**DOUGLAS ROBERTO GUIMARAES SILVA**

# EFFECTS OF GAMMA IRRADIATION ON RESTRUCTURED COOKED HAM FORMULATED WITH LOW NITRITE CONTENT

**LAVRAS – MG**
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Prof. Dr. Eduardo Mendes Ramos
Orientador

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# EFEITO DA IRRADIAÇÃO GAMA EM APRESUNTADOS FORMULADOS COM BAIXA CONCENTRAÇÃO DE NITRITO

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ciências dos Alimentos, área de Concentração Ciência e tecnologia de produtos de origem animal, para a obtenção do titulo de Doutor.

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**LAVRAS – MG**
**2018**

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**Abstract**

The objective of this study was to analyze the effects of gamma radiation applied at different doses against the survival of inoculated *Clostridium sporogenes* and *Listeria monocytogenes* in restructured cooked ham with low sodium nitrite content, as well as on the technological and sensorial characteristics of the products. Higher irradiation doses reduced ($P < 0.05$) the amount of *C. sporogens* spores, independent to the addition of sodium nitrite, with a lethality up to 5 log reductions. Gamma irradiation also reduced ($P < 0.05$) the residual nitrite levels, but had no effect ($P > 0.05$) on the pH, CIE color parameters or the texture profile, but improved their odour and flavor, according to sensorial analysis. The color of uncured samples was more yellowish (higher hº value) than cured ones, and this difference was perceived by the sensory panel. Irradiation also induced slight protein damage, characterized by the total sulfhydryl group reduction. However, irradiation did not cause any increase in the cyto or genotoxic (DNA strand breaks) effects in intestinal Caco-2 cells. Gamma irradiation had no effect ($P > 0.05$) on the pH, residual nitrite, synereses, TBARS, water activity or CIE color parameters on sliced ham, although significant ($P < 0.05$) interactions occurred with sodium nitrite level and storage days on residual nitrite, TBARS, and a*, C* and h° color parameters. The results indicated that irradiation at 6 kGy of cooked ham combined with low levels of nitrite can be used to improve the sensory quality and ensure their microbiological safety against *Clostridium* spores and the results indicated that irradiation at 1.5 kGy of RTE ham combined with 50 mg/Kg of sodium nitrite can be used to improve quality and ensure microbiological safety against *Listeria mococytogenes*

**Keywords:** *food safety, Clostridium sporogenes, Listeria monocytogenes.*

**Summary**

## 1. Introduction

Sodium nitrite is essential for developing specific characteristics in cured meat products like color and flavor. However, the important function of sodium nitrite is to control oxidative rancidity and principally to ensure the control of the development of the microorganism *Clostridium sporogenes.*

*Clostridium sporogenes* is a gram-positive, spore-producing bacillus, frequently found in soil, food, human faces, and animals. They are straight or slightly curved rods with peritrichous flagella, a capsule and are mobile and anaerobic, with oval and sub-terminal spores. To produce the toxin they need a basic or near neutral pH.

Nevertheless, the use of sodium nitrite in meat products is related to the development of N-nitrous compounds, especially N-nitrosamines that are potentially toxic, mutagenic, and carcinogenic. N-nitrosamines are linked to the development of the risk of leukemia in children as well as stomach, esophagus, liver and brain tumors in adults.

Usually, it is accepted that ready-to-eat (RTE) products are characterized by an increased shelf life at refrigeration temperature, appropriate pH, and low water activity compared to fresh meats, with consumption without further cooking required. These RTE meat products, such as ham, have been frequently reported in listeriosis outbreaks and as a food at risk of *Listeria monocytogenes* contamination. *Listeria monocytogenes* is a gram-positive, motile, rod-shaped bacteria, causing severe foodborne disease.

Because of the potential risks arising from nitrite addition and contamination for different microorganisms in RTE meat products, studies have proposed alternative ways to replace sodium nitrite or at least to reduce the quantity of sodium nitrite applied in the curing process. However, because of the important function that nitrite has in cured meat product quality and microbiological safety, changes in the products need to be carefully tested.

An alternative for control against *Clostridium sporogenes* and *Listeria monocytogenes* and to reduce sodium nitrite in ready-to-eat meat products is the use of the irradiation process. Irradiation has some benefits such as low or no heat generation, a low energy requirement, conservation of food in a single operation, irradiation of packaged or frozen products, and does not cause large changes in the nutritional value of foods. One of the main objectives of irradiation is the elimination of pathogenic and deteriorating microorganisms, and according to many studies, the irradiation dose required for this application is usually lower than 10 kGy.

Therefore, the objective of this study was to analyze the effects of gamma radiation applied at different doses against the survival of inoculated *Clostridium sporogenes* or *Listeria monocytogenes* in restructured ready-to-eat ham with low sodium nitrite content, as well as the effect on the technological and sensorial characteristics of the products.

In paper 1 the objective was to determine the effects of low-dose gamma radiation (1.5 to 6.0 kGy) on the survival of *C. sporogenes* spores inoculated into restructured cooked ham formulated with and without different sodium nitrite contents (50 and 150 mg/kg), as well as on the technological and sensorial characteristics of the products.

In paper 2 the objective was to investigate the effects of gamma irradiation in restructured cooked ham formulated with different concentrations of sodium nitrite (0, 50 and 150 mg/kg) on the potential cytotoxic and genotoxic effects on intestinal epithelial cells in vitro and on protein oxidation of the hams.

In paper 3 the aim was to determine the effects of a low dose (< 2 kGy) of gamma radiation on the survival of *Listeria monocytogenes* inoculated into restructured cooked ham with different contents of sodium nitrite added, as well as on the technological characteristics of the products.

# 2. Literature Review

## *2.1 Cured in meat products*

The term ‘‘cured’’, relating to meat products, is the addition of nitrite and/or nitrate to the product (Pegg & Shahidi, 2000; Sebranek et al., 2007). Different ingredients including sugar, spices, phosphates and others are typically included in cured meats, but it is the addition of nitrite in one form or another that results in the distinctive characteristics the typical color, flavor, shelf life and safety for all cured meat products that are recognized by consumers (Cassens, 1990; Dutra et al., 2011; Pegg, 2004; Sindelar & Milkowski, 2011).

An example of cured meat product is restructured cooked ham. According to normative instruction from Brazil, restructured cooked ham is a processed meat product obtained from cuttings and/or cuts of muscular masses of the hind and/or front limbs of pork, addition of ingredients and subjected to the proper cooking process. In addition, ingredients must be used: maximum additions of 150 ppm of residual nitrite and/or nitrate and 2.5% non-meat proteins in the aggregate form (Brasil, 2000). Table 1 contains the physico-chemical characteristics determined by Brazilian legislation.

Table 1. Physico-chemical characteristics for restructured cooked ham.

| Characteristics | Maximum (%) |
|---|---|
| **Starch**[1] | 2.0 |
| **Carbohydrate**[1] | 5.0 |
| **Humidity** | 75 |
| **Fat** | 12 |
| **Protein** | Minimum 13 |

[1]The sum of maximum starch and total sugars (total carbohydrates) shall not exceed 5%

Source: Brazil, 2000

## 2.2 Importance of Nitrite in Processed Meat Production

### 2.2.1 Cured color

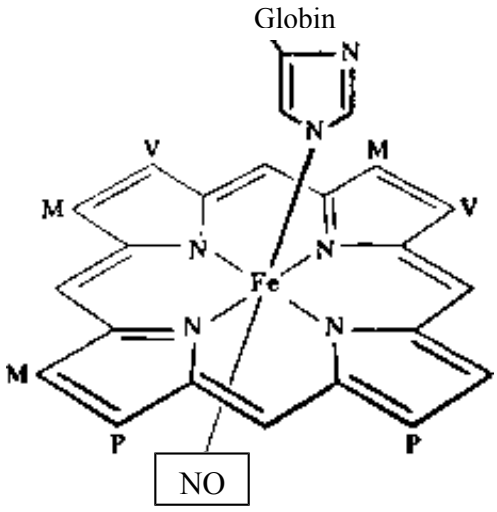
The first and most obvious effect is that of cured color development. Sodium nitrite is an additive used widely in meat products and it is involved in a number of functions including color formation (Honikel, 2008).

Myoglobin is a structure containing a protein and non-protein portion and this structure can readily undergo changes in color (Romans et al., 2001). The non-protein portion

is the heme group and it is composed of a porphyrin ring and contains an iron atom (Fe) (Price & Schweigert, 1987; Romans et al., 2001). Figure 1 shows the heme group with the six available binding sites (ligands), for the iron (Fe) atom. Of these sites, four are used to stabilize the porphyrin ring, one is used to bind the globin (protein portion) to the heme group and the other site is free to interact with a number of chemical elements (Romans et al., 2001; Feiner, 2006).

The color of the cured meat is developed by supplying nitric oxide (NO) for binding at the sixth position of the heme protein. The supply of nitric oxide for the development of cured color occurs through the direct addition of nitrite or nitrate by different methods, depending on the type of product being produced (Schrader, 2010). Nitrite ($NO_2$) is the active agent and nitrate ($NO_3$) is converted to nitrite by the naturally occurring reducing bacteria present in the meat during the curing process (Varnan & Sutherland, 1995).

Figure 1. Schematic representation of the heme complex of myoglobin.




Source: Reprinted from C.E. Bodwell and P.E. McClain, Proteins, in The Sciences of Meat Products, 2nd ed., J.E. Price and B.S. Schweigert, eds., 1971, W.H. Freeman & Co.

The first step for cure process is the conversion of the chemical forms of myoglobin into metmyoglobin and the meat is brown (Ramos & Gomide, 2017). Because of the mildly acidic meat conditions (pH 5.5 - 6.0), nitrite is converted to nitrous acid ($HNO_2$) and nitric oxide (NO). Nitric oxide reacts with myoglobin to produce cured meat color, first producing nitrosylmetmyoglobin that is unstable, and immediately producing nitrosylmyoglobin with red-pink pigment characteristic of raw cured products. The appreciated characteristic pink

color of cured meats is not developed until the meat product is heated (50 – 60 °C). The globin portion of the protein denatures and detaches from the iron atom resulting in the formation of the stable cured meat pigment, nitrosohemochrome (Schrader, 2010).

The pigment nitrosohemochrome is stable under certain conditions, however, can have discoloration resulting from chemical, physical and microbiological sources. Color degradation from bacterial contamination and growth involves an undesirable formation of brown and green pigments that are a result of hydrogen peroxide production and build-up from catalase positive bacteria (Price & Schweigert, 1987).

There are other factors known to cause discoloration of cured products when biological and biochemical factors such as bacterial growth and enzymes are excluded, which include oxygen, light, and dehydration. When cured meat products are exposed to high-intensity light or oxygen, they can develop a tan or grayish-brown pigment. Color fading process occurs in two steps: light accelerates the dissociation of nitric oxide from the myoglobin pigment and is then catalyzed in the presence of oxygen, resulting in dissociation and subsequent oxidation of nitric oxide. When there are oxygen penetrations, oxygen presence becomes a significant factor in discoloration of cured meat products, especially if light is present (Ramos & Gomide, 2017; Schrader, 2010), so it is necessary to use packages that give protection to light and oxygen.

The residual nitrite is also very important for the color characteristic. According to Cassens (1997) only 10% - 20% of nitrite is present after the curing process and during storage. Pardi et al. (1995) observed after seven days of storage only 10% residual nitrite of total put. In order to obtain a cured meat product with desirable color, it is necessary to maintain a residual nitrite content of 30 to 50 ppm according to Herson & Hulland (1984) and Muller (1991).

### 2.2.2 Cured flavor

The characteristic flavor of cured meat products can also be attributed to the chemical reactions of nitrite and its associated reactions as described above. The principal mechanism and the compounds responsible for this unique flavor remain unknown (Sindelar & Milkowski, 2011). Shahidi (1998) proposed that this characteristic feature could be due to the nitrite-related suppression of oxidation products, which manipulates the development of rancid-flavor compounds.

Sindelar and Milkowski (2011) suggested that cured meat flavor could be the result of a combination of nitrite related flavors and aroma. Hydrocarbons, ketones, alcohols, phenols, esters, furans, pyrazines, aldehydes, and other nitrogen-containing compounds, and increased carboxylic acids, sulfur, and nitrite/nitrate containing compounds have been found in cured meat compared to uncured meat (Ramarathnam, Rubin, & Diosady, 1993). Alcohols and phenols undergo nitrosation reactions and could impact volatile compounds as well. Increases in sulfur compounds could be attributed to S-nitrosothiol formation and reduction to disulfide bonds during meat curing (Ramarathnam et al., 1993).

According to Muller (1991), a residual nitrite content of 20 to 50 ppm is necessary to develop a desirable flavor for panelists.

### 2.2.3 Antioxidant effect

Another notable property of nitrite is its capacity to delay the development of rancidity during storage and the further warmed-over flavors developed upon heating of meat and meat products (Parthasarathy & Bryan, 2012; Pegg & Shahidi, 2000). The antioxidant activity of nitrite is assigned to the possibility of nitric oxide to bind to and stabilize heme iron of meat pigments during the curing process. Oxygen and other reactive oxygen species rapidly react with and are removed by nitric oxide (Ford & Lorkovic, 2002). Nitric oxide, as a free radical, can also terminate lipid autoxidation (Pegg & Shahidi, 2000).

According to Bergamaschi & Pizza (2011), in addition, it binds free ions and stabilizes heme iron which can reduce lipid oxidation by limiting pro-oxidant activity of iron. This lowers the amount of free iron released during cooking and chelates free radicals including lipid-derived alkyl, alkoxyl, and peroxyl radicals that accelerate lipid oxidation in meat products (Parthasarathy & Bryan, 2012).

Al-Shuibi & Al-Abdullah, (2002) reported the antioxidant effect of nitrite at levels as low as 40 ppm. Nitrite has been shown to inhibit warmed-over flavor development at relatively low levels. Sato & Hegarty (1971) reported significant inhibition of warmed-over flavor development at a 50 ppm nitrite level with complete inhibition at a 220 ppm level. Investigating the effect of nitrite on lipid oxidation in various muscle systems, Morrissey & Tichivangana (1985) reported as little as 20 ppm nitrite was sufficient to significantly ($P < 0.01$) inhibit oxidation of lipid in fish, chicken, pork, and beef systems. These researchers also reported that 50 ppm ingoing nitrite provided a significant ($P < 0.001$) reduction whereas

a 200 ppm ingoing level caused a 12-fold reduction in thiobarbituric acid (TBA) values for chicken, pork, and beef suggesting complete inhibition occurred.

### 2.2.4 Antimicrobial effect

Nitrite is strongly inhibitory to anaerobic bacteria, most importantly *Clostridium botulinum*, and contributes to control of other microorganisms such as *Listeria monocytogenes*. The effects of nitrite and the likely inhibitory mechanism differ in different bacterial species and are dependent on several environmental factors including pH, sodium chloride concentration, reductants and iron content among others (Tompkin, 2005).

Nitrite alone or in combination with other salts can inhibit the growth of several aerobic and anaerobic microorganisms. Nitrite targets bacteria at multiple sites by inhibiting metabolic enzymes, limiting oxygen uptake, and breaking the proton gradient. In addition, nitric oxide bound to iron thus limits iron availability which is necessary for enzyme functionality and bacterial metabolism and growth (Tompkin, 2005). Iron-sulfur complexes and heme ion centers of enzymes are often the targets of nitrite due to the high reactivity of iron and nitrite (Cui, Joannou, Hughes, & Cammack, 1992).

Furthermore, nitrite is well-known to suppress the outgrowth of *C. botulinum* spores in cured meat products (Sindelar & Milkowski, 2011) and to completely control botulism. Nitrite has been reported to contribute to controlling the growth of several other pathogens such as *Listeria monocytogenes, Bacillus cereus, Staphylococcus aureus* and *Clostridium perfringens* (Parthasarathy & Bryan, 2012; Pradhan et al., 2009).

#### *2.2.4.1 Clostridium sp.*

*Clostridium sp.* are rod-shaped, catalase-negative, proteolytic organisms which produce heat-resistant spores, and, except for a few aero tolerant species, are anaerobic.(Rood et al., 1997). The main sources of clostridia are soil and water (Jay et al., 2005).

Proteolytic clostridia are the principal causative agents of the spoilage of canned foods at neutral and slightly acidic pH, as they can form heat-resistant spores. The species involved are toxin-producing strains of *C. botulinum* and *C. perfringens*. Studies with *C. botulinum* can require stringent containment facilities, therefore *C. sporogenes* has been extensively studied as a model organism due to its similar metabolism to *C. botulinum* and lack of toxin formation (Cammack et al., 1998).

The clostridial toxins are water soluble, heat sensitive and acid stable proteins. They act on the nervous system by preventing the secretion of neurotransmitter vesicles. The first symptoms and signs of botulism are anxiety or agitation, drowsiness or blurred vision, and nausea or vomiting. Later, substernal burning or pain, abdominal distension and decreased bowel activity may occur, and death is usually the result of respiratory paralysis. Clostridial endospores are more resistant to heat, radiation or germicides than vegetative cells, and cannot be easily destroyed. Spores survive heating at 60 °C for several seconds, which kills vegetative cells of *C. sporogenes*. Germination of the dormant spore requires heat or specific activating substances (Ahnert-Hilger, 1995; Cammack et al., 1998; Jay et al., 2005).

Vacuum packaged processed meat products serve as an appropriate medium for the development of *C. botulinum*, as this pathogen grows in conditions without oxygen. The excellent conditions are beginning between a pH of 4.6 and 8.8 and a temperature of 3.3 °C to 55 °C with an optimum growth temperature near 37 °C (Jay et al., 2005).

Nitrite is especially effective against *Clostridium botulinum*, and its use in processed meat production has practically eliminated the risk of this deadly pathogen. As with the case of color and flavor development, the anti-botulinal mechanism is not clear, but microbial inhibition is thought to be a result of nitrous acid derived from nitrite in the acid environment of meat systems. The efficiency of nitrite against *C. botulinum* is dependent upon some factors including spore level, ingoing nitrite level, pH, the concentration of sodium chloride, iron content, the presence of reducing agents and residual nitrite levels in the meat (Tompkin, 2005; Archer, 2002).

According to Pierson & Smooth (1982), the anti-botulinal effects of nitrite occur at two different steps, the first in controlling the inhibition of vegetative cells emerging from surviving spores and the second by preventing cell division and growth in any vegetative cells that do emerge from surviving spores. The reactions of nitric oxide appear to be responsible for this effect, further emphasizing the importance of nitrite chemistry and vegetative cells of *C. botulinum* were found to contain iron-sulfur proteins (Reddy et al., 1983) and the reactions of nitric oxide have been shown to reduce germination by inactivating iron-sulfur complexes that are essential for growth (Payne, 1990). Muller (1991) observed that the nitrite level content between 75 to 150 ppm and combined with salt (NaCl) concentration between 1.5 to 2.0% and cooking until 71 °C is great against *Clostridium botulinum*.

## 2.3 Risks of using nitrite

### 2.3.1 Toxicity

Nitrate and nitrite are compounds that can be toxic to humans when not appropriately used, for example, levels above 300 mg/kg body weight is considered the dose for nitrite and is the reason for the strict regulations in place to control and monitor nitrite usage in meat products (Schrader, 2010).

According to Pierson & Smooth (1982), low levels used in cured meat production and consumption of these products do not present a known health hazard. However, if nitrite is consumed by itself can cause serious health implications or even death through the condition known as methemoglobinemia. Characteristics of methemoglobinemia include the distinguishing blue color of the skin that is a direct result of amounts of unoxygenated hemoglobin present, indicating lack of oxygen in the blood as well as other organs and tissues in the body. Hemoglobin is unable to transport and release oxygen to organs and various tissues in the body as a result of becoming oxidized from the ferrous to the ferric state. Newborn infants are particularly susceptible to methemoglobinemia as the enzymes needed to counteract the effects of nitrite poisoning are not fully developed. This leads to "blue baby syndrome" in which the infants turn blue in color due to lack of oxygen to internal organs and, if severe enough, can be fatal (Cammack et al., 1998).

### 2.3.2 Nitrosamines and Cancer

Nitrite can act as a nitrosating agent in the formation of nitroso compounds (Cassens, 1995). N-nitroso compounds belong to six fundamental categories: volatile N-nitrosamine, non-volatile N-nitrosamine, N-nitrosamide, N-nitrosated heterocyclic carboxylic products, N-nitrosated glycosylarnines, and Amadori compounds (Hiramoto, Kido, & Kikugawa, 1993). A number of epidemiological studies have confirmed a potential relationship between nitrate, nitrite, and N-nitroso compounds and the risk of cancer (Alexander & Cushing, 2011). Certain nitroso compounds belong to a family of potent human carcinogens known as N-nitrosodimethylamine. These nitrosamines are easily formed by the interaction of a secondary amino compound with nitrite under favorable conditions such as near acidic pH and a product temperature of more than 130 °C (Cassens, 1995).

Although general health risks associated with nitrite are known, Alexander & Cushing (2011) have observed that there is no supportive evidence to prove the relationship between processed meat consumption and cancer risk. The exposure to overly high doses of nitrite and nitrate from different sources has been associated with increased incidence of health risks (Gangolli et al., 1994; Sanchez-Echaniz, Benito-Fernandez, & Mintegui-Raso, 2001).

Ahn et al. (2002) confirmed that the breakdown products of N-nitrosamines did not reform in a model human stomach condition in vitro system, thus the authors proposed the possibility of using this effect in a real food system. N-nitrosamines found in processed meat are usually from added $NaNO_2$, which plays the role of a coloring agent or a preservative in cured meat products, but if the concentration of $NaNO_2$ is high enough, it oxidizes hemoglobin to methemoglobin, resulting in various adverse effects in humans (Peter, 1975).

The 2015 Food & Health Survey by the International Food Information Council Foundation (International Food Information Council Foundation, 2015) provides an example of consumers' contrary relationship with food preservatives such as nitrite: Nearly equal numbers (36% vs. 34%) believe that “chemicals in foods” and “foodborne illness from bacteria”, respectively, are the most important food safety issues at this time. Consumers worry about the same ingredients (chemical preservatives and antimicrobials) that protect them from something else that they fear (foodborne pathogens).

More specifically, consumers have developed an illogical relationship with nitrite (and its precursor, nitrate) in food. However, sales of bacon, arguably the food most commonly associated with nitrite, continue to grow 10% annually (Sax, 2014). Meanwhile, hot dogs containing celery powder (a rich natural source of nitrate) are erroneously touted in the popular press as being "by default healthier than nitrate-filled dogs" (Myers, 2014). Consumers seeking clean-label products will virtuously add celery, spinach, uncured bacon, and beet juice (popularized as an antidote for metabolic syndrome because of its high level of nitrate) to their shopping baskets, not realizing that all contain the same chemical that they assiduously avoid when added in the form of a purified chemical to foods.

Many studies have suggested alternative ways to substitute nitrite or at least to reduce the amount of nitrite applied in the curing process because of the potential risks of nitrite addition. However, because of the essential role that nitrite plays in cured meat quality and microbiological safety, changes in the products need to be carefully examined (Sebranek & Bacus, 2007).

## 2.4 Ready-to-eat products

According to 9 Code of Federal Regulations, part 430, ready-to-eat (RTE) meat is defined as a meat or poultry product that is edible without additional preparation to achieve food safety. It can receive an additional preparation to make the product more palatable (Nikmaram et al., 2018).

RTE meat products have got increasingly common due to the commodity in preparation, time-saving and taste. RTE products are made at intermediary temperatures with the final product temperatures typically between of 65–75 °C. They are different from tinned products that are processed at high temperatures for sterility/total microbial lethality. Thus, RTE foods and particularly muscle foods require refrigeration and are usually relate to as cold cuts. Despite the popularity, RTE meat and poultry are vulnerable to the foodborne outbreak related with the pathogens such as *Listeria monocytogenes* and spoilage microorganism such as lactic acid bacteria (Horita et al., 2018).

It is usually recognized that foodstuffs characterised by extended shelf life at refrigeration temperature, appropriate pH and water activity levels and consumed without further cooking (RTE) are most frequently involved in outbreaks of listeriosis (Ianneti et al, 2016).

### 2.4.1 *Listeria monocytogenes*

*Listeria monocytogenes* is a catalase-positive bacillus which can grow in milk products such as soft cheeses, and in meat and fish (Ryser & Marth, 1991). There are hemolytic (pathogenic) and non-hemolytic (non-toxic) strains and they are able to grow aerobically or anaerobically, over a range of temperatures between 34 to 50 °C, and survive for long periods of time. *L. monocytogenes* grows at pH values between 4.7 and 9.2. Temperature, pH, and concentration of preservatives are the most important factors in the inhibition of *L. monocytogenes* growth in food (Cammack et al., 1998).

*L. monocytogenes* has been isolated from many different settings including soil, water, vegetation, sewage, animal feed, farm environments, and food processing environments (Wiedmann & Sauders, 2009) in addition to the intestinal tracts of healthy animals and humans (Jay et al., 2005).

The control of *L. monocytogenes* represents a considerable challenge to processors of ready-to-eat (RTE) meat and poultry products especially those that are produced under uncured, natural, or organic methods. Prevention of post-processing contamination and reformulation of meat products to inhibit growth have been identified as critical strategies to reduce the risk of listeriosis (International Life Sciences Institute Research Foundation, 2005).

In addition, pregnant women are upon 20 times more probable than other sadio adults to take listeriosis (Tappero et al.1995). Although the mother is rarely affected, the disease is harmful to the fetus, as listeriosis can result in spontaneous abortion, still birth or premature birth.

**2.5 Technologies**

The challenge for the meat industry is to search for strategies to reduce supplemented and residual nitrite in cured meat in order to minimize the nitrite intake. There is a considerable interest in the development of alternatives from natural sources and other preservation techniques that are considered to be comparatively healthier. This interest is further accelerated by the pressure generated from consumer demand for salt- and nitrite-reduced meat products.

The residual nitrite concentration in most of the uncured products is generally lower than that in conventionally cured products (Sebranek & Bacus, 2007; Sindelar, Cordray, Sebranek, Love, & Ahn, 2007). Although it is difficult to replace nitrite by a single antimicrobial agent owing to its broad-spectrum activity (Pegg & Shahidi, 2000), a combination of nitrite and different antimicrobial agents may be effective. However, any improvement measures with regards to consumer safety should be undertaken without altering the unique characteristics of the natural and organic processed meat products.

Using ionizing radiation is known to be the best method to destroy pathogenic and spoilage microorganisms without compromising the nutritional properties and sensory quality of the food (WHO, 1999) and its use is gradually increasing worldwide. In addition, the application of irradiation for reducing the toxic or undesirable compounds such as volatile N-nitrosamines (Ahn et al., 2002), food allergy (Lee et al., 2001), and production of low-salted fermented foods (Lee, Ahn, Jo, Yook, & Byun, 2002) has been reported recently besides the sanitary purpose of the technology.

### 2.5.1 Food radiation

According to Mariano (2004) irradiation consists of exposing the food to ionizing radiation and there are examples of high energy charged particles such as electrons or photons of energy, X-rays and gamma rays. Ionizing radiation is defined as any type of radiation that ionizes atoms of substances subjected to it, where only those with energy below the threshold of nuclear reactions are considered (Pereira, 2004).

Potter (1968) reported that the ionizing radiations can be classified as particles (alpha and beta) and as electromagnetic waves (gamma- and X-rays). Gamma rays stand out among the other types of ionizing radiation, for their efficiency in the destruction of microorganisms in food, since they possess a high energy content and high penetration and lethality. Penetration is instant, uniform and deep, and wavelength is short, similar to ultraviolet light and microwaves, being generated by radioactive isotopes such as cobalt-60 ($^{60}Co$) and cesium-137 ($^{137}Cs$) (Passos, 2009).

When irradiating a food, it is desired to use radiation that has good penetration, in a way that the entire foodstuff is affected instead of only the microorganisms and enzymes that remain on the food surface. On the other hand, high-energy radiation such as neutrons are not used, as they could result in radioactivity of food (Potter, 1968). According to Miyagusku et al. (2003), years of research reached a consensus on the application of different doses of irradiation for food irradiation.

Radappertization at high doses of radiation (> 10 kGy), can be used to sterilize food, and at medium doses (1-10 kGy) can increase the shelf life of the product via a pasteurizing effect, and at low doses (< 1 kGy) can effectively control microorganisms in fresh or aged meat and can destroy insects and parasites in grains and fruits (Mariano, 2004).

The irradiation of foods has the same benefits as heat treatment, chemical treatment and freezing. These processes are used for inactivation of microorganisms, viruses and/or, insects; preventing the germination or sprouting of potatoes, onions and garlic; retarding the ripening and aging of fruits and vegetables; prolonging shelf life, reducing pathogens and, thus, protecting consumer health (Parlato et al., 2014). Another advantage of radiation is its flexibility, allowing its application in the great diversity of foods of different shapes and sizes (Andrade, 2013).

The radiation response in microbial populations can be expressed by the decimal reduction dose ($D_{10}$-value). Table 2 shows typical radiation resistances of a number of bacteria in non-frozen foods.

Table 2. Typical radiation resistances of some bacteria in non-frozen foods of animal origin

| **Bacteria** | **$D_{10}$ value (kGy)** |
|---|---|
| **Vegetative cells** | |
| ***Aeromonas hydrophila*** | 0.14–0.19 |
| ***Bacillus cereus*** | 0.17 |

| | |
|---|---|
| ***Brucella abortus*** | 0.34 |
| ***Campylobacter jejuni*** | 0.08–0.20 |
| ***Clostridium perfringens*** | 0.59–0.83 |
| ***Escherichia coli* (incl. O157:H7)** | 0.23–0.35 |
| ***Lactobacillus spp.*** | 0.3–0.9 |
| ***Listeria monocytogenes*** | 0.27–1.0 |
| ***Moraxella phenylpyruvica*** | 0.63–0.83 |
| ***Pseudomonas putida*** | 0.06–0.11 |
| ***Salmonella spp.*** | 0.3–0.8 |
| ***Streptococcus faecalis*** | 0.65–1.0 |
| ***Staphylococcus aureus*** | 0.26–0.6 |
| ***Vibrio spp.*** | 0.03–0.12 |
| ***Yersinia enterocolitica*** | 0.04–0.21 |
| **Bacterial spores** | |
| ***Bacillus cereus*** | 1.6 |
| ***Clostridium botulinum* types A and B** | 1.0–3.6 |
| ***Clostridium botulinum* type E** | 1.25–1.40 |
| ***Clostridium sporogenes*** | 1.5–2.2 |

Source: Farkas, 2001- Reprinted.

#### 2.5.1.1 Legislation

The use of irradiation is limited but authorized in many countries, and formally approved in 55 countries around the world (Parlato et al. 2014). According to Kume & Todoriki (2013), food irradiation showed a wide spread of the technique around the world. Data for the year 2010 indicate that some 285,223 tonnes of food were irradiated in Asia, 103,000 tonnes in the United States and 9,264 tonnes in the United European Union (Kume & Todoriki, 2013).

Irradiation is a process that has been studied for several years, regulated by the Food and Drug Administration (FDA), provided since 1963 for wheat flour and wheat intended for human consumption, and applications guided under the rules of Good Manufacturing Practices (GMP). Then, in the 1980s and 1990s, new regulations emerged in order to extend

the use of this technology to other foods (Ornellas et al., 2006). However, the use of ionizing radiation in conservation came after the discovery of the X-rays by Roentgen, and of radioactivity by Becquerel, in approximately 1895 (Oliveira, Soares, & Alves, 2012). However, it was not until 1990 that the FDA used irradiation to control food-borne pathogens; the same body approved the use of irradiation in frozen red meat in 1997 (Andrade, 2013).

In Brazil, the first researchers with food irradiation were made in the 1950s by the Center for Nuclear Energy in Agriculture (CENA). Even though it has been allowed since 1985, irradiation for food preservation was used only in studies which were restricted almost exclusively to the institutions, since the country had a limited number of specialists (Oliveira, Soares, & Alves, 2012).

The first Brazilian legislation for irradiated food was Decree no. 72718 of August 29, 1973 (Brasil, 1973), which was later complemented by ordinance nº 9, of March 8, 1985, and nº 30, of 25 September 1989 (Brasil, 1985, 1989). In the year 2001, these Ordinances were rescinded by Resolution - No. 21 of January 26, 2001 (Brasil, 2001), which included the Technical Regulations for Food Irradiation, which is in force until the present day. Legislation following the international recommendations suggested by Food and Agriculture Organization (FAO), International Atomic Energy Agency (IAEA) and Codex Alimentarium (Ornellas et al., 2006).

Accordingly, pursuant to Resolution No. 21 of January 26, 2001, irradiation of food is a physical treatment process consisting of subjecting the food, whether entire or in bulk, to controlled doses of ionizing radiation for sanitary, phytosanitary and/or technological purposes. Still, according to the Regulation, irradiation can be applied to any food, with the proviso that the minimum dose absorbed must be sufficient to achieve the intended purpose, and the maximum absorbed dose should be lower than the dose that would compromise the functional properties and/or sensory aspects of food (Brasil, 2001).

### 2.5.1.2 Irradiation in meat products

According to Ahn et al. (1999) surges of pathogenic bacteria in meat and meat products have the expanded application of irradiation technique because is important to improve safety and public confidence. However, according to these authors, attention has been paid to these quality aspects (safety and public confidence) of meat in irradiation studies, especially at low irradiation dose (< 10 kGy).

In the majority of countries, gamma radiation can be applied at any dose, as long as it does not compromise the functional and/or sensorial properties of the product (Dutra et al., 2011).

Dutra et al. (2017) evaluated the effects of different doses of gamma radiation (0 to 20 kGy) on the color and lipid oxidation of mortadella prepared with increasing nitrite levels (0 to 300 ppm) and they observed that higher radiation doses increased the redox potential, promoted lipid oxidation and elevated the hue color of the mortadella. However, they noted that increased levels of sodium nitrite addition elevated the residual nitrite content, reduced lipid oxidation and promoted the increase of redness and reduced hue color of mortadella, independent to the radiation dose applied.

Although many studies have evaluated the effect of gamma radiation on the quality of sausages, Ahn et al. (2003) evaluated the effects of irradiation (0, 5, and 10 kGy) prior to modified atmosphere packaging storage for 4 weeks on emulsion-type cooked pork sausage, measuring residual nitrite, residual ascorbic acid, nitrosomyoglobin (NO-Mb), color values, and their correlations. The authors observed that irradiation reduced the residual nitrite content and caused partial reduction of NO-Mb during storage and indicated that the proper combination of irradiation and modified atmosphere packaging could reduce the residual nitrite in sausage with minimal color change.

Byun et al. (2002), evaluated the effects of gamma irradiation (< 10 kGy) on the processing properties of meat products, emulsion-type sausage, beef patties and pork loin ham were manufactured and they observed that most contamination bacteria were killed by 3 kGy irradiation. A dose of 5 kGy was observed to be as effective as the use of 200 ppm $NaNO_2$ to provide and maintain the desired color of the product during storage.

Jo et al. (2003) studied emulsion-type cooked pork sausage made with or without 156 ppm $NaNO_2$; packaged in aerobic, vacuum or $CO_2$ (100%) conditions; and irradiated at 0 and 5 kGy. They observed that $NaNO_2$ addition to sausage reduced lipid oxidation and increased redness (Hunter a* values); residual nitrite content was the lowest in the sausage with $CO_2$

packaging; but no effect of irradiation at 5 kGy was found to reduce the content of nitrosodimethylamine at 0 weeks and nitrosopyrrolidine at 4 weeks.

Dutra et al. (2016) studied the effects of applying different doses of gamma radiation (0, 10 and 20 kGy) on *Clostridium botulinum* spores inoculated into mortadella with different nitrite levels (0, 150 and 300 ppm). They observed that in the non-irradiated raw batters, almost all spores could be recovered when no nitrite was added and the use of 150 ppm of nitrite was sufficient to inhibit the germination or growth of *C. botulinum* in non-irradiated cooked mortadella after 48 h of processing but not after 30 days of chilling storage (4 °C).

#### 2.5.1.3 Effect of irradiation on the quality of meat products

Irradiation induces quality changes in meats such as off-odor production and color change, and consumer responses to these quality changes are quite negative (Ahn et al., 2000; Du et al., 2002; Patterson & Stevenson, 1995).

When foods rich in fats, such as meat and its derivatives, are exposed to gamma radiation, the formation of free radicals may occur during the process (Ahn et al., 1998), due to the radiolysis of the water molecules, with consequent lipid oxidation of the meat, which tends to increase over time during storage. Hexanal is a good indicator of lipid oxidation in meat (Ahn & Nam, 2004), being a compound that contributes to the unpleasant odor of the oxidized flesh.

Free radicals generated during the irradiation process not only result in lipid oxidation, but also contribute to the radiolysis of the amino acids present in meat, and the degradation of these compounds is the main cause of the formation of unpleasant odor of irradiated meat, which can produce odors of cabbage, sulfur, putrid and/or rotten vegetable spoilage- (Brewer, 2009; Nam et al., 2003).

Yang et al. (2011) reported that all irradiated meat produces an odor characteristic of the radiation, regardless of the degree of lipid oxidation. The compounds responsible for the undesirable odor are the sulfur compounds, such as dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide, originating from radiolytic degradation of proteins.

In addition to the radiolysis of proteins giving rise to undesirable flavor compounds, radiation can cause damage to proteins, including deamination (with production of propionic and pyruvic acid), decarboxylation (with production of ethylamine and acetaldehyde), reduction of disulfide bonds, oxidation of sulfhydryl groups, the hydrolysis of peptide bonds and valence of the metal ions present in the enzymes. The main products formed by the

interaction of the irradiation process with the protein material are the carbonyl groups, ammonia, free amino acids, hydrogen peroxide, organic peroxides and others. When using high doses, some crosses may occur leading to the formation of new proteins by the binding of amino acids with proteins and by the aggregation of proteins. These changes occur mainly with the primary structure of proteins, however, the irradiation process can affect the structures secondary and tertiary levels as well (Giraux & Lacroix, 1998).

## 3. General Considerations

It was concluded that gamma irradiation applied at different doses had effects against the survival of inoculated *Clostridium sporogenes* and *Listeria monocytogenes* in restructured cooked ham with low sodium nitrite content, and according to chemical, physical chemistry, sensorial and microbiological analysis, it was possible to reduce levels of sodium nitrite.

In paper 1 it was concluded that gamma irradiation was effective in reducing the *Clostridium sporogens* spores and the residual nitrite levels, without any significant effects on the other physicochemical and technological parameters of cooked hams. These results also indicate that gamma irradiation of cooked hams only 6 kGy elaborated with 50 mg/kg sodium nitrite can be used as an alternative to add 50 mg/kg sodium nitrite, not only to ensure microbiological safety but also to improve the product sensory quality.

In paper 2 it was confirmed that gamma irradiation treatment is a promising and safe technology for restructured cooked ham with different concentrations of sodium nitrite. Irradiation did not affect lipid oxidation levels. However, protein oxidation increased with doses of irradiation. This is in agreement with the low oxidative defects found for proteins after irradiation, confirming the gamma irradiation decontamination system of pork products as a feasible application. Irradiation did not cause measurable changes in the cytotoxic or genotoxic effects of restructured cooked ham.

In paper 3 it was concluded that irradiation was efficacious in reducing the Listeria monocytogenes inoculated in cooked hams without significantly affecting the other physicochemical and technological parameters. Similarly, a lower dose of nitrite (50 mg/kg) can be used in combination with gamma irradiation (1.5 kGy).

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**ARTICLE 1**

Article drafted in accordance with the Meat Science journal standard

"Preliminary version"

# Gamma irradiation as hurdle technology to control *CIostridium* growth in uncured and cured restructured cooked ham

**Abstract**

*The objective was to evaluate the effects of gamma radiation (0, 1.5, 3, 4.5, 6 KGy) on the survival of Clostridium sporogenes and on technological and sensorial characteristics of restructured cooked hams, cured with sodium nitrite (0, 50 and 150 mg/kg). Higher irradiation doses reduced the amount of C. sporogenes spores, independent of the addition of sodium nitrite, with a lethality up to 5 log reductions. Gamma irradiation also had an effect ($P < 0.05$) improved sensory scores of odour and flavor, but had no effect on pH, residual nitrite, CIE colour parameters or the texture profile. The colour of uncured samples was more yellowish (higher b* and h° value) than cured ones, and this difference was perceived by the sensory panel. The results indicated that irradiation at 6 kGy of cooked ham elaborated with low levels of nitrite can be used to improve the sensory quality and ensure their microbiological safety against Clostridium spores.*

**Keywords:** *Lipid oxidation, residual nitrite, quality, sensorial*

## 1. Introduction

Nitrite and nitrate salts are food additives used to confer the characteristic pink colour and cured flavor in cooked processed meat products, such as ham, which also act against oxidative rancidity and impact on the product texture (Honikel, 2008; Sindelar & Milkowski, 2011; Dutra et al., 2014; Dutra et al., 2017). However, the concern about the use of nitrites in meat products has intensified, due to its association with the formation of N-nitrous compounds, especially N-nitrosamines (Eichholzer & Gutzwiller, 2003; Abid, Cross, & Sinha, 2014), which are potentially carcinogenic, toxic or mutagenic to humans (Bedale, Sindelar, & Milkowski, 2016). Therefore, it has been suggested to replace or at least reduce

the amount of nitrite applied in the curing process. However, the exclusion or even reduction of nitrite content involves meat safety concerns, since the most important function of the nitrite salt in meat products is the preservative action against deteriorating and pathogenic microorganisms, particularly its high effectiveness against *Clostridium botulinum* and its toxin (Dutra et al., 2016; Sindelar & Milkowski, 2011).

To reduce nitrite addition in meat products, several alternative approaches have been proposed, including the use of natural antimicrobials, like essential plant oils (Oliveira, Soares, Ramos, Cardoso, Alves & Piccoli, 2011; Dias, Rodrigues, Palhares, Ramos & Piccoli, 2015), and non-thermal treatments such as high pressure (Akhtar, Paredes-Sabja, Torres & Sarker, 2009) and gamma radiation (Szczawinski & Szulc, 1989; Ahn, Jo, Lee, Kim & Byun, 2003; Dutra et al., 2016). Gamma irradiation is a potential alternative as it is recognized as the best technology for the destruction of pathogenic and deteriorating microorganisms in food (Ahn, Jo, Lee, Kim & Byun, 2003; Galan, García, & Selgas, 2011; Hoz, Cambero, Cabeza, Herrero & Ordonez, 2008), it also may induce the radiolysis of residual nitrites (Szczawinski & Szulc, 1989; Dutra et al., 2011) and N-nitrosamines (Jo, Ahn, Son, Lee & Byun, 2003), reducing the concentrations of these components in the final product.

Recently, Dutra et al. (2016) reported that doses as high as 10 and 20 kGy of gamma radiation had a positive effect on the inactivation of *C. botulinum* spores inoculated ($10^7$ spores/g) in mortadella, independent of the sodium nitrite level used. However, Dutra et al. (2017) evaluated the combined effects of gamma irradiation (0–20 kGy) and nitrite addition (0–300 mg/kg) on the quality attributes of mortadella using a central composite rotatable design and reported that higher radiation doses (> 10 kGy) promoted lipid oxidation and colour changes of the products. Moreover, these authors reported that nitrite addition had a greater effect than irradiation on product quality parameters, reducing lipid oxidation and promoting an increase in the redness of the mortadellas.

Due to the possible deleterious effects on product quality, the use of lower doses of irradiation should be considered in meat products; especially in those products that have a lower colour and flavor intensity, such as cooked ham type products. However, the concern with microbiological safety, especially against *C. botulinum,* should also be maintained. To our knowledge, no study has evaluated the effects of low- dose gamma irradiation (< 5 kGy) on the survival of *Clostridium* spores inoculated in meat products with lower levels of nitrite content. Since *C. botulinum* is of high biological risk, *Clostridium sporogenes* has been widely used as the model for proteolytic strains *of C. botulinum* on the validation of food processes (Brown, Tran-Dinh & Chapman, 2012). Therefore, the objective of this study was to determine the effects of low-dose gamma radiation (1.5 to 6.0 kGy) on the survival of *C. sporogenes* spores inoculated into restructured cooked ham formulated with and without different sodium nitrite contents (50 and 150 mg/kg), as well as on the technological and sensorial characteristics of the products.

## 2. Materials and methods

The experiment was conducted at the Laboratory of Meat Technology (LabCarnes), Laboratory of Food Microbiology and Laboratory of Sensorial Analysis at the Department of Food Sciences of the Federal University of Lavras (DCA/UFLA), Brazil.

### *2.1. Microorganism and inoculum production*

A lyophilized strain of *Clostridium sporogenes* ATCC 11437 was activated in Reinforced Clostridial broth and incubated at 37 °C for 24 h in a vacuum using mineral oil, then 0.1 mL aliquots were transferred to tubes containing the same culture medium and

incubated in the same manner, for 24 h. Aliquots of 0.1 mL of the culture were transferred to Petri dishes containing AK # 2 agar (Himedia®) and incubated anaerobically at 37 °C for 120 h (Probac do Brasil®) to obtain the endospores.

The number of endospores was standardized after washing the agar surface with 10 mL of 0.9 % (w/v) saline solution and observing the optical microscopy of the endospores stained by the Wirtz-Conklin technique using Malachite Green dye solution 5 % (w/v) and the slaframine counter dye 0.5 % (w/v). When the presence of endospores was verified, the suspension was subjected to thermal shock (70 ºC, 15 min) and fast cooling in an ice bath. Serial dilutions were performed in 0.1 % (w/v) peptone water and plated in Reinforced Clostridial Agar, with the plates incubated anaerobically at 37 °C for 48 h.

The endospores were kept frozen in a freezing medium with a double concentration of glycerol (30 mL of glycerol, 0.5 g of bacterial peptone, 0.3 g of yeast extract, 0.5 g of NaCl and 10 0mL of distilled water) until use.

### *2.2. Restructured cooked ham processing*

Restructured hams were manufactured according to a Brazilian traditional formula as described by Dutra, Cardoso, Ramos, Ramos, Pinheiro & Fontes (2012). Three batches of each product were formulated with 56% lean boneless pork ham (14 mm grinder), 35% water and a commercial mixture of ingredients (New Max Industrial Ltd.; Americana, SP, Brazil) containing: sodium chloride (1.4%); cassava starch (2.0%); soy isolated protein (2.0%); maltodextrin (1.1%); carrageen (0.5%); monosodium glutamate (0.3%); sodium erythorbate (0.06%); sodium nitrite (0, 50 and 150 mg/kg, as previously described); sodium polyphosphate (0.5%); and spices (0.8%). The materials were mixed, and the meat batter was vacuum packed (TM300, TecMaq, São Paulo, SP, Brazil) in flexible multilayer film

(SpelFlex/E; Spel Embalagens, São Paulo, Brazil), formed into 1 kg metallic forms and stored (4 °C) for 12 h. After curing, samples were cooked in a water-bath until the internal temperature of the product reached 72 °C (checked with a flexible thermocouple probe), cooled in an ice water-bath and stored at 4 °C for 24 h. Samples were unpacked, cut into 2.5 cm thick portions, weighed and vacuum packaged again in nylon-poly bags prior to irradiation.

In samples destined for microbiological analysis, part of the raw meat batter was divided into 25 g portions, placed in nylon-poly bags, inoculated with $10^6$ spores/g of *C. spororgenes* and then homogenized for 2 min in a stomacher (490 strokes/min; Metroterm, Brazil). After vacuum sealing (TM300, TecMaq, SãoPaulo, SP, Brazil), the samples were subjected to the equivalent cooking process for the restructured cooked ham (water bath).

### *2.3. Irradiation process*

All hams (packaged, cooked and stored at 4 °C) were placed in Styrofoam thermal boxes (Kanauf Isopor, São Paulo, Brazil) and subjected to different irradiation doses (1.5, 3.0, 4.5 and 6.0 kGy) in a Gamma GB-127 Irradiator (IR-214, MDS Nordion, Ottawa, Canada; with cobalt-60 sources and flow rate of 5 kGy/h) at the Laboratory of Gamma Irradiation in the Center for Nuclear Technology Development (CDTN) of the National Commission of Nuclear Energy (CENEN). The non-irradiated samples (0 kGy) were maintained under the same conditions and for similar periods of time as the irradiated samples. The entire irradiation process lasted 48 hours after the samples were cooked, being samples stored at 4 °C until analysis.

### *2.4. Enumeration of C. sporogenes endospores*

The enumeration of *C. sporogenes* endospores was carried out at 1 and 30 days of storage (4 °C) post-irradiation. The 25 g packs of cooked ham inoculated with spores were opened aseptically and 225 mL of 0.1% (m/v) peptone water was added, after which the mixture was homogenized in Stomacher Metroterm® (490 strokes/min for 2 min). After homogenization in 0.1 % (m/v) peptone water, the suspensions were subjected to thermal shock at 75 °C for 15 min for inactivation of the viable cells. Subsequently, serial dilutions were performed in 0.1% peptone water, and then 1 mL aliquots were plated in depth with overlay on *Clostridium* base agar, and the plates incubated at 45 °C for 24 h. After incubation, the survivor populations were calculated with the results expressed as log colony forming units per gram of meat (log CFU/g).

### *2.5. Technological analyses*

The pH values and residual nitrite content ($NO_2R$) were also carried out at 1 day of storage (4 °C) post-irradiation process. The pH values were measured using a potentiometer (Digimed, model DM20, São Paulo, SP, Brazil) by inserting a penetration-type combined Ag/AgCl reference electrode into five different points of the product. The $NO_2R$, expressed as sodium nitrite (mg/kg), was quantified according to official method no. 973.31 of the Association of Official Analytical Chemists (AOAC, 2012).

The instrumental colour and texture analysis were conducted 24 h after the irradiation process. The colour of the cooked hams was evaluated with a CM-700d colourimeter-spectrophotometer (Konica Minolta Sensing Inc., Osaka, Japan), with 8 mm aperture, using a specular component excluded (SCE) mode, a D65 standard illuminant and an observer angle of 10°. Samples were removed from packaging and the mean value of five readings

performed at different surface points, being recorded lightness ($L^*$) values. The angular coordinates chroma ($C^*$) and hue angle ($h$, degrees) were calculated from the chromaticity indexes ($a^*$ and $b^*$ values) as: $C^* = (a^{*2} + b^{*2})^{0.5}$; and $h = \tan^{-1} (b^*/a^*)$. Samples colour were expressed in the CIE LCh space: $L^*$ indicates sample lightness; higher $C^*$ values suggests more vivid colour; and $h$ values near 0 are red and near 90 are yellow (Ramos & Gomide, 2017).

The texture analyses were conducted by the texture profile analysis (TPA) method according to Pereira, Ramos, Teixeira, Cardoso, Ramos & Fontes (2011), using a universal TA.XT2i Texture Analyzer (Stable Micro Systems Ltd., Surrey, England) with a 50 kg load cell. Six cores (cubes with 10 mm edge) were obtained and compressed twice to 50% of their original height, at room temperature, with a compression flat cylindrical aluminum probe (36 mm diameter). A cross-head speed of 180 mm/min was applied. There was no rest time between the two cycles of compression. Force-time curves were recorded and the attributes were calculated as follows (Ramos & Gomide, 2017): hardness (N), peak force required for first compression; springiness (mm), distance sample recovers after first compression; adhesiveness (N×mm), the negative force area for the first bite representing the work necessary to pull the compressing plunger away from the sample; cohesiveness, ratio of positive force area during the second compression to that in the first compression; and chewiness (N×mm), the product of hardness, cohesiveness and springiness.

### *2.6. Sensorial analysis*

Sensory characteristics were evaluated 7-days after the post-irradiation process, in two sessions, by 30 non-trained panelists using a multiple-comparison test as described by Dutra et al. (2012). A non-irradiated ham with 150 mg/kg of sodium nitrite added was taken

as a “reference” sample (labeled R) and presented to the panelists with the other six (irradiation x nitrite added treatments) code samples (1.0 cm x 2.5 cm x 2.5 cm) per sessions. The panelists were asked to compare each coded sample (including non-irradiated ham with 150 mg/kg) with the labelled R (reference) sample, classifying them on a five-point hedonic scale (where 1 corresponded to “equal to the reference” and 5 corresponded to the “extremely different from the reference”) for flavor, odour, colour and texture.

### *2.7 Statistical Analysis*

The experiment was conducted in a completely randomized design with three repetitions. For *Clostridium* endospores, pH value and $NO_2R$ analysis, the treatments were arranged in a split-plot design, with the 3 (nitrite added) x 5 (irradiation doses) as whole plot. The technological analysis (colour and texture) were arranged in a 3 (nitrite added) x 5 (irradiation doses) factorial design. For sensory analysis, treatments were arranged in a 2 (nitrite added) x 5 (irradiation doses) factorial design, with panelists (n = 30) as repetitions.

The resulting data were tested by an F-test (ANOVA) and when significative ($P < 0.05$) means were separated by the Tukey test. All statistical analysis was conducted using the SAS statistical (SAS Institute Inc., Cary, NC, USA) package, version 9.2.

## 3. Results and Discussion

### *3.1. C. sporogenes survival*

Nitrite addition did not affect ($P > 0.05$) the spore germination of *C. sporogenes*, regardless of the storage time, but the interaction between irradiation dose and storage time

was significant ($P < 0.05$). Increased gamma irradiation and storage time (0 and 30 days) decreased the survival of *C. sporogenes* spores in restructured cooked hams (Fig. 1). The behavior of the regression curves was similar at 0 and 30 days, in which the numbers of log-reduction grew according to the dose of radiation, but with a larger reduction in product aged for 30 days. At higher radiation doses (6 kGy), the observed lethality resulted in close to a 5-log reduction in samples stored for 30 days. Even at doses of 3 kGy, the observed lethality was close to a 2-log reduction, which is a considerable microbial control, especially in a sporulated microorganism.

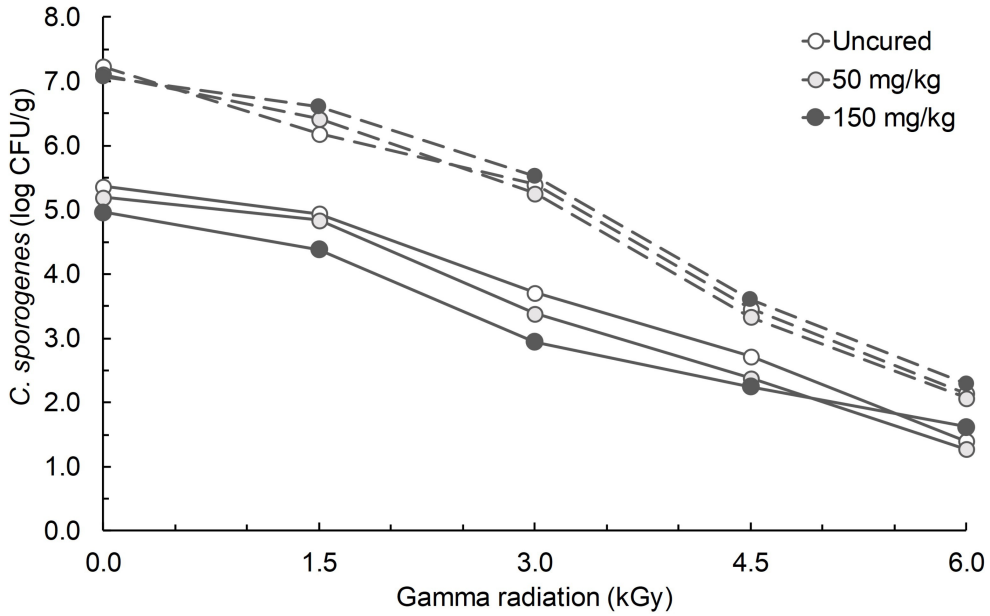


**Fig. 1.** Effect of gamma irradiation dose (G) in the *C. sporogenes* survival spores in restructured cooked hams after 0 (solid lines) and 30 (dashed lines) days of storage (4 °C).

The mechanism of microbial inactivation by ionizing irradiation is mainly due to direct damage to nucleic acids, by energy deposition in its macromolecule (DNA), or indirect damage acting through the oxidizing power of free radicals from the radiolysis of water, which can diffuse into the microbial cell (Farkas, 2006; Dutra et al., 2016). Microbial

inactivation by denaturation of enzymes and cell membrane alterations can also occur (Aymerichet, Picouet & Monfort, 2008).

Dutra et al. (2016) investigated the effects of different doses of gamma radiation (0, 10 and 20 kGy) on *Clostridium botulinum* spores inoculated into mortadellas with different nitrite additions (0, 150 and 300 mg/kg) and observed that 150 mg/kg of sodium nitrite was able to inhibit the germination or growth in non-radiated samples after 48 h of processing, but after 30 days of storage (4ºC) it was possible to recover $10^5$ CFU/g. According to their results, irradiation as high as 10 kGy had a positive effect on the inactivation of *C. botulinum* in mortadellas, independent of the sodium nitrite level used. Szczawinski & Szulc (1989) reported that high-dose radiation (10 to 50 kGy) considerably decreased both the level of residual nitrite ($NO_2R$), the main reason for this seems to be the decomposition of nitrite by ionizing radiation and the inhibition of *C. botulinum* spores in cured meat.

### *3.2. Technological effects*

The results for the effect of irradiation dose and sodium nitrite addition on the chemical and physicochemical characteristics of the hams are shown in Table 1. There was not effect ($P > 0.05$) on pH and this was considered normal for cooked ham, according to Santos (2005). The $NO_2R$ was the effect ($P < 0.05$) by the level of sodium nitrite.

**Table 1.** Sodium nitrite (N), gamma irradiation (G) effects on technological characteristics of restructured cooked hams.

| Effects | Source of Variation | pH | $NO_2R$ (mg/kg) |
|---|---|---|---|
| Sodium nitrite, mg/kg (N) | 0 | 6.32 ± 0.10 | 2.04 ± 0.85[a] |
| | 50 | 6.32 ± 0.10 | 4.33 ± 1.89[b] |
| | 150 | 6.34 ± 0.10 | 11.94 ± 5.77[c] |
| Irradiation dose, kGy (G) | 0 | 6.29 ± 0.10 | 5.08 ± 3.38 |
| | 1.5 | 6.33 ± 0.09 | 6.55 ± 5.64 |
| | 3.0 | 6.34 ± 0.09 | 6.79 ± 6.96 |
| | 4.5 | 6.34 ± 0.10 | 5.97 ± 6.07 |
| | 6.0 | 6.34 ± 0.10 | 6.11 ± 5.33 |
| Pr > F[1] | N | *0.583* | ***<0.001*** |
| | G | *0.553* | *0.675* |
| | N x G | *0.986* | *0.855* |

$NO_2R$ = residual nitrite; and *n.a.* = not analyzed.

[1]Significant values are shown in bold.

[a,b]-means in the same column, for each effect, followed by different letters differ ($P < 0.05$).

Many studies have observed lower $NO_2R$ values with the application of gamma irradiation and according to these authors, this could be due to its reaction with the hydroxyl radical produced in the radiolysis of water (Simie, 1983). Still, Shahidi, Pegg & Shamsuzzaman (1991) and Byun, Lee, Yook, Lee & Kim (1999) suggested that irradiation could increase the redox potential (Eh) of added reducing agents, which would imply lower values of $NO_2R$ in irradiated cured products. When the environment is in the reduced state, nitrite can easily be converted to nitric oxide, resulting in lower $NO_2R$ levels (Ahn et al., 2002b). However, Dutra et al. (2017) reported that the formation of highly oxidizing free radicals, promoted by the radiolysis of water due to the radiation process, led to an increase in Eh values. According to Gesi & Takagi (1964), the primary effects of irradiation on the nitrate or nitrite ions are its ionization and decomposition into $O_2$, which, in addition to inducing the increase of Eh, most likely contributed to the reduction in the $NO_2R$ values.

In several studies a significant reduction in the $NO_2R$ values has been reported in sausages (Ahn, Jo, Kim, Chung, Lee & Byun, 2002a; Ahn et al., 2003; Ahn, Kim, Jo, Lee, Yook & Byun, 2004a; Dutra et al., 2011) and cooked hams (Houser, Sebranek & Lonergan, 2003; Houser, Sebranek, Maisonet, Cordray, Ahn & Dixon; 2005b) after the application of gamma irradiation doses. In the majority of these studies, however, greater reductions in $NO_2R$ values between the control and the irradiated samples were observed in the samples with longer storage times. Other studies (Ahn et al., 2002a; Ahn, Kim, Jo, Lee & Byun, 2002b; Jo et al., 2002; Dutra et al., 2017) did not observe any effect of irradiation dose on $NO_2R$ values in sausages, even when doses as high as 30 kGy were used.

The mean values for lightness ($L^*$), chroma ($C^*$) and hue angle ($h°$) of cooked hams are shown in Table 2. All CIE values were affected ($P < 0.05$) by nitrite addition, with the uncured samples appearing more yellowish (higher $h°$) and also lighter and less red than cured ones. This agrees with the observations of Dutra et al. (2011) and Moura et al. (2017),

who reported higher *h*° values in uncured sausages and cooked hams, respectively. Although the differences were considered low, these authors also reported higher *L** values in uncured samples than the cured ones.

**Table 2.** Nitrite and gamma radiation dose effects on colour characteristics of restructured cooked hams.

| **Effects** | **Source of Variation** | ***L**** | ***C**** | ***h*** |
|---|---|---|---|---|
| Sodium nitrite, mg/kg | 0 | 68.84±0.79$^{a}$ | 13.16±0.58$^{a}$ | 74.54±4.69$^{a}$ |
| | 50 | 67.53±1.10$^{b}$ | 12.14±0.62$^{b}$ | 58.73±3.63$^{b}$ |
| | 150 | 67.52±0.91$^{b}$ | 12.55±0.78$^{a}$ $^{b}$ | 58.97±3.49$^{b}$ |
| Irradiation dose, kGy | 0 | 67.83±1.13 | 12.86±0.90 | 63.02±7.56 |
| | 1.5 | 67.91±1.36 | 12.42±0.59 | 64.42±8.13 |
| | 3.0 | 67.97±0.97 | 12.60±0.72 | 63.65±9.63 |
| | 4.5 | 68.15±0.92 | 12.69±0.84 | 65.25±9.55 |
| | 6.0 | 67.94±1.35 | 12.50±0.91 | 64.07±8.96 |
| Pr>F[1] | N | ***0.002*** | ***0.003*** | ***<0.001*** |
| | G | *0.980* | *0.750* | *0.854* |
| | N x G | *0.993* | *0.963* | *0.853* |

[1]Significant values are shown in bold.

$^{a-b}$ Means in the same column, for each effect, followed by different letters differ ($P < 0.05$).

Irradiation had no effect ($P > 0.05$) on CIE colour indexes (Table 2). Using a surface response methodology to evaluate the joint effects of irradiation doses (0-20 kGy) and nitrite addition (0-300 mg/kg) in the colour of mortadella, Dutra et al. (2017) reported only lower

*a** and *b** values and, consequently, also lower *h*° values. Many other authors (Byun et al. 1999; Ahn et al. 2004b; Dutra et al., 2011; Jo el al., 2003; Zhu et al., 2003; Houser et al., 2003) also did not observe effects of increased irradiation doses in cured meat products.

There was no effect of irradiation ($P > 0.05$) on any texture attributes (hardness, adhesiveness, springiness, cohesiveness and chewiness), however adhesiveness and springiness were both affected ($P < 0.05$) by different levels of sodium nitrite (Table 3). Theoretically, changes in texture are possible by altering the gel properties of proteins and polysaccharides, either by direct or indirect effects of radiolysis or by nitrite-induced interactions. Reactions of nitrite with sulphide groups (-SH) of amino acid residues of proteins result in the formation of disulphide bonds (-SS-) between the proteins, which contribute to the firm texture of the cured meat products (Dutra et al., 2014).

According to Dutra et al. (2014), any contribution of nitrite and/or irradiation is quite subtle, being detected instrumentally only under particular conditions. This demonstrates the complexity of understanding the characteristics of texture in cured meat products.

**Table 3.** Nitrite and gamma irradiation dose effects on texture characteristics of restructured cooked hams.

| **Effects** | **Source of Variation** | **Hardness (N)** | **Adhesiveness (N×mm)** | **Springiness (mm)** | **Cohesiveness** | **Chewiness (N×mm)** |
|---|---|---|---|---|---|---|
| Sodium nitrite, | 0 | 10.70±1.76 | 0.11±0.03$^{a}$ | 4.28±0.28$^{a}$ | 0.47±0.05 | 21.69±5.75 |
| mg/kg | 50 | 10.94±1.77 | 0.10±0.03$^{a}$ | 3.94±0.33$^{b}$ | 0.46±0.05 | 20.24±6.02 |
| | 150 | 10.31±1.55 | 0.07±0.02$^{b}$ | 4.17±0.26$^{ab}$ | 0.45±0.04 | 19.49±5.17 |
| Irradiation | 0 | 10.95±2.10 | 0.09±0.02 | 4.23±0.29 | 0.47±0.06 | 22.28±7.44 |
| dose, kGy | 1.5 | 10.75±1.66 | 0.10±0.05 | 4.29±0.34 | 0.46±0.04 | 21.43±5.56 |
| | 3.0 | 9.98±1.90 | 0.09±0.03 | 3.90±0.24 | 0.45±0.02 | 17.60±3.64 |
| | 4.5 | 10.59±1.51 | 0.10±0.02 | 4.08±0.31 | 0.45±0.05 | 19.73±4.96 |
| | 6.0 | 10.98±1.29 | 0.08±0.03 | 4.15±0.32 | 0.46±0.06 | 21.32±5.78 |
| Pr>F[1] | N | *0.635* | ***0.007*** | ***0.010*** | *0.525* | *0.599* |
| | G | *0.770* | *0.674* | *0.073* | *0.939* | *0.500* |
| | N x G | *0.814* | *0.254* | *0.9354* | *0.877* | *0.846* |

[1]Significant values are shown in bold.

[a-b] Means in the same column, for each effect, followed by different letters differ ($P < 0.05$).

### *3.3. Sensory effects*

The results for sensory characteristics (odour, colour, flavor, and texture) of the products are shown in Table 4. The colour and texture of cooked hams were only affected ($P < 0.05$) by nitrite addition. The panelists failed to differentiate the texture and colour of samples where 50 mg/kg sodium nitrite was added (cured) from samples where 150 mg/kg (reference) was added, but these samples were significantly lower in colour and texture than the uncured samples ($P < 0.001$). These results contradict the statement of Byun et al (2002) that the desired colour could be obtained in irradiated uncured cooked hams or with the combination of irradiation and curing with low concentration (50 mg/kg) of sodium nitrite.

**Table 4.** Nitrite and gamma irradiation dose effects on scores of sensorial characteristics of restructured cooked hams.

| Effects | Source of Variation | Colour | Flavor | Odour | Texture |
|---|---|---|---|---|---|
| | Reference[1] | 1.5 ± 0.6 | 1.7 ± 0.83 | 1.5 ± 0.8 | 1.9 ± 1.0[b] |
| Sodium nitrite, | 0 | 4.3 ± 0.9[a] | 3.0 ± 1.04 | 3.1 ± 1.3 | 2.7 ± 1.2[a] |
| mg/kg | 50 | 1.7 ± 0.9[b] | 1.9 ± 0.95 | 2.0 ± 1.0 | 2.0 ± 1.1[b] |
| Irradiation dose, | 0 | 4.2 ± 0.9 | 3.2 ± 1.1 | 3.4 ± 1.4 | 2.8 ± 1.3 |
| kGy | 1.5 | 4.4 ± 0.8 | 2.9 ± 1.1 | 3.2 ± 1.2 | 2.7 ± 1.2 |
| | 3.0 | 4.0 ± 1.3 | 2.9 ± 0.8 | 2.5 ± 1.1 | 2.6 ± 0.9 |
| | 4.5 | 4.5 ± 0.9 | 2.7 ± 1.0 | 3.2 ± 1.3 | 2.7 ± 1.2 |
| | 6.0 | 4.5 ± 0.8 | 3.4 ± 1.2 | 2.9 ± 1.1 | 2.9 ± 1.1 |
| Pr>F[2] | N | ***<0.001*** | ***<0.001*** | ***<0.001*** | ***<0.001*** |
| | G | *0.439* | *0.310* | ***0.001*** | *0.483* |
| | N x G | *0.187* | ***0.002*** | ***0.043*** | *0.848* |

Scores:1 corresponded to "equal to the reference"; and 5 corresponded to the "extremely different from the reference".

[1]Non-radiated cooked ham elaborated with 150 mg/kg of sodium nitrite.

[2]Significant values are shown in bold.

For both odour and flavor, significant effects ($P < 0.05$) were observed for the interaction of nitrite level and irradiation dose, while cured samples (50 mg/kg) obtained scores closer to the reference (150 mg/kg) than uncured ones (Fig. 2).

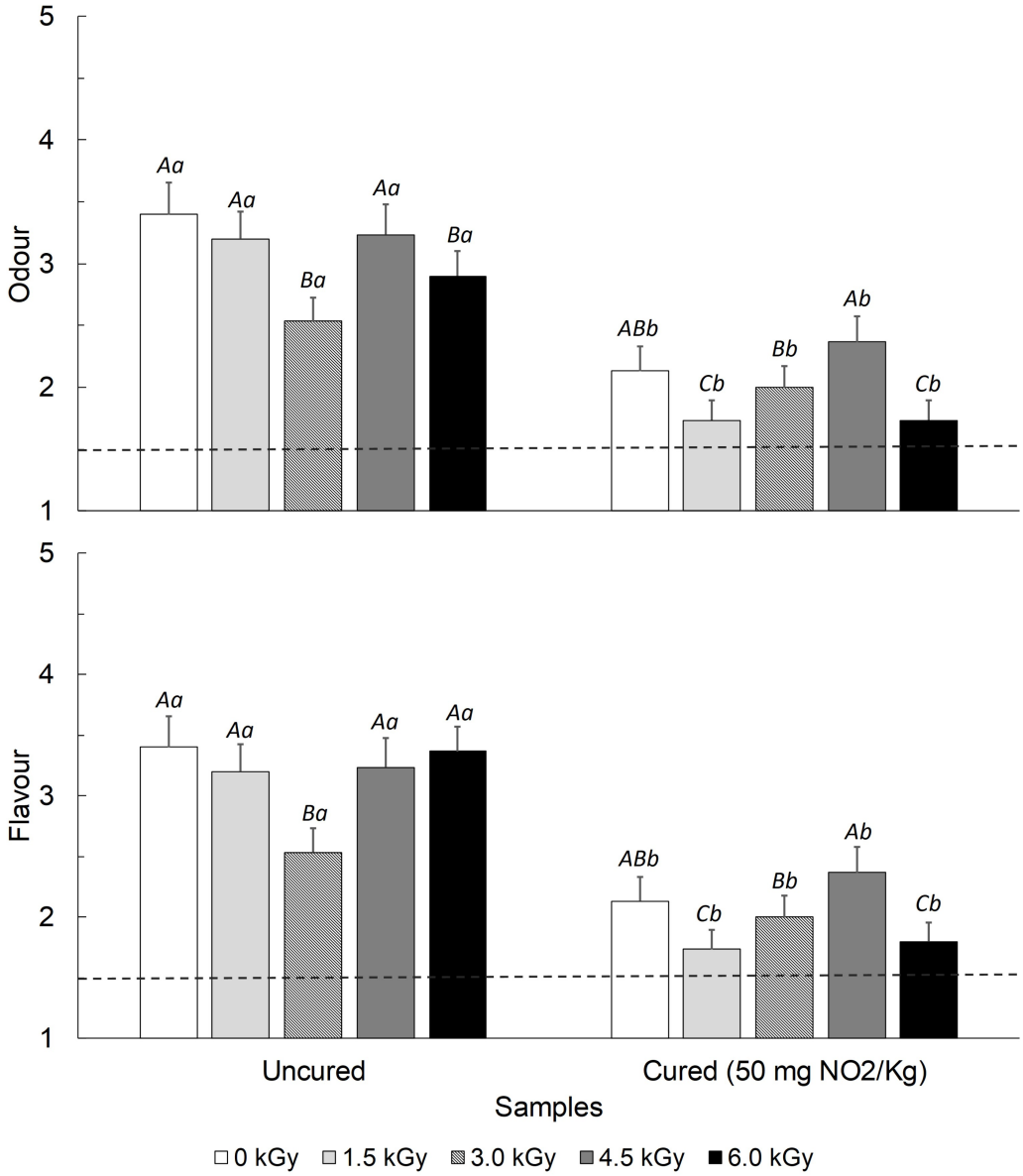


**Fig. 2.** Means of odour and flavor scores of cured (50 mg/kg of sodium nitrite added) and uncured (no sodium nitrite added) restructured cooked hams against a reference sample (un-radiated sample with 150 mg/kg of sodium nitrite added). Score 1 corresponded to "equal to

the reference" and score 5 corresponded to the "extremely different from the reference". The dotted line represents the means scores for coded reference sample against the reference. Bars (means a ± standard error of means) with a common superscript letter, lowercase (*ab*) for irradiation doses within samples (nitrite content) and uppercase (*AB*) for samples within each irradiation dose, do not differ ($P > 0.05$) by the Tukey test.

Overall, for both treatments, irradiation improved the sensorial attributes of the samples. Doses of 1.5 and 6.0 kGy improved the odour and flavor of cured samples, approaching those of the reference samples. For uncured cooked hams, the same improvement was achieved in samples irradiated with 3 kGy for odour and either 3 kGy or 6 kGy for flavor. Although these findings corroborate the observation of Byun et al. (2002) that the flavor of uncured ham or with a low concentration of sodium nitrite was improved by irradiation without adverse effects, the uncured irradiated samples were not even able to reach the scores obtained for the cured ones.

## 4. Conclusions

Gamma irradiation was effective in reducing the *Clostridium sporogens* spores and the residual nitrite levels, without any significant effects on the other physicochemical and technological parameters of cooked hams. In uncured hams, the colour was more yellowish than cured ones, as detected by the sensory panellists. Also, irradiation improved the odour and flavor of the hams, with the low cured (50 mg/kg sodium nitrite) samples obtaining scores closer to the reference (150 mg/kg sodium nitrite) than the uncured samples.

These results also indicate that gamma irradiation of cooked hams only 6 kGy elaborated with 50 mg/kg sodium nitrite can be used as an alternative to add 50 mg/kg

sodium nitrite, not only to ensure microbiological safety but also to improve the product sensory quality.

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**ARTICLE 2**

Article drafted in accordance with the Innovative Food Science and Emerging Technologies journal standard

"Preliminary version"

# Effects of Gamma-irradiation on oxidative and toxicological parameters of restructured cooked ham formulated with different concentrations of sodium nitrite


## Abstract

*Irradiation treatment of food is a non-thermal processing technique to improve food safety and preservation. Most of the chemical constituents of food absorb irradiation that can lead to chemical modifications and quality changes. The aim this study was to investigate the effects of different doses (0, 1.5, 3, 4.5 and 6 KGy) of gamma irradiation treatment on restructured ham, cured with different levels of sodium nitrite (0, 50 and 150 mg/kg) on and protein oxidation, and potential cytotoxic and genotoxic effects on intestinal epithelial*

*cells in vitro. Irradiation induced slight protein damage, characterized by the total sulfhydryl group reduction. However, gamma irradiation did not cause any increase in the cytotoxic or genotoxic (DNA strand breaks) effects in intestinal Caco-2 cells.*

**Keywords:** *Cytotoxicity, genotoxicity, protein oxidation, lipid oxidation, quality*

## 1. Introduction

The term ‘‘cured’’ is defined, in relation to meat products, to mean the addition of nitrite and/or nitrite (Sebranek & Bakus, 2007). Different ingredients including sugar, spices, phosphates and others are typically included in cured meats, but it is the addition of nitrite in one form or another that results in the distinctive characteristics such as the typical color, flavor, shelf life and safety for all cured meat products that are recognized by consumers (Cassens, 1995; Dutra, Ramos, Ramos, Fontes, Cardoso & Leal, 2011; Sindelar & Milkowski, 2011).

Nitrite is strongly inhibitory to anaerobic bacteria, most importantly *Clostridium botulinum*, and contributes to control of other microorganisms such as *Listeria monocytogenes*. The effects of nitrite its inhibitory mechanism differ between different bacterial species (Tompkin, 2005). Therefore, nitrite can act as a nitrosating agent in the formation of N-nitroso compounds (Cassens, 1997). N-nitroso compounds belong to six fundamental categories: volatile N-nitrosamine, non-volatile N-nitrosamine, N-nitrosamide, N-nitrosated heterocyclic carboxylic products, N-nitrosated glycosylamines, and Amadori compounds (Hiramoto, Kido, & Kikugawa, 1993). Several epidemiological studies have demonstrated a potential relationship between nitrate, nitrite, and N-nitroso compounds and the risk of cancer (Alexander & Cushing, 2011).

The challenge for the meat industry is to search for strategies to reduce supplemented and residual nitrite in cured meat in order to minimize the nitrite intake. There is a considerable interest in the development of alternatives from natural sources and other preservation techniques that are considered to be comparatively healthier. This interest is further accelerated by the pressure generated from consumer demand for salt- and nitrite-reduced meat products. So gamma irradiation has been studied as an alternative to reduce the quantity of nitrite and maintain shelf-life in cured meat products including ham, mortadella and sausage (Szczawinski & Szulc, 1989; Ahn, Jo, Lee, Kim & Byun, 2003; Dutra et al., 2016; Dutra et. al, 2017).

Prior studies measured *in vitro* cytotoxicity and genotoxicity in foods: retention of antimutagenic activity in high pressure treated fruits and vegetables (Butz, Garcia, Lindauer, Dieterich, Bognar & Tauscher, 2003); the innocuous effect of ultraviolet C (UV-C) on the mutagenicity of meat products (Sommers, Geveke, Pulsfus & Lemmenes, 2009); effects of UV-C treatment of liquid egg products on lipid and protein oxidation and potential cyto- and genotoxic effects on intestinal epithelial cells *in vitro* (Souza, Briviba, Müller, Fernández & Stahl, 2013); and evaluation of the toxicological and radiological safety of chicken meat that had been irradiated at 30 kGy with 7.5 MeV X-rays (Song et al., 2017).

Therefore, the objective of this study was to investigate the effects of gamma-irradiation in restructured cooked ham formulated with different concentrations of sodium nitrite (0, 50 and 150 mg/kg) on the potential cytotoxic and genotoxic effects on intestinal epithelial cells *in vitro* and on protein and lipid oxidation of the hams.

## 2. Materials and methods

### *2.1. Preparation of the restructured cooked ham*

The products were prepared at the Laboratory of Meat Science and Technology (LabCarnes) at the Department of Food Sciences of the Federal University of Lavras (DCA/UFLA), Brazil. Restructured hams were manufactured and irradiated according to Restructured hams were manufactured according to a Brazilian traditional formula as described by Dutra, Cardoso, Ramos, Ramos, Pinheiro & Fontes (2012). Three batches of each product were formulated with 56% lean boneless pork ham (14 mm grinder), 35% water and a commercial mixture of ingredients (New Max Industrial Ltd.; Americana, SP, Brazil) containing: sodium chloride (1.4%); cassava starch (2.0%); soy isolated protein (2.0%); maltodextrin (1.1%); carrageen (0.5%); monosodium glutamate (0.3%); sodium erythorbate (0.06%); sodium nitrite (0, 50 and 150 mg/kg, as previously described); sodium polyphosphate (0.5%); and spices (0.8%). The materials were mixed, and the meat batter was vacuum packed (TM300, TecMaq, São Paulo, SP, Brazil) in flexible multilayer film (SpelFlex/E; Spel Embalagens, São Paulo, Brazil), formed into 1 kg metallic forms and stored (4 °C) for 12 h. After curing, samples were cooked in a water-bath until the internal temperature of the product reached 72 °C (checked with a flexible thermocouple probe), cooled in an ice water-bath and stored at 4 °C for 24 h. Samples were unpacked, cut into 2.5 cm thick portions, weighed and vacuum packaged again in nylon-poly bags prior to irradiation.

### *2.2. Irradiation process*

All hams (packaged, cooked and stored at 4 °C) were placed in Styrofoam thermal boxes (Kanauf Isopor, São Paulo, Brazil) and subjected to different irradiation doses (1.5, 3.0, 4.5 and 6.0 kGy) in a Gamma GB-127 Irradiator (IR-214, MDS Nordion, Ottawa, Canada; with cobalt-60 sources and flow rate of 5 kGy/h) at the Laboratory of Gamma

Irradiation in the Center for Nuclear Technology Development (CDTN) of the National Commission of Nuclear Energy (CENEN). The non-irradiated samples (0 kGy) were maintained under the same conditions and for similar periods of time as the irradiated samples. The entire irradiation process lasted 48 hours after the samples were cooked, being samples stored at 4 °C until analysis.

### *2.3. Cyto and genotoxicity*

#### *2.3.1 Caco-2 cell culture*

The human colon adenocarcinoma cell line Caco-2 was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cells were routinely cultivated in 75 $cm^2$ cell culture flasks from Corning (Corning, USA) in EMEM containing 10 % (v/v) FCS, 1 % NEAA, 1 % glutamine, 50 $units.mL^{-1}$ penicillin G/50 $\mu g.mL^{-1}$ streptomycin. Cells were maintained in a humidified atmosphere with 5 % $CO_2$ at 37 °C. Cell culture medium was replaced three times.

#### *2.3.2. Cytotoxicity in cell viability assays*

Calcein assay Caco-2 cells were seeded into 96-well cell culture plates (4500 cells/well) and grown in EMEM containing 10 % (v/v) FCS, 1 % NEAA, 1% glutamine, 50 $units.mL^{-1}$ penicillin G/50 $\mu g.mL^{-1}$ streptomycin until they reached confluence. Subsequently, cells were incubated during 24 h in a 96-well plate (in a humidified atmosphere with 5 % $CO_2$ at 37 °C) with ham diluted to 2.5 %, 5 %, 10 % and 20 % (v/v) with cell culture medium. The effect of ham on cell viability was determined by the calcein assay. According to the manufacturer's protocol cells were incubated in the presence of calcein-AM (4 μM) for 45

min. Thereafter, the fluorescence was measured at 485 nm (extinction) and 535 nm (emission) using a microplate reader (Biosystems, Bioteck Instruments, Model EPOCH, Brazil). Incubation of cells with hydrochloric acid/methanol in the medium for 30 min was used as a positive control.

### *2.3.3. Genotoxicity: Comet assay (DNA damage)*

DNA strand breaks were determined by the Comet assay (single cell gel electrophoresis assay) as described by Souza et al., 2012. Caco-2 cells were seeded into 6-well cell culture plates (105 cells/well) and grown in EMEM containing 10 % (v/v) FCS, 1 % NEAA, 1 % glutamine, 50 units.mL$^{-1}$ penicillin G/50 µg.mL$^{-1}$ streptomycin until they reached confluence. Thereafter, cells were incubated for 24 h with Ham diluted to 5 % (v/v) 112 with cell culture medium. Control cells were incubated in medium only. Incubation of cells with hydrogen peroxide (30 µM) for 60 min was used as positive control. After treatment, the cells were washed with PBS, trypsinized, and viability was determined microscopically by the trypan blue exclusion method.

Cells (2 x 10$^5$) were mixed with 85 µL low melting point agarose and placed between two layers of agarose on a microscopic slide. After lysis (lysis solution: 100 mM Na2EDTA, 1 % Triton X 100, 2.5 mM NaCl, 1 % (w/v) lauroyl sarcosine sodium salt, 10 mM Tris, 10 % DMSO) followed electrophoresis for 1 h (gel: Gel tray, Renner; electrophoresis buffer: 1 mM Na2EDTA, 300 mM NaOH, pH 13; 25 V, 300 mA, 40 min). DNA was stained with 100 µL ethidium bromide solution (20 µg.mL$^{-1}$). The percentage of fluorescence in the tail (tail intensity, %) was assessed fluorimetrically (Biosystems, Bioteck Instruments, Model FLX800, Brazil) and then quantified using Perceptive Instruments imaging software (Halstead, UK).

### *2.4. Protein oxidation*

Protein oxidation, as measured by total carbonyl content, was evaluated by derivatization with dinitrophenylhydrazine (DNPH) according to the method described by Oliver, Ahn, Moerman, Goldstein, and Stadtman (1987) with slight modifications (Ganhão, Morcuende, et al., 2010). Samples (1 g) were minced and then homogenized 1:10 (w/v) in 20 mM sodium phosphate buffer containing 0.6 M NaCl (pH 6.5) using an ultraturrax homogenizer for 30 s. Two equal aliquots of 0.2 mL were taken from the homogenates and dispensed in 2 mL Eppendorf tubes. Proteins were precipitated by cold 10% TCA (1 mL) and subsequently centrifuged for 5 min at 620 *g*. One pellet was treated with 1 mL 2 M HCl (protein concentration measurement) and the other with an equal volume of 0.2% (w/v) DNPH in 2 M HCl (carbonyl concentration measurement). Both samples were incubated for 1 h at room temperature. Afterward, samples were precipitated by 10% TCA (1 mL) and washed twice with 1 mL ethanol: ethyl acetate (1:1, v/v) to remove excess DNPH. The pellets were then dissolved in 1.5 mL of 20 mM sodium phosphate buffer containing 6 M guanidine HCl (pH 6.5), stirred and centrifuged for 2 min at 620 *g* to remove insoluble fragments. Protein concentration was calculated from absorption at 280 nm using bovine serum albumin (BSA) as standard. A number of carbonyls were expressed as nmol of carbonyl per mg of protein using an absorption coefficient of 21.0 $nM^{-1}$ $cm^{-1}$ at 370 nm for protein hydrazones.

### *2.5. Statistical Analysis*

The experiment was conducted in a completely randomized design with three repetitions. For analysis, the treatments were arranged in a split-plot design, with the 3 (nitrite added) x 5 (irradiation doses) as whole plot.

The resulting data were tested by an F-test (ANOVA) and when significative ($P < 0.05$) means were separated by the Tukey test. All statistical analysis was conducted using the SAS statistical (SAS Institute Inc., Cary, NC, USA) package, version 9.2.

## 3. Results and Discussion

### *3.1. Effects on the vitality of the intestinal Caco-2 cells*

The viability of Caco-2 cells was different between cured and uncured restructured ham (Fig. 1), it being higher fluorescence for samples uncured. It was observed difference on cell viability to ham concentration, in low concentrations of ham the fluorescence was higher than high concentrations of ham. According to results irradiation had no effect ($P > 0.05$) on the vitality of the intestinal Caco-2 cells. However, the concentration of nitrite had an effect ($P < 0.05$).

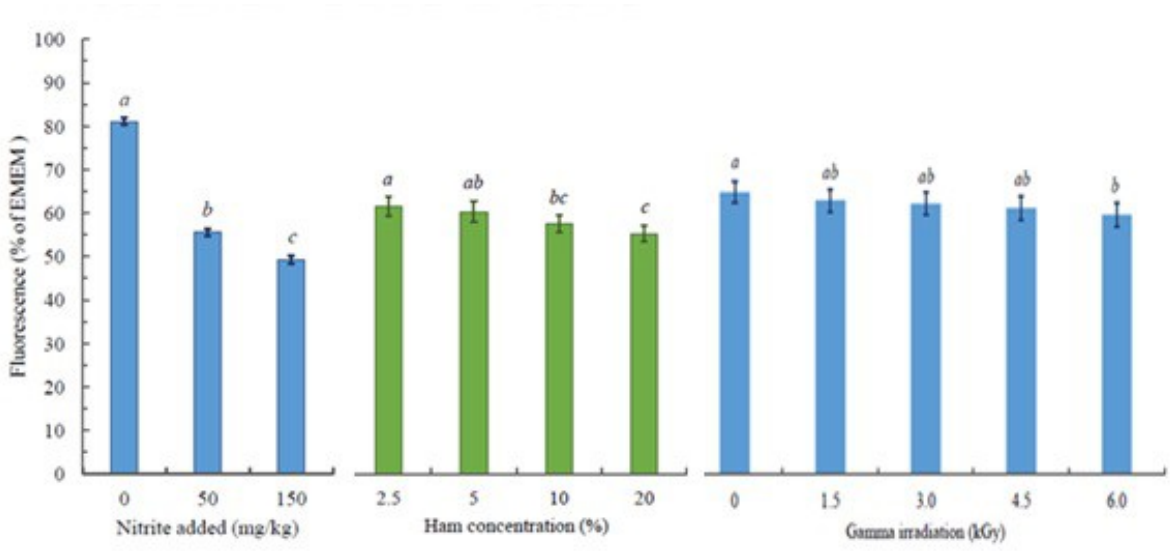

**Fig. 1.** Effect of sodium nitrite (a) and ham concentration (b) and gamma irradiation (c) in restructured cooked ham on the viability of Caco-2 cells. Control cells were incubated in medium only (EMEM). Results are the mean of triplicate ± standard deviation. Different superscripts for each concentration and control indicate difference statically significant at 95% confidence level.

Gamma irradiation can impair oxidative stress due to the formation of ions and free radicals, and might also accelerate the oxidation of important food components (proteins and lipids). Regarding the oxidative damage in cooked ham, the impact of gamma irradiation treatment is relatively low, but oxidized residues might have physiological implications which can be assessed *in vitro*. The production of furan in other food systems, such as UV-C treated apple juice and cider (Bule et al., 2010; Fan & Geveke, 2007) is of main concern, being mandatory to discard cytotoxic effects in UV-C decontaminated food products. According to Feng & Ahn (2016), irradiation processing resulted in the formation of new volatile compounds, including heptane, trans-1-butyl-2-methylcyclopropanone, 2-octene, and toluene in the ham, and 2-butanone in frankfurters. Therefore, ham components increasingly affected cell metabolism, resulting in cellular death. Several mechanisms could be implied. For example, low cytotoxic effects have been reported for pork meat patties in contact with Caco-2 cells after simulated gastric digestion (Kenny, O'Callaghan, & O'Brien, 2008).

Remarkably, differences in cytotoxicity between non-treated and gamma-irradiated cooked ham preparations were not statistically significant ($P < 0.05$). Consequently, the presence of new cytotoxic compounds or variations in the concentration of essential food components cannot be confirmed at the investigated treatment levels *in vitro*.

However, according to Souza (2012), the presence of new cytotoxic compounds, or variations in the concentration of essential food components, cannot be confirmed at the

investigated treatment levels *in vitro*. Further chemical analyses are however recommended to completely discard the presence of contaminants.

### *3.2 Effects on DNA damage*

For restructured hams, the incubation of cells with different doses of irradiation (0, 1.5, 3, 4.5, 6 KGy), did not significantly increase DNA strand breaks in Caco-2 cells ($P > 0.05$).By contrast, the curing of restructured ham using different sodium nitrite concentrations (50 and 150 mg/kg) had a significant effect ($P < 0.05$) upon DNA strand breaks (Fig. 2).

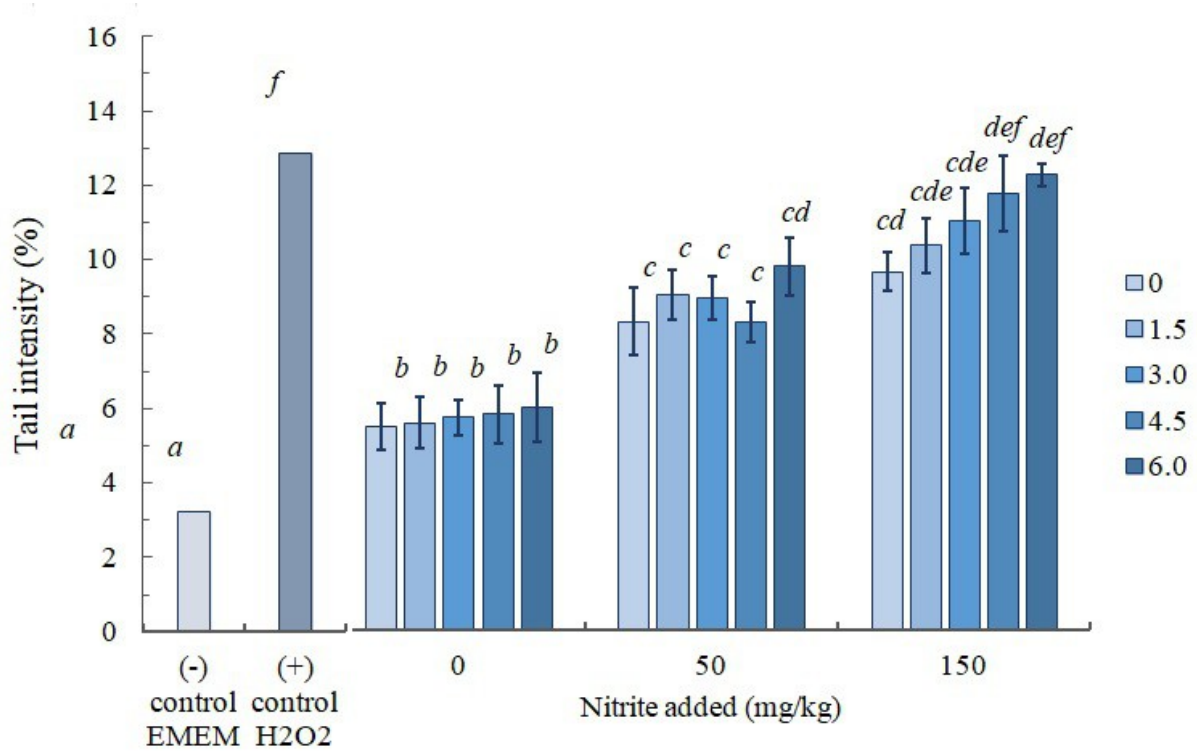


**Fig. 2.** Effect of gamma irradiation in restructured cooked ham with different concentrations of sodium nitrite addition on DNA strand breaks (% Tail intensity) in Caco-2 cells. Results are the mean of triplicates ± standard deviation. Different superscripts for bars indicate statistically significant differences between means at 95% confidence level.

Song et al. (2017) investigated chicken meat irradiated at 30 kGy with 7.5 MeV X-rays and did not observe any genotoxic effect. According to this study and that of Jung, Huang, Song, Byun & Kang (2014), this occurred as a result of the bacterial reverse-mutagen, the *in vitro* chromosomal aberration and *in vivo* micronucleus assays exhibiting dose-independent responses, similar to those of the negative control.

According to EC (2003), 60 studies on the induction of mutagenesis by irradiated foods were reviewed by the FDA and 20 were designed to assess the potential to induce dominant lethal mutations in rats and mice. Consequently, some reported positive results after feeding wheat irradiated at 0.75 kGy; while other studies, using much higher doses, gave negative results. However, many studies were concerned with investigating the production of polyploidy following the reported observation that malnourished Indian children consuming wheat irradiated at 0.75 kGy for 4-6 weeks showed polyploid cells in their blood.

In contrast, no mutagenic activity was detected in earlier studies with *Drosophila melanogaster* and mice fed chicken meat irradiated at 55.8 and 59 kGy (Lusskin, 1979).

According to the present study, nitrite can produce cytogenetic and genotoxic effects in restructured ham. It is therefore important to observe that the mutagenic potential of commercially processed food products depends on many factors: cooking conditions (i.e. temperature and time), equipment used and ingredients; as attested by considerable differences in the mutagenic activity among equivalent products from different manufacturers or restaurants (Knize, Dolbeare, Carroll, Moore, & Felton, 1994; Tikkanen, Sauri, & Latva-Kala, 1993).

### *3.3 Protein oxidation*

Gamma irradiation increased ($P < 0.05$) protein oxidation in restructured ham. Howeverdifferent levels of sodium nitrite had no effect on protein oxidation (Fig. 3). Many factors can affect the extent of protein oxidation in meat systems including the temperature of the system, packaging strategy, oxygen availability, protein concentration, and irradiation dose and rate, among others (Giroux & Lacroix, 1998; Lee & Song 2002).

Although irradiation of food up to overall dosage of 10 kGy has been identified to present no toxicological or nutritional hazard to the consuming public, irradiation of meat at this dose range has been found to result in various biochemical changes, including disruption in the ordered structure of protein (change in tertiary and secondary structure of protein) along with fragmentation, crosslinking, and aggregation of protein polypeptide chains (Giroux & Lacroix 1998; Lee & Song 2002).

Since water is similarly a major component of meat, ionizing radiation also triggers its radiolysis, releasing free radical species for initiation of protein oxidation in meat (Giroux & Lacroix 1998). Among the various major products formed by irradiation of protein, the main materials are carbonyl groups, free amino acids, hydrogen peroxides, and organic peroxides (Brault, D'Aprano & Lacroix, 1997). Sulfur-containing compounds, as well as aromatic amino acids, are the most susceptible to irradiation damage (Giroux & Lacroix, 1998).

Several other protein oxidation markers derived from these susceptible amino acids could be identified in meat systems following the irradiation process (i.e., formylnurenine from tryptophan and 3,4-dihydrophenylalanine from tyrosine, among others). Other studies have observed increased protein oxidation in poultry (Xiao, Zhang, Lee, Ma, & Ahn, 2011) and beef meat (Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004) with exposure to irradiation.

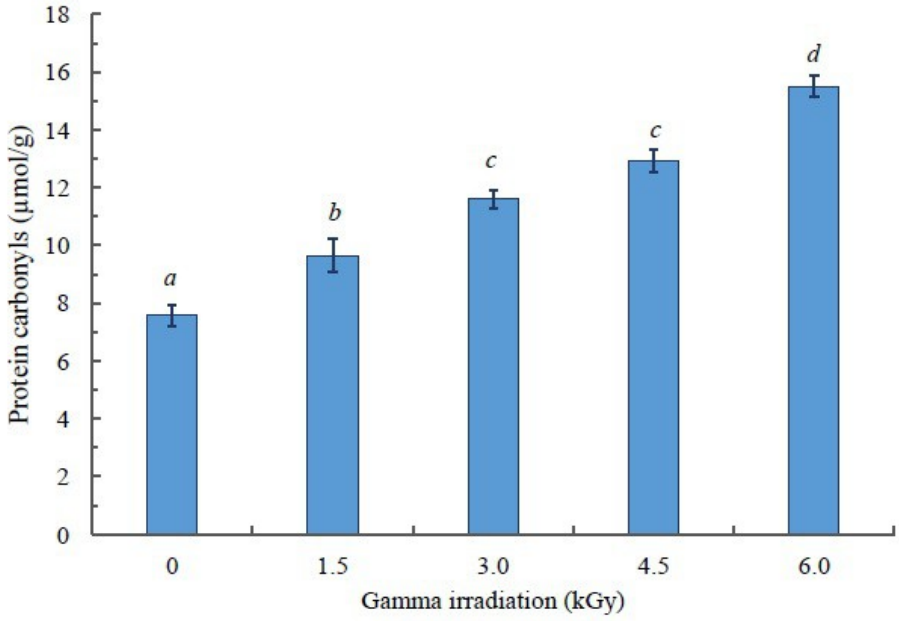


**Fig. 3.** Effect of different doses of gamma irradiation in restructured ham on the protein oxidation. Results are the means of triplicate ± standard deviation. Different superscripts for bars indicate statistically significant differences at a 95% confidence level.

## 4. Conclusion

According to the results obtained, it can be confirmed that gamma irradiation treatment is a promising and safe technology for restructured cooked ham with different concentrations of sodium nitrite. However, protein oxidation increased with doses of irradiation. This is in agreement with the low oxidative defects found for proteins after irradiation, confirming the gamma irradiation decontamination system of pork products as a feasible application. Irradiation did not cause measurable changes in the cytotoxic or genotoxic effects of restructured cooked ham.

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# ARTICLE 3

Article drafted in accordance with the Meat Science journal standard

"Preliminary version"

## Reducing sliced safety risk of low-nitrite restructured cooked ham by gamma radiation


## Abstract

*The aim of this study was to evaluate the effects of gamma radiation (0.5 to 2.0 kGy) on the survival of Listeria monocytogenes and on technological characteristics of sliced restructured cooked hams, cured with different levels (0, 50 and 150 mg/kg) of sodium nitrite. Irradiation doses reduced the amount of L. monocytogenes, independent to the addition of sodium nitrite, with a lethality of up to 7 log reductions. Gamma irradiation had no effect on the pH, residual nitrite, TBARS, water activity or CIE color parameters of ready-to-eat ham. The results indicated that 1.5 kGy irradiation combined with low nitrite level (50 mg/kg) of RTE ham can be used to improve its quality and ensure its microbiological safety against Listeria monocytogenes.*


**Keywords:** *Listeria monocytogenes, ready-to-eat product, lipid oxidation.*

## 1. Introduction

Around the world, food preservation and production techniques are being studied, including genetic engineering, modified atmospheric packaging, and irradiation. Food contamination by pathogenic microorganisms is a significant problem that can lead to

spoilage and deteriorate the quality of food products and, or, cause infection and disease (Lv et al., 2011; Celiktas et al., 2007). A major concern specifically associated with meat products would be the post-thermal manipulation occurring in slicing operations of ready-to-eat products, consumed without further cooking, which may result in an enhanced risk (sliced safety risk) and increase spoilage rates. For refrigerated sliced vacuum-packed processed meats, pathogens such as psychotropic *Listeria monocytogenes* and spoilage flora mainly composed of lactic acid bacteria represents the core control targets (EFSA, 2009; Oliveira et al., 2015).

*Listeria monocytogenes*is a gram-positive, motile, rod-shaped bacterium (Iannetti et al., 2016); which can cause severe foodborne infection (listeriosis) at a rate of 0.44 cases per 100,000 people in the European Union in 2013, 8.6% greater than the rate from 2012 (EFSA, 2015). The ability of *L. monocytogenes* to multiply in various foods at temperatures as low as 2 °C makes its occurrence in foods characterized by a long shelf-life at refrigeration temperature of particular concern (Luber, 2011). A considerable number of studies EFSA/ECDC, 2015b; Terentjeva et al., 2015) demonstrated that ready-to-eat meat products are one of the main sources of listeriosis for humans.

Growth of *L. monocytogenes* in ready-to-eat meats is possible at refrigeration temperatures and under anaerobic environmental conditions but is slowed by nitrite salt used in cured products (Duffy et al., 1994; Krause et al., 2011; Lee et al., 2018). Nitrite salts are preservative food additives used extensively to enhance the meat colour and flavor, to retard the development of rancidity and off-odours and off-flavors and to extend the shelf life during storage (Sindelar & Milkowski, 2011). However, the replacement or at least reduction of the amount of nitrite applied in the meat curing process has been suggested, due to its association with potentially carcinogenic compounds (N-nitrosamines) formation (Eichholzer & Gutzwiller, 2003; Abid, Cross, & Sinha, 2014). Beyond the effects on the sensory aspects,

this additive plays a key role on the microbiological stability of meat products, and its exclusion or even content reduction involves meat safety concerns (Article 01), specially its preservative action against *Clostridium botulinum* and its toxin (Dutra et al., 2016; Sindelar & Milkowski, 2011).

An alternative approach to reduce nitrite addition in meat products without enhance the safety risk or spoilage rates is the use of gamma radiation (Dutra et al., 2016), a non-thermal treatment recognized as the best technology for the destruction of pathogenic and deteriorating microorganisms in food. Irradiation is an efficient process for reducing or eliminating the growth of *L. monocytogenes* post-packaging and therefore assuring food safety (Thayer & Boyd, 2000; Feliciano et al., 2014).

The aim of this study was to determine the effects of a low dose (< 2 kGy) of gamma radiation on the survival of *L. monocytogenes* inoculated into restructured cooked ham elaborated with different contents of sodium nitrite, as well as on the technological characteristics of the products.

## 2. Material and methods

### *2.1. Microorganism and inoculum production*

The microorganism used was *Listeria monocytogenes* (ATCC 19117), purchased from the Collection Section of Cultures (Division of Medical Biology, Adolfo Lutz Institute, São Paulo, Brazil). The lyophilized culture was reactivated in HIMEDIA® brain heart infusion broth supplemented with 0.6% HIMEDIA® yeast extract (BHI-YE) and incubated at 37 °C for 24 hours. After incubation, the above procedure was repeated for a second cell activation. The bacterial cells were pelleted by centrifugation (605*g*/5 min) in microtubes and covered

by freezing culture media (15% glycerol, 0.5% bacteriologicall peptone, 0.3% yeast extract and 0.5% NaCl; pH 7.2-7.4). Stock cultures were kept frozen (-20 °C) throughout the experiment.

For work culture production prior to inoculations, an aliquot was transferred to BHI-YE media grown and incubated at 37°C. The overnight cultures were centrifuged (605*g*/5 min) and the pelleted cells resuspended with saline solution 0.85% (w/v). The cells were washed 2x with saline solution 0.85% (w/v), before being resuspended in a 50 mL of saline solution 0.85% (w/v) that was standardized by the McFarland Standard Scale to 107 CFU/mL. The cell suspensions were plated on BHI-YE for exact population quantification and confirmation prior inoculation to sliced restructured cooked hams.

### *2.2. Restructured cooked ham formulation*

The meat product used to conduct the studies was the restructured, cured and cooked ham, commercially available in Brazil with commercial name of "Apresuntado". The products were elaborated to contain different levels (0, 50 and 150 mg/kg) of sodium nitrite.

Hams were prepared at the Laboratory of Meat Science and Technology (LabCarnes) at the Department of Food Sciences of the Federal University of Lavras (DCA/UFLA), Brazil. Restructured hams were manufactured according to a traditional formula as described by Dutra, Cardoso, Ramos, Ramos, Pinheiro & Fontes (2012). Three batches of each product were formulated with 56% lean boneless pork ham (ground in a 14 mm disc), 35% water and a commercial mixture of ingredients (New Max Industrial Ltd.; Americana, SP, Brazil) containing: sodium chloride (1.4%); cassava starch (2.0%); soy isolated protein (2.0%); maltodextrin (1.1%); carrageen (0.5%); monosodium glutamate (0.3%); sodium erythorbate (0.06%); sodium nitrite (0, 50 or 150 mg/kg, as previously described); sodium polyphosphate

(0.5%); and spices (0.8%). The materials were mixed, and the meat batter vacuum packed (TM300, TecMaq, São Paulo, SP, Brazil) in flexible multilayer film (SpelFlex/E; Spel Embalagens, São Paulo, Brazil) and formed into 1 kg metallic shapes. The products were stored at 4°C for 12 h for curing before cooking in a water bath until the internal temperature of product reached 72 °C (checked with a flexible thermocouple probe). The cooked hams were cooled in an ice water-bath for 10 min and stored (at 4 °C) for further procedures.

### *2.3. Sample slicing and inoculation*

After 24 h of storage (4 °C) post-processing, the restructured cooked hams were aseptically opened, sliced and packaged according to the analysis purposes. For the technological analysis, the analytical units were composed of three slices of 5-mm thickness, which were vacuum-packaged in nylon-poly bags (Coex Ind. e Com. Ltda, PR, Brazil) and appropriately identified. For microbial inactivation purposes, 5-mm thickness slices (approximately 10 g) were placed in an aseptic laminar flow chamber under UV light for 10 min on each side to eliminate the residual flora that might influence the tests. Thus, 1 mL of the standardized cell suspension (obtained as described in item 2.1) was spread on each slice and dried for 15 min (in the laminar flow chamber without UV light) prior to vacuum packaging in nylon-poly bags. The microbiological analytical units were composed of three slices of 5-mm thickness (with $10^6$ CFU/g). Samples were kept under refrigeration (4 °C) for 24 hours until radiation process.

### *2.4. Gamma radiation process*

The sliced packaged cooked hams (at 4 °C) were placed into styrofoam thermal boxes (Kanauf Isopor, São Paulo, Brazil) and subjected to different gamma irradiation doses (0, 0.5, 1.0, 1.5 and 2.0 kGy) by a GammaBeam-127 gamma irradiator (IR-214, MDS Nordion, Ottawa, Canada; with cobalt-60 source and flow rate of 1.218 kGy/h) at the Laboratory of Gamma Irradiation at the Center for Nuclear Technology Development, National Nuclear Energy Commission (CDTN/CENEN). Non-irradiated samples were maintained under the same conditions and for similar periods of time as the irradiated samples.

### *2.5. Enumeration of Listeria monocytogenes*

The enumeration of *Listeria monocytogenes* was carried following 1 and 30 days of storage at 4 °C post radiation processing. The traditional methodology of the FDA (Food and Drug Administration), described by Hitchins (2003), was used for the enumeration of *Listeria monocytogenes*. The packages were opened aseptically, and samples were homogenized in 225 mL 0.1% m/v peptone water in a Stomacher Metroterm® homogenizer (490 strokes/min) for three minutes. After serial decimal dilutions in 0.1% peptone water, 100 µL aliquots were sowed from the surface in Oxford agar. The analyses were performed in triplicate and the plates were incubated at 37 °C/24-48 h. After incubation, the survivor populations were calculated with the results expressed as a log CFU/g.

### *2.6. Chemical and physicochemical analyses*

The quality and technology attributes were evaluated included pH, water activity (aw), residual nitrite content ($NO_2R$), lipid oxidation (TBARS index) and CIE colour after 24 h of refrigerated storage (4 °C) post radiation processing.

The water activity (aw) was determined directly (at 25 °C) using an AquaLab® water activity meter (model 4TE; Dacagon Devices, Inc., USA) following manufacturer recommendations. The pH measurements were obtained using a potentiometer (Digimed, modelo DM 20, São Paulo, Brasil) after initial homogenization of samples in distilled water at a ratio of 1:10 (Matos et al., 2007).

The residual nitrite content, expressed as sodium nitrite (mg/kg), was quantified according to official method no. 973.31 of the Association of Official Analytical Chemists (AOAC, 2005).

The effect of irradiation treatments on lipid oxidation was determined by the thiobarbituric acid reactive substances (TBARS) index according to the methodology proposed by Raharjo, Sofos, & Schmidt (1992), with some adaptations. Approximately 2 g of sample was homogenized in 10 mL of 5% trichloroacetic acid (TCA) and 1 mL of 0.15% butylhydroxytoluene (BHT) and 10 mL of 0.08 M thiobarbituric acid (TBA). The aliquot was incubated in a boiling water bath for 5 min, and then 5 min in cold water. The extract was centrifuged (3,000*g*/15 min) and the absorbance of the supernatant read at 531 nm. The malondialdehyde (MDA) concentration was determined from an analytical curve with 1,1,3,3-tetraethoxypropane (TEP), and the results were expressed as mg MDA/kg of sample.

The CIE colour of the cooked hams was evaluated 1 day of storage at 4 °C post radiation processing. Colour measurements were taken with a CM-700d colorimeter-spectrophotometer (Konica Minolta Sensing Inc., Osaka, Japan), with 8 mm aperture and using a specular component excluded (SCE) mode, a D65 standard illuminant and an observer angle of 10°. CIE lightness ($L^*$), redness ($a^*$) and yellowness ($b^*$) were recorded from the average of five readings taken from various surface points of ham slices. Samples colour were expressed in the CIELCH space, where the angular coordinates of chroma ($C^*$) and hue angle ($h$, degree) were calculated using the following formulas: $C^* = (a^{*2} + b^{*2})^{0.5}$;

and $h$ = arctan ($b^*/a^*$). Higher $C^*$ values suggesting a more vivid colour, and $h$ values near 0 denoting red and near 90 denoting yellow tons (Ramos & Gomide, 2017).

### *2.7. Statistical Analysis*

The treatments were arranged in a split-plot design, with the 3 (nitrite added) x 5 (irradiation doses) factorial as a whole plot, being conducted in a completely randomized design with three repetitions. The resulting data were tested by an F-test (ANOVA) at a significance level of 5%, and means were separated by the Tukey test. All statistical analysis was conducted using the SAS statistical package (SAS Institute Inc., Cary, NC, USA), version 9.2.

## 3. Results and Discussion

### *3.1 Effects on Listeria monocytogenes growth*

The *L. monocytogenes* growth was affected ($P < 0.05$) by the interactions nitrite added x storage time and by nitrite added x radiation dose.. The growth of *L. monocytogenes* was reduced ($P < 0.05$) with higher gamma irradiation doses, being this reduction greater ($P < 0.05$) in the cured samples (with 50 or 150 mg/kg) when doses above 1.0 kGy were used (Fig. 1).. Samples irradiated at 1.5 kGy resulted in complete lethality of listeria on cooked hams with 150 ppm of sodium nitrite, while at 2.0 kGy no listeria survived to the applied treatment, independent of the sodium nitrite content.

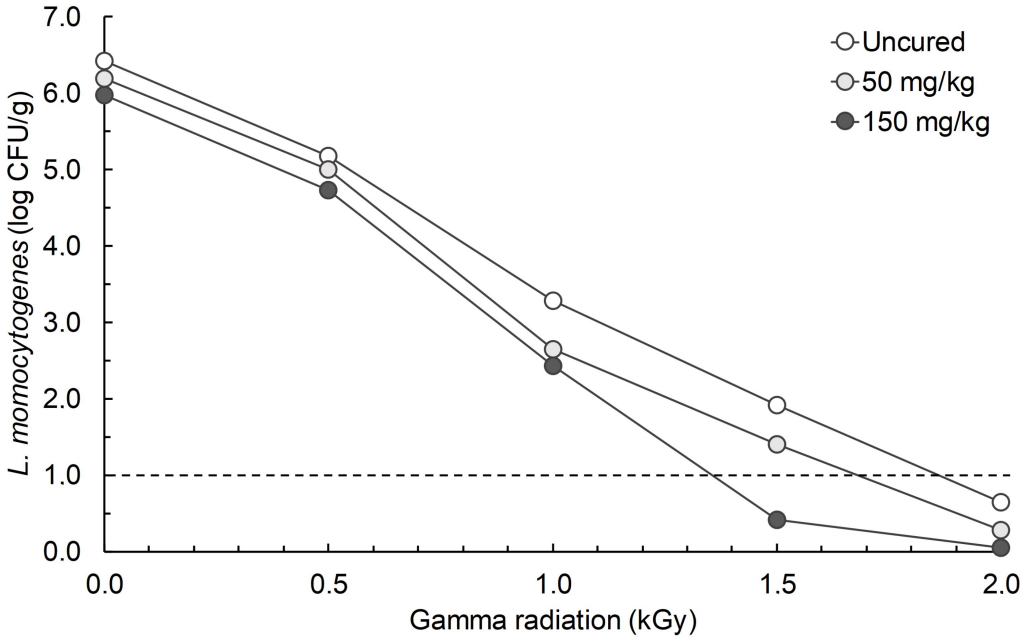


**Fig 1.** Effect of gamma irradiation dose (kGy) on the *Listeria monocytogenes* growth in vacuum-packed, cooked hams slices with (50 and 150 mg/kg) and without (uncured) sodium nitrite added.. Dashed line represents the reliable detection limit.

The decreased of *L. monocytogenes* growth as the dose of radiation increased was expected and agrees with what has been observed in other papers. The mechanism of microbial inactivation by ionizing irradiation is mainly due to direct damage to nucleic acids, denaturation of enzymes and cell membrane alterations or indirect damage acting through the oxidizing power of free radicals from the radiolysis of water (Farkas, 2006; Aymerichet, Picouet & Monfort, 2008). Turgis et al. (2012), observed a linear reduction of *L. monocytogenes* growth in sausages with increasing radiation doses (up to 4 kGy). Their results showed a 2-log reduction when irradiation of 1.5 kGy was used and the radiation dose required to eliminate *L. monocytogenes* was 3.9 kGy. Foong et al. (2004) found that the growth of *L. monocytogenes* on several types of irradiated ready-to-eat meat products, including uncured products, was inhibited for 5 weeks at 4 °C when irradiated by 2.0 kGy.

Some studies have indicated that sodium nitrite in cured ready-to-eat meat products has an inhibitory effect on *L. monocytogenes* growth. Birzele et al. (2005) reported that a concentration of residual nitrite above 40 mg/kg inhibited the listeria growth on fresh spreadable hams. Duffy et al. (1994) reported that the presence of sodium nitrite in the product formulation increased the lag time and reduced the growth rate of *L. monocytogenes* in vacuum-packed slices of inoculated cooked beef, pork, chicken or turkey stored at 0 and 5°C, but this effect was affected by temperature, pH, water activity (aw) and ascorbate content. According to these authors, in the presence of ascorbate, relatively small amounts of residual nitrite (35 mg/kg) in cooked meats of pH 6.3 and water activity (aw) 0.968 permitted less than a 1000-fold increase in numbers of *L. monocytogenes* cells during 21 d storage at 5°C. These conditions (ascorbate presence, temperature ~4 °C, pH ~6.3 and aw ~0.97) were present in the restructured cooked hams of this experiment and explain the higher effects against listeria observed in higher irradiation doses.

In the present experiment, high initial inoculum level was necessary to ensure that sufficient cells numbers survived the process, recovered and grew during cold storage. It would be expected that such levels would not normally be present in naturally contaminated meat products. For ready-to-eat meat products, recontamination during post-processing manipulation (e.g., during slicing) is assumed to be 10 CFU/g in the worst case (ICMSF, 2002) and, therefore, a 1.0 kGy (~ 2-log reduction) should be considered a considerable microbial control to reduce the slice safety risk in these products, even in an uncured one. The US Food Safety and Inspection Services (FSIS/2003) recommends a post-lethality treatment higher to 2-log reductions in ready-to-eat sealed package product to reduce or eliminate the level of pathogens for control of *L. monocytogenes*.

The exclusion or reduction of nitrite in meat products involves safety concern, due to its preservative action against *Clostridium botulinum* and its toxin (Dutra et al., 2016).

Therefore, the fundamental ability of irradiation process to inhibit germination of *C. botulinum* spores should also be considered. Silva (Article 01) reported a *C. sporogenes* lethality close to a 2-log reduction in low-nitrite (50 mg/kg) restructured cooked ham when irradiated with 1.5 kGy dose. Appling this dose, a listeria lethality observed in this experiment was close to a 4-log reduction in uncured and low-nitrite added (50 mg/kg) hams and no listeria survived in samples added with 150 mg/kg sodium nitrite. This lethality (4-log reductions) is recommended to ensure a maximum of 1 CFU/kg after an in-package pasteurization treatment in ready-to-eat meat products, considering the European microbiological criteria of $10^2$ CFU/g as maximum tolerance of *L. monocytogenes* (Oliveira et al., 2015).

*L. monocytogenes* can proliferate in vacuum-packaged irradiated or nonirradiated restructured hams during 30 days of cold storage (4 °C), but the use of sodium nitrite affected ($P < 0.05$) the listeria growth (Fig. 2). Overall, after 1- and 30-days of cold storage lower growth was observed ($P < 0.05$) in samples cured with 150 mg/kg than the others. Nitrite level of 50 mg/kg did not affect ($P > 0.05$) the listeria growth at 1-day post radiation process, but implied lower growth after 30-days of cold storage when compared to the uncured samples. According to Weiss at al. (2010), nitrite in processed meats can inhibit the growth of foodborne pathogens through various mechanisms, including oxygen uptake and oxidative phosphorylation interruption, formation of nitrous acid and nitric oxide compounds (NOs), and interruption of critical enzymes in bacterial metabolism such as aldolase.

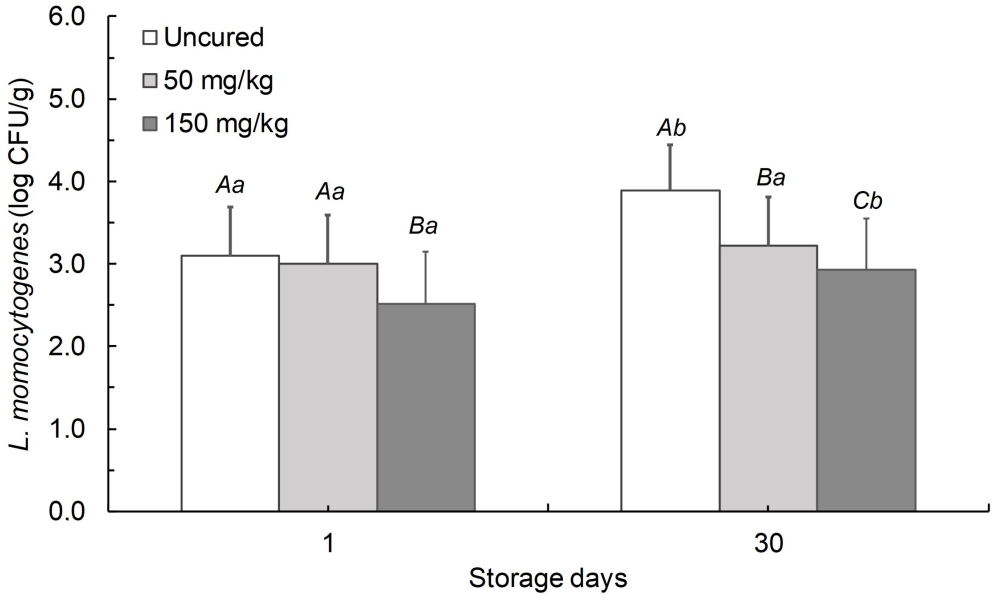


**Fig 2.** Growth of *Listeria monocytogenes* in vacuum-packed, cooked hams slices, elaborated with (50 and 150 mg/kg) and without (uncured) sodium nitrite, during cold storage (4 °C) for 1 and 30 days. Different capital letters $^{(A,B)}$, within storage day, and lower case letters $^{(a,b)}$, within each treatment, differ ($P < 0.05$).

### *3.2 Chemical and physicochemical analysis*

The effects of sodium nitrite, gamma irradiation and cold storage day on pH, residual nitrite, TBARS index, and water activity are reported in Table 1.

The water activity of restructured cured hams were measured only at day one, and they were not affected ($P > 0.05$) by irradiation process or nitrite content. Samples had an average water activity 0.97. According to Schrader (2010), *L. monocytogenes* grows best at $a_w \geq 0.97$ but can survive in environments $a_w < 0.90$ under refrigeration conditions. Therefore, the mean values of aw observed in the products were in the optimal range for listeria growth.

The pH values observed, average 6.36, are considered normal for this type of processed food, and the same results were found by Dutra et al. (2017), indicating that the pH values were not affected by the nitrite levels or irradiation dose.

**Table 1.** Sodium nitrite (N), gamma irradiation (G) effects on residual nitrite ($NO_2R$) and TBARS of vacuum-packed, cooked hams slices.

| **Effects** | **Source of Variation** | **$NO_2R$ (mg/kg)** | **TBARS (mg MAD/kg)** |
|---|---|---|---|
| Sodium nitrite (N), mg/kg | 0 | 3.03±0.15[a] | 0.14±0.03[a] |
| | 50 | 3.77±0.27[a] | 0.10±0.03[b] |
| | 150 | 11.42±1.87[b] | 0.12±0.02[a] |
| Radiation dose (G), kGy | 0 | 4.46±0.98 | 0.11±0.03 |
| | 0.5 | 6.77±2.16 | 0.11±0.02 |
| | 1.0 | 4.79±0.64 | 0.13±0.01 |
| | 1.5 | 7.50±2.70 | 0.12±0.03 |
| | 2.0 | 6.87±2.29 | 0.15±0.02 |
| Pr>F[1] | N | ***<0.001*** | ***0.001*** |
| | G | *0.770* | *0.080* |
| | N x G | *0.984* | *0.313* |

[1]Significant values are shown in bold.

[a,b] Means in the same column, for each effect, followed by different letters differ according to the Tukey test ($P < 0.05$)

The residual nitrite content was not affect by the irradiation process, which does not corroborate the observation of other studies (Houser et al. 2003; Ahn et al.,2004; Dutra et al., 2011) that gamma radiation process implies in lower residual nitrite values in cooked ham and sausages. Ahn et al. (2003) and Simie (1983) reported that residual nitrite in sausage was significantly reduced by irradiation, probably due to its reaction with the hydroxyl radical produced by the radiolysis of water. However, Dutra et al (2017) reported that residual nitrite values depended on the type of packaging used during the process and the dose applied. When the environment is in the reduced state, the nitrite can be easily converted to nitric oxide, resulting in lower residual nitrite levels in samples with anaerobic packaging (Ahn et al., 2003).

As observed for residual nitrite content, TBARS index was not affect by the irradiation process. Houser et al. (2003) also reported no effect ($P > 0.05$) on TBARS values in cooked hams irradiated with 4.5 KGy. They also reported that lipid oxidation did not increase during extended storage regardless of the application of irradiation at the different ham production processing steps. Studies conducted by Ahn et al. (1998, 1999), can support this explanation, observing that irradiation (2.5 to 4.5 kGy) did not affect oxidation in cooked ground pork.

The TBARS values was very small in the present study ($P = 0.08$) and should be related to the oxidation induced by free radicals generated by irradiation (Zhu et al., 2003). Furthermore, all TBARS values were very low and well below the suggested threshold of 0.5 to 1.0 for oxidative rancidity in pork as reported by Tarladgis et al. (1960), which was expected according to Shahidi et al. (1991) because nitrite is recognized as a strong antioxidant and prevents lipid oxidation.

### *3.3 Instrumental colour analysis*

Samples CIE colour were not affected ($P > 0.05$) by the irradiation process (Table 2), which are consistent with observed by others authors for Houser et al. (2003), who found no effect ($P > 0.05$) on L* from 4.5 kGy gamma irradiation between raw and cooked ham, with each other or with control (non-irradiated) ham. In addition, Fu et al. (1995) found no difference in L* values for cooked ham irradiated with 0.90 and 1.8 kGy and Shahidi et al. (1991) reported that L* values did not change for cured, cooked pork homogenate irradiated with 5 kGy compared with a non-irradiated control.

**Table 2.** Sodium nitrite (N), gamma irradiation (G) and storage day (D) effects on CIE colour characteristics of vacuum-packed, cooked hams slices.

| **Effects** | **Source of Variation** | ***L**** | ***a**** | ***b**** | ***C**** | ***h*** |
|---|---|---|---|---|---|---|
| Sodium nitrite (N), | 0 | 63.68±1.15$^{a}$ | 1.86±0.17 | 15.77±0.39$^{a}$ | 15.92±0.38 | 82.9±0.72 |
| mg/kg | 50 | 63.09±1,13$^{ab}$ | 4.58±0.32 | 13.58±0.27$^{b}$ | 14.48±0.17 | 70.95±1.54 |
| | 150 | 62.34±1.17$^{b}$ | 4.70±0.3 | 13.73±0.33$^{b}$ | 14.68±0.23 | 70.60±1.63 |
| Irradiation dose (G), | 0 | 62.37±1.49 | 3.80±0.47 | 14.23±0.45 | 14.90±0.36 | 74.54±2.09 |
| kGy | 0.5 | 63.47±1.56 | 3.82±0.51 | 14.34±0.47 | 15.05±0.33 | 74.38±2.34 |
| | 1.0 | 63.00±1.49 | 3.75±0.49 | 14.45±0.46 | 15.11±0.36 | 74.87±2.18 |
| | 1.5 | 63.15±1.47 | 3.78±0.51 | 14.45±0.5 | 15.14±0.38 | 74.66±2.32 |
| | 2.0 | 63.20±1.54 | 3.41±0.44 | 14.34±0.59 | 14.92±0.47 | 75.64±2.29 |
| Pr>F[1] | N | ***0.007*** | ***<0.001*** | ***<0.001*** | ***<0.001*** | ***<0.001*** |
| | G | *0.105* | *0.368* | *0.913* | *0.806* | *0.794* |
| | N x G | *0.101* | *0.323* | *0.126* | *0.147* | *0.321* |

[1]Significant values are shown in bold.

$^{a,b,c}$ Means in the same column, for each effect, followed by different letters differ according to the Tukey test ($P < 0.05$).

As opposed to irradiation, all colour indexes were affected ($P < 0.05$) by nitrite content. Lightness ($L^*$) were also affected ($P < 0.05$) by storage time. Overall, with uncured samples appearing more yellowish (higher $b^*$ and $h°$ values) and more reddish (higher $a^*$ and $C^*$ values) than cured hams. Increasing the dose of irradiation had no effects ($P > 0.05$) on any color characteristics (Table 1).

Redness values for restructured RTE ham treatments showed no differences, indicating that a* was not affected by irradiation treatments. This is coherent with results from Houser et al. (2003) and Fu et al. (1995) on hams. Nevertheless, Shahidi et al. (1991) observed increased a* between 0 and 5 KGy irradiation on day 0 but decreasing a* values for cured, cooked pork homogenate regardless of irradiation dose over a 21 day storage period, but and this decrease can be attributed to the dissociation of NO from the nitroso pigment and the consequent formation of brown metmyoglobin (Dutra et al., 2017).

Regarding b* values, Houser et al. (2003) and Fu et al (1995) reported no difference ($P < 0.05$) due to irradiation treatments, which are in agreement with the results found in this experiment.

The lack of difference in Chroma and hue angle values were similarly observed by Byun et al., 1999; Dutra et al., 2011 and Jo el al., 2003.

## 4. Conclusions

Irradiation was efficacious in reducing the *Listeria monocytogenes* inoculated in ready-to-eat hams without significantly affecting the other physicochemical and technological parameters. Similarly, a lower dose of nitrite (50 mg/kg) can be used in combination with gamma irradiation (1.5 kGy) to not only ensure the microbiological safety of RTE hams.

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