Incidence, Radioresistance, and Behavior of *Psychrobacter* spp. in Rabbit Meat

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ABSTRACT

The relative incidence of *Psychrobacter* spp. in rabbit meat, the radioresistance of these bacteria, and the growth of nonirradiated and irradiated psychrobacter isolates, alone and in coculture, during chilled storage of inoculated sterile rabbit meat was investigated. *Psychrobacter* spp. accounted for 4.2% of the storage psychrotrophic flora of 30 rabbit carcasses. The radiation $D_{10}$-values of 10 *Psychrobacter* isolates, irradiated at 4°C in minced rabbit meat, ranged from 0.8 to 2.0 kGy, with significant ($P < 0.05$) differences among strains. Over 12 days of storage at 4°C, pure cultures of two nonirradiated psychrobacter strains ($D_{10} = 2$ kGy) were capable of substantial increases (up to 3 log CFU/g) in sterile rabbit meat, but when the fastest growing strain was cocultured with *Pseudomonas fluorescens* and *Brochothrix thermosphacta* isolates, maximum cell densities and growth rates were significantly ($P < 0.01$) lower. After irradiation (2.5 kGy) of pure cultures in sterile rabbit meat, surviving cells of both *Psychrobacter* strains decreased for a period of 5 to 7 days and then resumed multiplication that, at day 12, resulted in a similar increase (1.6 to 1.7 log CFU/g) over initial survivor numbers. When irradiated in combination with the spoilage bacteria, one of the strains required 12 days to reach initial numbers. In conclusion, *Psychrobacter* spp. are radioresistant nonsporeforming bacteria with a low relative incidence among the storage flora of rabbit meat, unable to compete with food spoilage bacteria in this ecosystem and apparently not a major contributor to the spoilage of rabbit meat after irradiation.

The genus *Psychrobacter*, currently included in the family *Moraxellaceae* (12), comprises psychrotolerant and halotolerant gram-negative, coccolid, aerobic, nonmotile, oxidase-positive bacteria, which were originally allocated to “Achromobacter” or *Acinetobacter* or referred to as *Moraxella-Acinetobacter* (M-A) group or *Moraxella*-like (23, 24). These organisms are isolated from aquatic and terrestrial cold environments, including food commodities such as fish, poultry, meat, and several meat products (2, 13, 14, 16, 24, 33, 35). Earlier studies reported that in contrast to most of the vegetative cells found in these flesh foods, psychrotrophic coccocacilli of the M-A group were the main residual bacterial populations after irradiation for preservation purposes and constituted the predominant flora of certain spoiled irradiated foods (1, 21, 23, 27, 40).

Since *Psychrobacter* spp. can occur in chilled raw meat stored in air, they have been associated with meat spoilage (3, 20). However, there are no available data on psychrobacter development in foods, and the few studies from which their relative incidence can be determined suggest that they constitute a small proportion of the storage flora (11). One exception is that observed by Shaw and Latty (35), who found that *Psychrobacter immobilis* comprised up to 50% of the flora on a high-pH tissue (fat surfaces). Also, in poultry meat, M-A strains are isolated more frequently from leg muscle (high pH) than from breast muscle (28, 29).

In previous work (34), we have observed that ultimate pH of rabbit meat is close to 6.0 and that its storage microflora is somehow different from that on the meat of other food animals.

This work was undertaken to determine (i) the incidence of *Psychrobacter* spp. on a high-pH meat (rabbit meat), (ii) the sensitivity of these bacteria to irradiation when inoculated in rabbit meat, and (iii) the growth of nonirradiated and irradiated strains in pure cultures and cocultures during chilled storage of sterile rabbit meat.

MATERIALS AND METHODS

Incidence of *Psychrobacter* spp. on rabbit meat. At intervals, between 0 and 7 days of storage at 3°C (shelf life of 1 week), 30 samples from rabbit meat carcasses in polystyrene trays overwrapped with oxygen-permeable films were tested for psychrotrophic counts at 4.5°C and pH values as described elsewhere (35). Subsequently, a total of 95 randomly selected isolates were submitted to a phenotypic examination. Gram staining, cellular morphology, colonial pigmentation, and oxidase and catalase production were examined as described by Downes and Ito (4). Motility and ability to grow anaerobically were investigated as described by García-López et al. (10). *P. immobilis* strain CECT (Spanish Collection of Type Cultures) 5008 and one *Pseudomonas aeruginosa* strain from our laboratory collection were used as controls.

Definitive identification of suspected *Psychrobacter* isolates was achieved on the basis of a transformation assay (24, 25) by using the hypoxanthine and thiamine-requiring mutant *P. immo-
bilis strain ATCC 43117, the M9A medium (24), and the type strain P. immobilis CECT 5008 as control.

Irradiation conditions. In all cases, the irradiation was carried out at 4°C. Samples were irradiated using a cobalt-60 source (Gammabeam 650, Nordion International Inc., Ottawa, Ontario, Canada) with a dose rate of 6.32 kGy/h. Gammachrome YR dosimeters (AEA Technology, Harwell, UK) were used to confirm the dose received by samples. The change in absorbance of the dosimeters at 530 or 603 nm was spectrophotometrically measured and the corresponding doses obtained from a calibration graph provided by the National Physical Laboratory, Teddington, UK.

Irradiation resistance of Psychrobacter spp. in inoculated rabbit meat: test strains. Ten Psychrobacter strains were used. P. immobilis LMG 7062 (clinical specimen), LMG 7551 (fish), LMG 5008 (green bean), and LMG 7550 (cod) were obtained from the Belgian Coordinated Collections of Microorganisms; three Psychrobacter spp. strains were obtained in this study (strains RM1, RM2, and RM3); and three Psychrobacter spp. strains were recovered from shellfish specimens (strains SLL1, SLL2, and SLL3). Working cultures were maintained on tryptone soya agar (TSA; Oxoid, Basingstoke, UK) slants at 4°C.

Preparation, inoculation, and treatment of rabbit meat. Frozen rabbit carcasses were purchased from a local supermarket, thawed, deboned, minced, and packaged (10 g) in polyether-polyethylene bags as described by Patterson (32). After heat sealing, the bags were irradiated to 20 kGy to remove microflora and stored at 4°C until required.

Strains were grown in 10 ml of tryptone soya broth (Oxoid) for 48 h at 25°C to obtain cells in the late exponential phase of growth. Sterilized bags were partially opened and 100-μl volumes containing ca. 10⁷ cells, were mixed with 10 g of rabbit meat mince samples. Bags were resealed and treated within 1 h of inoculation, in triplicate with doses of 0 (control), 1, 2, 3, 4, 5, and 6 kGy.

Determination of radiation D₁₀⁻values. After irradiation, each sample was mixed with 90 ml of peptone saline water (Oxoid), homogenized for 2 min with a Colworth 400 stomacher (A. J. Seward, London, UK), and serially diluted in 0.1% peptone solution (Oxoid). Spread plating on TSA (Oxoid) containing 0.6% yeast extract (TSAYE; Oxoid) was used to enumerate surviving bacterial populations. Two plates per dilution were incubated at 25°C for 48 h.

The logarithm of surviving fractions of the populations was plotted against the radiation dose to determine the death rate of each Psychrobacter strain. Individual regression lines were calculated (Statistica V.5.5., Statsoft, Inc., Tulsa, Okla.) and D₁₀⁻values obtained by taking the negative reciprocal of the slope.

Pure cultures and cocultures studies in sterile rabbit meat mince: strains and inocula. Pure culture experiments were performed with P. immobilis strain LMG7062 and Psychrobacter spp. strain RM1, which showed the highest radioresistance (“Results” section). For coculture experiments, Pseudomonas fluorescens strain NCTC 10038 and Brochothrix thermophila strain NCTC 10822 were selected. All strains were grown separately in tryptone soya broth at 25°C for 48 h.

Inocula for pure culture experiments were 100-μl aliquots of culture broth, containing about 10⁷ CFU of each Psychrobacter strain in the late exponential growth phase. Two-strain inocula were 200-μl volumes containing approximately equal populations (ca. 10⁷ cells) of P. fluorescens and B. thermophila. Three-strain inocula were 300-μl volumes containing approximately equal populations (ca. 10⁷ cells) of Psychrobacter strain RM1, P. fluorescens, and B. thermophila.

Growth of nonirradiated and irradiated pure cultures of Psychrobacter strains. To investigate the behavior of nonirradiated and irradiated pure cultures of Psychrobacter in rabbit meat mince, 24 bagged sterile samples (10 g) were spiked with each Psychrobacter strain and half of them treated at 2.5 kGy. All samples were maintained at 4°C for up to 12 days and 4 bags (2 treated and 2 untreated) examined at intervals of 0, 2, 5, 7, 9, and 12 days of storage for microbiological analysis.

Growth of irradiated three-strain inocula. Growth of the three-strain mixtures after irradiation was studied by inoculating each combination in 12 sterile samples of rabbit meat mince, which were then irradiated at 2.5 kGy and chilled stored, two of them being examined each sampling day.

Growth of nonirradiated two-strain inocula. Twelve bags of sterile rabbit meat mince were inoculated with the combination of P. fluorescens and B. thermophila, stored, and examined as above.

Growth of nonirradiated three-strain inocula. Twelve sterile rabbit meat mince samples were spiked with the three-strain mixture, chilled stored, and examined at intervals. There are no differential media available for psychrobacters, so 12 additional samples were inoculated and kept refrigerated. Each sampling day, two of them were irradiated at 2.5 kGy to eliminate P. fluorescens and B. thermophila and analyzed.

Microbiological analysis. Each sample was mixed with 90 ml of peptone saline water, homogenized for 2 min, and serially diluted in peptone water as described above. In monoculture experiments, 100-μl aliquots of the appropriate dilutions were surface plated in duplicate on TSAYE plates, which were incubated at 25°C for 48 h.

In coculture experiments, total counts were determined on TSAYE, P. fluorescens on cetrimide-fucidin-cephaloridine (Oxoid) medium, and B. thermophila on streptomycin-thallous-acetate agar (Oxoid). Numbers of Psychrobacter strain RM1 were estimated on the basis of colony counts, D₁₀⁻value, and treatment dose after incubation of appropriated dilutions on TSAYE. All incubations were done at 25°C for 48 h. After 12 days of storage, the relative incidence of each inoculated species was estimated by random selection, using a Harrison disc (19), of 60 colonies from total count plates. Subcultured colonies were Gram stained and tested for oxidase and catalase production, oxidation-fermentation test, and motility (19).

Growth rate of the different strains. The daily growth rate of each strain was calculated from counts on solid media (log CFU per gram) using the following formula: daily growth rate between days a and b = (counts on day b − counts on day a)/number of days between a and b.

Replication of the experiments. All experiments about the behavior of pure cultures and cocultures in sterile rabbit meat mince were repeated twice (two lots). Each sampling day two bags were analyzed from each lot.

Statistical analysis. Mean D₁₀⁻values, mean log numbers, maximum numbers, and growth rates were compared by the t test (Statistica V.5.5.).
TABLE 1. Radiation $D_{10}$-values of Psychrobacter spp. inoculated in irradiated rabbit meat mince

<table>
<thead>
<tr>
<th>Strain of Psychrobacter</th>
<th>$D_{10}$-value (kGy)$^a$</th>
<th>$R^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>RM1</td>
<td>2.0 ± 0.2 A$^c$</td>
<td>0.96</td>
</tr>
<tr>
<td>LMG 7062</td>
<td>2.0 ± 0.1 A</td>
<td>0.96</td>
</tr>
<tr>
<td>SLL2</td>
<td>1.6 ± 0.2 AB</td>
<td>0.99</td>
</tr>
<tr>
<td>LMG 7550</td>
<td>1.3 ± 0.2 B</td>
<td>0.99</td>
</tr>
<tr>
<td>SLL3</td>
<td>1.3 ± 0.1 B</td>
<td>0.92</td>
</tr>
<tr>
<td>LMG 7551</td>
<td>0.8 ± 0.2 c</td>
<td>0.98</td>
</tr>
<tr>
<td>SLL1</td>
<td>1.0 ± 0.2 c</td>
<td>0.98</td>
</tr>
<tr>
<td>RM3</td>
<td>0.9 ± 0.1 c</td>
<td>0.98</td>
</tr>
<tr>
<td>RM2</td>
<td>1.0 ± 0.1 c</td>
<td>0.99</td>
</tr>
<tr>
<td>LMG 5008</td>
<td>1.1 ± 0.1 c</td>
<td>0.98</td>
</tr>
</tbody>
</table>

$^a$ Negative reciprocal of the slope of each regression line (log CFU per gram of surviving population versus radiation dose).

$^b$ Coefficient of determination of the regression line.

$^c$ Values with no letter in common are significantly different ($P < 0.05$). Each $D_{10}$-value is the mean of three sets of data (not significantly different, $P > 0.05$).

RESULTS

Incidence of Psychrobacter spp. on rabbit meat.

Only four isolates (4.2%) gave positive results when tested for the transformation assay. They were recovered from four different carcasses at different storage days.

Radiation resistance of Psychrobacter. Radiation $D_{10}$-values of the tested strains are shown in Table 1. The coefficients of determination of the regression lines ($R^2$) were between 0.92 and 0.99, all but one being higher than 0.96. The $D_{10}$-values ranged from 0.8 to 2.0 kGy. There were statistically significant differences ($P < 0.05$) between strains, which could be divided into three groups with average $D_{10}$-values of 0.96 (LMG 7551, SLL1, RM3, RM2, LMG 5008), 1.40 (SLL2, LMG 7550, SLL3), and 2.00 kGy (RM1, LMG 7062). These groups were not related to the origin of the strains.

Pure cultures and cocultures experiments in sterile rabbit meat mince. Figure 1 shows growth of nonirradiated and irradiated pure cultures of Psychrobacter strains LMG 7062 and RM1 in sterile rabbit meat mince over 12 days at 4°C. The maximum increase of nonirradiated strain LMG 7062 was 2.1 log CFU/g at the end of storage, with a daily increase of 0.17 log CFU/g. For nonirradiated strain RM1, maximum numbers (9 log CFU/g) were attained at day 9. Between days 0 and 12, population increase was 2.7 log units per g at a daily rate of 0.22 log CFU/g. After irradiation (2.5 kGy) in rabbit meat, both strains decreased in numbers for a period of 5 to 7 days and then resumed multiplication that resulted in a similar increase over initial survivor numbers (1.6 to 1.7 log CFU/g).

The effect of 2.5 kGy on the three-strain mixtures is shown in Figure 2. After treatment of sterile rabbit meat spiked with both cocultures, P. fluorescens and B. thermosphacta were under the detection level (1.7 log CFU/g) and remained so until the end of the storage period. At this time, all randomly selected colonies were psychrobacters. During storage, TSAYE counts on rabbit meat inoculated with the coculture containing strain LMG 7062 increased by 2.4 log CFU/g at a daily rate of 0.20 log CFU/g. For samples inoculated with the coculture containing strain RM1, the values were 0.1 log CFU/g and 0.01 log CFU/g, respectively.

When P. fluorescens and B. thermosphacta were grown in sterile rabbit meat for 12 days (Fig. 3), Pseudomonas numbers increased by 3.7 log CFU/g at a daily rate of 0.30 log CFU/g and B. thermosphacta increased by 2.7 log CFU/g at a daily rate of 0.22 log CFU/g. At the end of storage, the relative proportions of P. fluorescens and B. thermosphacta among the total flora were 59.3 and 40.7%, respectively.

Growth in sterile rabbit meat of the three-strain mixture...
FIGURE 4. Growth of cocultured food spoilage bacteria (P. fluorescens plus B. thermosphacta) and Psychrobacter strain RM1 in sterile rabbit meat stored at 4°C. ■, total counts; ▲, P. fluorescens counts; ×, B. thermosphacta counts; ○, estimated Psychrobacter counts.

consisting of the above spoilage bacteria and Psychrobacter strain RM1 is shown in Figure 4. Again, P. fluorescens attained its maximum population (9.7 log CFU/g) after 12 days, with numbers increasing by 3.9 log CFU/g at a daily rate of 0.32 log CFU/g. B. thermosphacta reached its maximum levels (8.4 log CFU/g) after 9 days of storage with an increase of 2.2 log CFU/g at a daily rate of 0.25 log CFU/g. Between days 9 and 12, B. thermosphacta population declined by 1.3 log CFU/g at a daily rate of −0.42 log CFU/g. The maximum counts of Psychrobacter (7.3 log CFU/g) were obtained after 7 days of storage, with an increase in numbers of 1.4 log CFU/g at a daily rate of 0.19 log CFU/g. Between days 7 and 12, Psychrobacter numbers declined by 0.1 log CFU/g at a daily rate of −0.03 log CFU/g. Only P. fluorescens was recovered from total count plates after 12 days of storage.

DISCUSSION

The relative incidence of Psychrobacter spp. among the storage flora of rabbit meat (4.2%) compares well with findings in other chilled raw proteinaceous foods (beef, lamb, poultry, freshwater fish, milk, and soft and fresh cheeses) in which they accounted for less than 1 to 5% (5, 9, 13, 16, 33). These bacteria appear to be more prevalent in fresh marine fish, particularly in herring, sardine, and cod (2, 14, 15, 30), although their highest relative incidences have been reported on fat surfaces (up to 50%) and in rehydrated salt-cured and dried salt-cured cod (up to 90%). A musty off odor has been associated with psychrobacters growth in rehydrated salted cod products (2, 35).

Because members of the M-A group and Moraxella-like strains have been found surviving in irradiated foods, psychrobacters are recognized as radiation-resistant organisms (24). A number of reports on irradiation sensitivity of M-A and Moraxella-like isolates were published in the 1970s and early 1980s, but only one (21) was performed with strains tested for the definitive transformation assay. Eleven psychrotrophic coccobiacilli, certainly psychrobacters, had $D_{10^5}$ values in phosphate buffer at 20°C in the range of 0.25 to 0.66 kGy. In similar conditions, the $D_{10^5}$ values of four M-A isolates from radurized Vienna sausages ranged from 0.44 to 0.54 kGy (23). More variation in radiation resistance existed among the M-A isolates treated in culture broth by Tiwari and Maxcy (38), who reported $D_{10^5}$-values ranging from 0.15 to 1.12 kGy. When isolating the most radiation-resistant vegetative cells from raw beef, Welch and Maxcy (40) found the extreme radiation resistance at −30°C of two M-A cultures ($D_{10^5}$-values of 2.73 and 4.80 kGy), and when a combination of drying and subfreezing temperature of irradiation was assayed, the $D_{10^5}$ value of representative M-A isolates was as high as 28.3 kGy (27). One strain of Moraxella phenylpyruvica (now Psychrobacter phenylpyruvica) treated in minced poultry meat had a $D_{10^5}$-value of 0.86 kGy (32). Analyzing data in the literature, van Gerwen et al. (39) identified several members of the M-A group as having very high resistance at very low temperatures (≤−30°C), estimating an average $D_{10^5}$-value of 3.65 kGy under this condition.

In this study, $D_{10^5}$-values obtained for P. immobilis and Psychrobacter spp. irradiated at 4°C in rabbit meat varied between 0.8 and 2.0 kGy depending on the strain. However, even the most sensitive strain was more resistant to gamma radiation than the majority of vegetative cells in fresh foods of animal origin, and there were isolates with radiation resistance comparable to that of some bacterial spores (7, 8, 31). Although sensitivity to irradiation depends on several factors, high resistance in nonsporulating bacteria is attributed to the ability to repair DNA damage caused by irradiation (8).

An early study (36) reported that radioresistant M-A isolates from beef were unable to multiply in sterilized ground beef, ground chicken meat, or fresh beef exudates incubated for 14 days in the range 5 to 32°C. In contrast, the two Psychrobacter isolates tested in the present study, especially that obtained from rabbit meat (RM1), were capable of substantial growth at 4°C in pure culture rabbit meat experiments (Fig. 1). When cocultured with P. fluorescens and B. thermosphacta, growth of isolate RM1 was affected, as its maximum cell density and growth rate were significantly lower ($P < 0.01$) than when grown alone (Figs. 1 and 4). In this three-strain experiment (Fig. 4), growth of the P. fluorescens isolate was similar to that observed in absence of strain RM1 (Fig. 4). But the behavior of the B. thermosphacta isolate differed at day 12, and although it grew faster and reached a higher maximum level than the Psychrobacter isolate, its final population was slightly smaller. Thus, at the end of storage, B. thermosphacta, which accounted for 40.7% of the total flora in the two-strain experiment, could not be detected in the three-strain experiment.

The relative proportions of the different organisms that comprise the storage flora of chilled meat in air are related to their relative growth rates, but this alone is not responsible for success (3). Part of the inhibitory activity of certain gram-negative bacteria, particularly pseudomonads of the fluorescent group, has been attributed to competition for iron and production of antibacterial compounds (17, 18). Although most Psychrobacter strains produce siderophores, as did strain RM1 (data not shown), this study shows that it is unlikely for this bacterium to be an important contributor to the spoilage of rabbit meat or to any of the other proteinaceous foods where Pseudomonas predominates.
Since radioresistant strains of the M-A group have been found to be responsible for the spoilage of irradiated Vienna sausages at 10°C (22) and predominating on irradiated stored fish and poultry (26, 37), it was suggested that these organisms (probably psychrobacters) could play a more direct role in spoilage of irradiated muscle foods (35). In this study, numbers of irradiated (2.5 kGy) pure cultures of *Psychrobacter* in rabbit meat were affected initially and, after a considerable lag time, increased by less than 2 log CFU/g (Fig. 1). When irradiated in combination with spoilage bacteria, surviving cells of strain LMG 7062 showed a shorter lag time and reached identical maximum number, but those of RM1 took 12 days to reach initial numbers (Fig. 2). This unexpected behavior cannot be related to surviving spoilage bacteria, since they were not detected over the storage period. Overall, our data suggest that radioresistant *Psychrobacter* strains irradiated in rabbit meat need prolonged refrigerated storage to repair sublethal damage (6) and to attain high numbers. This and their low spoiling potential (15, 33) make unlikely that these bacteria have a major role in spoilage of irradiated meat.

In conclusion, *Psychrobacter* spp. are radioresistant nonsporing bacteria with a low incidence among the storage flora of rabbit meat that apparently are unable to compete with common spoilage bacteria in this ecosystem. It also seems unlikely that they are responsible for the spoilage of irradiated rabbit meat.

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