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Effect of Low Temperature, Radiation and their Combination Treatments on Microorganisms Associated with Fresh Water Mola Fish, *Amblypharyngodon mola* (Hamilton-Buchanan 1822)

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ABSTRACT

This study was conducted to assess microbiological quality of a local fresh water fish of Bangladesh named mola, *Amblypharyngodon mola*. The effects of freezing temperature, irradiation and their combination treatments on the associated microorganisms during storage of the fish were also evaluated. For this purpose, fish samples were collected from a local market and irradiated with different doses (2.5, 5.0, 7.5 and 10.0 kGy) of gamma radiation and kept at -20°C for 6 months followed by microbiological assay after each month interval. The average Total Viable Bacterial Count (TVBC), Total Staphylococcal Count (TSC), Total Coliform Count (TCC), Total Faecal Coliform Count (TFCC), Total Aeromonas Count (TAC) and Total Fungi Count (TFC) of fresh mola fish samples were 9.74×10^{07}, 3.60×10^{06}, 9.70×10^{04}, 6.40×10^{05}, 4.00×10^{05} and 2.30×10^{03} cfu g^{-1}, respectively. Seventy six bacterial strains were isolated and identified from these samples which include *Staphylococcus aureus*, *Micrococcus varians*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella ozaenae*, *Bacillus subtilis*, *Bacillus megaterium*, *Klebsiella edwardsii* and *Micrococcus radiodurans*. When treated with gamma radiation dose, the total coliform, faecal coliform, *Aeromonas* sp. and fungi of the samples were eliminated at 2.5 kGy and total viable bacteria and staphylococci were eliminated at 7.5 kGy. During frozen storage, microbial counts were gradually decreased with time. However, the combination of these two treatments was found more effective than any of the single treatment for elimination of the associated microorganisms. The moisture contents of mola fish were not changed significantly at different stage of storage periods and also at different radiation doses. On the other hand, losses of total protein contents were also very low with increasing storage time and radiation dose. The findings indicated that, the combination treatment can be applied for long time preservation of mola fish in Bangladesh without any significant loss of moisture and protein values.

Key words: Low temperature, radiation, mola fish, preservation, decontamination

INTRODUCTION

Fish is a non-tetrapod chordate, i.e., an animal with a backbone that has gills throughout its life and has limbs in the shape of fins (Nelson, 2006). This is an important source of food for

mankind throughout the world and is a very important source of animal protein (Ravichandran *et al*., 2010; Ojewola and Annah, 2006; Chowdhury and Maharjan, 2001; Sutharshing and Sivashanthini, 2011). The important constituents of fish are water (70.0-85.0%), protein (15.0-20.0%), lipid (1.0-10.0%), ash (1.0-1.5%) and carbohydrate (0.3-1.0%). Protein fraction is the 50-95% of the total solids of the muscle tissue and is composed of sarcoplasmic, myofibrillar and stroma proteins (Panuncio, 2001; Moghaddam *et al*., 2007; Sutharshing and Sivashanthini, 2011). Fish is also a very good source of vitamins and minerals (Edem, 2009; Moghaddam *et al*., 2007). Most noticeably, the high nutrient containing biochemical composition classified fish as highly perishable food. According to Frazier and Westhoff (1988), fish is the most susceptible animal to autolysis, oxidation and hydrolysis of fats and microbial spoilage. The deterioration is believed to cause mainly by the bacterial activity, which brings about very noticeable changes in the texture, flavor, odor and general appearance of the product. For this reason, we are concerned primarily with the deterioration of fish by microorganisms and microbial enzymes. Huss *et al*. (2003) and Abraham (2011) reported that the microorganisms present on fish include those which are associated with the raw material and acquired during harvesting, handling and processing.

Fishes are in constant interaction with microorganisms which cause spoilage (Gram and Dalgaard, 2002) and their metabolic activities result in the appearance of slime, gross discoloration and strong odors and which become offensive to customers (Paulsen and Smulders, 2003). Spoilage of fish not only occurs at room temperature but also at the refrigeration temperature (5-7°C). At room temperature fish spoils within 8-20 h and when stores in ice, they can be kept for 27-30 days. Spoilage is the result of series of complicated changes brought about in the dead fish mainly by the enzymes and bacteria. Enzymes remain active after the death of the fish and result in autolysis (Shahidi and Kamil, 2001) which affect flavor, texture and sometimes the appearance of fish. When the fish dies, bacteria present on the surface and the guts multiply rapidly and invade the flesh, which provides an ideal medium for growth and multiplication (Gram and Dalgaard, 2002). The bacteria can breakdown the muscle itself and will also feed on the smaller units produced by autolytic action (Gram and Dalgaard, 2002). Some bacteria such as *Staphylococcus* sp., *Clostridium botulinum*, *Salmonella* spp., *Shigella* sp., can produce toxin in the spoiling fish which pose serious health hazard. Although, fish possesses bioactive molecules having antimicrobial activity as a part of their defense system (Ravichandran *et al*., 2010; Shanmugam *et al*., 2008; Bragadeeswaran and Thangaraj, 2011) presence of pathogenic bacteria is not uncommon in this protein source (Akinyemi and Buoro, 2011; Wefky and Ghobrial, 2008; Raja *et al*., 2006).

Most kind of fishes are readily decomposed by microorganisms unless special methods are used for their preservation. The goals of fish preservation methods are to increase the shelf life of the food and ensure the safety for human consumption. Various food preservation methods include use of low temperature, high temperature, drying, desiccation or dehydration, lyophilization, salting, chemical preservatives, anaerobiosis, controlled atmosphere, radiation etc. Exposure of microorganisms to low temperatures reduces their rates of growth and reproduction. This principle is used in refrigeration and freezing. Microbes are not killed in refrigeration at 5°C. Low temperatures are also used to retard chemical reactions and action of food enzymes and to slow down or stop growth and activity of microorganisms in fish. Low temperature slows the enzymatic action, chemical action and microbial growth. However, some are able to survive, such as, *Salmonella* spp. and Streptococci survive in freezing condition (Archer, 2004; Beal *et al*., 2001). Deep freezing at -20°C forms smaller crystals. It reduces biochemical activities of microbes. In most

cases, different retardation of the activity of enzymes at low temperature can change metabolic pathway and end products. This method of preservation does not kill the microorganisms but reduces microbial metabolism which is responsible for spoilage (Bakermans and Skidmore, 2011).

Gamma rays are used for some fish products storage which reduces surface contamination on fish products. It is highly lethal but the dose may be adjusted to yield pasteurizing or sterilizing effects. Most studies indicate that lethal damage to microbial DNA resulting in loss of ability to reproduce is a primary cause of lethality but damage to other sensitive and critical molecules (e.g., membranes) may also have an effect. Dose level lethal for microorganisms does not cause inactivation of most enzymes or major changes to proteins and other large molecules. Free radicals and other reactive molecules are formed particularly in water. However, radiation sensitivity differs among the strains. Removal of oxygen or water increases the resistance of microorganisms to ionizing radiation. Gram negative bacteria are more sensitive than Gram positive bacteria. Spores of bacteria show resistance to irradiation effect. The resistance of bacterial spores is little affected by freezing, so it is advantageous to irradiate a food in the frozen state (International Commission for Microbiological Specifications for Foods, 1998; ESFA, 2011). Gamma radiation is also effective in growth inhibition of fungi (Aziz and Moussa, 2002).

Mola fish (*Amblypharyngodon molai*) is a small fresh water fish which is cheap, tasty, easily available and highly nutritious. It is a very popular fish among the variety of fresh water fishes. It is found abundantly everywhere in Bangladesh. In case of fish like mola, the unwanted spoilage microbes may cause severe undesired change, resulting in considerable spoilage and economic loss. In Bangladesh, a very toxic chemical, formaldehyde, is commonly used for preserving fish without knowing about the health hazards of formaldehyde consumption with fish (Shahdat Hossain *et al*., 2008). Continuous ingestion of formaldehyde containing fish can even cause cancer and a variety of unknown pathology (Hildesheim *et al*., 2001; Vaughan *et al*., 2000). Therefore, with the help of a sustainable technique to avoid fish spoilage, this study was aimed to isolate and identify microorganisms associated with mola fish and their disinfection by low temperature, gamma radiation and their combination treatments. The effects of these treatments on biochemical properties and shelf life extension of mola fish were also investigated.

MATERIALS AND METHODS

Collection of samples: Fresh water mola (*Amblypharyngodon mola*) fishes were purchased from a local market of Savar, Bangladesh in the year of 2010. All the samples were aseptically packed in pre-sterilized polythene bags. The polythene bags were sealed air tightly after collecting the samples. The samples were brought out immediately in chilled condition (in insulating foam box with ice) to laboratory for microbiological analysis.

Sample processing and microbiological analysis: Collected fishes were thawed by keeping at room temperature and distributed in separate five small sterile polythene bags. Each bag contained 10 g fish taken from different parts of the fish. These samples were then macerated by using a blender machine under aseptic conditions. To determine the single effect of storage at freezing temperature (-20°C), samples (non-irradiated) were kept at -20°C for 6 months and microbial counts were determined each month interval. Again, to investigate the single effect of gamma radiation, samples were treated with different doses of gamma radiation (2.5, 5.0, 7.5, 10.0 kGy) at a dose rate of 12.5 kGy h^{-1} by Co^{60} gamma radiation source (50,000 Ci) at the Institute of Food and Radiation Biology, Atomic Energy Research Establishment, Savar, Dhaka, Bangladesh

followed by microbial counts determination. Finally, to observe the effect of combination of these two treatments, gamma radiated samples were kept at -20°C for 6 months and microbial counts were determined every month.

For microbiological analysis, Total Viable Bacterial Count (TVBC) was done by the standard spread plate method according to the APHA (1984). Total Coliform Count (TCC), Total Faecal Coliform Count (TFC), Total Staphylococcal Count (TSC) and Total Aeromonas Count (TAC) were done in the same way using McConkey agar medium, mFc agar medium, *Staphylococcus* agar medium and Starch ampicillin agar medium (Akinjogunla *et al*., 2011; Mahin *et al*., 2011), respectively. All the viable counts were the average of at least three independent experiments. Bacterial isolates were then identified according to the Bergey's manual of determinative bacteriology (Buchanan and Gibbons, 1974) and manual for the identification of medical bacteria (Barrow and Feltham, 1993).

Malt Yeast Glucose (MYG) chloramphenicol agar was used for fungal count and their isolation. The plates were incubated at 28°C and counts were recorded after 5 days of growth. Viable fungal counts were the average of at least three independent experiments.

Determination of moisture content: Fish sample (4 g) was taken in the crucible and dried at 105°C for 3 h. Then it was taken at desiccators until it reached at room temperature and weight of the sample was taken. The process was continued until constant weight was obtained. The loss of moisture from the sample was then determined and expressed in percentage as follows:

$$\text{Moisture content}(\%) = \frac{\text{Initial weight} - \text{Final weight (i.e., weight loss)}}{\text{Original weight of the sample}} \times 100$$

Determination of protein content: The total nitrogen was determined by the Micro-Kjeldahl procedure (Miller and Houghton, 1945).

RESULTS

Determination of microbial load associated with the fish sample: First the initial microbial load in non-irradiated samples was determined. The Total Viable Bacterial Counts (TVBC) were varied from 8.8×10^{7} cfu g^{-1} in sample 5 to 1.27×10^{8} cfu g^{-1} in sample 1, with the average of 9.7×10^{7} cfu g^{-1} (Table 1). The Total Staphylococcal Counts (TSC) were varied from 2.7×10^{6} cfu g^{-1} in sample 5 to 4.2×10^{6} cfu g^{-1} in sample 1, with the average of 3.6×10^{6} cfu g^{-1}. The Total Coliform

Table 1: Initial microbial load of fresh water mola fish samples

	Different types of microbial count (cfu g^{-1})					
Sample No.	TVBC	TSC	TCC	TFCC	TAC	TFC
1	1.27×10^{08}	4.20×10^{06}	7.30×10^{05}	9.70×10^{04}	5.00×10^{05}	3.50×10^{03}
2	9.20×10^{07}	3.90×10^{06}	7.10×10^{05}	1.03×10^{05}	4.30×10^{05}	2.80×10^{03}
3	8.90×10^{07}	3.20×10^{06}	6.90×10^{05}	9.00×10^{04}	3.80×10^{05}	1.80×10^{03}
4	9.10×10^{07}	4.00×10^{06}	4.80×10^{05}	9.50×10^{04}	2.70×10^{05}	1.60×10^{03}
5	8.80×10^{07}	2.70×10^{06}	5.90×10^{05}	9.90×10^{04}	4.20×10^{05}	1.80×10^{03}
Average	9.74×10^{07}	3.60×10^{06}	6.40×10^{05}	9.70×10^{04}	4.00×10^{05}	2.30×10^{03}

TVBC: Total viable bacterial count, TSC: Total staphylococcal count, TCC: Total coliform count, TFCC: Total faecal coliform count, TAC: Total aeromonas count and TFC: Total fungi count

Counts (TCC) were varied from 4.8×10^5 cfu g^{-1} in sample 4 to 7.3×10^5 cfu g^{-1} in sample 1, with the average of 6.4×10^5 cfu g^{-1}. The Total Faecal Coliform Counts (TFCC) were varied from 9.0×10^4 cfu g^{-1} in sample 3 to 1.03×10^5 cfu g^{-1} in sample 2, with the average of 9.7×10^4 cfu g^{-1}. The Total *Aeromonas* Counts (TAC) were varied from 2.7×10^5 cfu g^{-1} in sample 4 to 5.0×10^5 cfu g^{-1} in sample 1, with the average of 4.0×10^5 cfu g^{-1}. The Total Fungal Counts (TFC) were varied from 1.6×10^3 cfu g^{-1} in sample 4 to 3.5×10^3 cfu g^{-1} in sample 1, with the average of 2.3×10^3 cfu g^{-1} (Table 1).

Effect of refrigeration, low temperature (-20°C) and their combination treatment during storage period on the associated microorganisms: The average initial Total Viable Bacterial Count (TVBC) of mola fish samples was 9.7×10^7 cfu g^{-1}. Non-irradiated samples stored at -20°C showed only negligible change (9.7×10^7 to 5.6×10^6) after 6 months storage (Fig. 1a). Both of the radiation dose 2.5 and 5.0 kGy reduced TVBC count by 3 logarithmic cycle. However, when these radiation treated samples were stored at low temperature, TVBC counts were further reduced by 1 (1.3×10^4 to 1.3×10^3) and 3 (1.1×10^4 to 6.7×10^1) logarithm cycle respectively after 6 months. Radiation dose 7.5 kGy or more completely eliminated TVBC in the samples without low temperature treatment and when these samples were kept at low temperature, no bacterial growth was found until the end of the study period (Fig. 1a).

The initial average Total Staphylococcal Count (TSC) of non irradiated mola fishes (3.6×10^6 cfu g^{-1}) was reduced 2 logarithmic cycle (3.6×10^6 to 4.2×10^4 cfu g^{-1}) after low temperature storage for 6 months (Fig. 1b). Radiation dose of 2.5 and 5.0 reduced the TSC count by 1 and 2 logarithm cycle, respectively without low temperature treatment. However, when both of these radiation treated samples were kept at -20°C, no TSC was found after 3 months storage. Like TVBC, single effect of 7.5 kGy or more completely eliminate TSC counts in the fish samples without any requirement of low temperature treatment (Fig. 1b).

A very negligible change (6.40×10^5 to 5.2×10^5 cfu g^{-1}) in Total Coliform Count (TCC) was found when non-irradiated samples were stored for 6 months in -20°C (Fig. 1c). No viable coliform was found when the samples were treated with irradiation dose 2.5 kGy or more. TCC was not detected even after 6 months of these irradiated samples during their storage at low temperature (Fig.1c).

In case of Total Faecal Coliform Count (TFCC), the average initial count (9.7×10^4 cfu g^{-1}) of non-irradiated fish samples was not significantly changed when the samples were kept at -20°C for 6 months (Fig. 1d). However, the faecal coliforms were completely eliminated with a very low radiation dose of 2.5 kGy. No faecal coliform was detected in any of the irradiated samples throughout the storage period at -20°C (Fig. 1d).

Non-irradiated fish samples also showed only negligible change in Total Aeromonas Count (TAC). This count was not detected when the samples were treated with radiation dose 2.5 kGy or more just after irradiation and even after 6 months of storage at low temperature (Fig. 1e).

Unlike to the other cases, no Total Fungal Count (TFC) was detected in the non-irradiated fish samples after 2 months of storage at -20°C (Fig. 1f). In case of irradiated samples, radiation does of 2.5 kGy or more was enough from total elimination of fungal count. Fungal count was also absent in the irradiated samples kept at low temperature even up to the end of the studied storage period stored (Fig. 1f).

Identification of bacterial isolates from the fresh and stored fish samples: Morphological and biochemical characteristics of the bacteria isolated from fresh mola fish samples revealed that

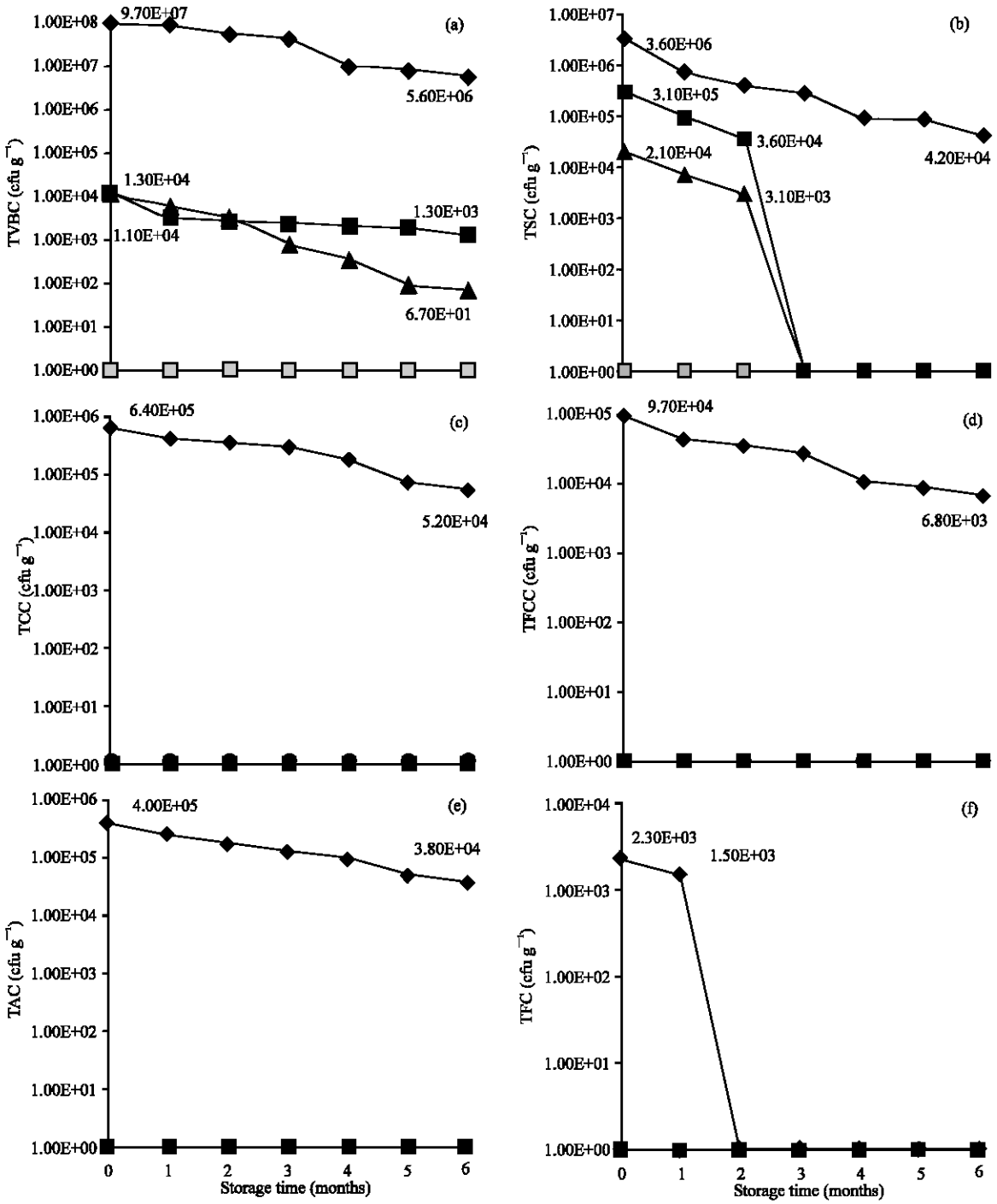

Fig. 1(a-f): Effect of combined treatment of low temperature and irradiation on (a) Total Viable Bacterial Count (TVBC), (b) Total Staphylococcal Count (TSC), (c) Total Coliform Count (TCC), (d) Total Faecal Coliform count (TFCC), (e) Total Aeromonas Count (TAC) and (f) Total Fungi Count (TFC) of mola fish samples. Different microbial counts after treatment with 0 kGy (closed diamonds), 2.5 kGy (closed squares), 5.0 kGy (closed triangles), 7.5 kGy (open squares) and 10 kGy (open circle) and stored at -20°C up to 6 months of storage are shown

Table 2: Distribution of isolated bacteria (n = 76) in fresh mola fish

Bacterial isolates	Isolation frequency No. (%)
Staphylococcus aureus	16 (21.06)
Micrococcus varians	11 (14.47)
Aeromonas hydrophila	9 (11.82)
Escherichia coli	8 (10.53)
Pseudomonas aeruginosa	7 (9.21)
Klebsiella ozaenae	6 (7.89)
Bacillus subtilis	6 (7.89)
Bacillus megaterium	6 (7.89)
Klebsiella edwardsii	4 (5.26)
Micrococcus radiodurans	3 (3.95)

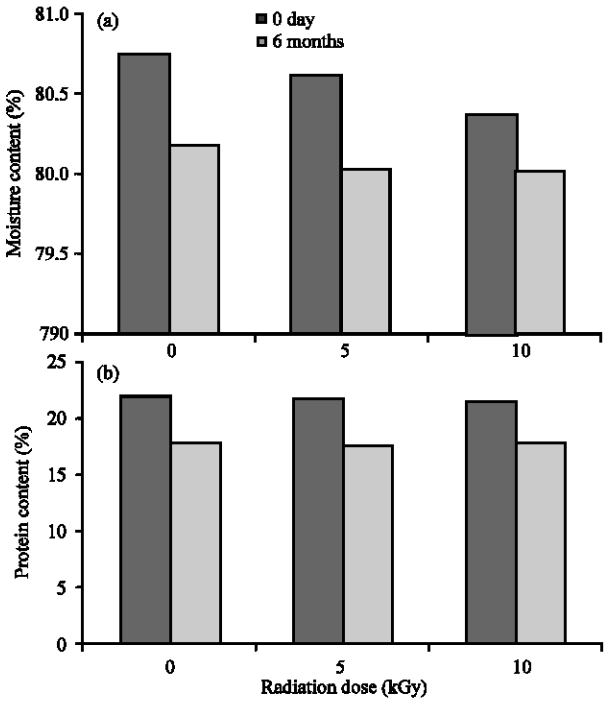

Fig. 2 (a-b): Changes in moisture (a) and protein (b) contents in the radiation treated fish samples stored at -20°C

among 76 bacterial isolates, 16 (21.06%) were *Staphylococcus aureus*, 11 (14.47%) were *Micrococcus varians*, 9 (11.82%) were *Aeromonas hydrophila*, 8 (10.53%) were *Escherichia coli*, 7 (9.21%) were *Pseudomonas aeruginosa*, 6 (7.89%) were *Klebsiella ozaenae*, 6 (7.89%) were *Bacillus subtilis*, 6 (7.89%) were *Bacillus megaterium*, 4 (5.26%) were *Klebsiella edwardsii* and 3 (3.95%) were *Micrococcus radiodurans* (Table 2). However, after 6 months of storage *Klebsiella edwardsii* and *Micrococcus radiodurans* were not found in the stored fish samples (data not shown).

Physicochemical analysis of irradiated and non-irradiated fish samples: The initial moisture content of the non-irradiated, 5.0 and 10 kGy irradiated samples were 80.75, 80.63 and 80.38%, respectively (Fig. 2a). After six months of storage this was lowered to 80.18, 80.03 and

80.01%, respectively. Alternatively, the initial protein content of the non-irradiated, 5.0 and 10 kGy irradiated samples were 21.89, 21.65 and 21.47%, respectively (Fig. 2b). After six months of storage this was lowered to 17.87, 17.53 and 17.76%, respectively.

DISCUSSION

In the present investigation, a total of five samples of fresh water mola fish were analyzed for microbial content. The total viable bacterial counts of the sample were varied from 8.8×10^{7} to 1.27×10^{8} cfu g^{-1}. This may be due to indigenous microbial contaminants, water source, processing or handling and selling condition of the fish sample. Similar results have been reported by Cho *et al.* (1992). World Health Organization (WHO) documented the primary contamination occurred up to the limit of harvesting, whereas, secondary contamination began at the start of processing as a result of contact with workers and processing equipments. Rashid *et al.* (1996) studied about microorganisms associated with different types of frozen fresh water fish sample and reported the variable counts in the frozen fish which is almost similar to the present findings.

The total *staphylococcal* counts were varied from 2.7×10^{6} to 4.2×10^{6} cfu g^{-1} in the fresh water mola fish. Presence of *Staphylococcus* spp. suggests that there was higher level of environmental contaminants and its presence indicates the possible risks of food poisoning as found by Mhlambi *et al.* (2010).

Total coliform and faecal coliform counts in samples were varied from 4.8×10^{5} to 7.3×10^{5} cfu g^{-1} and 9.0×10^{4} to 1.03×10^{5} cfu g^{-1}, respectively. Presence of coliform and faecal coliform in food is linked with the practice of inadequate hygienic measure, mishandling, improper storage and use of dirty water during marketing and all unhygienic condition of the shops. Munce (1980) and Rashid *et al.* (1996) also delineated the presence of coliform and faecal coliform in fish samples due to poor hygienic measure during their processing and storage.

The total Aeromonas counts were varied from 2.7×10^{5} to 5.0×10^{5} cfu g^{-1}. Similar result has been reported by Cho *et al.* (1992). Aeromonas is dangerous because it can cause food borne diarrhea (Janda and Abbott, 1999; Wadstrom and Ljungh, 1991). Total fungi count was varied from 1.6×10^{3} to 3.5×10^{3} cfu g^{-1} in the fish samples. Presence of fungi in raw and fish samples has also been reported by Rashid *et al.* (1996). The presence of *Bacillus megaterium, Bacillus subtilis, Micrococcus varians, Escherichia coli, Pseudomonas aerugenosa, Klebsiella ozaenae, Klebsiella edwardsii, Micrococcus radiodurans* in fresh water mola fish was also supported by Islam *et al.* (2001) and Rashid *et al.* (1996). However, their presence is alarming since the bacterial species are the possible source of food poisoning (WHO, 1992).

Total viable bacterial counts, total coliform counts and total faecal coliform counts were reduced one log in all studied samples after six months of storage. Our previous study on mackerel fish (Scomberomorus guttatus) preservation also showed the similar result (Hossain *et al.*, 2008). Microorganisms maintained at freezing or sub-freezing temperature may be considered dormant and they perform no detectable metabolic activity. Here the initial freezing kills a fraction of the population, where the survivors may remain viable for long period (Pelczar and Reid, 1986). Total Aeromonas were gradually decreased and fungi were totally eliminated in all samples during storage. Slow freezing is more detrimental than quick freezing because of the formation of large ice crystal which disrupts cell membranes as well as brings out solute of the cell. Thus freezing causes the death of the bacterial cell (Fung, 1987). The bacterial genus those survived after six months of storage such as *Escherichia, Pseudomonas, Bacillus, Micrococcus*-all are more resistant to freezing than other microorganisms (Canada *et al.*, 1964; Trakulchang and Kraft, 1977).

In response to various radiation doses total viable bacterial count was reduced three logs at 2.5 and 5.0 kGy of irradiation doses and no viable count was found when treated with the radiation dose of 7.5 kGy. Total staphylococcal count reduced to one to two logs in all studied samples at 2.5 and 5.0 kGy of irradiation doses and no staphylococcal count was found above these radiation doses. Total coliform, total faecal coliform, total Aeromonas and total fungus were eliminated after irradiated at 2.5 kGy.

This study reveals that microorganisms resistant to freezing may remain associated with the frozen fish samples and can exist during frozen storage since only the fungi were eliminated without need of radiation treatment (Fig. 1f). So, only cold storage or low temperature preservation is not enough for reduction of total bacterial count and controlling bacterial pathogens in the fish samples. Again, only radiation treatment of 7.5 kGy or more was required for complete elimination of TVBC and TSC. But when combination of these treatments was used it was found more detrimental (Fig. 1a, b). Thus, the combination method offers a promising approach for storage life extension of fish at freezing temperature. However, microorganisms associated with raw beef and beef kebab were more sensitive to the radiation treatment as we reported in our previous study (Mahin *et al*., 2011). Only single effect of 2 kGy gamma radiation was enough for total elimination of TCC, TFCC and TFC in these food samples. No TSC was detected in these samples when treated with 2 kGy radiation dose and kept at -20°C for 2 months. These differences indicated that fish is more vulnerable to microbial spoilage than raw or processed beef. Gomez-Guillen and Montero (2007) also reported that fish is a highly perishable food and its freshness loses much more quickly than in the case of mammal and avian meat.

CONCLUSION

The study clearly indicated that the combination treatment of irradiation and freezing temperature is more powerful than the single treatment of either radiation or freezing for microbial decontamination and long term preservation of a local fish of Bangladesh like mola. Thus, the method can be applied for large scale preservation of mola and any other local fish of Bangladesh for long time without any significant loss of moisture and protein values.

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