer flask) was made according to the thin layer chromatography procedure of Pons et al.(1968).

Statistical analysis of the data was performed using two way analysis of variance and Duncan's multiple range test at 0.05 significance level (Snedecor and Corhran, 1980) with the aid of SAS program.

RESULTS AND DISCUSSION

Effect of Antioxidants on Growth, Sporulation, and Aflatoxin Production on Solid Medium

Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tertiary butylhydroquinone (TBHQ) were applied to three toxigenic strains of A. parasiticus (NRRL 2999, NRRL 4123, NRRL 5835) and three nontoxigenic strains of A. flavus (NRRL 5521, NRRL 5917, NRRL 5918). At all levels tested (0.001, 0.005, 0.01, 0.02 g per plate), BHT and PG did not inhibit growth, sporulation and toxigenesis of all six cultures. However, in the presence of BHA and TBHQ (alone or in combination) all cultures were affected. These results agree with Fung et al. (1977), who found BHA (0.005-0.02 g per plate) had an inhibitory effect on growth and toxigenesis of A. flavus while BHT had no visible effects. Three distinct zones appeared on the plate containing BHA or TBHQ or a combination of BHA and TBHQ in equal amounts: (1) an inner zone of no growth, (2) a middle zone with growth and no sporulation, (3) an outer zone with growth and sporulation. Fig. 1 shows the inner inhibition zone increases in size as the amount of BHA applied increases. Good growth, sporulation, and toxigenesis were observed for all six tested cultures in the absence of antioxidants.

Effect of Antioxidants on A. parasiticus NRRL 2999

The effect of BHA, TBHQ and combination of BHA and TBHQ combined (BHA/TBHQ) on growth, sporulation and toxigenesis

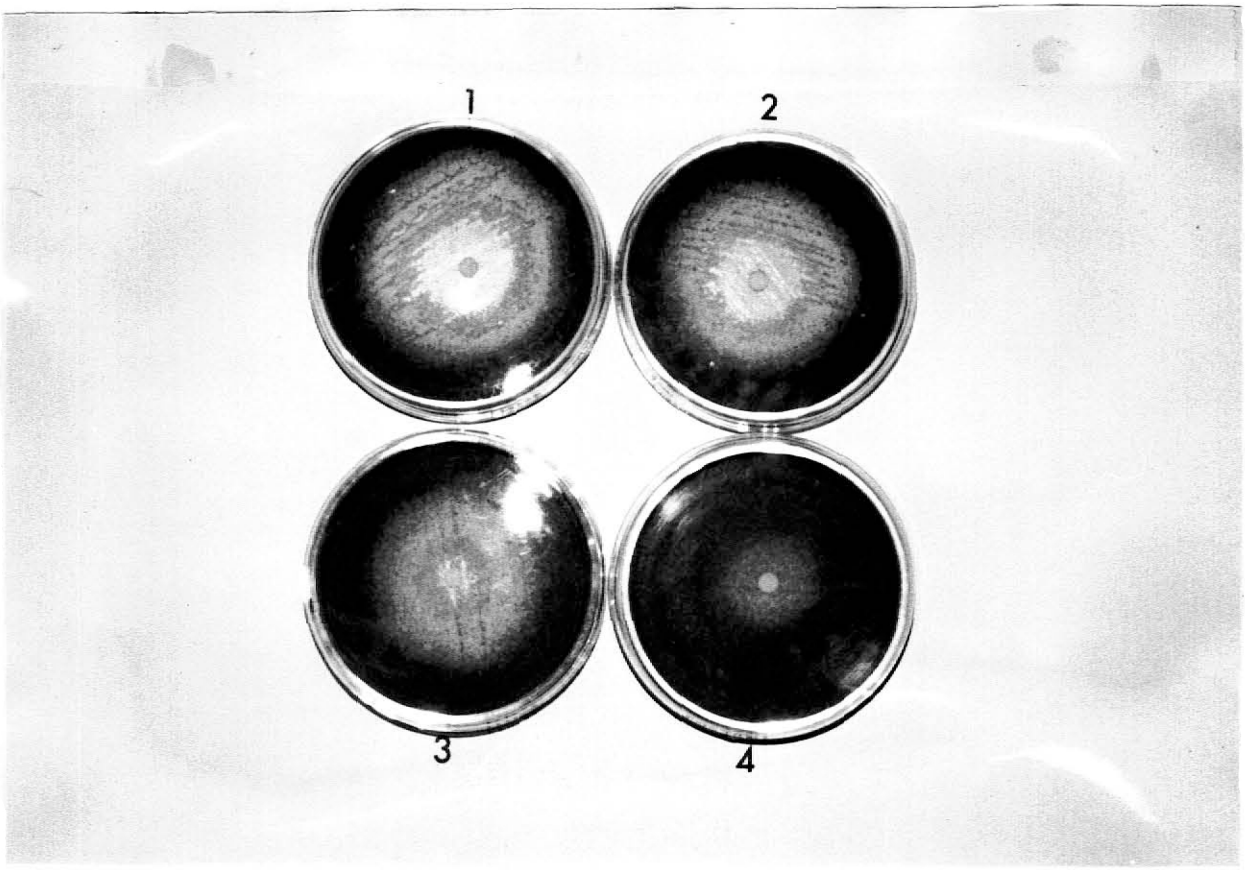


Figure 1. Growth of A. parasiticus NRRL 2999 on solid medium in the presence of different amount of BHA disk at the center area: (1) 0.01 g, (2) 0.005 g, (3) 0.001 g, (4) 0.0005 g.

of A. parasiticus NRRL 2999 is presented in Table 1. As was expected, the higher the application levels, the greater the inhibitory effect of BHA, TBHQ, and BHA/TBHQ on the test microorganism. The inner and middle zones increased in size as the amount of BHA, TBHQ and BHA/TBHQ applied increased. The outer zone showed growth and sporulation of the organism indicating that antioxidants has little effect at that region. The minimum inhibitory concentration (MIC) of BHA and TBHQ was 0.0005 g and 0.001 g per plate, respectively, while 0.0003 g per plate of BHA/TBHQ was inhibitory to NRRL 2999. In comparison with the inhibition size of three antioxidants, BHA/TBHQ gave the greatest result and TBHQ was the smallest one. The difference between the effectiveness of 0.01 g and 0.02 g per plate was limited. At 0.005 g application of BHA or TBHQ, plates showed brighter fluorescence at the outer zone than the middle and inner zones indicating that more aflatoxins were produced by the organism at a distance from the antioxidants. Fung et al. (1977) reported that sporulation is not necessary for the production of aflatoxin for toxigenic strain of A. flavus, thus the reason for occurrence of fluorescence zones in both middle and outer zone. The inner zone without any growth, exhibits slight fluorescence. Apparently aflatoxins produced at the outer and middle zones migrated toward the center area to cause fluorescence. Further increased of BHA to 0.01 and 0.02 g, and TBHQ to 0.02 g resulted in no fluorescence in the inner zone of the plates. Combination of BHA and TBHQ was the most efficient group

Table 1

Effect of BHA, TBHQ, and combination of BHA and TBHQ on growth, sporulation, and toxigenesis of A.parasiticus NRRL 2999 on solid medium[a]

amount of antioxidants per plate(g)	BHA			TBHQ			BHA&TBHQ[b]		
			fluorescence			fluorescence			fluorescence
	I[c]	M[d]	I M O[e]	I	M	I M O	I	M	I M O
0.0200	2.60	5.20	- + +	1.97	3.80	- + +	3.18	5.50	- + +
0.0100	2.58	5.20	- + +	2.03	3.91	+ + +	3.13	5.30	- + +
0.0050	2.16	4.80	+ + +	1.77	3.92	+ + +	3.02	5.30	- + +
0.0020	1.70	4.30	+ + +	0.95	3.15	+ + +	2.30	4.70	+ + +
0.0010	0.93	2.70	+ + +	0.11	1.09	+ + +	1.88	3.93	+ + +
0.0005	0.10	1.38	+ + +	0	0.62	+ + +	0.84	2.90	+ + +
0.0004	0	1.33	+ + +	0	0.11	+ + +	0.18	0.57	+ + +
0.0003	0	0	+ + +	0	0	+ + +	0.13	0.37	+ + +
0.0002	0	0	+ + +	0	0	+ + +	0	0	+ + +

[a] values indicate the mean of 8 samples.

[b] BHA&TBHQ were combined in equal amounts.

[c] I = "inhibition zone" without growth. A 100 mm diameter petri dish was used in this study. The diameter of the zone is expressed in cm.

[d] M = "middle zone" with growth but no sporulation.

[e] O = "outer zone" with growth and sporulation.

among three treatments. At 0.005 g per plate level, no fluorescence could be detected in the inner zone of the plates.

To confirm the presence of aflatoxin in the plates, the agar of control plates without antioxidants, and plates with 0.0005, 0.001, 0.005, and 0.01 g of BHA or TBHQ or BHA/TBHQ were analyzed for all four primary aflatoxins. Data in Table 2 indicate that in the presence of BHA, TBHQ and BHA/TBHQ, aflatoxin production by A. parasiticus NRRL 2999 was inhibited. The inhibition of aflatoxin production was generally proportional to the amounts of the antioxidants added. The amounts of all four aflatoxins decreased in a similar manner due to the depression of growth. At 0.0005 g per plate level, BHA showed selective effect on aflatoxin G_2 only, while TBHQ and BHA/TBHQ resulted in a selective inhibition on the production of aflatoxin G_1 and G_2. The effect of these antioxidants on aflatoxin production was greater in the G group (G_1 and G_2) than in the B group (B_1 and B_2). Aflatoxin G_1 was almost totally inhibited by 0.005 g per plate of BHA and BHA/TBHQ, whereas B_1 was only approximately 40% inhibited by 0.01 g per plate of BHA and BHA/TBHQ. Combination of BHA and TBHQ showed a slightly synergistic effect on aflatoxin production by A. parasiticus NRRL 2999. The results for BHA and BHT agree with Foudin et al. (1978), who reported that BHT did not appreciably affect growth and aflatoxin production of A. parasiticus NRRL 2999 at concentrations of 25-150 ppm in YES medium while BHA was fungistatic. They found that aflatoxin B_1 decreased

Table 2

Production of aflatoxins of A.parasiticus NRRL 2999 in the presence of BHA, TBHQ, and combination of BHA and TBHQ on solid medium[a]

grams of antioxidant per plate	Aflatoxin (µg/ml of agar)			
	B_1	B_2	G_1	G_2
Control	71.43	3.75	12.50	2.14
BHA				
0.0005	71.43	3.75	12.50	0.36
0.0010	65.00	2.60	8.30	0.05
0.0050	45.00	1.20	0.50	0.39
0.0100	45.00	2.55	4.00	0.45
TBHQ				
0.0005	71.43	3.75	9.40	0.45
0.0010	65.00	1.65	4.00	0.15
0.0050	55.00	2.70	2.40	0.33
0.0100	52.50	2.25	1.90	0.33
BHA&TBHQ[b]				
0.0005	71.43	3.75	6.25	0.27
0.0010	62.50	2.50	1.60	0.12
0.0050	40.00	1.80	0.40	0.06
0.0100	40.00	1.65	0.40	0.06

[a]values indicate the means of 6 samples.

[b]BHA&TBHQ were combined in equal amounts.

from 67 µg/ml in the controls to 0.3 µg/ml at 150 ppm added BHA, and G_1 decreased from 91 µg/ml to 0.06 µg/ml. The large difference in aflatoxin G_1 production between two control studies (91 µg/ml vs 12.5 µg/ml) could be attributed to the different medium used (liquid YES medium vs solid fluorescent agar) or a different physiological state of culture used.

Effect of Antioxidants on A. parasiticus NRRL 4123

The effects of BHA, TBHQ and BHA/TBHQ on growth, sporulation, and toxigenesis of A. parasiticus NRRL 4123 are shown in Table 3. Each of the three treatments showed an inhibitory effect against NRRL 4123. The inner and middle zones increased in size as the amount of BHA, TBHQ, and BHA/TBHQ applied increased from 0.0005 g to 0.01 g per plate. The growth and sporulation were profuse in the outer zone. The minimum inhibition concentration of BHA, TBHQ and BHA/TBHQ is 0.0005 g per plate. The combination of BHA and TBHQ revealed predominant inhibition in comparison with either antioxidant applied alone. At the 0.002 g application of BHA or BHA/TBHQ, plates showed brighter fluorescence at the outer zone than at middle and inner zones; 0.005 g of TBHQ gave a similar result. Further increased amounts of BHA and BHA/TBHQ from 0.005 to 0.02 g, and TBHQ from 0.01 to 0.02 g resulted in no fluorescence in inner zone of the plates.

Data reported in Table 4 is the production of aflatoxin by NRRL 4123 in the presence of BHA, TBHQ, or BHA/TBHQ on a solid medium. The amount of aflatoxins B_1, B_2, G_1 and G_2 generally decreased as the amounts of antioxidant applied

Table 3

Effect of BHA, TBHQ, and combination of BHA and TBHQ on growth, sporulation, and toxigenesis of A.parasiticus NRRL 4123 on solid medium[a]

amount of antioxidants per plate(g)	BHA			TBHQ			BHA&TBHQ[a]		
			fluorescence			fluorescence			fluorescence
	I[c]	M[d]	I M O[e]	I	M	I M O	I	M	I M O
0.0200	2.88	6.13	- + +	2.00	4.50	- + +	3.33	5.93	- + +
0.0100	2.70	5.40	- + +	2.12	4.66	- + +	3.12	6.10	- + +
0.0050	2.38	5.40	- + +	1.87	3.97	+ + +	3.32	5.85	- + +
0.0020	2.00	5.10	+ + +	0.80	3.03	+ + +	2.28	5.40	+ + +
0.0010	1.10	3.37	+ + +	0.80	1.82	+ + +	1.95	4.76	+ + +
0.0005	0.33	3.10	+ + +	0.60	0.83	+ + +	1.20	3.87	+ + +
0.0004	0	0	+ + +	0	0	+ + +	0	0.50	+ + +
0.0003	0	0	+ + +	0	0	+ + +	0	0	+ + +
0.0002	0	0	+ + +	0	0	+ + +	0	0	+ + +

[a] values indicate the mean of 8 samples.

[b] BHA&TBHQ were combined in equal amounts.

[c] I = "inhibition zone" without growth. A 100 mm diameter petri dish was used in this study. The diameter of the zone is expressed in cm.

[d] M = "middle zone" with growth but no sporulation.

[e] O = "outer zone" with growth and sporulation.

Table 4

Production of aflatoxins of A.parasiticus NRRL 4123 in the presence of BHA, TBHQ, and combination of BHA and TBHQ on solid medium[a]

grams of antioxidant per plate	Aflatoxin (ug/ml of agar)			
	B_1	B_2	G_1	G_2
Control	55.600	4.200	12.500	2.140
BHA				
0.0005	50.000	4.200	3.500	0.300
0.0010	40.000	3.900	4.600	0.300
0.0050	40.000	3.600	1.750	0.225
0.0100	20.000	1.650	0.600	0.050
TBHQ				
0.0005	53.300	4.200	10.000	0.800
0.0010	40.000	2.550	6.000	0.600
0.0050	36.000	2.250	3.670	0.260
0.0100	25.000	2.100	1.700	0.210
BHA&TBHQ[b]				
0.0005	30.000	1.650	1.600	0.275
0.0010	30.000	1.650	1.600	0.054
0.0050	30.000	2.550	1.600	0.250
0.0100	20.000	2.400	1.400	0.200

[a]values indicate the means of 6 samples.

[b]BHA&TBHQ were combined in equal amounts.

increased. The production of aflatoxin B_2 and G_2 were slightly variable in their response to various amounts of BHA/TBHQ. However, the general trend was a decrease in the quantities of aflatoxin B_2 and G_2 produced. At 0.0005 g per plate level, BHA and TBHQ showed selective inhibition on aflatoxin B_1, G_1, and G_2 while BHA/TBHQ inhibited all four aflatoxins. Antioxidants of BHA, TBHQ, and BHA/TBHQ at 0.01 g per plate level caused 90% inhibition in aflatoxin G_1 and G_2 production by NRRL 4123, while only 50-60% inhibition was obtained in aflatoxin B_1 and B_2.

Effect of Antioxidants on A. parasiticus NRRL 5835

The levels of BHA, TBHQ, and combined of BHA and TBHQ found to inhibit growth, sporulation, and toxigenesis of A. parasiticus NRRL 5835 are shown in Table 5. By the end of the incubation period, the outer area of plate showed abundant growth and sporulation, they emitted fluorescence under UV light. All three treatments affected the inner and middle zone of culture plates, the inhibition of growth was generally proportional to the amounts of the antioxidants applied. The MIC of BHA, TBHQ and BHA/TBHQ are 0.0004 g, 0.0005 g, and 0.0003 g, respectively. BHA/TBHQ show good synergism in inhibition growth of NRRL 5835. At all application level of BHA, TBHQ, and BHA/TBHQ, NRRL 5835 had weak fluorescence in both outer and middle zones when compared with other two toxigenic strains NRRL 2999 and NRRL 4123. No detectable fluorescence was shown in the inner zone when 0.01 and 0.02 g of antioxidants were used.

Table 5

Effect of BHA, TBHQ, and combination of BHA and TBHQ on growth, sporulation, and toxigenesis of A.parasiticus NRRL 5835 on solid medium[a]

amount of antioxidants per plate(g)	BHA			TBHQ			BHA&TBHQ[b]		
			fluorescence			fluorescence			fluorescence
	I[c]	M[d]	I M O[e]	I	M	I M O	I	M	I M O
0.0200	2.68	5.85	- + +	2.25	4.83	- + +	3.23	6.08	- + +
0.0100	2.43	5.84	- + +	2.06	5.00	- + +	3.00	6.30	- + +
0.0050	2.30	5.53	± + +	2.04	4.47	± + +	2.98	6.30	± + +
0.0020	1.78	5.00	+ + +	0.48	3.43	+ + +	2.48	5.60	± + +
0.0010	1.05	3.74	+ + +	0.38	1.93	+ + +	1.45	4.00	+ + +
0.0005	0.31	3.64	+ + +	0.14	1.93	+ + +	1.05	4.58	+ + +
0.0004	0.40	2.57	+ + +	0	0.87	+ + +	0.33	3.00	+ + +
0.0003	0	1.60	+ + +	0	0.90	+ + +	0.33	0	+ + +
0.0002	0	0	+ + +	0	0.45	+ + +	0	0	+ + +

[a] values indicate the mean of 8 samples.

[b] BHA&TBHQ were combined in equal amounts.

[c] I = "inhibition zone" without growth. A 100 mm diameter petri dish was used in this study. The diameter of the zone is expressed in cm.

[d] M = "middle zone" with growth but no sporulation.

[e] O = "outer zone" with growth and sporulation.

Table 6 showed aflatoxin production of NRRL 5835 was inhibited by the presence of BHA, TBHQ, and BHA/TBHQ on fluorescent agar. The amounts of aflatoxin production are small, this corresponds directly to the weak fluorescence under UV light. Due to the small amounts of aflatoxin G_2 in the control, aflatoxin G_2 was not detectable at any test level. Butylated hydroxyanisole at 0.005 g per plate almost totally inhibited all four aflatoxins, whereas for TBHQ slight inhibition was observed in aflatoxin B_1 production. At 0.01 g per plate level, combined of BHA and TBHQ completely inhibited aflatoxin B_2, G_1 and G_2 while only 90% of aflatoxin B_1 was inhibited.

Effect of Antioxidants on A. flavus NRRL 5521

Table 7 depicts the effect of BHA, TBHQ and BHA/TBHQ against NRRL 5521. All three treatments were found to be effective in inhibiting growth and sporulation. The inner and middle zones increased in size as the amount of BHA, TBHQ and BHA/TBHQ applied increased. Antioxidants have no effect on outer area of plates. No fluorescence was detected under UV light. The MIC of BHA and BHA/TBHQ were 0.0005 g while TBHQ was 0.001 g per plate. A good deal of synergetic inhibition was shown for the BHA/TBHQ group; the greatest inhibition zone (3.75cm) was observed in 0.02 g per plate level. In all levels applied, BHA was better than TBHQ in growth inhibition of NRRL 5521.

Table 6

Production of aflatoxins of A.parasiticus NRRL 5835 in the presence of BHA, TBHQ, and combination of BHA and TBHQ on solid medium[a]

grams of antioxidant per plate	Aflatoxin (ug/ml of agar)			
	B_1	B_2	G_1	G_2
Control	0.9000	0.1500	0.3300	0.0060
BHA				
0.0005	0.5500	0.1200	0.0300	Tr[d]
0.0010	0.3500	—[c]	0.0150	—
0.0050	0.0090	—	—	—
0.0100	0.0070	—	—	—
TBHQ				
0.0005	0.6000	0.1500	0.3200	Tr
0.0010	0.9000	0.0900	0.1500	Tr
0.0050	0.6000	0.0525	0.1250	—
0.0100	0.1700	—	0.0800	—
BHA&TBHQ[b]				
0.0005	0.9000	0.0900	0.1500	Tr
0.0010	0.5000	0.0800	0.1200	—
0.0050	0.1880	0.0450	0.1000	—
0.0100	0.0800	—	—	—

[a] values indicate the meams of 6 samples.

[b] BHA&TBHQ were combined in equal amounts.

[c] — = not detectable.

[d] Tr = trace amounts.

Table 7

Effect of BHA, TBHQ, and combination of BHA and TBHQ on growth, sporulation, and toxigenesis of A.flavus NRRL 5521 on solid medium[a]

amount of antioxidants per plate(g)	BHA			TBHQ			BHA&TBHQ[b]		
			fluorescence			fluorescence			fluorescence
	I[c]	M[d]	I M O[e]	I	M	I M O	I	M	I M O
0.0200	3.10	5.00	- - -	2.50	5.20	- - -	3.75	6.70	- - -
0.0100	2.95	5.10	- - -	2.55	5.56	- - -	3.65	6.40	- - -
0.0050	2.80	4.25	- - -	2.00	4.60	- - -	3.40	5.80	- - -
0.0020	2.15	3.20	- - -	1.10	3.20	- - -	1.45	2.50	- - -
0.0010	0	0.90	- - -	0.40	2.60	- - -	0.60	2.00	- - -
0.0005	0.30	0.80	- - -	0	1.00	- - -	1.00	1.70	- - -
0.0002	0	0	- - -	0	0	- - -	0	0	- - -

[a] values indicate the mean of 8 samples.

[b] BHA&TBHQ were combined in equal amounts.

[c] I = "inhibition zone" without growth. A 100 mm diameter petri dish was used in this study. The diameter of the zone is expressed in cm.

[d] M = "middle zone" with growth but no sporulation.

[e] O = "outer zone" with growth and sporulation.

Effect of Antioxidants on A. flavus NRRL 5917

The levels of BHA, TBHQ and BHA/TBHQ were found to inhibit growth and sporulation of A. flavus NRRL 5917 on solid medium (Table 8). As expected, the higher the application levels, the greater the inhibition zone of BHA, TBHQ, and BHA/TBHQ in the inner and middle zones. This nontoxigenic strain (NRRL 5917) revealed no fluorescence under UV light. The MIC of BHA, TBHQ, and BHA/TBHQ were 0.001 g, 0.002 g, and 0.005 g, respectively. Combination of BHA and TBHQ showed slightly synergetic effects, especially at lower concentration.

Effect of Antioxidants on A. flavus NRRL 5918

Table 9 shows A. flavus NRRL 5918 growth inhibition by BHA, TBHQ and BHA/TBHQ. The inner and middle zones increased in size as the amount of BHA applied increased. Growth inhibition of A. flavus NRRL 5918 had variable responses to various levels of TBHQ and BHA/TBHQ. However, the general trend was a increase in inhibition zone as amount of antioxidants increased. In comparison with BHA and TBHQ applied alone, combination of BHA and TBHQ showed synergetic effectiveness, the greatest inhibition size (3.55cm) was obtained at 0.01 g per plate level.

The effect of BHA, TBHQ and BHA/TBHQ at all treatment levels against three toxigenic strains of A. parasiticus and three nontoxigenic strains of A. flavus, as compared to the control, was analyzed using two-way analysis of variance and

Table 8

Effect of BHA, TBHQ, and combination of BHA and TBHQ on growth, sporulation, and toxigenesis of A.flavus NRRL 5917 on solid medium[a]

amount of antioxidants per plate(g)	BHA			TBHQ			BHA&TBHQ[b]		
			fluorescence			fluorescence			fluorescence
	I[c]	M[d]	I M O[e]	I	M	I M O	I	M	I M O
0.0200	3.40	6.50	- - -	2.75	6.45	- - -	3.60	7.2	- - -
0.0100	3.20	6.20	- - -	2.20	5.50	- - -	3.45	7.0	- - -
0.0050	2.95	5.60	- - -	1.65	5.50	- - -	3.25	6.0	- - -
0.0020	2.65	4.45	- - -	0.95	5.00	- - -	1.20	3.2	- - -
0.0010	1.40	2.00	- - -	0	1.10	- - -	0.60	1.6	- - -
0.0005	0	0	- - -	0	0	- - -	0.95	2.1	- - -
0.0002	0	0	- - -	0	0	- - -	0	0	- - -

[a] values indicate the mean of 8 samples.

[b] BHA&TBHQ were combined in equal amounts.

[c] I = "inhibition zone" without growth. A 100 mm diameter petri dish was used in this study. The diameter of the zone is expressed in cm.

[d] M = "middle zone" with growth but no sporulation.

[e] O = "outer zone" with growth and sporulation.

Table 9

Effect of BHA, TBHQ, and combination of BHA and TBHQ on growth, sporulation, and toxigenesis of A.flavus NRRL 5918 on solid medium[a]

amount of antioxidants per plate(g)	BHA			TBHQ			BHA&TBHQ[b]		
			fluorescence			fluorescence			fluorescence
	I[c]	M[d]	I M O[e]	I	M	I M O	I	M	I M O
0.0200	2.95	7.45	- - -	1.95	4.40	- - -	3.30	8.15	- - -
0.0100	2.70	7.50	- - -	1.95	4.50	- - -	3.55	8.00	- - -
0.0050	2.65	7.00	- - -	2.05	4.90	- - -	3.10	7.70	- - -
0.0020	2.50	6.00	- - -	1.00	4.00	- - -	2.40	6.70	- - -
0.0010	0	5.00	- - -	0	0.50	- - -	1.00	5.00	- - -
0.0005	0	4.10	- - -	0.40	2.00	- - -	1.00	4.65	- - -
0.0002	0	0	- - -	0	0	- - -	0	0	- - -

[a] values indicate the mean of 8 samples.

[b] BHA&TBHQ were combined in equal amounts.

[c] I = "inhibition zone" without growth. A 100 mm diameter petri dish was used in this study. The diameter of the zone is expressed in cm.

[d] M = "middle zone" with growth but no sporulation.

[e] O = "outer zone" with growth and sporulation.

Duncan's multiple range test. All three treatments (BHA, TBHQ, and BHA/TBHQ) were found to significantly ($p<0.05$) inhibit growth, sporulation and aflatoxin production of the test cultures. Kinds of antioxidants and cultures, levels of antioxidants used and type of aflatoxins resulted in different ($p<0.05$) inhibitory effects. The higher the amounts of antioxidants applied, the greater the inhibitory effect observed. Antioxidants are known to have synergistic antioxidant effects when used in combination with other antioxidants (Dugan, 1976). For inner zone development and aflatoxin production, combination of BHA and TBHQ showed significantly ($\alpha=0.05$) better inhibition than two antioxidants applied alone. The inhibitory effect of BHA on growth, sporulation and aflatoxin production was significantly better than TBHQ at all levels applied. Antioxidants had no significantly different effect on inner inhibition zone among three nontoxigenic strains of A. flavus. The inhibitory effect of antioxidants on the middle zone was significant in culture NRRL 5918. Antioxidants are more effective against non-toxigenic strains than toxigenic strains. Statistical correlation indicates that cultures producing higher amounts of toxin were more resistant to antioxidants ($\alpha=0.05$). Antioxidants have selective inhibitory effects on the production of the four primary aflatoxins, with B_1 (35%) least inhibited while B_2 (41%), G_1 (66%) and G_2 (82%) were more inhibited. In a solid medium study, plate which fluoresce under UV light also yielded aflatoxin in the thin layer

chromatography test indicating the reliability of the UV test as a presumptive test for aflatoxin production. Samples from nontoxigenic strains show no fluorescence and aflatoxin production in comparable studies.

This study indicated that BHA and TBHQ alone or in combination are effective in inhibiting growth, sporulation and aflatoxin production of Aspergillus on laboratory medium. The combination of BHA and TBHQ had, in general, synergistic effectiveness.

The importance of growth inhibition studies on laboratory medium was to evaluate the antifungal activities of BHA, BHT, TBHQ and PG alone or in combination. The information can be used as a reference for applying the compounds to a complex food system.

Growth Inhibition Studies of Antioxidants in Cooked Beef Salami

Three toxigenic strains of A. parasiticus (NRRL 2999, NRRL 4123, NRRL 5835) and one nontoxigenic strain of A. flavus (NRRL 5521) were applied to salami for this study. The salami with cultures and 100 ppm antioxidants were stored for varying lengths of time at room temperature (23 ± 1°C) and refrigerator temperature (7°C). Mold growth was estimated visually. The growth of species of Aspergillus was readily distinguishable by characteristic color and appearance (Bullerman, et al., 1969).

Salami with cultures incubated at room temperature for

5 days (RT) showed abundant growth and sporulation at the end of storage. On the second day of incubation growth and sporulation of A. parasiticus NRRL 2999 was observed whereas A. parasiticus NRRL 4123 and 5835 and A. flavus NRRL 5521 started growth at the third day. During 5 days incubation, bacteria and yeasts were present causing slimy appearance of salami. This may exist as an interference factor for growth and aflatoxin production of Aspergillus at room temperature.

No growth of any test cultures was observed at refrigeration temperature for 8 weeks; likewise, no aflatoxins were found at this temperature. These results agree with Oldham et al. (1971), who found that no detectable aflatoxin was obtained in luncheon-meat samples when kept at normal refrigeration temperature. About one-third of the samples were contaminated with other molds and yeasts, but few bacteria were observed. Salami tends to loose moisture during long term incubation at refrigerator temperature, this may relate to high fungi growth on salami samples. The contaminated plates were discarded, plates with no visible contamination were used for further studies. After 8 weeks in refrigerator, salami with cultures were then incubated at room temperature for 5 days (RR) to study the effect of temperature shift on growth and toxigenesis of test cultures on salami. All cultures began to grow at the third day. Profuse growth and sporulation were obtained at the end of 5 days incubation.

All salami samples from two different storage periods

were analyzed by thin layer chromatography. Salami inoculated with A. flavus NRRL 5521 shows no aflatoxin in a comparable study.

Results of the effects of antioxidants against aflatoxin production of A. parasiticus NRRL 2999 are presented in Table 10. The 100 ppm of BHA, TBHQ and combination of BHA and TBHQ (BHA/TBHQ) were found to suppress aflatoxin production of NRRL 2999 at both storage periods. An inhibitory effect of BHT and PG against aflatoxin production of NRRL 2999 was not noted at RR storage period, as compared to the controls. Although reaction of BHT and PG at two storage periods are not consistent, no significant (α=0.05) different between the control and the antioxidants (BHT or PG) was observed. Salami with culture (NRRL 2999) produced larger amounts of aflatoxins with RR incubation period than at room temperature for 5 days. Effects of BHA, TBHQ, BHA/TBHQ on the production of 4 aflatoxins (B_1, B_2, G_1, and G_2) by the test organism were greater after RT storage than RR storage. Butylated hydroxyanisole was the most effective in suppressing aflatoxin production by NRRL 2999 than the other two treatments (TBHQ and BHA/TBHQ). Due to the small amounts of aflatoxin B_2 and G_2 produced by NRRL 2999 at room temperature for 5 days incubation period, productions of aflatoxin B_2 and G_2 by NRRL 2999 were almost totally inhibited by BHA, TBHQ and BHA/TBHQ. None of the antioxidants tested were highly effective against aflatoxin B_1 and G_1 production by A. parasiticus NRRL 2999. Foudin et al. (1978) reported that 100 ppm of BHA almost

Table 10

Production of aflatoxins by A. parasiticus NRRL 2999 in the presence of antioxidants on salami[a]

Treatment	Storage period	Aflatoxin (µg/g of salami)			
		B_1	B_2	G_1	G_2
Control	RT[b]	7.0671	0.0050	11.7786	0.0050
	RR[c]	21.1268	0.0391	21.1268	0.0391
BHT	RT	7.2674	0.0045	7.2674	0.0045
	RR	21.1370	0.0395	21.1370	0.0395
PG	RT	6.1475	0.0050	6.1475	0.0050
	RR	21.3068	0.0393	21.3068	0.0393
BHA	RT	5.3097	Tr[d]	7.9646	Tr
	RR	18.3150	0.0332	18.3150	0.0332
TBHQ	RT	6.1224	Tr	9.1837	Tr
	RR	20.2156	0.0387	20.2156	0.0387
BHA&TBHQ[e]	RT	5.6818	Tr	11.3636	Tr
	RR	19.1327	0.0335	19.1327	0.0335

[a] values indicate the means of 6 samples.

[b] RT = incubation at room temperature (23 ± 1°C) for 5 days.

[c] RR = incubation at refrigeration temperature (7°C) for 8 weeks and then room temperature (23 ± 1°C) for 5 days.

[d] Tr = trace amounts.

[e] BHA&TBHQ were combined in equal amounts.

totally inhibited aflatoxin production by A. parasiticus NRRL 2999 in YES medium, while only slight inhibition was obtained in our salami sample study at 100 ppm BHA. The antimicrobial effectiveness of the antioxidants tested was markedly reduced in a food system containing lipid content.

The effects of antioxidants on the production of aflatoxin by A. parasiticus NRRL 4123 in salami are shown in Table 11. As expected, BHT and PG did not appreciably affect aflatoxin production by NRRL 4123 at 100 ppm concentration when compared with controls, while BHA, TBHQ and BHA/TBHQ showed inhibitory effects. Aspergillus parasiticus NRRL 4123 in salami sample produced larger amounts of aflatoxins at RR storage period than at room temperature for 5 days. Antioxidants (BHA, TBHQ, and BHA/TBHQ), at 100 ppm showed high inhibition on aflatoxin B_2 and G_2 at both incubation periods. This may relate to the trace amount of aflatoxin B_2 and G_2 production by NRRL 4123. Combining of BHA and TBHQ showed 56-71% inhibition to the production of aflatoxin B_1 and G_1 by the test culture at both incubation periods, while TBHQ only inhibited 11-29% of aflatoxin production. About 33% of the production of aflatoxin B_1 and G_1 by NRRL 4123 was inhibited by BHA at RT storage period, while the inhibitory effect of BHA was limited at RR storage period.

The data of Table 12 show the inhibitory effect of antioxidants on the production of aflatoxins by A. parasiticus NRRL 5835 on cooked beef salami. The production of aflatoxin B_2 and G_2 by NRRL 5835 was not detectable. Except for

Table 11

Production of aflatoxins by A. parasiticus NRRL 4123 in the presence of antioxidants on salami[a]

Treatment	Storage period	Aflatoxin (µg/g of salami)			
		B_1	B_2	G_1	G_2
Control	RT[b]	3.5714	0.0064	8.5700	0.0064
	RR[c]	9.2784	0.0232	15.4639	0.0257
BHT	RT	3.5500	Tr[d]	8.6200	Tr
	RR	9.5162	0.0242	16.0256	0.0258
PG	RT	3.5500	Tr	9.0000	Tr
	RR	9.0154	0.0201	15.2500	0.024
BHA	RT	2.3438	Tr	5.8594	Tr
	RR	9.5517	0.0053	12.9310	0.0054
TBHQ	RT	3.0612	Tr	7.0093	Tr
	RR	6.5934	0.0049	13.7363	0.0051
BHA&TBHQ[e]	RT	1.1819	Tr	2.4810	Tr
	RR	4.1034	Tr	5.1039	Tr

[a] values indicate the means of 6 samples.

[b] RT = incubation at room temperature(23 ± 1°C) for 5 days.

[c] RR = incubation at refrigeration temperature(7°C) for 8 weeks and then room temperature(23 ± 1°C) for 5 days.

[d] Tr = trace amounts.

[e] BHA&TBHQ were combined in equal amounts.

Table 12

Production of aflatoxins by A. parasiticus NRRL 5835 in the presence of antioxidants on salami[a]

Treatment	Storage period	Aflatoxin (μg/g of salami)			
		B_1	B_2	G_1	G_2
Control	RT[b]	0.0045	—[d]	0.0081	—
	RR[c]	0.1310	—	0.0570	—
BHT	RT	—	—	0.0050	—
	RR	0.1290	—	0.0560	—
PG	RT	0.0040	—	0.0072	—
	RR	0.1350	—	0.0620	—
BHA	RT	—	—	—	—
	RR	0.1290	—	0.0415	—
TBHQ	RT	—	—	—	—
	RR	0.0033	—	0.0308	—
BHA&TBHQ[e]	RT	—	—	—	—
	RR	0.0025	—	0.0083	—

[a] values indicate the means of 6 samples.

[b] RT = incubation at room temperature(23 ± 1°C) for 5 days.

[c] RR = incubation at refrigeration temperature(7°C) for 8 weeks and then room timperature(23 ± 1°C) for 5 days.

[d] — = not detectable.

[e] BHA&TBHQ were combined in equal amounts.

appearance of B_2, concentrations of BHA, TBHQ and BHA/TBHQ at 100 ppm inhibited the production of aflatoxin by NRRL 5835 at both incubation periods, while the effect of BHT or PG is not obvious. The antioxidants TBHQ and BHA/TBHQ were highly effective against aflatoxin production by NRRL 5835 at both incubation periods. Butylated hydroxyanisole totally inhibited the production of aflatoxin at RT storage period, while little inhibition was obtained at RR incubation period.

Antioxidants are known to have less antimicrobial activity when applied to foods than when used in laboratory media (Robach et al., 1977; Klindworth et al., 1979). The presence of as little as 0.25% lipid decreased inhibition of 200 ppm BHA by approximately 30% (Ahmad, 1979). The same author also found that BHA was more effective in preventing mold growth in low fat product (applesauce) than in high fat products (cheese). The fat content of this salami was 10.25%; this was assumed to be high enough to cause a decrease in the antifungal activity of BHA or TBHQ or BHA/TBHQ against Aspergillus. Unsaturated lipids are extremely reactive compounds which undergo autoxidation with the subsequent formation of free radicals and hydroperoxides (Dugan, 1976). Antioxidants such as those tested are active in preventing autoxidation by donating a hydrogen atom to the unstable lipid free radical. The antimicrobial activity of the antioxidants may be destroyed due to the reaction between the antioxidant and a lipid molecule that uses up the antioxidant molecule, making it no longer effective. Also, the hydrophobic

nature of antioxidants and their solubility in lipid compounds might cause the antioxidants to be localized within the lipid portion of foods. If this happens, the antioxidant would be unavilable to act against microorganisms in other portions of the food.

The heating step in the production of cooked beef salami destroys the typical raw meat flora except spores. Thermoduric organisms such as Micrococcus, Streptococcus, Microbacterium, Arthrobacter, Lactobacillus and Bacillus may survive (Nelson, 1976), also some yeasts will be present (Jay, 1970). Standard plate counts of plain salami show 1×10^3 organisms/g of sample. Under good refrigeration, the growth of these microorganisms is suppressed, but given a favorable temperature (such as room temperature), profuse growth will occur and cause sliminess of salami. Abundant growth of thermoduric organisms and yeasts at room temperature tended to use up the nutrients in salami, which limited the growth of Aspergillus. This may be related to the low aflatoxin production by Aspergillius at room temperature for 5 days (RT) set. Also, the presence and growth of these microorganism in salami may influence the overall effectiveness of antioxidant against the test microorganisms. The antioxidants (BHA and TBHQ) have been shown to be effective against types of spoilage bacteria present in meat (Davidson et al., 1981; Robach et al., 1980; Erickson and Tompkin, 1977). The effectiveness of these antioxidants against the test organisms could be reduced due to presence of the other microorganisms. Although PG and BHT did not inhibit

growth of Aspergillus on solid medium, PG and BHT reduce the growth of coliform, fecal coliform and psychrotrophs (Gailani, 1981), thus salami with PG and BHT may contain higher amounts of aflatoxin produced by Aspergillus as compared to control (without any antioxidant added).

Previous studies have shown the presence of curing salts ($NaNO_2$, $NaNO_3$, and NaCl) and spices to inhibit growth and aflatoxin production of Aspergillus. Bullerman (1968) found that pepper, nitrite and the complete spice mix (sodium chloride, ground white pepper, garlic powder, sucrose, sodium nitrate, sodium nitrite and MSG) reduced growth and aflatoxin production by A. flavus growing in glucose, ammonium-nitrate broth. Meier and Marth (1977) reported that the effect of individual curing salts or curing salt mixtures on growth and toxin elaboration by Aspergillus was substrate dependent. Hitokoto et al.(1980) tested inhibitory effects of 29 commercial powdered spices on the growth and toxin production of three toxigenic Aspergillus. Of the 29 samples tested, cloves, star anise seeds and allspice completely inhibited the fungal growth and aflatoxin production whereas most of the others show complete or minor inhibitory effects on toxin production of all three Aspergillus strains. Bullerman(1968) also found that smoking salami inhibited growth and aflatoxin production by A. flavus. The ingredients of salami in this study are salt, sugar, Heller salami seasoning and Prague Powder (contains nitrite). The constitents of Heller salami seasoning are pepper, coriander,

mustard, garlic powder, thyme, bay leaves, savory and extractives of cloves. All these factors may relate to the lower aflatoxin production by A. parasiticus in the control salami sample compared to control laboratory medium.

It is unlikely that BHA or TBHQ or BHT or PG could be used alone to control the growth of microorganisms in foods, since the required amounts appear to far exceed the legally allowed amounts(0.02% based on weight of fat). However, the antioxidants could be useful in combination with other antimicrobials, especially those such as sorbic acid and monoglycerides which show synergistic action with the antioxidants (Branen, et al.,1980).

The effect of antioxidants against the test microorganisms on salami, as compared to the control, was analyzed using two way analysis of variance and Duncan's multiple range test. No difference occurred ($p>0.05$) between control and antioxidants BHT and PG at the concentration tested, while BHA, TBHQ and BHA/TBHQ inhibited ($p<0.05$) growth of all test strains. The combination of BHA and TBHQ are the most effective treatment ($\alpha=0.05$) against aflatoxin production by Aspergillus. No difference was noted between BHA and TBHQ alone. Statistical analysis indicates that cultures producing higher amount of toxins were more resistant to antioxidants ($\alpha=0.05$). Although Ahmad (1979) found no effect of temperature on the inhibition of A. flavus by BHA, the test antioxidant showed slightly better effect at RT storage period than RR storage period.

Little work has been done on elucidation of mechanisms of microbial inhibition by the phenolic antioxidants. It is most likely that they act on the cytoplasmic membrane of the microorganisms. Surak et al. (1976b) reported that BHT caused leakage of intracellular contents from Tetrahymena pyriformis. Davidson (1979) found that BHA caused leakage of intracellular protein from Pseudomonas fluorescens. The mechanism of microbial inhibition might also be related to the destruction or inactivation of essential enzyme and/or genetic material (Branen et al., 1980).

Butylated hydroxyanisole is an analogue of one of the five rings of aflatoxin. It may be directly interfering with the formation of aflatoxin. Each aflatoxin contains a phenol ring linking several carbon rings. The third phenol ring contains an $O-CH_3$ group at the C-6 position. Butylated hudroxyanisole is a phenolic compound containing an $O-CH_3$ at the C-4 position. Butylated hydroxyanisole, when present in the growth medium, may be incorporated by the organism into precusors of aflatoxins instead of the correct component in the biosynthetic pathway. This activity probably accounted for the antifungal effect of BHA against aflatoxin production A. flavus (Fung, et al., 1977).

CONCLUSION

The results of growth inhibition studies on fluorescence agar demonstrated that BHA and TBHQ alone or in combination were effective in inhibiting growth, sporulation and aflatoxin production by three toxigenic strains of A. parasiticus and three nontoxigenic strains of A. flavus. Combination of BHA and TBHQ was effective as a synergistic inhibitor. Propyl gallate and BHT did not show an inhibitory effect on the growth and aflatoxin production of Aspergillus. The higher the amounts of antioxidants used, the greater the inhibition zone obtained on solid medium.

The antioxidants added to salami were less inhibitory to the growth of the test microorganism than were laboratory medium. The diminished antimicrobial effectiveness was due to the antioxidants being used up in the control of antioxidation of the lipid content of the cooked beef salami and losing their antimicrobial activity. Thermoduric organisms and yeasts present in the salami also limited the effectiveness of antioxidants. The presence of curing ingredients and the smoking process caused less aflatoxin production by Aspergillus in salami than in fluorescence agar. All three treatments (BHA, TBHQ, and BHA/TBHQ), at 100 ppm tested level, in salami were found to significantly inhibit ($p<0.05$) growth of the test organisms when compared to control samples. Combination of BHA and TBHQ showed synergistic effect.

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EFFECT OF BHA, BHT, TBHQ AND PG ON GROWTH AND TOXIGENESIS OF *ASPERGILLUS PARASITICUS* AND *ASPERGILLUS FLAVUS*.

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ABSTRACT

The antifungal effect of butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ) and Propyl gallate (PG) alone or in combination on three toxigenic strains of Aspergillus parasiticus (NRRL 2999, NRRL 4123, NRRL 5835) and three nontoxigenic strains of A. flavus (NRRL 5521, NRRL 5917, NRRL 5918) was examined. A method similar to antibiotic sensitivity test was used in this study. Fluorescent agar was used to study the effect of several levels of antioxidants (0.0002-0.02g per plate) on growth, sporulation and toxigenesis of the test cultures. Diameter of zone of inhibition was measured in cm and the presence of aflatoxin was first screened under UV light and then confirmed and quantitated by thin-layer chromatography. Salami with or without added BHA, BHT, TBHQ and PG were used to study the effects of these additives on growth and aflatoxin production in this food item.

BHT and PG (0.001, 0.005, 0.01, 0.02g per plate) did not inhibit growth, sporulation and toxigenesis of all six cultures. However, in the presence of BHA and TBHQ all cultures were affected. Three distinct zones appeared on the plate containing BHA or TBHQ or combination of BHA and TBHQ (BHA and TBHQ combined in equal amount) on solid medium: (1) an inner zone of no growth, (2) a middle zone with growth and no sporulation, (3) an outer zone with growth and sporulation. Both inner and middle zones increased in size as the amount of antioxidants

increased. The amount of aflatoxins production by three strains of toxigenic A. parasiticus (B_1, B_2, G_1 and G_2) in the presence of BHA, TBHQ and combination of BHA and TBHQ was significantly ($p<0.05$) reduced in comparison with the absence of antioxidants on solid medium. When more antioxidants were applied the amount of aflatoxins production were reduced. Although the antifungal effectiveness of antioxidant in salami was markedly diminished by the presence of lipid and thermoduric organisms, tests in salami showed that BHA, TBHQ alone or in combination at 100 ppm significantly ($p<0.05$) decreased the amounts of aflatoxin production by Aspergillus when compared to control samples. Combination of BHA and TBHQ showed synergetic inhibition in both studies (solid medium and salami studies).