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Microbiological Analysis Of Fish Feeds With The Demonstration Of The Antibiotic Susceptibility Of The Isolates And The Antibacterial Activity Of The Feeds

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Abstract: *The present study was done to examine the bacteriological quality of fish feed samples collected from various local markets of Dhaka city, Bangladesh. A total number of six fish feed samples were analyzed during the period from February 2018 to April 2018. The analysis encompassed enumeration of total viable bacterial count (TVBC), presumptive detection of other pathogenic bacteria and fungus from these samples. The higher counts of TVBC, Escherichia coli, and Staphylococcus aureus were recorded as 2.9×10^6 cfu/g, 3.3×10^5 cfu/g and $1.1x10^6$ cfu/g respectively. On the other hand, Salmonella spp., Shigella spp., Vibrio spp. and Pseudomonas spp. were also detected in several samples. Fungal count was also noticed in all samples with a maximum load of $2.8x10^6$ cfu/g and a minimum load of 2×10^4 cfu/g. In order to observe the antibiotic sensitivity pattern, the antibiogram assay was carried out. All isolates found from fish feed samples were 100% sensitive against Gentamycin and Ceftriaxone. The bacterial isolates also showed varying degree of resistance against other antibiotics tested in this study. However the fish feed samples didn't show any antimicrobial activity.*

Keywords: *Antibiogram, Antibacterial activity, Bacteriological quality and fungal count*

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I. Introduction

Fish and fish products are a popular source of protein and animal fat to the people and with the overgrowing people, the demand for protein is increasing. To meet the demand, fisheries sectors have been established and are increasing rapidly worldwide. To grow properly and rapidly, the fishes need proper nutrients in the artificial environment as they are removed from natural environment [1]. Fish is cheaper than other animal sources of protein such as poultry, pork, cow and accepted by the people from all religions [2].

Commercially available fish feeds are provided to the cultured farm fishes which can meet the protein demand of the fishes. Being an energy source the feeds not only help the fish to grow but also responsible for causing disease in some conditions if they harbor pathogenic bacteria. To get better quality fish, it is important to provide good quality and pathogenic microorganisms free feeds to the fishes [3]. The better the quality of fish, the higher the economic condition of the fisheries industries and the better health impacts on the consumers. Feed types can be divided into three groups, such as industrially compounded feeds, farm-made feeds and raw organisms. Artificial diets may be either complete or supplemental [1].

Fish feeds are prepared commercially mainly using the animal byproducts (extreta, bones, meat) as well as plant originated components (cereal seeds, bran, rapeseed or soybean meal or cake, legume seeds) which are better in delivering more nutrition. But the byproducts can readily transmit the associated pathogenic and opportunistic pathogenic bacteria in the feed and consequently they are responsible to cause illness by producing toxins in the fish [4,5,6].

Fish feeds are constantly in contact with environmental organisms and become readily colonized by various microbial species. Environmental factors during storage cause the microbial spoilage of the fish feeds. The presence of bacteria in feeds causes their decomposition and subsequently, fish diseases. Bacteria such as *Salmonella, E. coli* and other bacteria strains have been reported to contaminate fish feeds. Fungal contamination of fish feed has been reported to result in aflatoxicosis. Aflatoxins are chemical produced by fungi like *Aspergillus flavus* and *A. parasiticus* commonly known as mold. Other fungal toxins include patulins and trichotecens which are strongly cancinogenic and mutagenic. Fungal contamination occurs mainly during the storage in poor environmental conditions [1]. Any equipments, production stages, entire production plant can be responsible for potential contamination as a source. The growth and proliferation in the feed depends on numerous factors, such as moisture, temperature, type of feed, aerobic and anaerobic conditions, chemical and physical properties of raw material, feed pH value, presence of feed supplements, storage periods and conditions as well as feed decomposition products [7].

Public health can be hampered by the infection as well as the intoxications after consuming fishes which have diseases and intoxications. Such problems can become worse if the fish provide multi drug resistant pathogenic bacteria to the consumers which are difficult to treat by applying commonly prescribed antibiotics [8, 9].

The purpose of this study was to determine the microorganisms harboring the commercially available fish feeds with the antibiotic drug resistant traits of the isolated microorganisms. Antibacterial activity was also determined to assume the presence of any antimicrobial agents added during the manufacturing procedure.

II. Materials And Methods

2.1 Sampling and sample collection

The fish feeds used in this study were collected from different districts in Bangladesh within the period of February, 2018 to April, 2018. All the samples were transported to the laboratory after collection in sterile plastic bags and processed for microbiological analysis as soon as possible. The samples were labeled properly.

2.2. Sample processing and enrichment of samples

In case of every sample, 10 g sample was weighted and then homogenized in 90 ml normal saline (NS) to make a 100 ml sample suspension for the microbiological examinations. For enrichment purposes, 1 ml of each sample was added to 9 ml alkaline peptone water (APW) ((Difco Laboratories, Detroit, Mich.) for the enrichment of *Vibrio* spp. and the selenite cysteine broth (SCB) ((Difco Laboratories, Detroit, Mich.) for both *Salmonella* and *Shigella* spp. Culture suspensions were incubated for 4 hours at 37 ^{0}C [10].

2.3. Enumeration of total viable bacterial count (TVBC), total fungal count (TFC) and total coliform count (TCC)

For the enumeration of total viable bacteria, coliforms (especially *E. coli* and *Klebsiella* spp.) and fungi, an aliquot of 0.1 mL from the dilution of 10^{-3} and 10^{-4} was introduced onto the nutrient agar (NA) plates, MacConkey agar plates and Sabouraud dextrose agar (SDA) plates, respectively. After spreading 0.1 ml suspension from the dilution 10^{-3}, the NA and MacConkey agar plates were incubated at 37 ^{0}C for 24 hours and the SDA plates were incubated at 25 ^{0}C for 48 to 72 hours [10].

2.4. Detection of Vibrio spp., Salmonella spp., and Shigella spp.

Thiosulfate citrate bile salt (TCBS) agar plates were used to isolate the contaminating *Vibrio* spp. within the examined samples while Salmonella-Shigella (SS) agar was used both for the isolation and enumeration of *Salmonella* spp. and *Shigella* spp. 0.1 ml sample from the dilution of 10^{-3} and 10^{-4} of SCB and APW was spread onto SS agar and TCBS agar respectively. After incubation at 37 ^{0}C for 24 hours, characteristic colonies were noticed and enumerated [10,11,12].

2.5. Isolation of Staphylococcus spp. and Pseudomonas spp.

For the isolation of *Staphylococcus* spp. and *Pseudomonas* spp. 0.1 ml sample from the dilution of 10^{-3} and 10^{-4} was spread onto the Mannitol Salt Agar (MSA) & Pseudomonas agar (PA) & then incubated at 37 ^{0}C for 24 hours [9,11].

2.6. Biochemical tests

Identification of the isolates was done by major biochemical tests, for example- Triple Sugar Iron (TSI), Motility Indole Urease (MIU), Methyl-Red (MR), Voges-Proskauer (VP) and Citrate Utilization were performed following the standard methods [13].

2.7. Antibiotic susceptibility test

Antibiotic susceptibility testing or antibiogram was performed using the disc diffusion test on Mueller-Hinton agar (Difco, Detroit, MI) against frequently used antibiotics following the standard protocol. The isolates were screened for their resistance or sensitivity to the following antibiotics discs that include Gentamycin (10 µg), Nalidixic acid (30 µg), Ceftriaxone (30 µg), Novobiocin (30 µg), Rifampicin (5 µg), Amoxicillin (10 µg), Chloramphenicol (10 µg), Tetracycline (10 µg), Erythromycin (10 µg), Streptomycin (10 µg) [10,11].

2.8 Determination of antibacterial activity

The antibacterial activity of the samples was performed by using agar well diffusion method. At first, the suspensions (with standard turbidity compared to that of McFarland standard of 0.5) of each of the test bacteria; *Escherichia coli, Bacillus spp., Staphylococcus* spp., *Vibrio* spp., *Salmonella* spp., *Pseudomonas* spp., *Klebsiella* spp. were spread evenly over the MHA using cotton swab which in turn resulted into uniform lawns. Wells were made in the MHA using cork-borer. Each of the homogenized fish feed samples was then introduced

separately in the specified well along with a positive control Gentamicin (GEN-10μg) and a negative control (normal saline). Presence of clear zone around the sample solution (if any) was indicative of the presence of antibacterial activity of the samples tested [14, 15].

III. Results

3.1. Total Viable Bacteria (TVB)

In this study, the total viable bacterial counts ranged from $2.3x10^5$ cfu/g in sample 3 to $2.9x10^6$ cfu/g as shown in Table 1.

3.2. Escherichia coli count

E. coli was found only in one sample with a load of 3.3×10^5 cfu/g. The result is presented in Table 1.

3.3. Staphylococcus aureus count

Staphylococcus aureus count was observed in four samples within the margin from 3×10^4 cfu/g – $1.1x10^6$ cfu/g which is given in Table 1.

3.4. Salmonella spp. and Shigella spp. count

Table 1 indicates the presence of *Salmonella* spp. and *Shigella* spp. *Salmonella* spp. was noticed from $1.2x10^5$ cfu/g to 2.9×10^6 cfu/g in Sample no 4 and Sample no 6 respectively whereas *Shigella* spp. showed the load of 4×10^5 to 6×10^5 cfu/g in Sample no 4 and S ample no 5 consecutively. *Salmonella* spp. was absent in Sample no 2 and *Shigella* spp. was absent in sample 1, 2, 3 & 6 respectively.

3.5. Vibrio spp. count

The highest count of *Vibrio* spp. was recorded as 2.8×10^6 cfu/g in Sample no 1 and the lowest count was 1.9×10^5 cfu/g found on Sample no 3. These are shown in Table 1. On the other hand, sample 2, 4, 5 and 6 were free from *Vibrio* spp.

3.6. Pseudomonas spp. count

All fish feed samples of this current study indicated the presence of *Pseudomonas* spp. except sample 4. The observable recorded counts shown in Table 1 are in between $2x10^4$ cfu/g and 2.8×10^6 cfu/g found on Sample no 2 and Sample no 6 respectively.

3.7. Total Fungal count

The health hazards of mycotoxins to humans or animals have been reviewed broadly in recent years [16]. The viable fungal growth as recorded in Table 1 in this study was $2x10^4$ cfu/g to 2.8×10^6 cfu/g.

Table No 1: Microbial load found in fish feed samples (per gram)

Sample	(TVB) Total Viable Bacteria	*Escherichia coli*	*S. aureus*	*Salmonella* spp.	*Shigella* spp.	*Vibrio* spp.	*Pseudomonas* spp.	Total Fungal count
S1	6.8×10^5	0	8×10^4	2.5×10^6	0	2.8×10^6	2.3×10^5	2×10^4
S2	2.5×10^5	0	1.3×10^5	0	0	0	2×10^4	9×10^4
S3	2.3×10^5	0	3×10^4	5.1×10^5	0	1.9×10^5	1.3×10^6	1.9×10^5
S4	2.5×10^6	0	0	1.2×10^5	4×10^5	0	0	1.6×10^6
S5	2.8×10^6	0	0	5.5×10^5	6×10^5	0	5×10^4	2.12×10^6
S6	2.9×10^6	3.3×10^5	1.1×10^6	2.9×10^6	0	0	2.8×10^6	2.8×10^6

3.8 Biochemical identification of the isolates

Biochemical tests were performed to identify the microorganisms that were isolated from six fish feed samples. The isolates such as- *Shigella* spp., *Salmonella* spp., *Staphylococcus aureus*, *Pseudomonas* spp. and *Vibrio* spp., and *E. coli* were confirmed after their biochemical identification. The results are shown in Table 2.

Table No 2: Biochemical identification of the bacterial isolates from different fish feed samples

Presumptive Organism	TSI			H_2S creation	MR test	VP test	Citrate test	MIU	Oxidase	Catalase	Grams stain
	Slant	Butt	Gas								
Vibrio spp	Y	Y	_	_	_	+	_	+	_	+	_
Salmonella spp	Y	Y	_	_	_	+	+	+	_	+	_
Pseudomonas spp	Y	Y	_	_	+		_	_	+	_	-
Staphylococcus aureus	R	Y	_	_	+	_	+	+	_	+	+
Shigella spp.	Y	Y	+	_	+	+	-	-	_	+	_
Escherichia coli	Y	Y	+	_	+	-	+	+	_	+	_

TSI =Triple Sugar Iron, Y=Yellow (Acid), R=Red (Alkaline), MR=Methyl red, VP=Voges-Proskauer, MIU= Motility Indole urea. Key + = Positive; - = Negative; GP= Gram Positive; GP; GN= Gram negative

3.9 Antibiotic susceptibility patterns of pathogens found in Fish Feed samples

Table 3 shows the antibiotic susceptibility pattern of the isolates found in the fish feed samples. 10 selected antibiotics were used to determine drug sensitivity pattern against the isolated bacteria which were *E. coli*, *Salmonella* spp., *Shigella* spp., *Pseudomonas* spp., *Vibrio* spp. and *Staphylococcus aureus*. The degree of susceptibility of these organisms against each antibiotic was determined and interpreted as either sensitive (S) or resistant (R) by calculating zones of inhibition around the antibiotic discs. Study of antibiogram showed that all bacterial isolates were 100% sensitive against Gentamycin (10 µg) and Ceftriaxone (30 µg). The other antibiotics showed varying degree of sensitivity against these isolates.

Table No 3: Antibiogram of different the bacterial isolates collected from various fish feed samples

Isolates / Antibiotics	*E. coli* N = 2		*Pseudomonas* spp. N = 4		*Salmonella* spp. N=2		*Vibrio* spp. N=3		*Staphylococcus aureus* N=3		*Shigella spp.* N=4	
	S	R	S	R	S	R	S	R	S	R	S	R
GEN (10µg)	100%	0%	100%	0%	100%	0%	100%	0%	100%	0%	100%	0%
NA (30µg)	90%	10%	70%	30%	80%	20%	90%	10%	0%	100%	90%	10%
CRO (30µg)	100%	0%	100%	0%	100%	0%	100%	0%	100%	0%	100%	0%
NVB (30µg)	40%	60%	80%	20%	100%	0%	40%	60%	80%	20%	40%	60%
RFM (5µg)	80%	20%	80%	20%	80%	20%	30%	70%	10%	90%	40%	60%
AMO (30µg)	20%	80%	20%	80%	80%	20%	70%	30%	10%	90%	40%	60%
C(10 µg)	20%	80%	90%	10%	60%	40%	70%	30%	20%	80%	70%	30%
TE (10 µg)	20%	80%	90%	10%	60%	40%	70%	30%	20%	80%	70%	30%
E (10µg)	10%	90%	90%	10%	30%	70%	30%	70%	70%	30%	70%	30%
S (10µg)	10%	90%	90%	10%	30%	70%	30%	70%	70%	30%	70%	30%

GEN= Gentamicin, NA= Nalidixic Acid, CRO= Ceftriaxone, NV= Novobiocin, AMO= Amoxicillin, RFM= Rifampin, C=Chloramphenicol, TE= Tetracycline, E= Erythromycin, S= Streptomycin.

3.10. Determination of antibacterial activity of the fish feed samples

Homogenized fish feed samples were used to detect antibacterial activity against selected bacterial isolates. The previously stocked laboratory isolates of Department of Microbiology, Stamford University Bangladesh were used in this study. The selected isolates were *Pseudomonas* spp., *Staphylococcus* spp., *Vibrio* spp., *Escherichia* spp. and *Klebsiella* spp. But no antibacterial activity of the fish feed samples was found against these bacteria.

IV. Discussion

The microbiological analysis of fish feeds showed the presence of pathogenic bacteria like *Staphylococcus aureus, E. coli, Salmonella* spp., *Shigella* spp., *Vibrio* spp. *and Pseudomonas* spp. The presence of *E. coli, Staphylococcus aureus* and *Vibrio* spp. in the feeds suggests the contamination may be due to the mishandling of the feeds by the market sellers. Similar isolates were identified in the work of Ubeibi, 2017 [7] who found *Staphylococcus aureus, Bacillus substilis, Psuedomonas aeruginosa, Proteus vulgaris, Micrococcus spp.* and *E. coli* from feeds in Umuahia. The presence of these organisms in fish feeds may also arise from poor hygienic and sanitary practices employed in the manufacturing, processing and packaging of fish feed. Moreover several isolates found in this study showed multidrug resistance property which is also a matter of great concern.

V. Conclusion

There is very little information based on the microbiological analysis of the fish feed samples in Bangladesh. On the basis of gained results we can conclude that the most of the fish feed samples had high total viable bacterial and fungal count. Moreover these fish feeds were harboring various types of indicator and pathogenic isolates which may have negative health effects to fish. That's why the fish feed producers need to have very strict control on its quality production and safe distribution system until it reaches the hands of the users. Presence of fungus is also a matter of concern as some of these strains may have the capability of producing toxins, and which can also result with high fish mortality rate, or damage the whole production. Besides varying degree of drug resistance among the isolates is also a hazard from the environmental point of view.

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