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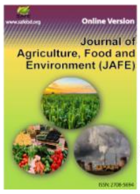

Research Article

Seasonal variation of bacteriological parameters of rui (*Labeo rohita*) and kalibaus (*Labeo calbasu*) collected from the Hakaluki Haor, Bangladesh

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A B S T R A C T

The fish *in situ* bacterial flora correlated with the aquatic environment. The higher initial bacterial flora in fish ultimately affects the storage life and quality of the products. The present study aimed to collect 36 samples of Rui (*Labeo rohita*) and Kalibaus (*Labeo calbasu*) from three locations in four seasons (pre-monsoon, monsoon, post-monsoon and winter) of Hakaluki haor to investigate their seasonal bacteriological changes. The samples were investigated for total bacterial load in term of Total Viable Count (TVC) and Total Coliform Count (TCC) as sanitary index The prevalence of *E. coli*, *Salmonella* spp. and *Vibrio* spp were also investigated. The higher viable bacterial loads (logarithmic scale) were observed in Kalibaus than Rui in all study seasons. In Rui, the bacterial load was 5.56±0.078, 5.51±0.043, 5.36±0.065, and 5.48±0.091 (CFU/g±SD) whereas in Kalibaus, those were 5.68±0.050, 5.58±0.057, 5.52±0.040, and 5.63±0.067 in monsoon, post-monsoon, winter, and pre-monsoon, respectively. For total coliform (TCC), Rui were 73, 72, 28, and 48 MPN/g, while in Kalibaus, the values were 78, 96, 65, and 86 MPN/g in monsoon, post-monsoon, winter, and pre-monsoon, respectively. *E. coli*, *Salmonella* spp., and *Vibrio* spp. were also present in both fish at each season. The results indicate that Rui had a lower prevalence of pathogenic bacteria than Kalibaus. The occurrence of *E. coli* and *Salmonella* spp. in both fish samples indicates fecal contamination, which may be linked to unhygienic environmental conditions.

INTRODUCTION

Bangladesh is a riverine country. Fish and fisheries are closely related to the Bangladeshi people for their contribution to providing animal proteins at low prices as well as income. It is also well-praised for providing sources of foreign currency (Hossain, 2015; Azim *et al.*, 2003; Alam and Thomson, 2001). "Haor" fishery contributes a considerable portion of the total fish production of inland captured fishery. *Haors* are bowl-shaped shallow depressions covering about 25% of the northeastern part of Bangladesh (Hossain and Nishat, 1989). In the case of fishery products, biological hazards like pathogenic bacteria and spoilage associated with pathogenic bacteria are important for food safety and human health (Herrera *et al.*, 2006). Due to their higher moisture and protein content, fish are very susceptible to spoilage. The initial freshness of fish is primarily hampered by enzymatic and chemical reactions, whereas microbial activity is accelerated the spoilage after death of fish (Pal and Mahendra, 2015; Rhea, 2009; Huss, 1998). Basically, live fish is sterile. The spoilage from enzymes and bacteria starts when it commences to death. Bacteria grow rapidly after the death of fish and accelerate the spoilage of fish (Gram and Huss, 1996; McMeekin *et al.*, 1992). Contamination in the fishery product mainly occurs due to the bursting and splitting of fish intestines during handling, abject processing, or unhealthy washing. The quality of harvested fish is greatly influenced by the degree of adulteration by pathogenic and spoilage bacteria during handling or processing (Adebayo-Tayo *et al.*, 2012). It is reported that fish contaminated with pathogenic bacteria from different sources, such as intestinal microflora of enteric bacteria of human or animal origin, are responsible for spoilages (Geldreich and Clarke 1966). Both fresh and spoiled fish and seafood have been reported with many spoilage bacteria and fungi. Among spoilage bacteria, *Aeromonas, Alcaligenes, Bacillus, Enterobacter, Enterococcus, Escherichia coli, Listeria, Pseudomonas, and Shewanella* are well documented, while fungi *Aspergillus, Candida, Cryptococcus, and Rhodotorula* are also isolated from both fresh and spoiled fish and other sea foods (Church, 1998). Other spoilage organisms are *Pseudomonas putrifaciens, Pseudomonas fluorescens, Bacillus, Moraxella* etc; and pathogenic bacteria are *Escherichia coli, Salmonella* spp., *Staphylococcus* spp., *Vibrio* spp., *Aeromonas* spp., *Clostridium botulinum, Listeria monocytogenes* (Abrahim *et al.*, 2010; David *et al.*, 2009; Ayulo *et al.*, 1994; Hood *et al.*, 1983). Food-borne infection and intoxication are caused by *Salmonella spp., Staphylococcus spp., Vibrio spp.,* and *Aeromonas spp,* which may also transmitted by fish if contaminated with those pathogens (Emikpe *et al.* 2011; Gold and Salit, 1993). Last few decades, Bangladesh has been exporting fish and fishery products and has earned foreign currencies. Hence, the fisheries and aquaculture sectors have been reported as the third most important contributors to export earnings (Ghose, 2014). In a few cases, exported products also were returned back due to the low microbiological quality (e.g., coliform, fecal coliform, Pseudomonas, Staphylococcus, etc.), which have caused some controversy about Bangladeshi fishery products (Noor *et al.*, 2013). As a consequence, importers are much more concerned about food safety in terms of the microbial quality of the products (Pal and Mahendra, 2015). To ensure the best fishery products and consumer acceptance, maintaining the quality of fish and fish products is essential (Okoro *et al.*, 2010; Begum *et al.*, 2010; Prabakaran *et al.*, 2011; Anbudhasan *et al.*, 2012). Fish a highly perishable foodstuff. It requires care and attention in every case of handling until it reaches the consumption table (Czerwińska *et al.*, 2008; Poli *et al.*, 2005; Clucas and Ward, 1996). During handling, bacteria present on the body surface or in the intestine can enter the fish flesh and cause contamination (Nerín *et al.*, 2016; Chowdhury *et al.*, 2010; Ivanek *et al.*, 2004). Nowadays, the pollution of waterbodies and the possible occurrence of different pollutants (physical, chemical, and biological) is a matter of concern for public health (Adedeji *et al.*, 2012: Ababouch 2006). The Hakaluki *haor* is situated in the Sylhet district of Bangladesh and is one of the prominent sources of fish. Though a huge amount of fish is collected from the *haor* area, there is no fish quality (physical or microbiological) monitoring in the *haor* area. In Bangladesh, studies about microbial contamination of fish and information on the bacteriological population are available, but information about the distribution of bacteria in fish from the open environment is patchy. Among the captured fishes from the Hakaluki *haor*, Rui and Kalibaus are two popular and available fish species among fish lover because of their taste and reasonable price. In this study, Rui and Kalibaus were collected from Hakaluki *haor* for comparative bacteriological assessment not only for their local demand and export market. It can give a clear idea about the microbial status of these fishes related to the *hoar* environment in terms of food safety and health concerns.

MATERIALS AND METHODS

The research investigated the bacterial load and isolation of bacteria from Rui and Kalibaus of Hakaluki *haor* in the Sylhet region of Bangladesh.

Study area, sample, and planning

Pre-selected three fishing points of the Hakaluki *haor*, namely Ghilachora of Fenchuganj Upazila, Islamganj area of Kulaura Upazila, and Kanungo area of Barlekha Upazila were used to collect samples. For the bacteriological status assessment of Rui and Kalibaus, a total of 72 individual fish (36 for each) were collected from this area. These fish species of Hakaluki Haor were selected because these species are the most valuable and highly demandable for the domestic. Four seasons (winter, pre-monsoon, monsoon, and post-monsoon) were also considered for the comparative analysis of the bacterial status of the selected fish samples. During this study period, temperature was recorded on all dates of sample collection.

Preparation of sample

A muscle sample of 20g, including skin, was collected for both fish. The samples were blended and homogenized in sterile alkaline peptone water aseptically. The samples were dilution for plate counts using serial dilutions. Briefly, 1mL of the samples were diluted into 9ml sterile distilled water. Hence, it is a ten-fold of dilution. Subsequently, dilution was prepared serially until 10^{-6} according to protocol (ISO, 1995). Then, 100µl of samples were plated into an agar plate, incubated in an incubator for 24h, and counted the colonies for total viable counts.

Calculation of Total Viable Count (TVC)

A sample of 100µl was taken from each serial dilution and inoculated into a plate using spread plate techniques. The plates were incubated in an inverted position in an incubator at 37°C for 24h. After 24h, the plates having 30 to 300 colonies were considered for counting. No. of bacteria per gram of the sample (CFU/g) was calculated by using the following formula:

$$\text{CFU/g} = \frac{\text{No. of colonies on petridish} \times 10 \times \text{dilution factor} \times \text{Volume of total sample solution}}{\text{Wt. of fish sample (g)}}$$

Total Coliform Count (TCC)

The total coliform of a sample is measured by the MPN (Most Probable Number) method, which estimates the viable population density of microorganisms in the sample. It was calculated based on the method described by Benson, 2002. Gas production is the fermentation tubes indicated its positive growth response. Interestingly, fewer positive cultures should be in such a manner that results from higher dilutions of the sample.

Isolation and identification of bacteria

Morphological characteristics such as size, shape, arrangement, and motility were investigated by Gram's staining reaction. The colony characteristics, biochemical reaction, catalase test, and motility test were also done for further isolation and identification of the bacteria. Briefly, the pure colonies from the streak agar plate were used as inoculum and subcultured in nutrient agar, VRB, TCBS, EMB, MacConkey, SS, and BGA to promote the growth of a particular type of bacterium. In all cases, the aseptic condition was maintained via inoculating the samples under laminar airflow. Afterward, the test results were analyzed, and the isolated bacteria present in the samples were identified. For Sugar fermentation, five basic sugars are lactose, dextrose, maltose, sucrose, and mannitol used. Change of color from red to yellow and the presence of gas bubbles were used for the test Acid and gas production, respectively. No color and gas indicated a negative reaction.

RESULTS

Bacterial load of Rui and Kalibaus in different seasons

Table 1: Bacterial load of Rui and Kalibaus in different seasons. Values showing different superscripts are significantly different (P<0.05). Values are mean ± standard deviation.

Species / Season	Rui (***Labeo rohita***) TVC (Log CFU/g±SD)	Kalibaush (***Labeo calbasu***) TVC (Log CFU/g±SD)
Monsoon	5.56± 0.078^a	5.68 ± 0.050^b
Post-monsoon	5.51 ± 0.043^a	5.58 ± 0.057^a
Winter	5.36± 0.065^a	5.52 ± 0.040^b
Pre-monsoon	5.48 ± 0.091^a	5.63± 0.067^b

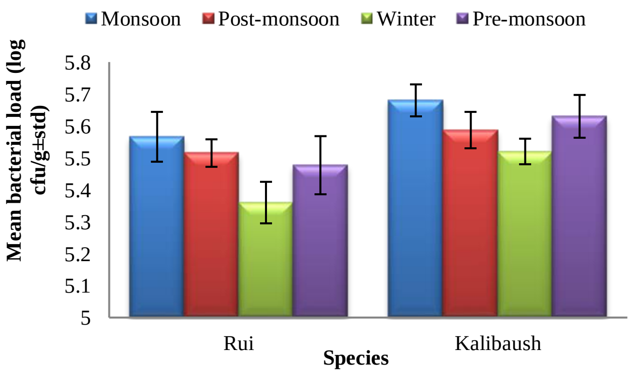

Figure 1: Bacterial load of Rui and Kalibaus in different seasons.

The mean of bacterial load was calculated on a logarithmic scale as CFU/g ± SD. Table 3.1 shows the overall seasonal result of the bacterial load of both fish. Table 1 and Figure 1 described that the significantly highest bacterial load was found in Rui 5.56±0.078 CFU/gm in the monsoon season, and the lowest was 5.36±0.065 in the winter season. Likewise, in Kalibaus, the highest (mean Log CFU/gm ± SD) bacterial load was 5.68±0.050 in monsoon, and the lowest bacterial load was 5.52±0.040 in winter. In post-monsoon, no significant differences were observed between Rui and Kalibaus.

3.2 Effect of temperature on bacterial load of Rui and Kalibaus

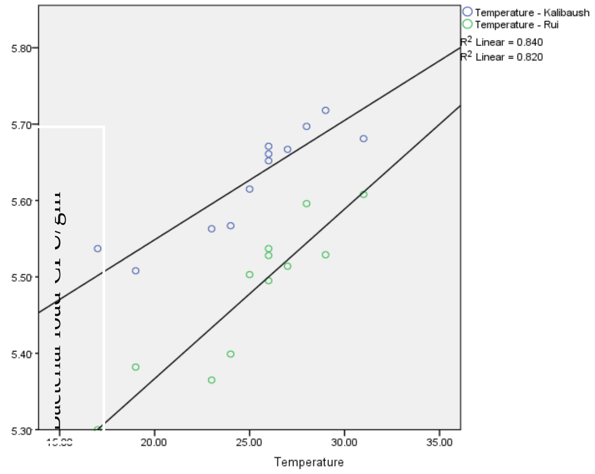

Figure 2: Temperature effect on bacterial load of Rui and Kalibaus

During this experiment period, the temperature remained (17^{0}C-31^{0}C) from May to April. Temperature showed an impact on bacterial load that occurred in both Rui *(Labeo rohita)* and Kalibaus *(Labeo calbasu)*. Food, temperature, and other environmental conditions have an influence on the growth and proliferation of bacteria. In this study period, bacterial load was highest at the temperature of 31^{0}C, and the lowest was at 17^{0}C for both types of species. The temperature was correlated positively with the bacterial load of Rui (correlation, r = 0.904) and Kalibaus (correlation, r = 0.916). Temperature was highly correlated with microbial load in both fishes of Rui and Kalibaus (Fig. 2).

Seasonal Variation of Total Coliform Count (TCC) in Rui and Kalibaus

MPN index was used for calculating total coliform. In post-monsoon, the total coliform was higher in both species. However, the mean Total Coliform of Rui was higher (73 MPN/g) in the monsoon season, indicating contamination of *hoar* from the surface runoff and/or flood during the monsoon season (Fig. 3). On the other hand, in Kalibaus (*Labeo calbasu*)**,** it was higher (96 MPN/g) in the post-monsoon season (Fig. 3). Interestingly, the Total Coliform of all seasons in both fish complied with the ICMSF standard.

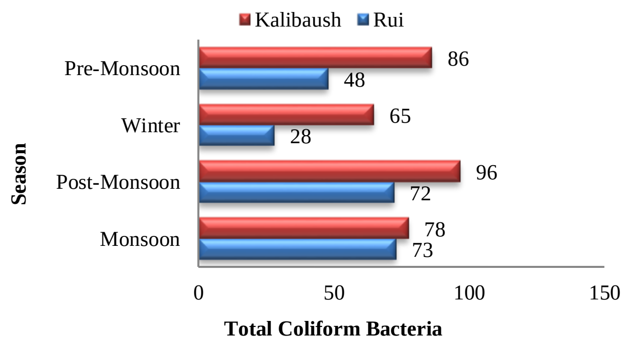

Figure 3: Seasonal variation of Total Coliform Count in Rui and Kalibaus

Pathogenic bacteria isolation and identification based on cultural characteristics, Gram staining, and biochemical test

Morphological characteristics

Three (3) types of bacteria were isolated from Rui and Kalibaus (Table 2). The isolated bacteria are *Escherichia coli*, *Salmonella* spp. and *Vibrio* spp. Gram staining was also done to identify *Escherichia coli*, *Salmonella* spp., and Vibrio spp. according to their staining characteristics (Table 3).

Table 2: Cultural characteristics (morphological) of *Salmonella* spp., *Escherichia coli*, and *Vibrio* spp.

Sl. no.	Name of bacteria	Selective media	Colony characteristics
1	*E. coli*	EMB agar	Metallic sheen (greenish black) colony.
2	*Salmonella* spp.	SS agar	Opaque, smooth, round with black centered.
3	*Vibrio* spp.	TCBSS agar	Yellow and green colony.

Table 3: Gram's staining properties of *Salmonella* spp., *Escherichia coli* and *Vibrio* spp.

Characteristics			Identified bacteria
Shape	**Arrangements**	**Gram's staining reaction**	
Short plump rods	Single, paired or in short chain	Negative	*E. coli* (d)
Very short plump rods	Single	Negative	*Salmonella* spp. (e)
Curved rod shaped	Single	Negative	*Vibrio* spp. (f)

3.4.2 Biochemical characteristics of *Escherichia coli, Salmonella* spp. and *Vibrio* spp.

Five basic sugars such as dextrose, maltose, lactose, sucrose, and mannitol were used for sugar fermentative test. Change of color from red to yellow and the presence of gas bubbles were used for the test Acid and gas production, respectively. No color and gas indicated a negative reaction. The results of the biochemical tests are presented in Table 4.

Table 4: Biochemical properties of *Salmonella* spp., *Escherichia coli* and *Vibrio* spp.

Carbohydrate fermentation test					TSI test	MR test	VP test	Indole test	Interpretation of results
DX	ML	L	S	MN					
AG	AG	AG	AG	AG	+	+		+	*E. coli*
A	A	_	_	A	+	+	_	_	*Salmonella* spp.
A	A	_	A	A	+	+	_	+	*Vibrio* spp.

Prevalence of pathogenic bacteria of Rui at different seasons

A spider web analysis was performed for the prevalence of pathogenic bacteria of Rui in different seasons. The availability of pathogenic bacteria in Rui was estimated by staining, motility test, catalase test, and different biochemical tests (sugar fermentation test, indole test, TSI test, Cimon Citrate test, MR-VP test). *E. coli* bacteria was found to be higher in monsoon and pre-monsoon seasons at 55.55% and lowest in winter at 33.33%. Whereas, *Salmonella* spp. steady in (the monsoon, post-monsoon and winter) seasons at 22.22% then marginally increase in pre-monsoon at 33.33%. On the other hand, *Vibrio* spp. was not found in the Winter season, but 22.22% in pre-monsoon. The percentage of *E. coli* was higher than *Salmonella* spp. and *Vibrio* spp. in Rui in all cases.

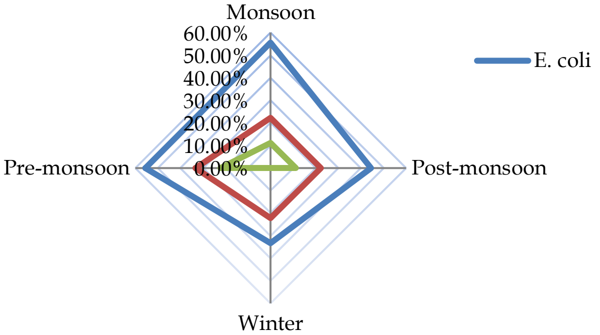

Figure 4: Prevalence of pathogenic bacteria in Rui (*Labeo rohita*)

Prevalence of pathogenic bacteria in Kalibaus at different seasons

A spider web analysis was performed for the prevalence of pathogenic bacteria of Rui in different seasons. The availability of pathogenic bacteria in Kalibaus was estimated by staining, motility test, catalase test, and different bio-chemical tests (sugar fermentation test, indole test, TSI test, Cimon Citrate test, MR-VP test).

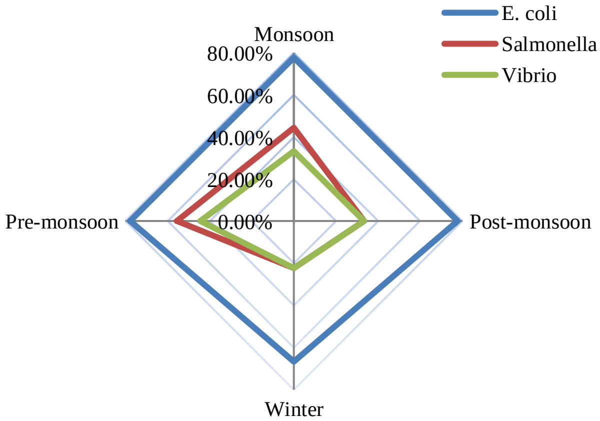

Figure 5. Prevalence of pathogenic bacteria in Kalibaus (*Labeo calbasu*)

E. coli bacteria was found static and higher in (monsoon, pre-monsoon and post-monsoon) season as 77.77%, similarly lower in winter as 66.66%. Whereas, *Salmonella* spp. was found higher in pre-monsoon at 55.55% and lower in winter at 22.22%. On the other hand, *Vibrio* spp. was found in lower amounts than the other two bacteria. It was the lowest in winter at 11.11%. In winter, these three bacterial species' percentages were found to be lower (Fig. 5). Overall, *E. coli* is found with a high prevalence during the monsoon season.

DISCUSSION

The results indicate that there was a seasonal variation in bacterial load. It was higher in Kalibaus than in Rui during the study period (Table 1). Statistical analysis between these two fish of TVC shows that results were highly significant ($p<0.05$). Though viable bacterial counts in Kalibaus were higher than the Rui, viable bacterial counts from both fishes comply with ICMSF standards (ICMSF 1986). For white fish, the standard plate count (SPC) accepted by ICMSF is 5×10^5 cfu/g, while it is 100 MPN/g and <3 MPN/g for total coliform and fecal coliform, respectively. However, the samples must be absent with *Salmonella* spp. and *Vibrio cholera* (including O group-01/non O group-139) (ICMSF 1986). It was reported that the total viable count of bacteria ranged from 9.9 x 10^6 to 1.4 x 10^7 CFU/g of intestine in different age groups of Rui fish (Hossain, 1993). Alternatively, it showed that the month of July had the highest bacterial load while it was lowest in the month of January.

Bandekar *et al.* (2004) studied the bacteriological screening of processed Rui (24 samples) and whole Rui (6 samples) obtained from fish processing factories, where TVC in 3 samples of whole Rui out of 6 samples exceeded $5x10^5$ cfu/g and 3 samples of processed Rui out of 24 samples exceeding $5x10^5$ cfu/g. Seasonal variations of bacterial load between two selected fish species were observed. In the monsoon season, both Rui and Kalibaus showed higher bacterial load (log cfu/g±sd) 5.56±0.078 and 5.68±0.050, respectively. The lowest bacterial load (Log CFU/g±SD) in both fishes was found in the winter season, 5.36±0.065 in Rui and 5.52±0.040 in Kalibaus, which are significantly ($P< 0.05$) different. In post-monsoon and pre-monsoon, bacterial load (Log CFU/g±SD) was observed as 5.51±0.043 and 5.48±0.091 in Rui, whereas 5.58±0.057 and 5.63±0.067 in Kalibaus. In post-monsoon, in the case of bacterial load between Rui and Kalibaush, non-significant differences were observed. Ahmed H. Al-Harbi (2003) mentioned in tilapia intestine that the total viable count based on seasons ranged from 1.6±2.0×106 to 5.1±2.5×107 cfu/g in summer, 6.8±1.9×106 to 7.5±1.4×107cfu/g in early summer, and $8.9\pm1.8\times10^5$ to $1.3\pm0.9\times10^7$ cfu/g in winter. Bisth *et al.* (2014) found that sediment of pond (6.40 x 10^4 cfu) had higher (10 times) bacterial load in comparison to water of the pond (6.93 x 10^3 cfu/ml), while microbial load in the intestinal of winter season was higher around 100 times (6.67 x10^5 cfu/g) while it was higher in 100 times (2.33 x10^6 cfu/g) during summer season in comparison to the surficial skin of fish during winter and summer (3.39 and 8.87 x10^3 cfu/cm^2) respectively. Total coliform of Rui and Kalibaus in all seasons complied with the ICMSF standard. The highest TCC was found (73 MPN/g and 96 MPN/g) in Rui and Kalibaus in the Post-monsoon season. Whereas, the lowest (28 MPN/g and 65 MPN/g) was in Rui and Kalibaus in the winter season. The result of the present study is more or less similar to Begum *et al.* (2010), where she found the highest total coliform (>240 MPN/g) in a number of samples from local markets, whereas the lowest count (0.9 MPN/g) was in Rui fish of super shop. The load of TCC in this fish sample indicates a range of contamination. These results are also more or less similar to the other work of Das *et al.* (2007) and Fatema (2005), where they found a comparable range of counts. Rui and Kalibaus were identified with three different types of pathogenic bacteria and it was *E. coli*, *Salmonella* spp., and *Vibrio* spp. Our finding aligned with the findings of Adebayo-Tayo *et al.* (2012), Truong *et al.* (2008), and Zaman (2013). Eosin Methylene Blue (EMB) agar plate showed characteristics of colony color with a metallic green sheen, indicating the *E. coli* (Sharada *et al.* 1999; Shorif, 2013). Morphologically, *E. coli* is a single or paired gram-negative and motile bacteria (Thomas, 1998). *Salmonella* was examined using selective media and found in almost all sample fishes. In SS agar plate colonies were observed with opaque and translucent having black in the centre (Hossain 2002), indicating the colonies of *Salmonella* spp. In gram staining, bacteria were observed with short rods, gram-negative, motile, single, or paired arrangement under the microscope (Samad, 2005). present investigation, *Vibrio* spp. was found in three out of four seasons in Rui samples that were collected from the *haor*. The morphology of *Vibrio spp.* on the TCBSS agar plate showed yellow and green color colonies in this study (Khan *et al*, 2007). Begum *et al.*, (2010) also found the same bacteria in examined fish but not in Rui fish. The recent study highlighted the seasonal difference in standard bacterial count and occurrence of *E. coli*, *Salmonella* spp., and *Vibrio* spp. collected from open water bodies of Hakaluki haor. To tackle the situation, hygienic conditions should be maintained at every step of fish handling, such as catching, landing, transporting, processing, and marketing. This information will help in controlling bacterial contamination, reducing foodborne disease, and increasing the fish and fishery products' self-life.

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