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Research Paper

Elimination of coliforms and *Salmonella* spp. in sheep meat by gamma irradiation treatment

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Abstract

This study aimed at evaluating the bacteriological effects of the treatment of sheep meat contaminated with total coliforms, coliforms at 45 °C and *Salmonella* spp. by using irradiation at doses of 3 kGy and 5 kGy. Thirty sheep meat samples were collected from animals located in Rio de Janeiro State, Brazil, and then grouped in three lots including 10 samples: non-irradiated (control); irradiated with 3 kGy; and irradiated with 5 kGy. Exposure to gamma radiation in a ^{137}Cs source-driven irradiating facility was perfomed at the Nuclear Defense Section of the Brazilian Army Technological Center (CTEx) in Rio de Janeiro. The samples were kept under freezing temperature (-18 °C) until the analyses, which occurred in two and four months after irradiation. The results were interpreted by comparison with the standards of the current legislation and demonstrated that non-irradiated samples were outside the parameters established by law for all groups of bacteria studied. Gamma irradiation was effective in inactivating those microorganisms at both doses tested and the optimal dose was achieved at 3 kGy. The results have shown not only the need for sanitary conditions improvements in slaughter and processing of sheep meat but also the irradiation effectiveness to eliminate coliform bacteria and *Salmonella* spp.

Key words: food irradiation, microbiology, sheep meat quality.

Introduction

The sheep industry is expanding as a result of changes in dietary habits of consumers, who have been seeking for products with higher quality, flavor, tenderness and lower fat contents (Neres *et al.*, 2001). Such factors justify the need for more researches investigating new conservation methods, since foods rich in protein and fatty acids, such as meats and its derivatives, are more susceptible to quality loss during storage (Leonel, 2008).

Food preservation methods have been improving together with sciences advances. Among the alternative technologies that are being adopted for food treatment

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worldwide, irradiation should be highlighted. Researches show that this conservation method could apply to both industry and consumers interests (Silva, 2008).

Data about the gamma radiation effects on microorganisms found in sheep meat are still limited, especially those who present standards set by a Board Resolution called "Resolução da Diretoria Colegiada" (RDC) N°12, dated 02/01/2001 (Brasil, 2001). This study aimed at evaluating the bacteriological effects of irradiation at gamma doses of 3 kGy and 5 kGy in the treatment of sheep meat contaminated with coliform bacteria and *Salmonella* spp. Its findings might help the control and prevention of foodborne diseases.

Material and Methods

Sampling

Thirty sheep meat samples (250 g each) were randomly collected from animals located in properties in Rio de Janeiro State, Brazil. The samples were removed immediately after slaughter and chilling of carcasses, individually vacuum packed and identified according to the treatment to be applied as follows: control (non-irradiated), irradiated with 3 kGy and irradiated with 5 kGy. All samples were then transported under refrigeration to the Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF), where they were kept at freezing temperature (-18 °C) until irradiation.

Irradiation and storage of frozen meat

The samples exposure to gamma radiation from a ^{137}Cs source was performed in a research irradiator at the Nuclear Defense Section of the Army's Technological Center in Guaratiba - RJ. The ^{137}Cs source had 47 kCi of activity and the average dose rate of 1.3 kGy/h, where the irradiation of the samples lasted 2.3 and 3.8 h at doses of 3 kGy and 5 kGy, respectively. Transportation to CTEx and return to UENF were made under refrigeration.

The control and irradiated samples were kept at -18 °C at UENF. Prior to analyses, they were subjected to slow thawing at a temperature of 4 °C in refrigerator for 12 h. Two and four months after irradiation, five samples from each treatment were analyzed at a time. The analyses followed the methodology described by Silva *et al.* (2001) and the results were interpreted by comparison with the standards of the RDC N°12, dated 02/01/2001 (Brasil, 2001).

Preparation of dilutions and culture media

For the dilutions preparation, the vacuum packages were aseptically opened. Each analytical unit had 25 g, which was aseptically removed from different points of each sample and added to 225 mL of sterile 0.1% peptone water for future analyses of total coliforms and coliforms at 45 °C. For the *Salmonella* spp. analyses, another 25 g analytical unit of each sample was removed by using the same procedure and added to 225 mL of Lactose Broth (pre-enrichment broth). The homogenization of the analytical unit and the diluent was performed in a *Stomacher* (60"/230 rpm) (Seward®), where the initial dilution was obtained (10^{-1}). For the preparation of the second dilution (10^{-2}), 1.0 mL of previous dilution (10^{-1}) was aseptically transferred into a tube containing 9 mL of the same diluent. The process was repeated until the 10^{-3} dilution was obtained. For the *Salmonella* spp. analyses, only a 10^{-1} dilution was prepared. The preparation of culture media followed the manufacturer recommendations.

MPN/g determination of total coliforms

An inoculation of 1.0 mL of the dilutions 10^{-1}, 10^{-2} and 10^{-3} was performed in three sets of three tubes containing 8.0 mL of Lauryl Sulfate Tryptose Broth (LST) which were incubated at 35 °C for 48 h. After this time, the tubes that showed turbidity and gas production (positive) in the interior of the Durham tubes were transferred to the analyses of total coliforms and coliforms at 45 °C. For total coliforms confirmation, a aliquot was transferred to tubes containing 8.0 mL of Brilliant Green Bile Broth (BG), which were then incubated at 35 °C for 24-48 h. Positive results were interpreted in the appropriate table of MPN considering the inoculated dilutions and then the MPN/g was calculated.

MPN/g determination of coliforms at 45 °C

An aliquot was transferred from LST Broth tubes with positive results to the corresponding tubes containing 8.0 mL of *Escherichia coli* Broth (EC). Incubation was performed in water bath (Tecnal®) at 45.5 °C for 24 h. The number of tubes with gas production in Durham tubes (positives) was recorded and compared with an appropriate MPN table of inoculated dilutions.

Salmonella spp. analysis

After Lactose Broth incubation at 35 °C for 20 h, 1.0 mL was transferred from this broth into two tubes (previously boiled for 10 min with the following media): one containing 10 mL of Tetrathionate Broth (TT), and another containing 10 mL of Selenite Cystine Broth (SC). By using the TT Broth, 0.2 mL of iodine solution was added to each tube. Both broths were incubated at 35 °C for 24 h. Differential plating was performed by shaking the tubes in a shaker vortex (Quimis®) and then by sowing using exhaustion from an aliquot of TT Broth on Hektoen Enteric Agar plates (HE) and Xylose Lysine Deoxycholate Agar (XLD) to obtain pure colonies. The same procedure was repeated with the SC Broth in the same media previously cited. The plates were inverted and incubated at 35 °C for 24 h in an incubator.

The api® 20 E test (bioMérieux®) was performed. Strains with *Salmonella* spp. biochemical characteristics were sent for serotyping confirmation to Laboratório de

Enterobacterias / Instituto Oswaldo Cruz / FIOCRUZ, Rio de Janeiro. Their antigenic characteristics were analyzed with somatic and flagellar antisera according Grimont and Weill (2007).

Statistical analysis

Statistical tests included variance analysis (ANOVA) with a 5% significance level and mean comparisons according to Tukey's test (SAS, 1999).

Results and Discussion

MPN/g determination of total coliforms and coliforms at 45 °C

The results for the total coliforms analyses of sheep meat performed after two and four months of storage counted in the control (non-irradiated) and in samples treated with 3 and 5 kGy are shown in Table 1.

RDC N°12 does not establish microbiological standards for total coliforms in any food category. However, in the present investigation, it was observed that all non-irradiated sheep meat samples were contaminated by total coliforms, detected as colony growth and gas production in the culture media, both in two and four months. It was also observed that there was no significant change in counts for this microorganism group between the two studied periods ($p > 0.05$).

No growth and/or gas production by total coliforms were observed in the samples irradiated with 3 and 5 kGy doses on both dates (Table 1), evidencing the effectiveness of irradiation for the control of total coliforms in sheep meat. Because it is more economical, faster to apply and has lower chances to cause sensory changes in the meat, the dose of 3 kGy should be preferred.

The presence of high concentrations of coliforms in foods is indicative of failures during processing, heat treatment or inadequate hygiene (Pardi *et al.*, 2001). However within the vast group of total coliforms, which includes about 20 species, not only bacteria from the gastrointestinal tract of humans and warm-blooded animals are present, but also non-enteric species like *Serratia* and *Aeromonas* genera. Thus, their presence in foods is less representative as an indicator of fecal contamination than coliforms at 45 °C or *E. coli* (Silva *et al.*, 2001).

The results from the analyses of coliforms at 45 °C in the non-irradiated samples of sheep meat and samples treated with doses of 3 and 5 kGy are shown in Table 2.

According to RDC No. 12, in a group analysis of five not matured vacuum packed meat samples, only two may present coliforms at 45 °C counting between 10^3 and 10^4 to be considered acceptable. In this study, of five non-irradiated samples, evaluated in two months, three had scores within this range, whereas in four months, four samples were between these values (Table 2). Therefore, these results demonstrate that the non-irradiated samples were outside the parameters established by current legislation and inadequate for human consumption from a microbiological standpoint. There was no significant difference in the microorganisms counting from this group between the two periods studied ($p > 0.05$).

Table 1 - Most Probable Number per gram (MPN/g) of total coliforms in non-irradiated (control group), and irradiated sheep meat with doses of 3 and 5 kGy groups.

	Analysis			
	2 months		4 months	
	Samples	Total coliforms (MPN/g)	Samples	Total coliforms (MPN/g)
Control group	1	2.4×10^3	6	2.4×10^3
	2	2.4×10^3	7	1.1×10^3
	3	2.4×10^3	8	2.4×10^3
	4	2.1×10^2	9	2.4×10^3
	5	2.1×10^2	10	2.4×10^3
Irradiated at 3 kGy	1	-	6	-
	2	-	7	-
	3	-	8	-
	4	-	9	-
	5	-	10	-
Irradiated at 5 kGy	1	-	6	-
	2	-	7	-
	3	-	8	-
	4	-	9	-
	5	-	10	-

Table 2 - Most Probable Number per gram (MPN/g) of coliforms at 45°C in non-irradiated (control group), and irradiated sheep meat with doses of 3 and 5 kGy groups.

	Analysis			
	2 months		4 months	
	Samples	Coliforms at 45 °C (MPN/g)	Samples	Coliforms at 45 °C (MPN/g)
Control group	1	2.4×10^3	6	2.4×10^3
	2	2.4×10^3	7	93
	3	2.4×10^3	8	2.4×10^3
	4	7	9	2.4×10^3
	5	28	10	2.4×10^3
Irradiated at 3 kGy	1	-	6	-
	2	-	7	-
	3	-	8	-
	4	-	9	-
	5	-	10	-
Irradiated at 5 kGy	1	-	6	-
	2	-	7	-
	3	-	8	-
	4	-	9	-
	5	-	10	-

In contrast, there was no growth or gas production of coliforms at 45 °C in samples irradiated with 3 kGy and 5 kGy in both analyzed time intervals, thus, the effectiveness of the treatment of sheep meat by exposure to gamma radiation for the control of such microorganisms has been clearly observed.

The presence of coliforms at 45 °C in foods suggests the presence of fecal contamination, since there is a high incidence of *E. coli* in this group, which has as its primary habitat humans and animals intestines. However, coliforms at 45 °C also include some strains that can be found in other habitats (plants and soil) like *Enterobacter* and *Klebsiella*. For that reason, the direct correlation between this group and the one originating from fecal contamination should be considered cautiously (Silva *et al.*, 2006).

Information about the effect of gamma radiation on microbial population as well as shelf life extension of sheep meat are still limited, especially by taking into account microorganisms that have standards established by the RDC N°12. Therefore, for comparison, the results of this study are consistent with those reported for other species.

Sedeh *et al.* (2007) conducted a survey in which beef samples were irradiated with doses of 0.5; 1.0; 2.0 and 3.0 kGy and then kept under refrigeration (4-7 °C) for three weeks and then frozen (-18 °C) for 8 months. Microbiological analyses showed that the number of coliforms decreased with increasing doses for both refrigerated and frozen samples, but this effect was more highlighted in those that were kept frozen. It was also observed that the optimal dose found was 3 kGy, *i.e.* equal to the lower dose tested in this study.

Oliveira *et al.* (2009) tested the effect of irradiation (1.5 and 3.0 kGy) and two package types (conventional or vacuum ones) in MPN/g determination of total coliforms and coliforms at 45 °C in chicken meat chilled for 30 days. Irradiation at both doses was found to be efficient at both doses in reducing microbial population, both in the group of total coliforms and coliforms at 45 °C, although no significant differences were found among the treatments of thermo tolerant coliforms group. The survey also found that there was no effect between the irradiation dose and the package used. Mantilla *et al.* (2010) also tested the effect of irradiation with doses of 3 kGy and a modified atmosphere (80% CO_2 / 20% N_2) on the growth of coliforms bacteria in chicken meat. It was observed that total coliforms only developed in samples packed in air and in non-irradiated and non-modified atmosphere. Coliforms at 45 °C, however, only grew in the non-irradiated air packages, which showed that, regardless of the atmosphere used, there was no growth of coliforms in irradiated samples.

In tests with camel meat samples, Al-Bachir and Zeinou (2009) showed that irradiation extended the shelf life of the samples from less than two weeks (in non-irradiated ones) to over six weeks (in those irradiated with doses of 2, 4 or 6 kGy) by taking into account the group of total coliforms.

Byun *et al.* (2001) reported that the washing process and irradiation with doses of 3 kGy eliminated coliforms

bacteria in natural sausages casings made from lamb and pigs intestines. In addition a 5 kGy dose sufficed to destroy such microorganisms without the need of the washing procedure.

Kanatt *et al.* (2010) used a variety of meat products ready for preparation and available in Indian grocery stores such as mutton and chicken bites, and reported that the samples irradiated with 2.5 kGy had their shelf life significantly extended and that coliforms at 45°C were destroyed by the irradiation treatment.

Yildirim *et al.* (2005) tested the effect of irradiation (2.4 and 7 kGy) in a group of coliforms bacteria present in a traditional Turkish dish (Cig Köfte), commonly consumed as an appetizer, having raw beef or lamb meat in its composition. Their study showed that doses of 2 kGy were sufficient to eliminate these microorganisms in that type of food.

Salmonella spp. analyses

The results obtained for *Salmonella* spp. analyses of sheep meat performed after two and four months of storages both for control samples and samples irradiated with doses of 3 and 5 kGy, are described in Table 3.

The analyses performed after two months of storage both for control and samples irradiated with doses of 3 and 5 kGy showed that there were no contamination by *Salmonella* spp. Thus, all samples met the standards established by the RDC N° 12, which determines the absence of this microorganism in a 25 g sample. However, in the analyses performed after four months of storage, in spite of the absence of *Salmonella* spp. in irradiated samples, both *Salmonella enterica* subsp. *houtenae* and *Salmonella* ser. Albany were isolated in a non-irradiated sample.

Table 3 - Presence of *Salmonella* spp. in sheep meat in non-irradiated (control group), and irradiated sheep meat with doses of 3 and 5 kGy groups.

	Analysis			
	2 months		4 months	
	Samples	*Salmonella* spp.	Samples	*Salmonella* spp.
Control group	1	-	6	-
	2	-	7	-
	3	-	8	-
	4	-	9	Present
	5	-	10	-
Irradiated at 3 kGy	1	-	6	-
	2	-	7	-
	3	-	8	-
	4	-	9	-
	5	-	10	-
Irradiated at 5 kGy	1	-	6	-
	2	-	7	-
	3	-	8	-
	4	-	9	-
	5	-	10	-

Salmonella spp. could cause serious food poisoning. Its presence in non-irradiated samples indicates the need of control measures and food security guarantee.

Jakabi *et al.* (1999) and Jones *et al.* (2000) stated that *S. enterica* subsp. *houtenae* has cold-blooded animals and the environment as a natural habitat. Some researchers, however, describe some findings that contradict such statements. As mentioned by Tabarani *et al.* (2010), this serotype has been isolated from foods of animal origin, pets, wild birds, mammals including wild possums and many reptile species.

Salmonella enterica subsp. *houtenae* was isolated for the first time in 1978 in an avian species (Phillips and Hatkin, 1978). Runkel *et al.* (1991) established that wild possums are reservoirs of this subspecies, since it corresponds to all isolates of the biliary tract of these animals. Ma *et al.* (2003) believe they have described the first report of human infection by *S. enterica* subsp. *houtenae* and Lourenço *et al.* (2004) described the first human bacteremia caused by *S. enterica* subsp. *houtenae* in Brazil associated with a patient infected by HIV. The latter authors claim that it is important to consider the fact that the patient used to live with birds and dogs, which may have served as a reservoir for *Salmonella* spp., and also could not discard the possibility of foodborne contamination. In a contamination assessment of chilled gooat meat sold *in natura* in the city of Recife, Brazil, it was observed that among seven samples contaminated by *Salmonella* spp., four were identified as *S. enterica* subsp. *houtenae* (Moura *et al.*, 2007). In supermarkets and butcher shops in Porto Alegre, Brazil, from 333 pork sausage samples analyzed, the presence of *Salmonella* spp. was detected in 82 (24.4%). In this study, however, only one isolate did not belong to *Salmonella enterica* subspecies, but the *Salmonella houtenae* subspecies. The authors also noted that human infections associated with this subspecies are rare (Mürmann *et al.*, 2009). This fact was also observed in a study conducted by Tavechio *et al.* (2002). From approximately 123 different serotypes of *Salmonella* spp. observed in various food sources, less than 1% corresponded to *S. enterica* subsp. *houtenae*.

Regarding *Salmonella ser.* Albany, literature reports that this serovar is not often correlated to infections in humans and animals. However, it has been isolated from chickens, as described in the studies by Luiz *et al.* (2004), Moreira *et al.* (2008) and Brito *et al.* (2010). Besides those correlations, Zaidi *et al.* (2006) monitored the *Salmonella* serotypes most commonly isolated from infected people and food supplies in Yucatan, Mexico, between 2000 and 2002. Their observations showed that *Salmonella* ser. Albany was the first serotype most isolated in poultry, the

sixth in asymptomatic children, and the eighth in patients with diarrhea and also in beef. In pork, however, the isolation was not observed. Despite the few isolation reports of these strains in humans, molecular studies of *Salmonella* ser. Albany in food (Doublet *et al.*, 2003) demonstrated the presence of genes that confer multiple resistance to antibiotics in those strains, which according to the authors, can difficult the treatment of intestinal infections caused by this microorganism.

In *Salmonella* spp. analysis in beef, the optimal dose of gamma irradiation to eliminate this microorganism was 3 kGy (Sedeh *et al.*, 2007). Costa *et al.* (2004) observed that the dose of 2.5 kGy was sufficient for effective control of *Salmonella* spp., regardless of the presence of fat and oxygen. Oliveira *et al.* (2009) reported that the control samples of chicken meat, both in conventional and vacuum packages, were contaminated by *Salmonella* spp. and that the dose of 1.5 kGy was sufficient for its elimination, regardless of the atmosphere packing used. However, Santos *et al.* (2003) stated that the recommended dose to ensure the safety of chicken meat against the presence of *Salmonella* spp. is 3.8 kGy. In chilled rabbit meat samples, *Salmonella* spp. was detected in control and irradiated samples at 1.5 kGy, but not at 3 kGy (Badr, 2004).

Some studies, however, did not detect the presence of *Salmonella* spp. both in irradiated and control samples, as observed by Yildirim *et al.* (2005) and Parlato *et al.* (2007), in a typical Turkish dish (Cig Köfte) and chicken, respectively.

The results obtained in the present study, it has been found the need of improvements in the sanitary conditions during slaughter and processing of sheep meat and the effectiveness of the irradiation treatment in the elimination of coliform bacteria and *Salmonella* spp.

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