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

XA0100966

Z. TORRES*, B. BERNUY**, G. ZAPATA***,
M. VIVANCO****, G. KAHN**, E. GUZMAN****, R. LEON****

* Instituto Peruano de Energía Nuclear, Peru
** Universidad Nacional Agraria La Molina, Peru
*** Universidad Nacional Agraria de la Selva, Peru
**** Universidad Federico Villarreal, Peru

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°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°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°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°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°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°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°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°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.

ACKNOWLEDGEMENT

The authors wish to thank the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency (IAEA), Vienna, Austria, and the Pan American Health Organization (PAHO), Washington, DCDC for sponsoring the Co-ordinated Research Project on Irradiation as a Public Health Intervention Measure to Control Foodborne Diseases (Cysticercosis / Taeniasis and *Vibrio* Infections) in Latin America and the Caribbean, which made this study possible.

REFERENCES

AOAC. (1990).Official Methods of Analysis, 15 th Ed., Vol. 1. International Association of Official Analytical Chemists, Arlington, Virginia, p. 684.

Carvajal, C., Ayala. G.M., Sirvas, C.S. (1991). Microbiología de Alimentos Marinos, Consejo Nacional de Ciencia y Tecnología (CONCYTEC), Lima, pp. 55–57.

CDC/NCD/OPS. (1995). Métodos de laboratorio para el diagnóstico de *Vibrio cholerae*. Organización Panamericana de la Salud, Washington, DC.

FDA. (1995). Bacteriological Analytical Manual, 8th Ed. AOAC International., Gaithersburg, MD.

Figueroa, C. (1979). Efecto del pretratamiento con soluciones de tripolifosfato en filetes de merluza radurizados. Comisión Chilena de Energía Nuclear, Santiago de Chile, p. 48.

Gallardo, S.M. (1978). El contenido de bases volátiles como índice del grado de frescura en productos pesqueros. Informe Técnico del Instituto de Investigaciones Pesqueras, Lima, p. 58.

IAEA. (1992). IAEA/FAO/PAHO (WHO) Technical Consultation on Irradiation as a Public Health Intervention Measure to Control Foodborne Disease in Latin America and the Caribbean, Washington, DC, 1992. International Atomic Energy Agency, Vienna.

IAEA/FAO/PAHO-WHO. (1997). Final Report of the Second Research Co-ordination Meeting of the FAO/IAEA/PAHO (WHO) Coordinated Research Project on Use of Irradiation as a Public Health Intervention Measure to Control Foodborne Disease (Cysticercosis/Taeniasis and *Vibrio* Infections) in Latin America and the Caribbean, Tampa, Florida, February, 1997, International Atomic Energy Agency, Vienna.

Killoran, J.J. (1974). Chemistry of food packaging. Adv. Chem. Ser. 135: 87.

Maza, R.S. (1986). Manual de Procesamiento y Control de Calidad del Langostino, CATC Publicaciones, pp. 1–30.

Muñoz, B.R., Ugáztegui, A.E., Vaca, F.C. (1985). Preservación de Alimentos por Irradiación. Instituto de Ciencias Nucleares, Quito, p. 160.

OPS/OMS. (1991). Riesgo de transmisión del cólera por los alimentos. RIMSA 7/22, 10.A, Pan American Health organization, Washington, DCDC

OPS/OMS. (1996). Contaminación Microbiana de los Alimentos Vendidos en la Vía Pública. Almeida, C. (Ed.), Organización Panamericana de la Salud, Washington, DCDC, pp. 3–4.

Singson, C. (1992). Technoeconomic feasibility of food irradiation in the Philippines. IAEA STI/PUB/883, International Atomic Energy Agency, Vienna, p. 159.