Combinations of nisin and γ irradiation to improve microbiological quality of minced chicken during refrigerated storage

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Abstract: This study evaluated the effect of nisin at supplementation levels of 100 or 200 µg/g, γ irradiation at dose of 1 or 2 kGy and their combination, on microbiological quality of minced chicken and minced chicken artificially inoculated with *Bacillus cereus* and *Escherichia coli*, during refrigerated storage. Samples were analyzed for total bacterial, mold & yeast counts at 0-time, 7 and 14 days and the log number of microorganisms were determined. Treatment of minced chicken with nisin alone at 100 or 200 µg/g showed no significant *(p> 0.05)* improvement in microbiological quality of minced chicken during storage. The combination of nisin and γ irradiation improved *(p< 0.05)* the microbiological quality of minced chicken together with the antibacterial activity against *Bacillus cereus* and *Escherichia coli*. The use of nisin combined with γ irradiation would be promising to control food-borne pathogens, improve microbiological safety and extend shelf-life.
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1. Introduction

Microorganisms control in meat products is the major concern in the preparation of high quality foods. Fresh chicken and chicken products can be easily contaminated with microorganisms and, if not properly handled and preserved, support growth of spoilage and pathogenic bacteria, leading to loss of quality and potential public health problems (Vernozy-Rozand *et al.,* 2002). Spoilage of fresh chicken products is an economic burden to the producer, consequently developing methods to prolong the shelf-life and overall safety/quality represents a major task of the chicken industry.

A recent trend in food processing research has focused on meeting consumer demands for more natural and fresher food. The application of combinations of different non-thermal hurdles, used at sub-lethal levels, could maintain the organoleptic and nutritional quality while still ensuring the safety and stability of the food product (Leistner and Gorris, 1995). One method as a means to non-thermally inactivate pathogens in food products is gamma (γ) irradiation. Irradiation offers a great potential for application in food preservation (Farkas, 1998, Farkas, 2006). New attempts in food irradiation technology consist to combine radiation treatment with other agents are of utmost importance in enhancing the microbiological effectiveness, reducing the energy requirement for food preservation and improving product quality (Farkas, 1989). It has been previously demonstrated that the irradiation in presence of various natural or synthetic active compounds would help to increase the radiosensitization of food pathogens (Lacroix and Ouattara, 2000). Radiosensitization is a well-known biological phenomenon which may be of practical utility in enhancing the technical effectiveness and feasibility of irradiation. The spectrum of applications of food irradiation could be expanded in combination with bacteriocins, (Galvez *et al.* 2007).

The empirical use of microorganisms and/ or their natural products for preservation of foods (biopreservation) has been a common practice in the history of man kind (Ross *et al.,* 2002). Nisin, the only bacteriocin with food and drug administration (FDA) –approved GRAS (Generally Regarded As Safe; US 21 CFR170.30-Food Additives), is successfully used as antibacterial agent in various food products (Jay, 2000). Nisin is more effective against spores than against vegetative cells (Bell and deLacy, 1985). It is effective against Gram positive bacteria but does not inhibit the majority of Gram-negative bacteria. Nisin acts mainly by permeating the cytoplasmic membrane with the formation of transient pores. The mechanism of action of this polypeptide bacteriocin involves binding to the peptidoglycan layer, causing destabilization of the membrane by formation of transient pores, which allow leakage of intracellular metabolites and dissipation of membrane potential (interference with membrane function) (Bruno *et al.*, 1992). According to Budde and Rasch (2001), the pore formation do not necessarily lead to cell death as cells may repair when transferred to a rich growth media without bacteriocin.. Nisin is stable under

refrigerated conditions, demonstrates heat stability, but is degraded by gut enzymes. By now, the antimicrobial activity of various combinations of nisin with γ irradiation in minced chicken has not been examined.

Therefore the aim of this work was to evaluate the inhibitory effect of nisin applied singly or in combination with γ irradiation to improve the microbiological quality of minced chicken and the antimicrobial effect against artificially inoculated *Bacillus cereus* and *Escherichia coli* in minced chicken during refrigerated storage.

2. Materials and Methods

2.1. Nisin preparation

Pure nisin with a label activity of 10^6 IU/g was provided by Aplin and Barrelt Ltd. Trobridge, UK. Nisin was solubilised in 0.02 M HCl at a concentration of 10 mg/ml with heating (60-70 ^oC) to aid solubilisation. The solution was sterilized by filtration through 0.22 µm membrane filters prior to use.

2.2. Preparation of minced chicken and treatments

Chicken carcasses were purchased from a local poultry processing plant immediately after evisceration. They were transported to the laboratory in an ice box and stored at 4^oC for no longer than 2h before use. Meat was separated from the bones and minced in an electrical mincer. Ten gram samples were aseptically sealed in polyethylene bags after the treatment with different nisin concentration and/ or γ irradiation according to the scheme explained in Table 1. Packages were then stored on the refrigerator (4± 1^oC) shelf until analysis. Three packages from each group were examined every analysis.

2.3. Irradiation process:

Packaged minced chicken samples were irradiated according to the scheme in Table 1 with 0, 1 and 2 kGy gamma irradiation at dose rate 3.49 kGy/ h using the "Indian Gamma Chamber 4000 A" with a ^{60}Co source. The irradiation process was conducted at the National Center for Radiation Research and Technology (NCRRT), Nasr city, Cairo, Egypt. After irradiation, all samples were kept in refrigerator at 4± 1^oC until examination. Three packages from each group were analyzed immediately after irradiation (0-time), 7 and 14 days.

2.4. Microbiological analysis:

For determination of microbial counts, 10 g of each sample were homogenized in 90 ml sterile physiological saline solution (0.85% NaCl), then serial 10-fold dilutions were prepared. Appropriate serial dilutions were duplicate plated (spread method) with plate count agar medium for total bacterial count (TBC) and czapek yeast extract agar medium for mold & yeast counts (M&YC). Plates were incubated at 37^oC for 48 h for TBC and 25 ^oC for 5 days for M&YC (APHA 2001).

2.5. Bacterial strains and inoculation:

Local strain of *Bacillus cereus* and *Escherichia coli* (previously isolated and identified in the Food Hygiene Dept., Animal Health Research Institute, Dokki, Egypt) maintained on nutrient agar (Oxoid) were used to artificially contaminate minced chicken samples. Each organism was grown separately in 50 ml nutrient broth (Oxoid) broth for 24 h at 37 ^oC. Then the cultures were serially diluted in sterile saline (0.85% NaCl) for standardization by pour plate assay in duplicate using nutrient agar plates incubated at 35 ^oC for 18 h. A final *Bacillus cereus* and *Escherichia coli* inoculum of 10^6 and 10^8 CFU/ ml, respectively were used separately for the inoculation of samples.

Samples used for artificial inoculation test were first sterilized using 20 kGy by accelerated electrons in the NCRRT (energy: 1.5 Mev and current: 0.9 m A). One ml of the inoculum of each organism was inoculated individually in a sample (3 packages for each treatment for each organism) after the confirmation of sterilization test by plating on plate count agar (PCA) medium. Survivors of *Bacillus cereus* and *Escherichia coli* were enumerated on PCA using spread technique after incubation at 35^oC for 24 h.

2.6. Statistical analysis:

Minced chicken samples from 9 (microbiological analysis) and 5 (antimicrobial effect) different groups were analyzed. Microbial populations were converted to $\log_{10}$ CFU/g. The values given in each treatment category are the mean values of three individual samples. Analysis of variance (F-test) was done for samples in each storage period using the General Linear Model (SAS, 2002). Least significant difference (LSD) ($P< 0.05$) was performed on the examined organisms.

3. Results

3.1 Microbiological analysis

The inhibitory effects of nisin at 100 or 200 µg/g, γ irradiation at 1 or 2 kGy and their combination on total bacterial (TBC) and mold & yeast (M&YC) counts in minced chicken samples stored at refrigerated (4± 1^oC) temperature for 14 days are shown in Figs. 1 and 2, respectively. Addition of nisin to minced chicken samples at 100 or 200 µg/g (groups B and C) resulted in TBC (Fig. 1) and M&YC counts (Fig. 2) not significantly ($p> 0.05$) different than those of the control samples neither at 0-time nor at the end of storage, indicating no antimicrobial activity of nisin alone at this level. Using γ irradiation at 1 or 2 kGy (group D and G) significantly ($p<0.05$) reduced both TBC (ranging from 1 to 1.4 log CFU/ g) and M&YC (ranging from 0.89 to undetected level) compared

with groups A, B and C at 0-time. While at the end of storage, this reduction ranged from 1.86 to 2.99 and from 1.81 to 3.32 log CFU/ g, respectively.

The combination treatment of nisin (100 or 200 µg/g) and γ irradiation (1 kGy) (group E and F) resulted in a decrease in the TBC and M&YC but this decrease was non significant ($p > 0.05$) compared with group D (1 kGy γ irradiation only) throughout all storage period except M&YC for group F at the end of storage. The same trend was found in M&YC for group H and I compared with group G. On the contrary, there was a significant ($p < 0.05$) reduction in TBC of group I compared with group G throughout all storage period.

At the end of storage, TBC and M&YC of the control (group A) reached 7.83 and 5.55 log CFU/ g, respectively. While these counts reached 3.92 and 1.68 log CFU/g, respectively, in group I. Although there was 0.55 log CFU/ g reduction in M&YC in samples treated with combination of 2 kGy and 200 µg/g (group I) compared to those treated with 2 kGy alone (group G), but this reduction was non significant ($p > 0.05$).

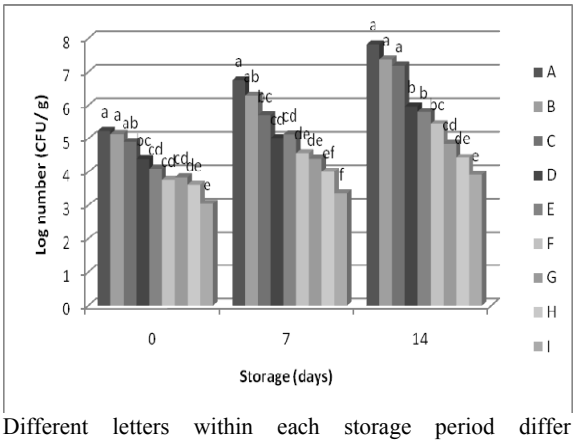
3.2 Antimicrobial activity in artificially inoculated minced chicken

The effects of combination treatments of nisin (100 or 200 µg/g) and γ irradiation (1 or 2 kGy) on *Bacillus cereus* and *Escherichia coli* in minced chicken samples stored at refrigerated (4± 1°C) temperature for 14 days are shown in Figs. 3 and 4, respectively. Due to psychotropic nature, the initial population of *Bacillus cereus* (5.35 log CFU/ g) in control samples increased reaching 8.12 log CFU/ g by the end of storage. It was clear that there was significant ($p < 0.05$) reduction in the survivors of *Bacillus cereus* (Fig. 3) in all combination treatments compared with the control throughout storage period. Although there was a non significant ($p > 0.05$) decrease in number of *Bacillus cereus* survivors in group H and I at 0-time and after 7 days of storage, but this decrease was significant ($p < 0.05$) at the end of storage. At the end of storage (after 14 days), *Bacillus cereus* was undetected in group I.

Concerning artificially inoculated *Escherichia coli*, it was clear from Fig.4 that there was a highly significant ($p < 0.05$) reduction in the number of survivors in all treatments throughout storage period compared with the control. *Escherichia coli* was undetected in group H at the end of storage and in group I after 7 days indicating its sensitivity to the combination treatments under investigation.

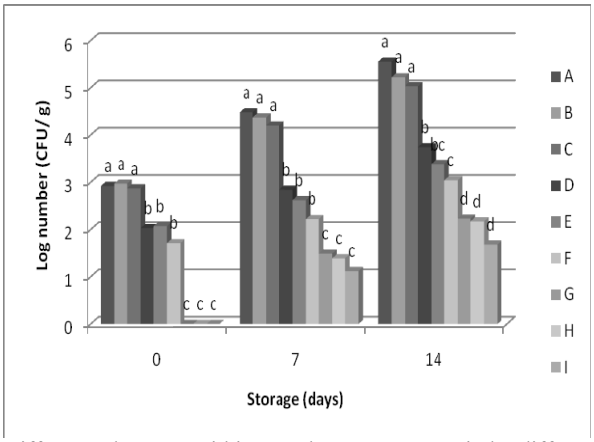
Table 1. Explanation of group names, nisin concentration and irradiation dose.

Irradiation dose (kGy)	Nisin (µg/g)		
	0	100	200
0	A	B	C
1	D	E	F
2	G	H	I



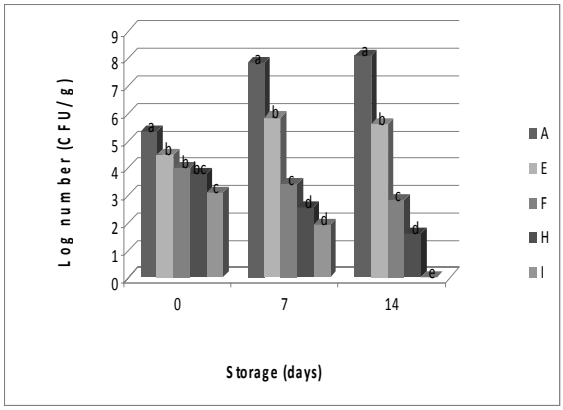
Different letters within each storage period differ significantly *(p< 0.05)*.

Figure 1 Total bacterial count of minced chicken (n=3) treated with (A) 0 kGy γ irradiation +0 µg/g nisin, (B) 0 kGy γ irradiation + 100 µg/g nisin, (C) 0 kGy γ irradiation + 200 µg / g nisin (D) 1 kGy γ irradiation + 0 µg/g nisin, (E) 1 kGy γ irradiation + 100 µg/g nisin, (F) 1 kGy γ irradiation + 200 µg / g nisin , (G) 2 kGy γ irradiation + 0 µg/g nisin, (H) 2 kGy γ irradiation + 100 µg/g nisin and (I) 2 kGy γ irradiation + 200 µg / g nisin during storage.



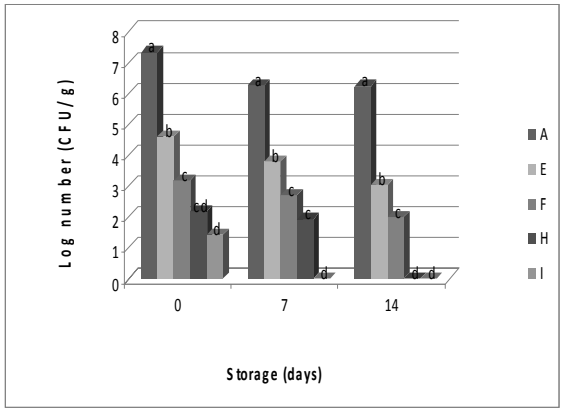
Different letters within each storage period differ significantly *(p< 0.05)*.

Figure 2 Mold & yeast count of minced chicken (n=3) treated with (A) 0 kGy γ irradiation + 0 µg/g nisin, (B) 0 kGy γ irradiation + 100 µg/g nisin, (C) 0 kGy γ irradiation + 200 µg/g nisin (D) 1 kGy γ irradiation + 0 µg/g nisin, (E) 1 kGy γ irradiation + 100 µg/g nisin, (F) 1 kGy γ irradiation + 200 µg/g nisin , (G) 2 kGy γ irradiation + 0 µg/g nisin, (H) 2 kGy γ irradiation + 100 µg/g nisin and (I) 2 kGy γ irradiation + 200 µg/g nisin, during storage.



Different letters within each storage period differ significantly *(p< 0.05)*.

Figure 3 Artificially inoculated *Bacillus cereus* survivors of minced chicken (n=3) treated with (A) 0 kGy γ irradiation + 0 µg/g nisin, (E) 1 kGy γ irradiation + 100 µg/g nisin, (F) 1 kGy γ irradiation + 200 µg/g nisin , (H) 2 kGy γ irradiation + 100 µg/g nisin and (I) 2 kGy γ irradiation + 200 µg/g nisin, during storage.



Different letters within each storage period differ significantly *(p< 0.05)*.

Figure 4 Artificially inoculated *Escherichia coli* survivors of minced chicken (n=3) treated with (A) 0 kGy γ irradiation + 0 µg/g nisin, (E) 1 kGy γ irradiation + 100 µg/g nisin, (F) 1 kGy γ irradiation + 200 µg/g nisin , (H) 2 kGy γ irradiation + 100 µg/g nisin and (I) 2 kGy γ irradiation + 200 µg/g nisin, during storage.

4. Discussion

The results of this paper clearly demonstrate the effectiveness of using combination of nisin and γ irradiation against TBC, M&YC, *Bacillus cereus* and *Escherichia coli.* At 0-time, TBC of the control (group A) was 5.23 log CFU/g indicating a need for improving microbiological quality of minced chicken by using some preservatives that could be easily applied in both large and small scale productions. After 14 days of storage, TBC of the control (group A) reached 7.83 log CFU/ g which was higher than the Egyptian standards (4178/ 2005) and should be rejected. The TBC is considered an index of quality which gives an idea about the hygienic measures during processing and helps in the determination of the keeping quality of the product (Aberle *et al.,* 2001). High TBC might be attributed to the contamination of the product from different sources or unsatisfactory processing as well as it may be due to unsuitable condition during storage (Cox *et al.,* 1998, ICMSF, 1980).

Mold and yeast count of group A was 2.92 log CFU/g at 0-time which increased to reach 5.55 log CFU/g after 14 days. Mold and yeast are used as index of sanitation and their high counts accelerate spoilage. Yeast may grow under aerobic condition on the surface of meats causing sliminess, lipolysis and pink to brown coloration due to the pigments in the yeast. Mold can assist in the putrefactive processes, they can grow over a wide range of temperature and may cause a moldy odor and taste to food, they may also produce mycotoxins which are harmful to man and animal (Frazier and Westhoff, 1983).

The addition of nisin at 100 or 200 µg/g to minced chicken did not cause any inhibitory *(p< 0.05)* effect on TBC and M&YC during refrigerated storage compared to the control. This finding was similar to that reported by Barboza De Martinez *et al.* (2002). Chung *et al.* (1989) found that nisin can delay the growth of Gram-positive bacteria that are attached to meat. However, nisin alone may not be sufficient to prevent spoilage, since gram-negative and nisin-resistant gram-positive bacteria such as lactic acid bacteria are often associated with meat spoilage. It is also known that the antibacterial activity of nisin is weakened in minced meat due to its binding to proteins and fat, or its reaction with meat proteases (Aasen *et al.,* 2003, Stergiou *et al.,* 2006).

The use of γ irradiation at 1 or 2 kGy resulted in a significant *(p< 0.05)* reduction in TBC and M&YC compared with the control. The immediate reduction after γ irradiation may be mainly due to the direct effect of gamma rays on the microbial cell by causing lesions in the genetic material of the cell, effectively preventing it from carrying out the biological processes necessary for its continued existence (Murano, 1995). He added that microorganisms which are responsible for spoilage of red meat, as well as those that are pathogenic, are easily destroyed by the dose used in cold pasteurization (1 to 10 kGy). Generally, it is known that Gram-negative bacteria which include many spoilage and pathogenic organisms are quite sensitive to radiation. The spoilage flora of meat is usually dominated by the

bacteria which grow most rapidly under the storage conditions of the product. Under aerobic conditions, the dominant spoilage organisms are strictly *Pseudomonas* spp. (Kanatt *et al.,* 2005). The sensitivity of these organisms to radiation explains the significant decline (*P< 0.05*) in their counts. By refrigerated storage, the microbial counts were increased progressively (*P< 0.05*). This finding agreed with that reported by Balamatsia *et al.* (2006) in chicken meat. Giroux *et al.* (2001) mentioned that gamma irradiation lowered the rate of increase in the number of microorganisms in irradiated beef patties than that in non-irradiated control.

Bacillus cereus is a spore forming, Gram positive, aerobic, rods bacteria. It has been long known as ubiquitous organism found in air, soil and water (Claus and Berkeley, 1986). *Bacillus cereus* is the aetiologic agent of two distinct types of food poisoning (Thayer and Boyd, 1994). They also stated that controlling stationary phase cells and endospores may be more important for food safety than controlling logarithmic phase cells because of the high radiation resistance of the endospore and because the stationary phase would probably be reached in most abused foods. In a study done by Zahran *et al.* (2008), the incidence of *Bacillus cereus* in minced chicken was 60% of examined samples.

Presence of *E. coli* in food products indicate direct or indirect contamination of faecal origin or might be due to handling under unhygienic conditions (Frazier and Westhoff, 1983). Several authors (Cleveland *et al.* 2001, Solomakos *et al.,* 2008) reported that nisin did not possess antimicrobial activity against Gram-negative *Escherichia coli* in minced beef. This might be due to inability of nisin to attack Gram-negative bacteria, due to the protective role of the outer membrane, which covers the cytoplasmic membrane and peptidoglycan layer of Gram-negative cells (Fang and Tsai, 2003). The 3 kGy gamma irradiation had a significant (*P<0.05*) effect on elimination of *E.coli* (Jo *et al.* 2005). The response of bacteria to irradiation in different environments has shown that various food matrices can offer different degrees of protection to the organism, also differences in recovery techniques (Diehl, 1995).

Since irradiation acts on DNA (Urbain, 1986) and nisin acts on the bacterial cytoplasmic membrane (Bruno *et al.,* 1992), the effectiveness (*p< 0.05*) of the combination of nisin (100 or 200 µg/g) and γ irradiation (1 or 2 kGy) in reducing TBC, M &YC, *Bacillus cereus* and *Escherichia coli* in the present study was not surprising. The enhanced antimicrobial activity was explained by several investigators (Ricke *et al.,* 2005, Galvez *et al.,* 2007) that the combination of more than one intervention treatment has been found to produce a greater antimicrobial effect than any single treatment (which is referred to as hurdle technology), often working synergistically, with lower doses compared to their individual application and very low probabilities for survival and proliferation for cells confronted with multiple hurdles. Ayari *et al.* (2012) observed that the inhibitory effect of γ irradiation (1 kGy) increased significantly *(p< 0.05)* in presence of nisin. The pre-treatment of bacterial cells with nisin, produced greater log reduction than the only irradiated control, as it rendered cells more susceptible to the sub-lethal ionizing radiation at 1 kGy.

5. Conclusion

The use of natural additives in food represents additional hurdle technologies that may complement or act synergistically with traditional intervention approaches. In addition, some of these natural compounds may exhibit antimicrobial properties that differ mechanistically from other antimicrobials being used. Treatment of minced chicken with nisin at 100 or 200 µg/g did not show any antimicrobial activity against TBC and M&YC. The combination of nisin with γ irradiation showed an additional effect *(p< 0.05)* against TBC and M&YC during refrigerated storage. It also had significant *(p< 0.05)* antimicrobial activity against *Bacillus cereus* and *Escherichia coli* in minced chicken. Finally, this combination should not be used as a substitute for complying with general hygienic practices when handling and preparing food.

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