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Irradiation to control vibrio infection from consumption of raw seafood and fresh produce

Results of a co-ordinated research project
organized by the
Pan American Health Organization
and the
Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture



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FOREWORD

Vibrio spp. comprises an important group of pathogenic bacteria in food that often causes human illness and even death when the contaminated food is consumed raw or improperly cooked. The most dangerous member of this group, the El Tor strain of *V. cholerae*, was responsible for the cholera pandemic which started in Peru in 1991 and spread to nearby countries, resulting in hundreds of thousands of cases and thousands of deaths.

Recognizing the role of irradiation to ensure the microbiological safety of food, the Pan American Health Organization of the World Health Organization and the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture jointly sponsored a Co-ordinated Research Project (CRP) on the Use of Irradiation as a Public Health Intervention Measure to Control Foodborne Diseases in Latin America and the Caribbean, to assess the efficacy of this technology for food protection. The CRP was initiated in 1993 and concluded in 1998.

The results of this CRP demonstrated that irradiation is effective for ensuring the microbiological safety of food naturally contaminated by *Vibrio* spp. This process offers unique benefits for decontamination of seafood, often contaminated with this group of aquatic bacteria at the source, and fresh vegetables that may be contaminated during production and handling, especially when these products are consumed raw or not thoroughly cooked. Because of the sensitivity of this group of bacteria to radiation, the dose required to ensure microbiological safety of food against them is not more than 1 kGy. The CRP also generated data on the effectiveness of irradiation to control infection by pork tapeworm (*Taenia solium* metacestode). However, the results of these studies were not conclusive enough for publication.

This publication presents the research results reported at the final Research Co-ordination meeting on this CRP held in Havana, Cuba, 16–20 November 1998.

The scientific secretaries for this CRP were P. Loaharanu and R. Molins of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture. The latter was responsible for finalizing the report of the final RCM including preparation of its proceedings for publication.

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CONTENTS

SUMMARY OF THE CO-ORDINATED RESEARCH PROJECT

1. BACKGROUND

The incidence of food borne diseases in recent years has increased at an alarming rate around the world, causing consumer outcries and prompting governments and industry to evaluate various technological options that could enhance the hygienic quality of the food supply, including techniques for decontaminating food of potentially pathogenic microorganisms. Although the risk of microbial contamination is high along the entire food chain, from production to consumption, the hazards posed by microbiological contaminants in solid and semisolid foods that are consumed raw are a major concern for public health authorities because, in most instances, no decontamination processing steps stand between contamination and consumption of such foods.

In essence, the search for food safety assurance technology has focused on processing techniques that could accomplish control of human pathogens in solid and semisolid foods, especially those consumed raw and including pre-cut, "ready-to-eat" produce, similar to that provided liquid foods by thermal pasteurization. In this context, the unique potential of irradiation as a physical method for eliminating disease-causing bacteria that contaminate solid and semisolid food has been recognized. Because irradiation can eliminate pathogenic bacteria in food without substantial increases in temperature, it is being referred to as "cold pasteurization." When used for pathogen control, irradiation has the same purpose as the thermal pasteurization treatment of milk and other liquid foods, except that ionizing radiation is used in the process in lieu of heat.

Advocates of the modern preventive food safety control methodology for use in food processing and handling operations known as hazard analysis and critical control point (HACCP), designed to prevent, eliminate or reduce to acceptable levels the risks posed by the presence of disease-causing bacteria in food, are faced with the need to consider food irradiation as possibly the only critical control point presently available for raw foods of animal origin such as poultry, meat, fish and other seafood, and fresh produce. By definition, only a cold decontamination treatment like irradiation, able to inactivate bacteria in food without heating it, can be used on food that is to be sold and consumed in raw form.

Foodborne diseases that may be transmitted by many shellfish, fish, and other seafood include some that have also been attributed to poultry and meats (e.g. salmonellosis), as well as others caused by aquatic microorganisms, particularly bacteria belonging to the *Vibrionaceae*. Among these, *Vibrio cholerae* has been implicated in the cholera pandemic that afflicted many South and Central American countries from 1991 to 1993, with a toll of more than 300 000 cases and 30 000 deaths. Other members of this troublesome group of bacteria are *Vibrio vulnificus* and *Vibrio parahaemolyticus*, frequently found in shellfish. Irradiation at relatively low doses (2–3 kGy) has long been advocated to eliminate these contaminants and allow for continued enjoyment of raw fish dishes as the popular South American "ceviche," as well as fresh, live oysters and clams. However, although irradiation of fish and seafood is approved in 14 countries and is being considered for approval in several more, there are countries such as Uruguay that require such fresh mollusks in the shell as mussels to be marketed live. Little is known about the survivability of irradiated mollusks.

To address the above issues, the Pan American Health Organization (PAHO) Regional Program for Technical Cooperation in Food Safety adopted irradiation as a new technology that could improve food safety. This involved studies on the efficacy of applying irradiation

as a public health intervention measure to prevent foodborne diseases from consumption of raw seafood contaminated with *Vibrio* spp. and cysticercosis/taeniasis from eating pork. Consequently, PAHO and the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture of the International Atomic Energy Agency (IAEA), convened a Joint Technical Consultation on Irradiation as a Public Health Intervention Measure for Foodborne Diseases in Latin America and the Caribbean, at the headquarters of PAHO in Washington, DC, from 19 to 21 October 1992.

During the technical consultation the epidemiological and economic repercussions of irradiation as a method to inactivate pathogenic bacteria and parasites in food were discussed in depth. A 5-year plan of action was recommended to carry out investigations to ensure that concrete results could be obtained on this important subject. The plan of action contemplated the development of an IAEA/FAO/PAHO/WHO Co-ordinated Research Project on Irradiation as a Public Health Intervention Measure to Control Foodborne Diseases, which provided the orientation framework for the joint studies to demonstrate the efficiency and efficacy of this technology in food protection. The research was oriented towards foodborne diseases caused by bacteria and parasitic pathogens (viz. *Vibrio* spp. and *Taenia solium*) that are considered of importance as a public health problem and as cause of human suffering in Latin America and the Caribbean. To make the 5-year programme operational, it was agreed that periodic Research Co-ordination meetings (RCMs) of principal scientific investigators would be convened. The purpose of these meetings would be to discuss the results, plan the future development, and co-ordinate the research being conducted by the participating investigators. The plan of action was endorsed by the VIII Inter-American Meeting at the Ministerial level on Animal Health (RIMSA VIII) convened in Washington, DC, in April 1993.

Apart from the investigations that were carried out, both agencies continued to provide the necessary co-operation to its Member Governments to develop the regulations needed in matters of irradiation, taking into consideration the principles embodied in the *Codex Alimentarius* General Standard for Irradiated Foods and its associated Recommended International Code of Practice for the Operation of Radiation Facilities Used for the Treatment of Foods. Thus, in 1997 the International Consultative Group on Food Irradiation (ICGFI), jointly sponsored by the FAO, the IAEA and WHO, conducted a workshop in Lima, Peru that produced a Model Regulation for Irradiated Food for Latin America and the Caribbean to be used in harmonization of food irradiation regulations in the Americas. Similar models have been developed through the efforts of the IAEA and/or ICGFI for Asia, Africa and the Near East, thus facilitating the goal of global harmonization of such regulations.

The first co-ordination meeting of this programme was convened at the WHO Collaborating Center for Reference and Training in Remote Sensing and Geographical Information Systems for Veterinary Public Health, Louisiana State University, School of Veterinary Medicine, Baton Rouge, Louisiana, USA, from 14–16 September 1994. The second one was held in Tampa, Florida, USA, from 1–5 April, 1997. Thehe third and final was held in Havana, Cuba, from 16–20 November, 1998.

2. OBJECTIVES

The general objective of this CRP was to assist institutions in Latin America and the Caribbean to investigate the efficacy of using irradiation as a public health intervention measure to control *Vibrio* infections through consumption of raw or semi-cooked seafood, and cysticercosis/taeniasis through pork consumption. In particular, the CRP aimed at:

(1) Determining the effectiveness of irradiation to control infections caused by seafood contaminated with *Vibrio* spp., both for domestic markets and for export.

(2) Determining the infectivity of cysticerci of *Taenia solium* in pork muscle after irradiation.

(3) Conducting studies on the potential epidemiological and economic repercussions of using irradiation as a method to control these foodborne diseases in Latin America and the Caribbean.

An additional specific objective in the original programme consisted of implementing pilot-scale projects on irradiation to control infectivity of cysticerci of *T. solium* in pork muscle in endemic areas. This objective was abandoned at the second RCM in view of the impossibility of identifying suitably isolated localities to conduct such projects.

In view of the requirement in some South American countries that live oysters and mussels in the shell be marketed live, and the lack of information about the survivability of mollusks upon irradiation, this aspect of the irradiation treatment was incorporated in the studies dealing with live mollusks.

Because all attempts at isolating bacteria belonging to the *Vibrionaceae* in Chilean shellfish were unsuccessful throughout the first three years of the programme, indicative of a very low prevalence or absence of these organisms in Chilean coastal waters, and considering that the relatively few cases of cholera reported in Chile during the 1991-1993 pandemia were attributed to fresh produce irrigated with contaminated water, the second RCM approved the inclusion of a new specific objective: Determining the potential of radiation decontamination of *Vibrio cholerae* inoculated on fresh produce for raw consumption.

3. RESULTS AND CONCLUSIONS

The results of the eight studies on the use of irradiation as a public intervention measure to control *Vibrio* infections in Latin America and the Caribbean successfully concluded as part of the CRP can be summarized as follows:

Effect of irradiation on Vibrio spp. and other pathogenic bacteria in live and shucked mollusks

- Potentially pathogenic bacteria belonging to the *Vibrionaceae* occur naturally in shellfish throughout Latin America, the Caribbean, and the Gulf of Mexico. A notable exception to this general distribution is the Pacific coast of Chile, possibly as a result of low seawater temperatures.

- Higher seawater temperatures consistently correlate with higher mesophilic aerobic bacterial counts in raw shellfish and fish, and with a higher rate of isolation of strains of *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio fluvialis*, and *Vibrio vulnificus*. This tendency is apparent also in relation to other potentially pathogenic bacteria such as *Salmonella* spp., *Shigella* spp., *Aeromonas hydrophila,* and *Escherichia coli*, and with a higher total coliform population in shellfish during the summer months

- Non-toxigenic strains of *Vibrio cholerae* are present in oysters from Cuba, the United States, and Brazil; in Uruguayan mussels; and in mussels and other shellfish from Peru. In

contrast, *V. cholerae* strains belonging to the toxigenic serotype O1 were not isolated during the present project by any of the studies. However, the presence of endemic, non-toxigenic strains of *V. cholerae* points to the potential for virulent strains to thrive and contaminate the mollusks if conditions conducive to such contamination are given.

- The radiation decimal reduction dose (D_{10}) for *Vibrio cholerae* O1 El Tor Inaba *in vitro* and *in vivo* (i.e. in inoculated sea snails, oysters, clams, and mussels) varies between 0.08 and 0.17 kGy, depending on the medium. These D_{10} values indicate that this pathogen is highly radiation sensitive in relation to other pathogenic bacteria.

- Radiation resistance is low in *Vibrio* spp., *Aeromonas hydrophila*, and *E. coli* O157:H7 inoculated on various seafood products, whereas *S. enteritidis* and *S. typhimurium* have a relatively high radiation resistance. Radiation doses in the range 1.5–2.0 kGy effectively control all pathogenic bacteria tested in shellfish except *Salmonella* spp., particularly, *S. enteritidis*, which requires 3.0 kGy.

- Refrigerated shucked oyster meat and frozen half-shell oysters can be made *Vibrio vulnificus*-safe by as low a radiation dose as 1.0 kGy.

- Radiation doses of up to 3.0 kGy are not lethal to oysters and allow marketing of these mollusks in live form in ways similar to current commercial practices. Mussels survive 1.0 kGy without adverse effects.

Effect of irradiation on vibrio spp. in crustaceans and fish

- The radiation D_{10} value for *Vibrio cholerae* O1 El Tor inoculated on shrimp (*Penaeus vannamei*) tails and on fillets of lisa (*Mugil cephalus*) is 0.13 kGy. In saurel (*Trachurus picturatus murphyi*) fillets this value is very similar, 0.12 kGy.

- A dose of 1.0 kGy is sufficient to make shrimp tails and lisa or saurel fillets cholera-safe even for consumption in raw form as "ceviche."

- The microbiological shelf-life of fish ("lisa" and "saurel") fillets kept at 0–1^{o}C can be extended two-fold by irradiation at 1.0 kGy in relation to untreated fillets.

Effect of irradiation on vibrio cholerae in fresh vegetables

- Irradiation provides an effective intervention measure for improving the hygienic quality of fresh lettuce, cabbage, and celery contaminated with *Vibrio cholerae*. A dose of less than 0.75 kGy inactivates up to 10^5 cells/g of toxigenic *V. cholerae* in these products.

- Irradiation at a dose of up to 1.0 kGy does not adversely affect the sensory quality of fresh lettuce, cabbage, and celery or their vitamin C content. These results challenge the long-held notion that irradiation cannot be applied to fresh produce because of texture damage, and justify future research.

- PAHO considered that the co-operative research programme with FAO/IAEA successfully demonstrated that food irradiation is a viable technology for use as an intervention measure in control of pathogenic bacteria in seafood. PAHO expressed its intention to

consider food irradiation a public health intervention measure in the future and to promote this technology among the Governments of the Americas.

- Because of the high relevance to public health of the research conducted under the CRP, and in view of the results, it was also concluded that new research work should be conducted on uses of low-dose irradiation to decontaminate whole fresh produce, pre-cut salad components, and minimally-processed foods considered not to be amenable to radiation treatment based on studies conducted in the 1950s and 1960s.

DECONTAMINATION OF CUBAN OYSTERS USING IRRADIATION

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Abstract

Oysters (*Crassostrea virgínica*) collected on the Cuban coast near Havana were examined for contamination with *Vibrio cholerae* and other potentially pathogenic *Vibrio* species. The strains thus isolated were characterized and identified to species following standard methods, and their radiation resistance (D_{10}) was determined in pure culture. The *Vibrio* species most often isolated were *V. cholerae*,*V. parahaemolyticus* and *V. Alginolyticus*. Representative cultures from each species were later used to inoculate shucked oysters to determine the optimal radiation dose that would ensure elimination of 10^8 colony forming units (CFU)/g. The highest proportion of isolates were identified as *Vibrio parahaemolyticus* and *V. algynoliticus.* Non-O1strains of *Vibrio cholerae* were isolated from 50% of samples, but no *V. cholerae* O1 was identified. D_{10} values calculated for the various strains were low in relation to those in the literature. The radiation dose for decontaminating heavily inoculated (10^8 CFU/g) oysters was 1.2 kGy.

INTRODUCTION

Many *Vibrio* species are recognized as human pathogens. These bacteria are native to the aquatic environment, including *Vibrio cholerae*, the etiological agent of cholera, which has been found as a natural inhabitant of waters in areas free of cholera.

The seventh cholera pandemia arrived in the American continent through Peru, in 1991, rapidly expanding to various countries in South and Central America, but not to the Caribbean area. In Cuba, a surveillance programme of fish, lobster, crab, shrimp, and oysters for contamination with *V. cholerae* was established during the cholera pandemia, to detect any appearance of the pathogen in products consumed by Cubans, used for export, or supplied to the tourism industry. Because of normal consumption patterns of most of these products in Cuba, which call for cooking, they do not present a serious epidemiological risk; the exception is oysters, which are marketed shucked and consumed raw. Thus the interest in studying the potential use of irradiation to decontaminate oysters should these be found to carry *Vibrio cholerae* O1 or other potentially pathogenic *Vibrio* spp.

The objectives of the study were: a) To determine the degree of natural contamination of Cuban raw oysters with bacteria of the genus *Vibrionaceae*; b) to characterize and identify the *Vibrio* isolates to species; c) to establish the radiation resistance (D_{10}) of the potentially pathogenic *Vibrio* species isolated; and d) to determine the optimal dose for radiation decontamination of oysters artificially contaminated with the potentially pathogenic *Vibrio* species previously isolated and identified.

All microbiological work was conducted at the Department of Food Microbiology, Institute for Nutrition and Food Hygiene (INHA), Public Health Ministry. This was done in co-ordination with the Laboratory for Irradiation Techniques (LTI), National Center for Plant and Animal Health (CENSA), where irradiation of samples and dosimetry were carried out.

MATERIALS AND METHODS

A series of 48 composite samples of oysters belonging to the species *Crassostrea virginica* were collected from oyster beds on the North Coast of the province of Havana, which was deemed to be a more likely area for contamination of sea water with sewage effluents than other areas of the island. The oysters were transported in the shell to the laboratory, where they were shucked manually using aseptic techniques, placed in polyethylene bags, and refrigerated until used. Each bag contained 50 g of oyster meat. To determine the possible presence of *Vibrio cholerae* and other potentially pathogenic species of *Vibrio*, the standard methodology described in the 8th Edition of the Bacteriological Analytical Manual published by the US Food and Drug Administration, scheme No. 1 (FDA, 1995), was followed. This methodology prescribed alkaline peptone water as enrichment medium, and TCBS and gelatin agars for isolation media. Various series of biochemical test were also used for characterization and identification of *Vibrio* isolates (ICMSF, 1978; Alcina and Anicet, 1994).

The radiation sensitivity of strains of *Vibrio parahaemolyticus*, *V. alginolyticus*, and *V. cholerae* non-O1 isolated from oyster samples was determined in 18- to 24-h pure cultures. Cultures of the isolates were suspended in physiological saline so as to contain 10^8 CFU/mL according to the McFarland scale, followed by 100-fold dilution to final suspensions containing 10^6 cfu/mL. The initial concentration of the suspensions was ascertained by plating on TCBS and gelatin agars. Ten-mL aliquots of each suspension were placed in quadruplicate in 20-mL glass vials, transported to the irradiation center, and irradiated at doses in the range 0.0-2.0 kGy using a Gamma Cell 500–002 having a 30-kCi ^{60}Co source and a capacity of 80 L, at the Laboratory for Irradiation Techniques (LTI) of the National Center for Plant and Animal Health (CENSA). Viable *Vibrio* cells were enumerated post-irradiation by surface plating appropriate dilutions of each suspension on TCBS and gelatin agars. Tubes of tryptone soy broth were also inoculated for MPN enumeration of survivors.

To determine the optimal radiation dose for decontamination of artificially inoculated oysters, 50-g oyster meat samples in polyethylene bags were irradiated at 10 kGy to ensure absence of naturally-occurring *Vibrionaceae*. This was ascertained by macerating samples with tryptone soy broth and plating the appropriate dilutions on TCBS and gelatin agars as before. Sets of five samples were inoculated for each *Vibrio* culture. To prepare the inocula, separate suspensions of each isolate having a McFarland value of 10, equivalent to 10^9 colony forming units (CFU)/mL, were prepared in physiological saline using 18- to 24-h cultures grown on tryptone soy agar at 37^{o}C. As before, inoculum concentration was determined by plating.

Five 50-g sample bags were inoculated with 5 mL of a *Vibrio* sp. culture, followed by 3-h incubation at 37^{o}C. The inoculation level was determined as before. The bags were subsequently taken to the LTI irradiation center and irradiated at 200, 400, 600, 800, 1000 and 1200 Gy. Separate non-irradiated bags inoculated with each *Vibrio* strain were used as controls (0.0 Gy). Fricke and ceric sulfate dosimeters placed among the bags were used to determine absorbed doses in the range 200–400 Gy and 1000–1200 Gy, respectively.

After irradiation, the samples were transported back to the Department of Food Microbiology (INHA), were they were tested for surviving *Vibrio* cells as before. The contents of bags subjected to the two highest radiation doses were also incubated with tryptone soy broth to ensure absence of survivors.

RESULTS

All oyster samples tested were positive for *Vibrionaceae*, and 50% of samples contained non-O1 strains of *Vibrio cholerae*. This suggests that non-pathogenic strains of *V. cholerae* may be ubiquitous in waters of the north coast of Cuba as they are in other marine environments (Prazeres, 1986). Some samples contained more than one strain of *Vibrio*. The identity of the *Vibrio* strains isolated throughout the study and the total number of isolations of each strain are shown in Table 1.

Table 1: *Vibrio* spp. Isolated from Oysters

***Vibrio* spp. Isolated**	**No. of Isolates**
Vibrio cholerae non-O1 (Heiberg Group I)	39
V. cholerae non-O1 (Heiberg Group other than I)	30
V.parahaemolyticus	131
V. alginolyticus	120
V.harveyi	18
V. vulnificus	3

Initial studies designed to determine the radiation resistance of pure cultures of *Vibrio* strains isolated from oysters resulted in D_{10} values considerably lower (i.e. . 0.05 kGy) than those reported by other authors (0.10–0.20 kGy). This was likely due to the mediun used for preparing the test suspensions, physiological saline; NaCl may have potentiated the lethal effect of ionizing radiation on the cultures.

Considering the non-pathogenicity of the *V. cholerae* isolates, all of which were non-O1 serotypes, it appears that *V. parahaemolyticus* would be a much more likely foodborne disease hazard in Cuban oysters. The prevalence of *V. vulnificus* was low but nevertheless a matter of concern. These three strains were used in the second phase of the study to determine the optimum dose for radiation decontamination of the oysters. The concentration of inoculum in oysters artificially contaminated with pure cultures of *Vibrio cholerae* non-O1, *Vibrio parahaemolyticus*, and *Vibrio vulnificus* isolated from the oysters during the first phase of the study were 4×10^7, 5×10^7, and 2×10^7, per gram, respectively.

The results of dosimetry measurements are shown in Table 2. The slope of the calibration curve was 28.62 ± 0.16; the Y intercept was 0.0 ± 0.45; and the value of R was 0.9989.

Table 3 shows the doses required for the complete elimination of the three *Vibrio* spp. in oyster meat, at an inoculation level of 10^7 CFU/g. Considering that such seemingly high level of *Vibrio* spp. contamination was found to occur naturally in oysters collected during the summer months, these results offered a promising option for decontamination.

Table 2: Dosimetry Parameters

Dose	Expected Dose (Gy)	Time (Min.)	Measured Dose (Gy)	Error (Gy)
D1	200	7.14	199.44	3.87
D2	400	14.25	401.42	6.88
D3	600	21.43	-	-
D4	800	28.57	-	-
D5	1000	35.71	998.09	4.56
D6	1200	42.86	1181.61	53.88

NOTE: P = 0.05.

Table 3: Radiation Dose Required to Eliminate 10^7 CFU/g *Vibrio* spp. in Oysters

Type of Microorganism	Extinction Dose (kGy)
1.1.	
1.2. Vibrio cholerae	1.0
Vibrio alginolyticus	0.8
Vibrio parahaemolyticus	1.2

CONCLUSIONS

The study indicated that *Vibrio* spp. including non-pathogenic strains of *V. cholerae* and potentially pathogenic strains of *V. parahaemolyticus* and *V. vulnificus*, but not pathogenic strains of *V. cholerae,* are prevalent in Cuban oysters collected in waters of the North Coast of Cuba. Therefore, consumption of such oysters in raw form may pose serious health hazards.

The results of radiation treatment of artificially contaminated oysters confirmed that a dose of 1.2 kGy would be appropriate to eliminate numbers as high as 10^7 CFU/g *Vibrio* spp. in oysters. Considering the safety and effectiveness of irradiation to control foodborne microbial diseases (OMS/FAO, 1982; FAO/OIEA/OPS/OMS, 1992), and the absence of adverse effects from radiation treatment at low doses on the nutritional and sensorial properties of foods, this conclusion confirms that irradiation could be used as a public health intervention measure to ensure the hygienic quality of raw oyster meat.

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STUDY ON THE RADIATION SENSITIVITY OF PATHOGENIC *VIBRIONACEAE* AND *ENTEROBACTERIACEAE IN VITRO* AND AFTER INOCULATION INTO OYSTERS (*CASSOSTREA BRASILIANA*)

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Abstract

In vitro studies were conducted to evaluate the effects of ionizing radiation on various biotypes and serotypes of *Vibrio cholerae* (different biotypes and serotypes from group O1 and one strain from group O139); *V. parahaemolyticus*, *V.vulnificus*, *V. fluvialis*, *Aeromonas hydrophila*, *Plesiomonas shigelloides*; *Salmonella* typhi, *S. enteritidis*, *S. typhymurium*, *Shigella flexneri* and *Escherichia coli* O157:H7. *In vivo* tests were also conducted in oysters allowed to self-contaminate with *V. cholerae* and *S. enteritidis* cultures in sea water tanks through the natural feeding process of the mollusks. Bacterial populations irradiated (0.5–3.0 kGy) in pure culture in liquid broth or in oysters varied from 10^6 to 10^{10} colony forming units per mL or gram (CFU/g or mL), respectively. The decrease in viable cells through the radiation dose range applied varied from 4 to 10 $\log_{10}$. The lowest radiation resistance was found in *Vibrio* spp. *Aeromonas hydrophila*, and *E. coli* O157:H7, whereas *S. enteritidis* and *S. typhimurium* proved to be the most resistant species tested. A dose of 1.5 kGy was determined to be appropriate for elimination of up to 10^{10} of bacteria tested except *Salmonella* spp. particularly, *S. enteritidis*, which required 3.0 kGy for complete elimination. Radiation doses of up to 3.0 kGy were not lethal to oysters.

1. INTRODUCTION

Bivalve mollusks are marine organisms which are often consumed in raw form while still alive (De Paola *et al*.,1983; Varnam and Evans, 1991). They inhabit coastal areas and are considered to be "sentinels" of the environment in which they live because of their food intake pattern, which involves an hourly filtration of up to 6 L of water that causes mollusks to concentrate microorganisms, including potential human pathogens, in their digestive system (Madden *et al.* 1982; Costa, 1983; Rodriguez *et al.* 1990; Donini *et al*. 1993). As a result, bivalve mollusks are potential vehicles for foodborne diseases (Saliba and Helmer, 1990), and are thus frequently implicated in outbreaks of cholera, salmonellosis, shigellosis, and other serious diseases (Klontz *et al.* 1987; IOM, 1991; Varnam and Evans, 1991; Madden *et al*. 1982; PAHO, 1991a). Therefore, decontamination processes that may contribute to reduce the microbiological hazard posed by bivalve mollusks are or should be of interest to public health (Eyles and Davey, 1984; Saliba and Helmer, 1990).

Ionizing radiation is a technology that can be used to eliminate microbiological hazards in foods (Ley, 1966; ICMSF, 1980; Sang *et al*., 1987; FAO/OIEA/OPS, 1992; Jay, 1992; Loaharanu, 1994). Moreover, ionizing radiation is one of the most effective available technologies for control of microbial hazards in bivalve mollusks for human consumption (Ley, 1966; Wood, 1970; ICMSF, 1980; Kilgen *et al*., 1987; WHO, 1994; Jay, 1992; FAO/OIEA/OPS (OMS), 1997). In 1984, the Codex Alimentarius issued a standard to regulate the treatment of food with ionizing radiation (Anonymous, 1984). Several countries have since enacted regulations allowing this technology (IAEA, 1995). Varnam and Evans (1991) reported that a radiation dose of 3 kGy was sufficient to completely eliminate

pathogenic bacteria belonging to the *Vibrionaceae*, as well as *Salmonella* spp., in mollusks. Sang *et al.* (1987) used ionizing radiation to control *Salmonella* spp. in frog legs.

Laboratory experiments designed to study the efficiency of ionizing radiation to decontaminate bivalve mollusks, in general, have been conducted after sterilizing the mollusks prior to controlled inoculation, or using the naturally occurring bacterial flora for determining treatment effects. In contrast, experimental models using live oysters and natural feeding patterns to contaminate the mollusks, allow an evaluation of irradiation effects in the presence of the natural background flora (Gelli *et al*., 1998). Moreover, these models allow contamination of oysters with high levels of pathogenic enteric bacteria which are less frequent, but possible, in natural environments.

The seventh cholera pandemia reached South America in 1991, establishing its devastating presence first in Peru (Tauxe and Blake, 1990; PAHO, 1991b; Ries *et al*., 1992; Quevedo, 1993). This epidemic highlighted the need for urgent sanitary measures to control microorganisms having ample environmental dissemination (Loaharanu, 1994; Levine, 1991; PAHO, 1991b; Ries *et al*., 1992; Swerdlow *et al*., 1992). Such measures must be scientifically sound, technologically feasible, and economically viable. Irradiation has been proven to fulfill all of these conditions (WHO, 1994). For that reason, the International Atomic Energy Agency (IAEA) and the Pan American Health Organization (PAHO) organized a Co-ordinated Research Project to evaluate the applicability of irradiation as a public health intervention measure which, among other objectives, would evaluate the effect of irradiation as a decontamination method of bivalve mollusks (FAO/OIEA/OPS, 1992). The present study was undertaken as part of that project.

The objectives of the present study were: a) To evaluate the *in vitro* effects of ionizing radiation on selected pathogenic strains of *Vibrionaceae* and *Enterobacteriaceae*; b) to evaluate the effects of irradiation on some of the same bacterial strains (i.e. *V. cholerae* and *S. enteritidis*) *in vivo* after incorporation into live oysters (*Crassostrea brasiliana*) via natural oyster feeding patterns; and c) to determine oyster survival post-irradiation.

2. MATERIALS AND METHODS

1. *Bacterial strains and culture preparation*:

The strains used in the study were *Vibrio cholerae* O1, El Tor, Ogawa, non toxigenic; *V. cholerae* O1, El Tor, Ogawa, toxigenic; *V. cholerae* O1, El Tor, Inaba, toxigenic; *V.cholerae* O1, Classic, Ogawa, toxigenic; *V. cholerae* O139; *V. parahaemolyticus*; *V. fluvialis; V. vulnificus*; *Aeromonas hydrophila*; *Plesiomonas shigelloides; Salmonella typhi*; *S. enteritidis*; *S. typhimurium*; S*higella flexneri;* and *Escherichia coli* O157:H7, verotoxigenic. All bacterial strains were obtained from the Bacterial Culture Collection Section of the Instituto Adolfo Lutz, Sao Paulo. The non-toxigenic strain of *V.cholerae* was isolated from sea water from the coast of the state of Sao Paulo. The El-Tor Inaba strain was isolated from a cholera patient in Peru, in 1991, whereas the El-Tor Ogawa strain was similarly isolated in Bolivia, in 1992. The *Salmonella enteritidis* and *S. typhimurium* strains used were isolated at the Instituto Adolfo Lutz from foods implicated in foodborne disease outbreaks.

Bacterial cultures were kept lyophilized until used. Prior to use, all cultures except the *Enterobacteriaceae* were grown in alkaline peptone water; the *Enterobacteriaceae* were grown in buffered peptone. Upon incubation of cultures at 37^{o}C for 18–24 h, cultures were

reinoculated into the same media and incubated as before to ensure that actively growing cultures were used.

2. *Inoculation of oysters*:

Oysters from the genus *Cassostrea brasiliana* were obtained from a depuration plant in Cananeia, on the southern coast of the state of Sao Paulo. Oysters were inoculated by placing them in 40-L tanks filled with sand-filtered sea water in which the desired bacterial cultures were diluted according to the procedure described by Gelli *et al*. (1998). Oysters were left in the tank and allowed to feed in a natural way for 24 h.

3. *Irradiation*:

A ^{60}Co Gammacell II 220 irradiator (Atomic Energy of Canada, Ltd., AECL) belonging to the Coordenadoria de Aplicaciones en Inginieria e Industria, Instituto de Pesquisas Energeticas (IPEN/CNEN), Sao Paulo, was used to irradiate the samples. Fricke dosimeters were used to determine the absorbed dose in oysters.

4. *In vitro studies*:

Three tubes, each containing 3 mL of the suspension of one of the pure cultures described earlier, were prepared. One of the tubes was used to determine the initial numbers of viable cells. The second tube was irradiated, and the third served as non-irradiated control to determine possible adverse effects of transportation and time of irradiation on the cultures. Irradiation doses tested were 0.0 (control), 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 kGy.

5. *In vivo studies*:

Twenty oysters inoculated as described earlier were used for every experiment. Five oysters were used to determine the initial bacterial load, which provided a measure of inoculum uptake by the oysters through natural feeding; the remaining oysters were irradiated as described earlier for pure cultures.

6. *Microbiological analyses*:

Standard methods were used for the microbiological analyses. Oysters were aseptically shucked from the shell, homogenized in a blender, and a 25-g sample was taken for dilution with 225 mL alkaline peptone water (*Vibrio* spp., *Aeromonas* and *Plesiomonas*) or buffered peptone water (*Enterobacteriaceae*). A sample of non-inoculated, non-irradiated oysters from each batch received in the lab was examined to determine the possible presence of naturally occurring microorganisms of the same species as those being inoculated.

One-mL aliquots were taken from the initial oyster meat homogenate and from the 5-mL tube containing the pure culture of each bacterium, for the *in vivo* and *in vitro* studies, respectively, to prepare serial dilutions according to standard methods. Serial dilutions were incubated at 35^{o}C for 18–24 h. For *Vibrio* spp., the methods used were those described in APHA (1992) and AOAC (1994). For the *Enterobacteriaceae*, dilutions were incubated at 35^{o}C for 24h, except for *Shigella flexneri* and *E. coli* O157, which were kept at that temperature for 6–8h and 18–24h, respectively.

Selenite-cysteine and modified Rappaport-Vassiliades media, and 42°C, were used for *Salmonella* spp. enrichment. No selective enrichment was used for *S. typhi*, and serial dilutions were plated directly on Salmonella-Shigella (SS) agar. This medium was also used for *Shigella*, whereas *E. coli* was recovered in Mac-Conkey-Sorbitol agar. Characteristic colonies of each bacterium were isolated from the corresponding media and identified using biochemical and serological profiles (APHA, 1992; AOAC, 1994).

V. cholerae cultures isolated from irradiated oysters (*in vivo* studies) or pure cultures (*in vitro* studies) were examined for possible radiation-induced changes in specific characteristics, such as biotype and toxin producing capacity, following AOAC (1994) methods.

7. *Evaluation of radiation effects on oysters*:

Non-inoculated oysters were irradiated at the same doses used for inoculated samples to test the lethality of various radiation doses. Oysters thus irradiated were kept under refrigeration (5°C) for up to 7 days, and their survival was determined daily.

All experiments were replicated three times.

3. RESULTS AND DISCUSSION

The oyster samples used in this study were found to be free of pathogenic microorganisms which were used for the artificial inoculation of the test samples. The data also indicated that transportation of cultures and oyster samples to the irradiation facility, as well as the time necessary for irradiation, did not have any effect on bacterial numbers. There were no alterations in *V. cholerae* biotype and toxin producing capacity, or on the biochemical characteristics of the cultures, due to irradiation.

A comparison of the data on bacterial counts obtained from *in vitro* studies (Tables 1 and 2) indicated wide variation in radiation resistance among the cultures selected for the study. As a group, the *Vibrionaceae* were more radiation sensitive than the *Enterobacteriaceae*. Whereas a dose of 1.5 kGy was enough to eliminate an initial contamination of 10^{10} colony forming units (CFU)/mL in pure culture suspensions of *Vibrio* spp., 2.5 kGy were necessary to achieve similar reductions in *Enterobacteriaceae* cultures. These results agreed with those of Kilgen *et al.* (1987). A comparison of Tables 1 and 2 show that the radiation resistance of *Salmonella typhi*, *Shigella dysenteriae*, and *E. coli* 0157:H7 was no different from that of the *Vibrio* spp., *Aeromonas* and *Plesiomonas* cultures tested. However, S*almonella enteritidis* surviving cells were found in culture suspensions irradiated at doses as high as 2.0 kGy.

Similar results were obtained from *in* vivo studies using inoculated oysters (Table 3). No surviving cells of non-toxigenic *Vibrio* spp. culture tested were detected in oysters treated at doses above 1.5 kGy. In contrast, 3.0 kGy were needed to ensure complete elimination of 10^6 CFU/g *S. enteritidis* similarly inoculated into oysters.

A comparison of the data in Tables 1 and 2 with those in Table 3 highlights the protective effect of the oyster matrix on inoculated *Salmonella enteritidis* but not on *Vibrio cholerae* O1 Ogawa against radiation injury. A radiation dose of 3.0 kGy was necessary to achieve elimination of 10^6 CFU/g *S. enteritidis* in oysters than was needed to bring 10^{10} CFU/mL cells of *Vibrio cholerae* to non-detectable levels in pure culture suspensions.

Table 1: *In vitro* Effects of Ionizing Radiation on Potentially Pathogenic *Enterobacteriaceae*

Culture	Initial Count (CFU/mL)	Radiation Dose (kGy) and Bacterial Counts (CFU/mL)					
		0.5	1.0	1.5	2.0	2.5	3.0
S. *typhi*	10^8	10^5	10^3	10^1	ND	ND	ND
S.tm.	10^{10}	10^6	10^4	10^3	ND	ND	ND
S.e.	10^{10}	10^7	10^{-4}	10^3	10^1	ND	ND
Sh.f.	10^8	10^3	10^1	ND	ND	ND	ND
O157	10^9	10^4	10^2	ND	ND	ND	ND

S.tm — *Salmonella typhimurium*.
Sh.f. — *Shigella flexneri.*
S.e. — *Salmonella enteritidis*.
O157 — *Escherichia coli* O157:H7.
ND – None detected.

To determine the optimal radiation dose necessary to ensure absence of these pathogens in naturally contaminated oysters, it is essential to ascertain their naturally occurring levels, which may be seasonal and variable depending on the potential for fecal pollution of sea water in each area. This information may be specially important when oyster beds are located in coastal waters close to urban areas. According to Varnam and Evans (1991), 10 CFU/g *S. enteritidis* could cause salmonellosis in the most susceptible groups of consumers; thus the importance of tight control of water quality in bivalve mollusk production and extraction areas (Wood, 1970; IOM, 1991; APHA, 1992). Assuming that the mollusks are grown and collected using Good Primary Production Practices, doses lower than 3.0 kGy should provide a reasonable level of safety against *S. enteritidis*, and hence, against potentially pathogenic *Vibrionaceae*.

In relation to *Vibrio cholerae* O1, human ingestion of bacterial cells in numbers lower than 10^3/g are reported not to result in infection (Levine *et al*., 1981). Irradiation at a dose of 1.5 kGy would suffice to eliminate high numbers of this pathogen, something desirable because, being a halophilic, aquatic microorganism, it can proliferate in sea waters rich in nutrients, and is able to also grow in numbers during commercialization of mollusks (Cook & Ruple, 1986).

Table 2: *In vitro* Effects of Irradiation on Pathogenic *Vibrionaceae*, *Aeromonas hydrophila* and *Plesiomonas shigelloides*

Culture	Initial Count (CFU/mL)	Radiation Dose (kGy) and Bacterial Counts (CFU/mL) 0.5	1.0	1.5	2.0	2.5	3.0
V.c. NT	10^{-10}	10^{-6}	10	ND	ND	ND	ND
V.c. El Tor Inaba	10^{-10}	10^{-6}	10^{-2}	ND	ND	ND	ND
V.c. El Tor Ogawa	10^{-10}	10^{-5}	10	ND	ND	ND	ND
V.c. Classic	10^{-10}	10^{-5}	10^{-1}	ND	ND	ND	ND
V.c. O139	10^{-10}	Not done	Not done	ND	ND	ND	ND
V.v.	10^{-10}	10^{-5}	ND	ND	ND	ND	ND
V.p.	10^{-10}	10^{-5}	10^{-2}	ND	ND	ND	ND
V.f.	10^{-9}	Not done	10	ND	ND	ND	ND
P.s.	10^{-8}	Not done	10	ND	ND	ND	ND
A.h.	10^{-9}	Not done	10	ND	ND	ND	ND

V.c – *Vibrio cholerae.* NT – Non toxigenic.
V.v.- *Vibrio vulnificus.* V.p. – *Vibrio parahaemolyticus.*
V.f.- *Vibrio fluvialis.* P.s. – *Plesiomonas shigelloides.*
A.h.- *Aeromonas hydrophila.*
ND – None detected.

Table 3: Effect of Ionizing Radiation on Numbers of *Vibrio cholerae* O1 Ogawa and *Salmonella enteritidis* Inoculated into Oysters via Natural Feeding

Bacterial Culture	Initial Count (CFU/g)	Radiation Dose (kGy) 0.5	1.0	1.5	2.0	2.5	3.0
3. V.c.[a]	10^6	10^3	10^2	ND	Not done	Not done	Not done
S.E.[b]	10^6	10^4	10^3	10^2	10	+ [c]	ND

V.c. – *Vibrio cholerae* O1 Ogawa, non toxigenic.
S.E. – *Salmonella enteritidis*.
[a] – Mean of 3 replications.
[b] – Mean of 2 replications.
+ = <10 but positive.
ND – None detected.

Irradiation tests conducted using non-inoculated oysters revealed that their viability, as well as their common organoleptic characteristics, are not affected by even the highest radiation dose used in this study, 3.0 kGy, which would allow live, refrigerated post-irradiation commercialization. The survival of oysters belonging to the species used in this study, *Crassostrea brasiliana*, was determined on the basis of difficulty to open the valves; this species of oyster only opens its valves during feeding or after death.

4. CONCLUSIONS

Used in conjunction with Good Primary Production and Processing Practices, as required by international standards, irradiation allows hygienization of oysters without affecting the survival of the mollusks for subsequent live, in-the-shell commercialization. A radiation dose of 1.5 kGy is sufficient to ensure the safety of raw *Crassostrea brasiliana* against pathogenic *Vibrionaceae*, including *V. cholerae*, as well as against *Aeromonas hydrophila, Plesiomonas shigelloides, Shigella flexneri*, and *Escherichia coli* O157:H7, but may not ensure elimination of *Salmonella typhi*, *S. enteritidis* or *S. typhimurium* if initial contamination is high (10^8–10^{10} CFU/g). To achieve safety levels against *Salmonella* spp., particularly *S. enteritidis*, in raw oysters having high initial contamination (10^8–10^{10} CFU/g), a dose of 3.0 kGy is advisable.

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EFFECT OF IONIZING RADIATION ON FRESH VEGETABLES ARTIFICIALLY CONTAMINATED WITH *VIBRIO CHOLERAE*

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Abstract

Lettuce, cabbage and celery were artificially contaminated with *Vibrio cholerae* El Tor 01 Inaba, and irradiated at 0.50, 0.75 and 1.00 kGy. Non-irradiated samples were used as controls. The effect of irradiation was measured during 7-days storage under refrigeration, from the viewpoints of microbiological (MPN), nutritional (Vitamin C content), and sensory quality. Irradiation proved to be an effective technique to eliminate *V. cholerae* in fresh vegetables. Doses of less than 0.75 kGy were sufficient to eliminate an initial contamination of 10^5 cells/g of *V. cholerae*; neither sensory properties or nutritional quality (Vitamin C content) were adversely affected by the treatment. The cost of irradiating the vegetables at 0.5 kGy under the conditions of the study was US $ 0.121, 0.067 and 0.445 per unit of lettuce, cabbage and celery, respectively.

INTRODUCTION

Cholera has been endemic in India for centuries, but devastating epidemics in other parts of the world have occurred from time to time, as the one that began in January 1991 in the Latin American region (Mossel *et al*., 1992; Quevedo, 1993). The impact of this cholera pandemic, beginning with its epidemiological characteristics, has been different in various Latin American countries because of such factors as sanitary conditions, population concentration, food habits, and ecological variability (FAO/OPS/OMS, 1992a).

Cholera infection is produced by the ingestion of viable *Vibrio cholera* cells, whose source is the excreta of infected people. The same direct and indirect mechanisms of transmission that operate in other enteric infections apply also to this disease. The main vehicles of *Vibrio cholerae* are water and a variety of foods. Among the foods associated with the initiation of cholera epidemics, the literature mentions various seafood, meats, and fresh vegetables (Sang *et al*., 1987; De Paola, 1981; 1992; PAHO/WHO, 1991).

During the cholera epidemic in Chile, initiated in 1991, water was identified as the main vehicle of cholera. However, a potential hazard from foods such as seafood and fresh vegetables has been recognized (Astorga, 1998; INTA, 1991; FAO/OIEA/OPS, 1992b). This, together with the fact that Chilean food consumers prefer cooked seafood, whereas green vegetables are consumed fresh, uncooked, make it possible that vegetables that grow close to the ground and that are irrigated with contaminated water may present a higher of risk of being cholera vehicles than is seafood. In this regard, during an epidemic in Israel, *Vibrio choleare* biotype El Tor was isolated from vegetables collected from fields irrigated with sewage water, and the epidemiological evidence, together with the bacteriological findings, indicated that contaminated vegetables were the main vehicle for the spread of the infection (Gerichter, *et al*., 1975).

Food irradiation could be a safe method for decontaminating vegetables of *Vibrio cholerae* and other pathogenic microorganisms. The safety and effectiveness of this technology has been amply demonstrated (Diehl, 1991).

The objectives of the present study were to determine the effect of ionizing radiation on *V. cholerae* in three green vegetables marketed fresh as ready-to-eat: Lettuce, cabbage and celery, and to evaluate selected physical, chemical and organoleptic quality parameters of irradiated vegetables over a period of storage and simulated distribution. In addition, the study aimed at evaluating the cost of irradiating the vegetables at the doses needed to achieve decontamination of *V. cholera*.

MATERIALS AND METHODS

Three green vegetables were used: Lettuce (*Lactuca sativa*), cabbage (*Brassica oleracea*), and celery (*Apium graveolens*), obtained from one of the main distributor in Santiago. All vegetables came from the same producer no more than 24 hours post-harvest. The lettuce came packaged in polyethylene bags, while the cabbage and celery had been wrapped ia a retractable PVC film.

A strain of *Vibrio cholerae* biotype El Tor serotype 01 Inaba was used. This strain was provided by the Instituto de Salud Pública de Chile, and cultured overnight in alkaline peptone water (pH 86) at 35°C. The culture was grown to a concentration of 10^9 colony forming units (CFU)/mL, diluted with 0.1% peptone water (pH 7), and used to contaminate the green vegetables so as to obtain 10^4–10^5 CFU/g in the vegetables. To inoculate the samples, 10 g of each separate vegetable were introduced in a sterile flask to which 100 mL of the pure culture suspension of *V. cholerae* were added, followed by shaking for 5 min. The samples were withdrawn from the bottles using tweezers, and placed in sterile plastic bags which were then sealed in air.

The samples were irradiated at 0.25, 0.75, and 1.00 kGy in an experimental irradiator provided with a ^{137}Cs source. The activity of the source was 53 430 Ci (1.98×10^{15} Bq) and the dose rate was 0.44 Gy/s. All treatments were carried out before 24 hours post-harvest. Non-irradiated samples were used as a controls.

Dosimetry was conducted for each vegetable studied. Ferrous-sulfate cupric sulfate dosimeters were prepared to measure the absorbed doses (ASTM D 2954-71).

The number of viable microorganisms in irradiated and control samples was determined by the most probable number method (MPN), with the aid of standard tables. The presumptive MPN was determined using peptone alkaline water (pH 86) (FDA, 1992), incubated 48 h at 35°C. Positive tubes were confirmed using TCBS agar (Oxoid), and 35°C incubation for 48 h.

To prove the absence of *V. cholerae* in the samples treated with the highest doses applied (0.75 and 1.00 kGy), new tests were conducted using 20-g samples of each one of the fresh vegetables under study, contaminated with 180 mL of pure culture, following the methodology already described.

Sensory evaluation of control and irradiated samples was carried out by a trained panel consisting of 11 staff members of the Comisión Chilena de Energía Nuclear, on days 1, 5, and 7 after irradiation. The quality of the vegetables was measured using the following

parameters: Appearance, color, aroma, sweetness, acidity, bitterness, texture, and flavor, using the Scoring method and a scale of 1 to 9 points. Quality and acceptability tests were replicated three times, and the data were analyzed using ANOVA and the Duncan Test (5% level).

The vitamin C content of vegetables was determined using high-performance liquid chromatography (HPLC); ascorbic and dehydroascorbic acid contents were considered to calculate the vitamin C content. A Merck Hitachi LaChrom chromatograph equipped with a UV detector (LaChrom L-7400) and an integrator (Merk Hitachi D-2500) was used. A Lichrosob RP-18 (5µ) column was used, and a solution of monopotassium phosphate was the mobile phase. Samples were freeze-dried using a Chris model Delta 1–20 KD freeze dryer.

The cost of irradiation was calculated following the methodology described in the Handbook for Conducting Feasibility Studies published by the ICGFI (1986). The parameters used were those specified in an 1995 pre-feasibility study for the installation of a multi-purpose gamma irradiation facility conducted in Chile; the study envisioned the plant as a contract irradiation facility that would treat foods as well as medical supplies, pharmaceutical products, and cosmetics. The study considered 22 industrial sectors, and identified nine types of users willing to readily apply the technology or to adopt it in the near future. Based on such estimates, it was determined that the processing potential on the fourth year of operation was 3500 metric tons of foods per year. The initial capital investment and the annual operation costs of the facility were calculated at US$ 2.5 million and US$ 417 thousand, respectively.

The total horticultural area dedicated in Chile to the produce under study comprises some 112 thousand hectares, according to estimates provided by the Chilean Ministry of Agriculture. The principal production regions are the Metropolitan, IV, V, VI, and VII (Oficina de Planificación Agrícola, 1998). For calculation purposes, it was assumed that the fresh vegetables would be sold in individual packages such as those presently used in supermarket chains: lettuce would be packaged in polyethylene bags, whereas cabbages and celery would be presented individually packaged in retractable PVC.

The multipurpose plant considered as the basis for the calculation was of the batch type, and designed to handle simultaneously four 1.8-m^3 pallets capable of carrying 1900 heads of cabbage, 2848 celery units, or 1 048 lettuces. The yield of the facility for products having densities of 0.15 (lettuce), 0.28 (cabbage), and 0.19 (celery), would be 0.38 h/batch, considering a ^{60}Co source with an activity of 500 KCi. This figure would increase to 0.50 h/batch when loading and unloading are taken into account.

RESULTS AND DISCUSSION

Dosimetry measurements indicated that the uniformity rate of the irradiation process was 1.04, 1.08, and 1.20 for lettuce, celery and cabbage, respectively. Irradiation times varied between 7 min, 47 s, and 44 min, 4 s, depending on the dose and product.

The effects of irradiation at 0.0, 0.25, 0.75, and 1.00 kGy on *V. cholerae* inoculated on the selected vegetables are shown in Table 1. The number of viable cells of *V. cholerae* in vegetable samples was reduced not only because of the irradiation treatment, but also because of the subsequent storage at refrigeration temperatures (5–10^oC). The effect of low-temperature storage alone was measured in non-irradiated, control samples, which had a reduction of 2–4-$\log_{10}$ cycles in the number of surviving *V. cholerae* after four days of

storage. This results also demonstrated that the pathogen remained viable in fresh vegetables after several days at low temperature, in agreement with data published in the literature suggesting that *V. cholerae* survives in this type of foods 1–7 days at ambient temperature (30–31°C), and 7–10 days under refrigerated conditions (5–10°C) (PAHO/WHO, 1991).

Table 1: Effect of Irradiation on Survival of *Vibrio cholerae* El Tor O1 Inaba Inoculated on Fresh Lettuce, Cabbage and Celery During Subsequent Storage at 5–10°C (MPN/g)

	Product and Storage Time (h)								
	Lettuce			Celery			Cabbage		
Dose (kGy)	0	48	96	0	48	96	0	48	96
Control	23 000	6400	460	20 000	24 000	93	460 000	24 000	2400
0.25	75	9	4	4	<3	<3	<3	<3	<3
0.75	<3	<3	<3	<3	<3	<3	<3	<3	<3
1.00	<3	<3	<3	<3	<3	<3	<3	<3	<3
Control	460 000	460	93	1 400 000	7500	900	46 000	3900	15
0.25	<3	<3	<3	<3	<3	<3	<3	<3	<3
0.75	<3	<3	<3	<3	<3	<3	<3	<3	<3
1.00	<3	<3	<3	<3	<3	<3	<3	<3	<3
Control	39 000	4600	460	240 000	12 000	240	150 000	4600	240
0.25	4	<3	<3	<3	<3	<3	<3	4	<3
0.75	<3	<3	<3	<3	<3	<3	<3	<3	<3
1.00	<3	<3	<3	<3	<3	<3	<3	<3	<3

Irradiation was shown to be an effective method for eliminating *V. cholerae* in the fresh vegetables studied. All samples irradiated at 0.75 or 1.00 kGy presented MPN value <3/g, and none of the additional tests to determine the presence/absence of *V. cholerae* was positive. It is worth highlighting that this result was obtained in samples that had an initial contamination of 7×10^5 to 1.5×10^6 viable cells/g, as determined in control samples. A radiation dose of 0.25 kGy was not enough to eliminate *V. cholerae* under the experimental conditions used. These results agreed also with those of earlier studies in which the D_{10} was 0.076 kGy for a pure culture of *V. cholerae* serotype Inaba (Rubio, 1997). Therefore, a dose not lower than 0.5 kGy would be needed to eliminate a burden of 10^4 CFU/g in fresh vegetables.

The effects of radiation treatment on the vitamin C content of vegetables are shown in Table 2. Vitamin C was chosen as an index of radiation-induced losses in the nutritional value of lettuce, celery and cabbage because it is known to be one of the most radiation sensitive vitamins (Jaddou, 1990).

Table 2: Ascorbic Acid Content of Irradiated and Control Fresh Lettuce, Cabbage, and Celery (mg/100 g)*

Dose (kGy)	PRODUCT		
	Lettuce	Cabbage	Celery
0.00	2.357	3.085	0.549
0.50	2.007	7.028	1.272
0.75	2.149	5.250	0.549
1.00	2.036	5.018	0.616

*Means of 3 replications.

There was a marked difference in the natural total ascorbic acid content of the vegetables studied; cabbage was highest. Irradiation did not decrease these initial concentrations, and in the case of cabbage, it actually increased them. A similar phenomenon has been reported in some citrus fruit and has been attributed to radiation-induced formation of ascorbic acid from precursors. The literature is not consistent in this regard, since some authors have reported minimal vitamin C losses from irradiation while others have measured high losses. This divergence may be attributed to differences in analytical approaches. While some studies have measured only ascorbic acid, others have measured the total vitamin C content (GCIIA, 1992).

Sensory evaluation scores received by the irradiated vegetables are presented in Table 3. In terms of quality**,** cabbage was the most radiation resistant vegetable tested, since it did not suffer changes in any quality attribute upon irradiation at the doses tested. The only significant differences ($p < 0.05$) detected between control and irradiated samples, i.e. in the appearance of lettuce and in the colour of celery, were not judged by produce experts to have "commercial" significance.

Moreover, all control and irradiated samples received good overall acceptability scores, not significantly different from non-irradiated samples; the lowest mean overall acceptability score was 6.78 in a scale of 1 to 9 (Table 4). Given these results, it can be asserted that irradiation at doses <1.0 kGy might well be applied for decontamination of fresh lettuce, cabbage, and celery without adversely affecting their quality attributes. These results contradict reports in the literature published during the 1960's and 1970's, concerning alleged unsuitability of irradiation processing for treating fresh vegetables because of such radiation-induced deleterious effects as wilting and softening of lettuce, unsightly browning of celery, appearance of distasteful flavors in various vegetables, and changes in aroma and texture (Urbain, 1986).

Table 3: Sensory Scores of Lettuce, Cabbage and Celery Irradiated at Low Doses (<1.0 kGy)

Parameters	Product and Dose (kGy)											
	Lettuce				Cabbage				Celery			
	0.0	0.5	0.75	1.00	0.0	0.5	0.75	1.00	0.0	0.5	0.75	1.00
Acidity	2.31	2.32	2.26	2.24	2.65	2.70	2.63	2.55	2.98	2.99	3.05	3.15
Bitterness	3.27	3.56	3.51	3.37	3.50	3.49	3.45	3.72	3.35	3.52	3.61	3.66
Appearance	7.42	7.02*	6.76*	6.65*	7.53	7.57	7.59	7.52	7.29	7.07*	7.21*	7.25
Aroma	4.42	4.21	4.39	4.44	4.08	4.13	4.01	4.22	4.97	5.07	5.23	4.79
Color *	5.23	5.19	5.14*	5.07*	4.79	4.66	4.73	4.66	5.28	5.03*	4.89*	4.98*
Sweetness	5.22	4.88	4.58	4.50	4.30	4.34	4.23	4.23	4.04	3.86	3.85	3.99
Flavor	5.27	5.05	5.02	5.00	5.16	5.24	5.15	5.21	5.17	5.17	5.35	5.12
Texture	7.05	6.69	6.72	6.67	7.53	7.48	7.46	7.54	7.42	7.38	7.39	7.31

*Significantly different ($p < 0.05$); means of three replications.

Table 4: Overall Acceptability of Lettuce, Cabbage, and Celery Irradiated at Low Doses (<1 kGy)

Product and Dose (kGy)												
	Lettuce				Cabbage				Celery			
	0.00	0.25	0.50	1.00	0.00	0.25	0.50	1.00	0.00	0.25	0.50	1.00
Mean Overall Acceptability Score	7.27	6.87	7.00	7.05	6.81	6.78	6.79	6.82	7.32	7.33	7.26	7.14
Acceptability (%)	100.0	93.9	90.9	90.9	81.2	81.2	81.2	78.8	97.0	97.0	100.0	93.9
Indifference (%)	0.0	6.1	6.1	9.1	15.2	12.1	18.2	15.2	3.0	3.0	0.0	6.1
Rejection (%)	0.0	0.0	3.0	0.0	3.0	6.1	0.0	6.1	0.0	0.0	0.0	0.0

With regard to the cost of irradiating the selected fresh vegetables, and considering a dose of 0.5 kGy, the cost of irradiation under the conditions of this study was estimated at US$ 0.121, US$ 0.067 and US$ 0.045 for a head of lettuce, a head of cabbage and a head of celery, respectively. Considering the unit price of these vegetables in trade, in Chilean pesos, wholesale: Chilean $170, for the lettuce, Chilean $190 for the cabbage, and Chilean $249 for the celery, and an exchange rate of Chilean $465/1 $US, the cost of irradiation would represent 33, 16, and 7% of the wholesale price of the vegetables, respectively. This could be a very high cost, although the benefit in terms of public health could eventually compensate for it. According to estimations made in 1991, cholera could have caused internal economic losses to Chile in the order of US $ 300–400 million. However, the data gathered during the first phase of the present co-ordinated research project indicated that such losses were substantially smaller (i.e. approximately US$100 million; Rubio, 1997).

CONCLUSIONS

Low-dose irradiation (0.75 kGy) proved to be an effective processing technique to decontaminate fresh lettuce, cabbage, and celery artificially contaminated with 10^4–10^5/g viable cells of toxigenic *Vibrio cholerae* O1 El Tor Inaba. At this dose, no adverse effects from irradiation were detected on sensory quality attributes of the vegetables studied, on their overall acceptability, or on their vitamin C content.

The cost of irradiating the vegetables at 0.5 kGy using the parameters and assumptions of an earlier pre-feasibility study for a commercial, batch type, multi-purpose irradiation facility, was estimated at US$ 0.121, US$ 0.067, and US$ 0.045 for lettuce, cabbage and celery, respectively. This cost was relatively high in relation to the wholesale price of the vegetables.

The results of the present study suggest that further research is needed into the potential application of irradiation to decontaminate fresh produce, particularly cut-up products marketed as ready-to-eat, of microbial contaminants posing a health hazard to the consumer. Modern processing and dosimetry techniques may disprove long-held beliefs that radiation treatment is not suited for fresh produce.

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RADIATION DECONTAMINATION OF PERUVIAN MARINE "LEAD SNAIL" (*THAIS CHOCOLATA*) INOCULATED WITH *VIBRIO CHOLERAE* O1 EL TOR

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Abstract

In vivo studies were conducted using marine snails (*Thais chocolata*) artificially contaminated in a tank containing sea water inoculated with a pure culture of *Vibrio cholerae*, such that 10^5 colony forming units per gram (CFU/g) were uptaken by the mollusks in 1.5 h. A radiation D_{10} value of 0.12 kGy was determined for *V. cholerae* upon subsequent irradiation of the live snails at doses in the range 0.0–4.0 kGy. A second series of tests were conducted using naturally contaminated, non-inoculated snails, shelled and packaged simulating commercial procedures, irradiated at 0.0–3.0 kGy, and stored at 2–4°C. These tests indicated that a dose of 2.0 kGy was optimal to extend the microbiological shelf-life of the snails to 21 days without inducing significant adverse sensory or chemical effects. Non-irradiated snails similarly treated and stored spoiled after only seven days.

INTRODUCTION

The Peruvian "lead snail" (*Thais chocolata*) is a mollusk having a single, heavy, large and wide valve, uniformly brown in color, from which an orange internal columnella and bluish interior can be observed. Its average size is 88-mm long by 35-mm dia. (Rosales, 1988). This snail is typically found in marine rocky beds, where it forms banks at a depth of some 30 m, affixing itself to rocks in temperate waters (15–17°C). The snail often shares its habitat with a large species of mussel popularly called choro (*Aulacomya ater*), and it is found on the Pacific coast touched by the Humbolt current, from Ecuador to northern Chile (IMARPE, 1996; Quiroz *et al*., 1996). Capture of this snail is done mostly by local artisan fishermen using motorized boats provided with refrigerated storage bins, although some of the total volume comes from individual divers who operate from the shore. Thus, there is wide variation in the microbiological quality of the snails during commercialization, and contamination of these mollusks with *Vibrionaceae*, including toxigenic *Vibrio cholerae*, is a distinct possibility.

Cholera constitutes a serious public health concern in many countries of the world. The spread of the cholera pandemia to Latin America and the Caribbean in 1991, and the dissemination of a new strain of *Vibrio cholerae* in Asia (O139), suggest that the disease will continue to be a global problem in future years (CDC/NCD/OPS, 1995). To minimize the incidence of cholera, therefore, effective intervention methods will be needed. Aware of this need, the Pan American Health Organization (PAHO), the International Atomic Energy Agency (IAEA), and the Instituto Peruano de Energía Nuclear (IPEN), in 1991, proposed the use of ionizing radiation to decontaminate raw seafood of *Vibrio cholerae* O1 El Tor, other pathogenic serotypes of *V. cholerae*, and various potentially pathogenic *Vibrionaceae*, and organized a Co-ordinated Research Project to study the potential public health benefits of applying this technology in countries where consumption of raw shell fish, fish, and other seafood is traditional. The present study is part of this project.

MATERIALS AND METHODS

Product Preparation

Live, fresh marine snails belonging to the species *Thais chocolata* were purchased at the harbor in Pisco (Laguna Grande), and at the seafood terminal market in Villa María del Triunfo, Lima. The snails were transported to the laboratories of IPEN using styrofoam boxes filled with ice, at 2–5^{o}C, to slow the biological activity of the mollusks and minimize microbial growth. The snails were washed using clean sea water to eliminate the natural mucosity of the shell and other impurities, and microbiological tests were conducted to determine initial bacterial counts and the possible presence of *Vibrio cholerae* as described below.

Inoculum

A toxigenic strain of *Vibrio choleare* O1 El Tor, serotype Inaba, was obtained from the Peruvian Health Ministry collection. The pure culture was characterized as Gram-negative, curved to straight rods, facultatively anaerobic, asporogenous, motile, halophilic, oxidase positive, which reduced nitrates to nitrites (CDC/NCD/OPS, 1995).

The culture was suspended in sterile alkaline peptone water (AP) and incubated at 37^{o}C for 8 h. A loopful from the top portion of the suspension was streaked onto thiosulfate citrate bile and sacarose agar (TCBS) and the plate was incubated at the same temperature for 18 h, after which a characteristic colony was transferred to a slant of brain heart infusion agar (BHIA) and reincubated for 24 h. The slant was washed and the cell suspension was diluted into 300 mL AP, which resulted in a suspension of 10^{9}–10^{10} colony forming units per mL (CFU/mL), as determined by plate count on TCBS before using it to inoculate the snails.

Sample Inoculation, Preparation and Irradiation

A 30-L tank containing previously ozonated sea water was inoculated with the pure culture of *V. cholerae* so that the concentration of viable cells in the water was 10^{6} CFU/mL. Live snails were placed in this simulated habitat and allowed to contaminate themselves with the suspended *V. cholerae* through their natural feeding mechanism, to a level of 10^{5} CFU/g. The time required to reach this level of contamination was only 1.5 h.

(1) Determination of D_{10}: Inoculated snails were placed in high-density polyethylene bags, 25 to a bag, and the bags were heat sealed. The bags were then irradiated at doses in the range 0.0–0.4 kGy, in increments of 0.1 kGy, in a Gammacell 220 irradiator provided with a ^{60}Co source having a dose rate of 2.41 kGy/h; non-irradiated controls were also prepared.

(2) Shelf-life Determination: The shell was removed from fresh, non-inoculated snails, and the meat was washed and packaged in polyethylene bags as before. Radiation doses of 0.0, 1.0, 2.0, and 3.0 were applied, and the bags were labelled T_1 through T_4, respectively. Absorbed radiation doses were measured using Fricke dosimeters strategically placed in the product. The bags were stored at 2–4^{o}C, and microbiological analyses were conducted on days 1, 7, 14, and 21 post-irradiation. Concurrent sensory analyses were conducted to determine acceptability.

Microbiological Analyses

Inoculated samples were examined for survival of *V. cholerae* by macerating 25 g of snail meat in 225 mL peptone water, preparing serial dilutions following standard methods, and surface plating on TCBS plates as described by the FDA (1995). Bacterial counts were transformed into logarithms and plotted against radiation dose to draw the bacterial survival curve of *V. cholerae* in snails, and to establish the radiation D_{10} (i.e. the dose necessary to reduce the *Vibrio* population by 90%), given by the negative inverse value of the slope of the survival curve (Muñoz *et al.*, 1985). For shelf-life studies, serial dilutions were pour plated with BHIA, and the plates were incubated at 22°C for 24 h to enumerate total aerobic bacteria.

Sensory Analyses

An eight-person trained panel was used to establish the acceptability of irradiated snail samples over the 21-day refrigerated storage at 2–4°C. A 5-point hedonic scale was used to rate appearance (color), odor, and texture of irradiated and control snail samples, raw or cooked in boiling water for 3 min without addition of salt or any other additive. The experimental design was a square block with a 4 × 4 factorial (four treatments, four storage times) and three replications; the results were compared using Tukey's test ($p = 0.5$).

Chemical Analyses

On sampling days, pH values were determined potentiometrically in a 1:10 solution of snail meat homogenate in distilled water (Maza *et al.*, 1984). Freshness of snails was quantified on the basis of total volatile basic nitrogen (VBN) according to the method of Conway, as described by Muñoz *et al.* (1985). Drip losses were also determined throughout the storage period (AOAC, 1995).

RESULTS AND DISCUSSION

Figure 1 shows the survival curve of toxigenic *Vibrio cholerae* in inoculated snails. The D_{10} value thus determined, 0.12 kGy, agreed with equivalent values reported by other researchers (Matches and Liston, 1970). This value is relatively low compared with those of other pathogenic bacteria, and indicative of low radiation resistance by this microorganism, but it is higher than the D_{10} of other *Vibrionaceae* such as virulent and non-virulent *V. vulnificus*, reported to be 0.06 and 0.04 kGy, respectively (Dixon and Rodrick, 1998).

Figure 2 shows aerobic bacterial growth in raw snails irradiated at doses of 0.0 (controls), 1.0, 2.0, and 3.0 kGy during subsequent refrigerated storage at 2–4°C. Bacterial numbers on day 0, the day samples were irradiated, indicated that irradiation brought about an immediate reduction in bacterial numbers equivalent to 2.0–2.5 $\log_{10}$ cycles, from an initial 10^5 CFU/g down to 10^1–10^2 CFU/g. During storage, control samples reached 10^8 CFU/g after 14 days, indicative of spoilage, whereas snails irradiated at 2.0 and 3.0 kGy did not reach that level even after 21 days. There was no significant difference in bacterial numbers for the 2.0 and 3.0 kGy treatments, suggesting that a dose of 2.0 kGy was enough to extend the microbiological shelf-life of the snails at 2–4°C beyond 21 days.

A radiation dose of 2.0 kGy was deemed optimal for delaying aerobic bacterial growth in the snails and hence prolonging their microbiological shelf-life during post-irradiation refrigerated storage at 2–4°C. While samples irradiated at 2.0 kGy sustained little bacterial growth during the first 7 days of storage, total aerobes in control samples reached spoilage levels of 10^8 CFU/g levels in the same period. Even after 21 days refrigerated storage, total aerobic counts in irradiated snails were only 10^6 CFU/g.

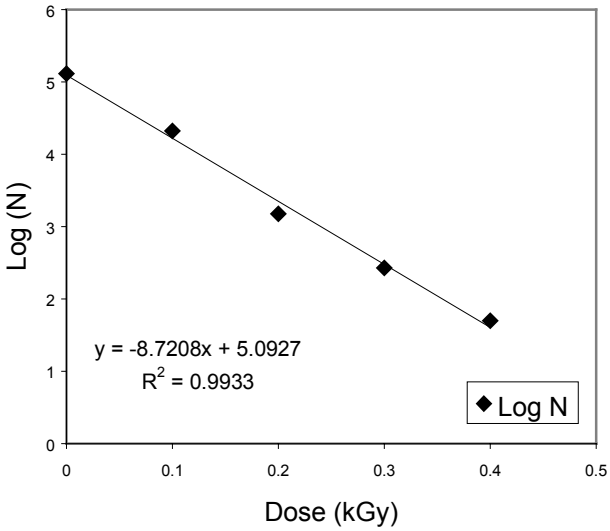

Fig. 1. Survival curve for *Vibrio cholerae* inoculated into marine snails

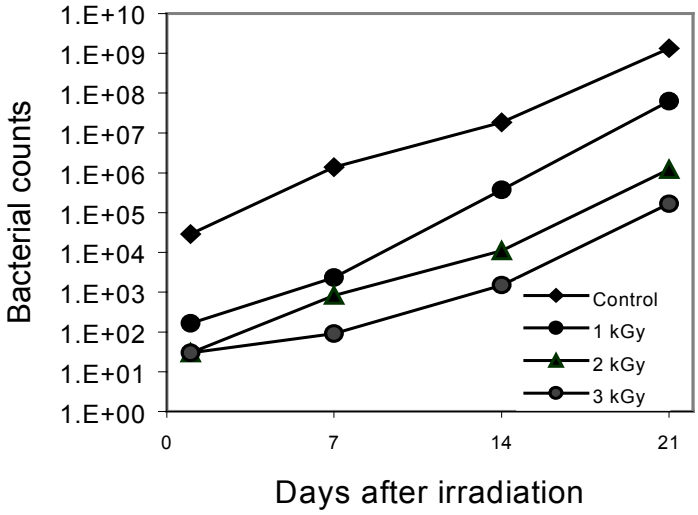

Fig. 2. Aerobic bacterial growth in irradiated marine snails during storage at 2–4°C

Tables 1 and 2 present the results of sensory analyses conducted on irradiated raw and cooked snails, respectively. Irradiated samples, raw or cooked, did not undergo significant ($p < 0.0.5$) changes in appearance (i.e. color), odor, flavor, or texture when compared to non-irradiated controls on the day irradiation was applied or after seven days refrigerated storage at 2–4°C. However, after 14 days, irradiated raw snails received favourable odor scores whereas non-irradiated control samples had deteriorated severely due to spoilage. Some irradiation effects on the typical odor of fresh fish fillets reported elsewhere (Torres *et al*., 1988), consisting of a slight smoke odor that disappeared during post-irradiation storage, were not present in irradiated snails. By day 21 of storage, raw snails irradiated at 2.0 or 3.0 kGy had significantly better scores for all attributes than those treated at 1.0 kGy, although the scores for all attributes in the latter had decreased significantly compared to the corresponding values on day 14. These results were similar for cooked snails, confirming that 2.0 kGy would be the optimum dose for prolonging the shelf-life of these mollusks to 21 days at 2–4°C, and suggesting that no additional quality gains would be obtained using 3.0 kGy.

Table 1: Effect of Ionizing Radiation on Sensory Attributes of Raw Marine Snails During Storage at 2–4°C

	Appearance score and radiation dose				Odor score and radiation dose				Texture score and radiation dose			
kGy:	0	1	2	3	0	1	2	3	0	1	2	3
Days												
0	4.3	4.3	4.5	4.3	4.4	4.4	4.3	4.3	4.3	4.5	4.1	4.1
7	4.2	4.2	4.0	4.0	3.7	4.0	4.1	3.8	4.1	4.0	4.0	4.2
14	3.2	3.7	3.8	3.7	2.7	3.6	3.7	3.8	3.2	3.5	3.4	3.4
21	1.7	3.3	3.5	3.5	1.5	2.9	3.5	3.5	2.0	3.2	3.5	3.5

Table 2: Effect of Ionizing Radiation on Sensory Attributes of Cooked Marine Snails During Storage at 2–4°C

	Odor score and radiation dose				Flavor score and radiation dose				Texture score and radiation dose			
kGy:	0	1	2	3	0	1	2	3	0	1	2	3
Days												
0	4.4	4.4	4.5	4.2	4.6	4.5	4.5	4.2	4.2	4.1	4.1	4.1
7	4.4	4.3	4.2	4.3	4.3	4.2	4.1	4.1	4.2	4.1	4.1	4.2
14	3.2	3.9	4.1	4.1	2.8	3.8	3.9	4.0	3.7	4.0	4.0	3.8
21	1.8	3.5	3.7	3.5	1.0	3.4	3.8	3.7	2.4	3.7	3.7	3.6

Volatile basic nitrogen (VBN) is an index of fish and seafood freshness (Maza *et al.*, 1984). Values between 20 and 23 mg VBN/100 g are usually indicative of product freshness, whereas values in the range 30–45 mg/100 g indicate spoilage (IAEA, 1982). Irradiated snails reached a maximum VBN value of 24 mg/100 g only at the end of the storage period, while non-irradiated controls surpassed the rejection limit (30 mg VBN/100 g) after the first evaluation (7-day storage at 2–4°C; data not shown).

Determination of drip losses in snails showed that irradiated samples tended to loose more water during storage than non-irradiated controls. Drip losses in snails treated at 2.0 kGy were 1.1% shortly after irradiation, but reached 7.6% on day 21 of refrigerated storage; in contrast, control sample drip losses were 1.3% on irradiation day and 2.9% at the onset of spoilage on day 7. This differences could be attributed to rapid increases in pH value and in volatile basic nitrogen content in non-irradiated controls due to microbial spoilage. As the pH value increases due to microbial-induced proteolysis, water retention also increases in most muscle foods. On the other hand, there are reports of increased water losses as absorbed radiation doses increase in products such as poultry. Kumta and Sreenivasan (1970) reported that drip loss increased linearly in proportion to irradiation dose, from 20 to 24%, in Bombay duck (*Harpodon nehereus*) treated at radiation doses lower than 10 kGy; this effect was not observed in product irradiated at 20 and 30 kGy.

CONCLUSIONS

The radiation D_{10} for *Vibrio cholerae* O1 biotype El Tor in marine snails is 0.11 kGy. A 2.0-kGy radiation dose is optimal for extending the shelf-life of raw snails of the species *Thais chocolata* during post-irradiation refrigerated storage at 2–4°C. This dose extends the microbiological shelf-life of raw snails 3-fold to 21 days or more, and causes no significant effects on sensory attributes (color, odor, flavor, and texture) of raw or cooked snails, or on

chemical characteristics (pH, VBN), although it also increases drip losses during subsequent storage at refrigeration temperatures.

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INACTIVATION OF *VIBRIO CHOLERAE* O1 EL TOR INOCULATED INTO PERUVIAN "CHORO" MUSSELS (*AULACOMYA ATER*) AND TWO SPECIES OF CLAMS (*ARGOPECTEN PURPURATUS* AND *GARI SOLIDA*) USING MEDIUM-DOSE IRRADIATION

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Abstract

The radiation decimal reduction dose (D_{10}) for *Vibrio cholerae* O1 biotype El Tor inoculated through the natural feeding system into three species of bivalve mollusks from the Peruvian Pacific coast: "choro" mussels (*Aulacome ater*), "abanico" clams (*Argopecten purpuratus*), and common clams (*Gari solida*), was determined *in vivo*. The D_{10} value obtained *in vivo* was 0.14 kGy in all mollusks tested. Concurrent studies conducted to determine the potential use of irradiation to extend the microbiological shelf-life of the mollusks during post-irradiation storage at 0–1°C indicated that a dose of 1.0 kGy was optimal for choro mussels and abanico clams, whereas 2.0 kGy produced the best results when treating common clams. Shelf-life extension thus achieved was 31 days for choro mussels, 16 days for abanico clams, and 21 days for common clams. Non-irradiated control samples of all mollusks spoiled after 7–11 days of refrigerated storage. There were no significant ($p<.05$) adverse effects from the application of the optimal radiation treatments on the sensory characteristics (i.e. appearance, odor, flavor, and texture) of the mollusks. Total volatile basic nitrogen (VBN) and pH values were examined for use as indexes of seafood freshness.

INTRODUCTION

Bivalve mollusks are among the favorite dishes for Peruvians and many other South and Central Americans, who may consume them raw or cooked, in the form of a typical lime juice marinade preparation know as "ceviche." This seafood is frequently harvested from coastal habitats near urban discharges of raw sewage or along sea currents that may transport fecal contaminants to growing areas far from the actual discharge zones. On the other hand, the natural feeding system of bivalve mollusks, based on the filtration of large volumes of sea water from which organic particles, including potentially pathogenic bacteria, are separated and consequently concentrated, makes this seafood potential vehicles for transmission of serious human enteric diseases. As a result, consumption of these mollusks, particularly in raw form, present a serious human health hazard. Since the appearance of cholera in Peru in 1991, which initiated a continental pandemia that caused massive loss of lives, placed unbearable strains on the publish health systems of the Americas, and brought about international embargoes on important South American marine export products, concerned public health authorities in various countries have searched for viable decontamination treatments that might be used as intervention measures to eliminate pathogenic bacteria from such raw seafood (OPS/OMS, 1991).

Treatment of food with ionizing radiation has been advocated as a viable alternative to ensure elimination of *Vibrio cholerae* and other pathogens from raw shellfish and other

seafood (IAEA, 1992). The present study was conducted as part of a 5-year co-ordinated research project sponsored by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, and the Pan American Health Organzation (PAHO). The study was designed to determine the radiation decimal reduction dose (D_{10}) of toxigenic *Vibrio cholerae* in pure culture, *in vitro*, and in various shellfish, *in vivo*, and to use these data to evaluate the potential of irradiation as an intervention measure to eliminate this pathogen in the selected seafood. A secondary objective of the study was to examine potential additional benefits from such intervention in terms of shelf-life extension of the shellfish, for marketing under refrigerated conditions.

MATERIALS AND METHODS

Product Preparation

The choro (*Aulacome ater*) is a species of large mussel that is widely distributed along the Peruvian and Chilean coasts, while the "abanico" clam (*Argopecten purpuratus*), a valuable export crop, is cultivated under intensive methods in salt water farms. The *Gari solida* or common clam is a bivalve pelecypod that lives buried up to 5 m in sand and gravel within the inter-tidal zone; it is found along the Pacific coast from Pocusana (Peru) to the Chonos archipelago (Chile). The latter two shellfish are frequently used raw to prepare "ceviche," while choros are usually cooked prior to consumption.

Live, fresh choros for the study were purchased at the marketplace in Villa María del Triunfo, Lima, some 12–15 h after harvest from the growing beds, and transported to the laboratory. The common clams were purchased immediately after harvest at a commercial growing area in Laguna Grande, Pisco, so that they were not refrigerated for only 30 minutes. Abanico clams, in turn, were purchased at a production farm near Lima, and were taken to the laboratory within 3 h of harvest. The shellfish were transported to the laboratories of IPEN using Styrofoam boxes filled with ice, at 2–5^{o}C, to slow the biological activity of the mollusks and minimize microbial growth. All samples were washed using clean, ozonated sea water. In addition, the common clams were placed in an aquarium containing 40 L ozonated sea water after cleansing the outside of mud and sand that commonly adheres to the valves, and left there for 50 h to depurate their insides of excess organic matter. Microbiological tests were conducted before inoculation or irradiation on composite samples of each type of shellfish to determine initial bacterial counts and the possible presence of naturally occurring *Vibrio cholerae*.

Inoculation of Samples

A toxigenic strain of *Vibrio cholerae* O1 El Tor, serotype Inaba, was obtained from the Peruvian Health Ministry collection. The pure culture was characterized as Gram-negative, curved to straight rods, facultatively anaerobic, asporogenous, motile, halophilic, oxidase positive, which reduced nitrates to nitrites (CDC/NCD/OPS, 1995).

The culture was suspended in sterile alkaline peptone water (AP) and incubated at 42^{o}C for 8 h. A loopful from the top portion of the suspension was streaked onto a slant of brain heart infusion agar (BHIA), and incubated at 37^{o}C for 24 h (Carvajal *et al*. (1991). A loopful of the culture was then transferred to three bottles, each containing 50 mL sterile alkaline peptone water, and the bottles were incubated at 42^{o}C for 24 h. The content of the bottles was poured into an aquarium containing 40 L ozonated water in which the choros,

abanico clams, or common clams had been previously immersed, which resulted in a concentration of ca. 10^6 CFU/mL in the water. This inoculation procedure took advantage of the natural filtering system of feeding characteristic of mollusks, and resulted in inoculation of *Vibrio* cholerae into the choros and abanico clams at a level of 10^8 CFU/g, and of 10^6 *Vibrio* CFU/g for the common clams, after only 3 h in the aquarium. Bags made out of Nylon 6 (25-μ thick), recommended for use in irradiation (Killoran, 1974) because of their low residual odor upon irradiation, were used to package separately the inoculated choros, abanico clams, and common clams, 25 to a bag, followed by heat sealing in an air atmosphere. Similar packages were prepared with non-inoculated shellfish for the shelf-life extension studies.

Sample Irradiation

(a) Determination of D_{10}: Bags containing inoculated choros, abanico clams, and common clams, were irradiated at ambient temperature (ca. 20^0C) at doses in the range 0.0 (controls) — 0.4 kGy, in increments of 0.1 kGy, in a Gammacell 220 provided with a ^{60}Co source having a dose rate of 2.41 kGy/h. Absorbed radiation doses were measured using Fricke dosimeters strategically placed in the product. The D_{10} of the pure culture of *Vibrio cholerae* grown in tubes containing saline peptone water was determined simultaneously for reference purposes.

(b) Shelf-life determination: bags containing non-inoculated choros, abanico clams, and common clams, were irradiated at ambient temperature (ca. 20^0C) as before. Microbiological analyses were performed to determine the optimal dose for treating each type of mollusk under study. The dose(s) used were 1.0 kGy for choros, 1.0 and 2.0 kGy for abanico clams, and 1.0, 2.0, and 3.0 kGy for common clams. All bags were stored at 0–2^{o}C post-irradiation, and a total aerobic mesophilic bacterial population level of 10^6 CFU/g, equivalent to a $\log_{10}$ value of 7.00, was adopted as index of microbial spoilage (Carvajal *et al*., 1991).

Microbiological Analyses

Inoculated samples were examined for survival of *V. cholerae* by macerating 25 g of the meat in 225 mL peptone water, serially diluting with sterile peptone water following standard methods, and surface plating on TCBS plates as described by the FDA (1995). Bacterial counts were transformed into logarithms and plotted against radiation dose to draw the bacterial survival curve of *V. cholerae* in each type of mollusk, and thus determine the radiation D_{10} (i.e. the dose necessary to reduce the *Vibrio* population by 90%); the D_{10} value is given by the negative inverse value of the slope of the survival curve (Muñoz *et al.*, 1985). For shelf-life studies, serial dilutions were pour-plated with BHIA, and the plates were incubated at 22^{o}C for 24 h to enumerate total aerobic bacteria (Carvajal *et al*., 1991). Earlier studies involving Chilean seafood indicated that higher bacterial recoveries were obtained using this rather low temperature than the more conventional one for aerobic bacteria, 33–37^{o}C, probably because it corresponds to the temperature of their natural habitat (Figueroa, 1979).

Sensory Analyses

An eight-person trained panel was used to evaluate the acceptability of irradiated samples of each type of mollusk over the 21-day refrigerated storage at 2–4^{o}C. A 5-point hedonic scale was used to rate appearance (color), odor, and texture of irradiated and control snail samples, as described by Singson (1992). A score of 3 was adopted as the lowest limit

for acceptance. On sampling days (day 0, 7, 14, and 21 after irradiation), raw and cooked samples of each product were evaluated. The choros were lightly cooked in boiling water for 3 min, without salt or any additive, and presented to the panel on coded plates. The abanico clams were steamed for 3 min without adding salt or any additive, and presented to the panel as were the choros. The common clams were cooked for 3 min in a 2% salt solution. The experimental design was a square block with a 4 × 4 factorial (four treatments, four storage times) and three replications; the results were compared using Tukey's test ($p = 0.5$).

Chemical Analyses

On sampling days, the pH of the meat was determined potentiometrically in a 1:10 dilution of mollusk meat homogenate in distilled water (Maza, 1986). Freshness of snails was quantified on the basis of total volatile basic nitrogen (VBN) according to the method of Conway, as described by Muñoz *et al.* (1985), based on micro-diffusion in Conway plates using boric acid and saturated potassium carbonate, incubation at 37°C for 90 min, and titration with 0.02N HCl. Drip losses were also determined throughout the storage period (AOAC, 1995).

RESULTS AND DISCUSSION

D_{10} Values:

The D_{10} value determined *in vivo* for *Vibrio cholerae* O1 El Tor serotype Inaba inoculated into choros, abanico clams, and common clams, was 0.14 kGy for all shellfish. This result was slightly higher than the corresponding value determined *in vitro*, 0.13 kGy, and closely agreed with the 0.15 kGy reported by Gelli *et al*. in oysters (IAEA/FAO/PAHO, 1997). Consequently, treating raw choro mussels, abanico clams, and common clams with ionizing radiation at doses in the range 1.0–1.5 kGy would be appropriate to bring a contamination level as high as 10^8 CFU/g of *Vibrio cholerae* to extinction; this high number of viable cells of toxigenic *Vibrio cholerae* has been reported to be necessary to induce the cholera disease in humans (OPS/OMS, 1991).

Microbiological Shelf-life Extension:

The initial microbiological quality of the selected shellfish, in general, and of abanico clams in particular, was low, since pre-irradiation total aerobic bacterial populations were in the high $\log_{10}$ 5.00 CFU/g level for choros and common clams, and in the high $\log_{10}$ 6.00 CFU/g level (very close to the defined spoilage level of $\log_{10}$ 7.00) for abanico clams (Table 1). Irradiation at 1.0 kGy reduced these numbers only by one to one and one-half $\log_{10}$ cycles, which was sufficient to prolong the shelf-life of choros to more than 21 days and that of the common clams to 14 days compared to the corresponding non-irradiated controls; however, abanico clams did not benefit from the application of this low dose due to the already high initial bacterial counts of the samples. This confirmed that irradiation does not improve seafood having poor initial quality. It is noteworthy that none of the non-irradiated control samples of any shellfish had a shelf-life at 0–2°C greater than 7 days, a rather short time for marketing in fresh form.

Table 1: Total Aerobic Mesophilic Bacteria in Irradiated Choros, Abanico Clams, and Common Clams During Storage at 0–2°C (Log_{10} CFU/g)

Days at 0–2°C	Radiation Dose (kGy)								
	0.0			1.0			2.0		
	A[a]	B[b]	C[c]	A[a]	B[b]	C[c]	A[a]	B[b]	C[c]
0	5.79	6.93	5.56	3.95	6.18	4.00	-	4.00	3.91
7	6.48	7.80	6.20	4.18	6.91	4.60	-	5.12	4.00
14	6.90	8.53	8.08	4.72	7.18	5.62	-	6.57	4.48
21	8.88	9.73	9.20	4.81	7.66	6.60	-	6.26	5.53

[a]A = Choro mussels.
[b]B = Abanico clams.
[c]C = Common clams.

In choros, which had an acceptable initial microbiological quality, 1.0 kGy appeared to have injured surviving bacteria in ways that inhibited their growth almost entirely during the 21-day refrigerated storage at 0–2°C post-irradiation (Table 1). While the microbiological acceptability of irradiated choros outlasted the study period, non-irradiated samples reached spoilage levels on day 21. This result and that from the D_{10} value determination led to the conclusion that the optimal radiation dose for extending the microbiological shelf-life of choros was 1.0 kGy; hence, no higher doses were tested for this mollusk.

The optimal dose for microbiological shelf-life extension of common clams was 2.0 kGy. Although this dose did not result in significantly lower bacterial counts than 1.0 kGy up to day 14 of refrigerated storage at 0–2°C, a significant difference was noted on day 21 in that bacterial counts were one log_{10} lower than in clams treated at 1.0 kGy. Therefore, 2.0 kGy was deemed optimal for these mollusks.

Sensory Evaluation:

The results of appearance, odor, flavor, and texture evaluations in terms of time at 0–2°C during which mean panel scores were 3.0 or above, are presented in Table 2 for all products tested.

The results of the microbiological shelf-life extension phase of the study were determinant in selecting the experimental radiation dose(s) applied to each product. Consequently, in addition to 0.0 kGy (non-irradiated controls), only 1.0 kGy was used to treat choro mussels, and only 2.0 kGy were applied to common clams. These selections were justified by the results of sensory evaluations. Choro mussels, raw or cooked, irradiated at 1.0 kGy, were acceptable in appearance, odor, flavor (cooked only), and texture well beyond the experimental period of 21 days at 0–2°C, so that there was no need for higher doses. Following this reasoning, since 1.0 kGy had been found to result in unduly short microbiological shelf-life (Table 1) of common clams, only a 2.0-kGy dose was used to treat the common clams. Sensory evaluations of the clams treated at 2.0 kGy was invariably favourable in all the parameters measured (Table 2).

With regard to abanico clams, sensory evaluation results were mixed. Cooked samples were deemed acceptable in terms of appearance, flavor, and texture throughout the refrigerated storage period when either 1.0 or 2.0 kGy were applied. In contrast, even 2.0 kGy did not provide acceptable scores beyond 14 days at 0–2°C, reflecting the poor initial quality of the samples.

Table 2: Acceptability of Appearance, Odor, Flavor, and Texture of Raw and Cooked Irradiated (0.0–2.0 kGy) Choro Mussels, Abanico Clams, and Common Clams During Post-irradiation Storage at 0–2°C

Type of Product		Radiation Dose (kGy)											
		0.0	1.0	2.0	0.0	1.0	2.0	0.0	1.0	2.0	0.0	1.0	2.0
		Acceptable[a] Appearance (days)			Acceptable[a] Odor (days)			Acceptable[a] Flavor (days)			Acceptable[a] Texture (days)		
Choros	Raw	14	21+	*	7	14	*	--	--	*	14	21+	*
	Cooked	14	21+	*	--	--	*	7	21+	*	14	21+	*
Abanico Clams	Raw	7	14	14	14	14	14	--	--	--	--	--	--
	Cooked	14	21	21+	--	--	--	14	21	21	21	21	21
Common Clams	Raw	7	--	21+	7	--	21+	--	--	--	<7	--	21+
	Cooked	14	--	21+	14	--	21	7	--	21+	14	--	21

NOTE: * denotes that the radiation dose was not applied; — denotes sensory evaluation parameter not measured.
[a] Acceptable scores are mean values in the range 3.5–5.0.

Chemical Analyses:

Total volatile nitrogen (TVN) is a measure of freshness in raw fish and other seafood (Gallardo, 1978). In addition to chemical breakdown of proteins in fish muscle, TVN values have been reported to frequently correlate with microbial growth. The results of TVN measurements in raw choros indicated that irradiation at 1.0 kGy, the only radiation dose used to treat this product, effectively prevented TVN increases after 7 days refrigerated storage at 0–2°C (Table 3). Although TVN values in irradiated choros were somewhat higher than the usually accepted upper limit for freshness in fish (30 mg TVN/100 g), corresponding values in non-irradiated controls were much higher throughout the experimental period. It is interesting to note that TVN values in choros were consistently higher than in abanico clams or common clams, despite the fact that the microbiological quality of choros was the highest among all products tested (Table 1). On the other hand, abanico clams that had a particularly poor microbiological quality from the beginning, had the lowest TVN values. These results not only suggest that there was no direct correlation between TVN values and microbial counts, but also may point to an inverse correlation, if any. It is conceivable that rapidly growing microorganisms may deplete the bases that are measured by this test, or that volatile nitrogen, basic in nature, may be neutralized by increases in organic acids, as suggested by decreasing pH values in spoiling shellfish (Table 4).

According to Maza (1986), good-quality, fresh choros, abanico clams, and common clams should register a pH value of 6.3–6.9; values below 5.8, in turn, would indicate spoilage. The decrease in the pH of fish and shellfish muscle as spoilage progresses contrasts with pH increases in red meats and poultry as microbial spoilage sets in.

On the basis of pH values (Table 4), and on that of initial total aerobic microbial counts (Table 1), it must be concluded that the quality of the samples used in this study was not optimal, a likely reflection of inappropriate handling techniques during capture and transportation of seafood not uncommon in some developing countries. Nevertheless, pH

Table 3: Total Volatile Nitrogen (TVN) Content of Raw Irradiated Choros, Abanico Clams, and Common Clams During Storage at 0–2°C (mg N/100 g)

Days At 0–2°C	Radiation Dose (kGy)								
	0.0			1.0			2.0		
	A[a]	B[b]	C[c]	A[a]	B[b]	C[c]	A[a]	B[b]	C[c]
0	30	5	25	30	5	*	*	--	25
7	50	7	28	42	7	*	*	--	26
14	48	18	28	42	8	*	*	--	28
21	75	32	43	45	17	*	*	--	36

NOTE: * denotes that the radiation dose was not applied; — denotes parameter not measured.
[a]A = Choro mussels.
[b]B = Abanico clams.
[c]C = Common clams.

Table 4: pH Value in Homogenates of Raw Irradiated Choros, Abanico Clams, and Common Clams During Post-irradiation Storage at 0–2° C

Days At 0–2°C	Radiation Dose (kGy)								
	0.0			1.0			2.0		
	A[a]	B[b]	C[c]	A[a]	B[b]	C[c]	A[a]	B[b]	C[c]
0	6.1	6.1	6.5	6.1	6.1	*	*	--	6.5
7	6.1	6.0	6.0	6.1	6.1	*	*	--	6.5
14	6.0	5.9	5.5	6.1	6.1	*	*	--	6.4
21	5.6	5.7	5.0	6.0	6.0	*	*	--	6.3

NOTE: * denotes that the radiation dose was not applied; — denotes parameter not measured.
[a]A= Choro mussels.
[b]B= Abanico clams.
[c]C= Common clams.

values, as well as all the other quality parameters measured during this study, confirmed that irradiation at 1.0 or 2.0 kGy effectively contributed significantly to prolong the chemical shelf-life of the selected mollusks.

CONCLUSIONS

The D_{10} value *in vivo* for *Vibrio cholerae* O1 El Tor serotype Inaba inoculated into choros, abanico clams, and common clams, is 0.14 kGy for all shellfish. Therefore, radiation doses in the range 1.0–2.0 kGy would effectively eliminate the potential hazard posed by *Vibrio cholerae* in these mollusks when consumed raw.

Although the initial microbiological quality of the selected shellfish, in general, and of the abanico clams in particular, was low, irradiation at 1.0 kGy prolonged the shelf-life of choros to more than 21 days and that of the common clams to 14 days compared to the corresponding non-irradiated controls. Abanico clams did not benefit from the application of this low doses due to high initial bacterial counts of the samples, which confirmed that irradiation does not improve seafood having poor initial quality.

Choro mussels, raw or cooked, irradiated at 1.0 kGy, were acceptable in appearance, odor, flavor (cooked only), and texture well beyond the experimental period of 21 days at 0–2°C, so that there was no need for higher doses. For common clams, a dose of 2.0 kGy was optimal. With regard to abanico clams, cooked samples were acceptable in appearance, flavor, and texture when treated at either 1.0 or 2.0 kGy, but even 2.0 kGy did not provide acceptable scores beyond 14 days at 0–2°C because of poor initial quality of the samples.

All the quality parameters measured during this study confirmed that irradiation at 1.0 or 2.0 kGy effectively prolong the shelf-life of the selected mollusks.

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SHELF-LIFE EXTENSION AND DECONTAMINATION OF FISH FILLETS (*TRACHURUS PICTURATUS MURPHYI* AND *MUGIL CEPHALUS*) AND SHRIMP TAILS (*PENAEUS VANNAMEI*) INOCULATED WITH TOXIGENIC *VIBRIO CHOLERAE* O1 EL TOR USING GAMMA RADIATION

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Abstract

The radiation decimal reduction dose (D_{10}) of toxigenic *Vibrio cholerae* O1 El Tor, Inaba was determined *in vitro* (0.13 kGy) and in inoculated fresh fillets of saurel (*Trachurus picturatus murphyi*) (0.12 kGy) and another Pacific fish species known in Peru as "lisa," *Mugil cephalus* (0.13 kGy), both of which are frequently consumed raw in "ceviche." The D_{10} value was similarly determined in tails of the shrimp species *Penaeus vannamei* (0.13 kGy). In a second phase of the study, radiation doses in the range 1.0–4.0 kGy were evaluated for use in microbiological shelf-life extension of the selected seafood, and for adverse effects on various sensory attributes (appearance, odor, flavor, and texture). A dose of 1.0 kGy doubled the microbiological shelf-life of fish fillets during post-irradiation storage at 0–1°C to approximately 30 days. This dose was deemed optimal also for preserving all sensory characteristics evaluated except appearance, due to a darkening of fillets. Best results in shrimp tails were obtained using 2.0 kGy, which doubled their microbiological shelf-life to 20 days at 0–1°C. Dipping the fillets in a 10% solution of sodium tripolyphosphate before irradiation prevented radiation-induced drip losses.

INTRODUCTION

The cholera epidemic suffered by Peru in the 1990's increased public awareness on the serious environmental sanitation problems faced by a large sector of the population and on the need to establish preventive and corrective measures to control the spread of *Vibrio cholerae*. World Health Organization figures published in 1992, the second year of the epidemic, cited by CEPIC (1992), estimated the number of cases in Peru at over 300 000, including nearly 3000 deaths. The first cases of cholera were diagnosed in coastal areas near Lima, but the epidemic soon spread to interior provinces and beyond the national borders.

Common sources of infection with the dreaded disease during the cholera epidemic were fish and other seafood captured in contaminated coastal areas and consumed raw or insufficiently cooked, as it is the case with "ceviche," a highly popular dish. Among the fish species commonly used in ceviche preparation because of their relatively low cost and abundance are saurel and "lisa" (a sardine-like, small fish). These fish are frequently captured

in coastal areas contaminated with urban sewage, and thus could present a vehicle for transmission of *Vibrio cholerae.* Also of concern because of the economic implications of potential embargoes of seafood exports would be the potential presence of *Vibrio cholerae* in frozen shrimp. Such an embargo caused massive economic disruptions on the Peruvian economy during the first months of the cholera epidemic.

"Cold pasteurization" of fish and other seafood through radiation processing has been advocated as a potential intervention measure to control pathogenic bacteria such as toxigenic *Vibrio cholerae* in marine products, especially those destined for consumption in raw form (ICMSF, 1983). The present study was conducted with the main objective of evaluating the survival of *Vibrio cholerae* in fish fillets and shrimp irradiated at medium doses, and the potential adverse effects of the radiation treatment on various quality characteristics of these products. The study was part of a research program co-sponsored by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Vienna, Austria, and the Pan American Health Organization (PAHO), Washington, DC. The program originated from a joint technical consultation held in Washington, DC in 1992 (IAEA, 1992), as a response to the growing continental cholera epidemic, and was implemented from 1993–1998.

MATERIALS AND METHODS

Determination of *Vibrio cholerae* D_{10} values:

A toxigenic strain of *Vibrio cholerae* El Tor, Inaba, responsible for the Peruvian 1991–1993 epidemic of cholera, was selected for the study. The strain, obtained from the collection of the Ministry of Health, had been isolated from a cholera patient. The pure culture was first suspended in alkaline peptone water and incubated 8 h at 42°C, followed by streaking on slants of brain heart infusion agar (BHIA) and 24-h incubation at 37°C (Carvajal *et al.*, 1991); this procedure was repeated to obtain a fast growing culture. The cell growth obtained on the slants was then transferred into 250 mL alkaline peptone water to prepare a suspension of *Vibrio cholerae* containing 10^8 colony forming units (CFU)/mL.

Fresh saurels having an average weight of 500 g, and fresh "lisas," a sardine-like, small fish, were purchased at the Lima seafood market, transported to the laboratory of the Instituto Peruano de Energía Nuclear (IPEN) in iced Styrofoam boxes, washed, degutted, and filleted. The fillets were cut into whole muscle pieces weighing ca. 25 g, placed in high-density polyethylene bags, heat sealed in air, and irradiated at 10 kGy to eliminate most of the natural microbial flora. The pieces were then submerged in the suspension of *Vibrio cholerae* for 2.5 min, which had been previously found to result in an inoculation level of 10^7 CFU/g. The inoculated fish pieces were allowed to drain excess inoculum before being placed in high-density polyethylene bags and heat sealed in air. A similar procedure was followed to inoculate shrimp (*Penaeus vannamei*) tail samples, although for this product the immersion time in the culture suspension was 5 min.

The bags containing inoculated fish fillet pieces or shrimp tails were irradiated at ambient temperature (25–27°C) at doses between 0.0 and 0.4 kGy, in 0.01 kGy increments, using a Gammacell 220 irradiator having a ^{60}Co source with a dose rate of 2.41 kGy/h. Non-irradiated samples were used as controls. In addition, tubes containing the *Vibrio cholerae* suspension were irradiated alongside the products to determine the D_{10} of the pure culture *in vitro*. The radiation dose absorbed by the samples and culture suspension tubes was measured

used Fricke dosimeters strategically placed between and around the samples (ASTM, 1972). All experiments were replicated three times.

After irradiation, 25-g composite samples of saurel muscle, lisa muscle, or shrimp tails were aseptically taken and homogenized in 225 mL alkaline peptone water. Serial dilutions were prepared from the homogenate using standard methods. The dilutions were pour plated with BHIA, and the plates were incubated at 37°C for 48 h. Typical colonies were enumerated, and the counts were transformed into logarithms to draw the extinction curve by plotting them (Y axis) against radiation dose (X axis); the inverse slope of the resulting fitted curve represented the decimal reduction dose, D_{10} (IAEA, 1982). A similar plating procedure was followed with the irradiated culture suspension to establish the reference, *in vitro* D_{10}.

Determination of the optimal radiation dose

Samples of saurel, "lisa," and shrimp such as those used in the determination of the D_{10} were bought at the Ventanilla maritime terminal (Callao). Considering the distance between the fishing areas and the Callao harbor, it was estimated that the catch would be approximately three days old on arrival in the market terminal. However, during the time from capture to marketing, the fish and shrimp were kept frozen on board the fishing vessels. The samples were transported to the laboratories of the IPEN and prepared for irradiation as before, but without the inoculation step. Fish parts known to be high in fat content were excised to minimize radiation-induced lipid oxidation problems. A second set of saurel fillets were dipped in a 10% (w/v) solution of sodium tripolyphosphate (STP) for 1.5 min to test the potential of this added treatment to reduce radiation-induced drip losses; the treatment was applied before packaging the fillets in Nylon 6 bags, heat sealing the bags, and cooling them to <10°C before irradiation.

Microbial growth in control and irradiated saurel fillets and shrimp tails, but not in lisa fillets, was monitored according to standard methods (FAO, 1981) after 1, 5, 10, and 15 days of refrigerated storage at 0–1°C, with a view to determining the microbiological shelf-life. A total aerobic bacterial count of 10^7 CFU/g was defined as the spoilage level (Maza, 1986). BHIA was the enumeration medium for total aerobic mesophilic bacteria, but the incubation temperature used in this study, 27°C, was lower than the standard one (i.e., 37°C) because such temperature had been found earlier to allow higher bacterial recoveries in Peruvian marine products in our laboratory.

Drip losses in fish fillets and shrimp were measured during the storage period (AOAC), as was the total volatile nitrogen content (TVN), a measure of seafood freshness widely used throughout the world. TVN values were measured following the method of Conway, as described by Muñoz *et al.* (1985), based on micro-diffusion in Conway plates using boric acid and saturated potassium carbonate, incubation at 37°C for 90 min, and titration with 0.02N HCl. Drip losses were also determined throughout the storage period (AOAC, 1995).

Sensory evaluations were performed alongside the other tests to define the overall shelf-life of products. A 6-person trained panel was used to evaluate appearance (color), odor, and texture of raw and cooked, irradiated and non-irradiated (control) samples. To evaluate flavor and texture in cooked samples, raw fish filletes were washed in water and then exposed to steam for 10 min. A 7-point hedonic scale was used to score the various attributes, and a score of 4 was defined as the lowest acceptability score for any attribute. Evaluations were

conducted on days 1, 5, 10, and 15 after irradiation. Mean scores of two replicates were analyzed using minimum squares and a confidence level of 95% (Gacula, 1975).

RESULTS AND DISCUSSION

The D_{10} value determined *in vitro* for the toxigenic *Vibrio cholerae* O1, Inaba strain used in the study was 0.13 kGy. The corresponding value in saurel fillets was 0.12 kGy (Fig. 1), while 0.13 kGy was obtained in lisa fillets and shrimp tails. These results agreed with those obtained by Gelli (1997) in nutrient broth and in inoculated oysters, and by Alcántara (1993) in lisa fillets, and suggested that there was no detectable protection afforded to *Vibrio cholerae* against radiation injury by any component of the fish or shrimp muscle tested, as was described by Jay (1994) in other media. These D_{10} values are very low in relation to those of other non-spore forming pathogenic bacteria (Loaharanu, 1996), and confirm that a radiation dose of 1.0 kGy, equivalent to ca. eight times the D_{10}, would be sufficient to eliminate a *Vibrio cholerae* contamination level as high as 10^7 CFU/g in the fillets and shrimp.

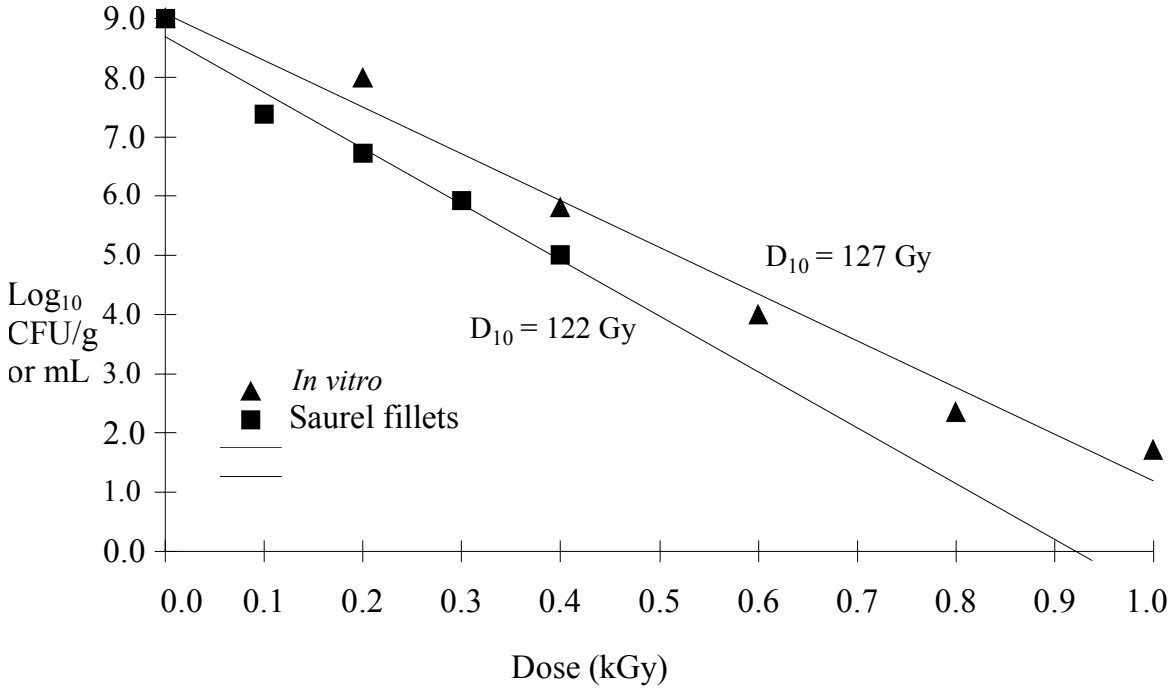

Fig.1. Survival curve of *Vibrio cholerae* irradiated in physiological serum or in lisa filletes

Irradiation effected an immediate reduction on the bacterial flora of saurel fillets. A radiation dose of 1.0 kGy applied to saurel fillets decreased the initial microbial population by 2 $\log_{10}$ cycles, and injured surviving bacteria enough to inhibit their growth for as long as 6 days at 0–1^{0}C (Fig. 2). As a result, the microbiological shelf-life of refrigerated saurel fillets irradiated at this relatively low dose was extended to 15 days, which compared very favorably with only two days for non-irradiated controls. It is worth mentioning that the initial microbiological quality of the fillets was low, since a bacterial content of 10^6 CFU/g has been proposed as the threshold for appearance of spoilage signs in fish and other seafood (Carvajal, *et al.*, 1991). Earlier observations by De La Sierra (1970) suggested that 3.0 kGy would be

needed to prolong to 21 days the shelf-life of refrigerated fillets of *Merluccius merluccius* that had an initial microbial load of 10^5 colony forming units (CFU)/g.

Microbial growth in irradiated and control shrimp samples was similar to that in saurel fillets (data not shown). Initial total aerobic bacterial counts in shrimp were at levels approaching 10^6 CFU/g, indicative of poor quality. Irradiation at 1 kGy reduced the levels by one $\log_{10}$ cycle, while 2 and 3 kGy effected a 3-$\log_{10}$ decrease in bacterial counts. However, only the highest dose tested on shrimp (3.0 kGy) provided a shelf-life extension of 10 days over that of non-irradiated controls or of samples irradiated at the lower doses, all of which spoiled by day 8 of storage at 0–1°C. This result confirmed the tenet that irradiation, like other food processing techniques, cannot be used to improve poor quality seafood.

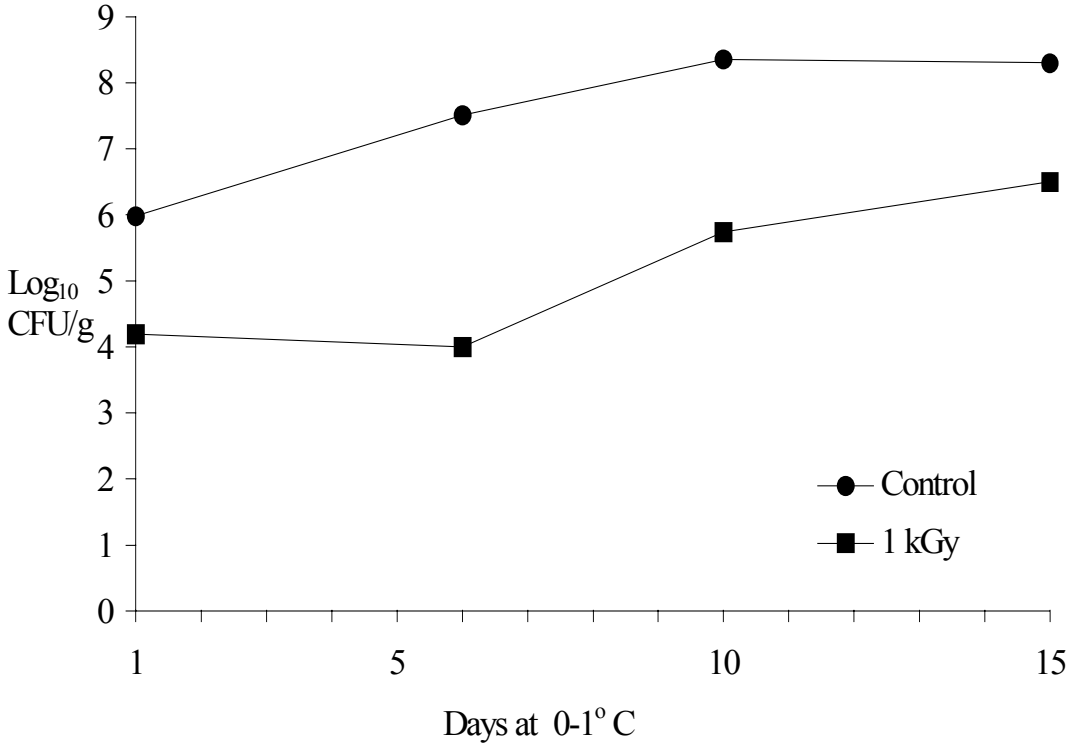

Fig. 2. Aerobic mesophilic bacterial counts in irradiated and non-irradiated saurel fillets during storage at 0–1°C

Shelf-life extension of lisa fillets was not measured in terms of numbers of bacteria in the samples during storage, but only on the basis of sensory and chemical (TVN) evaluations.

Sensory evaluation of saurel fillets irradiated at 1.0 kGy, the only dose used for this product because of its efficacy in extending shelf-life, indicated that a smoke-like odor was present immediately after treatment; this off-odor disappeared after a few hours of refrigerated storage. There was also a slight rancid odor which persisted in the fillets, that was likely due to radiation effects on the lipids of this rather high fat-containing fish; this affected the taste and brought about lower scores for cooked saurel fillets than those received by control samples on the first evaluation period after irradiation, but not thereafter. The color of fillets also was impaired to some extent by irradiation, consisting of a slight darkening of the muscle, but this effect was not strong enough to influence appearance scores. Based on odor and texture scores, the two most important quality attributes of fish that is to be consumed

raw as "ceviche," it was concluded that the shelf-life of saurel fillets was extended more than 15 days by 1.0 kGy irradiation, as opposed to a shelf-life of only eight days in non-irradiated, control samples (Table 1). Pre-irradiation treatment of fillets with a 10% STPP solution greatly improved texture and decreased drip loss throughout refrigerated storage. In general, the time in refrigerated storage during which the various quality attributes of saurel fillets monitored in this study were found acceptable by the trained panel doubled in irradiated fillets compared to non-irradiated samples.

In shrimp, non-irradiated raw tail samples were rejected after 8 days at 0–1°C, whereas those irradiated at 1.0 kGy were judged acceptable for as long as 15 days. There was an additional extension of shelf-life to 22 days when the dose applied was 2.0 kGy but no additional benefits were obtained when the dose applied was 3.0 kGy; this agreed only partially with earlier reports by Kumta and Sreenivasan (1979) of increases in shelf-life of shrimp with increasing radiation doses. In fact, when shrimp samples irradiated at 3.0 kGy were cooked for sensory evaluation, the panelists detected an objectionable flavor that prompted rejection; a similar result was reported by Muñoz *et al.* (1985). Therefore, although the best microbiological results in shrimp were achieved with a 3.0 kGy radiation dose, 1.0 and 2.0 kGy only were chosen for further evaluation.

Texture was found to be a poor indicator of quality in raw, irradiated shrimp. Therefore, more importance was given to texture determination in cooked shrimp. A similar approach was found to be more accurate to evaluate shrimp odor and flavor. Shrimp tails irradiated at 1.0 or 2.0 kGy and cooked scored significantly higher for odor and flavor than their non-irradiated counterparts (Table 1). These results translated into a shelf-life extension of one and two weeks for the 1.0 and 2.0 kGy treatments, respectively, although the benefits of the higher dose over 1.0 kGy were not conclusive.

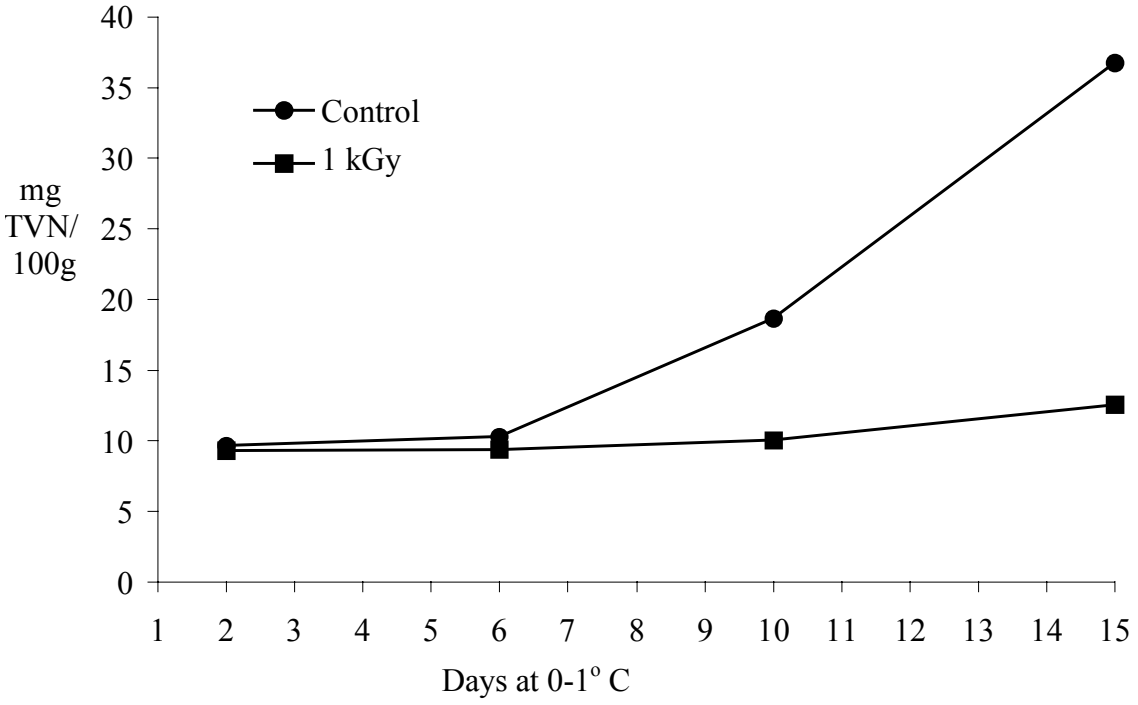

Fig. 3. Total volatile nitrogen (TVN) in saurel fillets during storage at 0–1°C

TABLE 1: ACCEPTABILITY OF APPEARANCE, ODOR, FLAVOR, AND TEXTURE OF IRRADIATED (0.0–2.0 kGY) RAW AND COOKED SAUREL FILLETES, SHRIMP TAILS, AND LISA FILLETS DURING POST-IRRADIATION STORAGE AT 0–1°C

Type of Product		Radiation Dose (kGy)											
		0.0	1.0	2.0	0.0	1.0	2.0	0.0	1.0	2.0	0.0	1.0	2.0
		Acceptable[a] Appearance (days)			Acceptable[a] Odor (days)			Acceptable[a] Flavor (days)			Acceptable[a] Texture (days)		
Saurel Fillets	Raw	15	22	--	8	15	--	--	--	--	15	22+	--
	Cooked	--	--	--	--	--	--	8	22+	--	15	22	--
Shrimp Tails	Raw	8	15	22	8	15	22	--	--	--	--	--	--
	Cooked	--	--	--	1	15	15	1	15	22	22	22	22
Lisa Fillets	Raw	8	8	8	8	15	15	--	--	--	8	15	8

NOTE: * denotes that the radiation dose was not applied; -- denotes that sensory evaluation parameter was not measured.
[a] Acceptable scores are mean values in the range 3.0–5.0.

The shelf-life of lisa fillets, as measured by texture (an extremely important attribute in fish muscle for use in the preparation of ceviche), was extended to 15 days at 0–1°C by irradiation at 1.0 kGy; this was twice the shelf-life of non-irradiated samples (Table 1). A higher dose (2.0 kGy) did not result in additional shelf-life. Fillets dipped pre-irradiation in STPP solution, as well as non-irradiated controls, received the highest texture scores throughout the storage period and had the lowest volume of drip (data not shown). Taking into account all the parameters monitored during refrigerated storage (0–1°C), the shelf-life of lisa fillets irradiated at 1.0 was prolonged to 15 days from only 8 in non-irradiated ones. Irradiation had an adverse effect on the color of this product that was evident immediately after treatment but became less obvious as control samples underwent rapid microbial spoilage.

TVN values in irradiated (1.0 kGy) saurel fillets remained below the limit for acceptability (i.e. 23 mg/100g) beyond the 15-day period of shelf-life defined by the sensory and microbiological quality attributes discussed earlier, whereas the controls surpassed it shortly after 12 days of refrigerated storage (Fig. 3). Similar results were obtained in shrimp tails irradiated at 2.0 kGy and in lisa filletes treated also at 2.0 kGy (data not shown).

Drip losses in irradiated fillets of lisa showed a tendency to increase as the radiation dose increased. However, although such losses were not significantly higher in irradiated fillets, a pre-irradiation immersion in 10% STPP eliminated any differences.

CONCLUSIONS

Toxigenic *Vibrio cholerae* O1 El Tor Inaba is quite susceptible to injury by gamma radiation.

The radiation D_{10} value for this microorganism *in vitro*, in artificially contaminated tails of the shrimp species *Penaeus vannamei,* and on lisa (*Mugil cephalus*) fillets, was 0.13 kGy; in saurel (*Trachurus picturatus murphyi*) fillets the D_{10} values was very similar, 0.12 kGy. These results suggested that a dose of 1.0 kGy would be sufficient to ensure inactivation of a 10^7 CFU/g of the pathogen in these products, making them cholera-safe even if consumed raw as these fish frequently are.

A radiation dose of 1.0 kGy was optimal for extending the microbiological shelf-life of saurel filletes to twice that of control samples, whereas 2.0 and even 3.0 kGy where needed to achieve similar results in shrimp tails because of their low initial microbial quality. In general, irradiation at medium doses (1.0 and 2.0 kGy) preserved the sensory attributes of fish fillets and shrimp tails and extended their acceptability in refrigerated storage (0–1°C) to twice as long as non-irradiated samples.

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COLLABORATIVE EVALUATION OF COMMERCIAL IRRADIATION FOR *VIBRIO VULNIFICUS* CONTROL IN LOUISIANA OYSTERS[1]

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Abstract

Oysters commercially harvested September 1994 and April 1995 from Black Bay, Louisiana were processed by industry collaborators as: 1) live shellstock boxed 200 per 60 lb (27 kg) box in Florida; 2) shucked 12 oz (310 g) containers in Florida; and 3) frozen half-shell in Virginia. The oysters were then shipped by each processor in commercial refrigerated trucks to Food Technology Services, Inc. in Mulberry, Florida for commercial irradiation processing. Live shellstock were held at a temperature of about 7°C from harvest through sampling. Ambient levels of *Vibrio vulnificus* in the oysters were 4.6×10^5 MPN/g in September 1994 (harvest water 29°C) and 1.5×10^2 MPN/g in April 1995 (harvest water 20°C). Two 60-lb (27 kg) boxes of live oysters were each treated at minimum levels of 0.0 (controls), 0.5, 1.0, 1.5, and 2.0 kGy, 400 oysters per dose. The dose ratios were calculated to be about 2:1 in the commercial box packaging. Levels of *V. vulnificus*, total aerobic plate counts, and percent cumulative mortality were enumerated every three days through day 14 post-harvest for the September 1994 samples, and through day 28 post-harvest for the April 1995 samples. September 1994 live shellstock oysters treated with doses of 0.5 kGy and 1.0 kGy had a very significant 4-$\log_{10}$ reduction in levels of *V. vulnificus* at 0.5 kGy, and a 5-$\log_{10}$ reduction at 1.0 kGy 2 days post-irradiation (4 days post-harvest), without a significant difference in mortality from the non-irradiated control group, which lost 1 $\log_{10}$ after storage at 3°C for 4 days. The April 1995 samples were reduced to below detectable levels of *V. vulnificus* at all doses. Commercial shucking and washing of fresh product, and freezing in the half-shell, also reduced numbers of *V. vulnificus* 4–5 $\log_{10}$ by day 14 post-harvest. Additional irradiation processing of these products reduced *V. vulnificus* to below detectable levels at the lowest dose of 1.0 kGy.

INTRODUCTION AND BACKGROUND

The rich coastal estuaries of the State of Louisiana on the Gulf of Mexico have traditionally had a commercial oyster industry with an economic impact of approximately $100 million dollars annually. However, between 1988 and 1991, there was a devastating economic decline of approximately 50% in the commercial industry and in the wholesale price of Gulf oysters. This was largely due to loss of consumer confidence in the product, which has never fully recovered (Kilgen, 1993; Levy and Fein, 1995; Lin *et al*., 1991). This loss in consumer confidence was mainly due to public concern over the presence of *Vibrio vulnificus*, a naturally occurring bacterium that is found in estuarine and marine waters (Farr, 1990; Kilgen, 1991; National Academy of Sciences, 1991). Increase in consumer confidence

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and marketability could increase the Louisiana industry value over a 2–3 year period by $20 million annually, with a final impact of $33 million annually. This value would be much greater for the entire Gulf Coast industry (Voisin, 1998).

Vibrio vulnificus is a naturally occurring, halophilic, Gram negative, rod-shaped bacterium that ferments lactose and is found in estuarine and marine waters (Oliver, 1989; Kilgen, 1991; National Academy of Sciences, 1991). *V. vulnificus* is considered a major component of the normal microflora of warm (>25°C) estuarine waters, and thus, of filter-feeding molluskan shellfish. It has been isolated from estuarine and marine waters of the US gulf coast, east coast, and west coast, and on various continents (Wright *et al.*, 1986; Kaysner *et al.*, 1987; Oliver *et al.*, 1992; O'Neill *et al.*, 1992; Hoi *et al.*, 1998). It has also been isolated from many seafood samples, including filter-feeding mollusks, where these bacteria can adhere and multiply in the gut region (Oliver, 1989; Ruple and Cook, 1992; DePaola *et al.*, 1994; Groubert and Oliver, 1994). It can be transferred from this natural environment to man directly through wound infections in estuarine waters, and this is the most common route of transmission. It is also transmissible through the consumption of raw or undercooked oysters and other seafood by immunocompromised or "at risk" individuals, and has thus become the main microorganism associated with seafood-borne mortalities in recent years. These mortalities have been a result of primary septicemia only in "at risk" individuals through consumption of raw oysters or other seafood. These deaths are very rare, and occur only in individuals who are immunocompromised or have preexisting liver disease (Oliver, 1989; CDC, 1993; Kilgen, 1991; National Academy of Sciences, 1991). Even these "at risk" individuals have only about a 1 in 25 000 chance of contracting this serious disease if they consume raw oysters contaminated with *V. vulnificus* (CDC, 1993); 99% of at-risk raw oyster consumers with underlying medical conditions do not acquire this disease (Kilgen and Hemard, 1996). However, although individuals who are considered normal and healthy have never been reported to have contracted the potentially fatal primary septicemia disease, a great deal of negative and alarming national media coverage over the last 10 years has resulted in a serious economic impact on the Gulf of Mexico oyster industry, and that of the entire country (Kilgen, 1991; Kilgen, 1993; Kilgen and Hemard, 1996).

Because of this problem, many post-harvest techniques and strategies have been evaluated to reduce the numbers of *Vibrio vulnificus* in live and processed oysters over the last 10 years. They have included time and temperature for reduction of numbers (Cook, 1994), cold, freezing, low heat/cold pasteurization treatments (Cook and Ruple, 1992; Cook, 1994; Cook, 1997; Kilgen and Hemard, 1996), vacuum packaging (Parker *et al.*, 1994), use of additives (Tamplin and Capers, 1991), suspension relaying into offshore waters (Motes and DePaola, 1996), ultra violet (UV) light (Tamplin and Capers, 1991), food condiment treatment (Sun and Oliver,1995), and hydrostatic high pressure processing (Hoover *et al.*, 1989; Farr, 1990; Rovere, 1995). Some of these strategies have been shown to be effective under experimental conditions, but few have been shown to be economically viable for commercial use. Of all the post-harvest elimination strategies for foodborne pathogens, ionizing radiation has been studied the most. The technology has been approved by the US Food and Drug Administration for many foods, from wheat and flour in 1963 to fresh fruits, vegetables, dry spices, seasonings, enzymes, pork, poultry, and most recently, meats (IAEA, 1998; Henkel, 1998), but not yet on fish and seafood, including mollusks.

Man has sought to preserve his food supply and control disease and insect infestation from earliest times. However, despite the fact that we are approaching the 21st century, the Food and Agriculture Organization of the United Nations (FAO) estimates that 25% of all food production worldwide is lost post-harvest due to insects, bacteria and rodents (ICGFI,

1991). In the United States, the Council for Agricultural Science and Technology (CAST), and a May, 1997 presidential report, "Food Safety from Farm to Table," estimated that foodborne disease caused by bacterial pathogens and parasites cause 6–33 million cases of diarrheal disease and approximately 9,000 deaths annually in the United States, and is on the increase. In addition to this loss of lives, it is estimated that the annual economic losses associated with foodborne illness is as high as $5–6 billion (Thayer *et al.*, 1996; Henkel, 1998). This does not include the economic losses due to food spoilage post-harvest.

Many national and international agencies have actively investigated, supported or approved the health and safety of ionizing irradiation technology to address the issues of foodborne disease throughout the world. They include the World Health Organization (WHO), the International Atomic Energy Agency (IAEA), the American Medical Association (AMA), and the American Dietetic Association (ADA). Some US industry trade groups supporting the technology include the National Meat Producers Association, the Grocery Manufacturers of America, and the National Food Processors Association (Henkel, 1998). Many experimental studies in the past have indicated that gamma radiation processing of seafood, especially molluskan shellfish, can enhance safety by elimination of potentially pathogenic bacteria (Josephson and Peterson, 1982; Kilgen *et al.*, 1987; Mallet *et al.*, 1987; Grodner and Andrews, 1991; ICGFI, 1991; National Academy of Sciences, 1991; Kilgen, 1993; Pszczola, 1993; Kilgen and Hemard, 1996; Osterholm and Potter, 1997; Thayer *et al.*, 1996; Henkel, 1998; IAEA, 1998).

The current estimated added cost of approximately $0.05/lb for large volume commercial irradiation would certainly be considered a cost beneficial value-added processing step to the oyster industry in Louisiana and the entire country (Thayer *et al.*, 1996). The incentives are to offer an even safer product to all consumers and to regain public confidence and the large commercial markets.

MATERIALS AND METHODS

This study was a university-industry collaborative effort to evaluate the feasibility and effectiveness of commercial level harvesting, processing, packaging, shipping, and irradiation processing of several commercial oyster products from Louisiana estuaries for *Vibrio vulnificus* elimination or control. These commercial oyster products included: 1) live shellstock in 60-lb (27- kg), 200-count boxes; 2) cases of shucked 12-oz (310 g) and 8-oz (230 g) containers of fresh oysters (12 per case); and 3) cases of 6 half-shell shrink-wrapped packaged quick frozen (PQF oysters).

Microbiological Methods

Oyster products irradiated at a commercial irradiation facility were processed according to APHA standard methods (APHA, 1985) for total aerobic plate counts (APC/g). Levels of *V. vulnificus* were enumerated by the three-tube most probable number (MPN) method, using enrichment in alkaline peptone water, presumptive identification with colistin-polymyxin B-cellobiose (CPC) selective and differential agar (Oliver *et al.*, 1982), and confirmation with a monoclonal antibody enzyme immunoassay (EIA) (Tamplin *et al.*, 1991).

Methods for Processing and Treatment of Commercial Oyster Products

Live shellstock oysters were commercially harvested from Black Bay, Louisiana on September, 1994 and stored at 7^{o}C (40^{o} F). A sample of 12 oysters from that harvest was

taken for ambient or control *Vibrio vulnificus* MPN/g and total aerobic plate count (APC)/g levels. Growing water temperature (29^{oC}, 85^{o}F) was taken at the harvest site. These shellstock oysters were shipped from Louisiana by refrigerated truck (7^{o}C, 40^{o}F) to Florida and Virginia for further processing and packaging, and from those states to Food Technology Services, Inc. in Florida for commercial irradiation processing.

Thirteen sacks of the oysters harvested in September, 1994 were picked up at the boat dock in Black Bay, Louisiana by commercial refrigerated truck and shipped to a Florida seafood company for processing into boxed live shellstock and fresh shucked 12-oz products. The processed live shellstock and shucked 12-oz products were shipped to Food Technology Services, Inc. in Florida. The oysters were processed using various doses of ionizing radiation three days post-harvest and returned to Nicholls State University (NSU) by commercial refrigerated truck (7^{o}C, 40^{o} F). They were analyzed for MPN/g *V. vulnificus*, total APC, and percent cumulative mortality (live shellstock only).

Three large sacks of shellstock oysters (about 680 oysters) from the same harvest site in Black Bay Louisiana in September, 1994 were shipped in a different refrigerated truck to a commercial seafood company in Virginia. The oysters were commercially shucked on the half shell, shrink-wrapped on a styrofoam tray, 6 half-shell oysters to a tray, and cryogenically "package quick frozen" (PQF) using liquid CO_2. These oysters were also shipped commercially to Food Technology Services, Inc. in Florida to be irradiated using several doses of ionizing radiation three days post-harvest. A "control" sample of 12 shucked, unwashed oysters was taken before processing, and shipped overnight on frozen gel packs to NSU for microbiological analyses (i.e. MPN/g *V. vulnificus* and total APC/g).

This procedure was repeated in April 1995 for live shellstock and fresh shucked oysters to evaluate potential differences in percent mortality at the beginning of the oyster spawning season. Only the live shellstock and shucked 8-oz containers of oysters were evaluated during this time period.

Live Shellstock Oysters

Live shellstock Louisiana oysters were commercially processed into ten 60-lb (27 kg) boxes of 200 shellstock oysters each at a Florida seafood processing plant. They were then shipped by commercial refrigerated truck to Food Technology Services, Inc. in Mulberry, Florida the same day for irradiation.

At Food Technology Services, Inc., two commercial 60-lb (27 kg) boxes of about 200 live shellstock oysters were each treated with minimum levels of 0.0 (controls), 0.5, 1.0, 1.5, and 2.0 kGy (for a total of 400 oysters per dose).

Shellstock product density was calculated to be 0.755 g/cc at Food Technology Services, Inc. The size of the commercial shellstock oyster box was 11 3/4 in. wide X 17 ¾ in. long × 9 5/8 in. high. The MAX/MIN dose ratio of live shellstock oysters in their commercial packaging was calculated to be about 2:1. The boxes could be stacked one deep and two wide in the tote, for a total of 12 boxes per overhead tote.

Irradiation LD_{50} for Louisiana oysters depends on the Condition Index of the oysters, but averaged about 2.25 kGy in a previous study using the experimental size irradiator at the Louisiana State University Nuclear Science Center (Kilgen *et al.*, 1987). These data was used to set the maximum dose of 2.0 kGy for live shellstock oysters.

One box of live shellstock oysters contained 192 oysters that were individually labelled with dosimeters and carefully arranged in the box to effect a location matrix. This was done to evaluate the exact doses obtained by the oysters in specific locations in the commercial box packaging. Eight oysters were laid across the front of the box and labelled 1 through 8 (eight columns). Eight columns of oysters were placed across the entire bottom of the box, four deep (labelled A, B, C ,and D planes). This made a total of 32 oysters (eight columns × four deep) on the bottom row or layer. There were a total of six complete rows or layers of 32 each, for a total of 192 oysters. A dose of 2.0 kGy was used. The exact radiation dose absorbed by each oyster in the box was recorded.

Oysters radiation-processed at Food Technology, Inc. were shipped back to Nicholls State University and stored at 7°C (40°F) in a walk-in cooler. A random sample of 12 oysters (six from each box at the same dose) was taken from each of the dose groups and analyzed for *V. vulnificus* MPN/g, APC/g and percent cumulative mortality at days 1, 4, 8, 11, and 14 post-irradiation. Day 11 post-irradiation was 14 days post-harvest. All the oysters in both 60-lb (27 kg) boxes (400 total oysters) at each dose were evaluated for percent cumulative mortality at each sampling period. The normal accepted rate of mortality in live oysters is 2–3%, and in normal commercial use, live shellstock oysters have a maximum shelf-life of 14 days post-harvest.

The experiment with live shellstock was repeated in April, 1995 to evaluate seasonal differences in oyster tolerance to irradiation processing.

Fresh Shucked and Packaged Oysters

In Florida, eight cases of 12 each 12-oz (310 g) containers of shucked fresh oysters harvested from Lousiana in September, 1994 were packed in ice and shipped by commercial refrigerated truck to Food Technology, Inc. for radiation processing. Two cases (12 in each) of 12-oz (310 g) containers of fresh shucked oysters were each treated with minimum dose levels of 0.0 (controls), 1.0, 1.5, and 2.0 kGy.

The experiment with fresh shucked product was repeated in April, 1995 to evaluate seasonal differences in oyster tolerance to irradiation processing. The oysters were processed in Florida and packed into 8-oz (230 g) containers.

Package Quick-Frozen (PQF) Half-Shell Frozen Oysters

In Virginia, live shellstock Louisiana oysters harvested in September, 1994 were commercially processed into a total of 80 trays of six half-shell oysters each. They were shucked on the half shell, shrink-wrapped on a styrofoam tray for six half-shell oysters, cryogenically "package quick frozen" (PQF) using CO_2, and packed in commercial boxes. The boxes were shipped frozen to Food Technology, Inc., in Florida, for radiation processing while frozen.

At Food Technology, Inc., sixteen PQF trays of six half-shell oysters/tray were each treated with minimum dose levels of 0.0 (controls), 1.0, 2.0, 3.0 and 5.0 kGy, for a total of 96 oysters/dose. Following irradiation, the frozen oysters were shipped back to NSU by commercial refrigerated truck and stored at –20°C. Two trays (12 frozen oysters) from each of the five dose levels were analyzed weekly for MPN/g *V. vulnificus* and APC/g for two months following irradiation. A total of 80 trays of six oysters was analyzed.

RESULTS AND DISCUSSION

Live Irradiated Shellstock Oysters

Live shellstock oysters were commercially harvested from Black Bay, Louisiana in two seasons, September and April, and irradiated for *V. vulnificus* control to evaluate whether the difference in the oyster quality and *Vibrio* levels in these different seasons would affect oyster mortalities or radiation effectiveness. Ambient levels of *V. vulnificus* in the oysters from both seasons were very different initially, 4.6×10^{5} MPN/g in September (water temperature of 29°C, 85°F), and 1.5×10^{2} MPN/g in April (water temperature of 20°C, 69°F). However, results showed that levels of *V. vulnificus* in both samples only dropped one $\log_{10}$ cycle after two weeks of storage at 7°C (40°F). This indicates that in live shellstock oysters, levels of *V. vulnificus* only drop approximately one $\log_{10}$ MPN/g after harvest and cold storage at 7°C (40°F) for the two-week shelf-life, regardless of original harvest water temperature.

In April, when ambient levels of *V. vulnificus* were only 1.5×10^{2} MPN/g, they were easily reduced to below detectable levels (<0.3 MPN/g) at the lowest dose of 0.5 kGy. The high ambient levels of 4.6×10^{5} MPN/g in September were reduced four $\log_{10}$ cycles to 46 MPN/g at 0.5 kGy.

Ambient mortalities were actually greater in April (5%) than in September (1%). This was probably due to the physiological condition of the oysters in April, when they are ready to spawn. After 14 days post-irradiation at 1.0 kGy, mortalities were 12.0% for controls and 17.0% for treated oysters in April, and 8.3% for controls and 9.5% for treated oysters in September.

Effect of Irradiation on V. vulnificus Levels and APC in Live Shellstock Oysters Harvested in September, 1994

Doses of 0.5 and 1.0 kGy showed a significant four and five $\log_{10}$ reduction in levels of *V. vulnificus*, from 4.6×10^{5} MPN/g in control shellstock to 46 and 2.3 MPN/g, respectively, at two days post-irradiation for September oysters. Doses of 1.0 kGy reduced the *V. vulnificus* level to undetectable (<0.3) by day 14 of cold storage. Doses of 1.5 and 2.0 kGy reduced *V. vulnificus* levels five and six $\log_{10}$ cycles initially, and to undetectable levels (<0.3) by 11 days cold storage at 7°C (40°F) (Fig. 1).

In the box of live shellstock oysters containing 192 oysters individually labelled with dosimeters and irradiated at 2.0 kGy, the maximum dose of 2.3 kGy was in a location at the top left, on the outside row. The minimum dose of 1.3 was in a location toward the lower mid left, in the interior of the box. This resulted in an average dose of 1.8 kGy in the commercial packaging for live shellstock oysters treated at 2.0 KGy.

Ambient levels of total aerobic plate counts (APC/g) were an extremely high 1.17×10^{6} in the 9/94 live shellstock samples (water temperature 29°C (85°F), one day post-harvest. By 14 days at 7°C (40°F), they were still 1.1×10^{6}/g. The lowest irradiation dose of 0.5 kGy reduced the APC/g in these samples by 3 $\log_{10}$ cycles to 1.4×10^{3} initially; by 14 days storage, APC/g increased to 2.8×10^{5}/g. At 1.0 kGy, the APC/g was only 1.1×10^{4} after 14 days of storage. This indicates a definite shelf-life extension for warm water shellstock oysters.

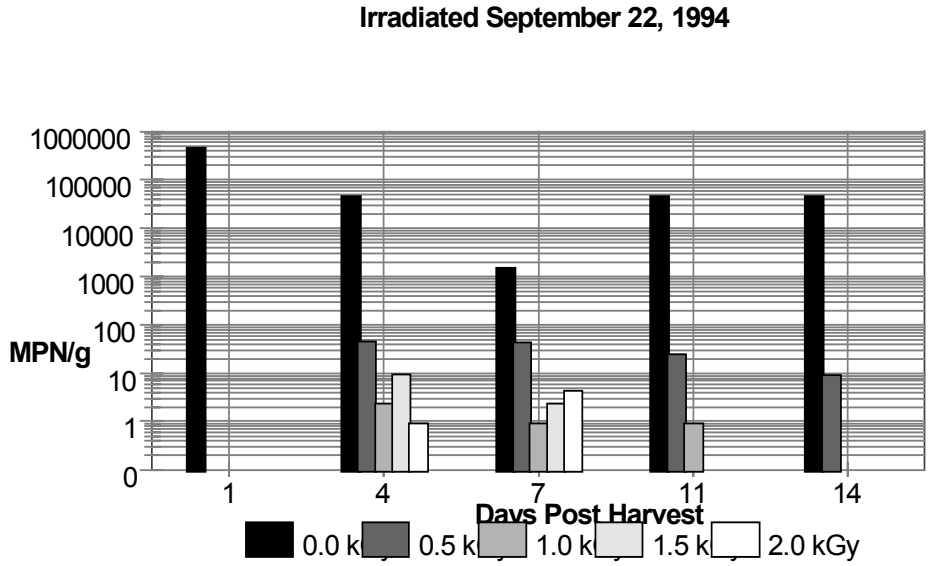

Figure 1. Effect of ionizing radiation on *Vibrio Vulnificus* populations in live shellstock Louisiana oysters from September, 1994.

Cumulative Mortality for Irradiated Live Shellstock from September, 1994

Control and treated oysters were within the expected 2–3% normal levels of mortality of live oysters removed from warm waters and stored at 7°C (40°F). Controls showed slightly more mortality than treated oysters at 5 days post-harvest. There were no significant increases in mortality of treated live shellstock as compared to the non-treated controls through 14 days post-harvest (shelf-life for live oysters). In fact, control oysters showed the highest mortality at 5 and 7 days post-harvest, and were still higher than mortalities at dose levels of 0.5 kGy and 1.0 kGy at 9 days post-harvest. At day 14 post-harvest, the dose level of 0.5 kGy showed the lowest mortality. At the end of the approved shelf-life of 14 days, and a dose of 1.0 kGy, mortalities were 8.3% for controls and 9.5% for treated oysters in September (Fig. 2).

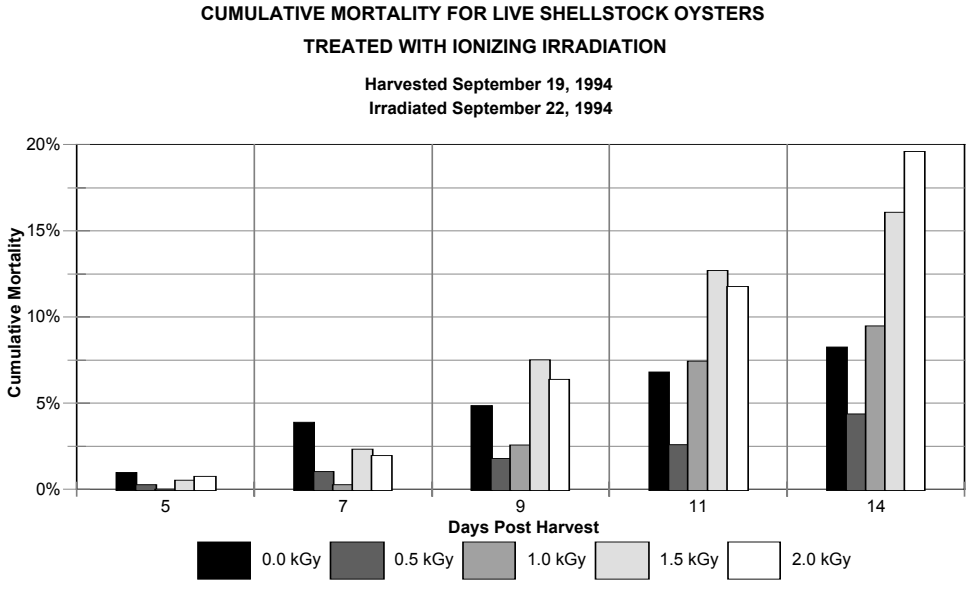

Fig. 2. Cumulative mortality for irradiated live shellstock from September, 1994.

In April 1995, the ambient levels of *V. vulnificus* in live shellstock was only 150 MPN/g. This was expected at an ambient water temperature of 20°C, 69°F. At these low levels, even the lowest dose (0.5 kGy) showed no detectable levels (<0.3 MPN/g) of *V. vulnificus* throughout the 28-day evaluation period (Fig. 3). Ambient total APC/g was only 1.2×10^3 in live shellstock oysters one day post-harvest, and 1.5×10^5 after 14 days storage at 7°C (40°F). Irradiation at 0.5, and 1.0 kGy reduced APC/g to 5.1×10^3 and 3.4×10^3, respectively after 14 days cold storage post-irradiation.

Cumulative Mortality for Irradiated Live Shellstock Oysters From April, 1995

Ambient mortalities of oysters were greater in April (5%) than in September (1%). This was probably due to the physiological condition of the oysters in April, when they are ready to spawn. At 7 days post-harvest, control oysters and those treated with 0.5 and 1.0 kGy showed the same mortality rates. At the end of the approved shelf-life period of 14 days, mortalities at 1.0 kGy were 12% for controls and 17% for treated oysters (Fig. 4).

Fresh Shucked Irradiated Oysters

Oysters commercially harvested from Black Bay, Louisiana during two seasons (September and April), and commercially shucked, washed and packaged in 12-oz (310g) or 8-oz (230 g) containers had very different ambient levels of *V. vulnificus*: 4.6×10^5/g in September (water at 29°C (85° F), and 1.5×10^2/g in April (water at 20°C (69° F). Levels of *V. vulnificus* and APC/g were evaluated every three and seven days, respectively, for a total of 35 days. *V. vulnificus* dropped five $\log_{10}$ cycles to 2 MPN/g by 14 days post-processing in September, 1994, and 2 $\log_{10}$ cycles, to 9 MPN/g, 14 days post-processing in April, 1995.

Effect of Irradiation on *V. vulnificus* Levels and APC in Fresh Shucked 12-oz (310-g) Containers from Oysters Harvested in September, 1994

Oysters commercially shucked, washed, and packed in 12-oz plastic containers were stored in ice under commercial conditions. Levels of *V. vulnificus* dropped one $\log_{10}$ cycle, from 4.6×10^5/g to 2.4×10^4/g in control oysters after shucking, washing and packing in ice.

There was a very low 0.4 MPN/g *V. vulnificus* level at 1.0 kGy four days post-irradiation. There were no other detectable levels of *V. vulnificus* in irradiated fresh shucked oysters, even at the lowest dose of 1.0 kGy, throughout the 35-day sampling period.

Even non-irradiated controls dropped from 4.6×10^5 MPN/g to 2 MPN/g by 14 days post-harvest on ice in cold storage. There were no detectable *V. vulnificus* in non-irradiated controls by 17 days at 7°C (40° F). Surface *V. vulnificus* are extremely sensitive to the commercial processing of shucking and washing or "blowing" in ice water. The additional packing of the shucked, washed products in ice also has a lethal effect on the microorganisms after about 14 days. However, that is the shelf-life for the product (Fig. 5).

Initial levels of 1.2×10^6 APC/g in control shucked oysters increased to 2.0×10^7 APC/g at the end of the 14-day shelf-life. At 14 days, doses of 1.0, 1.5, and 2.0 kGy had only 4.1×10^4, 4.4×10^4, and 3.2×10^4 MPN/g, respectively. The National Shellfish Sanitation Program (NSSP) guidelines recommend 500 000 (i.e., 5.0×10^5 total APC/g in shucked processed oysters (FDA, 1993).

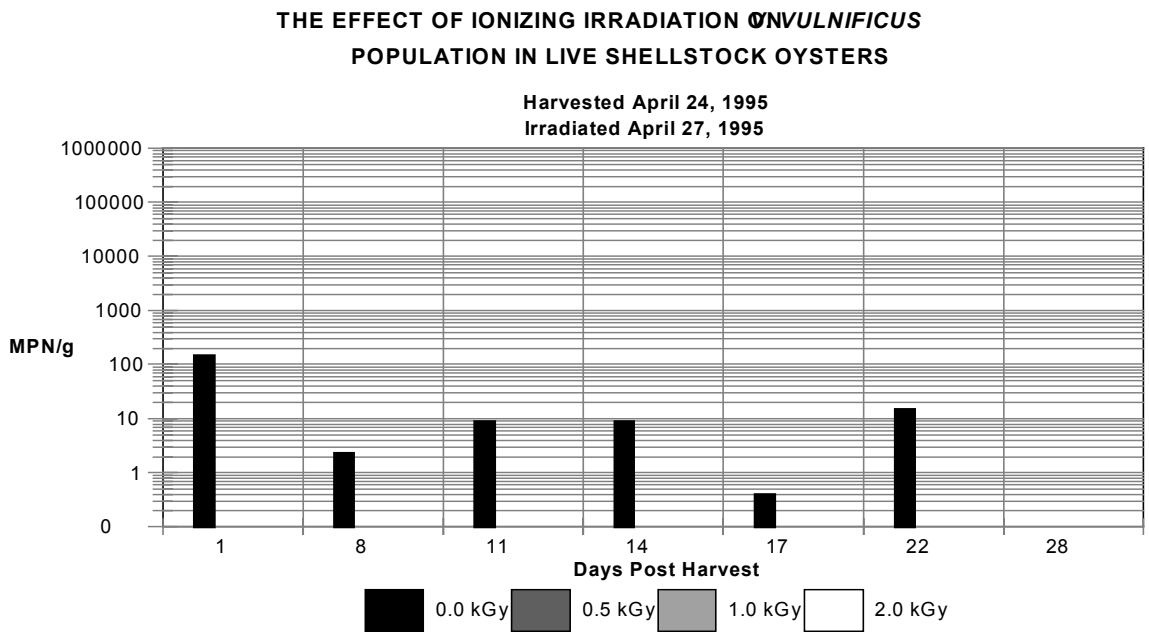

Fig. 3. Effect of radiation on *V. vulnificus* in live shellstock oysters from April 1995.

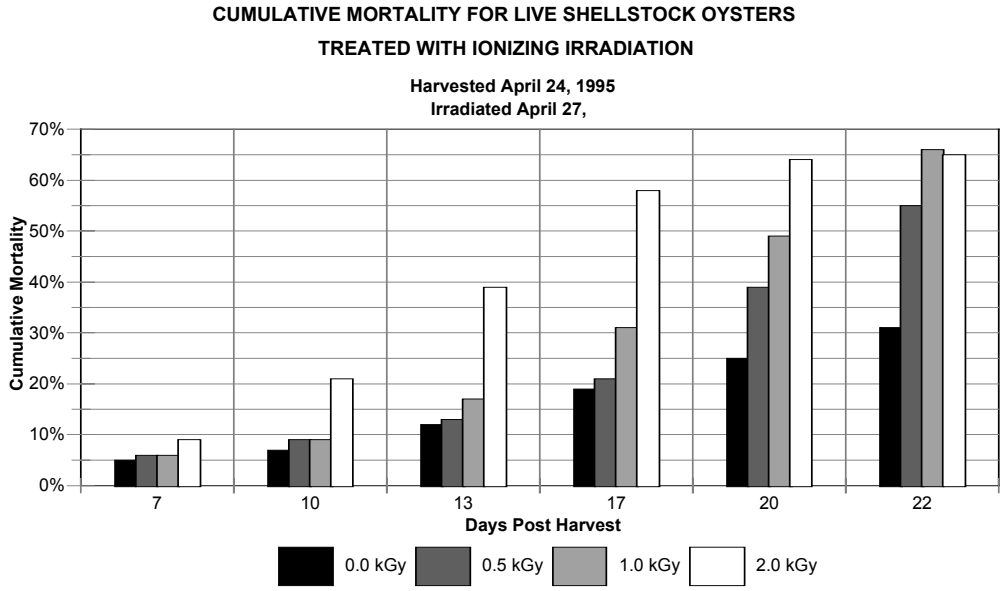

Fig. 4. Cumulative mortality for irradiated live shellstock from April 1995.

Effect of Irradiation on *V. vulnificus* Levels and Total APC/g in Fresh Shucked 8-oz (230-g) Containers from Oysters Harvested in April, 1995

Oysters commercially shucked, washed, and packed in 8-oz plastic containers were stored in ice under commercial conditions. Levels of *V. vulnificus* dropped one $\log_{10}$ cycle, from 1.5×10^2/g to 2.4×10^1/g in control oysters after shucking, washing and packing in ice. Oysters were sampled every three to five days for 28 days post-harvest.

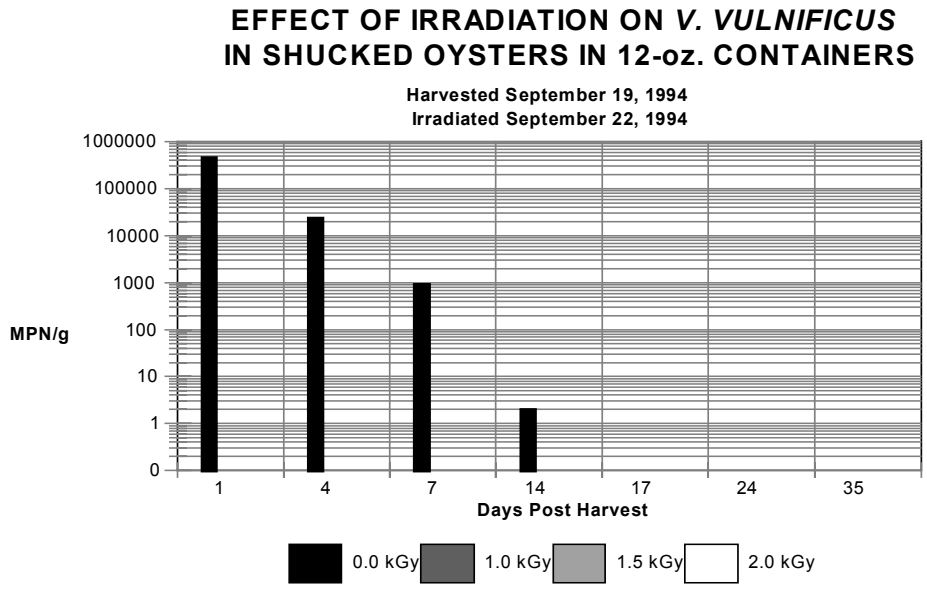

Fig. 5. Effect of ionizing radiation on *V. vulnificus* in 12-oz containers of oysters processed in September, 1994.

There were no detectable levels of *V. vulnificus* in irradiated fresh shucked oysters, even at the lowest dose of 1.0 kGy, throughout the 28-day sampling period. Even non-irradiated controls dropped from 1.5×10^2 MPN/g to a non-detectable level (<0.3 MPN/g) by 14 days post-harvest on ice in cold storage. There were no detectable *V. vulnificus* in non-irradiated controls by 17 days at 7°C (40°F) (Fig. 6). Initial levels of 1.2×10^3 APC/g in control shucked oysters increased to 7.7×10^4 APC/g at the end of 17 days of storage. Initial doses of 1.0, and 1.5 kGy reduced APC/g to 8.0×10^2, but they rose to 3.3×10^5 and 4.2×10^5 at 17 days post-harvest. As mentioned earlier, the National Shellfish Sanitation Program (NSSP) guidelines recommend 500 000 total APC/g in shucked processed oysters (FDA, 1993).

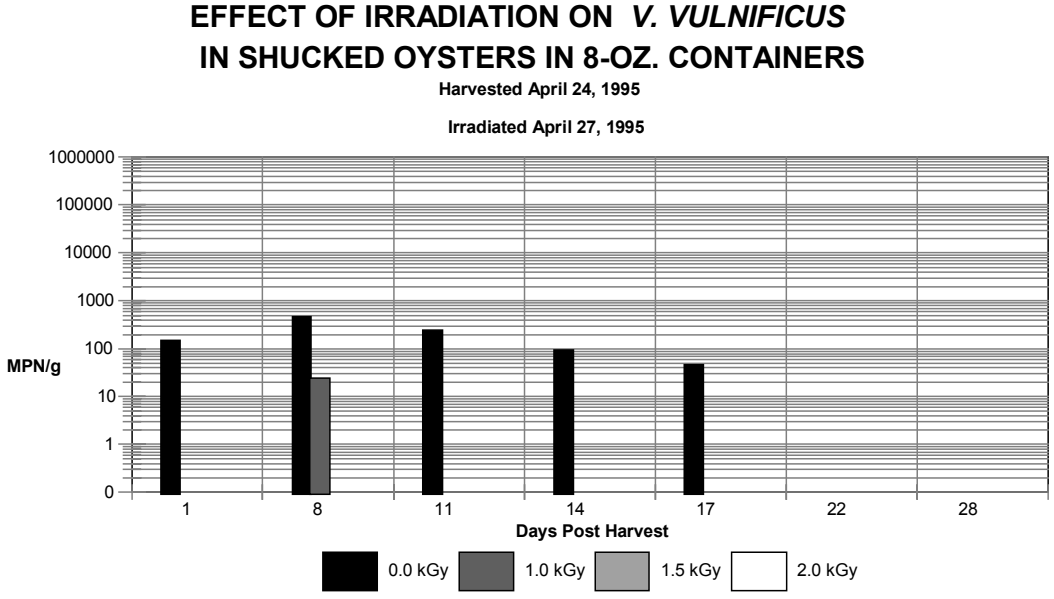

Fig. 6. Effect of irradiation on *V. vulnificus* in 8-oz oyster containers processed in April, 1995.

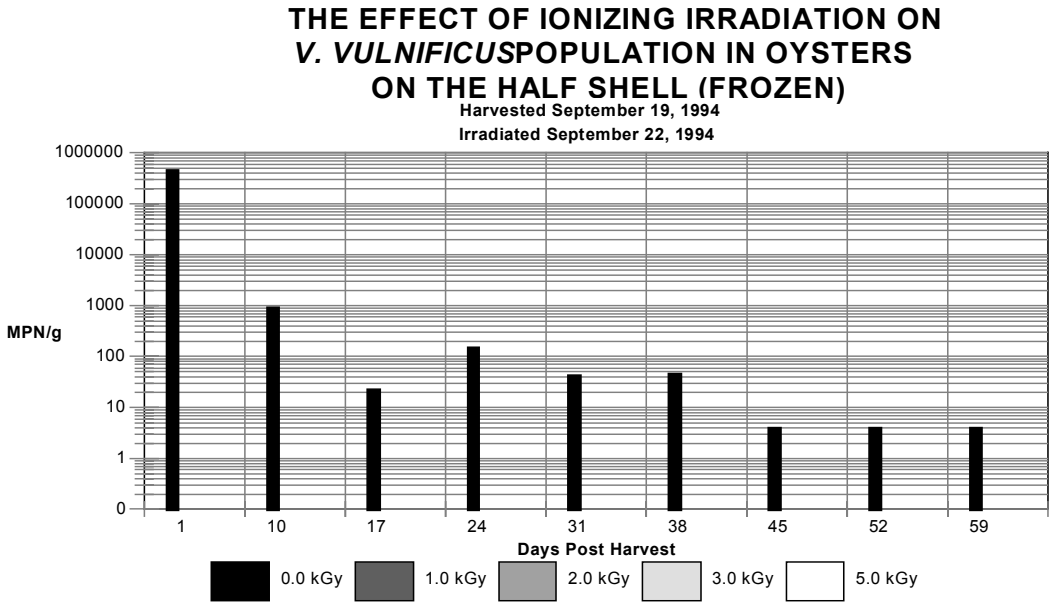

Fig.7. Effect of irradiation on *V. vulnificus* in frozen half-shell oysters from September 1994.

Half-Shell Frozen Irradiated Oysters

There were no detectable levels of *V. vulnificus* in any of the radiation-processed frozen half-shell oysters, even at the lowest dose of 1.0 kGy; non-irradiated controls dropped to <10 $\log_{10}$ MPN/g after two months (Figure 7).

CONCLUSIONS

Low-dose (0.5–1.0 kGy) irradiation is extremely effective in greatly reducing or eliminating populations of *Vibrio vulnificus* from live, fresh shucked, or frozen oysters under commercial conditions.

Higher mortalities in control live oysters harvested in April were mainly attributed to the weakened physiological state of the oysters when preparing to spawn, and to the cold storage temperature of 7°C (40°F). Storage at 10°C+ (50°F) might be less physiologically stressful to the live oyster. However, mortalities of oysters treated at 0.5 to 1.0 kGy were not significantly higher than those for the non-irradiated controls.

Frozen shrink-wrapped half-shell oysters irradiated even at the lowest dose level of 1.0 kGy have no detectable levels of *V. vulnificus*, and have practically unlimited shelf-life. Low doses of 0.5-1.0 kGy also greatly reduce total aerobic bacterial counts initially, and these counts in live shellstock remain lower than in controls throughout a 14-day cold storage period.

Overall, it is suggested that low-dose irradiation at 0.5 or 1.0 kGy would be extremely effective and commercially feasible as a post-harvest intervention method to control or eliminate *V. vulnificus,* and to lower total aerobic plate counts, in warm water live shellstock oysters without killing the oysters. Doses of 1.0 kGy or higher would also help eliminate *V. vulnificus* and possibly other potential foodborne pathogens in packaged fresh shucked and frozen oysters.

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EVALUATION OF THE NATURAL PREVALENCE OF *VIBRIO SPP.* IN URUGUAYAN MUSSELS (*MYTILUS* SP.) AND THEIR CONTROL USING IRRADIATION

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Abstract

The presence of potentially pathogenic bacteria belonging to the *Vibrionacea*, especially *Vibrio cholerae*, and of *Salmonella* spp., was examined in fresh Uruguayan mussels (*Mytilus* sp.) during two annual seasons. The radiation decimal reduction dose (D_{10}) of various toxigenic strains of *Vibrio cholerae* was determined to vary *in vitro* between 0.11 and 0.19 kGy. These results and those from the examination of natural *Vibrio* spp. contamination in mussles were used to conclude that 1.0 kGy would be enough to render Uruguayan mussels *Vibrio*-safe. Mussels irradiated in the shell at the optimal dose survived long enough to allow the eventual introduction of irradiation as an effective intervention measure without affecting local marketing practices, and making it possible to market the fresh mussels live, as required by Uruguayan legislation.

INTRODUCTION

The *Vibrionaceae* are a family of facultatively anaerobic, halophilic, Gram-negative rods, polarly flagellated, motile bacteria that comprises 28 species, of which 11 are potential human pathogens (De Paola, 1981). Most of the *Vibrio* spp. are marine microorganisms, hence their natural occurrence in many raw seafood. Among the most prevalent species of *Vibrio* is *V. parahaemolyticus*, a fast growing bacterium that resists high salt concentrations (Battisti, R. and Moretto, E., 1994). It is reported to be the main cause of gastroenteritis in Japan, where there is a large consumption of raw fish. *Vibrio vulnificus*, another pathogenic species of the *Vibrionaceae*, is a pleomorphic, short rod that requires high salt concentrations for growth. This characteristic is likely a factor in restricting the presence of this potential pathogen to seafood. In contrast, the most dreaded of all the *Vibrionaceae*, *V. cholerae*, a straight to curved, motile, short rod, the etiological agent of cholera, is transmitted most frequently through contaminated water. Its rapid growth in alkaline media (pH > 9) is frequently used as an aid to isolate it and selectively promote its growth. The enterotoxin produced by toxigenic strains of *V. cholerae* causes massive secretion of liquids towards the intestinal lumen, thus producing the often deadly diarrhea typical of the disease. Two main biotypes of *V. cholerae* have been identified: the classic type and the El Tor; the latter was the causative agent of the Lain American pandemia.

Uruguay escaped the cholera pandemia that afflicted most of South and Central America during the early 1990's. However, the location of the coastal areas of the country in which mussels are grown and captured, close to large urban centers and consequently to areas of discharge of raw sewage, present a potential risk of contamination of these mollusks with enteropathogens and with other pathogens that may be transmitted via fecal contamination, including *Vibrio cholerae* and other members of the *Vibrionaceae* (Odizzio *et al*., 1997). This potential human health hazard becomes even more likely in view of the fact that mussels in Uruguay are consumed raw by nationals and by very large numbers of tourists during the southern summer months. Besides the public health implications of a potential cholera

outbreak from mussels on the national health system, the importance of the tourist industry to the Uruguayan economy makes it imperative that the microbiological safety of mussels be studied, and that alternative decontamination intervention measures be examined (FAO/IAEA/OPS, 1992; 1994; 1997).

The present study was undertaken from March 1996 to October 1998 to fill an information void concerning the microbiological safety of Uruguayan mussels in terms of the prevalence of *Vibrio* spp. and *Salmonella* spp. A second objective was to evaluate the feasibility of using gamma radiation to reduce the microbial load of raw mussels and thus improve their hygienic quality (Fink and Rehmann, 1994). In addition, the study seeked to establish the optimal radiation dose to be applied to minimize the potential risk posed by *Vibrio* spp. and *Salmonella* spp. in these mollusks, and the survival of the mussels post-irradiation (FAO/IAEA/OPS, 1997).

MATERIALS AND METHODS

Sample Collection

Mussel samples for the study were collected directly from production areas on the coast of Montevideo, Piriápolis, Punta del Este, and La Paloma, where the largest Uruguayan mussel banks are located: the island of Lobos, Punta Ballena, and the island of Gorriti. Sample collection took place on a monthly basis during May–August, and biweekly throughout the remainder of the year. During April–December 1997 samples were also purchased from commercial outlets on the coast of Montevideo; these samples originated mostly from growing areas east of the capital city.

The samples were placed in polyethylene bags immediately after purchase, and the bags were covered with ice inside styrofoam boxes to maintain a temperature of 10–15^{o}C during transportation to the laboratory. The mussels arrived in the laboratory within 12 h of collection or purchase.

Microbiological Analyses

Once in the laboratory, the mussels were washed and cleaned using a sterile brush to remove dirt deposited on the outside of the shell, taking care not to damage the joint between the valves. The mussels were then placed on sterile trays and allowed to dry at ambient temperature before aseptically separating the valves using a sterile knife and removing the meat with tweezers and a scalpel. Composite samples weighing 25 g of meat and mussel juices were weighed and homogenized for 2 min in 225 mL alkaline peptone water using a Stomacher 400. The homogenate (10^{-1} dilution) was used to prepare serial dilutions in alkaline peptone water following standard methods.

Total mesophilic aerobes were enumerated using the pour plate method and plate count agar, and incubating the plates for 48 h at 37^{o}C (APHA, 1992; FDA, 1992). Colonies were counted and the results were recorded as colony forming units (CFU)/g. Total coliforms were used as indicators of fecal contamination because there were already limited data on the presence of this group of bacteria in raw mussels from an earlier study. Enumeration of total coliforms was by the most probable number (MPN)/g technique; the incubation temperature was 37^{o}C, and results were recorded at 24 and 48 h.

To determine the presence of members of the *Vibrionaceae* in mussels, the samples were placed in alkaline peptone water (pH 8.6) for 8 h. After this period of enrichment, homogenates and serial dilutions were prepared as before, plated on thiosulfate citrate bile salt sucrose agar (TCBS), and the plates were incubated at 37°C for 18–24 h. Colonies formed on the plates were examined for Gram stain, oxidase reaction, and oxidation/fermentation of glucose. Identification of the isolates was done using the BBL CRYSTAL system consisting of 30 biochemical and enzymatic dehydrated substrates.

Experiments were replicated three times. Bacterial counts were transformed into $\log_{10}$ for statistical analysis.

Determination of D_{10} and Irradiation of Mussels

To determine the radiation decimal reduction dose, several strains of *Vibrio cholerae* which had been tyed by the Institute of Hygiene (Instituto de Higiene) of the Universidad de la República were obtained from the National Fisheries Institute (Instituto Nacional de Pesca, I.N.A.P.E.). The culture was grown in TCBS and typical colonies were confirmed using the BBL-Crystal System. The D_{10} was determined in 24-h cultures of the typical colonies in alkaline peptone water, incubated at 37°C, and adjusted to a density equivalent to the MacFarland No. 1, which corresponds to approximately 10^8 CFU/mL. The tubes to be irradiated contained approximately 1 mL of culture in 2 mL of alkaline peptone water. For irradiation, the tubes were placed in a foam box surrounded by crushed ice. Irradiation was done in a ^{60}Co Gamma Chamber 4000A owned by the Nuclear Research Center (Centro de Investigaciones Nucleares, C.I.N.), of the Sciences School (Facultad de Ciencias), Universidad de la República, having a dose rate of 0.85 kGy/h. The radiation dose absorbed by the cultures were determined using Fricke dosimeters placed around the tubes. The dosimeters were read by the Ezeiza Atomic Center (Centro Atómico de Ezeiza, Argentina).

A set of five tubes was irradiated at each radiation dose, from 0.05 to 0.30 kGy, in increments of 0.05 kGy. Control, non-irradiated tubes were prepared that (a) remained in the laboratory to test any adverse effects due to transportation to the irradiator and back, and (b) travelled with the tubes to the irradiator and back, but were not irradiated. The time elapsed for transportation of the tubes to the irradiator was 7 min. After irradiation, the cultures were streaked on the surface of TCBS plates to determine whether or not there were surviving cells. All experiments were replicated three times.

A 40-L aquarium was used to inoculate mussels with a pure culture of *Vibrio cholerae*. The mussels were placed in the tank after filling it with salt water prepared using a commercial salt mixture to simulate Uruguayan coastal seawater. Oxigenation of the tank was accomplished using an underwater pump that also produced a circular current over the mussels. The water was inoculated by pouring into it a pure culture of the pathogen calculated to give a suspension of ca. 10^3 CFU/mL. After immersing the live mussles in the contaminated water and allowing them to feed naturally through their filter feeding system, mussels contained a load of *Vibrio cholerae* in the 10^5 CFU/g level. Mussels thus inoculated were irradiated at doses in the range 0.0 (non-irradiated controls) — 1.3 kGy to evaluate survival of *Vibrio cholerae*.

In addition to the inoculated studies, non-inoculated mussels were irradiated as before to test their survival to radiation doses necessary to eliminate up to 10^5 CFU/g *Vibrio cholerae*. This aspect was deemed essential, since Uruguayan law requires that fresh mussels must be alive during marketing (Reglamento Bromatológico Nacional, 1994). Therefore, after

irradiation, mussels (0.0–1.3 kGy) were placed in a tank containing salt water similar to the one used to inoculate them, and their viability was recorded over a period of 48 h post-irradiation.

RESULTS AND DISCUSSION

Out of 34 lots of mussels examined, 12 had total aerobic plate counts (APC) of 10^3 CFU/g, 13 had 10^4 CFU/g, and 9 had a level of 10^5 CFU/g. Fig. 1 shows the APC as a function of sampling dates, in mussels collected from the growing areas.

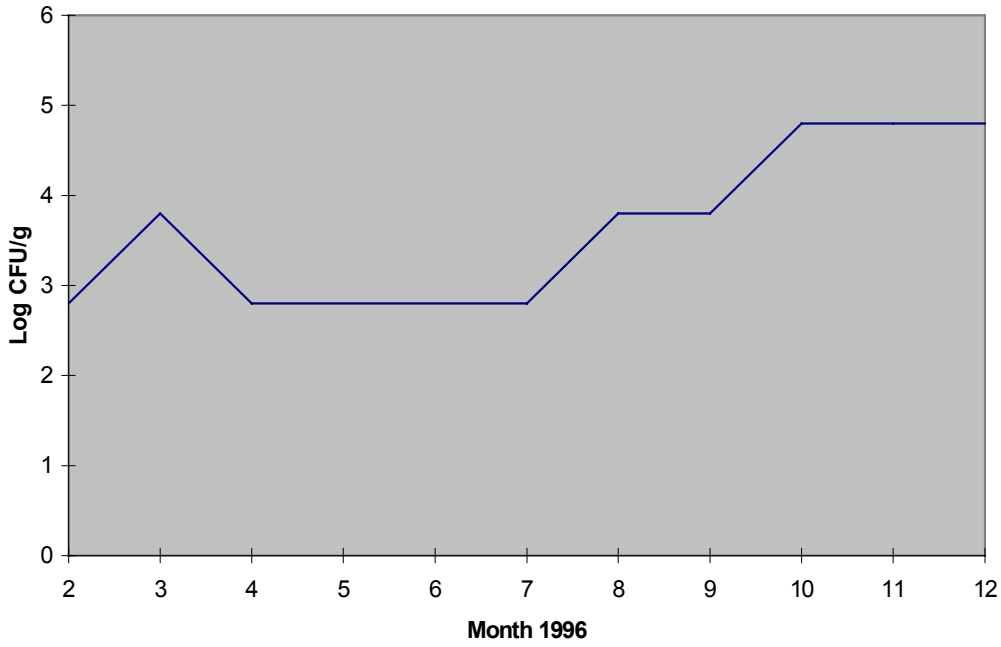

Fig.1. Mesophilic aerobic bacterial counts in fresh mussels

Plate counts for total coliforms were between <3 and 93 most probable number (MPN)/g depending on the season. As before, numbers of coliforms increased during the southern hemisphere summer months (October-December), and were consistently ca. one $\log_{10}$ cycle higher in mussels purchased in port than in those collected from the growing areas. Pathogenic microorganisms of the two groups examined, *Vibrio* spp. and *Salmonella* spp., were detected in 51% of the samples. *Vibrio* spp. were isolated from 32% and *Salmonella* from 19% of mussels. When the strains of *Vibrio* isolated from samples were subjected to primary and biochemical tests to identify them to species, 22% were identified as *Vibrio cholerae*, while the remaining 10% could only be identified as *Vibrio* spp. other than *V. cholerae*. However, 100% of the *V. cholerae* strains isolated were serologically confirmed as non-O1, suggesting that non-toxigenic strains of *V. cholerae* are part of the natural aquatic flora of the Uruguayan coastal waters. *Vibrio parahaemolyticus* was not detected in any of the samples, which is not surprising in view of the reportedly more common prevalence of this *Vibrio* in warm waters than in cold seas (Garcia Moreno and Landgraf, 1997). These results were similar for the two years studied.

Table 1: Survival of *Vibrio cholerae* Cultures Irradiated at 0.0–0.30 kGy

Radiation Dose (kGy)	No. Survivors (CFU/mL)*
0.00	1.0×10^8
0.05	1.4×10^3
0.10	2.3×10^2
0.15	0.6×10^2
0.20	0
0.25	0
0.30	0

*Means of three replications.

The results of the D_{10} value determination are shown in Table 1. A plot of the number of survivors against radiation dose produced a survival curve having a negative slope (the D_{10}) of 0.11–0.19 kGy, depending on the *Vibrio cholerae* strain tested. These results, when considered along those obtained from the examination of natural levels of *Vibrio* contaminants in the mussels (i.e. 10^3–10^5/g), indicated that a radiation dose of 1.0 kGy would be sufficient to render these mollusks *Vibrio* safe. This calculation was later confirmed by the results of the inoculated studies. Mussels artificially contaminated with *V. cholerae* to a level of 10^5 CFU/g through their natural feeding system were positive for *Vibrio* spp.after irradiation at 0.5 kGy, but had no detectable *Vibrionaceae* when the radiation dose applied was 1.0 kGy and above.

CONCLUSIONS

A total of 51% of raw mussels were found to be contaminated with one of the potentially pathogenic microorganisms examined: *Vibrio* spp. (32%) or *Salmonella* spp (19%). Among the *Vibrio* isolates, 22% were *V. cholerae*, but none were toxigenic O1 types. No *V. parahaemolyticus* were detected.

The D_{10} value calculated for *Vibrio cholerae* was between 0.11 and 0.19 kGy. Considering that the natural level of *Vibrionaceae* isolated from Uruguayan mussels over a period of three years fluctuated between 10^3 and 10^5 CFU/g, a radiation dose of 1.0 kGy would suffice to render these mollusks *Vibrio* safe.

Application of a radiation dose of 1.0 kGy allows survival of mussels for at least 48 h.

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