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Final Report - May 1976

Contract Title: Health Protection and Food Preservation by Gamma Irradiation

Contract Number: NAS9-11045

University of Nebraska - Lincoln

Project Director: Dr. T. E. Hartung

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Attention: Dr. Malcolm C. Smith
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General

The overall objective of the work carried out under this contract was to consider the application of gamma irradiation to food systems for manned space flight from the health protection and food preservation perspective. A series of applications has been considered and reported in detail in monthly reports and in publications. A general review will be made here, including relevant publications and a detailed report of a concluding study not previously reported in the closing year of the contract. The project director wishes to acknowledge the excellent cooperation afforded through Dr. Norman D. Heidelbaugh and Dr. Malcolm C. Smith as the technical representatives to the project.

The project involved several major activities, including providing support services for food systems on missions beginning with Apollo 12 through Apollo-Soyuz and investigations of the application of irradiation to food for manned space flight.

The activity for flight food systems involved the application of radurization (pasteurizing levels) doses of gamma irradiation to flour and bread supplied by Pepperidge Farms in advance of the missions. All flights from Apollo 12 through 17 carried irradiated fresh bread. On Apollo 17, cooperation with Natick Laboratories permitted the introduction of a ham sandwich using irradiated bread and irradiated sterile ham. Investigations centered on irradiated bread were conducted during the course of these missions.

The Skylab food system involved the use of a canned bread which was adapted by Natick Laboratories and was pasteurized using gamma

irradiation following the baking and sealing steps. The duration of the Skylab missions precluded the use of the irradiated fresh bread of the Apollo series. A series of studies was conducted just prior to and during the Skylab missions dealing with the application of pasteurizing doses (radurization) on dried food mixes.

The Apollo-Soyuz mission involved two food systems under this project, the Apollo radurized fresh bread and a radurized pastry. Studies on the application of irradiation to pastries were conducted prior to and following this mission. Several fresh food concepts have been under investigation looking toward the ASTP (Shuttle) missions. These have centered on radurized fresh sandwiches using a variety of meats. A concluding study is included with this final report.

Throughout the studies of the application of gamma irradiation under this contract, the emphasis was placed upon using low levels of irradiation in the pasteurizing or radurizing doses--under a Megarad. The primary goal was to determine if a public health benefit could be demonstrated using radurization along with food preservation and food quality improvements. The public health benefit would be parallel to that of pasteurization of milk as a concept.

Early studies were applied to the concept of improving fresh bread from the point of view of mold inhibition. The studies considered how irradiation could best be applied at what levels and on a variety of bread types. Two applications were considered--irradiation of the flour, irradiation of the bread and their combination. Benefits were found in both applications and bread types had a significant influence on the irradiation timing. Generally, irradiation under 200 Krads was found to be the best for microbial conditions as well as organoleptic acceptability. Extension of a mold-free time of fresh bread was demonstrated and some freshness improvement noted when irradiation was used. Publications are included providing the details of these observations, one dealing with the flour characteristics and the other dealing with the influence on fresh bread types. These demonstrate the major findings noted during the period of the studies examining bread.

A series of studies was developed to examine the influence of radurization on selected microorganisms. These studies included indicative organisms--bacteria and molds. The bacteria were selected to include spore and nonspore formers used as public health indicators. The molds were those identified as present in bread and

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APPLICATION OF LOW DOSE IRRADIATION TO A FRESH BREAD SYSTEM FOR SPACE FLIGHTS

INTRODUCTION

MANNED SPACE MISSIONS must achieve maximum life support capabilities. One component is the food system. The use of the fresh food concept permits ease of use and provides a satisfaction to the crew. One such item is fresh bread which can be used for sandwiches or snacks. Fresh bread spoilage due to molds even in the presence of mold inhibitors was noted in early missions. The application of low dose gamma irradiation to the fresh bread system was initiated to determine if benefits could be derived from its use.

The use of ionizing irradiation was most intensively examined in the 1950's. Its application to wheat and wheat products was noted by Milner and Yen (1956). They observed decreases of germination of wheat as radiation levels increased up to 1,000,000 rep, but no marked change in free fatty acids or protein solubility. There was a detrimental effect at the higher dose levels on dough characteristics. Milner (1957) reported that it was possible to control fungi growth in stored wheat which had been exposed to levels of gamma irradiation above 250,000 rep. The effect of gamma radiation on baking quality of flour (Brownell et al., 1955) was related to irradiation levels with detrimental effects noted above 50,000 rep. A flavor change was observed at 100,000 rep with an increased sweetness noted as a result of higher maltose values. Kretesz et al. (1959) observed that ionizing radiation depolymerized starch macromolecules. Most recently Ananthaswamy et al. (1971) reported that initial levels of reducing sugars and diastatic activity were a function of dose level in irradiated wheat.

It is quite clear that higher levels of irradiation of both wheat and flour will result in chemical changes that have detrimental effects upon dough and baking characteristics. However, with variation in the method of measuring the irradiation as roentgens and rep, it is difficult to establish the actual absorbed level of irradiation that is involved which is now expressed as rads. Ananthaswamy et al. (1971) reported their work in the range of 20–200 Krads which does permit interpretation of the level used in our work.

The influence of irradiation on wheat and flour as related to biological contamination has been primarily directed at insect control. Work with microbial changes has been reported by Stehlik (1967) wherein a retardation of mold was observed in irradiated bread at levels from 50–500 Krads. Specific mold identification as well as bacterial content were not reported.

EXPERIMENTAL

THE STUDY utilized two bread types, white and raisin, which were defined for the Apollo food system (NASA). The formula included a mold inhibitor. The gamma irradiation source was provided by a Cobalt 60 portable shipboard irradiator on loan to the University of Nebraska by the Atomic Energy Commission. Dosimetry for irradiating the flour and the bread slices was established using the Fricke dosimeter, ASTM D1671-63. One level of gamma irradiation was selected, 50,000 rads, for treating flour prior to making the bread and subsequently treating individually packaged slices of bread. The treatments identified by bread groups 1 through 8 were as follows:

		Irradiation treatment	
Group	Bread type	Flour	Bread
1	White	+	+
2	White	+	0
3	White	0	+
4	White	0	0
5	Raisin	+	+
6	Raisin	+	0
7	Raisin	0	+
8	Raisin	0	0

Flour used in the study was sealed into 404 x 700 cans by the contractor for the Apollo mission bread and shipped to the University of Nebraska. One-half of the flour was retained by the contractor as unirradiated flour, and the remainder was irradiated at 50,000 rads and returned to the contractor. The bread was prepared, sliced, quick frozen and shipped to the University of Nebraska in the frozen form.

Using a clean room procedure within 1 wk after baking, the frozen bread slices were placed in individual clear plastic pouches (PL540 polyethylene) which were flushed with nitrogen three times and heat sealed. Following packaging, the bread which was to be irradiated was given a 50,000 rad treatment. All bread slices were stored at 21–25°C. Random samplings of 12 slices per group were made initially and at 1, 2, 4, 8, 12, 16 and 20 wk.

Microbiological measurements included determination of percentages of samples with visible mold development of total remaining bread slices; and standard plate counts of total viable aerobic bacteria, coliform organisms, viable yeasts and viable molds for bread and flour (AACC, 1962; Christensen, 1946; Sharf, 1966).

Bacteria, yeast and mold isolates were classified according to standard methods using microscopic morphology and biochemical tests (Society of American Bacteriologists, 1957; Breed et al., 1957; Lodder, 1970; Gilman, 1957; Raper and Fennell, 1965; Raper et al., 1968).

All mold isolates were screened for their ability to produce several mycotoxins (Shotwell et al., 1966; Davis et al., 1966). The extracts were analyzed chemically for mycotoxins using thin layer chromatography (Scott et al., 1970). Mycotoxin standards included aflatoxins (Southern Utilization R&D Div., USDA, New Orleans, La.), penicillic acid (Northern Utilization R&D Div., USDA, Peoria, Ill.), patulin (Agronomy Dept., University of Nebraska, Lincoln, Nebr.), ochratoxin (Food & Drug Directorate, Dept. of National Health and Welfare, Ottawa, Canada) and sterigmatocystin (Calbiochem, Los Angeles, Calif.).

Measurements for chemical changes included TBA values (Tarladgis et al., 1960), pH, infrared spectra of extracted lipids and gas liquid chromatographic profile of volatile compounds.

Lipids for infrared spectroscopy were extracted from 20g of a finely blended and composited bread sample (4 slices) in a soxhlet extractor using ethyl ether:petroleum ether (1:1 v/v) as a solvent. Solvent was subsequently evaporated on a rotary evaporator. A 10% w/w solution of the extracted fat in carbon tetrachloride was prepared. This solution was placed in a 1 mm path length sealed sodium chloride IR cell. The infrared spectrum was obtained with a Beckman IR-5A infrared spectrophotometer operated in a double beam, slow scan mode.

The gas chromatographic profiles of the volatile components of composited bread samples (4 slices/group) were obtained using a Varian-Aerograph Model 1200 gas chromatograph equipped with a flame ionization detector using a stainless steel column 3.2 mm OD x 30.5 cm packed with 8% 1,2,3-tris (2-cyanothoxy) propane on 80/100 mesh Chromosorb G AW-DMCS. Flow rates were 350 ml/min for air, 35 ml/min hydrogen and 35 ml/min nitrogen carrier gas. A headspace sampling, on-column trapping procedure was used for volatile components. The oven was operated for 10 min at an initial temperature of 70°C, and then programmed at 4°C/min increase to 110°C.

Chromatograms of each sample were compared to those of similar type and to those from previous samples to determine changes in the composition of the volatile fraction resulting from treatment or storage of the samples.

A sensory evaluation of the bread was made by 12 trained judges evaluating aroma, appearance, flavor, texture and freshness using a 1 to 5 scale classifying from poor to excellent, respectively. Flavor determinations were terminated after 12 wk because of the development of mold on all slices of breads in some of the treatments. Other observations were continued using visual, aroma and finger evaluation for tactual properties after 12 wk. The results of the evaluations were subjected to analysis of variance (AOV) (Dixon and Massey, 1957).

RESULTS & DISCUSSION

Microbiological determinations

Low dose gamma irradiation reduces the microbial content of the flour but the effect was most striking in the bread. Total detectable aerobic bacteria per g of flour were 600 and 2400 for irradiated and nonirradiated flour, respectively. The total detectable viable mold per g of flour was 24 and 60 for irradiated and nonirradiated flour, respectively.

The percentage of bread slices showing visible mold is shown in Table 1. After 4 wk of storage, a marked difference existed between the nonirradiated white bread control (group 4) compared to all of the irradiated white bread treatments (groups 1, 2 and 3). The dual irradiation, e.g., flour and bread, had superior mold inhibition to that observed from the single irradiation process. Comparing the raisin bread treatments, the nonirradiated control (group 8) and the single irradiation of flour (group 6) were quite similar in the percent of visible mold. The dual irradiation treatment (group 5) and the irradiation of the bread singly (group 7) were quite similar and were substantially lower in mold incidence than groups 6 and 8. This demonstrated the irradiation process advantage in treating the bread which has a variety of ingredients that may contribute to the mold contamination of the finished product.

The microbial total counts substantiate the pasteurizing influence of the low dose irradiation process. Table 2 shows the total aerobic bacteria detected. The dual irradiation treatment (groups 1 and 5) had excellent control of bacterial content of both white and raisin bread. The raisin bread in this study had a very low bacterial content for all groups. On the other hand, the nonirradiated white bread control exhibited bacterial growth throughout the test period. The predominant bacteria type was gram positive cocci (88%), most of which proved to be *Staphylococcus epidermidis*. The remainder were gram positive rods. No coliform or gram negative organisms were detected in any of the samples.

The yeasts detected were primarily of the type used in the baking process, *Sacchromyces cervisiae*. The counts did not relate to the irradiation treatment but did show a marked difference between the bread types. The raisin bread did not have any measurable yeast counts throughout the test.

The molds detected are shown in Table 3. The irradiation process resulted in very low detectable mold counts in the white bread. The control nonirradiated white bread (group 4) had substantial counts throughout the test period. The raisin bread had considerable variation in molds from period to period. However, dual irradiation (group 5) generally resulted in lower mold counts in the raisin bread than the other irradiation treatments. Irradiation of the flour alone (group 6) had no consistent influence compared to the control group (8) insofar as viable mold counts. This is also borne out in Table 1. Irradiation of the raisin bread alone (group 7) did not show a consistent difference compared to group 8 (control) in mold counts but did show substantial differences in visible mold growth shown in Table 1, as previously noted. Stehlik (1967) studied mold inhibition in rye bread using irradiation of a similar dose (50,000 rads) and observed a benefit in that bread type. Stehlik (1967)

Table 1—Percentage of bread samples with visible mold growth

Sample no.	Percent moldy samples					
	4 Wk storage	6 Wk storage	8 Wk storage	12 Wk storage	16 Wk storage	20 Wk storage
1	2.0	2.0	2.1	2.8	2.9	3.6
2	12.1	14.4	9.5	16.9	16.1	20.7
3	11.0	14.5	17.8	26.0	27.1	30.9
4	36.7	44.2	43.0	46.4	47.8	54.0
5	9.0	12.2	12.0	12.3	14.0	16.0
6	30.3	41.2	43.1	39.7	39.4	46.4
7	11.2	13.5	13.0	17.1	16.1	15.0
8	28.7	34.6	37.2	45.2	43.4	48.2

Table 2—Average total viable aerobic bacteria detected/gram of sample as determined by standard plate count

Sample no.	Time							
	Initial	1 Wk	2 Wk	4 Wk	8 Wk	12 Wk	16 Wk	21 Wk
1	30^{1}	10^{1}	2	5	ND[a]	38^{2}	ND	ND
2	50	30^{2}	13^{2}	95^{3}	130^{3}	120^{3}	120^{3}	< 30
3	50	60^{1}	160^{3}	150^{3}	120^{3}	120^{3}	67^{3}	ND
4	25^{1}	43^{1}	60^{3}	310^{3}	140^{3}	52^{3}	51^{3}	84^{3}
5	ND	ND	ND	ND	ND	ND	ND	ND
6	10	49^{1}	2,000	ND	ND	190	ND	ND
7	ND	5	ND	ND	ND	ND	ND	ND
8	ND	190	ND	ND	ND	ND	ND	ND

[a]ND = none detected.

Table 3—Average total viable molds detected/gram of sample as determined by aerobic plate count[a]

Sample no.	Time							
	Initial	1 Wk	2 Wk	4 Wk	8 Wk	12 Wk	16 Wk	21 Wk
1	ND[b]	ND	ND	ND	ND	ND	ND	ND
2	ND	ND	ND	13^{6}	ND	ND	ND	ND
3	ND	ND	ND	5	5	ND	40^{1}	ND
4	ND	ND	ND	900^{3}	480^{3}	28^{5}	46^{2}	85^{2}
5	ND	ND	40^{1}	21^{1}	32^{3}	61^{3}	60^{3}	ND
6	ND	ND	33	29^{2}	15^{5}	ND	13^{5}	98^{3}
7	ND	ND	70^{3}	72^{3}	ND	370^{3}	ND	ND
8	ND	ND	68^{3}	75^{3}	24^{2}	ND	80^{4}	ND

[a]Acidified potato dextrose and pour plate method were used.

[b]ND = none detected.

Table 4—Percentages distribution of different molds and total molds isolated from bread samples

Sample no.	Percentage of molds			
	Aspergillus	Penicillium	Unclassified	Total
1	1.1	ND[a]	ND	1.1
2	ND	4.6	ND	4.6
3	ND	4.6	3.4	8.0
4	6.9	13.8	1.1	21.8
5	ND	8.0	4.6	12.6
6	5.7	14.9	ND	20.6
7	2.3	4.6	5.7	12.6
8	2.3	16.1	ND	18.4

[a]ND = none detected.

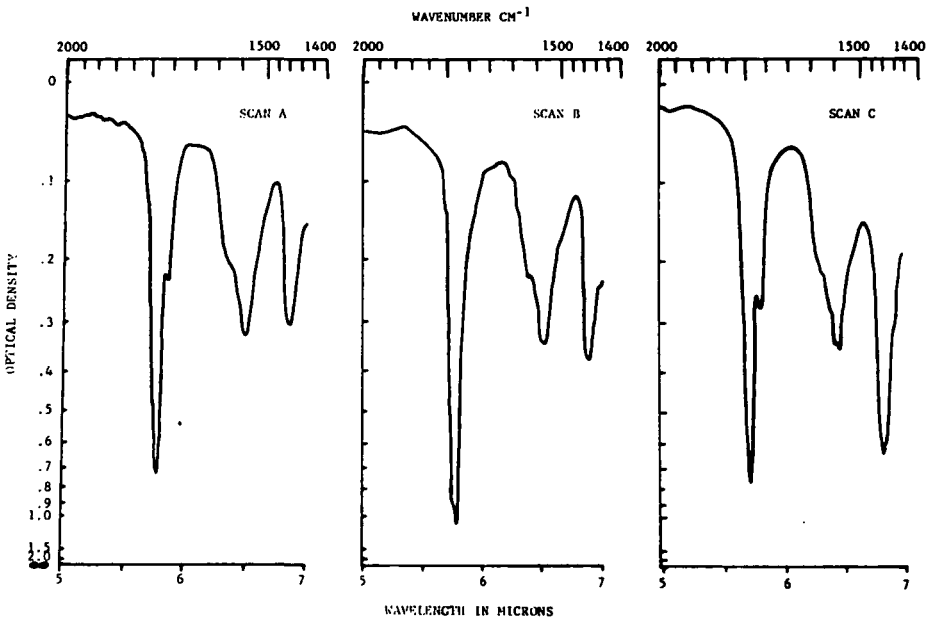

Fig. 1—Comparison of infrared scans of bread lipids from irradiated bread (Scan A) and nonirradiated bread (Scan B) at 4 wk storage and nonirradiated bread (Scan C) at 16 wk storage.

did not consider a dual irradiation process but did use a heating treatment (50°C) prior to irradiation which had an additive effect in retarding mold development.

As a means of estimating the influence of the low dose irradiation on mold types, the isolates were grouped for the total test period of each of the treatments. A comparative identification of mold types from the nonirradiated and irradiated flour indicated a shift in mold types as a result of irradiation. The irradiation process first reduced the total number of viable molds by approximately 50%. The molds were typed as to *Aspergillus, Penicillium* and Unclassified. In the nonirradiated flour, the distribution percentages of the molds typed were 85.3 of the combined *Aspergillus-Penicillium* and 14.7 Unclassified. In the irradiated flour, the distribution percentages of mold types were 44.6 and 55.4, respectively. This is of particular significance since these mold genera (*Aspergillus* and *Penicillium*) are among those capable of producing toxic and carcinogenic metabolites.

Identification of the mold isolates from the bread types is summarized in Table 4. This is expressed as a percent of total mold isolates. These data from the white bread types (groups 1–4) show a similar change to that observed in the irradiation treatment of flour. The influence of irradiation on the raisin bread types followed a similar pattern when comparing the nonirradiated control (group 8) to the dual irradiated treatment (group 5). Thin layer chromatography screening of the mold isolates did not identify any organisms capable of aflatoxin production. However, some isolates were noted which produced other mycotoxins. Penicillic acid was produced by 7.2% of the isolates and patulin was found to be produced by 4.3%. One isolate of *Aspergillus ochraceus* produced both ochratoxin and penicillic acid. Sterigmatocystin production was suspected in 4.3% of the isolates. This is currently under further study.

Chemical determinations

The influence of irradiation upon development of oxidative rancidity was measured using TBA values. No statistically significant difference was noted due to irradiation treatment in either the white bread or the raisin bread. There was an increase in TBA values with storage time; however, this was not large.

During the sampling procedures, measurement of bread pH was monitored and no statistical differences were noted due to irradiation, but differences were apparent between the bread types with white bread higher than raisin bread and a decreasing trend with storage time.

Comparisons of gas chromatography (GC) profiles within a given bread type were made initially and over each of the sampling periods to 20 wk. A general decrease occurred with time. The GC profiles of the irradiated white bread (groups 1, 2 and 3) could not be distinguished from the control (nonirradiated) bread (group 4).

The general GC profile for raisin bread was notably different from that of the white bread. However, again no distinguishable differences appeared when comparing any of the irradiation treatments to the control (nonirradiated raisin bread).

The characteristics of the lipids, as measured using infrared spectrophotometry, were compared within the bread types as influenced by irradiation. In the raisin bread comparisons, no distinguishing differences were noted due to irradiation treatment or storage time. The infrared scan of raisin bread did differ from the white bread.

On the other hand, the infrared scans of the white bread, lipids in early storage showed a major change in the irradiation treatments. However, this was also associated with storage time in the nonirradiated white bread. An absorption peak at 5.85μ (1700 cm^{-1}) appeared in groups 1, 2 and 3 at the 4 wk sampling. The region is in the area denoting a carbonyl peak. The control (nonirradiated) white bread began to show a similar second carbonyl peak after 12 wk of storage. The appearance of this peak was apparently triggered by the irradiation treatment but could not be explained as entirely due to irradiation because of its appearance later in the control bread. Figure 1 shows a typical infrared scan of nonirradiated and irradiated white bread with the carbonyl peak being noted. This component was noted by Chung et al. (1967) and was attributed to irradiation. However, as our study shows this carbonyl group appeared

Table 5—Average freshness scores in 1–5 hedonic scale of white bread samples

Bread group	1	2	3	4
Initial	3.0	3.2	3.0	3.2
1-wk	2.3	2.4	2.4	2.2
2-wk	1.9	2.1	1.9	1.6
4-wk	1.6	1.4	1.8	1.4
8-wk	1.7	1.6	1.6	2.1
12-wk	1.3	1.3	1.3	1.2
20-wk	1.2	1.1	1.1	1.0

in nonirradiated bread with sufficient time, which does not appear to be associated with gamma irradiation. Bisulfite derivatization was attempted in order to identify this carbonyl but could not be recovered in workable concentration. Known aldehydes were evaluated for absorbance and a similar response was observed when propionaldehyde was used. Further comparison was made of the lipid fraction using gas chromatography and similar retention times were observed with propionaldehyde and the unknown fraction. Thus the new carbonyl compound was tentatively identified as propionaldehyde.

Organoleptic determinations

The panel members were asked to rank randomly coded bread samples for aroma, appearance, flavor, texture and freshness. The comparisons were run separately for white bread and raisin bread. Panelists were not served bread which had an outward appearance of mold growth so the appearance score was not indicative of this condition. Further, it should be recognized that mold presence would have an influence upon aroma, flavor and freshness and yet in order to evaluate the bread, it was deemed desirable to exclude samples showing mold.

Statistical analysis (AOV) of the mean scores for each characteristic within each bread type over the total test period and within each sampling period did not show any significant difference due to irradiation. This is in contrast to findings reported by Miller et al. (1965) in which the products were noted as being less palatable using a comparable irradiation level. Nicholas et al. (1958) stated that a bread flavor threshold was noted at $1.0 \times 10^{4.6}$ rep in irradiated flour and that all panels distinguished an undesirable flavor at a level of 1×10^5 rep.

Measurement of bread quality was based upon general appearance of loaf volume and textural appearance of the treated groups versus the control or nonirradiated bread. The bread type defined by NASA is a more dense bread with smaller loaf volume than the typical bread found in U.S. retail markets. No apparent differences were noted in bread quality due to the irradiation treatments, initially.

Freshness scores were significantly (5%) different due to storage periods. Panel scores were generally higher for raisin bread than for white bread for aroma and flavor with little difference in texture and freshness. The raisin bread appearance was lowered as a brown tint spread from the raisins into the surrounding bread such that by 20 wk, the bread was totally tinted. This condition did not appear to be associated with the irradiation treatment.

The degree of acceptability of the bread in the study was not judged on the basis of when the panelist would reject but rather on a hedonic scale. In general, however, panelist comments on the ballots suggest that loss of total acceptability occurred after 4 wk. The application of low dose irradiation provides a means of extending general acceptability of fresh bread based primarily on microbiological characteristics.

An interesting trend appears in the freshness data for the white bread. These data appear in Table 5. The panel scores tend to favor the irradiation treatment of the samplings at 1 wk, 2 wk and 4 wk. The freshness scores would be comparative within treatments but would not necessarily be similar to commercial bread. Samples of the bread were examined for the amount of shear force using a Kramer shear press. No differences were noted in the shear values using a raisin bread comparing the control (nonirradiated—group 8) versus the irradiation treatments (groups 5, 6 and 7). However, the shear values were consistently lower for the white bread involving irradiation treatment (groups 1, 2 and 3) compared to group 4 nonirradiated white bread. These data suggest that an acceptability factor can be involved in favor of the irradiation process as used in this study. Improved bread quality was noted by Miller and Yen (1956), but specific characteristics were not reported.

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Mycotoxin-Producing Potential of Molds Isolated from Flour and Bread[1]

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Summary

Wheat flour, white bread, and raisin bread were examined for their mold content. Molds were isolated, classified to the genus level, and chemically screened for production of several mycotoxins. Total counts and specific incidences of toxic molds were generally low in both flour and bread. Flour contained more potentially toxic molds than did bread.

THE MOLD FLORA of flour is primarily limited to species of *Aspergillus* and *Penicillium* (1,2). Within these genera certain specific groups are usually present. In the genus *Aspergillus* these include members of the *A. candidus, A. glaucus, A. flavus-oryzae,* and *A. versicolor* groups; while in the *Penicillium* genus the commonly found series include *P. cyclopium, P. citrinum,* and *P. urticae (patulum)* (3). A number of molds within these groups have been shown to produce toxic metabolites (mycotoxins). Some of these metabolites are also carcinogenic. Strains of *A. flavus* and *A. parasiticus* of the *A. flavus-oryzae* group produce aflatoxins, and strains of *A. versicolor* produce sterigmatocystin, both of which are carcinogenic (4). Strains of *P. cyclopium* and *P. martensii* of the *P. cyclopium* series produce penicillic acid, and *P. patulum* has been found to produce patulin. These toxins are also carcinogenic (4-7). *Penicillium citrinum* has been shown to produce citrinin, a nephrotoxin (4), *A. ochraceus* produces ochratoxin, and a number of Penicillia produced leuteoskyrin. Both toxins cause liver damage in rats and mice (4). The mold flora of flour has been widely studied, but there are no reports relative to the mycotoxin-producing ability of the molds commonly found in flour and bread. For that reason, this study was initiated.

Preliminary examination of three brands of bleached wheat flour and three brands of white and raisin breads stored for different lengths of time showed various degrees of fungal contamination. The majority of the molds appeared to belong to the *Aspergillus* and *Penicillium* genera (8). Since certain members of these two genera have the potential to produce mycotoxins, it was decided to isolate representative strains of Aspergilli and Penicillia from wheat flour, as well as white bread and raisin bread, and to screen them for the ability to produce mycotoxins.

The microbiological examination of flour and bread was according to the AACC Method 42-50 for yeast and mold counts (9), except that the plating media used were malt agar plus 7.5% NaCl (10), and potato-dextrose agar (Difco) plus 30 p.p.m. sterile tetracycline-HCl added to inhibit bacterial growth. Both media were sterilized by autoclaving at 121°C. for 15 min. The tetracycline solution was sterilized by filtration and aseptically added to the potato-dextrose agar after sterilization. Plates were incubated at 25°C. for 3 to 5 days, then counted using either a Quebec Colony counter or a steromicroscope. Representative colonies of mold were isolated and purified by streaking on the same type of agar medium from which they were isolated. The purified cultures were transferred to potato dextrose agar (Difco) slants and Czapek's solution agar (Difco) slants for storage. The isolates were classified to the genus level using the keys of Raper and Fennell (11) and Raper and Thom (12). The molds were then chemically screened for mycotoxin production by growing the organisms in flask culture using yeast-extract sucrose (YES) broth (13) and sterilized long-grain white rice; extracting with chloroform and comparing the extracts on thin-layer chromatography (TLC) plates (20 X 20 cm., 0.25-mm.-thick Silica Gel G-HR; developing solvent, toluene:ethyl acetate:formic acid, 60:30:10) with standard mycotoxins. The extracts were screened for aflatoxins B_1 and G_1, sterigmatocystin, ochratoxin A, penicillic acid, patulin, citrinin, and leuteoskyrin. Aflatoxin standards were obtained from the Southern Utilization Research and Development Division, USDA, New Orleans, La.; penicillic acid from A. Ciegler, Northern Utilization Research and Development Division, USDA, Peoria, Ill.; patulin from T. M. McCalla, University of Nebraska, Lincoln; sterigmatocystin from Cal Biochem, Los Angeles, Calif.; ochratoxin A, citrinin, and leuteoskyrin from P. M. Scott, Food and Drug Directorate, Ottawa, Ont. The method used was essentially that of Scott et al. (14) with some additional steps. All culture extracts were compared to standards using three different TLC plates to make four

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observations. Developed TLC plates were observed under long- and short-wave ultraviolet (UV) lights to detect fluorescence of aflatoxins, sterigmatocystin, ochratoxin A, citrinin, and leuteoskyrin. One plate was then subjected to ammonia fumes for 5 to 6 min. and then observed again under long- and short-wave UV lights. This treatment increased the fluorescence of ochratoxin A and formed bluish fluorescent derivatives of penicillic acid and patulin. The two remaining TLC plates were sprayed with a *p*-anisaldehyde reagent and phenylhydrazine, according to the method of Scott et al. (14), then examined in visible and long-wave UV light. The color reactions of the various toxins are given by Scott et al. (14). No extracts were counted as positive for a specific toxin unless all tests were positive. When the method was tested using known toxin-producing molds its ability to detect the various toxins in culture extracts was verified.

The total mold counts of the flour samples examined ranged from 12 to 500 g. The mold counts of the bread were very low. A total of 70 molds were isolated from flour and bread samples. Of these isolates, 16 were classified as *Aspergillus* species, 48 as *Penicillium* species, and six as other genera. All of the Aspergilli and Penicillia were screened for mycotoxin production. No aflatoxin-producing molds were found from either flour or bread. However, nine strains of Penicillia isolated from flour produced penicillic acid, and two produced patulin. None of the Aspergilli isolated from flour produced any of the toxins in question. Four *Penicillium* isolates from bread produced leuteoskyrin. One isolate of *Aspergillus ochraceus* from bread produced both ochratoxin A and penicillic acid. No citrinin production was noted in any of the isolates from either flour or bread. The flour contained more potentially toxic molds than the bread. All of the toxin-producing molds isolated from flour were Penicillia, and they produced predominantly penicillic acid and patulin. One mold isolate from flour, identified as *P. patulum*, was found to produce very high levels (850 γ per ml.) of patulin in potato-dextrose broth. The authenticity of the compound was confirmed using UV and infrared spectral data as well as melting point determinations and TLC data.

Mold content of flour varies with brand, which probably reflects differences in sanitation in the mills. Although toxin-producing molds found in flour are not extremely high in number, they are of interest since they can be carried into other food products of which flour is an ingredient. If these food products are not properly processed, the molds may have an opportunity to survive, grow, and produce toxins. Also, during mixing of food ingredients flour can serve as a source of mold contamination to the atmosphere of the processing establishment, which may recontaminate products after processing. However, apparently the bread we examined was relatively free of total mold as well as specific toxic strains of mold. Since mold spores are not particularly heat-resistant, one can expect molds from the flour to be killed in the baking process. Mold contamination of the bread may therefore be expected to be primarily caused by recontamination after baking.

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that could have the potential of producing aflatoxins. The studies involved using broth and bread substrates. Publications are included that report the major findings of these studies. The general conclusions indicate that radurization reduces the potential of a public health concern for surviving bacteria and molds.

Prior to and during the Skylab missions a series of studies was initiated to consider the application of irradiation to dried food mixes. The general procedure for preparing the Skylab dried food mixes was the incorporation of a large variety of freeze dried or conventionally dried ingredients under a sterile room environment. These mixes were sealed and the resultant mix monitored for final microbial content. The approach considered under this contract was the desire to reduce the contamination or the contamination potential where a variety of ingredients are blended following thermal processing. These studies first examined the relative survival of selected organisms of public health concern on a dry substrate when exposed to radurization. As reported, the selected bacteria and molds were significantly reduced or eliminated when gamma irradiation was applied at the levels tested--50 to 200 Krads. This demonstrated that the radurization process would be a feasible treatment for dried food mixes. Selected Skylab dried food mixes were exposed to the radurization process. Microbial content was measurably reduced and no undesirable organoleptic characteristics were noted. Measurement for oxidative rancidity using TBA values indicated that no undesirable shifts occurred using the radurization process.

A side question was examined to determine how radurization might influence the rehydration of freeze dried foods. This related to concerns during the Skylab missions on poor acceptability of thermally stressed dried food mixes aboard during the high temperature cabin conditions. Radurization was applied to commercially freeze dried foods--individual and multiple food mixes. The process was reported as not having either a beneficial or detrimental effect on rehydration. A technique was tested reported as "moisture tempering." Freeze dried foods were moisture tempered--adding 10% by weigh water. The products were radurized and subjected to thermal stress. Several freeze dried foods--carrots and diced meats had improved rehydration characteristics as a result of the process. Details of these studies were contained in the monthly reports.

The desire to place pastries aboard for the Apollo-Soyuz led to several studies applying radurization to selected pastries. Those having high mold counts because of the nature of preparation or ingredients were chosen. These included donuts, filled pastries and

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USE OF γ-IRRADIATION TO PREVENT AFLATOXIN PRODUCTION IN BREAD

INTRODUCTION

THE USE OF low level or pasteurizing doses of gamma irradiation to extend the shelf life of certain foods has been studied by numerous workers (Bellamy, 1959; Hannesson, 1972; Niven, 1958). These levels of irradiation do not sterilize food products but rather reduce the microbial population and thereby extend storage life. This application of irradiation was employed by Hartung et al. (1973) to extend the shelf life of fresh bread used in food systems for manned space flight missions.

Molds are a common and important cause of spoilage of bread (Frazier, 1967). Mycostatic agents, principally propionates, are added to bread to delay mold growth, but molding still occurs after extended storage. Mold spoilage of bread containing mold inhibitors was noted in early manned space missions (Hartung et al., 1973). By applying a 50 Krad dose of irradiation to flour and subsequently to bread, Hartung et al. (1973) showed a significant reduction in the amount of visible and total mold that developed on the bread during storage up to 20 wk. However, a small percentage of molds survived and were capable of outgrowth during storage.

A number of common spoilage molds are capable of producing secondary metabolites that are both toxic and carcinogenic to a wide range of animals (Detroy et al., 1971). One such group of these compounds are the aflatoxins, which are produced by certain strains of *Aspergillus flavus* and *A. parasiticus*. Miyaki et al. (1967) reported that aflatoxins were resistant to ionizing irradiation doses as high as 30 Mrad. Jemmali and Guilbot (1969; 1970a, b) observed that exposure of *A. flavus* spores to gamma irradiation doses of less than 200 Krad tended to induce or increase aflatoxin production in subsequent cultures.

Hartung et al. (1973) found that *Aspergillus* and *Penicillium* species predominated among molds isolated from unirradiated flour and bread. While irradiation treatment lowered the incidence of these organisms, surviving spores had the capability of outgrowth. The possibility that such survivors might be capable of toxin production or might have altered patterns of toxin production raises concerns about the use of irradiation as a method of food preservation, not only for space food systems, but food systems in general. This work was initiated to study the effects of selected low levels of gamma irradiation on the ability of strains of *A. parasiticus* to survive and produce aflatoxin in a fresh bread system.

EXPERIMENTAL

Organisms

Aspergillus parasiticus strains NRRL 2999 and NRRL 3000, obtained from the culture collection of the USDA Northern Regional Research Laboratory, ARS, Peoria, Ill., were used in this study. Both are known aflatoxin-producing cultures. Strain 2999 is generally a more potent aflatoxin producer than strain 3000. Stock cultures were maintained at 2–4°C on slants of Difco potato dextrose agar in screw cap test tubes.

Inoculum

Cultures of the toxic molds were grown on potato dextrose agar slants for 10 days at 25°C until well sporulated. The spores were washed from the slants with a sterile 0.01% solution of Tween 80. The harvested spores were suspended in 100 ml of sterile Tween 80 and aseptically filtered through sterile cheesecloth to remove mycelial debris. The filtered suspensions were quantitated using a Petroff-Hausser counting chamber. Portions of the suspenions were diluted to obtain two levels of inoculum, approximately 10^2 and 10^6 spores/bread slice. The dilutions were made so that the required number of spores were contained in 0.1 ml of spore suspension. One-half of this amount was applied to each side of each slice of bread.

Inoculation, packaging and irradiation of bread slices

The bread used in this study was baked without mold inhibitor by a local bakery and was obtained and used within 12 hr of baking. The slices of bread were aseptically removed from the packaged loaves and placed on sterile towels inside a bacteriological glove box. The interior surfaces of the glove box had previously been sanitized with a 50% solution of household bleach and exposure to germicidal UV light for 1 hr before use. The bread slices were exposed to germicidal UV light for 15 min per side prior to inoculation. The slices were inoculated and the inoculum was spread over the surface of the bread by brushing it with a flamed inoculating loop. The inoculated slices were individually packaged in polyethylene pouches (PL540, W.R. Grace Co.) and the pouches were sealed under an air atmosphere. The inoculated slices were irradiated at 0, 100 and 200 Krad at ambient (ca 25°C) temperature using a Cobalt 60 source similar to the one described by Teeny and Miyauchi (1970). Dosimetry for irradiating the bread was established using a Fricke Dosimeter, ASTM 01671-63. The bread was stored for 10 days at 25°C, and then analyzed for aflatoxins and total mold content.

Extended storage studies were also included wherein white bread slices were inoculated, packaged and irradiated in the same manner as described above. The bread slices were then stored at 25°C, and examined at 0, 1, 2, 4 and 6 wk. Samples were evaluated for aflatoxin content, total molds, yeasts and bacteria.

In all treatments duplicate samples were used and the studies were replicated three times.

Analyses of bread slices

The bread slices were observed for visible mold growth during the storage periods. After storage, duplicate samples were composited by blending in a sterile blender. Total bacteria, yeast and mold counts were made using standard methods (Sharf, 1966), except that instead of acidifying potato dextrose agar for yeast and mold counts, 30 ppm tetracycline was used to prevent bacterial growth.

The composited samples were also analyzed for aflatoxin content using a modification of the extraction method of Pons et al. (1966), in which 100% ethyl alcohol was substituted for acetone in the extraction solvent. This resulted in cleaner extracts that did not as readily form emulsions during the subsequent chloroform extraction step. The aflatoxin concentration in the extracts were estimated by visual comparison of the fluorescence of the samples to known standards on exposure to long wave UV light (Chromatovue Cabinet, Model CS) using thin-layer chromatography (TLC) plates (20 × 20 cm, 0.25 mm thick Silica Gel G-HR, Brinkmann Instruments, Inc.). The TLC plates were developed in toluene/ethyl acetate/90% formic acid (60/30/10) according to the method of Scott et al. (1970). Standard aflatoxins were obtained from the USDA Southern Utilization R & D Div., New Orleans, La.

RESULTS & DISCUSSION

Growth

The amount of growth of toxic strains of *A. parasiticus* in bread was reduced by low dose gamma irradiation of inoculated bread (Table 1). Both 100 and 200 Krad doses resulted in lower amounts of detectable toxic mold in all treated bread

Table 1–Effect of irradiation on total number of mold propagules per gram of white bread inoculated with Aspergillus parasiticus NRRL 2999 and NRRL 3000 and stored at 25°C for 10 days

Strain	Inoculum level/slice	Number of mold propagules/g of bread		
		Control	100 Krad	200 Krad
NRRL 2999	10^2	8.3×10^7	2.8×10^6	2.1×10^4
	10^6	1.7×10^7	1.5×10^6	4.1×10^4
NRRL 3000	10^2	3.3×10^8	4.4×10^4	9.5×10^5
	10^6	1.1×10^5	5.0×10^5	9.1×10^4

Table 2–Effect of gamma irradiation on the production of aflatoxins by Aspergillus parasiticus NRRL 2999 and NRRL 3000 on white bread stored at 25°C for 10 days

Strain	Level of spores/slice	Aflatoxin	µg Aflatoxin/g of bread[a]		
			Control	100 Krad	200 Krad
NRRL 2999	10^2	B_1	33	–	–
		G_1	218	–	–
	10^6	B_1	29	22	–
		G_1	164	77	–
NRRL 3000	10^2	B_1	49	< 0.1	–
		G_1	249	2	<0.01
	10^6	B_1	5	2	–
		G_1	50	15	–

[a] – None detected

Table 3–Effect of gamma irradiation on aflatoxin (B_1 + G_1) production by Aspergillus parasiticus NRRL 2999 and NRRL 3000 on white bread stored at 25°C for various periods up to 6 wk

Storage time (wk)	µg Aflatoxins (B_1 + G_1)/g of bread[a]											
	Strain 2999						Strain 3000					
	10^2 spores/slice			10^6 spores/slice			10^2 spores/slice			10^6 spores/slice		
	Control	100 Krad	200 Krad	Control	100 Krad	200 Krad	Control	100 Krad	200 Krad	Control	100 Krad	200 Krad
1	182	–	–	16	111	–	105	–	–	15	6	–
2	46	0.03	–	540	640	0.05	370	–	–	24	122	0.02
4	70	–	–	113	41	13	16	–	–	5	81	–
6	4	–	–	132	1061	–	34	–	–	264	227	–

[a] – None detected

samples except in one case. Visible mold was evident to varying degrees on all control samples after storage at 25°C for 10 days. The control cultures of both strains produced more apparent growth at the 10^2 spore inoculum than the 10^6 spore level. The effect was greatest with strain NRRL 3000. This may represent some type of competitive effect or auto-inhibition that is related to spore concentrations and limited growth conditions. Visible mold was not evident to any appreciable degree on the slices treated with a 100 Krad dose and was completely lacking on slices given a 200 Krad dose with strain NRRL 2999. There appeared to be little difference between a light and heavy inoculum in terms of survival and amount of subsequent visible growth after irradiation treatment. Strain NRRL 3000 was more variable than strain NRRL 2999 in amounts of growth noted within and between treatments. Strain NRRL 2999 appeared to be more resistant to the 100 Krad dose than was strain NRRL 3000.

Bread slices that were inoculated, irradiated and stored for up to 6 wk showed similar trends, as the bread stored for 10 days, toward lower amounts of toxic mold during the first 2 wk of storage. However, as the storage time increased, the molds tended to overcome the effects of irradiation and the amounts of mold growth in treated slices at 6 wk of storage approached the amounts found in the control samples. Peak amounts of growth were reached in less time in the control samples than in the treated slices. Peak amounts of mold occurred later in the 200 Krad treated samples than in 100 Krad treatment. There did not seem to be a noticeable difference in amount of toxic mold growth between a light and heavy inoculum within any of the treatments. Apparently, the long storage time allowed sufficient time for surviving spores to grow out and produce approximately the same amount of mold mass whether a few spores were present initially or whether the numbers were greater. Other factors such as nutrients, moisture and available oxygen within a given package also might have affected the amount of growth produced.

Examination of inoculated bread slices for yeasts and bacteria showed only a very low incidence of yeasts. Bacterial numbers were initially very low but tended to increase with storage times. The patterns of bacterial numbers did not reflect any particular trends as a result of the various treatments. The numbers of bacteria found in irradiated samples were of a similar order of magnitude as in control samples. Reduction in mold growth did not result in an increase in bacterial numbers.

Aflatoxins

Gamma irradiation of bread slices inoculated with spores of *A. parasiticus* NRRL 2999 and 3000 reduced the amount of aflatoxins produced on subsequent storage and incubation (Table 2). The 200 Krad dose essentially eliminated aflatoxin production by either strain at both levels of spore inoculation during 10 days of storage. These results agree with those of Jemmali and Guilbot (1970a) who reported that gamma irradiation doses of 200 Krad and above tended to reduce the ability of *A. flavus* to produce aflatoxins.

The 100 Krad treatment level also reduced the amount of aflatoxins detected in inoculated bread at the end of the 10 day storage period (Table 2). With strain NRRL 2999 no aflatoxins were detected at the 10^2 spore inoculum level but with

strain NRRL 3000 small amounts of toxins were found. Higher amounts of aflatoxins were noted in samples inoculated with 10^6 versus 10^2 spores at the 100 Krad dose treatment with both strains. The total mold counts did not differentiate between mold types and since complete sterilization of the substrate before inoculation was not attempted, it is possible that other mold types may have added to the load detected at the lower inoculum levels. With the larger inoculum it is likely that more spores survived the irradiation treatment and were more capable of competing with any other microbial growth that occurred.

Aflatoxin production in inoculated bread samples irradiated and stored for up to 6 wk followed the same general pattern as observed with the bread stored for 10 days (Table 3). With the 200 Krad dose, no aflatoxins were detected in any bread samples that were inoculated with 10^2 spores of either NRRL 2999 or 3000. With 10^6 spores at 200 Krads, trace amounts of aflatoxins were found with both organisms at 2 wk of storage. And with 10^6 spores of strain NRRL 2999 at the 200 Krad treatment level significant amounts of aflatoxins were detected at 4 wk of storage. Except for small amounts of aflatoxins found at 2 wk of storage with strain NRRL 2999, no toxins were detected in any of the bread inoculated with 10^2 spores and irradiated at 100 Krads. But with 10^6 spores both organisms produced very high amounts of aflatoxins after irradiation at 100 Krad and subsequent storage.

The data suggest that stimulation of aflatoxin production may have occurred at the 100 Krad treatment level in samples inoculated with 10^6 spores after 1, 2 and 6 wk of storage (Table 3). This would be supported by the findings of Jemmali and Guilbot (1970). However, it should be remembered that there is considerable natural variation in aflatoxin production by a given mold strain and between strains. Also, because of the substrate and the conditions of the experiment it was impossible to determine the amount of aflatoxins produced per unit mass of mold mycelia, which would be necessary to conclusively prove stimulation of toxin production. At this point, stimulation of toxin production at the 100 Krad level in a bread substrate certainly appears probable, but further studies are needed for more conclusive evidence. There seemed to be more stimulation of toxin production in strain NRRL 2999.

Production of aflatoxins by *A. parasiticus* strains on bread was effectively eliminated by treatment of the inoculated bread with 200 Krad doses of irradiation. The mold strain as well as the initial load of spores were factors in the organisms' ability to produce aflatoxins after irradiation treatment. Strain NRRL 2999 seemed more capable of toxin production after irradiation treatment than strain NRRL 3000. With the lower irradiation dose, cultures from either strain using 10^6 spores/slice were more capable of aflatoxin production after treatment than were cultures from 10^2 spores. The 100 Krad dose appeared to stimulate aflatoxin production at 1, 2 and 6 wk of storage when 10^6 spores/slice were used for inoculum. But this could not be shown when an inoculum of 10^2 spores/slice was used. It is apparent that the higher irradiation dose provided a greater margin of safety in preventing the development of aflatoxins in bread under the conditions of this study.

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A Research Note
EFFECT OF LOW LEVEL GAMMA IRRADIATION ON GROWTH AND PATULIN PRODUCTION BY Penicillium patulum

INTRODUCTION

LOW DOSE gamma irradiation has potential to extend the shelf-life of certain foods (Hannesson, 1972). Hartung et al. (1973) applied a 50 Krad dose to flour and to bread made from the flour and obtained a reduction in the amount of visible and total mold that developed on the bread during storage up to 20 wk. However, a small percentage of molds survived and were capable of outgrowth during storage. The predominate molds found were species of *Aspergillus* and *Penicillium*.

Several workers have reported that gamma irradiation doses below 200 Krad may induce or increase aflatoxin production by *A. flavus* (Jemmali and Guilbot, 1969; 1970a, b; Applegate and Chipley, 1973a, b; Schindler and Noble, 1970). However, other reports show no stimulation of aflatoxin production by this same irradiation dose range in either *A. flavus* or *A. parasiticus* (Schindler et al., 1972; Bullerman and Hartung, 1974). These discrepancies may be due to strain and species differences.

If low level gamma irradiation is to be considered as a potential method of food processing, more information is needed concerning the effects of irradiation on microorganisms important to the public health, including mycotoxin-producing molds. Aside from the work with aflatoxins, little is known of the effects of low level gamma irradiation on molds capable of producing other mycotoxins. Applegate and Chipley (1974) reported increased ochratoxin production by *Aspergillus ochraceus* after irradiation at 150 and 200 Krads. The work reported herein was initiated to determine the effects of low level gamma irradiation on growth and patulin production by strains of *Penicillium patulum*. This organism had previously been isolated from irradiated bread raising concerns about possible effects of irradiation on patulin production in bread (Hartung et al. 1973).

EXPERIMENTAL

ALIQUOTS of 50 ml of potato dextrose broth (pH 5.6) prepared according to the method of Norstadt and McCalla (1969), were used to support growth and patulin production by *P. patulum* in still cultures in 250 ml Erlenmeyer flasks before and after irradiation. Two strains of *P. patulum* were used: strain M108 isolated in our laboratory from irradiated bread; and strain NRRL 989 obtained from the culture collection of the USDA Northern Regional Research Lab. ARS, Peoria, Ill.

Spore suspensions in sterile 0.01% Tween 80 were prepared from 10-day old potato dextrose slant cultures of the organism as previously described (Bullerman and Hartung, 1974). Aliquots of the spore suspensions were irradiated at 0, 100 and 200 Krad at ambient temperature (ca 25°C) using a Cobalt 60 source similar to the one described by Tenny and Miyauchi (1970). Dosimetry for irradiating the inocula was established using the Fricke Dosimeter, ASTMD1671-63. The irradiated and nonirradiated spore suspensions were used to inoculate the flasks of potato dextrose broth in amounts of approximately 10^6 spores per flask. Growing vegetative mycelia, on potato dextrose slants, were also irradiated at the same dosage levels as the spore suspensions. Portions of the mycelia were used to inoculate potato dextrose broth. All treatments were done in duplicate, and the study was replicated three times.

All cultures were incubated at 25°C for 7 days and then given a brief heat treatment (121°C for 30 sec) to kill spores and vegetative mycelia. The mold mats were collected, washed with distilled water, dried at 130°C for 2 hr and weighed. Mold growth was expressed as mg mycelial dry weight. The pH of the filtered broth was determined.

Patulin was extracted from the broth using ethyl acetate in two liquid-liquid extractions of 50 ml each. The extracts were concentrated and the patulin concentration was estimated by visual comparison of samples to known amounts of patulin on thin-layer chromatography (TLC) plates (20 × 20 cm, coated with a 0.25 mm thick layer of Silica Gel (G-HR, Brinkmann Instruments). The plates were developed in toluene/ethyl acetate/formic acid (60/30/10). Patulin was observed as a light-blue fluorescent spot under long wave UV light after exposure to ammonia fumes, and as a yellow colored derivative in natural light after spraying the plates with 4% phenylhydrazine in water and heating for 3 min at 110°C (Scott and Somers, 1968).

Table 1—Production of patulin by Penicillium patulum NRRL 989 and M108 on potato dextrose broth in 7 days of incubation at 25°C when grown from irradiated spores

Irradiation level (Krad)	NRRL 989		M108	
	Broth (μg/ml)	Dry Mycelia (μ/mg)	Broth (μg/ml)	Dry mycelia (μg/mg)
	Patulin			
0 (Control)	137	24	376	67
100	88	19	122	26
200	0.2	0.09	2	0.6

Table 2—Production of patulin by Penicillium patulum NRRL 989 and M108 on potato dextrose broth in 7 days of incubation at 25°C when grown from irradiated vegetative mycelia

	NRRL 989		M108	
Irradiation level (Krad)	Culture broth (μg/ml)	Dry mycelia (μg/mg)	Culture broth (μg/ml)	Dry mycelia (μg/mg)
	Patulin			
0 (Control)	32	9	13	3
100	ND[a]	–	0.7	0.2
200	ND[a]	–	ND[a]	–

[a] ND = None detected.

RESULTS & DISCUSSION

Growth

Low level gamma irradiation of spores of *P. patulum* reduced subsequent growth in potato dextrose broth of both strains studied. After 7 days of incubation, the growth of strain NRRL 989 from spores irradiated at 100 Krad was 81% of the control. Growth from spores irradiated at 200 Krad was 40% of the control. Strain M108 was somewhat more resistant to irradiation than strain NRRL 989. Growth of strain M108 from spores irradiated at 100 Krad and 200 Krad was 84 and 65% of the control, respectively. Irradiation of growing vegetative mycelia resulted in variable growth by subsequent cultures in potato dextrose broth. The growth of strain NRRL 989 from irradiated mycelia was equal to or greater than the control at both the 100 and 200 Krad doses. The growth of strain M108 was less than the control at the 100 Krad dose, but equal to the control at the 200 Krad dose. The final pH of all control cultures was lower than the final pH of cultures grown from irradiated inocula.

Patulin production

Production of patulin by cultures grown from irradiated spores was substantially less than the control cultures (Table 1). Strain NRRL 989 produced 36% less patulin after irradiation at 100 Krads and 99+ % less after irradiation at 200 Krads. Strain M108 produced 68% less patulin at 100 Krads and 99+ % less at the 200 Krads dose. Patulin production by cultures grown from irradiated vegetative mycelia produced even less patulin than cultures from irradiated spore cultures (Table 2). Strain NRRL 989 did not produce detectable amounts of patulin after either irradiation treatment of mycelia and strain M108 produced trace amounts of patulin after irradiation of mycelia at 100 Krads.

There was no stimulation of patulin production observed in strain NRRL 989 or M108 from spores or mycelia by either level of irradiation. The amount of patulin produced per mg of dry mycelia declined steadily as the irradiation dosage increased. Complete inhibition of growth or patulin production was not achieved by irradiation of spores at levels up to 200 Krads. Irradiation of mycelia likewise did not eliminate growth, but did eliminate patulin production at the 200 Krad dose level.

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EFFECT OF LOW DOSE GAMMA IRRADIATION ON GROWTH AND AFLATOXIN PRODUCTION BY *ASPERGILLUS PARASITICUS*[1]

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ABSTRACT

Spores and growing vegetative mycelia of *Aspergillus parasiticus* strains NRRL 2999 and NRRL 3000 were irradiated at 100 and 200 Krad, and the effects on growth and aflatoxin production in yeast-extract sucrose (YES) broth were measured. Irradiation of growing mycelia reduced subsequent growth in YES broth by a greater amount than irradiation of spores. Irradiation of spores at 100 Krad resulted in more B_1 and G_1 production by strain NRRL 2999 than the non-irradiated control, however, strain NRRL 3000 produced less aflatoxins B_1 and G_1 after irradiation at 100 Krad than its non-irradiated control. Spores of both strains irradiated at 200 Krad produced less aflatoxins B_1 and G_1 than non-irradiated controls. Irradiation of growing vegetative mycelia of both strains at 100 and 200 Krad resulted in a definite decline in both aflatoxins B_1 and G_1 in subsequent cultures at each irradiation level. Apparent stimulation of production of both B_1 and G_1 occurred after irradiation of spores of strain NRRL 2999 at 100 Krad. However, the variation of the values as determined by the standard deviation was such that one would conclude that no differences existed among means. The apparent stimulation was slight and of much less magnitude than that which has been reported by other investigators using *A. flavus*. No stimulation of toxin production was observed with the other strain when grown from irradiated spores or with either strain when vegetative mycelia were irradiated.

Low level ionizing irradiation, amounting to pasteurizing doses to extend shelf-life, have been studied extensively as a means of preventing bacterial deterioration of food products *(5)*. However, few reports of the effects of low level gamma irradiation on toxic molds are available. Mohyuddin et al. *(9)* studied inactivation of conidiophores and mycelia of *Aspergillus flavus* by gamma irradiation, but did not measure the effects on aflatoxin production. Jemmali and Guilbot *(6, 7, 8)* reported that gamma irradiation does below 200 Krad may induce or increase aflatoxin production in *A. flavus*, when grown on Czapek's broth fortified with yeast extract. They found the effects to be maximum at 100 and 150 Krad after 7 days incubation at 25 C. On the other hand, Applegate and Chipley *(1)* observed increased aflatoxin B_1 production by *A. flavus* at 150, 200, and 300 Krad of irradiation when the organism was grown on a cracked wheat substrate. They also found that only the 150 Krad dose stimulated aflatoxin B_1 production by *A. flavus* in a synthetic medium. Production of aflatoxin G_1 was affected in a similar manner *(2)*. Growth of toxic and non-toxic strains of the fungus in wheat and synthetic media was greatly reduced by exposure of the organisms to 300 Krad. Applegate and Chipley *(1, 2)* did not observe induction of the ability to produce either aflatoxin B_1 or G_1 in nontoxic strains by irradiation. Schindler and Noble *(10)* observed increased B_1 production by an *A. flavus* strain after exposure to 20, 100, 250, and 500 kr. In one instance the maximum was observed at 100 kr and in another at 500 kr. At 500 kr the increase was more than 50-fold over the non-irradiated control. They also observed stimulation of G_1 production at the 100 and 500 kr levels. However, in another study Schindler et al. *(11)* observed no stimulation of aflatoxin production at 49-53 Krad of irradiation of both *A. flavus and Aspergillus parasiticus*. Because of the difficulty of converting kiloroentgens (kr) to kilorads (Krad), it is not possible to directly compare these results with those of others. However, it is evident that in the studies of Schindler and Noble *(10)* the greatest stimulation of aflatoxin production occurred at a higher level of irradiation than observed by Jemmali and Guilbot *(6)*.

While stimulation of aflatoxin production by *A. flavus* after irradiation has been observed by several workers, there are differing reports as to the exact level of irradiation that will cause the phenomenon. Most of the work reported to date has involved the effects of irradiation on *A. flavus* rather than *A. parasiticus*, except in one instance where no stimulation of toxin production by *A. parasiticus* was observed at very low levels of irradiation *(11)*. Toxic strains of *A. parasiticus* are generally more potent aflatoxin producers than are toxic strains of *A. flavus*. Also, *A. flavus* strains frequently produce only the B group of aflatoxins, and not the G group. Schindler *(12)* has found that different strains of *A. flavus* vary greatly in their response to irradiation. It is not known whether *A. parasiticus* would react in the same manner to irradiation as *A. flavus*. While *A. flavus* and

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A. parasiticus are generally recognized as morphologically distinct, few physiological differences are known *(15)*. Yet, differences in patterns of aflatoxin production between the two organisms clearly exist *(4, 15)*.

Additional studies would be helpful to adequately assess the effects of low level ionizing irradiation on different aflatoxin producing mold strains, particularly strains of *A. parasiticus*. This work was initiated to study the effects of low level gamma irradiation on growth and aflatoxin production by known aflatoxinogenic strains of *A. parasiticus*.

MATERIALS AND METHODS

Media for growth and toxin production

Yeast-extract sucrose (YES) broth *(3)* was used to support growth and aflatoxin production by the organisms in still cultures before and after irradiation treatment. Aliquots of 50 ml of the broth contained in 250 ml erlenmeyer flasks were employed.

Organisms, inoculum, and treatments.

A. parasiticus strains NRRL 2999 and NRRL 3000 were obtained from the culture collection of the Northern Regional Research Laboratory, A.R.S., U.S.D.A., Peoria, Illinois, for this study.

Cultures of the toxic molds were grown on potato dextrose agar (Difco) slants for 10 days at 25 C until well sporulated. The spores were washed from the slants with a sterile 0.01% solution of Tween 80 and aseptically filtered through sterile cheese cloth to remove mycelial debris. Spores in the filtered suspensions were counted using a Petroff-Hausser counting chamber. A portion of each spore suspension was diluted with sterile phosphate buffer to obtain a spore concentration of 10^7 conidia/ml. Aliquots of 10 ml of the diluted spore suspensions were irradiated at 0, 100, and 200 Krad at ambient (ca 25 C) temperature using a Cobalt-60 source similar to the one described by Teeny and Miyauchi *(14)*. Dosimetry for irradiating the inocula was established using the Fricke Dosimeter, ASTM D1671-63. Flasks containing sterile YES broth were inoculated with 0.1 ml of the irradiated or non-irradiated spore suspensions, resulting in approximately 10^6 spores per flask culture.

The effects of irradiation on growing vegetative mycelia and subsequent growth and toxin production in broth cultures were also studied. Slant cultures of the same two strains were grown on potato dextrose agar at 25 C until mycelial growth had just become evident (24-48 h), but before sporulation had begun. Slant cultures were irradiated at the same levels and in the same manner as were the spore suspensions. Several transfer loops of irradiated or non-irradiated mycelia were used to inoculate flasks of YES broth.

Determination of growth and toxin production after irradiation

All cultures were incubated as still cultures at 25 C for 7 days, and then given a brief heat treatment (121 C for 30 sec) to kill spores and vegetative mycelia. The mold mats were separated from the broth by gravity flow filtration through Whatman #4 filter paper and washed with 20 ml distilled water, dried at 130 C for 2 h and weighed. Mold growth was expressed as milligrams of mycelial dry weight. The pH of the filtered broth was determined.

Aflatoxins were extracted from the broth by liquid-liquid extraction using two 50 ml portions of chloroform. The extracts of each culture were filtered, pooled, and dried over 5-10 g of anhydrous sodium sulfate. The pooled extracts were either diluted or concentrated as needed in preparation for aflatoxin analyses. The aflatoxin concentrations in the extracts were estimated by visual comparison of the fluorescence of the samples to known standards on exposure to long wave U.V. light using thin-layer chromatography (TLC). The TLC plates were 20 × 20 cm, and were coated with a 0.25-mm thick layer of Silica Gel G-HR (Brinkmann Instruments, Inc.). The TLC plates were developed in toulene/ethyl acetate/formic acid (60/30/10) according to the method of Scott et al. *(13)*. Standard aflatoxins were obtained from the Southern Utilization Research and Development Division, U.S.D.A., New Orleans, LA.

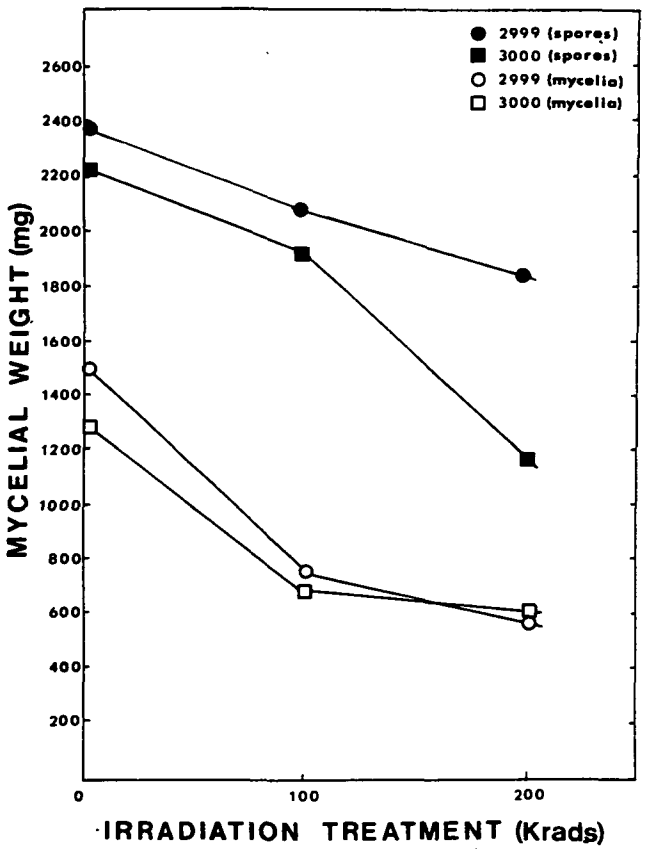

Figure 1. Effect of gamma irradiation of spores and vegetative mycelia of *Aspergillus parasiticus* NRRL 2999 and 3000, on subsequent amount of growth in yeast-extract sucrose broth after 7 days incubation at 25 C.

RESULTS

Growth

Low level gamma irradiation of both spores and growing vegetative mycelia partially inhibited growth of the two strains studied (Fig. 1). At the 200 Krad treatment, growth of strains NRRL 2999 from irradiated spores was reduced by 23% from the non-irradiated control. Irradiation of growing mycelia of the same strain at 200 Krad reduced growth by 62%. The growth of strain NRRL 3000 from spores irradiated at 200 Krad was reduced by 48% and from irradiated growing mycelia by 54% from the non-

TABLE 1. PRODUCTION OF AFLATOXIN B_1 BY *Aspergillus parasiticus* NRRL 2999 AND NRRL 3000 ON YEAST-EXTRACT SUCROSE BROTH IN 7 DAYS OF INCUBATION AT 25 C WHEN GROWN FROM IRRADIATED SPORES OR MYCELIA

Irradiation level (Krad)	NRRL 2999		NRRL 3000	
	Spores	Mycelia	Spores	Mycelia
	(Aflatoxin B_1, μg/ml broth)			
0 (Control)	136	88	60	17
100	150	13	43	5
200	120	4	17	<1

TABLE 2. PRODUCTION OF AFLATOXIN G_1 BY *Aspergillus parasiticus* NRRL 2999 AND NRRL 3000 ON YEAST-EXTRACT SUCROSE BROTH IN 7 DAYS OF INCUBATION AT 25 C WHEN GROWN FROM IRRADIATED SPORES OR MYCELIA

Irradiation level (Krad)	NRRL 2999		NRRL 3000	
	Spores	Mycelia	Spores	Mycelia
	(Aflatoxin G_1, μg/ml broth)			
0 (Control)	689	411	932	191
100	727	60	824	34
200	351	17	146	<1

TABLE 3. EFFECT OF LOW LEVEL GAMMA IRRADIATION OF SPORES AND MYCELIA ON THE SUBSEQUENT PRODUCTION OF AFLATOXIN B_1 PER UNIT WEIGHT OF DRY MOLD MAT BY *Aspergillus parasiticus* NRRL 2999 AND *NRRL* 3000 IN YEAST-EXTRACT SUCROSE BROTH AFTER 7 DAYS AT 25 C

Treatment (Krads)	NRRL 2999		NRRL 3000	
	Spores	Mycelia	Spores	Mycelia
	(Aflatoxin B_1, μg/mg dry mycelium)			
0 (Control)	2.9	2.6	1.3	0.6
100	3.6	0.7	1.1	0.3
200	3.1	0.3	0.7	<0.1

TABLE 4. EFFECT OF LOW LEVEL GAMMA IRRADIATION OF SPORES AND VEGETATIVE MYCELIA ON THE SUBSEQUENT PRODUCTION OF AFLATOXIN G_1 PER UNIT WEIGHT OF DRY MOLD MAT BY *Aspergillus parasiticus* NRRL 2999 AND NRRL 3000 IN YEAST-EXTRACT SUCROSE BROTH AFTER 7 DAYS AT 25 C

Treatment (Krads)	NRRL 2999		NRRL 3000	
	Spores	Mycelia	Spores	Mycelia
	(Aflatoxin G_1, μg/mg dry mycelium)			
0 (Control)	14.5	12.1	19.6	6.1
100	17.4	3.2	17.5	1.9
200	9.4	1.3	0.5	<0.1

irradiated controls. The final pH of the broth media in which the control cultures were grown was 5.3 while the pH of the cultures irradiated at 100 Krads were 6.0 and 6.2 for NRRL 2999 and NRRL 3000 respectively, and 6.2 and 6.5, respectively, for the cultures irradiated at 200 Krad. Growth of organisms in broth media is associated with a decline in pH as growth proceeds. The higher pH of the irradiated cultures reflects less growth which was borne out by the lower mycelial weights obtained.

Aflatoxin production

Aflatoxin B_1 production by cultures from spores of strain NRRL 2999 irradiated at 100 Krad was slightly more than the control (Table 1). At the 200 Krad treatment of spores, this strain produced less aflatoxin B_1 than the control. When growing vegetative mycelia of the same organism were irradiated at the 100 and 200 Krad levels, there was a definite decline in aflatoxin B_1 production at each level of irradiation (Table 1). Aflatoxin B_1 production by strain NRRL 3000 was decreased in cultures grown from spores irradiated at 100 Krad (Table 1). Irradiation of spores of strain 3000 at 200 Krad reduced B_1 production by more than one-half of the control. With growing vegetative mycelia, there was likewise a decline in aflatoxin B_1 production at each level of irradiation (Table 1).

More aflatoxin G_1 was detected in cultures of strain NRRL 2999 that were grown from spores irradiated at 100 Krad than control cultures (Table 2). However, irradiation of spores at 200 Krad resulted in almost 50% less G_1 production than the unirradiated control. Irradiation of growing vegetative mycelia of the same organism resulted in a decline in G_1 production at both the 100 and 200 Krad levels. Aflatoxin G_1 production by strain NRRL 3000 was decreased slightly after irradiation of spores at 100 Krad compared to the control (Table 2). At the 200 Krad treatment of spores, G_1 production was much less than the control cultures of the organism. Irradiation of strain NRRL 3000 mycelia caused a definite decline in aflatoxin G_1 production at both the 100 and 200 Krad treatments compared to the control (Table 2). At the 200 Krad treatment of growing mycelia very low levels of G_1 were produced.

To determine if there might have been stimulation of aflatoxin production by the irradiation treatments, the amount of toxins produced was converted to micrograms of toxins produced per milligram of dry mycelia. With strain NRRL 2999 spores, more aflatoxin B_1 was produced per milligram of dry mycelia at both the 100 and 200 Krad treatments than the control (Table 3). At 100 Krad the effect was greater than at 200 Krad. However, with irradiated growing mycelia of the same organism an opposite trend was noted, with a very definite reduction in amount of aflatoxin B_1 produced per milligram of dry mycelia. No stimulation of aflatoxin B_1 production by either irradiated spores or growing mycelia of strain NRRL 3000 was evident based on micrograms of toxin produced/milligram of dry mycelia (Table 3). There appeared to be a more or less steady decline in amount of B_1 production by this organism due to irradiation.

Aflatoxin G_1 production per unit weight of dry mycelia by cultures derived from irradiated spores of strain NRRL 2999 was greater than the control after irradiation at 100 Krad, but not after irradiation at 200 Krad (Table 4). Irradiation of growing mycelia

of NRRL 2999 caused a definite decline in the amount of G_1 produced per unit of dry mycelia at both the 100 and 200 Krad treatments compared to the control. Aflatoxin G_1 production per milligram of dry mycelia by irradiated spores of strain NRRL 3000 was less than the control cultures at both the 100 and 200 Krad treatments (Table 4). Irradiated growing mycelia of strain NRRL 3000 likewise showed a steady decline in the amount of G_1 produced per milligram of dry mycelia.

DISCUSSION

Growth

Strain NRRL 2999 spores seemed to be somewhat more resistant to irradiation at 200 Krad than those of strain NRRL 3000. Growth of strain NRRL 3000 from irradiated spores was reduced by a greater amount at this treatment than was growth of NRRL 2999 from irradiated spores. There did not appear to be much difference in the reaction of the two strains at 100 Krad since the decline in growth of both strains from that of controls appeared to be similar. The reduction of growth of *A. parasiticus* as a result of irradiation of spores supports and expands the observations with *A. flavus* of Applegate and Chipley *(1, 2)*. The retarding effect of irradiation on growth of cultures from irradiated growing mycelia was very evident at the 100 Krad level with further reductions at 200 Krad. Under the conditions of this study, the growing mycelia of both strains were more sensitive to irradiation in terms of reduced growth than were the spores.

Aflatoxin production

The only instance of possible stimulation of aflatoxin B_1 and G_1 production occurred with spores of strain NRRL 2999. The increased amounts were very small and were not of the same magnitudes as those observed by other workers with *A. flavus*. In addition the variation of these observations as determined by the standard deviation was such that it could be concluded that no actual differences existed between treatment means. The main effect was observed at 100 Krads, which is in agreement with the findings Jemmali and Guilbot *(6)*. However, the results of Schindler and Noble *(10)* and of Applegate and Chipley *(1, 2)* appear to be somewhat different since they observed increased aflatoxin production at higher levels of irradiation of *A. flavus*. However, Applegate and Chipley *(1, 2)* did not observe stimulation of aflatoxin production beyond 150 Krad when using a synthetic medium. Mohyuddin et al. *(9)* found only 0.15% survivors of *A. flavus* conidiophores irradiated at 125 Krad. Presumably higher irradiation levels would have resulted in almost complete inactivation of the particular strain of *A. flavus* they worked with, suggesting that aflatoxin production might also have been eliminated.

Strain NRRL 3000 did not exhibit any tendency toward stimulation of either B_1 or G_1 production at either the 100 or the 200 Krad treatment of spores. There seemed to be a more definite reduction in the amounts of both toxins produced by strain NRRL 3000 after irradiation at 200 Krad.

The irradiated growing mycelia of both strains did not exhibit any tendency toward increased production of either toxin as the result of irradiation treatment. The effect with growing vegetative mycelia was rather one of progressive decline in total toxin production as well as production of toxins on a per unit weight of mycelia. This might be explained by the fact that the mycelia are multinucleate structures, which would require a greater number of hits to produce genetic changes.

These studies show that low level gamma irradiation reduced the amount of growth of *A. parasiticus* in YES broth in a 7-day incubation period. In addition, aflatoxin production was markedly reduced in this same period. Obviously some of the inoculum was destroyed by the irradiation treatment, which might have delayed the amount of growth and aflatoxin production obtained in a 7-day period changing the growth cycle. However, the growth itself appeared to be affected in that the cultures that developed after irradiation did not grow as profusely as did the controls. Whether the effect is due to changes in the metabolism of the organism or simply a delay in the growth cycle is not clear. However, it is clear that growth and aflatoxin production are reduced or prevented within a given time frame. The incubation time of 7 days is comparable to the incubation time used by other workers, and permits comparison of data from different sources.

Obviously differences in results exist between several workers. The differences are too great to be accounted for on the basis of methodology. These differences range from 50-fold increases in toxin production by *A. flavus* at approximately 500 Krad reported by Schindler and Noble *(10)* to essentially no increases in toxin production by *A. parasiticus* in our studies at 100 Krad with definite reductions in toxin production occurring after irradiation at 200 Krad. In addition, Jemmali and Guilbot *(6)* obtained no increase in aflatoxin production by *A. flavus* at 200 Krad and a definite decline in toxin production at 350 and 500 Krad. Applegate and Chipley *(1, 2)* on the other hand observed stimulation of aflatoxin B_1 and G_1 production by *A. flavus* at 200 and 300 Krads

using a cracked wheat substrate, but no stimulation beyond 150 Krad using a synthetic medium. It would seem that these apparent discrepancies might be explained on the basis of differences between species, differences between strains within species, and substrate effects.

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INHIBITION OF AFLATOXIN PRODUCTION BY CINNAMON

INTRODUCTION

AFLATOXINS are secondary metabolites of *Aspergillus flavus* – Link and *Aspergillus parasiticus* – Speare, which have been shown to be both toxic and carcinogenic in test animals (Lillehoj et al., 1970). Recent evidence indicates that these toxins may also be involved in the etiology of human liver cancer in certain parts of the world (Shank and Wogan, 1972; Shank et al., 1972a, b, c, d). Presence of these toxins in food, therefore, constitutes a potential hazard to human health. Both *A. flavus* and *A. parasiticus* are widely distributed in nature and frequently contaminate human food resources (Detroy et al., 1971; Marth, 1967; Wogan, 1966). Experimentally, aflatoxins have been produced on numerous food products, including cereal grains, whole wheat and rye breads, cheese, meats, nut products and fruit juices (Detroy, 1971).

Recent work in our laboratory has been concerned with mold growth and toxin production in fresh breads (Hartung et al., 1973; Bullerman et al., 1973). During the course of this work, it was noted that raisin bread, containing cinnamon, did not support extensive mold growth. It has been known for some time that essential oils of certain spices possess antimicrobial activities (Frazier, 1967). Oil of cinnamon is said to be "fairly effective" against yeasts, and quite effective against bacteria. In high concentrations it is known to permit mold mycelial growth but inhibit asexual spore formation (Frazier, 1967).

While it is generally accepted that the essential oils of certain spices have antimicrobial activities, their actions against specific microbial metabolic processes have not been elucidated. This study was conducted to determine (a) if the cinnamon component of raisin bread was responsible for the reduced mold growth on that product, and (b) the effects of cinnamon on growth and aflatoxin production by known toxinogenic strains of *A. parasiticus*.

MATERIALS & METHODS

Bread types

White, raisin, rye and whole wheat breads were manufactured without mold inhibitor by a local baker. The breads were obtained the day of manufacture and were compared as substrates for mold growth and aflatoxin production. The raisin bread was made using the same formula as the white bread except that raisins and cinnamon were added. The amount of cinnamon used was 1.0% of the total mix.

Organisms

A. parasiticus, strains NRRL 2999 and NRRL 3000, were obtained from the culture collection of the USDA Northern Regional Research Lab., ARS, Peoria, Ill. and used in this study. Stock cultures were maintained at 5°C on slants of Difco potato dextrose agar.

Inoculum

Cultures of the toxic molds were grown on potato dextrose agar slants for 10 days at 25°C until well sporulated. The spores were washed from the slants with a sterile 0.01% solution of Tween 80. The spores from two slants of each organism were combined to form a single heavy spore suspension. The harvested spores were suspended in 100 ml of sterile Tween 80 and aseptically filtered through sterile cheese cloth to remove mycelial debris. The filtered suspensions were quantitated using a Petroff-Hausser counting chamber. Aliquots of the suspensions were diluted such that two levels of inoculum were used, approximately 10^2 and 10^6 spores/slice of bread. The dilutions were made so that the required number of spores were contained in 0.1 ml of spore suspension. One-half of this amount was applied to each side of each slice of bread.

Inoculation and packaging of bread slices

The slices of bread were aseptically removed from the packaged loaves and placed on sterile towels inside a bacteriological glove box. The glove box had previously been sanitized with a 50% solution of liquid household bleach and was exposed to germicidal UV light for 1 hr before use. The bread slices were exposed to germicidal UV light for 15 min per side prior to inoculation. The slices were inoculated using a sterile 1 ml tuberculin syringe. The inoculum was distributed over the surface of the bread as much as possible by brushing with a flamed inoculating loop, but most of the inoculum remained at the point of inoculation. The inoculated slices were individually packaged in polyethylene pouches (PL540; W.R. Grace Co.), which were sealed under an air atmosphere. The bread was stored for 10 days at 25°C. Duplicate bread slices were used, and the studies were replicated three times.

Analyses of bread slices

The extend of the mold growth was assessed visually throughout the storage period. Following storage, all duplicate samples were composited by dry blending in a sterile Osterizer blender. The composited samples were analyzed for aflatoxin content using a modification of the method of Pons et al. (1966), in which 100% ethanol was substituted for acetone in the initial extraction solvent. This resulted in cleaner extracts and did not form emulsions as readily during the chloroform extraction step. Extracts were separated on thin-layer chromatography (TLC) plates (20 x 20 cm, 0.25 mm thick Silica Gel G-HR, Brinkmann Instruments, Inc.). The TLC plates were developed in toluene/ethyl acetate/90% formic acid (60/30/10) according to the method of Scott et al. (1970). Aflatoxin concentrations in the extracts were estimated by visual comparison of the fluorescence of the respective aflatoxins of the samples to known standards on exposure to long wave UV light. Standard aflatoxins were obtained from the Southern Utilization Research and Development Division, USDA, New Orleans, La.

Aflatoxin production on raisins and cinnamon

Raisin bread contained raisins and cinnamon in addition to the standard dough ingredients. To determine the effects of raisins and cinnamon on aflatoxin production, these ingredients were studied separately. Sterilized and nonsterilized raisins were studied as well as four concentrations of cinnamon in yeast-extract sucrose (YES) broth (Davis et al., 1966). 50g of raisins were added to a 250 ml Erlenmeyer flask along with 10 ml of distilled water. The mixture was sterilized at 121°C for 15 min. Nonsterilized raisins were aseptically added to a sterilized 250 ml Erlenmeyer flask containing 10 ml of sterile distilled water. In addition, a raisin slurry was prepared by adding 50 ml of distilled water to 25g of raisins and blending in a blender until the raisins were finely ground, forming a homogeneous slurry. One lot was sterilized by autoclaving at 121°C for 15 min, and a second lot was prepared with nonsterilized raisins using sterilized water and utensils. A third set of raisin substrates was prepared by making a hot water extract of raisins in the following manner: 200 ml of hot (80°C) distilled water was added and the mixture was allowed to stand for 1 hr. The slurry was then filtered through Whatman No. 4 filter paper (gravity flow) to remove particulate matter. The filtrate was sterilized by autoclaving at 121°C for 15 min in 50 ml quantities in 250 ml Erlenmeyer flasks.

Four amounts of cinnamon (0.02, 0.20, 2.00 and 20.00%) were added to 50 ml of YES broth in 250 ml Erlenmeyer flasks and sterilized at 121°C for 15 min. Control flasks of only YES broth were also included. Spore suspensions, made by adding 10 ml of sterile Tween 80 to a slant culture of each of the test organisms were used for inocula. All flasks (raisin and cinnamon) were inoculated with 0.1

ml of an appropriate spore suspension. The cultures were incubated for 10 days at 25°C as still cultures.

Since the essential oil of cinnamon is alcohol-soluble (Parry, 1945), cinnamon in amounts corresponding to 0.02%, 0.20%, 1.0% and 2.0% levels in 50 ml quantities of broth were extracted for 1 hr at 25°C with 100% ethanol. The extracts were filtered to remove particulate matter and the solvent was evaporated. The residues were dissolved in 50 ml portions of glucose ammonium nitrate (GAN) broth (Brian et al., 1961). The GAN broth was used because it is a completely defined medium. The resulting solutions were sterilized by membrane filtration (Millipore®, 0.45μ) and placed in sterile 250 ml Erlenmeyer flasks. Flasks of GAN broth containing alcoholic extracts of cinnamon equivalent to 0.02, 0.20, 1.0 and 2.0% cinnamon levels in the broth substrate were thus obtained. All flasks were inoculated and incubated as described for ground cinnamon in YES broth.

Analyses of raisin, cinnamon and cinnamon extract cultures

After incubation, the cultures were given a brief heat treatment (121°C for 30 sec) to kill spores and vegetative mycelia. The mold mats were separated from the broth cultures by gravity flow filtrations through Whatman No. 4 filter paper and washed with 50 ml of distilled water. The washed mold mat was then transferred to predried and tared filter paper and dried at 70°C to constant weight. Growth on the raisins was estimated visually using the following scale: –, no growth; +, scant growth; ++, moderate growth; +++, extensive growth; ++++, very extensive growth.

The broth, including the filter paper used to separate the mold mat from the broth, and the raisin cultures were extracted in toto using two 50 ml portions of chloroform. The extracts of each culture were filtered through Whatman No. 2 filter paper, wetted with chloroform, pooled and dried over 5–10g of anhydrous sodium sulfate. The pooled extracts were either examined for aflatoxins without concentration, or were concentrated on a steam bath and transferred to screw cap vials, further evaporated to dryness and redissolved in known volumes of chloroform. Aflatoxin concentrations in the unconcentrated and concentrated extracts were then estimated in the same manner as described for the bread samples.

RESULTS & DISCUSSION

BOTH STRAINS of *A. parasiticus* grew abundantly on rye, whole wheat and white breads. However, growth on raisin bread was very scant, or not visible at all. Likewise, the quantities of aflatoxins B_1 and G_1 produced on rye, whole wheat and white breads were higher than those obtained on raisin bread, where little or no aflatoxins were detected except for the high inoculum level of strain NRRL 2999 (Tables 1 and 2). Strain NRRL 2999 produced greater quantities of aflatoxins on all substrates than strain NRRL 3000. Whole wheat bread provided the best substrate for aflatoxin production, but rye and white breads also supported rather high yields of toxins.

Table 1—Aflatoxin B_1 production on several types of bread by Aspergillus parasiticus NRRL 2999 and NRRL 3000 (μg toxin/g bread)

Bread type	Strain NRRL 2999		Strain NRRL 3000	
	10^2 Spores	10^6 Spores	10^2 Spores	10^6 Spores
Rye	28	54	1	3
Wheat	15	45	5	4
White	20	11	2	5
Raisin	<0.1	3	ND[a]	<0.1

[a] ND = None detected.

Table 2—Aflatoxin G_1 production on several types of bread by Aspergillus parasiticus NRRL 2999 and NRRL 3000 (μg toxin/g bread)

Bread type	Strain NRRL 2999		Strain NRRL 3000	
	10^2 Spores	10^6 Spores	10^2 Spores	10^6 Spores
Rye	71	131	2	23
Wheat	164	186	27	27
White	76	43	9	50
Raisin	0.2	10	ND[a]	0.3

[a] ND = None detected.

The lack of aflatoxin production on raisin bread raises questions concerning the reasons for this phenomenon. The possibility that ingredients contained in the raisin bread, but not in the other breads, might be inhibitory to growth and toxin production was considered. These ingredients were identified as raisins and cinnamon.

Raisins supported growth by both mold strains when sufficient moisture for growth was available. Whole raisins with 20% added moisture did not support visible growth by either strain. However, both the raisin slurry and raisin extract supported heavy growth by both mold strains. There was no difference between sterilized and nonsterilized raisins in terms of the amount of growth supported. Apparently the amount of inoculum supplied was sufficient to overcome all competing growth in the nonsterile raisin cultures. Aflatoxin production by the raisin cultures followed the same general pattern as growth. No aflatoxins were detected in whole raisins with 20% added moisture. The lack of growth and aflatoxin production on the whole raisins is most likely attributable to a lack of sufficient available moisture to support growth of the organisms. High levels of aflatoxins corresponding to heavy growth were observed on the raisin slurry and raisin extract cultures. These substrates had a higher moisture level than the whole raisins. The amounts of total aflatoxins ($B_1 + G_1$) produced were 150 and 170 μg/g by strains NRRL 2999 and NRRL 3000 respectively on the raisin slurry and 25 and 13 μg/g respectively on raisin extract.

When increasing levels of cinnamon were added to YES broth, an inhibitory effect was noted on the growth of both mold strains (Table 3). The effect was especially pronounced with strain NRRL 2999. With strain NRRL 3000, inhibition was also noted, but the organism was more variable in its reaction to the cinnamon. The growth of strain NRRL 2999 was affected by concentrations of cinnamon as low as 0.02% (200 μg/ml). Growth was reduced 13–31% by concentrations of cinnamon ranging from 0.02–2.0%.

When aflatoxin production was determined, the effect was even more striking

Table 3—Effect of ground cinnamon on growth and total aflatoxin ($B_1 + G_1$) production by Aspergillus parasiticus NRRL 2999 and NRRL 3000 in yeast-extract sucrose broth after 10 days at 25°C

Level of cinnamon	Strain NRRL 2999				Strain NRRL 3000			
	Mycelia		Aflatoxins		Mycelia		Aflatoxins	
	mg	Inhibition	μg/ml	Inhibition	mg	Inhibition	μg/ml	Inhibition
Control	2301	–	356	–	1896	–	292	–
0.02%	1943	16%	267	25%	1959	(+3%)	232	21%
0.2%	1768	23%	148	58%	1557	18%	49	83%
2.0%	1589	31%	11	97%	1658	13%	2	99%
20.0%	ND[a]	100%	ND	100%	100	95%	0.3	99.9%

[a] ND = None detected.

Table 4—Effects of an alcohol extract of cinnamon on growth and aflatoxin production in glucose-ammonium nitrate (GAN) broth by Aspergillus parasiticus NRRL 2999 after 10 days at 25°C

	Mycelial weight		Aflatoxins/ml							
			B_1		B_2		G_1		G_2	
Treatment	mg	% Inhibition	µg/ml	% Inhibition	µg/ml	% Inhibition	µg/ml	% Inhibition	µg/ml	% Inhibition
Control	534	–	5.52	–	1.01	–	4.11	–	1.01	–
0.02%	564	–	1.44	74	0.16	84	0.90	78	0.12	89
0.20%	466	13	0.83	98	0.007	99+	0.05	99	0.006	99
1.00%	126	76	0.008	99+	0.001	99+	0.01	99+	0.001	99+
2.00%	108	80	0.007	99+	0.001	99+	0.006	99+	0.001	99+

(Table 3). Increasing concentrations of cinnamon in YES broth resulted in detection of decreasing levels of aflatoxins. At the lowest level of cinnamon (200 µg/ml), reduction in aflatoxin production ranged from 21–25%, while at the 2% (20 mg/ml) level of cinnamon, the toxin production was reduced by 97–99%. At the 20% level of cinnamon, growth and aflatoxin production were essentially eliminated. These differences were judged to be significant differences between means as determined by the standard deviation and Student t Test.

The effects of an alcoholic extract of cinnamon on growth and aflatoxin production by strain NRRL 2999 in GAN broth, a completely defined medium, were similar to those obtained with ground cinnamon in YES broth (Table 4). Growth was reduced by levels of extracts ranging from 0.2 to 2% cinnamon. The 0.02% level did not reduce growth. However, the levels of aflatoxins B_1, B_2, G_1 and G_2 produced were all significantly reduced by the lowest level of cinnamon extract as determined by the t test. These reductions ranged from 74–89%. At the 0.2% level of cinnamon, aflatoxin production was inhibited by 98–99% and at the 1 to 2% level aflatoxin production was reduced by more than 99%.

The preferential inhibition of aflatoxin production over mold growth by cinnamon and cinnamon extract is significant. Spices are normally used as flavoring agents in levels of 0.5–1.0% in finished products (Furia, 1968). The raisin bread used in this study contained 1.0% cinnamon. The broth studies with ground cinnamon and cinnamon extract indicate that at 1–2% concentrations of cinnamon, the production of aflatoxins is approximately 99% eliminated. This accounts for the very low levels of alfatoxin found in the raisin bread containing cinnamon.

Cinnamon is one of the more effective bacteriostatic spices and is also effective against yeasts (Frazier, 1967). Heavy concentrations of cinnamon are known to permit mold growth but inhibit asexual spore formation (Frazier, 1967). These studies indicate that cinnamon is also an effective inhibitor of aflatoxin production even though mycelial growth may be permitted. While reduction in mold growth due to increasing cinnamon concentration was observed, the most striking effect was the reduction of aflatoxin production.

These data indicate that foods that contain cinnamon may not readily support mold growth and/or mycotoxin production. While the primary function of the cinnamon in these foods may not be preservative in nature, it has preservative properties, which result in built-in safety systems in these foods. Further studies dealing with specific compounds which may be responsible for these effects are in progress.

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frosted rolls. Radurization significantly reduced the microbial content of each without any organoleptic changes. Of particular advantage was treatment for the filled pastries. Details of the studies are found in the monthly reports.

Looking toward short term mission plans for Shuttle (ASTP), a series of studies was initiated to consider the application of radurization to fresh food systems. The emphasis was placed upon sandwiches. Early, just prior to Apollo 17, a complete sandwich concept was considered using irradiated sterile meat and combining with bread that was then totally radurized. This did not hold any great advantage to combining while in flight radurized bread with sterile meat. The greater problem was a moisture buildup in the bread during extended storage. The most recent studies concentrated on the concept of assembling the sandwich and subjecting it to radurization following packaging. These studies have considered single meat as well as sandwich spreads. The first phase involved the changes noted in the sandwich meats or spreads alone. Radurization did not result in any measurable change of the organoleptic characteristics. The sandwich spreads and fabricated luncheon meats were significantly improved as to microbial content. Radurization at the higher levels used in the studies, 200 Krad, resulted in a noticeable rise in TBA values initially. However, no accelerated rate was noted while the meat was stored under refrigerated conditions.

A series of sandwish types has been examined and the principle factors in each case were a significant improvement in microbial content of the radurized sandwiches and a rise in TBA values. The concluding study was initiated to seek to control the oxidative rancidity change as noted by the TBA value. This will be reported upon in the following section.

Sandwich Series - Concluding Study

Previous monthly reports have included the evaluation of radurization of luncheon meats and spreads and sandwich combinations. The primary advantage was the lowering of microbial content and providing an extension of the shelf life from the microbial point of view. The principle concern was an increase in oxidative rancidity in meat spreads that did not involve cured meat or salad mixes having low pHs. This series was designed to consider two approaches to reducing oxidative rancidity; first, irradiating the sandwich in the frozen state; and a second factor of packing the sandwiches under nitrogen prior to irradiation.

The design involved preparing sandwiches using commercially prepared bologna, fabricated turkey roll and Pepperidge bread. Sandwiches were assembled under sanitary conditions of a test kitchen and packaged in a laminated plastic pouch. Half of the sandwiches were sealed under a slight vacuum and the other half were sealed using a nitrogen fill following a triple purging process. Each group was subdivided, with half frozen at -20°C and exposed to irradiation and the other exposed to irradiation immediately following packaging. The sandwiches were stored at 2°C for a period of 14 days with samplings made initially, 3, 7, 10 and 14 days. Microbial determinations were made following the previously detailed procedures and TBA values determined. The measurements were made to indicate what microbial changes might occur while seeking to inhibit or retard oxidative rancidity.

Tables I, II and III report observations of the bologna sandwiches and Tables IV, V and VI, the turkey sandwiches. The bacterial content (Table I) of the bologna sandwiches was reduced initially and over a period up to 7 days of storage when irradiation was used. Nitrogen fill versus vacuum packaging did not alter the bacterial content within the treatments. The relative effectiveness of the irradiation on reduction of bacterial content was not altered comparing irradiation of the unfrozen versus the frozen sandwiches. The yeast determinations are shown in Table II. The highest level of irradiation markedly reduced the yeast content of the vacuum packaged sandwiches compared to the nonirradiated sandwiches. The nitrogen fill packages tended to permit more yeast growth than the vacuum packaging. Mold measurements were made but not shown in this report. Limited mold growth occurred in the nonirradiated sandwiches with no mold development in the irradiated sandwiches.

The TBA values of the bologna sandwiches are shown in Table III. Up through 7 days the irradiation influence was retarded using the frozen sandwiches treatment. Nitrogen fill was not consistent in its influence.

The measurements on the turkey sandwiches for bacterial content are shown in Table IV. Counts were considerably higher in the turkey sandwiches compared to the bologna sandwiches. Irradiation held an advantage through 10 days of storage. No difference was noted comparing nitrogen fill versus vacuum packaging. Again irradiation effectiveness was not apparently reduced comparing the frozen environment for the irradiation exposure to the unfrozen condition. The yeast content reported in Table V was generally higher in the turkey sandwiches versus the bologna sandwiches. Irradiation reduced the yeast counts.

Nitrogen fill did not alter the yeast content compared to vacuum packaging. Frozen versus unfrozen treatment did not alter the relative yeast content for either the bologna or the turkey sandwiches. Mold growth was substantially inhibited by irradiation.

The TBA measurements for the turkey sandwiches are shown in Table VI. All values were greatly higher compared to the bologna sandwiches. Neither the packaging nor the frozen treatments produced consistent differences. Irradiation tended to increase the TBA values; however, the variation was great within the treatment over storage time. Further work should be considered to lead to more definitive practices to retard the irradiation influence on oxidative rancidity while gaining the microbial reductions of the fresh product.

TABLE I
Bacterial Content of Bologna Sandwiches
Exposed to Irradiation and Stored

Irrad. Treatment		Package	Days of Storage (2°C)				
Environ	Level, Krad	Vac/N_2	0	3	7	10	14
Unfrozen	0	Vac	280×10^4	25×10^5	38×10^4	22×10^6	54×10^5
		N_2	116×10^3	140×10^3	117×10^4	46×10^5	76×10^5
	50	Vac	56×10^3	61×10^3	58×10^3	142×10^3	475×10^5
		N_2	139×10^3	202×10^3	76×10^4	61×10^5	99×10^5
	100	Vac	35×10^2	109×10^3	65×10^3	130×10^3	261×10^3
		N_2	200×10^2	113×10^3	94×10^4	285×10^4	48×10^4
Frozen	0	Vac	TNTC	29×10^3	157×10^3	91×10^3	66×10^3
		N_2	254×10^3	174×10^3	82×10^4	286×10^4	145×10^4
	50	Vac	25×10^2	64×10^3	176×10^3	57×10^3	166×10^4
		N_2	298×10^3	55×10^4	114×10^4	39×10^4	67×10^4
	100	Vac	35×10^1	107×10^3	79×10^4	73×10^4	64×10^5
		N_2	150×10^2	84×10^3	97×10^4	55×10^4	19×10^4

TABLE II

Yeast Content of Bologna Sandwiches
Exposed to Irradiation and Stored

Irrad. Treatment		Package	Days of Storage (2°C)				
Environ	Level, Krad	Vac/N_2	0	3	7	10	14
Unfrozen	0	Vac	130	19×10^1	54×10^2	29×10^3	6×10^2
		N_2	23×10^1	52×10^1	33×10^2	133×10^2	117×10^3
	50	Vac	26	216	84×10^1	46×10^2	276×10^3
		N_2	30×10^1	77×10^1	38×10^2	13×10^3	187×10^3
	100	Vac	41	28×10^1	71	27×10^1	48×10^3
		N_2	63×10^1	50×10^1	40×10^2	42×10^2	129×10^3
Frozen	0	Vac	27×10^1	30×10^2	45×10^3	105×10^3	166×10^3
		N_2	80×10^1	41×10^1	77×10^1	19×10^3	175×10^3
	50	Vac	15×10^1	55×10^2	27×10^1	33×10^2	53×10^2
		N_2	389	55×10^1	174×10^1	55×10^2	96×10^2
	100	Vac	22	51×10^2	29	19×10^2	5×10^2
		N_2	236	33×10^1	110×10^2	85×10^2	81×10^3

TABLE III
Measurement of TBA values of Bologna Sandwiches
Exposed to Irradiation and Stored

Irrad. Treatment		Package	Days of Storage (2°C)				
Environ	Level, Krad	Vac/N_2	0	3	7	10	14
Unfrozen	0	Vac	.195	.0	.058	.164	.078
		N_2	.156	.172	.039	.195	.164
	50	Vac	.156	.0	.312	.273	.172
		N_2	.234	.234	.390	.156	.039
	100	Vac	.195	.390	.234	.234	.351
		N_2	.312	.390	.312	.0	.140
Frozen	0	Vac	.117	.0	.234	.055	.156
		N_2	.156	.390	.195	.0	.078
	50	Vac	.078	.078	.078	.242	.234
		N_2	.156	.468	.078	.117	.140
	100	Vac	.156	.234	.234	.117	.686
		N_2	.390	.351	.546	.234	.101

TABLE IV
Bacterial Content of Turkey Sandwiches
Exposed to Irradiation and Stored

Irrad. Treatment		Package	Days of Storage ($2^{o}C$)				
Environ	Level, Krad	Vac/N_2	0	3	7	10	14
Unfrozen	0	Vac	43×10^3	249×10^4	24×10^5	189×10^6	31×10^7
		N_2	31×10^5	37×10^4	36×10^7	21×10^7	26×10^7
	50	Vac	107×10^4	0	129×10^4	87×10^6	22×10^7
		N_2	29×10^4	125×10^3	31×10^5	93×10^6	42×10^7
	100	Vac	45×10^3	78×10^3	109×10^3	70×10^6	72×10^6
		N_2	120×10^3	80×10^3	59×10^4	84×10^4	20×10^7
Frozen	0	Vac	51×10^3	73×10^4	106×10^5	104×10^6	41×10^7
		N_2	48×10^5	37×10^5	14×10^7	23×10^7	32×10^7
	50	Vac	48×10^3	80×10^3	16×10^5	84×10^6	169×10^6
		N_2	156×10^3	49×10^4	46×10^5	25×10^7	54×10^7
	100	Vac	23×10^3	64×10^3	151×10^5	108×10^5	75×10^6
		N_2	117×10^3	140×10^3	88×10^4	153×10^6	31×10^7

TABLE V

Yeast Content of Turkey Sandwiches
Exposed to Irradiation and Stored

Irrad. Treatment		Package	Days of Storage ($2^{o}C$)				
Environ	Level, Krad	Vac/N_2	0	3	7	10	14
Unfrozen	0	Vac	247	28×10^2	56×10^3	171×10^4	139×10^6
		N_2	69×10^2	16×10^2	30×10^3	175×10^4	60×10^4
	50	Vac	99×10^1	17×10^1	83×10^2	133×10^3	67×10^5
		N_2	29×10^1	38×10^1	255×10^1	67×10^3	152×10^3
	100	Vac	56	64×10^1	80×10^2	111×10^3	86×10^5
		N_2	11×10^1	25×10^1	274×10^1	90×10^3	62×10^3
Frozen	0	Vac	231	82×10^1	139×10^3	272×10^4	97×10^6
		N_2	82×10^2	49×10^2	81×10^3	72×10^4	55×10^4
	50	Vac	72	177×10^1	62×10^2	338×10^3	250×10^5
		N_2	40×10^1	30×10^1	18×10^3	76×10^4	93×10^3
	100	Vac	36	43	72×10^1	227×10^3	200×10^4
		N_2	34×10^1	37×10^1	53×10^2	64×10^4	2×10^3

TABLE VI
Measurement of TBA values of Turkey Sandwiches
Exposed to Irradiation and Stored

Irrad. Treatment		Package	Days of Storage (2°C)				
Environ	Level, Krad	Vac/N_2	0	3	7	10	14
Unfrozen	0	Vac	2.652	3.510	2.574	3.276	3.588
		N_2	3.666	2.808	2.496	3.822	2.886
	50	Vac	3.588	2.418	2.886	3.042	2.808
		N_2	3.510	3.510	3.276	2.262	4.012
	100	Vac	2.574	2.574	3.198	1.950	1.950
		N_2	2.886	4.056	3.900	2.340	4.290
Frozen	0	Vac	3.237	3.237	3.900	2.496	2.652
		N_2	3.354	2.964	3.354	4.368	4.446
	50	Vac	3.276	3.276	3.432	2.964	3.744
		N_2	2.106	2.730	3.900	2.028	3.744
	100	Vac	2.652	2.652	3.900	3.432	3.666
		N_2	2.340	2.964	3.042	4.212	3.510