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Effect of Gamma Radiation and Low Temperature on Pathogenic *Staphylococcus aureus* Isolated from Pizza

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ABSTRACT

Staphylococcus aureus, a common human pathogen, produces enterotoxin and causes intoxication when ingested through contaminated food. The aim of the present study was to investigate microbiological quality of pizza and to detect the presence of the pathogenic *S. aureus* in this food. Moreover, effects of gamma radiation and low temperature on inoculated pathogenic *S. aureus* in pizza were examined. For this purpose, 20 pizza samples were collected from 5 different shops to check the microbiological quality and the presence of *S. aureus*. Isolated *S. aureus* were then checked for toxin production by mice assay. Pre-sterilized pizza samples inoculated with toxin producing isolate were then subjected to different gamma radiation doses and kept at refrigerator followed by detecting the presence of viable *S. aureus*. Among the collected pizza samples, 18 samples showed the presence of high number of total bacterial count, coliform count and staphylococcal count. Microwave heating could completely eliminate the viable counts only after 2 min. Among the isolated *Staphylococcus* spp., 13 isolates were identified as pathogenic *S. aureus* and one isolate produced deadly toxin. Radiation dose of 8 kGy resulted in the total elimination of *S. aureus* inoculated in pizza samples. However, low temperature (4°C) storage after gamma radiation showed a drastic change on the growth of the organism. The shelf life of these pizza samples was also extended up to 14 days. Thus, irradiation at 8 kGy with combination of storage at 4°C could be the suggested treatment for the storage of such ready-to-eat food without presence of pathogenic *S. aureus*.

Key words: *Staphylococcus aureus*, gamma radiation, low temperature, pizza, preservation, decontamination

INTRODUCTION

Contaminated food consumption often resulted in the illness which is called Food borne illness or food poisoning. *Staphylococcus aureus*, a Gram positive bacterium, is a common cause of bacterial food borne disease worldwide (Biswas *et al.*, 2011). The effected person experiences vomiting and diarrhea within very short period of having the *S. aureus* toxin contaminated food. The toxin is a preformed enterotoxin and known as superantigen because of requiring very short incubation time to effect adversely on the immune system. The enterotoxin gene is found on prophages, plasmids and pathogenicity islands in different strains of *S. aureus*. (Pinchuk *et al.*, 2010; Stewart, 2008;

Ikawaty *et al.*, 2010). This organism is a pathogen of major concern as human is one of the major sources of this organism and is carried by about one third of the general population (Soomro *et al.*, 2003). It also has the capacity to adapt to adverse environmental conditions (Jeshina and Surekha, 2009).

Fast food shops have already become very popular thorough out the world because of having enough time to cook or prepare food in our daily busy life. The most popular fast foods in Bangladesh are burger, pizza, french fries etc., The consumption of these ready-to-eat foods has been reported to be associated with serious health problems (Adams and Moss, 2000; FDA, 2000). Recently, Pizza is becoming very popular in the country which is found both in the most expensive fast food shops and roadside food shops. One of the main components of making pizza is the dough. Pizza dough is comprised of flour, water, yeast, salt, sugar, oil and spices. When this dough is kept in refrigerator, it works as an excellent substrate for the growth of microorganisms (Cabo *et al.*, 2001). Sometimes pizza also includes cheese, vegetables and chicken/beef. Prevalence of *Campylobacter* spp., *Staphylococcus* spp., *Escherichia coli*, *Salmonella* spp., *Yersinia* spp. and *Listeria* spp. are found in the ingredients which are commonly used for preparation of pizza, such as, meat, chicken, sea foods, vegetables, commercial mayonnaise etc., Presence of *S. aureus* and enteric bacteria, like, *E. coli* on raw vegetables suggest the use of poor quality water for pizza preparation (Pelczar *et al.*, 2006). The high incidences of food poisoning outbreaks in developing countries (Koffi-Nevry *et al.*, 2011) are often referred to as the unavailability of potable water for preparing fast foods and washing the dishes used in fast food shops (Alvarado-Casillas *et al.*, 2007). Moreover, high water content of meat and soft cheeses allow bacteria, viruses or molds to grow and replicate quickly (Lemya *et al.*, 2006; Bichai *et al.*, 2008). Sometimes, excess pizza that are not sold out are kept in refrigerator for preservation (in case of normal restaurants) to sell on next day allowing the growth of psychrophilic microorganisms (De Freitas *et al.*, 2008). Health risks are also associated with subsequent contaminations by the workers during handling. Staphylococci are included in the bacterial group that contaminates food products in this way (Fadel and Ismail, 2009). Poached chicken are generally handled with bare hands, the natural source of *S. aureus*, thereby allowing the food item to be contaminated (Heymann, 2004). Following time and temperature abuses, *S. aureus* may grow and produce enterotoxin.

Food irradiation has been compared with pasteurization as a tool for assuring the safety of food. In combination with good manufacturing or processing practices, gamma irradiation can significantly reduce the chance of microbial food poisoning (ESFA, 2011). Decontamination of food by ionizing radiation is a safe, environmental friendly and energy saving process. Irradiation is mainly used to decontaminate end products. The process is very useful for decontamination of red meat, poultry egg, fishery products and spices (Fallah *et al.*, 2008). Radiation doses of 2-7 kGy can successfully eliminate well known non-sporeforming pathogenic bacteria including *Salmonella*, *Staphylococcus aureus* *Campylobacter*, *Listeria monocytogenes*, *Escherichia coli* O157:H7 etc., from food products without losing their food values. A very distinctive characteristic of radiation decontamination is that it can also be applied in frozen food. When the demand for high-quality convenience foods is increasing day by day, radiation treatment in combination with other processes is assuring to increase the safety of poorly processed foods. Moreover, radiation doses of 0.15-0.7 kGy are enough to control many food borne parasites under specific conditions (Farkas, 1998). When low radiation treatment is used on food products some microorganisms survive. The surviving microorganisms then become more susceptible to stresses or subsequent treatments than those were not exposed to irradiation (Farkas, 1998).

In this study, the degree of bacteriological contamination level of a very popular ready-to-eat food of Bangladesh named Pizza was determined to reveal the microbiological quality of the food. Further, the study also aimed to understand the effect of irradiation and refrigeration on the growth and survival of inoculated pathogenic *S. aureus* in pizza during storage period.

MATERIALS AND METHODS

Sample collection, processing and microbiological analysis: Five shops of Dhaka city were selected for sample collection where middle class and poor people are used to have fast foods, especially pizza, during different parts of the day. Twenty pizza samples (S1-S20) were purchased from these shops in March, 2010. All the samples were aseptically packed in pre-sterilized polythene bags and transferred immediately in the laboratory in an ice box in chilled conditions to prevent the bacterial multiplication during the transportation and microbiological analysis was done on the same day. All the samples were kept at -20°C until these were used for microbiological assay. The samples were cut into different pieces and kept into the sterilized polythene bags and used for determination of different bacterial counts.

Bacteriological analysis: Ten grams of each of the pizza sample was aseptically homogenized for 2 min in a sterile plastic bag containing 90 mL of sterile 0.1% peptone water using a stomacher (Seward Medical Co., UK). For microbiological analysis, appropriately diluted sample was used for Total viable Bacterial Count (TBC), Total Coliform Count (TCC) and Total Staphylococcal Count (TSC) in Nutrient agar medium, MacConkey agar medium and Mannitol Salt Agar (MSA) medium, respectively (Yeboah-Manu *et al.*, 2010; Akinjogunla *et al.*, 2011; Mahin *et al.*, 2011). All the viable counts were the average of three independent experiments.

Effect of microwave heating on the associated bacteria of pizza: To observe the effect of microwave heating on the associated bacteria, the pizza sample S1 and S2 were subjected to microwave heating for 2 min followed by total bacterial and staphylococcal counts determination as described earlier.

Identification of the Pathogenic *S. aureus*: All yellow colonies found on mannitol salt agar after 20 h of incubation were selected for further identification of *S. aureus* as described by Flayhart *et al.* (2004). Slide coagulase, exogenous nuclease and mannitol fermentation tests were used for identification of *S. aureus* where mannitol fermentation was determined using a peptone agar base with phenol red indicator (Flayhart *et al.*, 2004). An isolate positive by slide coagulase, exogenous nuclease and mannitol fermentation assay was identified as *S. aureus* (Bannerman, 2003). For further confirmation, blood hemolysis and novobiocin sensitivity tests were also performed (Kloos and Schleifer, 1975).

Pathogenicity test through Mouse Assay: Pathogenicity test of the identified *S. aureus* was done according to the method described by Matsuzaki *et al.* (2003) using five 10 to 21 days old Swiss white NIH/NMRI CV mice. *S. aureus* cells were grown in 100 mL in nutrient broth (Difco) at 37°C and centrifuged at 8000 g for 5 min at the early stationary phase. The cell pellets were washed with 100 mL saline, centrifuged again under the same conditions and finally resuspended in 5 mL saline. After appropriate dilutions, bacterial suspensions of 10⁷ to 10⁸ cfu mL⁻¹ in saline were prepared compared with standard turbidity unit (Matsuzaki *et al.*, 2003). Then 0.5 mL of the

bacterial cell suspensions were injected into the peritoneal cavities of mice through one side of the abdomen. Equal volume of bacterial suspension of *S. aureus* ATCC 6538 and saline alone were used as positive and negative control, respectively and were injected intraperitoneally in mice. The test animals were observed hourly for 10 h to detect any abnormality or death of the animal.

Inoculation of the pathogenic *S. aureus* in pizza samples: Pathogenic *S. aureus* which killed mouse in mice assay, was designated as PS-2 and used for inoculation in pizza samples according to the procedure described by Lee *et al.* (2006). The PS-2 cell preparation was spread aseptically on pizza samples (10 g) previously sterilized with 10 kGy gamma irradiation. The test pizza samples were kept in a sterile workstation for 1 min to allow it to be absorbed.

Effects of irradiation and refrigeration temperature on the inoculated *S. aureus*: To investigate the effect of gamma radiation on pathogenic *S. aureus*, inoculated pizza samples were irradiated in a cobalt-60 irradiator at the Gamma Source Division of the Atomic Energy Research Establishment in pre-sterilized sealed polythene bags. The source strength was approximately 51.15 kCi with a dose rate of 2.066 kGy h. Dosimetry was performed using amber Perspex Fricke solution and ceric cerous sulfate solution. The dosimeters were calibrated against an international standard set by the International Atomic Energy Agency (Vienna, Austria). The applied doses in this study were 2, 4, 6 and 8 kGy. Samples remained un-irradiated (0 kGy) was titled as 'control' in all the steps of the experiment. Moreover, to observe the effect of combination of low temperature and irradiation, gamma radiated samples were kept at 4°C for 8 days. Microbial assay for *S. aureus* count determination were done according to the procedure previously described.

RESULTS

Bacteriological analysis of pizza samples: All the pizza samples collected from 20 different shops in 5 different area of Dhaka city were found to have high number of total bacteria and coliform (Table 1). Except two pizza samples, S10 and S11, Staphylococci were also found in all the samples. The range of total bacterial counts were 1.26×10^3 cfu g⁻¹ in S12 to 3.02×10^7 cfu g⁻¹ in S3, total staphylococcus counts were 2.10×10^2 cfu g⁻¹ in S12 to 5.60×10^5 cfu g⁻¹ in S1 and total coliform counts were 2.00×10^1 cfu g⁻¹ in S5 cfu g⁻¹ to 9.60×10^2 cfu g⁻¹ in S8.

Effect of microwave heating on the associated bacteria of pizza: Application of microwave for 1 min resulted in the decrease of both total aerobic bacterial count and staphylococcal count. After 2 min, both types of bacterial concentration became below the detection limit of the used experimental procedure (Fig. 1).

Identification of the Pathogenic *Staphylococcus aureus*: Among the Staphylococci isolates found in MSA from 18 pizza samples, 13 isolates of their representative pizza samples fermented mannitol and produced golden yellow colonies in MSA. They were exogenous nuclease positive and showed agglutination on slides with blood coagulase. They also produced beta hemolysis and were novobiocin sensitive. These isolates were designated as PS-1 to PS-13, respectively. Then the productions of deadly toxin by these 13 isolates were checked by mouse assay. After peritoneal injection of mice with the *S. aureus* isolates, 4 mice (injected with isolate PS-8, PS-10, PS-11 and PS-12) were in normal condition but 9 mice (injected with isolate PS-1, PS-2, PS-3, PS-4, PS-5, PS-6, PS-7, PS-9 and PS-13) became weak and showed abnormal behaviors. The mouse injected

Table 1: Initial microbial load of pizza samples collected in Dhaka city

Sample no.	Different types of microbial count (cfu g ⁻¹) ^a		
	TBC	TSC	TCC
S-1	3.00×10 ⁰⁷	5.60×10 ⁰⁵	2.00×10 ⁰²
S-2	2.90×10 ⁰⁷	1.60×10 ⁰⁵	3.00×10 ⁰²
S-3	3.20×10 ⁰⁷	2.40×10 ⁰⁵	2.60×10 ⁰²
S-4	6.40×10 ⁰⁶	3.60×10 ⁰⁵	5.00×10 ⁰¹
S-5	3.70×10 ⁰⁷	6.20×10 ⁰⁵	2.00×10 ⁰¹
S-6	6.00×10 ⁰⁶	1.60×10 ⁰³	6.40×10 ⁰²
S-7	4.00×10 ⁰⁵	3.20×10 ⁰⁴	6.30×10 ⁰²
S-8	5.80×10 ⁰⁵	2.60×10 ⁰³	9.60×10 ⁰²
S-9	9.80×10 ⁰⁵	6.60×10 ⁰³	8.00×10 ⁰²
S-10	2.92×10 ⁰³	-	5.00×10 ⁰¹
S-11	1.30×10 ⁰³	-	8.00×10 ⁰¹
S-12	1.26×10 ⁰³	2.10×10 ⁰²	1.20×10 ⁰¹
S-13	5.30×10 ⁰⁵	4.00×10 ⁰³	1.00×10 ⁰²
S-14	4.20×10 ⁰⁵	3.30×10 ⁰³	1.40×10 ⁰²
S-15	4.00×10 ⁰⁶	2.30×10 ⁰⁴	1.00×10 ⁰²
S-16	2.09×10 ⁰⁵	1.60×10 ⁰³	3.00×10 ⁰²
S-17	3.20×10 ⁰⁵	2.20×10 ⁰³	2.80×10 ⁰²
S-18	3.00×10 ⁰⁵	2.20×10 ⁰³	2.60×10 ⁰²
S-19	5.10×10 ⁰⁴	3.80×10 ⁰²	1.10×10 ⁰²
S-20	1.50×10 ⁰⁴	2.20×10 ⁰²	8.10×10 ⁰²

^aValues are averages for three independent experiments. -: No viable count was detected, TBC: Total viable bacterial count; TSC: Total staphylococcal count; TCC: Total coliform count

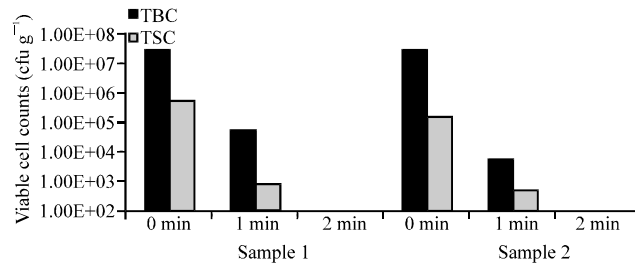


Fig. 1: Effect of microwave heating on the associated bacteria of pizza samples. TBC (total viable bacterial count) and TSC (total staphylococcal count) of pizza sample 1 and sample 2 after exposure to microwave heating are shown. Values are the average of three independent experiments

with *S. aureus* ATCC 6538 also showed abnormal behaviour. After 4 h of injection, the mice injected with PS-2 and *S. aureus* ATCC 6538 were died. Thus, strain PS-2 could be considered as the most deadly toxin producer like the control strain.

Effects of irradiation and refrigeration temperature on the inoculated *S. aureus*: After inoculation with pathogenic *S. aureus* PS-2, when pizza samples were irradiated with gamma radiation staphylococcus counts were reduced (Fig. 2a). The dose of 2.0 kGy reduced *S. aureus* population by 2 logarithmic cycles (10⁷ to 10⁵ cfu g⁻¹). The dose of 4.0 and 6.0 kGy reduced

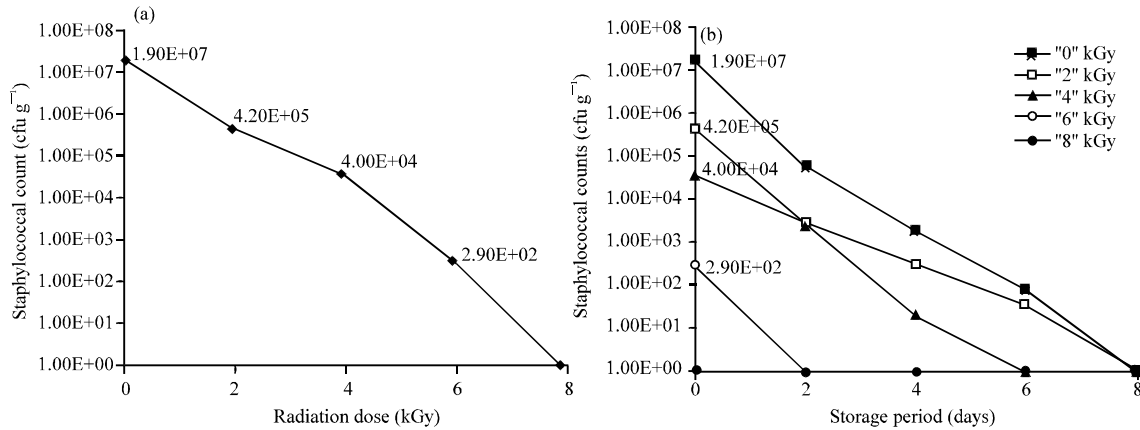


Fig. 2 (a-b): Effect of gamma radiation (a) and combined treatment of low temperature and gamma radiation (b) on pathogenic *S. aureus* inoculated in pizza samples. Staphylococcal counts of the samples irradiated at different doses of gamma radiation just after treatment (a) and storage at 40°C for 8 days (b) are shown. Values are the average of three independent experiments

S. aureus population by 3 and 5 logarithmic cycles, respectively. No staphylococcal count was detected in pizza irradiated with 8.0 kGy. The pizza samples irradiated with 8 kGy were completely putrefied after 6 days when they were kept at room temperature.

When the irradiated samples were stored at 4°C in a refrigerator, the combination of radiation and low temperature showed extensive effect on the viability of pathogenic staphylococcus in pizza samples (Fig. 2b). Staphylococcal count in the samples irradiated at the dose level 6.0, 4.0, 2.0, 0 kGy became below the detection level after 2, 6, 8 and 8 days, respectively. Shelf life of the sample irradiated at 8 kGy was extended up to 14 days with the complete absence of *S. aureus* (Data not shown).

DISCUSSION

The hygienic conditions of the shops from where pizza samples were collected were not as good as maintained by the high class fast food shops. Therefore, the presence of total bacteria, staphylococci and coliform were very much expected. Presence of bacterial contamination in pizza and other fast foods were reported earlier by Cabo *et al.* (2001) and De Freitas *et al.* (2008). The presence of staphylococci and coliform indicated the use of poor quality water and poor handling during different steps of pizza production (Pelczar *et al.*, 2006). The presence of *Staphylococcus* spp. was alarming since the bacteria is considered as the third most important cause of disease in the world amongst the reported food-borne illnesses (Zhang *et al.*, 1998).

The level of microbial contamination found in pizza samples is indicating a huge health hazard for the common country people who are having these foods. To ensure the safety of their health, these foods should be free of pathogenic microbes. Microwave heating is commonly used in fast food shops in our country to heat fast foods. Our results indicated that only enough heating (for 2 min) can eliminate the contaminating microorganisms. However, only 60% of the shops from where we collected the pizza samples had microwave oven. Moreover, the shops which had the oven were not used to heat the pizza for enough time to avoid burning. So, the people who are having fast food from these shops are very much likely to have food poisoning.

Among the 20 pizza samples 18 were contaminated with staphylococci. Thirteen of the staphylococcal isolates were CPS (Coagulase Positive Staphylococci). CPS are the effective indicator of the degree of contamination with potentially pathogenic strains, particularly from human sources (Desmarchelier *et al.*, 1999) and of poor hygienic practices (Soomro *et al.*, 2003; Nanu *et al.*, 2007). *S. aureus* isolates were identified using the following biochemical tests of slide coagulase, exogenous nuclease and fermentation of mannitol (Flayhart *et al.*, 2004). Human plasma (sensitivity of 91%) was used for the coagulase test in this experiment since it has more sensitivity for the test than sheep plasma (sensitivity of 81%). However, both plasmas have very low specificity (11 and 7%, respectively). The sensitivity and specificity of the coagulase test (human plasma) was markedly improved when Mannitol salt agar and DNase were introduced as a tri-combination test for routine identification of *S. aureus* (100% specificity and 67% sensitivity) (Kateete *et al.*, 2010).

Farkas (1998) suggested irradiation as an effective tool for food and end product decontamination and elimination of various pathogens. We also implemented the technique to check whether the technique is suitable for eliminating the microorganisms associated with pizza samples. However, at least 8 kGy gamma radiation dose was required for complete elimination of inoculated *S. aureus*. According to Thayer (1995), ionizing radiation of <3.0 kGy can eradicate or significantly lower the number of the most common enteric pathogens, such as, *Campylobacter jejuni*, *E. coli*, *S. aureus*, *Salmonella spp.*, *L. monocytogenes* and *Aeromonas hydrophila*. Thus, the treatment can be an effective way to kill enteric pathogens associated with meat and poultry products. Spoto *et al.* (2000) reported 6 logarithmic cycle reduction of pathogenic *S. aureus* at the dose level of 4.0 kGy and total absence of the pathogen at 6.0 and 8.0 kGy. The present study also revealed the similar type of result which was 3 and 5 logarithmic cycle reduction at the dose level of 4.0 and 6.0 kGy, respectively and complete elimination at 8.0 kGy. To make the process more effective we stored the irradiated pizza at 4°C. In case of refrigeration (4°C) storage, the pathogen became undetectable in the 0 and 2.0 kGy irradiated samples after 8 days. The samples showed no bacterial growth after 6 and 2 days when irradiated with 4.0 and 6.0 kGy radiation, respectively and kept at 4°C. Samples irradiated at 8.0 kGy radiation dose were remained pathogen free from the initial day of radiation and its shelf life was extended up to 14 days. The advantages of using combined treatment of radiation and low temperature for food preservation were reported by other authors (Murano *et al.*, 1995; ICMSF, 1998; ESFA, 2011). Lamb *et al.* (2002) also showed that combination of low-dose gamma irradiation and refrigeration is more effective method for reducing *S. aureus* in ready-to-eat foods than refrigeration treatment alone.

However, the findings of present study were contradictory with the reports of Spoto *et al.* (2000) where they observed the *S. aureus* counts of 10^8 to 10^4 and 10^1 to 10^2 CFU g⁻¹ in chicken breast kept in refrigerator for 28 days after radiation treatment at 2 and 4 kGy, respectively. In our study, *S. aureus* were completely absent in pizza samples irradiated at 2 and 4 kGy after 8 and 6 days. This can be explained by the presence of different spices which might help to limit the microbial count in pizza.

CONCLUSION

The study indicated that pizza found in common shops of Bangladesh are highly contaminated with microorganisms. Gamma radiation can eliminate the pathogenic *S. aureus* in the fast food. However, combination of gamma irradiation and refrigeration can be a useful tool for preservation of ready-to-eat food like pizza without presence of pathogenic *S. aureus*, which is one of the major causes of food born infection.

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