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Evaluation of Total and Faecal *Coliforms* and *Salmonella* spp. in Irradiated Mussels from Brazil

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Abstract: Ilha Grande Bay is one of the biggest producers of bivalves of Rio de Janeiro State. Statistics reports of foodborne diseases are quite low in Brazil, however, this fact is a matter of Public Health. In their majority concerning consumption of bivalves meat, the availability of safe products requires the use of technology as food irradiation. The objective of this paper was to evaluate the presence of bacteria resulting from the environmental contamination and epidemiological importance, *Salmonella* spp., total and faecal *coliforms* of mussel (*Perna perna*) from that region and the use of irradiation on the product *in natura*. Fifteen indicative samples of mussel were collected from five gr owing points in Ilha Grande Bay. A sample of each point was irradiated with doses of 1.0 and 1.5 kGy. The bacteriological analysis followed the instructions of the Brazilian legislation. The samples presented irregularities in relation to *Salmonella* spp. and faecal coliforms, the latter for the control group. The control group was noticed as not appropriate for consumption. The dose of 1.0 kGy was effective for the reduction of faecal coliforms, but ineffective for the extinction of *Salmonella* spp.

Key words: Mussel, bacteriological evaluation, Ilha Grande Bay, food irradiation.

1. Introduction

The rational culture of mussels, or mitiliculture, is one of the most productive types of aquaculture known. The main reasons are: mussels' ability to filtrate, which makes the supply of supplementary feed unnecessary; high ratings of feed conversion, which results in fast growing and high productivity; low-cost installations for the culture; facility in the handling and acquirement of young mussels for culture [1].

Due to the nourishing process a great quantity of water is filtered by the gills of the mussels. These bivalve mollusks concentrate many microorganisms in their tissues and the microbiota is a result of its environment. For this reason, the bivalves are known as reservoirs of many microbial pathogens [2].

According to data from the Hospital Information System of the Health Ministry (Sistema de Informações Hospitalares do Ministério da Saúde) there were more than 3,400,000 hospitalizations of foodborne diseases in Brazil during the years of 1999 to 2004. The average was of 570 thousand occurrences per year [3].

About 90% of the reports of diseases caused by the consumption of fisheries contamination result from

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bivalve intake [4]. The consumption of bivalves is registered as responsible for countless epidemic breakouts. This responds directly to problems of Public Health, especially when these mollusks are ingested raw or undercooked, and the sanitary quality of the aquatic environment where the mollusks were captured is under bad conditions [5].

The most important enteric pathogens, from water polluted with human and/or animal residues, include *Salmonella* spp. and enterotoxic strains of *Escherichia coli* [2].

The availability of safe products requires the development of conservation technology, as mollusks, as other fishing products in general, are highly vulnerable to deterioration. The use of food irradiation has been widely known among the conservation methods available to keep the quality and increase the commercial expiry date of food. Food irradiation has the aim of eliminating pathogens, reducing, thus, the risks for Public Health.

The process of gamma radiation interferes in bacteria's cell division through the rupture of its molecular structure. The doses of gamma radiation, when properly used, do not provoke toxicological effects. The levels of energy used are not sufficient to induce radioactivity in the food [6].

The aim of this paper was to evaluate the presence of bacteria resulting from the environmental contamination and epidemiological importance, *Salmonella* spp., total and faecal coliforms of mussel (*Perna perna)* of culture in Ilha Grande Bay/RJ and the use of irradiation on the *in natura* product.

2. Materials and Methods

2.1 Materials

The samples of mussel *Perna Perna* were taken from five different strategic points of culture of bivalve mollusks in Ilha Grande Bay, Rio de Janeiro, Brazil (023°04′46″ S, 44°17′01″ W) following the methodology of sampling used by the Fishing Sector of the Economical Activities Bureau of Angra do Reis City Hall (Setor de Pesca da Secretaria de Atividades Econômicas da Prefeitura Município de Angra dos Reis). The points selected were the following beaches: Praia de Araçatiba (A), Praia de Tapera (B), Praia de Passa Terra (C), Praia de Castelhanos (D) e Praia de Maciés (E).

2.2 Methods

Each sampling done at the studied points was composed of at least 60 adult animals alive, male and female, according to the norms of APHA [7] in order to reach the volume of meat and pallial liquid needed for the analysis of bivalve mollusks. For this sampling plan, one indicative sample of each point has been considered.

The samples alive were put in identified cool boxes for transportation. These cool boxes had the same seawater from where the samples had been collected. The samples were taken to the Laboratory of Nuclear Instrumentation of the Institute Alberto Luiz Coimbra of Post-Graduation and Engineering Research of Federal University of Rio de Janeiro (Laboratório de Instrumentação Nuclear do Instituto Alberto Luiz Coimbra de Pós-Graduação e Pesquisa de Engenharia da Universidade Federal do Rio de Janeiro-LIN-COPPE/UFRJ) for the irradiation procedure.

The samples arrived alive and in good conditions at the laboratory. From each point of collection, at least 20 specimen of mussel *Perna Perna* were randomly taken for each sample: control, irradiated at 1.0 kGy and irradiated at 1.5 kGy. The samples were put in previously identified polyethylene bags.

For the irradiation of the samples, the material used was a MDS Nordion, model Gammacell/220 Excel radiator, which uses a source of gamma radiation of cobalt 60. The tax of dose from the source during the day of the irradiation was 31.56 Gy/min.

During the process of irradiation, the samples were put in an expanded polystyrene box containing ice bags and keeping a mild temperature. Immediately

after the radiation the samples were taken to the Laboratory of Microbiological Control of Animal Origin Products, Department of Food Technology, Faculty of Veterinary Medicine, Federal Fluminense University (Laboratório de Controle Microbiológico de Produtos de Origem Animal, Departamento de Tecnologia de Alimentos, Faculdade de Veterinária, Universidade Federal Fluminense/UFF), in Niterói, Rio de Janeiro, Brazil for the analysis.

The laboratory analyses were done in an aseptic manner, according to the methodology of IN nº 62 [8].

For the research of Salmonella spp., the selective enrichment was done though the use of Caldo Tetrationato (CT-Tetrationato Broth) (OXOID® CM029) and of Caldo Rappaport Vassiliadis (CRV - Rappaport Vassiliadis Broth) (HIMEDIA® M880-500G). For confirmation by serology, it was used the Salmonella polyvalent serum (BIORED ®) which contains antibodies against the antigens of Salmonella groups A, B, C, D and E.

For the enumeration of total and faecal coliforms was used the quick method technique with broth Rapid Hi-Coliform (HiMed ® M1453-500G) (modification of the LMX broth). The dilution procedure was also used for counting of staphylococci following miniaturization technique [9].

2.3 Data Analysis

The data were statistically described through parametric means (average and standard deviations), tabular (statistical Tables) and graphs (sector graphs and box plots).

Comparisons between binary proportions were done through binomial test.

Comparisons between the average values of the samples and the standard values of legislation were done with the Student test t.

The analysis of variance (ANOVA) investigated the differences between the averages of three of the sample groups, complemented, for multiple comparisons between the groups, by the Turkey test.

The analysis of data was done with the help of computational resources: Microsoft Excel spreadsheet and SPSS v.10.0 program.

The statistical decisions were taken at the significance level $\alpha = 0.05$.

3. Results and Discussion

Ilha Grande Bay, due to its geographical characteristics, has a system of basic sanitation that is not satisfactory. Also, the area is highly explored for tourism and some of its beaches have high population density. Considering that some areas of collection are populated and the lack of basic sanitation and the mortality of mollusks of the last years, these animals may be high important for Public Health. The biota of fish is the result of their environment and especially in the case of bivalve mollusks that are filtering organisms [2, 10-13]. Some authors warn for the danger of consuming bivalve mollusks due to both the sanitation quality of the environment of the latter and the way they are ingested, generally raw or undercooked [5, 14-17], which was observed in the places of collection.

The presence of *Salmonella* spp. was identified in 21 colonies. The proportion of the presence of colonies positive for salmonela in the sample groups obeyed the following order: 100% of the samples of the control group and 40% of the samples in each of the two radiated groups (1.0 kGy e 1.5 kGy). In the control group, all of the collection points were positive for salmonela, while in the radiated group with 1.0 kGy only the points A and B were positive and, in the group radiated with 1.5 kGy, the groups B and D.

The pattern of Brazilian legislation [18] is the absence of salmonela in samples of 25 g. This way, there is no evidence of expressive differences between the proportions observed in the samples at 1.0kGy and at 1.5 kGy with the pattern (binomial test; $P > 0.05$). The control group, however, presented, with statistical significance ($P < 0.05$), a proportion superior to the

pattern legislation: binomial test; *P*-value = 0.031.

Riedel [19] reported that, despite the fact that the *Salmonella* (*S. typhi* and *S. paratyphi*) is of human and animal origin, it is frequently found in polluted waters and concentrated with oysters, mussels and shellfishes, that can be observed in this paper. In spite of it, the microorganisms worldly researched as indicative of environmental pollution in bivalve mollusks are total and faecal coliforms.

Jay [11] reported that the analysis of shellfishes of the coast of Florida presented *Salmonella* spp. in 43% of the samples. Mohamed Hatha and Lakshmanaperumalsamy [20] analyzed 730 samples of fish and 276 samples of crustacean in fish markets of south of India and *Salmonella* spp. were found in 14.25% of the samples of fish and 17.39% of the samples of crustaceans. Gil [21] analyzed samples of *sururu* (Mitilidae) and cockle in relation to the presence of *Salmonella* spp., bacterial incidence of 10% in sururu and 20% in cockle were found. Vieira [22] indicated that in the United States, in an analysis of the sample of fish eaten raw, the tax of prevalence was of 1% for oysters, 3.4% for other mollusks and 12.2% for raw fish. Farias [23] analyzed samples of oyster (*Crassostrea* spp.) of the coast of the state of Parana (Brazil) and no presence of *Salmonella* spp. was identified; the same happened with Damasceno [24], researching *Salmonella* spp. in gutted and cold salmon of retail establishments in the city of Belo Horizonte (Brazil). These results are highly inferior to this study, which occurred in presence of 100% of the non-irradiated samples.

Jay [11] indicated as D10 values of radiation for *Salmonella* spp. 0.13 kGy e 0.800 kGy, similar to Molins [2], who reported that the dose D10 for *Salmonella* spp. varies, according to the type of food and storage temperature, between 0.416 e 0.57 kGy, while Massaguer [25] indicated a higher dose of 4.75 kGy to reduce seven logarithmic cycles of *Salmonella* spp. in chicken. Molins [2] affirms that the dose for the extinction of *Salmonella* spp. in frozen fish is of 4.0 to 5.0 kGy and the dose for *Salmonella enteritidis* for live oysters is of 2.5 kGy. Corroborating the data, as the determination is qualitative (presence) of *Salmonella* the dose of 1.0 kGy and 1.5 kGy of mussel *in natura* used in this paper was not enough for the extinction of the bacteria.

The results of the total coliforms varied in control group from 90 to 930 NMP/g. In the point B (Tapera) of collection, no positive result was found. However, in the group irradiated with 1.0 kGy, the values were between 90 e 230 NMP/g. In the points D (Castelhanos) and E (Maciés), though, these microorganisms were not enumerated. Meanwhile, the group with 1.5 kGy showed positive results for the presence of *Salmonella* spp. Enumeration (230 NMP/g) occurred only in the points C (Passa Terra) and D (Castelhanos), as the other points did not grow up.

The average of the groups was: 336 NMP/g for control; 110 NMP/g for 1.0 kGy and 92 NMP/g for 1.5 kGy. In Fig. 1, the distribution of data according to the groups was observed through the use of a box plot.

Comparing the average per group using the analysis of variance (ANOVA), it was possible to notice the inexistence of differences that were statistically significative ($P > 0.05$) between the levels of radiation considered. A summary of the findings obtained through ANOVA is expressed in Table 1.

There is no specific pattern for the NMP of the total coliforms in Brazilian legislation. However, the presence of this microbiota is indicative of environmental contamination. According to *Codex Alimentarius* [26], the water for culture and/or the bivalve meat must be monitored in order to identify the presence of *E. coli*/faecal coliforms or total coliforms, since they are important bacteria to indicate the level of faecal contamination. The level found in the mussels analyzed in this paper can be considered low.

The results obtained through the NMP of the faecal coliforms for the control group varied from 40 to 230

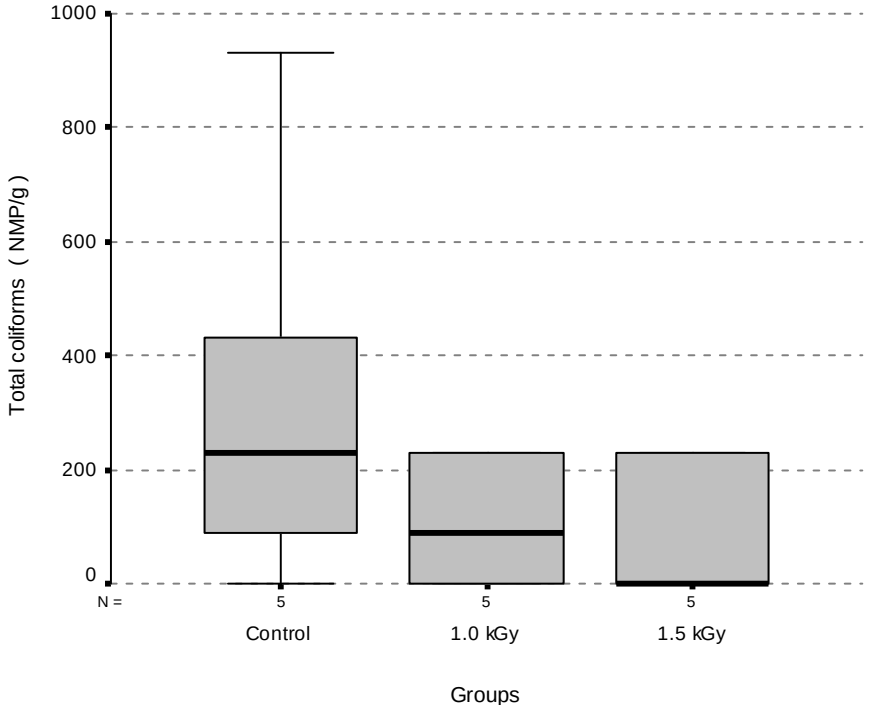

Fig. 1 Distribution of the count of total coliforms per groups of analysis.

Table 1 Description of the analysis of variance (ANOVA) between the groups for the results of NMP of total coliforms.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F_{obs}	*P*-value
Radiation levels	184,893.3	2	92,446.7	2.169	0.177
Points of sampling	322,226.7	4	80,556.7		
Residuals	340,973.3	8	42,621.7		
Total	848,093.3	14			

NMP/g of mussel. In the points B (Tapera) and E (Maciés), there was not bacterial growth, which resulted in an average of 72 NMP/g for the group. However, the group irradiated with 1.0 kGy did not present any growth in the five collection points. Thus, the average was equal to 0.0 NMP/g. In the group 1.5 kGy, a value a little bit above previous the average was found (average: 8 NMP/g), as there was a growth (40 NMP/g) in the collection point D (Castelhanos) (Fig. 2).

In the Brazilian legislation of microbiological pattern [18], the pattern adopted for the bivalve mollusks was of 50 NMP/g for faecal coliforms. The average of the control group was, thus, out of the pattern established in the legislation.

FAO [27] determines 10^1 to 10^3 as the minimum dose of infection for *E. coli*. According to *Codex Alimentarius* [28], live or raw bivalve mollusks have an international microbiological pattern of less than 230 NMP/g for *E. coli.* Considering the value following the international pattern, the averages of the groups found in this paper are within the pattern. The control group, though, presented a result in the limit (230 NMP/g) of the international pattern in the analysis of point C (Passa Terra). As the ICMSF [29] has a stricter pattern, of no more than 16 NMP/g de *E. coli*, according to this pattern, the irradiated groups would be within the limit permitted and the control group would be out of the limit.

According to Forsythe [30], the infectious dose estimated to *E. coli* is of 10^6 to 10^7 UFC/g, which is highly superior to the one found in this paper.

Gil [21] analyzed samples of sururu (Mitilidae) and cockle and determined the quantity of total and faecal

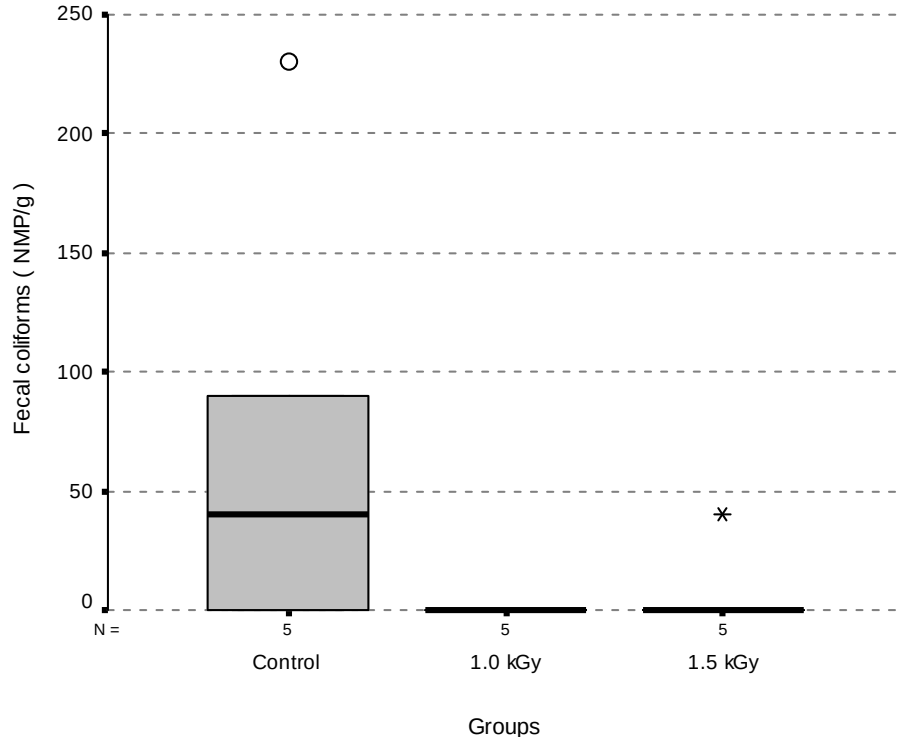

Fig. 2 Distribution of the count of faecal coliforms per group of analysis.

coliforms in the samples. For the sururu, the results were of no more than 3.0×10^7 NMP/g for the total coliforms, and of at least 7.0×10^4 NMP/g; 80% of the samples were out of the pattern of the legislation for faecal coliforms and presented high levels of faecal contamination. For the samples of cockle, the results were even higher: maximum of 5.0×10^8 for the total coliform and $> 6.5 \times 10^6$ for faecal coliforms. These values are superior to the ones found in this paper for both total and faecal coliforms.

Farias [23] researched the presence of total and faecal coliforms in oyster samples (*Crassostrea* spp.) collected in different points of the coast of Paraná (Brazil), especially in Guaratuba Bay. The presence of total coliforms was detected in all the points, with a percentage per sample that varied from 100% to 25% among the points, while just in half of the points the *E. coli* was identified with the percentage per sample that varied between 67% and 33%. This paper did not identify total or faecal coliforms in all the samples.

Forcelini [31] analyzed oyster samples in relation to the quantity of *E. coli* in different points, and obtained maximum values between 1176.276 NMP/g in the internal point and 413.576 NMP/g in the external point of Guaratuba Bay (Paraná, Brazil). These values are highly superior to the ones found in this paper. Morelli [32], on the other hand, analyzed oyster samples from two informal commercial establishments in the beach Praia do Futuro (Fortaleza, Brazil). These samples were analyzed in relation to the quantification of faecal coliforms the values found were between < 3 and > 110.000 NMP/g. The result found in the latter research is more similar to the minimum values found here.

In relation to the average presented, the control group is the only one that does not differ from the pattern established (t = 0.514; g.l. = 4; *P*-value = 0.635), being considered out of the acceptable limit. The other groups are expressively under the pattern established, being suitable for human consumption (group 1.0 kGy: t cannot be calculated for there is no variation in the values observed; and group 1.5 kGy: t = -5.250; g.l. = *P*-value = 0.006).

Table 2 Description of the analysis of the variance (ANOVA) between the groups for the results of NMP of total coliforms.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F_{obs}	*P*-value
Radiation levels	15,573.3	2	7,786.7	2.381	0.154
Points of sampling	11,800	4	2950		
Residuals	26,160	8	3270		
Total	53,533.3	14			

Despite being unable to be calculated in the group 1.0 kGy, the behavior of statistics t in the surroundings of the value 0.0 with variability tending to zero, indicates a highly expressive difference in relation to the value established as a pattern. Then, the treatment in 1.0 kGy expressively reduces the faecal coliforms.

By comparing the averages of the groups with restriction per point of sampling through the analysis of variance (ANOVA), a lack of a difference statistically significant ($P > 0.05$) between the groups was indicated. Table 2 presents a summary of the findings obtained through ANOVA.

According to Jay [11], the dose D_{10} for the elimination of *E. coli* and *E. coli* O157:H7 is of 0.20 kGy and of 0.307 kGy, respectively. The doses used in for this paper (1.0 e 1.5 kGy) were able to significantly reduce the NMP/g of *E. coli*.

Molins [2] indicated a dose of 1.5 kGy for the extinction of *E. Coli* in oysters (*Crassotrea virginica*) and of inferior to 2.0 kGy for shrimp, by using dry sepia, this value raises to 3.0 kGy. In this paper, the dose of 1.0 kGy had the same effect in mussels *in natura.*

Similarly to the results obtained, Marins [33] analyzing the effects of ionizing radiation in frog meat, observed a reduction in the average of the results of *E. coli* converted in logarithms with the increase of the dose of irradiation. The averages were 1.28 log NMP/g for the control group (0 kGY); 1.11 log NMP/g for the group irradiated with 2 kGY; 0.92 log NMP/g for the group irradiated with 5 kGy and 0.36 log NMP/g for 7 kGy. Valente [34] irradiating samples of mussel with 3, 5 e 7 kGy found significative statistical differences between the control group and the irradiated groups in the result of NMP of *E. coli*. The averages of NMP/g for mussel were 0.35 log NMP/g for the control group; 0.06 log NMP/g for 3 kGy; 0.04 log NMP/g for 5 kGy e 0 log NMP/g for7 kGy.

Siqueira [35], on the other hand, irradiating tilapia, concluded that the dose of 2.2 kGy was not effective to reduce the bacterial load for faecal coliforms.

4. Conclusion

The presence of *Salmonella* spp. was identified in the whole groups, and the doses of 1.0 kGy and 1.5 kGy were not effective to eliminate the *Salmonella* spp. The presence of total coliforms was relatively small. The group that presented the biggest enumeration was the control group. For the faecal coliforms, the control group presented itself out of the legislation and the doses used for the reduction of faecal coliforms were effective. The group radiated at 1.0 kGy was the one which presented the best bacteriological results. However, the doses used were not sufficient to make the food safe for consumption.

For this reason, the consumption of mussel *in natura* or undercooked from the region analyzed can be risky for Public Health, due to the presence of *Salmonella* spp. and the enumeration of faecal coliforms out of the safe limit.

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