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Bacterial and Fungal Population Assessment in Smoked Fish during Storage Period

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Abstract

Microbial contamination of food is the main obstacle of ensuring food safety. For this, the objective of this article was to determine the changes of microbial load of three smoked fish species (*Tenualosa ilisha, Oreochromis mossambicus, Pangasius hypophthalmus*) in fresh and stored condition.

To fulfill that objective, the freshly smoked fish samples were collected from the laboratory of Fisheries and Marine Science Department, Noakhali Science and Technology University of Bangladesh. Total Bacterial Counts (TBC), Total Coliform (TC), Fecal Coliform (FC) and the density of *Vibrio* spp. and yeasts and molds spp. in the fresh and stored smoked fish were determined by using serial dilution and spread plate technique. The microbial assessments of stored smoked fish species were performed in every seven days interval up to one month storage periods.

At the first sampling day of smoked fish species, TBC, TC, FC, *Vibrio* spp. and yeasts and molds spp. were not found in fresh smoked fish species. But the microbial loads in smoked fish species were consequently increases with the increasing of storage time. The highest densities of microbial loads were found in experimental smoked fish species in the 3rd week of smoked fish storage in refrigerator. This may be occurred due to the contamination of raw product and final smoked product from the polluted culture environment or processing environment or due to the improper processing during smoking.

The results of this research indicate that the new hygienic processing practices of smoked fish products can ensure the food safety for consumers by maintaining all the safety standards properly.

Keywords: Smoking; Smoked fish; Microbial load; Total bacterial count; Total coliform; *Vibrio* spp.; Food safety

Introduction

Fish and fisheries products are the most important nutritious food all over the world which represents about 15-20% of all animal protein on a global basis [1]. But the nutritional value of fish mostly depends on the freshness of fish. Spoilage of fish starts with the death of fish due to enzymatic digestion, oxidation of fat and bacterial decomposition. But Microbial action has been playing a large role in the spoilage of fish [2]. Each kind of foods has its natural characteristics such as appearance, texture, smell, taste and flavor. Therefore, any change in one or more of these characteristics of food indicates the food spoilage which may cause illness because of the presence of pathogenic microorganisms and their toxins [3]. After the death of fish, spoilage bacteria enter the muscle from the skin and gills, disintegrate the muscle cells and take necessary energy to grow. So, different types of processing and preservation methods have to be followed as soon as possible after the catching of fish to keep the freshness and nutritive value of fish flesh in a condition as near as possible to that of fresh fish [4].

The purpose of fish preservation is to reach the fish or fisheries product to an ultimate consumer in good and usable condition. Different types of fish preservation methods such as chilling and icing of fish, freezing of fish, sun drying of fish, smoking, salting, fermentation, canning of fish etc. have been followed mostly in all the regions of Bangladesh to reach the fish or fisheries product to an ultimate consumer in good and usable condition and prevent or reduce the post-harvest losses [5]. Smoked fish are well accepted food items in our country but have not practiced in all the regions of Bangladesh. Smoking is the method of fish preservation effected by a combination of drying and deposition of naturally produced chemicals resulting from the thermal breakdown of wood (Smoldering/smoke production) [6]. Smoking gives the product a desirable colour, taste and odour, a longer shelf-life through its anti-bacterial and oxidative effect, lowering of pH and acts as antagonist to spoilage [1,2,7-9]. The smoked fish products have gained a popular market at commercial basis due to its attractive colour, flavour and aroma and have a high potentiality as a processed item in Bangladesh for commercialization.

Smoking of fish is generally done in two methods-cold smoking and hot smoking. Cold smoking is generally done in 30°-40°C and hot smoking in 80°-90°C temperature [5]. Almost all microbes except

some pathogenic bacteria are destroyed due to the hot smoking because the fish is cooked and dried completely at high temperature. Most microbes present in the fishes are dried and destroyed completely due to the excessive heat and chemicals inherent in the smoke deprive microbes by removing the necessary growth factors of microbes.

But smoked fish and shellfish products can be a source of microbial hazards including *Listeria monocytogenes, Salmonella* spp., *Clostridium botulinum* etc. [10] due to the unhygienic handling, marketing and storage or due to the partial removal of water activity during production. Raw smoked fish are generally eaten in many countries. If the smoked fish are contaminated with pathogenic microbes, this can cause the fatal diseases in the human body [11].

For this reason, it is necessary to estimate the bacterial load (TBC, TC and FC) along with some pathogenic bacteria (*Vibrio* spp.) and fungal contamination in fresh and stored smoked fish.

Materials and Methods

Experimental fish

Three different smoked fish species (*Tenualosa ilisha, Oreochromis mossambicus, Pangasius hypophthalmus*) were examined in fresh and stored condition to enumerate the microbial load (Table 1). The methods applying in this research work were followed from ICMSF (International Commission of Microbiological Specification for Food) [12].

Study area and collection of samples

This study was conducted in the laboratory of the Department of Fisheries and Marine Science, Noakhali Science and Technology University of Bangladesh. Three experimental fish species were smoked at 80°-90°C in the laboratory of Department of Fisheries and Marine science. The fish samples were collected from laboratory in sterilized plastic bag after the hot smoking of fish samples. Then the microbial load of some fresh smoked experimental fish species were investigated and the rest of the smoked samples were transferred in the refrigerator at 4°C.The microbial assessment (Total bacterial load, total coliform, fecal coliform, *Vibrio* spp. and Yeast and Mold spp.) of stored smoked fishes were performed in every seven days interval up to one month storage periods by following the methods of ICMSF [12]. All the used glass wares such as conical flasks, beakers, measuring cylinder, test tubes, L-shaped glass rod, Petridish and inoculation tips were washed, dried and sterilized in autoclave (40B series, LDZX) at a temperature of 121°C for 15 min at 15 lb/inch^2 pressure. The area (bench) where the work was done was properly cleaned and disinfected with 75% ethanol.

S. No	Scientific name	Local name	Common name	Sample size	References
1	*Tenualosa ilisha*	Ilish	Indian River Shad	30	Hamilton [13]
2	*Oreochromis mossambicus*	Tilapia	Tilapia	30	Peters [14]
3	*Pangasius hypophthalmus*	Pangus	Thai Pangus	30	Sauvage [15]

Table 1: Name and sample size of experimental smoked fish species. A total of 90 smoked fish species (Tenualosa ilisha, Oreochromis mossambicus, Pangasius hypophthalmus) were collected from the Laboratory for enumeration of microbial load.

Processing of samples

The muscle samples were collected aseptically from each smoked fish sample and weighed with a sterile aluminum foil. Then 1 g muscle of each samples were homogenized and diluted separately in PBS solution by using subsequent serial dilution technique upto 10^{-5} using Vortex machine (Digisystem Laboratory Instruments INC., Model VM-1000) (Figure 1).

Inoculation of plates for enumeration of bacterial load

In this study, all the used media for enumeration of bacterial load was prepared media by Merck, Germany. For enumeration of bacterial load, the petri-dish containing culture media was inoculated with l00 µl of each diluted solution of each sample using spread plate method [16]. For enumeration of total aerobic bacteria in smoked fish sample, Nutrient agar media was used as culture media and incubated at 37°C for 18-24 h in the incubator after inoculation. For the enumeration of total and fecal coliform, Membrane fecal coliform (mFC) agar media was used and inoculated media were incubated at 37°C for 18 to 24 h in the case of total coliform and in the case of fecal coliform at 44 to 44.5°C for overnight following the methods of [17]. TCBS (Thiosulfate citrate bile sucrose) plate was used as selective media for identification of *Vibrio* spp. according to the [18] which were incubated at 37°C for 18-24 h to count the colonies of *Vibrio* spp. Yeasts and molds spp. were identified and counted on OGYEA (Oxytetracycline-Glucose-Yeast Extract Agar) plate [19] which were incubated at 22 ± 2°C and examined for growth up to 5 days of incubation.

Statistical Analysis

Bacterial density data were transformed into natural log before statistical analysis. The means of bacterial load were compared using ANOVA followed by Tukey's post hoc for multiple comparisons. Microsoft Excel 2010 and Statistical software SPSS version 16.0 was used to analyze the data with the level of significance at $p<0.05$.

Results

Total Bacterial Count (TBC) in smoked fish species at consecutive sampling days

The densities of TBC were similar in three experimental smoked fish species at 0 day of sampling (0 CFU/g) but were significantly ($p<0.05$) different in the 1st, 2nd and 3rd week (Figure 2). The densities of total aerobic bacteria in three experimental smoked fish species were 0 CFU/g at 0 day, which were consequently increases with the days. The highest densities of TBC were found in the experimental smoked *Tenualosa ilisha, Oreochromis mossambicus, Pangasius hypophthalmus* during 3rd week of storage of smoked fish samples,

which were 2.4 ± 0.45 × 10^5, 4.0 ± 0.56 × 10^4 and 1.2 ± 0.25 × 10^6 CFU/g respectively.

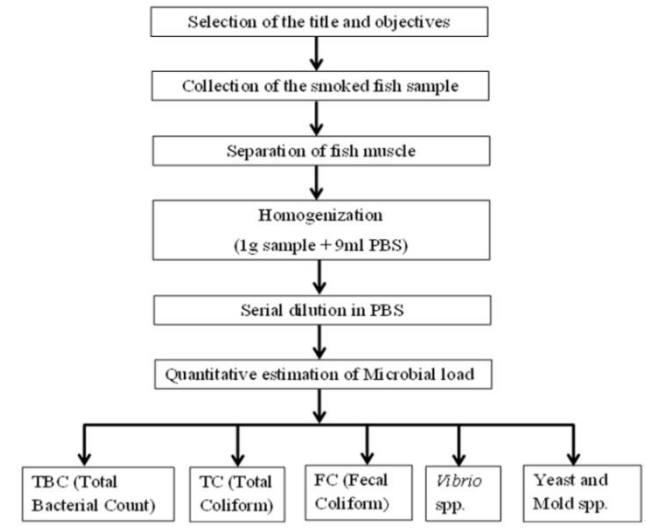

Figure 1: Flow diagram of the methodology applied in this research. The first step of this research work was selection of titles and objectives and then collection of smoked fish sample from laboratory aseptically. Then the muscle samples were collected and homogenized separately with PBS solution. Then the dilutions of samples were made separately upto 10^{-5} by using serial dilution technique. l00 µl from diluted solution of each sample were transferred to culture media containing petri-dish and inoculated using spread plate method for bacteriological analysis.

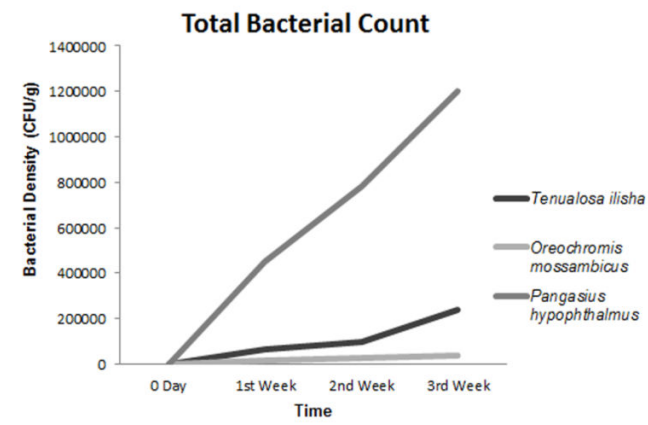

Figure 2: Comparisons of total bacterial density (CFU/g) of three experimental smoked fish species at 0 day of sampling (fresh condition) and in the 1st, 2nd and 3rd week of storage of smoked fish species in refrigerator (stored condition).

Densities of Total coliform (TC) in smoked fish species at consecutive sampling days

The densities of TC were similar in three experimental smoked fish species at both 0 day and 1st week (0 CFU/g) but were significantly ($p<0.05$) different in the 2nd and 3rd week (Figure 3). The highest densities of TC were found in smoked *Tenualosa ilisha, Oreochromis mossambicus, Pangasius hypophthalmus* during 3rd week of storage of smoked fish, which were 4.0 ± 0.54 × 10^3, 1.8 ± 0.18 × 10^4 and 5.7 ± 0.39 × 10^3 CFU/g respectively.

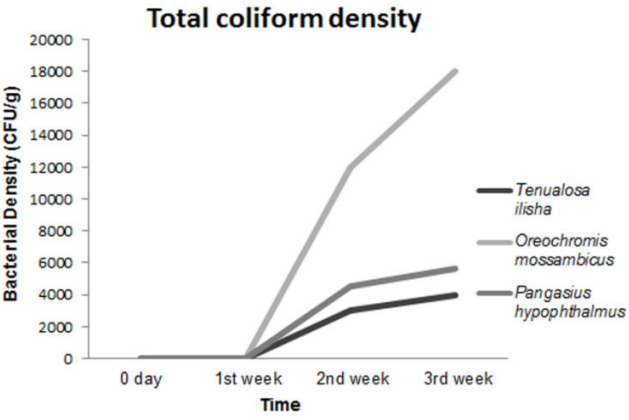

Figure 3: Comparisons of total colifom density (CFU/g) of experimental smoked fish species at 0 day of sampling (fresh condition) and in the 1st, 2nd and 3rd week of storage of those smoked fish species in refrigerator (stored condition).

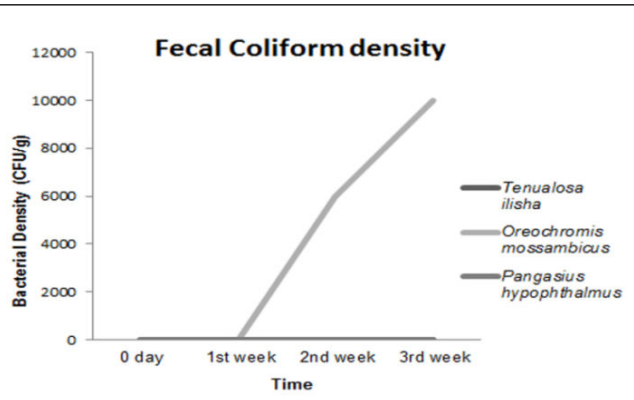

Figure 4: Comparisons of fecal coliform density (CFU/g) of experimental smoked fish species at 0 day of sampling (fresh condition) and in the 1st, 2nd and 3rd week of storage of those smoked fish species in refrigerator (stored condition).

Densities of Fecal Coliform (FC) in smoked fish species at consecutive sampling days

The densities of FC were similar in three experimental smoked fish species both at 0 days of sampling and in the 1st week of storage but were significantly ($p<0.05$) different in the 2nd and 3rd week (Figure 4). Among three experimental smoked fish species, no fecal coliform was found in *Tenualosa ilisha* and *Pangasius hypophthalmus* with the following days of sampling i.e. in fresh and stored condition. Fecal coliform was only found in *Oreochromis mossambicus* in the 2nd week (6 × 10^3 CFU/g) which was increased in the 3rd week (1 ± 0.03 × 10^4 CFU/g) of storage of smoked fish species in refrigerator.

Presence of *Vibrio* spp. in experimental smoked fishes at consecutive sampling days

The densities of *Vibrio* spp. were significantly ($p<0.05$) different in three experimental smoked fish species in the 0 days, 1st, 2nd and 3rd week of sampling (Figure 5). No *Vibrio* spp. was found in three experimental smoked fishes at 0 days of sampling i.e. in fresh condition of smoked fishes. Among three experimental smoked fishes, no *Vibrio* spp. was found in Thai Pangus at consecutive sampling days i.e. in both fresh and stored condition. The highest densities of *Vibrio* spp. were found in smoked *Tenualosa ilisha* and *Oreochromis mossambicus* during 3rd week of storage, which were $0.78 \pm 0.01 \times 10^2$ and $0.86 \pm 0.02 \times 10^2$ CFU/g respectively.

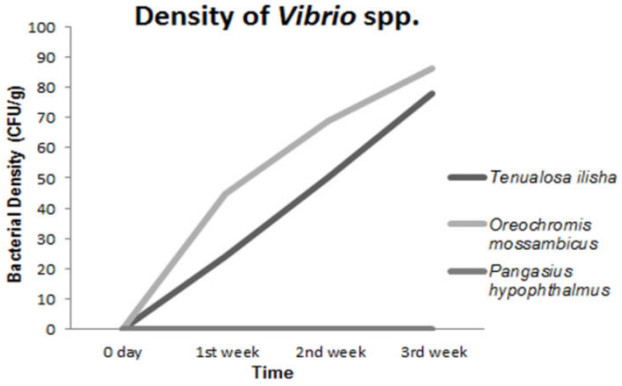

Figure 5: Comparisons of *Vibrio* spp. density (CFU/g) of experimental smoked fish species at 0 day of sampling (fresh condition) and in the 1st, 2nd and 3rd week of storage of those smoked fish species in refrigerator (stored condition).

Densities of Yeast and Mold spp. (CFU/g) in fishes at consecutive sampling days

The densities of Yeast and Mold spp. in the three experimental smoked fish species were 0 CFU/g at the 0 days of sampling but the densities were increase in stored smoked fishes with the increase of the storage day (Figure 6). The highest densities of Yeast and Mold spp. were found in smoked *Tenualosa ilisha*, *Oreochromis mossambicus*, *Pangasius hypophthalmus* in the 3rd week of storage of smoked fishes, which were $2.1 \pm 0.16 \times 10^2$, $5.0 \pm 0.12 \times 10^2$ and $3.0 \pm 0.21 \times 10^2$ CFU/g respectively.

Discussion

Smoked fish has been a popular product in some coastal districts like Cox's Bazar since ancient times. Generally in Southeast Asia, smoking is practiced not necessarily to impart desired color and flavor but mainly to accelerate the drying of fish [9]. But nowadays the extent of smoking of species has been reduced significantly with the reduction in catching of fish and increased availability of ice. But local people of Teknaf have been found to prepare this delicious dish at home for their own consumption. Brilliant color and delicious flavor have made it one of the cherished food items in this area [5].

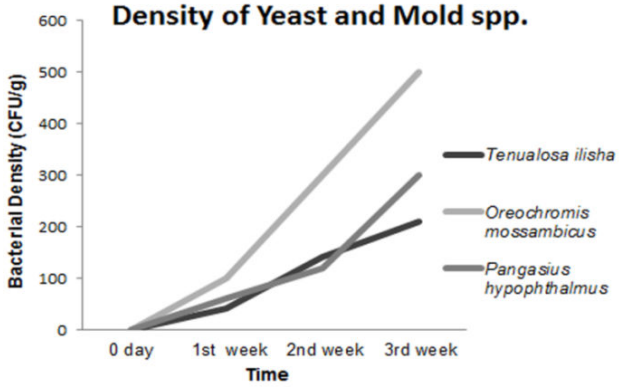

Figure 6: Comparisons of Yeast and Mold spp. density (CFU/g) of experimental smoked fish species at 0 day of sampling (fresh condition) and in the 1st, 2nd and 3rd week of storage of those smoked fish species in refrigerator (stored condition).

While fishery products play an important role in human nutrition worldwide [20], they can also act as a source of food-borne pathogens. The processed fish are easily contaminated with microorganisms in nature, through rough handling, improper processing and also through improper and unhygienic post-processing handling [21]. Smoked fish become excellent substrates for the growth of most common bacterial agents of food-borne disease when it is processed at inappropriate temperatures or in improper procedures and by those people who are not aware of proper sanitation and hygiene. Smoked fish become contaminated and spoiled by different microorganisms from their processing through storage to marketing. If the bacterial loads of smoked fish exceed the acceptable limit, that smoked fish become unacceptable and may cause serious diseases to human body. The quality of smoked products is dependent on several factors, including, the quality of the fish during smoking, the preparation of the raw material, the nature of wood and the type of the smoking procedure employed [22]. So to preserve food safety, every possible source of bacterial transmission should be removed before manipulation of food [23].

This study exposed that no aerobic bacteria were found in the smoked *Tenualosa ilisha, Oreochromis mossambicus* and *Pangasius hypophthalmus* samples in the fresh condition i.e. at 0 day of sampling (Figure 2). But the bacterial load in three experimental smoked fish species was increasing with the increase of days or storage time and the highest densities of TBC (Total Bacterial Count) were found in smoked fish species in the 3rd week of storage of smoked fishes in refrigerator. In the 3rd week, the highest density of TBC (total bacterial count) was found in smoked Thai Pangus ($1.2 \pm 0.25 \times 10^6$ CFU/g) and the lowest in smoked Tilapia ($4.0 \pm 0.56 \times 10^4$ CFU/g). The reasons of that finding could be the improper processing, contamination of raw fish or the unhygienic handling during smoking, partial removal of water activity during production or cross contamination. The higher levels of microorganisms identified from smoked fish which were purchased from the markets can be attributed to poor fish handling and improper smoking process adopted by fish mongers [24]. Ayuba et al. [25] found that the total bacterial load of smoked sardine (*Sardina pilchardus*) which was sold in five markets in Makurdi, Nigeria ranged from 0.988×10^3 to 2.632×10^3 CFU/g. The total bacterial load of smoke-dried *Clarias* species ranged from 109.00×10^5 CFU/g to 136.67×10^5

CFU/g which were collected from five different markets in Lafia, Nigeria [26]. Nyarko et al. [27] found that the total bacterial load of smoked sardine (*Sardinella aurita*) ranged from 6.2×10^4 to 3.3×10^5 CFU/g in three smoking sites while 7.2×10^4 to 4.1×10^7 CFU/g in three marketing centres of Tema municipality, Ghana. Oku and Amakoromo [28] found that the total bacterial load of fresh fishes ranged from 4.0×10^8-2.30×10^{10} CFU/g and 1.8×10^4-2.5×10^8 CFU/g of smoked fishes in Nigeria. The total bacterial count for the fresh fish was 1.84×10^6 CFU/ml. and for the smoked fish 2.06×10^6 CFU/ml [29]. According to Chakma et al. [30], the total bacterial load in fresh *Nappi* (fermented fish paste) ranged from 1.32×10^{10} CFU/g to 7.70×10^9 CFU/g while 1.63×10^{10}- 9.5×10^7 CFU/g in stored *Nappi*.

Smoking is a method of preservation but most of the time, spore of bacteria was not destroyed at the time of smoking due to the use of inappropriate or low temperature and then this spore was multiplied during storage period. Smoking can control the microbial contamination in fish at adequately high temperatures (>600°C), although, sometimes the use of high temperature might not be sufficient enough to kill all the microbial contaminants such as spores [31].

This study revealed that total coliform (TC) bacteria were not found in three experimental smoked fish samples in fresh condition i.e. at 0 days of sampling and in the 1st week of storage i.e. after 7 days of storage (Figure 3). The highest density of total coliform (TC) bacteria was found in *Oreochromis mossambicus* (1.8×10^4 CFU/g) and the lowest in *Tenualosa ilisha* (4×10^3 CFU/g) after 21 days of storage or in the 3rd week of storage of those smoked fish samples. From the present study, it was found that the densities of TC in smoked *Tenualosa ilisha, Oreochromis mossambicus* and *Pangasius hypophthalmus* increase with the increase of days or storage time, which may be due to the culture in most polluted water or due to the unhygienic handling, using of polluted water during processing and improper storage.

Coliforms are called 'Sanitary Index' organisms whose presence in food in large quantity indicates the probability of culturing the organism in unhygienic condition or the usage of polluted water during processing. Detection of coliform bacteria is used as an indicator of water sanitation or as a general indicator of sanitary condition of the culture area as well as the food-processing environment [32]. Therefore, fecal coliforms are considered more accurate indicator of food contamination by animal or human feces than the total coliforms.

In this research, no fecal coliform bacteria were found in fresh and stored *Tenualosa ilisha* and *Pangasius hypophthalmus* (Figure 4). The highest density of fecal coliform (FC) bacteria was found in *Oreochromis mossambicus* (1×10^4 CFU/g) in the 3 weeks of storage, which may be due to the culturing in polluted water or using polluted water during processing. The presence of higher amount of fecal coliforms in experimental smoked fish species indicates the sewage contamination of culture environment, and post-harvest technology is improper and is somehow contaminated by human or other warm blooded animal's excreta. The total coliform bacteria in smoked sardine (*Sardinella aurita*) ranged from 0.0 to 2.1×10^4 CFU/g in three smoking sites while 4.7 to 2.0×10^2 CFU/g in three marketing centres of Tema municipality, Ghana [27]. Majumdar et al. [33] found that the total and fecal coliform counts in different species of marine fish samples ranged from $2.18 \pm 1.49 \times 10^5$ to $4.18 \pm 4.01 \times 10^6$ CFU/g and $1.48 \pm 1.47 \times 10^4$ to $2.54 \pm 1.95 \times 10^5$ CFU/g.

Good quality fish should have counts of total bacteria of less than 10^5 per gram and fecal coliforms and total coliforms should not exceed 10/g and 100/g respectively [34]. The high quantity of total coliform and fecal coliform colonies made the experimental smoked fish species unacceptable, and humans may suffered from various diseases after consuming those smoked fish. *E. coli* can cause diarrhea and kidney damage as well as uncomplicated community acquired urinary tract infections in human [35].

This study exposed that no *Vibrio* spp. was found in three experimental smoked fish species at 0 days of sampling or in fresh condition (Figure 5). No *Vibrio* spp. was found in fresh and stored *Pangasius hypophthalmus*. The densities of *Vibrio* spp. in *Tenualosa ilisha*, and *Oreochromis mossambicus* increased with the increase of storage time. The highest density of *Vibrio* spp. was found in *Oreochromis mossambicus* (0.86×10^2 CFU/g) and the lowest in *Tenualosa ilisha* (0.78×10^2 CFU/g) after 21 days of storage of smoked fish species at 4°C. This may be due to the contamination of raw product and final product from the unhygienic culture environment of fish or the processing environment of smoked fish. Fishes could be infected by *V. cholerae* either due to sewage contamination of water or by consumption of aquatic vegetation and zooplankton infested with *V. cholera* [36]. Some other studies have also shown that zooplankton including copepods and other aquatic crustaceans such as crabs, shrimps, prawns, lobsters and blue green algae act as important reservoirs of *V. cholera* [37-39]. So the fishes may be contaminated by *Vibrio* spp. through the culture environment and food. But sometimes, aquaculture products may be contaminated by *Vibrio* spp. due to external cross-contamination.

The densities of Yeast and Mold spp. in the three experimental smoked fish species were 0 CFU/g at the 0 days or in fresh condition but the densities were increasing in stored smoked fishes with the increase of the storage day (Figure 6). This may be caused due to the improper processing and partial dehydration of fish during smoking. Due to the maintenance of inappropriate temperature and others parameters during smoking, smoked fish samples may have a relatively low water activity level which is a prerequisite for fungal growth. Fungus can grow in food which is refrigerated at temperatures of 4°C (39°F) or below [25]. The presence of *Aspergillus flavus* and *Aspergillus fumigatus* in the fish samples can cause serious health concern because of their mycotoxigenic potentials [35]. Essien et al. [40] reported that *Aspergillus flavus* and *Aspergillus fumigatus* produced aflatoxins, which destroyed the liver and kidney in man resulting to death. The density of yeast and moulds in smoked sardine (*Sardinella aurita*) ranged from 1.1×10^2 to 9.3×10^4 CFU/g in three smoking sites while 5.0×10^2 to 8.0×10^4 CFU/g in three marketing centres of Tema municipality, Ghana [27]. According to Chakma et al. [30], the total bacterial load in fresh *Nappi* (fermented fish paste) ranged from 5.05×10^6 CFU/g to 14.7×10^6 CFU/g while 5.70×10^6-6.85×10^6 CFU/g in stored *Nappi*. Oku and Amakoromo [28] found that the fungal counts for fresh fish samples ranged from 1.8 x 10^4-7.0×10^4 CFU/g while 1.0×10^4-4.0×10^5 CFU/g for smoked fish.

Although still there is no such document in Bangladesh, it is also truthful that there is very little work regarding the correlation of fish consumption and outbreak of these fatal diseases. Frequently, typhoid and cholera appeared as epidemic in Bangladesh but, unfortunately the reason behind this epidemic is nearly unknown and is considered to be the water-borne diseases. Another important notable thing is that people in our country did not habituate to consume raw fish which decreases the risk of diseases. But, still there is a risk of secondary

infection or cross-contamination which ultimately results in epidemics. Although species level determination of the microbes was out of scope in this study, but when these smoked fish will be consumed without proper processing, these high quantities of TBC, TC, FC, *Vibrio* and Yeast and Molds spp. can cause serious diseases in human.

Conclusion

Food safety is the scientific discipline describing handling, preparation, and storage of food in ways that prevent foodborne illness to avoid potentially severe health hazards. Generally, the density of microflora in fish and fishery products are related to the environmental factors such as water pollution, hygienic condition of processing environment, processing in appropriate methods, handling, transportation, commercialization and storage condition. So to preserve food safety, GAP (Good Aquaculture Practice), SSOP (Sanitation Standard Operating Procedure), HACCAP (Hazard analysis and critical control points) etc. should be maintained in every step from culturing through processing to marketing of product. To prevent the incidence of food contamination, there is a need to educate the related people such as fish processors, handlers, retailers or vendors about the importance of sanitation, hygienic measures and good food handling practices. Proper hygienic condition should be maintained at every step of culturing, catching, landing and transportation, processing, storing and marketing following HACCAP steps for producing good quality of fish and fishery products. And this good quality of fish and fishery products can play a significant role in fulfilling the nutrient demand of people of the country and earn foreign currency to improve the economic condition of the nation.

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