Distribution of Microorganisms in Spices and Their Decontamination by Gamma-irradiation

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The distribution of microorganisms in 15 samples of selected spices and the effects of irradiation of them were studied. The total aerobic bacteria in black pepper, white pepper, turmeric, rosemary and basil were determined to be 3×10^3 to 5×10^7 per gram. Coliforms were also determined in 8 samples to be 2×10^2 to 2×10^6 per gram. The main aerobic-spore-formers were identified as *Bacillus pumilus* and *B. subtilis*. Molds were determined in 10 samples to be 1×10^2 to 2×10^4 per gram which consisted mainly of the *Aspergillus glaucus*, *A. restrictus*, *A. flavus*, *A. fumigatus*, *A. niger* groups and *Penicillium*. A study on the inactivation of microorganisms in spices showed that gamma-irradiation doses of 1.2 to 1.5 Mrad were required to reduce the total aerobic bacteria to below a detectable level, while doses of below 1.0 Mrad were required to decrease the spore-forming bacteria to below 10^3 per gram, the Japanese hygienic standard. Coliforms were eliminated with 0.4 to 1.0 Mrad irradiation. In the storage study, at humidity levels higher than 84% at 30 or 35°C, mold counts increased more than 10^6 per gram in many kinds of powdered spices in polyethylene pouches during 1 to 2 months of storage, while samples subjected to 0.4 Mrad irradiation were free from molds.

The frequent contamination by microorganisms of imported spices has been causing serious problems for the food industry in Japan. The contamination of spices may occur during harvesting, handling, transportation and storage in the exporting countries.^{1,2)} Processed spices with a high bio-burden utilized in the food industry accelerate the putrefaction of manufactured food products. Mold growth under unfavorable conditions during long storage and shipment should also cause quality changes and occasionally mycotoxin production. In many countries, both fumigation with ethylene oxide and heat sterilization have been tried with varying degrees of success. However, these methods have several disadvantages for application to the sterilization of spices such as toxic residues are left and organoleptic properties are changed.^{3,4)} For these reasons, the spice

traders are required to seek alternative methods for decontamination. There have been many reports that radiation treatment is a suitable method for decontaminating spices,^{3~7)} and commercial scale use of radiation processing for spices has been successful in several European countries and the U.S.A.⁸⁾ in recent years. However, there have been only a few reports on the decontamination effect on spices of irradiation under Japanese conditions. A survey of 26 kinds of imported spices reported previously showed that more than 50% of the species showed levels of more than 10^4 spore-forming bacteria per gram, and gamma-irradiation doses of 0.5 to 1.5 Mrad were required to decrease the spore-forming bacteria to below a detectable level.⁹⁾ This report presents data on the distribution of microorganisms in, the efficacy of gamma-irradiation for decontamination of,

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and storage effects on many spices available in Japan.

MATERIALS AND METHODS

1. *Materials.* Nine kinds of spices were obtained from local companies in Japan. All of the 19 samples of these spices were imported from several countries.

2. *Enumeration of the microbial load.* The total aerobic bacteria were determined by the surface plate agar method with medium containing Difco-nutrient agar 23 g, glucose 5 g, yeast extract 5 g, and K_2HPO_4 2 g per liter (pH 7.2). Coliforms were counted on Difco-MacConkey agar. Fungi were counted on MYG-chloramphenicol agar containing malt extract 10 g, yeast extract 4 g, glucose 4 g, agar 20 g, and chloramphenicol 20 mg per liter (pH 6.0). Osmophilic molds were counted on 15% NaCl-malt agar containing malt extract 50 g, NaCl 150 g, and agar 20 g per liter (pH 6.0).

Five g of each sample was added to 50 ml of 0.01% Tween-20 sterile water and homogenized thoroughly in a plastic bag with a Coloworth stomacher Lab-Blender-400 for one minute. Each suspension was diluted 10^2 or 10^4 times with the same sterile water, and then 0.2 ml aliquots were spread on the surface of agar plates. For whole spices, the samples were ground in a blender under sterile conditions prior to mixing with sterile water. Total aerobic bacteria, fungi and osmophilic molds were counted after 3~7 days incubation at 30°C. Coliforms were counted after 16 to 18 hours incubation at 37°C.

Detection of salmonellae in some selected spices having heavy coliform loads was also performed by the same

method as that used for fish meal.¹⁰⁾

3. *Microbiological identification.* After enumeration of the microorganisms on the agar plates, representative strains of the total aerobic bacteria, coliforms, fungi and osmophilic molds were selected and transferred onto suitable agar slants. Identification to the levels of genus and species was performed with reference to "Bergey's Manual of Determinative Bacteriology"¹¹⁾ and "The Genus *Aspergillus*."¹²⁾

4. *Gamma-irradiation.* For irradiation, the spices were packed, from 5 to 20 g, into polyethylene pouches. The gamma-ray source used was the 100 kCi cobalt-60 irradiator in this Institute. The dose rate at the irradiation position was 0.5 to 1.0 Mrad per hour, as determined with a Fricke dosimeter.

5. *Storage studies.* Nine kinds of spices and one sample of fish meal were packed, 20 g each, into polyethylene pouches, and then stored after irradiation in a chamber kept at 30 or 35°C and a relative humidity of 78~98% for 28 to 56 days.

6. *Estimation of aflatoxin producing ability.* The conventional plate agar¹³⁾ containing $(NH_4)_2H_2PO_4$ 10 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KCl 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, sucrose 30 g, $HgCl_2$ 5×10^{-4} M, corn steep liquor 0.5 g, and agar 25 g per liter (pH 5.5) was used for estimation of aflatoxin production after incubating a large colony of the *A. flavus* group at 28°C for 7~10 days. The chloroform extract from the surrounding zone of a large colony was assayed by thin-layer-chromatography with development with chloroform-acetone (9:1).

TABLE I. DISTRIBUTION OF MICROORGANISMS IN SPICES (per gram)

Type of spice		Total bacteria	Coliforms	Osmophilic molds	Fungi
P. Black pepper	A	5.3×10^7	1.5×10^4	6.5×10^4	5.5×10^3
P. Black pepper	B	3.8×10^7	9.9×10^3	7.1×10^3	3.0×10^3
W. Black pepper	A	2.0×10^7	—	—	—
W. Black pepper	B	4.8×10^7	8.9×10^3	2.7×10^4	3.4×10^3
P. White pepper	A	9.2×10^4	1.7×10^2	6.3×10^3	3.1×10^3
P. White pepper	B	—	—	—	—
W. White pepper	A	9.3×10^4	—	2.6×10^3	2.5×10^2
W. White pepper	B	9.8×10^5	2.0×10^6	2.9×10^4	1.7×10^4
P. Turmeric	A	3.6×10^7	5.7×10^2	2.6×10^3	8.1×10^3
P. Turmeric	B	3.1×10^6	7.5×10^1	—	—
W. Turmeric	B	6.7×10^5	—	—	—
P. Rosemary	A	4.9×10^3	—	5.6×10^3	2.1×10^3
P. Rosemary	B	2.2×10^6	5.8×10^2	7.5×10^1	1.5×10^2
W. Rosemary	B	2.8×10^3	—	—	—
P. Basil	A	9.0×10^6	9.0×10^2	2.8×10^2	6.5×10^2

—, below the detectable limit; P, powder sample; W, whole sample; A, B, samples with different capital letters were obtained at different times.

TABLE II. SOME CHARACTERISTICS OF TYPICAL BACTERIA IN SPICES

Species	Morphology	Gram-reaction	Motility	Spore formation	Oxidase	Glucose	Starch hydrolysis	Nitrate reduction	Methyl red	No. of isolates
<i>Bacillus subtilis</i>	Rods	+	+	+	±	Oxidized	+	+	*	8
<i>Bacillus licheniformis</i>	Rods	+	+	+	±	Oxidized & fermented	+	+	*	1
<i>Bacillus pumilus</i>	Rods	+	+	+	+	Oxidized	—	—	*	4
<i>Bacillus megaterium</i>	Large rods	+	+	+	+	Oxidized	+	+	*	1
<i>Micrococcus varians</i>	Cocci	+	—	—	—	Oxidized	—	+	*	1
<i>Pseudomonas radiora</i>	Rods	—	+	—	+	Oxidized	±	+ or —	*	2
<i>Klebsiella</i> sp.	Short rods	—	—	—	—	Fermented	*	+	—	13
<i>Enterobacter</i> sp.	Short rods	—	+	—	—	Fermented	*	+	—	12
<i>Citrobacter</i> sp.	Short rods	—	+	—	—	Fermented	*	+	+	4

* Not tested.

RESULTS

1. *Distribution of microorganisms in spices*

As shown in Table I, total aerobic bacteria in black pepper, white pepper, turmeric, rosemary and basil were determined to be 3×10^3 to 5×10^7 per gram, except for white pepper B, in which no microorganisms were detected. The main aerobic bacteria were identified as *Bacillus subtilis* and *B. pumilus*, with lower frequencies of *B. megaterium*, *B. licheniformis*, *Micrococcus* and *Pseudomonas radiosa* (Table II).

Coliforms were also determined in 8 samples to be 2×10^2 to 2×10^6 per gram. The coliforms species mainly consisted of *Klebsiella*, *Enterobacter* and *Citrobacter*. No pathogenic species such as *Salmonella* and *Escherichia* were detected.

Molds were determined in 10 samples to be 1×10^2 to 7×10^4 per gram, which consisted mainly of the *Aspergillus glaucus*, *A. restrictus*, *A. flavus*, *A. fumigatus* and *A. niger* groups, and *Penicillium* and *Rhizopus*, and lower frequencies of the *A. ochraceus* and *A. wentii* groups, as shown in Table III. In this case, all of 11 isolates of the *A. flavus* group were identified as *A. flavus* var. *columnaris*, and 2

strains produced aflatoxins that gave blue fluorescent spots on thin layer chromatography (B_1 , B_2 etc.) by the conventional plate agar method, as shown in Fig. 1.

2. *Inactivation of microorganisms in spices*

Three kinds of spices highly contaminated by microorganisms were used for the inactivation study with gamma-irradiation. Figures 2~4 show the inactivation curves for microorganisms in turmeric, black pepper and white pepper. The estimated dose for eliminating the microorganisms to below a detectable level, especially the total aerobic bacteria, ranged from 1.2 to 1.5 Mrad. Coliforms in spices were eliminated with 0.4 to 1.0 Mrad. Generally, molds and coliforms required lower doses for inactivation compared to total bacteria because of the high frequency of *Bacillus* in the latter.

The D_{10} values in 0.067 M phosphate buffer under aerobic conditions of *B. pumilus* strains S2, S4 and S30 ranged from 0.16~0.17 Mrad, and those for *B. subtilis* S1, S14 and S18, and *B. licheniformis* S34 ranged from 0.13~0.14 Mrad. That for *B. megaterium* S31, the most radioresistant strain isolated from spices, was 0.24 Mrad. These D_{10} values are similar to

TABLE III. COMPOSITION OF MOLDS IN SOME SELECTED SPICES ISOLATED FROM MYG-CHLORAMPHENICOL AGAR

Spice	Treatment	Molds count (g)	Mycoflora	Relative frequency (%)
Black pepper	Before storage	1×10^3	<i>A. flavus</i> var. <i>columnaris</i>	56
			<i>A. wentii</i> group	14
			<i>A. fumigatus</i>	31
White pepper	Before storage	8×10^2	<i>A. flavus</i> var. <i>columnaris</i>	19
			<i>A. fumigatus</i>	75
			<i>Penicillium</i> sp.	6
Turmeric	Before storage	2×10^4	<i>A. chevarieri</i>	66
			<i>A. ruber</i>	32
			<i>A. flavo-furcatis</i>	1
			<i>A. ochraceus</i> group	0.3
Rosemary	Before storage	8×10^3	<i>A. chevarieri</i>	98
			<i>Penicillium</i> sp.	2
Thyme	Long storage	8×10^6	<i>A. chevarieri</i>	96

FIG. 1. Blue Fluorescent Spots of Extracts from *A. flavus* var. *columnaris* Isolated from Spices on Thin Layer Chromatography.

1, S46, 2, S74, 3, *A. flavus* IFO-30180.

FIG. 2. Inactivation Curves for Microorganisms in Powdered Turmeric A after Gamma-radiation.

●, total bacteria; ○, coliforms; ▲, molds.

FIG. 3. Inactivation Curves for Microorganisms in Powdered Black Pepper A after Gamma-radiation.

●, total bacteria; ○, coliforms; ▲, molds.

FIG. 4. Inactivation Curves for Microorganisms in Whole White Pepper B after Gamma-radiation.

●, total bacteria; ○, coliforms; ▲, molds.

those in the previous report for retainer type Kamaboko.¹⁴⁾ Figure 5 shows the survival patterns of two strains in dried spices with different water activities. *B. pumilus* S4 and *B. megaterium* S31 showed similar D_{10} values in spices with higher water activity (aw , 0.90~0.98) to as in the aerobic phosphate buffer. In contrast, when these organisms were irradiated in the spices with lower water activity (aw , 0.60), the survival curves tended to tail off at higher doses. This effect is similar to that seen in some inactivation curves for microorganisms in gamma-irradiated spices.

3. Storage effect on irradiated spices

Many kinds of spices are hygroscopic in

nature, which may encourage putrefaction by molds and cause quality changes of the products. For the storage study, black pepper, white pepper, turmeric and rosemary were used in powder and whole forms.

As shown in Fig. 6, mold growth occurred rapidly in powdered black pepper during the first month of storage and reached more than 10^6 per gram at 35°C and 93% R.H. In this case, the aerial gas volume in the polyethylene pouch decreased considerably and the shape of the pouch changed to as if it had been packed

FIG. 5. The Effect of Water Activity of Spices (*aw*) on the Radiation Sensitivity of *B. pumilus* S4 and *B. megaterium* S31.

○, *B. pumilus* in powdered black pepper; ●, *B. pumilus* in powdered turmeric; △, *B. megaterium* in powdered black pepper; ▲, *B. megaterium* in powdered turmeric.

FIG. 6. Effect of Irradiation on the Growth of Osmophilic Molds in Powdered and Whole Black Pepper during Storage at 35°C and 90~93% R.H.

P, powder; W, whole. ○ ●, non-irrad.; △ ▲, 0.2 Mrad; ◆ ◆, 0.4 Mrad.

FIG. 7. Effect of Irradiation on the Increase in Water Activity of Powdered and Whole Black Pepper during Storage at 35°C and 90~93% R.H.

P, powder; W, whole. ○ ●, non-irrad.; △ ▲, 0.2 Mrad; ◆ ◆, 0.4 Mrad.

under vacuum. When the aerial gas volume in the pouches of the stored samples decreased, color changes of the spices were observed with the naked eye. A delay in growth of molds was observed in irradiated samples with a dose of 0.2 Mrad. When powdered black pepper was irradiated with a dose of 0.4 Mrad, mold growth was not observed for more than 2 months of storage. In contrast to the powder form, the absorbed dose of 0.2 to 0.4 Mrad used for the whole black pepper was insufficient to suppress mold growth during storage. This different effect can be explained by that in powder samples, irradiation reduced the rate of increase in *aw*, as shown in Fig. 7, which therefore inhibited the rapid increase of molds. On the contrary, the increase of molds in whole-form samples was independent of the dose but related instead to the increase in *aw*. In this case, samples irradiated at 0.4 Mrad showed the highest rate of increase in *aw*.

Mold growth was also observed in turmeric (powder), rosemary (powder and whole) and white pepper (powder and whole) during 2 months of storage, and reached $10^4 \sim 10^6$ per gram. However, absorbed doses of 0.2 or 0.4 Mrad were sufficient to suppress the mold growth. A similar relationship of *aw* and mold growth was also observed for the other kinds of spices, except for a few samples, such as

TABLE IV. THE RELATIONSHIP BETWEEN THE INCREASE IN OSMOPHILIC MOLDS AND WATER ACTIVITY OF REPRESENTATIVE SPICES IN POWDER FORM AFTER 4 MONTHS OF STORAGE AT 35°C AND 90~93% R.H.

Spice	Dose (Mrad)	Before storage		After 4 months of storage	
		Molds count (g)	<i>aw</i>	Molds count (g)	<i>aw</i>
Black pepper	0	6×10^3	0.62	4×10^6	0.85
	0.2	—	0.62	7×10^5	0.81
White pepper	0	—	0.65	3×10^5	0.83
	0.2	—	0.65	—	0.82
Turmeric	0	1×10^2	0.61	3×10^5	0.83
	0.2	—	0.61	—	0.77
Rosemary	0	3×10^1	0.56	1×10^5	0.79
	0.2	—	0.56	—	0.79
Bay leaves	0	1×10^3	0.55	4×10^4	0.76
	0.2	—	0.55	—	0.70
Bacil	0	3×10^2	0.52	2×10^4	0.80
	0.2	—	0.52	—	0.76
Thyme	0	3×10^1	0.54	1×10^4	0.73
	0.2	—	0.54	—	0.73
Paprika	0	—	0.50	—	0.73
	0.2	—	0.50	—	0.70
Garlic	0	—	0.44	—	0.65
	0.2	—	0.44	—	0.68
Fish meal	0	3×10^2	0.66	—	0.73
	0.2	—	0.66	—	0.73

—, below the detectable limit.

FIG. 8. Effect of Different Relative Humidity Levels during Storage on the Growth of Osmophilic Molds in Powdered Black Pepper Stored in Polyethylene Pouches of Different Thickness.

A, 73 μ m thickness; B, 99 μ m thickness.

powdered paprika and garlic, in which no mold growth was observed as in the case of fish meal (Table IV). Even so, the *aw* of all kinds of spices increased rapidly compared with the fish meal.

The effect of different relative humidity levels on the growth of molds in powdered black

pepper was also investigated. Figure 8 shows that a relative humidity of over 84% allowed growth of molds regardless of the thickness of the polyethylene pouch. However, the growth rate was 73 μ m thickness polyethylene was higher than with 99 μ m material after 42 days of storage. A similar relationship of mold

growth and relative humidity was observed under the storage condition of 30°C.

In these studies, only the *Aspergillus glaucus* and *A. restrictus* groups propagated in spices, and the *A. flavus* group and other toxigenic molds tended to disappear during storage.

DISCUSSION

Spice consumption in Japan is increasing with the steady growth of the food industry, and approximately 30 thousand tons is consumed per year.⁴⁾ About 85% of spices are used in the food industry and 15% in the household sector. In Japan, nearly all spices are imported from overseas countries, and their contamination by microorganisms has been causing serious problems in food processing. Several methods have been tried for microorganism-decontamination of spices such as fumigation with ethylene oxide or steam treatment. Recently, super-heated steam treatment was also applied to some kinds of spices.^{15,16)} However, many spices always show losses to some degree of volatile oils and other important constituents. Radiation treatment has been found to be the most promising method for this purpose. In the study of Goto *et al.*,⁶⁾ the main contaminants of 11 kinds of spices consisted of *Bacillus subtilis* and *B. licheniformis*, and many contaminants were destroyed by irradiation with 0.5 Mrad. In our study, *B. subtilis* and *B. pumilus* were the common contaminants in many spices, and an irradiation dose of 1.0 Mrad appeared to be sufficient for decreasing spore-forming bacteria to below 10³ per gram, the Japanese hygienic standard. However, inactivation curves for microorganisms in irradiated spices tended to tail off at higher doses, and this effect can be explained as due to the increasing radiation-resistivity of spore-forming bacteria at lower water activity or residual radiation-resistant microorganisms such as *B. megaterium* in spices.

In this study, mold growth was observed in many kinds of spices during the summer condition of high humidity in polyethylene

pouches. These results show the possibility of mold propagation in spices under unfavorable conditions of storage and shipment, especially in tropical countries. The damage to spices caused by microorganisms should be considerable in Japan because the summer humidity and temperature are high enough for the growth of molds or bacteria. Irradiation can suppress the growth of molds in spices packed in polyethylene bags during storage and transportation with a dose of 0.4~0.6 Mrad. In this study, an amount of the *Aspergillus flavus* group was isolated from many kinds of spices. However, these toxigenic molds disappeared and the mycoflora became dominated by the *A. glaucus* and *A. restrictus* groups during storage under summer conditions. Further studies on the growth of *A. flavus* and other toxigenic molds during storage are required.

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