Application of Replica Plating and Computer Analysis for Rapid Identification of Bacteria in Some Foods

II. Analysis of Microbial Flora in Irradiated Dover Sole (Microstomus pacificus)

D. A. CORLETT, JR., J. S. LEE, AND R. O. SINNHUBER
Department of Food Science and Technology, Oregon State University, Corvallis, Oregon

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Abstract

Corlett, D. A., Jr. (Oregon State University, Corvallis), J. S. Lee, and R. O. Sinnhuber. Application of replica plating and computer analysis for rapid identification of bacteria in some foods. II. Analysis of microbial flora in irradiated dover sole (Microstomus pacificus). Appl. Microbiol. 13:818-822. 1965.—This investigation was carried out to determine the nature of the microbial flora shifts in dover sole as a result of irradiation and storage at 6 C. The relationship was determined between the microorganisms which initially survive irradiation and those making up the final spoilage flora. A total of 2,723 isolates were examined by use of the replica-plating and computer analysis method. The spoilage of the unirradiated control samples during storage at 6 C was almost entirely due to the growth of Pseudomonas. This group, which occupied 25% of the fresh flora, grew to nearly 100% in 2 days of storage. In contrast, irradiation doses of 0.1, 0.2, 0.3, and 0.4 Mrad favored the growth of Achromobacter and yeasts. The Micrococcus, which survived radiation, did not grow at 6 C. At 0.5 Mrad, spoilage of fish samples was due entirely to the growth of yeasts.

Radiation pasteurization eliminates a large number of microorganisms in fresh food. During subsequent storage of irradiated food at low temperature, however, the survivors eventually grow and produce spoilage.

The effects of irradiation and storage on the microbial flora have been reported for haddock fillets and shucked soft-shelled clams (Masurovsky, Voss, and Goldblith, 1963), Pacific cod fillets (MacLean and Welander, 1960), minced chicken meat (Thorley, 1957), chicken meat (Ingram and Thorley, 1959), chilled poultry treated with antibiotics in addition to irradiation (Thorley, Ingram, and Barnes, 1960), and beef (Wolin, Evans, and Niven, 1957). In these studies, only a qualitative distinction was made between the immediate survivors of irradiation and those that grew during storage at refrigeration temperature. This was perhaps due to the limited number of isolates that were studied.

In the consideration of the microorganisms producing the spoilage of an irradiated food, the relative radiation resistance of the mixed population is not the only factor. It is equally important to determine which of the microorganisms surviving irradiation actually are capable of producing spoilage. A group (or groups) must not only be reasonably resistant to radiation but must also be present in large enough numbers prior to irradiation so that radiation will not eliminate them completely; above all, they must be able to multiply under the given storage condition.

We have attempted in this study to analyze and compare the microbial flora of dover sole (Microstomus pacificus) according to the above criteria.

Materials and Methods

Fish sample preparation. Dover sole fillets, obtained from a commercial filleting plant in Astoria, Ore., were ground in a sterile meat grinder, mixed thoroughly in a sterile glass jar, weighed into sterile petri dishes, and transferred aseptically into glass vials, according to the procedure described by Lee, Shiflett, and Sinnhuber (1966). Each vial contained 10 g of ground fish.

Irradiation. After preparation, the vials were frozen and held frozen until the next day. The fish samples were defrosted at room temperature for 1
hr, packed in wet ice, and taken to the radiation facility. Samples were exposed to radiation doses of 0.1, 0.2, 0.3, 0.4, or 0.5 Mrad by a Co\textsuperscript{60} \(\gamma\)-radiation source. The source had an initial activity of 89,000 c and a dose rate of 1.3 Mrad/hr. Samples were irradiated at ambient temperature, repacked in ice, and subsequently returned to the laboratory. The transit and irradiation took less than 3 hr.

**Storage.** Control (unirradiated) and irradiated vials were stored at 6 C to simulate the conditions of an average household refrigerator.

**Sampling procedure.** The microbial counts of the control and irradiated samples were determined immediately after irradiation and at intervals during storage for up to 22 days. At the time of sampling, two vials were selected at random from a lot having the same radiation exposure level. Each vial was opened aseptically, and the entire contents were emptied into a bottle containing 90 ml of 0.2% peptone water and 25 g of glass beads. After shaking, to break up the tissue, further dilutions were made with 0.2% peptone blanks. Samples of 0.1 ml of the desired dilutions were spread on solidified Tryptone-peptone-NaCl (TPN) agar (Corlett, Lee, and Sinnhuber, 1965). Triplicate plates were made for each dilution. For plating a low dilution, pour-plating sometimes was necessary.

**Microbial identification.** Identification of microbial isolates was carried out according to the procedures reported earlier by Corlett et al. (1965). All colonies which developed on the primary isolation plates were picked. Usually, primary plates which had fewer than 100 colonies per plate were selected for picking, because the colonies on such plates were well separated. When colonies from all triplicate plates were pooled, the total number of isolates per sample ranged from 100 to 300 (Table 1). Care was taken to group colonies of similar size on the same master plates to eliminate difficulties encountered by replica-plating colonies with different growth rates. The practice of grouping colonies according to size may have resolved some of the earlier difficulties reported by Ayers (1960) in his attempt to adapt replica plating for identification of microbial flora in food.

**RESULTS AND DISCUSSION**

**Microbial flora of unirradiated fish.** The microbial flora of fresh, unirradiated dover sole were diverse. All 10 of the microbial groups commonly associated with fish products were found (Corlett et al., 1965). Shewan and Hobbs (1965) pointed out that fish fillets would be expected to be contaminated with psychrophiles from marine sources and mesophiles from landing and filleting processes. It was, therefore, expected that the fresher the fillets, the more diverse the kinds of flora. The microbial species that were found in fresh dover sole are listed in Table 2. The microorganisms in this sample, in order of predominance, were: *Pseudomonas, Flavobacterium, Achromobacter, Bacillus* gram-positive pigmented rods, yeasts, *Micrococcus*, and suspected coliforms.

**When this fresh sample was frozen, thawed, and transported on ice to and from the irradiation source, along with the experimental samples, the viable count increased to 3.8 \(\times\) \(10^4\) compared with 1.7 \(\times\) \(10^4\) for the unfrozen fillet. This increase in count was mainly due to the rapid growth of *Pseudomonas* species (Table 2). The *Pseudomonas* species initially occupied 25% of the

**Table 2. Microbial flora of unfrozen and control* dover sole**

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Per cent in flora</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Un-frozen</td>
</tr>
<tr>
<td>Coliforms</td>
<td>0.6</td>
</tr>
<tr>
<td><em>Pseudomonas</em> type I</td>
<td>14.6</td>
</tr>
<tr>
<td><em>Pseudomonas</em> type II</td>
<td>8.1</td>
</tr>
<tr>
<td><em>Pseudomonas</em> types III and IV</td>
<td>2.7</td>
</tr>
<tr>
<td><em>Achromobacter</em></td>
<td>2.7</td>
</tr>
<tr>
<td><em>Flavobacterium</em></td>
<td>29.2</td>
</tr>
<tr>
<td><em>Bacillus</em></td>
<td>17.9</td>
</tr>
<tr>
<td>Gram-positive pigmented rods</td>
<td>0.6</td>
</tr>
<tr>
<td><em>Micrococcus</em></td>
<td>4.6</td>
</tr>
<tr>
<td>Yeasts</td>
<td>0</td>
</tr>
<tr>
<td>Unidentified</td>
<td>0</td>
</tr>
</tbody>
</table>

* See text for explanation.

**Table 1. Number of isolates examined for flora analysis**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Length of storage</th>
<th>No. of isolates examined</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>days</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0 (Fresh)</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td>0 (Control)</td>
<td>189</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>195</td>
</tr>
<tr>
<td>0.1 Mrad</td>
<td>0</td>
<td>80*</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>144</td>
</tr>
<tr>
<td>0.2 Mrad</td>
<td>0</td>
<td>15*</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>185</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>128</td>
</tr>
<tr>
<td>0.3 Mrad</td>
<td>0</td>
<td>6*</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>172</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>312</td>
</tr>
<tr>
<td>0.4 Mrad</td>
<td>10</td>
<td>172</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>302</td>
</tr>
<tr>
<td>0.5 Mrad</td>
<td>10</td>
<td>6*</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>374</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>2,723</td>
</tr>
</tbody>
</table>

* All isolates examined.
Table 3. Microbial flora distribution among radiation survivors in Dover sole

<table>
<thead>
<tr>
<th>Microbial group</th>
<th>Per cent in each survivor after irradiation (Mrad)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>Coliforms</td>
<td>1.1</td>
</tr>
<tr>
<td>Total Pseudomonas</td>
<td>60.2</td>
</tr>
<tr>
<td>Pseudomonas I</td>
<td>48.1</td>
</tr>
<tr>
<td>Pseudomonas II</td>
<td>9.0</td>
</tr>
<tr>
<td>Pseudomonas III and IV</td>
<td>1.1</td>
</tr>
<tr>
<td>Achromobacter</td>
<td>7.4</td>
</tr>
<tr>
<td>Flavobacterium</td>
<td>15.8</td>
</tr>
<tr>
<td>Bacillus</td>
<td>9.5</td>
</tr>
<tr>
<td>Gram-positive pigmented rods</td>
<td>2.6</td>
</tr>
<tr>
<td>Micrococcus</td>
<td>1.1</td>
</tr>
<tr>
<td>Yeasts</td>
<td>1.6</td>
</tr>
<tr>
<td>Unclassified</td>
<td>2.6</td>
</tr>
<tr>
<td>No. of colonies examined</td>
<td>189</td>
</tr>
</tbody>
</table>

* There were no initial isolates irradiated at 0.4 and 0.5 Mrad.
† All isolates examined.

Flavobacterium species and gram-positive pigmented rods were recovered after 0.1 Mrad, but were no longer detected among the survivors of 0.2 and 0.3 Mrad samples. Pseudomonas species were highly sensitive to radiation, and none could be recovered after 0.1 Mrad, although they were the most numerous species in the unirradiated sample. This observation is in general agreement with the following reported effect of ionizing radiation on the microbial flora in other foods.

The ability of yeasts to survive radiation better than most bacteria in pure culture studies was reported by Bridges (1964), in beef by Wolin et al. (1957), and in chicken by Ingram and Thornley (1959). The high sensitivity of the Pseudomonas species to radiation was observed in beef by Wolin et al. (1957), and in chicken by Ingram and Thornley (1959). The latter authors also found that the Achromobacter species were quite resistant to radiation, but not as resistant as the yeasts.

The composite nature of the dose-survival curves shown in Fig. 1 for the total count, as well as for each surviving group, might have resulted from the species heterogeneity within each group.

Microbial flora change during storage at 6°C.

The graphic representation of the microbial flora shift in the nonirradiated Dover sole, stored for 2 days at 6°C, is given in Fig. 2. The Pseudomonas, Achromobacter, and, to a lesser degree, the Flavobacterium comprised 96% of the total flora after 2 days. The Achromobacter, yeasts, and gram-positive flora but increased to 58% of the flora in the frozen-thawed sample. Since the irradiated samples were also subjected to freezing and thawing, the values of a frozen-thawed sample were used as the control.

Microbial flora changes after irradiation. A comparison of the microbial flora in unirradiated and irradiated fish is given in Table 3. A dose of 0.1 Mrad reduced the original population to less than 0.1%. Of the 10 main groups of microorganisms, 5 were recovered after 0.1 Mrad. They were Achromobacter, Micrococcus, yeasts, gram-positive pigmented rods, and Bacillus. It was noted that the Pseudomonas and Flavobacterium species, which occupied 76% of the unirradiated flora, were all but eliminated by this irradiation dose.

Figure 1 shows the dose-survival curves for the surviving groups at 0.1, 0.2, and 0.3 Mrad levels. No survivors could be detected among 0.4 and 0.5 Mrad samples at the minimal dilution of 10⁸. The yeasts were most resistant to radiation among microorganisms found in Dover sole. Despite their low initial number, the proportion of surviving yeasts increased over the others as the radiation dose increased (Fig. 1).

The Micrococcus and Achromobacter species were second and third in their respective resistances, although higher numbers survived 0.1 Mrad. The number of survivors depended on their initial number in the unirradiated fish. This accounts for the large number of Achromobacter species surviving after 0.1 Mrad, despite their higher radiation sensitivity when compared with Micrococcus and yeasts. As the radiation dose increased, however, more yeasts and Micrococcus survived.

Fig. 1. Microbial flora change in Dover sole as a result of irradiation.
itive pigmented rods also increased very rapidly, but not the Bacillus and Micrococcus species.

The flora shift in Dover sole irradiated at 0.1 Mrad and stored at 6°C is illustrated in Fig. 3. After 6 days, only Achromobacter species and yeasts were detectable. The Micrococcus species, the gram-positive pigmented rods, and Flavobacterium species, which were detected among the initial survivors, were no longer recovered after storage. The potential for a given microbial species to survive radiation and its ability to grow at low storage temperatures are two distinct properties.

The postirradiation flora of the 0.2-, 0.3-, and 0.4-Mrad samples were similar to that of the 0.1-Mrad sample (Fig. 4). The rapid growth of the Achromobacter species allowed this group to multiply in the 0.2- and 0.3-Mrad samples. Nevertheless, yeasts grew in these samples equally well. In the 0.4-Mrad samples, the Achromobacter species and yeasts grew in approximately equal proportions. Irradiation at 0.4 Mrad apparently reduced the numbers of surviving Achromobacter species to a point where their growth could no longer overtake the growth of yeasts.

At 0.5 Mrad, yeasts and the Micrococcus species were the only detectable survivors (Fig. 4). The terminal spoilage flora in this sample, however, consisted entirely of yeasts. The Micrococcus species were unable to grow efficiently at 6°C, although they were among the principal radiation survivors (Fig. 4).

No attempt was made to identify yeasts in this study. It was expected that they would have been similar to those examined from Pacific crab meat by Eklund et al. (in preparation).

Effect of radiation on microbial population in fish. Fresh fish fillets may come from numerous sources and may contain a diverse assortment of microorganisms (Shewan and Liston, 1955). However, those microorganisms that possess the ability to grow under given storage conditions are responsible for the final spoilage of fish. The Pseudomonas species appear to have such an advantage in Dover sole stored at 6°C under aerobic conditions.

Irradiation introduces a different selective factor. It was noted in this study that yeasts were the most radiation-resistant group, followed by Micrococcus and Achromobacter species in Dover sole. On the other hand, the Flavobacterium and Pseudomonas species were among the most sensitive to radiation. Since the initial number of a given microbial species prior to irradiation determines the probability of its postirradiation recovery, a group's participation in the terminal spoilage flora is determined by the following factors: the initial number, the relative resistance to radiation, and the ability to grow at 6°C.

The Pseudomonas species, which predominated in the initial flora and grew readily at refrigeration temperature, nevertheless, were the most...
Fig. 4. Microbial growth in Dover sole irradiated (0.2, 0.3, 0.4, and 0.5 Mrad) and stored at 6 C. Symbols: T = total count, A = Achromobacter, Y = yeasts, M = Micrococcus, G = R = gram-positive pigmented rod. Radiation doses, given in Mrad, are shown after symbols.

sensitive to radiation and could be eliminated easily by 0.1 Mrad.

The Micrococcus species, which showed relatively higher resistance against radiation and were constantly recovered in postirradiation populations despite their low initial number, however, did not grow at 6 C. The Micrococcus species, thus, contributed least to the spoilage flora of irradiated Dover sole.

The Achromobacter species found in moderate numbers in the initial flora were moderately resistant to radiation and were also able to grow rapidly at 6 C. This group, therefore, contributed the most to the spoilage flora of Dover sole at 0.1 to 0.3 Mrad.

Yeasts were found in low numbers in the unirradiated sample. Their growth rate was somewhat slower than bacteria in Dover sole. Due to their high resistance to radiation, they contributed to the spoilage flora of samples irradiated at 0.4 and 0.5 Mrad. Since yeast growth may be reduced under anaerobic conditions, this generalization may not apply to vacuum-packed fish, as shown by Eklund et al. (in preparation).

The Flavobacterium species, Bacillus species, "coryneforms," and Gram-positive pigmented rods were found in irregular proportions in the fresh sample. Although they were recovered in the irradiated sample, it is not likely that these groups contribute significantly to the final spoilage, owing to their low numbers and slow growth rates at 6 C.

Acknowledgments

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Literature Cited


