Assessment of contamination of *Salmonella spp.* in imported black pepper and sesame seed and salmonella inactivation by gamma irradiation

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Abstract

This study shows the frequency of seeds samples contaminated by *Salmonella* spp. collected randomly from local markets; on 30 black pepper sample no contaminated sample was found while *Salmonella* spp. was detected in 3 of 36 (8.3%) analyzed sesame samples; three different serotypes were identified: *S.* Montevideo, *S.* Stanleyville e *S.* Tilene. The efficacy of gamma irradiation to inactivate *Salmonella* Montevideo in black pepper and sesame irradiated between 1 and 5 kGy was evaluated. 3 kGy is sufficient to reduce of 3-4 log CFU/g; whereas 5 kGy have been need to reduce 5.5-6 log CFU/g for samples of black pepper and sesame. No statistically significant differences were found between black pepper and sesame.

Introduction

Traditionally the spices have been used throughout the world mainly for their flavouring and aroma properties. However, demand for these foods products has grown in recent years as a consequence of continually increasing consumption of ready-to eat foods, that include spices and seeds as ingredients (Witkowska *et al*., 2011). Additionally, they have also been recognized to possess health beneficial properties. Many studies documented digestive stimulant action, hypolipidemic effect, antidiabetic influence, antilithogenic property, antioxidant potential, anti-inflammatory property, antimutagenic and anticarcinogenic potential of spices and seed (Srinivasan, 2005). Pepper (*Piper nigrum L.*) and sesame seeds (*Sesamum ìndicum*) are among the most widely used spices in the world (Gharby *et al*., 2017; Mošovská *et al*., 2018).

They are classified as low water activity (a_w) food (a_w<0.70) and they do not support the growth of either vegetative or spore-forming bacteria (Blessington *et al*., 2013). Therefore, they are generally considered a safe product for health, but literature data showed that they are highly susceptible to microbial contamination in the process of growing, harvesting, processing and transporting. Bacteria of public health importance, such as *Salmonella* spp. , *E. coli*, *B. cereus* etc. may be present and the presence of such organisms could potentially create a public health risk (Sagoo *et al*., 2009).

Salmonella is one of the most frequently isolated foodborne pathogens in pepper and in sesame seeds and in their products (tahini, halva, etc.) (Van Doren *et al*., 2013; Sagoo *et al*., 2009). Outbreaks associated to their consumption were identified (Paine *et al*., 2014; Zweifel and Stephan 2012; Van Doren *et al*., 2013b; Kase *et al*., 2017) and different serotypes were reported (*S. Typhimurium DT 104, S. Bovismorbificans, S. Mbandaka, S. Maastricht*) including often *S.* Montevideo (Willis *et al*., 2009; Unicomb *et al*., 2005).

The use of ionizing radiation, among the new technologies for bacterial decontamination, has been shown to eliminate various foodborne pathogens from seeds, spices and sprouts with low-water activity (Mendonça 2002; Qian *et al*., 2013). Food sterilization, at dose up to 10 kGy has been widely accepted (FDA 1990) and is now legally recognized in many countries, including Italy (Dlgs 94/2001). Radiation energy can penetrate the cracks, crevices and intercellular spaces of the seeds and sprouts that harbor the pathogens (CDC 2013b).

Several studies investigated the inactivation of microorganisms by gamma irradiation in black pepper; among the recent ones, Rico C. W. *et al*. (2010) observed that a dose of 10 kGy resulted in a 5-log reduction of the initial microbial load (6 Log CFU/g) of red pepper; Song *et al*. (2014) reported that gamma irradiation greatly reduces levels of microorganisms (*E. coli* O157:H7 and *S.* Typhimurium) in black and red pepper; Celale *et al*. (2014) demonstrated that microbial load was not detectable after 12 kGy irradiation of several spices, including black pepper; Wenwen *et al*. (2015) shown the irradiation at doses of 4.00 kGy and of 5.00 kGy was appropriate for eliminating almost all *E. coli* and *S.* Typhimurium in Chili e Sichuan pepper. Koo *et al*. (2015) valuated that the total aerobic microbe's population decreased by irradiation in a dose-dependent manner in red pepper powder.

To date, the gamma irradiation has been applied to study the radiosensibility of different microorganisms in pepper but no studies is available on inactivation of *Salmonella s*pp. in sesame. Furthermore, the microbiological inactivation by gamma irradiation could be affected by the some factors such different chemical composition and grain size of pepper and sesame (Mendonça, 2002; Song *et al*., 2014).

Therefore, the objectives of this study were to assess the contamination of *Salmonella* spp. in imported pepper and sesame seeds and to evaluate inactivation of *S.* Montevideo by gamma irradiation of sesame and pepper, in order to appraise a different radiosensibility between two spices.

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Materials and Methods

Sampling to microbiological analyses

A total number of 66 samples were collected randomly from local markets (Sicily, Italy). Samples (200 g/sample) were collected in sterilized polyethylene bags and transported to the laboratory where they were kept at 4°C until testing. Sampling was performed three times in each local market. Information on samples and markets was recorded on a standard questionnaire (Supplementary Material). The samples were imported from seven different non-EU countries (Table 1).

Microbiological analyses

50 grams of sample were pre-enriched in 450 ml sterile Buffered Peptone Water (Difco, USA) for 18 h at 37°C. 0.1 mL of the culture broth was added to 10 mL Rappaport Vassiliadis Soy broth (Oxoid, UK) and incubated at 41.5°C for 24 h. A loopful of the culture was streaked in Xylose Lysine Desoxycholate Agar (XLD) (Oxoid, UK) and incubated at 37°C for 24 h. The suspected *Salmonella* spp. colonies were subcultured for purity and identified by the Biolog automatic system (Biolog Inc., Hayward, CA). Serotyping of isolates was carried out by using commercial antisera (Staten Serum Institut, Denmark, and Denka-Seiken, Japan).

Sample preparation to irradiation treatment

Black pepper and sesame in whole grains with 4-5 mm and 2-3 mm diameter respectively were purchased at a local market (Palermo, Italy). Samples were irradiated using a Cobalt-60 gamma irradiator at doses 25 kGy to remove the natural microflora. All samples were stored under refrigerated conditions (5±1°C) until they used.

Culture preparation and bacterial inoculation

S. Montevideo was obtained from our laboratory culture collection. This strain was originally isolated from sesame seed samples imported from Nigeria. Strain was maintained at -20°C in Tryptic Soy Broth (TSB) (Difco, USA) supplemented with 20% glycerol. Working culture was prepared by tracking to Tryptone Soya Agar (TSA) (Difco, USA) and incubating for 24h at 37°C. One colony from TSA was cultured in 50 ml TSB for 24 h at 37°C to obtain an initial population level of 8-9 log CFU/ml. 5 ml of culture was mixed with 50 g of each samples irradiated at 25 kGy into a sterile polyethylene bags. The inoculated samples were mixed by hand for 1 min. and kept for 1h at room temperature (20°C) to facilitate microorganism attachment to the samples. After the excess culture was removed using a sterile pipette. The samples were placed on aluminium foils under a biosafety hood to dry at room temperature (20°C) for 24 h, until the a_w of the samples equalled that of non-inoculated sample, in order to not change the effect of gamma-ray irradiation on Salmonella inactivation.

The initial level of the pathogen in inoculated samples was confirmed by enumerating Salmonella and was 7.46 log CFU/g in sesame and 8.42 log CFU/g in pepper. After drying, all samples were transported to the irradiation facility under cold storage conditions (4±1°C).

Gamma irradiation treatment

100g of inoculated black pepper and 100g of inoculated sesame seed samples in stomacher bags were irradiated under electronic equilibrium conditions with a ^{60}Co panoramic irradiator IGS-3 at doses of 1, 3 and 5 kGy. The dose values were calculated using irradiator time and the dose rate (1.50 kGy/h) measured with Fricke dosimeter; the overall uncertainty in the absorbed dose in the irradiated samples was <±5%. The samples were stored at 4°C immediately after irradiation.

Water activity (a_w)

The water increases the radiation sensitivity of foodborne pathogens, due to the radiolysis of the water, which amplifies effects induced by radiation; since this affects on pathogen D_{10} value, the a_w value of each sample (10±1 g) were measured after 24 hours drying on same day of irradiation treatment and compared to samples not inoculated; the a_w values were replicated three times and measured using a calibrated water activity meter (HygroLab, Rotronic, Bassersdorf, Switzerland) according to the manufacturer's instructions.

Salmonella enumeration

90 ml of Buffered Peptone Water was added to the bags containing 10 g irradiated samples and then homogenized in a Stomacher (model 400, Serward, UK) for 60s. The initial suspension was stood for 1 h at 20°C in order to resuscitate the stressed microorganisms.

Serial dilution was prepared and 0.1 ml of appropriate dilutions were spread-plated on XLD Agar. Where low populations of microorganisms were anticipated, 1 ml of the initial homogenate were distributed on three plates. The plates were incubated for 24 h at 37°C.

Microbial counts were expressed as log CFU/g and each microbial count was the mean of three observations.

D-value calculation

Number of surviving pathogens was plotted on a logarithmic scale as a function of dose (kGy) "survivor curve". D-value (dose in kGy required to inactivate population of pathogen by 90%), was obtained by the following equation (Hvizdzak *et al.*,2010):

$$\text{Log}\ (N/N_0) = (-1/D)\ d$$

where N = viable counts after gamma irradiation dose d, N_o = initial viable counts and D = D-value (decimal reduction dose).

Statistical analysis

The D-values obtained were subjected to one-way ANOVA analysis, using Statistical Analysis System (SAS/ML v.8.2) and Duncan's multiple range test was employed to determine if there were significant differences (P<0.05).

Results and Discussion

Results of microbiological analysis

No contaminated black pepper sample was found while *Salmonella* spp. was detected in 3 of 36 (8.3%) analysed sesame samples. Three different serotypes were identified: *S.* Montevideo, *S.* Stanleyville e *S.* Tilene. However, Salmonella counts were <1 log CFU/g. All contaminated sesame seeds were imported from Nigeria.

S. Montevideo has often been responsible for food-poisoning. During period 2002 - 2015 outbreaks were identified in Australia, New Zealand, Asia, Europe and USA attributed to consumption of wide range of foods including spices and seed. (Lalsiamthara *et al.*, 2017). Between 2002 and 2003 three outbreaks were identified in Australia and New Zealand associated to sesamo seed product (tahini) and 68 infected individuals have been reported. (Unicomb *et al.*, 2005). In 2009 and 2010,

Table 1. Numbers samples pepper and sesame imported from non-EU countries.

Spices	No. samples	Country
Pepper (n=30)	15	Vietnam
	4	Mexico
	9	India
	2	Indonesia
Sesame (n=36)	16	India
	13	Nigeria
	4	Pakistan
	3	Egypt

in the USA two outbreaks related to consumption of salami products including pepper occurred and 272 patient-cases have been reported. Black pepper from Vietnam and red pepper from India and China used in the salami products were the source of contamination (Van Doren *et al*., 2013b). Moreover, in 2012 an outbreak linked to consumption of contaminated tahini imported from Turkey occurred in New Zealand (Paine *et al*., 2014).

Instead *S.* Stanleyville e *S.* Tilene were rarely cause of human infection. *S.* Stanleyville caused two outbreaks in Italy in 2015 and 2016: in the outbreak of 2015 cuttlefish and octopus were the source of infection while in 2016 the source was unknown (Cibin *et al*., 2019). *S.* Tilene is a rare serotype in Europe but human infection was reported in the United States, Africa and Asia mainly associated with hedgehog contact (Su-Jin *et al*., 2016).

Multiple *Salmonella* serotypes are isolated from sesame and pepper and no serotype is specific to or preferentially found in these two spices. In 2013 Van Doren *et al*. examined the results of the three-year FDA Salmonella surveillance activities (2007-2009) for imported spices in the USA. One purpose of the study was to evaluate serotype diversity in spices. They identified 94 serotypes among 205 isolates in particular 36 different serotypes in pepper and 39 in sesame. They did not find any data to support the hypothesis that spice contamination is limited to specific *Salmonella* serotypes. The wide diversity of *Salmonella* serotypes found may reflect a wide diversity of contamination sources, such as soil, water, rodents, birds, and insects. However, in literature *S.* Montevideo is indicated as a frequently isolated serotype in sesame and pepper while no data has been found for *S.* tilene and *S.* Stanleyville.

The Salmonella contamination values observed in this study, even if related to a few samples, can be compared to values determined by several authors (Van Doren *et al*. (2013), Brockmann *et al*. (2004), Garcìa *et al*. (2001), Witkowska *et al*. (2011) and Sospedra *et al*. (2010).

Performed investigations on the occurrence of *Salmonella* spp. in pepper and sesame seeds are summarized in Table 2. The Salmonella-positivity percentage observed in these studies ranged from 0 to 11.3% for sesame seeds and 0 to 18.2% for pepper. A lot of the values listed in Table 2 are from surveillance studies and most samples were collected from retail such as in present studies. These data demonstrate that Salmonella contamination in these two spices has a wide range of positivity percentage. The different values reported in different countries may reflect a real difference in microbiological quality of the spices examined or it may be the result from different sampling protocols or different analytical methods but, in any case, these results confirm the frequency of sesame seeds samples contaminated by *Salmonella* spp. The presence of Salmonella is a particular concern in spices because these products are often added to foods that are consumed raw (e.g. salad) or added to food after cooking. In the present study Salmonella counts were low (< 1 log CFU/g) in contaminated sesame seeds samples but low concentrations of *S.* Montevideo (from 0.03 to 0.46 organisms/g) in a sesame-seed product have been reported to cause a outbreak of salmonellosis in New Zealand (Unicomb *et al*. 2005). Therefore, the presence of Salmonella in these types of products may represent a hazard to health, because they can cause human illness.

Water activity (a_w) analysis

The a_w values measured for not inoculated black pepper and sesame seeds are 0.600±0.028 and 0.539 ± 0.016 respectively. After inoculation and drying for 24 hours the a_w values for black pepper is 0.620±0.028 and for sesame is 0.580±0.016; these values are not significantly different respect to samples not inoculated and consistent with literature data of 0.663 for black pepper (Ristori *et al*. 2007) and 0.51 for sesame (Borchani *et al*. 2010).

Effect of irradiation on *S. Montevideo*

The effect on inactivation of *S.* Montevideo in black pepper and sesame dried for 24 hours and after gamma irradiation doses of 1, 3, and 5 kGy and unirradiated was measured and the obtained results are shown in Table 3. The initial populations of *S.* Montevideo in inoculated black pepper and sesame seeds were about 8 and 7 log CFU/g (Table 3) respectively. Reduction of pathogen increased with an increasing radiation dose. Radiation dose of 3 kGy is sufficient to determine a reduction of 3.2 log CFU/g for black pepper and 4.2 log CFU/g for sesame seeds, whereas 5 kGy are needed to obtain a reduction of 5.5

Table 2. Selected studies on the occurrence of Salmonella spp. in pepper and sesame seeds.

Products	Country	Positive samples (total)	% positive samples	Sample collection point	References
Sesame seeds	USA	17 (177)	11.3	Imported	Van Doren (2013)
	UK	13 (771)	1.7	Retail	Willis C. *et al*. (2009)
	USA	0 (526)	0	Retail	Zhang *et al*. (2017)
	Germany	Sesame product 11(117)	9.4	Retail	Brockmann *et al*. (2004)
	UK	7 (367)	2.5	Retail	S. Surman-Lee, HPA, personal comunication Willis C *et al*. (2009)
Pepper	Mexico	0 (61)	0	Retail	García *et al*. (2001)
	USA	3 (1264)	0.24	Retail	Zhang *et al* (2017)
	Brazil	12 (66)	18.2	Retail	Moreira *et al*. (2009)
	EU	Unknown	8	/	Banach *et al*. (2016)
	Turkey	2 (58) 1 (59)	3.4 (black pepper) 1.7(red pepper)	Retail	Hampikyan *et al*. (2009)
	Ireland	0 (77)	0	Retail	FSAI (2005) 3rd Trimester National Microbiological Survey 2004
	Ireland	Unknown	0	Retail	Witkowska *et al*. (2011)
	Spain	0 (4)	0	Retail	Sospedra *et al*. (2010)
	USA	13 (291) 1 (87)	4.5 (black pepper) 1.1 (white pepper)	Imported	Van Doren (2013)

Table 3. Effects of gamma irradiation treatment on S. Montevideo in black pepper and sesame seeds (means of three replications ± standard deviation).

Dose (kGy)	Viable cell counts (log CFU/g) Black pepper	Sesame seeds
0	8.42±0.33	7.46±0.31
1	6.9±0.04	5.3±0.13
3	4.9±0.16	3.7 ±0.11
5	2.9±0.04	1.7±0.19

Table 4. Reduction of *S. Montevideo* in black pepper and sesame seeds after irradiation treatment (means of three replications ± standard deviation).

Dose (kGy)	Reduction of *Salmonella* spp. (log CFU /g) Black pepper	Sesame seeds
1	1.35±0.04	1.96±0.13
3	3.22±0.16	4.17±0.11
5	6.02±0.04	5.53±0.19

CFU/g for black pepper and 6.0 log CFU/g for sesame seeds (Table 4).

The decimal reduction dose (D-value) of Salmonella is 1.18±0.008 in black pepper and 1.12±0.04 in sesame seeds.

The *D*10 value provides information on the sensitivity of microorganisms to irradiation. Several critical factors can influence the radiation resistance of *Salmonella* spp. According to S. Jeong *et al*. (2012) the *D*-value is a function of water activity; *S.* Enteritidis PT30 and *S*. Tennessee were less resistant to irradiation on surface-inoculated almonds and walnuts when the nuts were in their driest state. Song W.-J *et al*. (2014) reported that the D10 values of *E. coli* O157:H7 and *S.* Typhimurium in whole black pepper were smaller than those in ground black pepper. Also, the D10 value of *E. coli* O157:H7 in over 1.19 mm red pepper was smaller than those of smaller particle sizes.

But these results conflict with the study of Thayer *et al*. (2003) in which alfalfa seed size, water activity and moisture did not significantly affect the D-value. But Thayer (2004) demonstrated that physical and chemical composition of food also affects microbial responses to irradiation. In a complex food system, some of the constituents, such as proteins and fat, are thought to compete with the cells for interaction with radiolytic free radicals, thereby reducing the net effect of radiation damage or making organisms more radiation resistant.

In this study, no significant differences were established in *S.* Montevideo D10 value of black pepper and sesame seeds, though they have different sizes and chemical composition (percentage of proteins, fat and carbohydrate of sesame is more elevate respect to pepper).

The D-values for *S.* Montevideo obtained in this study are comparable with D-value obtained for *S.* Typhimurium for red pepper (1.17 kGy with similar grain size of black pepper) analyzed in study by Song W.-J *et al*. (2014) and also for almonds for *S.* Enteritidis PT30 (1.25 kGy), *S.* Anatum (1.23 kGy), *S.* Hartford (1.06 kGy) and for a cocktail of *S.* Anatum, *S.* Infintis, *S.* Newport and *S.* Stanley (1.16 kGy) by Prakash *et al*. (2010).

Conclusions

The obtained results show that:

- Salmonella spp. was not detected in any pepper samples analysed while it was detected in sesame seeds. The Salmonella contamination values of sesame seeds are in low concentrations but the presence of Salmonella in sesame seeds indicate a potential public health hazard to consumers, once the sesame is added to uncooked foods.

To date European law doesn't identify salmonella such as 'food safety criterion' for this food categorie but according Regulation (EC) No 178/2002 food business operators have the obligation to place on the market safe food. In order to prevent the health risk, food business operators should employ good hygiene practice and procedures based on hazard analysis and critical control point (HACCP) principles and also use decontamination treatments such as irradiation that can minimized the risk of pathogens in spices.

- The irradiation dose of 5 kGy is enough to reduce the *S.* Montevideo contamination of 6.0 log CFU/g for sesame and of 5.5 log CFU/g for black pepper. Then no statistically significant differences were found between black pepper and sesame.
- Sure at typical irradiation dose of 7-8 kGy, used to irradiate herbs and spices, the use of ionizing radiation represents a safe and valid method to reduce Salmonella contamination of pepper and sesame.

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