16S rRNA sequence based identification of pathogenic gut microbiota of Rohu *Labeo rohita* (Hamilton-Buchanan 1822) and Silver carp *Hypophthalmichthys molitrix* (Valenciennes 1844)

A thesis submitted to the Department of Fisheries, University of Dhaka in the partial fulfillment of the requirements for the degree of **Master of Science (MS) in Fisheries**

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Dedicated

To

Department of Fisheries

University of Dhaka

Certificate

This is to certify that the research work embodying the results reported in this thesis entitled **"16S rRNA sequence based identification of pathogenic gut microbiota of Rohu *Labeo rohita* (Hamilton-Buchanan 1822) and Silver carp *Hypophthalmichthys molitrix* (Valenciennes 1844)"**submitted by **Nusrat Jahan Punom**, Roll: Curzon-4211, Session: 2013-14, Registration No: HA-2986, has been carried out under our supervision in the Aquatic Laboratory of Department of Fisheries in collaboration with Molecular Biology Lab, Department of Biochemistry and Molecular Biology, University of Dhaka.

It is further certified that the research work presented here is original and suitable for submission for the partial fulfillment of the Degree of Master of Science (MS) inFisheries, University of Dhaka, Bangladesh.

We wish her every success in life.

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Author

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Abstract

Rohu (*Labeo rohita*) and Silver carp (*Hypophthalmichthys molitrix*), both are tropical, most common, popular and commercially important fish of Bangladesh where pathogenic gastrointestinal bacteria of these fish, indicates the acceptability or quality as a whole the economic status of these fish. Current study was, therefore, carried out to investigate and compare the gastrointestinal pathogenic bacteria of these economically important fish of Bangladesh.

The experimental fishes were collected from three different fish markets of Dhaka metropolitan city *viz*. Nobabgonj Bazar, Palashi Bazar and Anando Bazar. In the present study, the gastrointestinal tract of experimental fishes were examined with three replicates. Homogenized samples were then used for bacteriological density enumeration using serial dilution and spread plate techniques with five selective media Luria agar (LA), Thiosulphate citrate bile salt sucrose agar (TCBS), *Salmonella- Shigella* agar (SS), *Aeromonas* agar, Mannitol Salt agar (MSA), Eosin Methylene Blue agar (EMB) plate to understand Total Bacterial Count (TBC), Total *Vibrio* like colony count, Total *Salmonella- Shigella* count, Total *Aeromonas* count, Total *Staphylococcal* count, Total coliform count. Besides conventional culture techniques, Biochemical as well as molecular techniques (16S rRNA sequencing) were performed for the purpose of isolation and identification of gut microbiota. The study was also investigated the antibiotic susceptibility of 14 antibiotics on selected isolates.

On an average, TBC of Rohu samples was $5.27 \pm 2.01 \times 10^7$ cfu/g and in Silver carp was $3.02 \pm 1.42 \times 10^7$ cfu/g; total *Vibrio* count from Rohu and Silver carp sample was $1.58 \pm 3.51 \times 10^6$ cfu/g and $2.38 \pm 3.63 \times 10^3$ cfu/g, respectively; total *Salmonella* and *Shigella* was $6.94 \pm 7.15 \times 10^6$cfu/g from Rohu and $1.11 \pm 0.97 \times 10^6$ cfu/g from Silver carp. In Rohu, total *Aeromonas* was $1.31 \pm 1.06 \times 10^7$ cfu/g and in Silver carp was $6.09 \pm 4.61 \times 10^6$ cfu/g; total *Staphylococcal* count was found $1.03 \pm 0.52 \times 10^7$ cfu/g in Rohu and $5.48 \pm 3.98 \times 10^6$ cfu/g in Silver carp. For Rohu sample, enteric bacteria with special reference to coliform count was $1.68 \pm 0.981 \times 10^7$ cfu/g and for Silver carp was $1.39 \pm 2.35 \times 10^7$ cfu/g.

Bacterial density (TBC, *Salmonella-Shigella* and *Staphylococcal* count) showed significant difference between species but among markets no significant difference were observed. All types of load counts exceed the ICMSF acceptable limit.

18 different (9 isolates of Rohu and 9 isolates of Silver carp) colonies were selected based on morphology for biochemical identification tests. Among 18 isolates, 3 were gram positive. From Rohu sample, 2 *Aeromonas* sp. and *Pseudomonas* sp., 1 *Vibrio* sp., 1 *Proteus* sp., 1 *Staphylococcus* sp., 1 *Enterobacter* sp. and 1 *Klebsiella* sp. was provisionally identified. In Silver carp, 1 *Vibrio* sp., 1 *Salmonella* sp., 1 *Pseudomonas* sp., 1 *Escherichia* sp., 1 *Klebsiella* sp. and 2 *Aeromonas* sp. and *Staphylococcus* sp. were provisionally identified.

10 representative isolates, named as njp1, njp2, njp3, njp4, njp5, njp6, njp7, njp8, njp9 and njp10 were selected for sequencing by 16S rRNA gene and identified as *Aeromonas hydrophila* subsp. *dhakensis*, *Proteus penneri* , *Pseudomonas plecoglossicida*, *Aeromonas caviae*, *Enterobacter* sp., *Pseudomonas aeruginosa*, *Aeromonas* sp., *Citrobacter freundii*, *Klebsiella pneumoniae* subsp. *rhinoscleromatis* and *Klebsiella pneumoniae* subsp. *rhinoscleromatis*, respectively. Multiple sequence alignment was performed to find out the polymorphic sites among the sequenced strains with considering 1341 bp nucleotides where 1.49% dissimilarities were observed among the identified 3 *Aeromonas sp.* and 3.88% in 2 *Pseudomonas sp.*All of the 2 group (njp9 and njp10) were absolutely similar in all positions of the sequence to each other except in 3 positions (338, 361 and 1038) were polymorphic. Phylogenetic analysis also confirmed the taxonomic relations among the identified species.

Finally, 6 different group of pathogenic gastrointestinal bacteria were identified by 16S rRNA sequencing whereas biochemical assay provisionally identified 9 different group of pathogenic bacteria.

The present study also revealed that, all the isolates including reference strain (*E. coli* DH5α) were sensitive to Ciprofloxacin and resistant to Sulphamethoxazole.

Findings of this study indicate the poor quality and unhygienic condition of the marketed fish which reflects the potential reservoir of pathogenic bacteria causing fish borne disease outbreaks.

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List of Symbols and Abbreviations

Symbols	Details
AA	*Aeromonas* agar (LAB)
APHA	Ameriacan Public Health Association
ANOVA	Analysis of Variance
bp	Base pair
cfu	Colony forming unit
cm	Centimeter
^0C	Degree centigrade
dATP	Deoxy nucleotide triphosphate
DNA	Deoxyribonucleic acid
EMB	Eosin Methylene Blue
et al	And others (et alliori)
eg	For example (exempli gratia)
E.	*Escherichia*
GDP	Gross domestic product
g	Gram
H_2S	Hydrogen sulphide
HSD	Honestly Significant Difference
hr	Hour
kg	Kilogram
L	Liter
LA	Luria agar
LB	Luria broth
ml	Millilitre
mg	Milligram
mm	Millimeter
µg	Microgram
µl	Microlitre
MSA	Mannitol Salt Agar
MEGA	Molecular Evolutionary Genetics Analysis
NaCl	Sodium Chloride

Symbols	Details
NaOH	Sodium hydroxide
pH	Negative logarithm of hydrogen ion concentration
rpm	Rotations per minute
rRNA	Ribosomal Ribonucleic Acid
SD	Standard Deviation
spp	Species
SPSS	Statistical Package for the Social Sciences
SS	*Salmonella- Shigella* agar
TAC	Total *Aeromonas* Count
Taq	*Thermus aquaticus*
TCC	Total Coliform Count
TBC	Total Bacterial Count
TCBS	Thiosulphate Citrate Bile Sucrose agar
TAE	Tris-acetate EDTA
TVC	Total *Vibrio* Count
TSSC	Total *Salmonella- Shigella* Count
TAC	Total *Aeromonas* Count
TSC	Total *Staphylococci* Count
V.	*Vibrio*
w/v	Weight/volume
(+)ve	Positive
(-)ve	Negative

Chapter 1
Introduction

1.1 Background

Fish are continuously exposed to microorganisms present in the aquatic environment. Being rich in nutrients, the digestive tract of fish confers a favorable growth environment for microorganisms. The intestinal tract of fish is generally colonized by a good number of heterotrophic bacteria (Kar *et al.* 2008).

1.2 Gut microbiota of fish

Gut microbiota of fish are the ecological community of commensal, symbiotic and pathogenic microorganism that literally share gastrointestinal tract of fish. They play an essential role in the development and health of fish. The gastrointestinal tract is home to an abundant and highly diverse community of microbes that have evolved important nutritional and physiological dependencies among them and with the host. The composition of gut microbiota can also be affected by the host's genetic background, lifestyle, feeding habit and selective pressures of gut habitats (Zoetendal *et al.* 2001; Rawls *et al.* 2006; Ley *et al.* 2008; Li *et al.* 2012; Yan *et al.* 2012). A poorly functional fish gut microbiota can be detrimental to their survival and fitness and a burst of studies is now attempting to understand its evolutionary dynamics with the host. Therefore, understanding of the fish gut microbiota is important. Information on pathogenic gut microbes can be used to improve fish health, thereby improving aquaculture. Culturable counts differ between studies on the gut microbiota, with numbers for both feces and rinsed intestine ranging from 10^2 to 10^9 cfu/g (Askarian *et al.* 2012). Direct microscopic counts range from 10^8 to 10^{11} cfu/g (Navarrete *et al.* 2010). In general, feces harbor greater diversity than rinsed intestine samples, but many of the abundant genera are similar between sample types. For instance in freshwater fishes, Aeromonas, Enterobacter, Pseudomonas, Micrococcus, and Bacillus are common in both feces and intestine (Di *et al.* 2013). However, studies identify Staphylococcus more frequently in rinsed intestine samples (Ringo *et al.* 2008). More recently, studies of microbial biodiversity, emphasis has been placed on molecular-based culture-independent

techniques, which have been generating some exciting data. The organisms may enter the mouth with water or food and pass through and/or colonize the digestive tract (Hovda *et al.* 2007). *Bacillus*, *Proteus*,*Pseudomonas*, *Klebsiella*, *Streptococcus*, *Salmonella*, *Staphylococcus*, *Micrococcus*, *Serratia* and *Escherichia* are found in the skin and intestine of fish (Tanu *et al.* 2012).

1.3 *Labeo rohita,* the study Fish

The Rohu (*Labeo rohita*) is a diurnal, column feeder and commercially important Indian major carp. It is a fast growing omnivorous fish, mostly feeds on the planktonic, filamentous algae, aquatic plant leaves, phytoplankton, zooplanktons and minor on small insects (Bairagi *et al*. 2002). It being an important source of proteins, vitamins and minerals is usually preferred by the people of Bangladesh. It contains vitamins like A, D, E, K and C as well as essential fatty acids like PUFA. ω -3 fatty acids like alpha linolenic acid, eicosapentaenoic acid, docosahexaenoic acid are mostly present in fat content of *Labeorohita*. It also contains amino acids, minerals like calcium, zinc, iron and thallium (Kaneko 1971; Silva *et al.* 1995).

The *Labeo rohita* frequently present in fresh water reservoirs, lakes, rivers, and is reported from all over Bangladesh and neighboring countries like India, Nepal, Pakistan and some regions of Myanmar and Srilanka (Kanwate *et al.* 2006; Bhat 2003; Menon 1987; Jayaram 2010). The *Labeo rohita* found only in south Asia and has huge demand of import from other countries like Japan due to its great nutritional value. For export fish should be of with long lasting nutritional value and should not harbor pathogenic microbes with it. Therefore it is impervious to study the microbes associated with fish.

1.3.1 Gut microbiota of *Labeo rohita*

Labeo rohita is widely preferred fish, its study of micro flora becomes impervious. The quantitative and qualitative aspects of intestinal bacteria of Rohu fish *(Labeo rohita)* showed that total viable count of bacteria ranged from 9.9×10^6 to 1.4×10^7 cfu/g of intestine in different age groups of fish (Hossain *et al.* 1999). Some researchers showed that strains of *Bacilli, Pseudomonas, Aeromonas, Enterobacter* (Ghosh *et al.* 2010), *Flavobacterium, Micrococcus, Achromobacter, and Vibrio* (Hossain *et al.* 1999) found in the gastrointestinal tract of *Labeo rohita*.

Some studies have isolated different bacteria species from the gut of one of the Indian major carps—Rohuand it has been suggested that the gut microbiota might be beneficial in the nutrition of the fish (Ghosh *et al*. 2002a, Kar and Ghosh 2008, Ray *et al*. 2010).Ghosh *et al.* (2002) isolated three strains of *Bacilli*, Lr1.1, Lr1.2 and Lr2.2 from the gut of Indian major carp Rohu, *Labeo rohita* and identified them as *B. circulans*, *B. pumilus* and *B.cereus* respectively, on the basis of morphological, physiological and biochemical characteristics.

1.4 *Hypophthalmichthys molitrix,* the study fish

The silver carp (*Hypophthalmichthys molitrix*) is a stomach-less, filter-feeder freshwater cyprinid, a variety of Asian carp native to China and eastern Siberia (Froese *et al.* 2006). The silver carp (*Hypophthalmichthys molitrix*) have been introduced as a food fish or a way to control plankton populations in many countries (Kolar 2007; Sampson *et al.* 2009). Silver carp feed on both phytoplankton and zooplankton (Radke and Kahl 2002).*Silver carp* (*H. molitrix*) was introduced in Bangladesh in 1969. The fish was imported from Hong Kong. Among exotic fishes, the silver carp has attracted more attention from ecologists and fishery managers, because of its impressive growth rate and ability to live harmoniously with other carp species. The silver carp naturally occurs in the river systems of south and central China and in the Amur Basin in USSR. The species has been introduced into many countries in recent years for aquaculture. It has become one of the most important fish species in the pond polyculture system in Bangladesh. The fish breeds naturally during April-July in the flowing waters of its natural habitat. (en.banglapedia.org).

The species has been introduced to or spread by connected waterways, into at least 88 countries around the world. The silver carp reaches an average length of 60–100 cm with a maximum length of 140 cm and weight of 45 kg. Silver carp, like all *Hypophthalmichthys* species, have no stomachs; they are thought to feed more or less constantly, largely on phytoplankton. They also consume zooplankton and detritus (https://en.wikipedia.org). It contains better balance of amino acid like as alanine, glutamic acid, phenylalanine and valine and also moisture, carbohydrates, lipid content (Ashraf *et al.* 2011).

1.4.1 Gut microbiota of *Hypophthalmichthys molitrix*

Rahman *et al.* (2010) significantly found higher density of *Vibrio*, coliform, Fecal streptococci from the gut of Silver carp.*Pseudomonas* (Khidhir *et al.* 2014), *Aeromonas*, *Brevundimonas*, *Massilia*, *Curvibacte*r and an unclassified *Sphingobacteriales* genus (Li *et al.* 2014), *Bacilllus*, *Enterobacter*, *Anoxybacillus*, *Clostridium*, *Actinomyces*, *Citrobacter* (Ye *et al*. 2014) were investigated from the gut sample of Silver carp.

1.5Different members of gut microbiota

1.5.1 *Vibrio* spp.

Vibrio are gram-negative, motile, short, curved, single or united into spirals. Heterotrophic organisms varying greatly in their nutritional requirements. Aerobic, facultative anaerobic and anaerobic species. Widely distributed as saprophytic forms in salt- and fresh-water and in soil; also occur as parasites and as pathogens. Ristori *et al*. (2007) isolated *Aeromonas spp., Plesiomonasshigelloides, Vibrio cholerae* 01, *Vibrioparahaemolyticus, and Vibrio vulnificus* from different organs of fishes. The antibiotic sensitivity pattern of shrimp bacterial isolates indicated that *V. harveyi* was found to be highly susceptible to chloramphenicol, ciprofloxacin, nalidixic acid and streptomycin (Selvin *et al.* 2005).

1.5.2 *Salmonella* spp.

Salmonella spp. are rods that are either motile or non-motile and gram-negative. Colonies are generally 2-4 mm in diameter. Most strains are aerogenic. Found in the bodies of warm-blooded animals, including man, and occasionally in reptiles; frequently found in the food eaten by these animals. Fish and shellfish appear to be passive carriers of *Salmonella,* demonstrate no clinical disease and can excrete *Salmonella* spp. without apparent trouble. The contamination of this organism derives from terrestrial sources and fish may serve as a vector for *Salmonella* spp. (Metz 1980; Minette 1986; Chattopadhyay 2000).Samad (2005)stated that *Salmonella* are facultative anaerobic, Gram negative bacilli and usually enter the body via the gastrointestinal tract where they can persist for long period of time. Chessbrough (1985) noted that on SS and MC agar *Salmonella* spp. produced lactose non- fermenting colony. Most strains showed blackening of the colony due to H_2S production. Chrisolite and Sugumar (2006) reported that drug resistance among bacteria depends on the amount and kind of drugs used in that particular

geographical area and he had noticed the resistant pattern of *Salmonella* from water, beach, sand and fish collected from the four fish landing centers of Thoothukudi.

1.5.3 *Shigella* spp.

Shigella spp. are rods that are non-motile, Gram-negative and aerobic. Possess distinctive antigenic structures. Pathogenic, causing dysenteries, or non-pathogenic species, all living in the bodies of warm-blooded animals. Found in polluted water supplies and in flies. Microbial species ordinarily involved in fish food spoilage and poisoning are of *Salmonella, Staphylococcus, Aeromonas, Listeria, Vibrio, Plesiomonas, Aeromonas, Shigella and Escherichia coli, Clostridium perfringens, Clostridium botulinum* (Akoachere *et al*. 2009; Nieto *et al.* 1984; Kraft 1992; Al Bulushi *et al.* 2008).

1.5.4 *Aeromonas* spp.

Aeromonas spp. are short (rarely more than 3 microns), rod-shaped cells. Motile by means of polar flagella, usually monotrichous; occasionally non-motile. Gram-negative and heterotrophic bacteria. The majority of species thus far described are from water or are known to be pathogenic to marine and freshwater animals such as fish and amphibians. Aeromonads are ubiquitous in fresh water, fish and shellfish, and also in meats and fresh vegetables (Isonhood and Drake 2002). The Gram-negative *Aeromonas* of the diseased fish were highly susceptible to many of the broad-spectrum antibiotics, except nitrofurantoin (Debasis *et al.*2004).

1.5.5 *Pseudomonas* spp.

Pseudomonas spp. are elongate, straight rods, occasionally coccoid, motile by means of polar flagella which are either single or in small or large tufts. A few species are non-motile. Gram negative may possess either water-soluble pigments that diffuse through the medium or no water soluble pigments. Usually found in soil or water, including sea water or even heavy brines. Many plant and a few animal pathogens. Izumi *et al.* (2004) reported that *Pseudomonas* spp. are gram negative rod-shaped aerobic and non-spore forming bacteria. The Gram-negative *Pseudomonas* of the diseased fish were highly susceptible to many of the broad-spectrum antibiotics, except nitrofurantoin (Debasis *et al.*2004). Kumar and Surendran (2005) isolated *Pseudomonas* resistant to nitrofurantoin and sulphamethizol from fish, prawn, brackish water and fresh water aquaculture farm environments.

1.5.6*Staphylococcus* spp.

Staphylococcus are non-motile, gram positive. Spherical cells occurring singly, in pairs, in tetrads and in irregular clusters, especially when growing in broth. Many strains produce an orange or yellow pigment, particularly on media containing high levels of NaCl. Enterotoxins produced by *Staphylococcus aureus* are another serious cause of gastroenteritis after consumption of fish and related products.

1.5.7 *Escherichia* spp.

Escherichia spp. are short straight rod, motile or non-motile, gram-negative and 1.1 -1.5 µm (living) (Luria 1960). Found in feces; occasionally pathogenic to man (enteritis, peritonitis, cystitis, etc.). Widely distributed in nature. *E. coli* is a classic example of enteric bacteria causing gastroenteritis. *E. coli* including other coliforms and bacteria as *Staphylococcus* spp. and sometimes enterococci are commonly used as indices of hazardous conditions during processing of fish. Such organisms should not be present on fresh-caught fish (Chattopadhyay 2000). The contamination of food of fish origin with pathogenic *E. coli* probably occurs during handling of fish and during the production process (Ayulo *et al*. 1994; Asai *et al*. 1999). Sharada *et al.* (1999) reported that *E. coli* colonies on nutrient agar (NA) are smooth, moist, low and convex. The colony on Eosine Methylene Blue (EMB) agar plate appeared as dark with characteristics metallic sheen. Resistance pattern of different antibiotics for *E. coli* isolates from various sources can be compared with isolates from river water, sediment and aquaculture pond samples (Hatha *et al*. 1999, Harish *et al.* 2003).

1.5.8 *Klebsiella* spp.

Klebsiella spp. arenon-motile, capsulated rods can be arranged singly, in pairs or short chains.It has more or less dome shaped, glistening colonies of varying degrees of stickiness, depending on the strain and the composition of the medium.

1.6 Biochemical assay for provisional identification of bacteria

Bacteria can be differed by their morphologies, besides the media test, biochemical tests can be used to identify bacterial isolates. These various tests were designed to identify various metabolic properties which helps to identify different bacterial species. For the identification of bacterial isolates different biochemical tests was performed by many

researchers (Dhruba *et al.* 1999 and Hossain 2002, Perez-Guzzi *et al.* 2000, Ali *et al.* 1998, Iqbal *et al.* 1998, Shetty and Ravishankar 1992, Manikandan *et al.* 2012).

1.7Molecular techniques for the identification of gut microbiota

Molecular technique lies in its increased discriminatory power. DNA can always be extracted from bacteria so that all strains are type able. Genomic DNA is a stable characteristic and its composition is independent of cultural condition or method of preparation. It allows for statistical data analysis and is amenable to automation (Bingen *et al*. 1993). To understand microbial community structure, dynamics and how organisms might influence or be influenced by their surroundings is to classify DNA sequences taxonomically or phylogenetically (Poretsky *et al.*2014).

1.7.1 16S rRNA sequencing

There is a conserved portion in the 16S rRNA gene of bacteria which can be amplified by the universal PCR method. In this method, only one set of universal primers is used for the amplification of the conserved region ofthe 16S rRNA gene, in common bacterial pathogens.

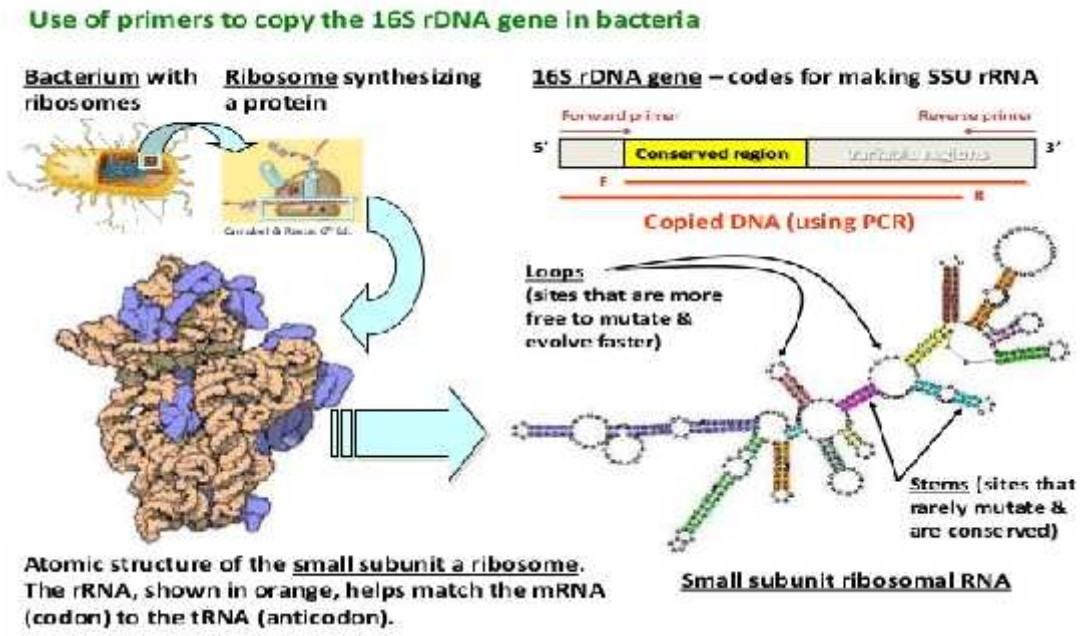


Fig 1.1. 16S rDNA gene in bacteria (Source: http://www.slideshare.net)

16S ribosomal RNA (rRNA) sequencing is a common amplicon sequencing method used to identify and compare bacteria present within a given sample. 16S rRNA gene

sequencing is a well-established method for studying phylogeny and taxonomy of samples from complex microbiomes or environments that are difficult or impossible to study.Besides this, Massively parallel sequencing methods are increasingly being applied to the characterization of microbial communities based on amplification of this gene and have led to a better appreciation of extant biodiversity (Sogin *et al.*2006).

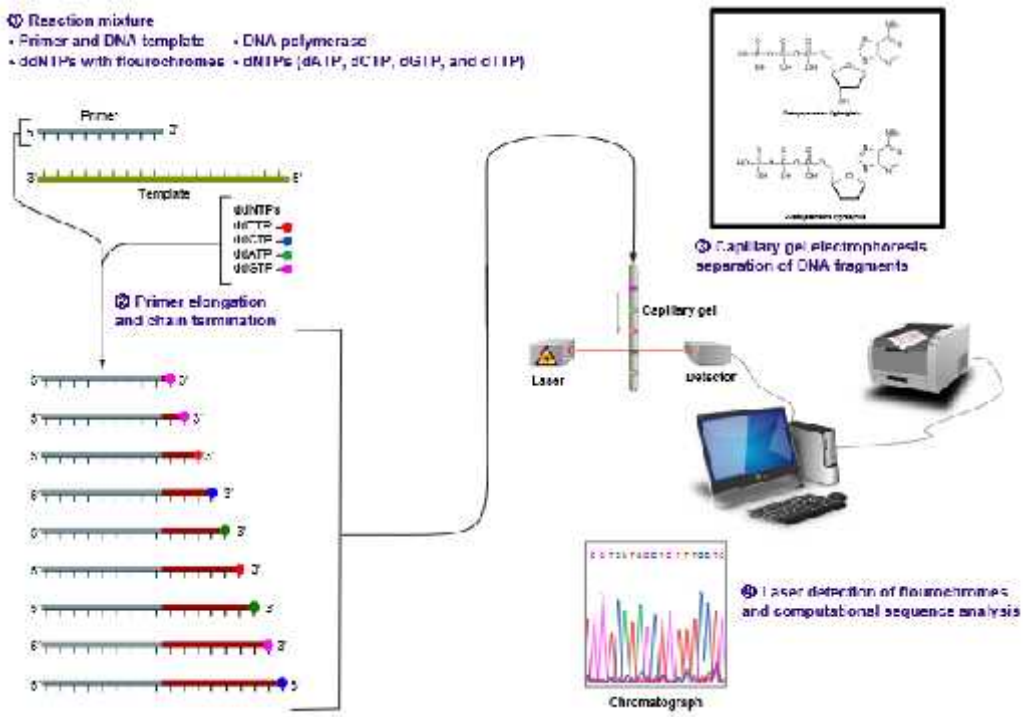


Figure 1.2.The Sanger (chain-termination) method for 16SrRNA sequencing.

(1) A primer is annealed to a sequence, (2) Reagents are added to the primer and template, including: DNA polymerase, dNTPs, and a small amount of all four dideoxynucleotides (ddNTPs) labeled with fluorophores. During primer elongation, the random insertion of a ddNTP instead of a dNTP terminates synthesis of the chain because DNA polymerase cannot react with the missing hydroxyl. This produces all possible lengths of chains. (3) The products are separated on a single lane capillary gel, where the resulting bands are read by an imaging system. (4) This produces several hundred thousand nucleotides a day, data which require storage and subsequent computational analysis**(https://en.wikipedia.org).**

However, the 16S rRNA based techniques are known to be limited by the short read lengths obtained, sequencing errors (Quince *et al*. 2009, 2011), differences arising from the different regions chosen (Youssef *et al.* 2009), and difficulties in assessing operational taxonomic units (OTUs) (Huse *et al*. 2010). Furthermore, the use of a single marker gene to assess diversity is challenging, given the prevalence of horizontal gene

transfer and the difficulty inherent in defining bacterial species (MacDonald *et al.* 2005; Rossello- Mora *et al.* 2001) as well as the limited resolution of the 16S rRNA gene among closely related species. Recently, 16S rRNA gene amplicon sequencing was compared to metagenomic data from synthetic communities (Shakya *et al*. 2013), but to our knowledge, there has been no systematic evaluation of high throughput 16S rRNA gene sequencing involving multiple sequencing and PCR replicates from natural microbial communities.

1.7.2Phylogenetic Analysis

Phylogenetics and sequence alignment are closely related fields due to the shared necessity of evaluating sequence relatedness (Ortet 2010). The field of phylogenetics makes extensive use of sequence alignments in the construction and interpretation of phylogenetic trees, which are used to classify the evolutionary relationships between homologous genes represented in the genomes of divergent species. The degree to which sequences in a query set differ is qualitatively related to the sequences evolutionary distance from one another. Roughly speaking, high sequence identity suggests that the sequences in question have a comparatively young most recent common ancestor, while low identity suggests that the divergence is more ancient.Progressive multiple alignment techniques produce a phylogenetic tree by necessity because they incorporate sequences into the growing alignment in order of relatedness.

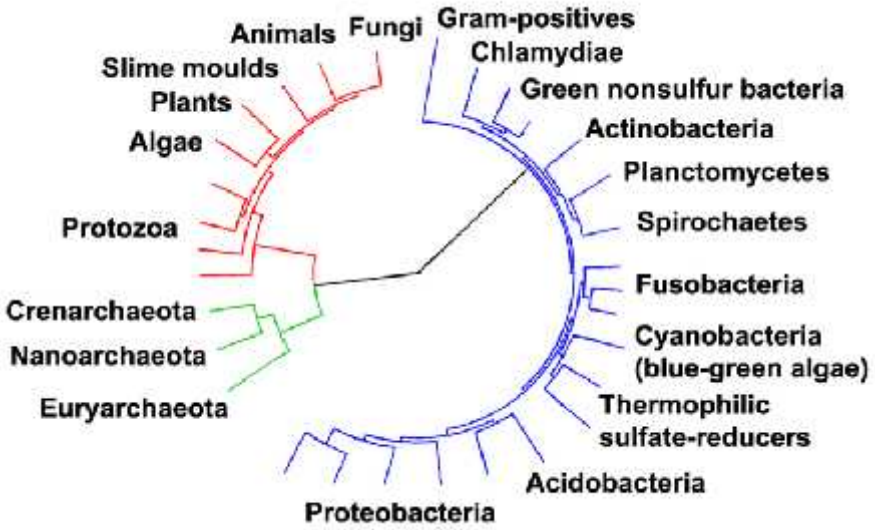


Fig 1.3. A highly resolved Tree of Life, based on completely sequenced genomes.

Eukaryotes are colored red, Archaea green and Bacteria blue. (Ciccarelli 2006)

1.8 Rationale

The gastrointestinal tract of *Labeo rohita* contain different pathogenic bacteria like *Pseudomonas, Aeromonas, Enterobacter* (Ghosh *et al.* 2010), *Flavobacterium, Micrococcus, Achromobacter, and Vibrio* (Hossain *et al.* 1999) whereas Silver carp carry *Pseudomonas* (Khidhir *et al.* 2014), *Aeromonas*, *Brevundimonas*, *Massilia*, *Curvibacte*r and an unclassified *Sphingobacteriales* genus (Li *et al.* 2014), *Bacilllus*, *Enterobacter*, *Anoxybacillus*, *Clostridium*, *Actinomyces*, *Citrobacter* (Ye *et al*. 2014) in their intestine. All types of pathogenic bacteria can act as health concern to human and fish, as a whole this can adversely disturb the economy of a country.

Fishes act as vector of infection for human beings as many fishes found to be amalgamated with infectious pathogens like *Salmonella* sp., *Staphylococcus aureus, Vibrio cholerae, V. parahaemolyticus, Yersinia enterocolitica, Listeria monocytogenes, Campylobacter jejuni* and *Escherichia coli* (Venugopal *et al.* 1999). These pathogenic bacteria could be transmitted from environment, feed to gastrointestinal tract of fish and after that to the human body. Sera and Ishida 1972 studied that the stomach and intestinal content of fish closely indicates the bacterial flora of their diet.

Many evidence confirm the presence of bacteria in the environment, usually they could be psychrophilic and mesophilic microbes like *Acinetobacter, Aeromonas, Alcaligenes, Flavobacterium, Moraxellaand Corynebacterium* (Akoachere *et al.* 2009; Nieto *et al.* 1984; Kraft 1992; Emikpe *et al.* 2011). Researchers reported that many food fishes like *Labeo rohita, Catlacatla, Cirrhinus cirrhosus, Cyprinus carpio* are spoiled by these microbes (Ghosh *et al.* 2007, Wahab *et al.* 2002, Nair *et al*. 1988). In Bangladesh, *Vibrio* was also observed in the intestine of Silver carp(Muniruzzaman 1993).
The contamination of fish can occur prior to harvest, during harvest and processing operations, distribution and storage and during preparation (Wekell *et al.* 1994). More specifically source of pathogens can come from feed, pond, soil, bird droppings and other live forms of surrounding ecosystems as well as the fish handlers can act as source of pathogens like *L. monocytogenes* (Jaysekaran *et al.*2002).

Microbiological screening of fish samples will help in understanding the types of contaminating organisms. In accordance with human health safety issues fishes should be free of infectious pathogens with long lasting nutritional value. Food spoilage and food borne infections are the foremost roots for deteriorating human health.

Identification of the causative agents of human pathogens up to the species level is also helpful for research and epidemiological studies as it can use in determining the exact source of any outbreak to reduce the severity of the disease.

Therefore, the present study was carried out to investigate, identify the pathogenic gastrointestinal bacteria from Rohu and Silver carp through microbiological assessment, Biochemical assay, and 16S rRNA gene sequencing and and characterize through antibiotic susceptibility test

1.9 Research gap

The gastrointestinal tract is reservoir to an abundant and highly diverse community of microbes that have evolved important nutritional and physiological dependencies among them and with the host. Both pathogenic and beneficial bacteria found from the gut. All of them pathogenic bacteria are major concern to human as well as for fish health. In silver carp, (*Hypophthalmichthys molitrix* Val.) Corneal opacity due to Gram-positive bacterium, *Staphylococcus aureus* was reported by Shah and Tyagi 1986. Mukherjee *et al.*2002 have recorded a mass mortality in farm reared silver carps and isolated *Staphylococcus aureus* from the affected eyes of diseased fish.Dropsy is another important fish disease in India were Rohu *(Labeo rohita)*, *Catla (Catla catla)* and Mrigal were affected mostly in the latewinter. Kumar *et al.*1986a revealed a mixed infection of *A. hydrophila* and myxosporidian parasite in the case of infectious dropsy in *Catla catla*.Recovery of humanenteric pathogenic bacteria indicates the extent of pollution by domesticsewage (Pradeep*et al.*1985). The presence of virulent strains of *Aeromonas* in healthy fish suggests their role as an opportunistic pathogen. Many studies of bacterial flora in the skin and intestine of fish have been conducted (Al-Harbi and Uddin 2004; 2005; Okoro *et al*. 2010; Yagoub 2009; Das, Trakroo and Agarwal, 2011). Bacteria such as *Pseudomonas fluorescens,Aeromonas hydrophila, Edwardsiella tarda, Vibrio spp.* and *Myxobacteria* are ubiquitous in the aquaticenvironment (Gilmour *et al.* 1976; Allen *et al*. 1983).

Outbreak of *Salmonella* (Fell *et al*. 2000; Senanayake *et al*. 2004; Sanyal *et al.* 1987; Ling *et al.* 2002; Gaulin *et al.*2002), diarrhoeal illness caused by ingestion of food contaminated with enterotoxigenic *E. coli* (Mitsuda *et al*. 1998; Vieira *et al*. 2001; Ayulo *et al*. 1994; Pierard *et al*. 1999; Asai *et al*. 1999; Semanchek and Golden 1998), faecal contamination by *Shigella* spp. (Wachsmuth and Morris 1989); furunculosis (Isonhood

and Drake 2002), meningitis (Ouderkirk *et al*. 2004), Fatal bacterial pneumonia (Kao *et al.* 2003), liver cirrhosis (Qu *et al.* 2003), eye infection (Tamura and Hida 2003), fatal septicemia (Shiina *et al*. 2004) by different isolates of *Aeromonas* spp. are found in different fish which causes life threating illness to human being.

Fishes are known to transmit human food borne infection and intoxication caused by *Salmonella* spp., *Staphylococcus* spp., *Vibrio* spp. and *Aeromonas* spp. Bacteria found in fish is classified into non-indigenous and indigenous bacteria. The nonindigenous bacteria include *Clostridium botulinum, Listeria monocytogenes,Staphylococcus aureus, Salmonella* spp., *Shigella s*pp. and *Escherichia coli.* On the other hand, indigenous bacteria include *Vibrio* spp., *Staphylococcus aureus, Salmonella, Shigella, Aeromonas, Yersenia* and *Pseudomonas* (Ward 1994).*Bacillus, Proteus, Pseudomonas, Klebsiella, Streptococcus, Salmonella, Staphylococcus,Micrococcus, Serratia* and *Escherichia* are found in the skin and intestine of fish. The walls of intestine do break down sufficiently for bacteria to move into the flesh through the muscle fiber (Kaneko 1971).

1.10 Objectives of the study

The overall objective of this study was to identify and characterize the pathogenic gut microbiota of indigenous Rohu and invasive Silver carp of three different markets.

The specific objectives were as follows-

- Quantitative enumeration of bacterial density using various selective agar media.
- Isolation and Identification of pathogenic gut bacteria by classical biochemical tests and molecular technique16S rRNA sequencing.
- Construction of phylogenetic tree using 16S rRNA sequences of selected isolates from this study and downloaded sequences from NCBI GenBank database.
- Antibiotic susceptibility test of the representative bacterial isolates of indigenous Rohu and invasive Silver carp.

1.11 Scope and Limitations

This study focused only on the identification of pathogenic gastrointestinal bacteria isolated by 5 selective medium of indigenous Rohu and invasive Silver carp of Bangladesh and characterization of them. Molecular identification of the isolates were also investigated in this study. The main limitation was that fishes were collected from fish markets that were not representing the real environmental sources. In the present study, 16S rRNA gene was used to identify gastrointestinal bacteria but Multi locus gene or Next generation sequencing was not use and this could be a future target of this study. So, there is a huge opportunity is waiting for the investigation of whole gut microbial community of the both type of studied fish.

CHAPTER 2

MATERIALS AND METHODS

2.1 Experimental Specimen

Rohu (*Labeo rohita*) and Silver carp (*Hypophthalmichthys molitrix*), both are tropical, most common, popular and commercially important fish species of Bangladesh, was considered as experimental fish.

2.1.1 Taxonomic study of Rohu

Phylum: Chordata

Class: Actinopterygii

Order: Cypriniformes

Family: Cyprinidiae

Genus: *Labeo*

Species: *Labeo rohita* (Hamilton-Buchanan 1822)

Plate 2.1. A photograph of *Labeo rohita*

2.1.2 Taxonomic study of Silver carp

Phylum: Chordata

Class: Actinopterygii

Order: Cypriniformes

Family: Cyprinidiae

Genus: *Hypophthalmichthys*

Species: *H. molitrix* (Valenciennes 1844)

Plate 2.2. A photograph of *Hypophthalmichthys molitrix*

2.2 Study location

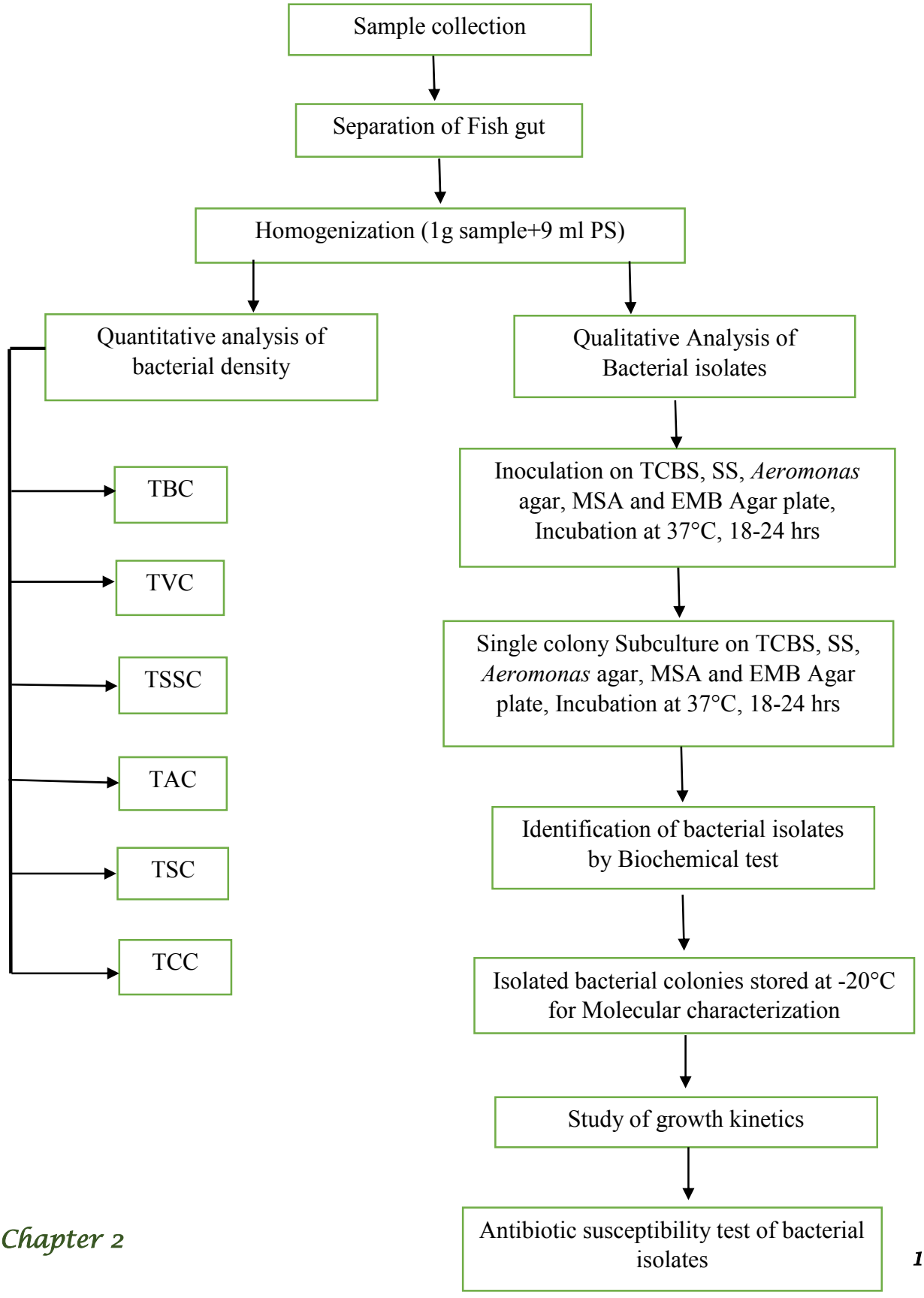
The study was carried out at Aquatic Laboratory of Department of Fisheries and Molecular Microbiology Laboratory in the Department of Biochemistry and Molecular Biology, University of Dhaka and the molecular identification of bacterial isolates through 16S rDNA gene were done on Invent, Life Science Laboratory, Dhaka. The sequencing was performed in theFirst BASE Laboratories Sdn Bhd, Malaysia.

2.3 Sampling Procedures

Fish samples of Rohu and Silver carp were collected in sterilized plastic bag aseptically following the methods of American Public Health Association (APHA) from 3 different fish markets (Nobabgonj Bazar, Palashi Bazar, Anando Bazar) of Dhaka metropolitan

city early in the morning. After that, samples were transported in the laboratory within 1hr.

Flow chart 1. Overview of Processing of fish, identification and characterization of Bacterial isolates



2.4 Media preparation and techniques for the enumeration and isolation of bacteria

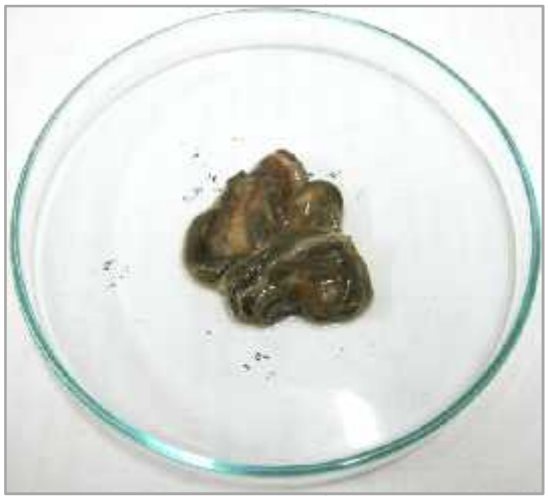
2.4.1 Processing of samples

The samples were washed with sterile 0.9% NaCl or physiological saline to remove sand, detritus as well as microorganisms attached to the surface of fish. Then the gut samples were collected aseptically following the method of APHA (1998). The collected samples were separately homogenized with saline solution using sterilized homogenizer and were then used for microbial load count and *Vibrio,* Coliform bacteria, *Salmonella-Shigella, Staphylococci*and *Aeromonas*specific enrichment.

A. Sample collection (Rohu)

B. Sample collection (Silver carp)

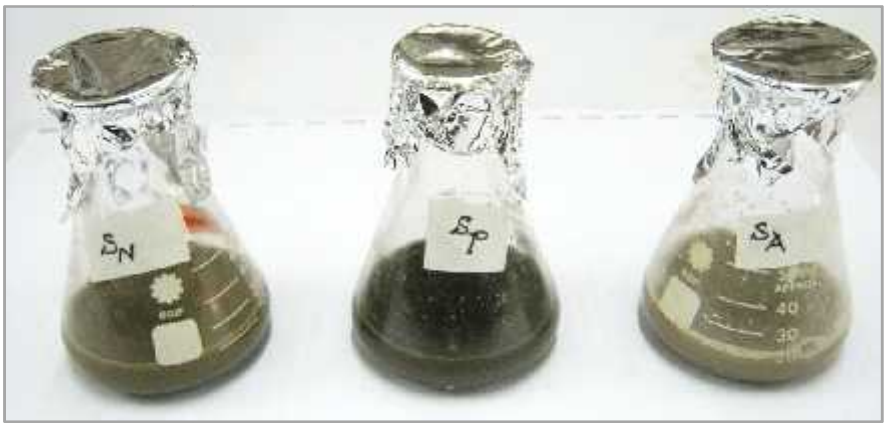
C. Gut sample of Rohu



D. Gut sample of Silver carp

Plate 2.3. Photographs of collection of fish (A-B) and gut sample (C-D).

A. Prepared gut sample (Rohu)



B. Prepared gut sample (Silver carp)

Plate 2.4. Photographs of prepared sample of gut (A-B).

2.5 Culture and media preparation

2.5.1 Total bacterial count (TBC) on LA media

Luria Broth Agar was used for the enumeration and isolation of total bacteria present in fish samples and the composition of the LB-Agar plate was Peptone (HIMEDIA, India) 1.0%, Yeast extract (LOBA Chemi, India) 0.5%, NaCl (UNI-Chemie, China) 1.0% and Agar (MERCK, India) 1.5% and the pH of the medium was adjusted to 7.0±0.2 and according to the protocol of ASM (www.microbelibrary.org).

A subsample of l g of each of the homogenized sample was taken and mixed with 9 mL physiological saline. Then 100µL of sample solution was diluted with 900µL sterile

physiological saline in a sterile test tube and shaken well and further diluted up to 10^5 for the plating in medium used.

An l00 µl of each homogenized diluted subsample was taken in a sterilized agar contained Petri plate by pipette. After that, the samples were spread by using a sterilized spreader. Then the plates were placed inverted position and incubated at 37°C for 24 h in incubator (Memmert, USA).

After 24 h of incubation, the plates having well discrete colonies were selected for counting. The plates were counted as cfu/g of sampled fish.

2.5.2 Total *Vibrio* like colonies on TCBS (Thiosulfate citrate bile salts sucrose) Agar

TCBS agar (Oxoid LTD, Basingstoke, Hampshire, England) medium were used to enumerate and isolate *Vibrio* spp. 88g of media was dispensed in 1 litre of deionized water and the adjusted pH was 8.6±0.2. After mixing and boiling, the sterilized media was poured into the sterilized petri plates under laminar air flow. A sub-sample of l00 µl of each of the homogenized diluted sample was taken in a sterilized TCBS agar contained Petri plate by sterilized pipette. Plating in duplicated plates were made for each diluted sample. After that, the samples were spread by using a sterilized spreader. After setting the medium the plates were placed inverted position and incubated at 37°C for 24 h in an incubator (Memmert, USA).

After 24 h of incubation, the plates having well discrete colonies were selected for counting. The selected plates with yellow, blue-green colonies were counted as cfu/g of sampled fish.

2.5.3 Total *Salmonella-Shigella* like colonies on SS Agar Media

SS agar (Himedia, India) medium were used to enumerate and isolate *Salmonella-Shigella* spp. 63.02g of media was dispensed in 1 litre of deionized water and the adjusted pH was 7.0±0.2. After mixing and boiling, the sterilized media was poured into the sterilized petri plates under laminar air flow. A subsample of l00 µl of each of the homogenized diluted sample was taken in a sterilized SS agar contained Petri plate by sterilized pipette. Plating in duplicated plates were made for each diluted sample. After that, the samples were spread by using a sterilized spreader. After setting the medium the plates were placed inverted position and incubated at 37°C for 24 h in an incubator (Memmert, USA).

After 24 h of incubation, the plates having well discrete colonies were selected for counting. The selected plates with pink color, cream pink color, colorless and colorless with black centre colonies were counted as cfu/g of sampled fish.

2.5.4 Total *Aeromonas* like colonies on *Aeromonas* Agar Media

Aeromonas agar (mass hall road, Heywood, Lancoshire,) medium were used to enumerate and isolate *Aeromonas* spp. 45.5g of media was dispensed in 1 litre of deionized water and the adjusted pH was 7.4±0.2. After mixing and boiling, the sterilized media was poured into the sterilized petri plates under laminar air flow. A sub-sample of l00 µl of each of the homogenized diluted sample was taken in a sterilized lab agar contained Petri plate by sterilized pipette. Plating in duplicated plates were made for each diluted sample. After that, the samples were spread by using a sterilized spreader. After setting the medium the plates were placed inverted position and incubated at 37°C for 24 h in an incubator (Memmert, USA).

After 24 h of incubation, the plates having well discrete colonies were selected for counting. The selected plates with tan to buff color and purple color colonieswere counted as cfu/g of sampled fish.

2.5.5 Total Staphylococcilike colonies on Mannitol Salt Agar Media

Mannitol Salt agar (Oxoid LTD, Basingstoke, Hampshire, England) medium were used to enumerate and isolate *Staphylococci* spp. 111g of media was dispensed in 1 litre of deionized water and the adjusted pH was 7.4±0.2. After mixing and boiling, the sterilized media was poured into the sterilized petri plates under laminar air flow. A sub-sample of l00 µl of each of the homogenized diluted sample was taken in a sterilized SS agar contained Petri plate by sterilized pipette. Plating in duplicated plates were made for each diluted sample. After that, the samples were spread by using a sterilized spreader. After setting the medium the plates were placed inverted position and incubated at 37°C for 24 h in an incubator (Memmert, USA).

After 24 h of incubation, the plates having well discrete colonies were selected for counting. The selected plates contained yellow colonies with yellow zone and pink colonies with no color change were counted as cfu/g of sampled fish.

2.5.6 Total coliform like colonies on EMB (Eosin Methylene blue) Agar Media

EMB agar (Oxoid LTD, Basingstoke, Hampshire, England) medium were used to enumerate and isolate coliform spp. 37.5g of media was dispensed in 1 litre of deionized water and the adjusted pH was 7.2±0.2. After mixing and boiling, the sterilized media was poured into the sterilized petri plates under laminar air flow. A sub-sample of l00 µl of each of the homogenized diluted sample was taken in a sterilized EMB agar contained Petri plate by sterilized pipette. Plating in duplicated plates were made for each diluted sample. After that, the samples were spread by using a sterilized spreader. After setting the medium the plates were placed inverted position and incubated at 37°C for 24 h in an incubator (Memmert, USA).

After 24 h of incubation, the plates having well discrete colonies were selected for counting. The selected plates contained greenish metallic sheen, dark purple, colorless to pinkish color colonies counted as cfu/g of sampled fish.

2.6 Isolation and Preliminary selection of bacterial isolates

After counting well discrete gastrointestinal bacterial colonies were selected immediately based on the colony morphology. 216 different colonies were selected primarily and finally 18 isolated colonies were selected.

2.7 Pure bacterial isolates preparation

On the basis of growth pattern, the selected isolates were purified through streaking plate technique. Only one type of colony was considered as pure.

2.8 Maintenance and preservation of isolates

Purified isolates of bacteria was transferred on Luria Broth agar, TCBS agar, SS agar, Mannitol Salt agar,Eosin Methylene Blue agar plates. The plates were kept in polythene bags with tags and preserved as stock culture in a refrigerator at 4°C for further study (Biochemical tests, Antibiogram and Growth kinetics). Periodical transfers of isolates on different agar plates were done for maintaining viability of the isolates. For molecular characterization selected bacterial colonies were stored in -20°C.

2.9 Morphological observation of isolates

For the identification of selected isolates, following morphological characters were studied and recorded.

2.9.1 Colonial morphology

Colony characters such as form, elevation, margin, shape, color, surface and arrangement of cells were observed under a phase contrast microscope from 18-24 hours old culture grown on LA, TCBS, SS, MSA, *Aeromonas* agar, EMB plates (Eklund and Lankford 1967).

2.9.2 Gram staining of bacterial isolates

The Gram stain is fundamental to the phenotypic characterization of bacteria. The staining method differentiates organisms of domain bacteria according to cell wall structure. Gram-positive cells have a thick peptidoglycan layer and stained blue to purple. Gram-negative cells have a thin peptidoglycan layer and stained red to pink.

The Gram staining method was performed according to the protocol of ASM MicrobeLibrary (www.microbelibrary.org).

The results were recorded as Gram positive (blue-violet) and Gram negative (red).

2.9.3 Motility test of bacterial isolates

Motility test was performed according to the protocol of ASM MicrobeLibrary (www.microbelibrary.org).

Red turbid area extending away from the line of inoculation recorded as positive result and red growth along the inoculation line but no further recorded as negative result.

2.10 Biochemical characteristics of bacterial isolates

All the biochemical characteristicswere performed for 18 isolates according to the protocol of ASM MicrobeLibrary (www.microbelibrary.org).

2.10.1 Catalase test

Catalase test was performed according to the protocol of ASM MicrobeLibrary (www.microbelibrary.org).

The reaction of catalase test-

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2 \uparrow$$

The evolution of bubbles indicated the positive result *i.e.* the organism having the enzyme catalase.

2.10.2 Oxidase test

The oxidase test assays for the presence of cytochrome oxidase, an enzyme sometimes called indophenol oxidase.

Oxidase test was performed according to the protocol of ASM MicrobeLibrary (www.microbelibrary.org).

Purple color indicated a positive result.

2.10.3 Gelatin hydrolysis test

The gelatin hydrolysis test detects the ability of bacteria to produce gelatinases.

Gelatin hydrolysis test was performed according to the protocol of ASM MicrobeLibrary (www.microbelibrary.org).

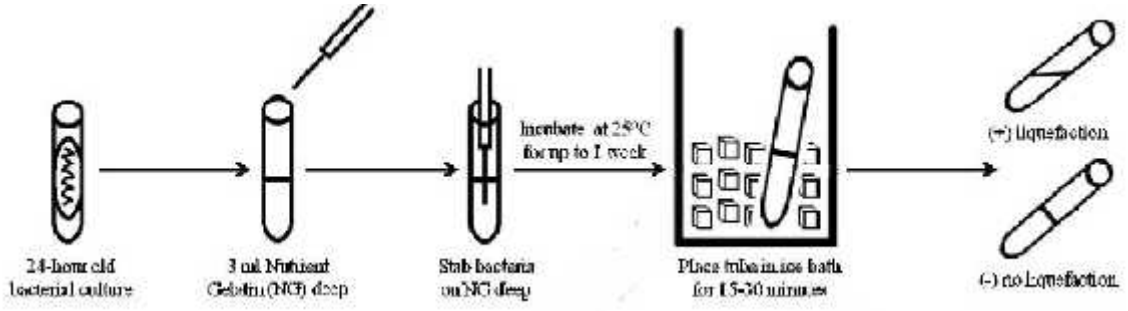
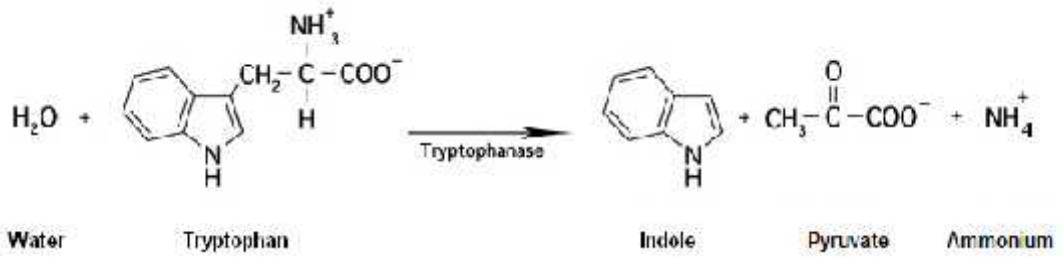


Fig 2.1.A schematic diagram of gelatin hydrolysis test using the Nutrient Gelatin stab Method (ASM MicrobeLibrary)

Liquefaction of the medium interpreted as positive result.

2.10.4 Indole test

Indole test was performed according to the protocol of ASM MicrobeLibrary (www.microbelibrary.org).The reaction of the indole test is-



A red color (cherry-red ring) indicated formation of indole as positive result.

2.10.5 Methyl red test

This test was performed according to the protocol of ASM MicrobeLibrary (www.microbelibrary.org).

Red color throughout the broth indicated positive reaction whereas yellow or any yellowish red indicated negative reaction.

2.10.6 Voges-Proskauer test

Voges-Proskauer test was performed according to the protocol of ASM MicrobeLibrary (www.microbelibrary.org).

Development of crimson to ruby red color indicates a positive reaction that is the production of acetyl-methyl carbinol.

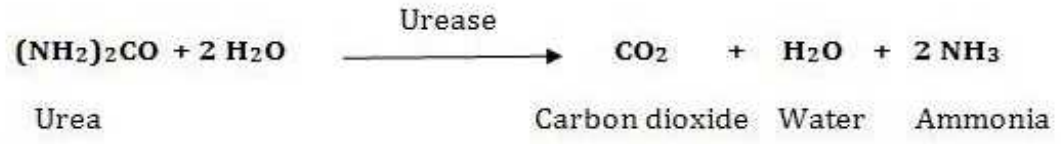
2.10.7 Citrate test

Citrate test was performed according to the protocol of ASM MicrobeLibrary (www.microbelibrary.org).

Utilization of citrate established by changing the color from green to blue.

2.10.8 Urease test

Urease test was performed according to the protocol of ASM MicrobeLibrary (www.microbelibrary.org). The reaction of urease test is-



Pink color allowed as positive reaction to urease hydrolysis test.

2.10.9 Starch hydrolysis test

Starch agar is a differential medium that tests the ability of an organism to produce extracellular enzymes α-amylase and oligo-1, 6-glucosidase that hydrolyze starch.

Starch hydrolysis test was performed according to the protocol of ASM MicrobeLibrary (www.microbelibrary.org).

Development of a clear zone around the growth indicated starch hydrolysis.

2.10.10 Triple Sugar Iron agar test

Triple sugar iron (TSI) agar is a tubed differential medium used in determining carbohydrate fermentation and H_2S production. TSI differentiates bacteria based on their fermentation of lactose, glucose, sucrose and on the production of H_2S.

Triple sugar iron (TSI) agar test was performed according to the protocol of ASM MicrobeLibrary (www.microbelibrary.org).

Acidic butt, alkaline slant (yellow butt, red slant): Glucose was fermented but not sucrose or lactose.

Acidic butt, acidic slant (yellow butt, yellow slant): Sucrose and/or lactose was fermented.

Alkaline butt, alkaline slant (red butt, red slant): neither Glucose, sucrose nor lactose was fermented.

Hydrogen sulfide production results in a black precipitate in the butt of the tube.

Gas production is indicated by splitting and cracking of the medium.

2.10.11 MacConkey agar test

MacConkey agar test was performed according to the protocol of ASM MicrobeLibrary (www.microbelibrary.org). Growth of the bacteria is indicated as gram negative bacteria.

2.11 Presumptive Identification of the isolates

Bergey's Manual of determinative Bacteriology, 9th edition. (Kreig and Holt. 1994) and www.tgw1916.net/bacteria_logare.html were used to identify the purified bacterial isolates.

2.12 Study of growth kinetics

Growth curves for 6 isolates were studied and the following steps were used-

- Isolates were grown in 10/15 ml Liquid media (LB) overnight at 37°C from a single colony.
- Then the OD was taken at 600 nm by UV Spectrophotometer (Shimadzu, UV-1800, Japan) and adjusted around 0.05 by diluting with autoclaved ddH_2O.
- Then the culture was used as seed culture.
- After that, 1 ml seed culture was inoculated into 50 ml media and the OD was also taken.
- The OD of different inoculates was followed every 30 min, 40 min or 60 min interval up to find out the death phage of the bacteria.

The same media (bacteria free) used as blank.

Calculation

For OD adjustment: $V_1 \times OD_1 = V_2 \times OD_2$

Where, V_1 =? OD_1 = Check, V_2 = 5 ml, OD_2 = ~ 0.05

Plate 2.5. A Photograph of UV Spectrophotometer

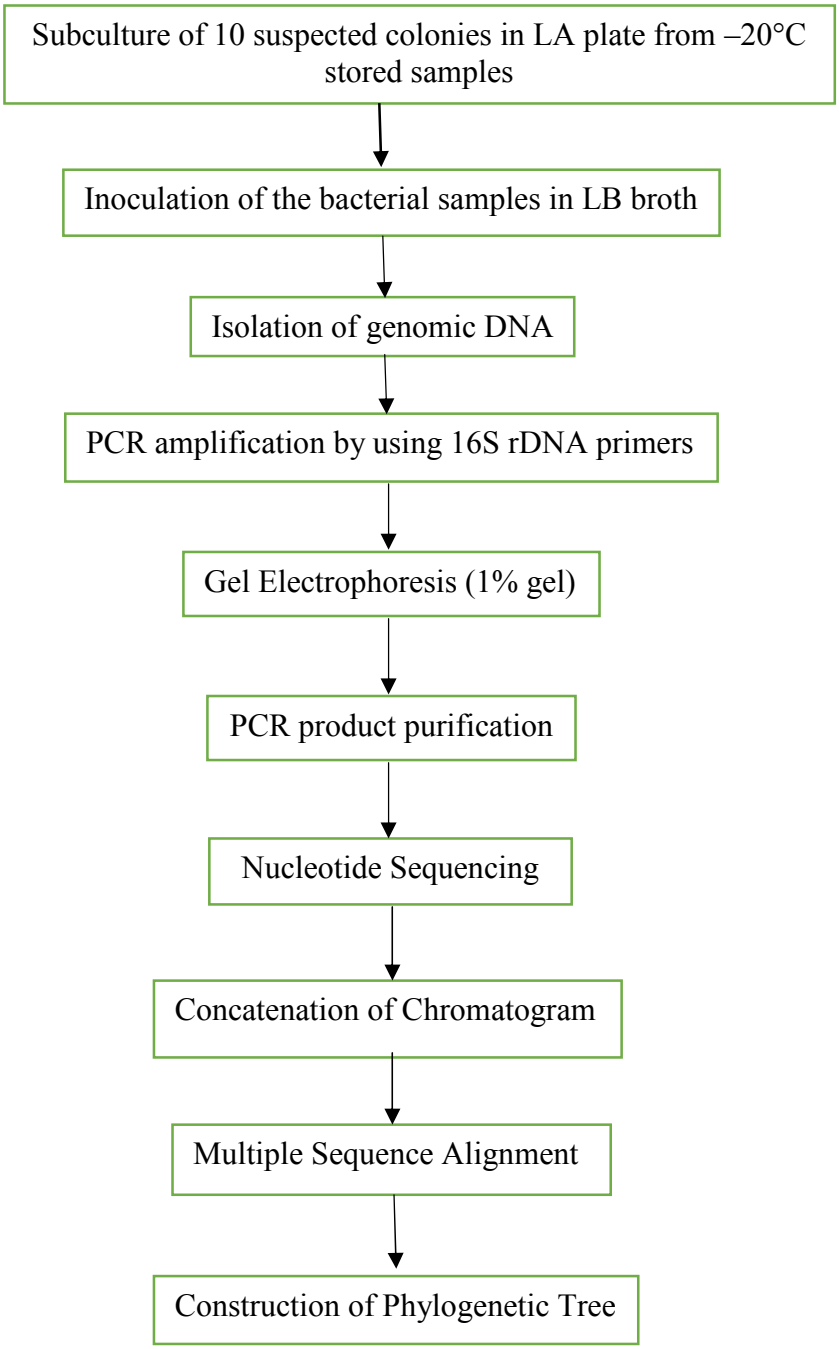
2.13 Molecular Techniques used for identification of bacterial isolates

Molecular techniques used for the identification and characterization of bacterial isolates shown in Flow chart 2.

2.13.1 Isolation of genomic DNA

A loop of fresh overnight grown colony of bacterial isolates incubated into 5 ml LB broth in shaking incubator (SHEL LAB, **Sheldon Manufacturing, Inc., North America)** at 37°C for overnight. 300µl of the overnight grown culture was taken into the DNA kits with plungers. After that, 300µL of elution buffer was taken into the elution tube. For washing 35 mins DNA kits were placed into the Automatic nucleic acid purification system (Maxwell® 16 MDx Research Instrument, Promega, USA).

Flow chart 2. Molecular identification of bacterial isolates



2.13.2 Purity of DNA

Absorbance of purified DNA by Nano Drop spectrophotometer (Nano Drop 2000 UV-Vis spectrophotometer, Thermo Fisher Scientific Inc., USA) was checked at 260 nm to determine the purity of DNA of bacterial isolates which was around 1.8. Ratios of less than 1.8 indicate that the preparation was contaminated, either with protein, RNA or with phenol.

A. Automatic nucleic acid purification system

B. Nano Drop spectrophotometer

Plate 2.6. Instruments for DNA extraction

2.13.3 Polymerase Chain Reaction (PCR)

Amplification of washed DNA samples were performed by Polymerase Chain Reaction (PCR)for further analysis.

a) Preparation of Reaction Mixture

The reaction mixture for PCR was prepared by mixing the specific volume of the components in an appropriate sized tube provided in the following table 2.1

- 12.5 µL of Hot Start Colorless Master Mix containing- dNTPs, Buffer, $MgCl_2$, Taq Polymerase (Cat: M7432, Origin: Promega, USA) without template DNA was prepared and aliquoted into PCR tubes.

- Specific template was added into a properly labeled PCR tube. The PCR tube containing reaction mixture and template DNA was capped properly followed by vortex and centrifugation briefly to mix the mixture gently and collect all components to the bottom of the tube respectively.
- The total mixture was then recollected, sealed and placed in a thermal cycler and the cycling was started immediately. PCR amplification was done in an oil-free thermal cycler (Applied Biosystems® 2720 Thermal Cycler).

Table 2.1. Components and Volume of Reaction Mixture for PCR (10 reactions)

Reaction components	Volume(µL)	Total volume(µL)
Master mix	12.5	12.5 × 10 = 125
Forward primer	1	1 × 10 = 10
Reverse primer	1	1 × 10 = 10
Template DNA	1	1 × 10 =10
Nuclease free water	9.5	9.5 × 10 = 95
Total Reaction volume	25	250

For the partial amplification of 16S rDNA gene the following primer pairs were used in PCR showed in table 2.2

Table 2.2. Primers and their sequence used in PCR

Primer	Type	Sequences
27F	Forward	5′- AGA GTT TGA TCM TGG CTC AG -3′
1492R	Reverse	5′- CGG TTA CCT TGT TAC GAC TT -3′

b) PCR conditions

The reaction mixtures containing PCR tubes were preheated at 95ºC for 5 minutes in the thermal cycler to ensure the denaturation of all DNA templates. The PCR reaction was then continued according to the following table 2.3

Table 2.3. Amplification cycle, temperature and required time for PCR

Number of Cycle	Step	Temperature	Time
1	Pre Heat	95°C	5 min
32 cycles	Denaturation	95°C	30 sec
	Annealing	48°C	30 sec
	Extension	72°C	1 min 30 sec
1	Final Extension	72°C	5 min
1	Hold	4°C	Overnight

After completion of cycling, the reactions were held at 4ºC.

c) Gel Electrophoresis of the amplified products and documentation

The successful amplification of the desired genes was visualized by resolving the PCR products in 1% agarose gel (w/v) depending on the size of amplicon.

- The gel was prepared using 0.32 g 1% Agarose powder (V3125, Promega, USA) and 40 ml 1X TAE buffer (V4251, Promega, USA).
- The mixture was heated in a hot plate and magnetic stirrer (VS- 130HS, Vision scientific Inc., LTD, Korea) for about 3 minutes to dissolve the agarose.
- The boiled mixture was allowed to cool to about 45°C and 2µL of Ethidium Bromide (H5041, Promega, USA) was added.
- The gel was poured onto gel casing preset with well former(comb) and allowed to set on a flat surface for about 15 minutes.
- After solidification of the gel, the comb was removed and buffer (1X TAE) was poured intotank to submerge the solidified gel.
- Samples were prepared by mixing 5µl PCR product with 1µl loading dye and each 6 µl prepared PCR product was loaded into the wells formed in the gel.
- Electrophoresis was conducted in 1X TAE buffer at 100 Volts for 60 mins.
- 1kb DNA ladder was also electrophoresed along the side of the amplified sample DNA.
- DNA bands were observed and photographed by AlphaImager MINI Gel - documentation system (ProteinSimple, USA).

A. Hot plate and magnetic stirrer

B. Centrifuge machine

Plate 2.7. Photograph of Hot plate and magnetic stirrerused for gel preparation (A) and Centrifuge machine used for PCR products purification (B)

2.14 Purification of PCR products

The PCR products were resolved by agarose gel electrophoresis to confirm successfulamplification of the desired sequence. The PCR products of specific genes were purifiedwith the Wizard PCR SV Gel and PCR Clean-Up System kit (Promega, USA) accordingto the manufacturer's instruction prior to sequencing. The steps of purification are givenbelow in brief:

a) Processing of PCR products

An equal volume of Membrane Binding Solution was added to the PCR amplification.Binding of DNA. A SV Minicolumn was inserted into Collection Tube.The prepared PCR product was transferred to the Minicolumn assembly and incubated at room temperature for 1 minute.The preparation was centrifuged at 16,000 × *g* for 1 minute using the centrifuge (Sigma, USA). The flow through was discarded and the Minicolumn was reinserted into Collection Tube.

c) Washing

700µL Membrane Wash Solution (ethanol added) was added into the Minicolumn and centrifuged at 16,000 × *g* for 1 minute. The flow-through was discarded and the Mini column was reinserted into Collection Tube.The previous step was repeated with 500µL Membrane Wash Solution and centrifuged at 16,000 × *g* for 5 minutes.The Collection Tube was emptied and the column assembly was re-centrifuged for 1 minute with the micro-centrifuge lid open (or off) to allow evaporation of any residual ethanol.

d) Elution

The Minicolumn was carefully transferred to a clean 1.5mL micro centrifuge tube.50µL Nuclease-Free Water was added to the Minicolumn and incubated at room temperature for 1 minute followed by centrifugation at 16,000 × *g* for 1 minute.The Minicolumn was discarded and DNA was stored at 4°C or –20°C.

2.15 16S rRNA sequencing

The PCR products were sent to First BASE Laboratories Sdn Bhd (Malaysia) where cycle sequencing was performed using BigDye® Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems®, USA) according to manufacturer's instruction and extension product was purified followed by capillary electrophoresis using ABI Genetic Analyzer (Applied Biosystems®, USA). Bidirectional (5′ to 3′ and 3′ to 5′) sequences were done for all the 10 representative isolates. The basic sequencing protocol involved amplification of target sequence by PCR and purification of desired amplicon followed by cycle sequencing reaction, cycle sequencing product purification and capillary electrophoresis.

2.16 Automated Sequencing and Bioinformatics analysis

The isolates were identified based on alignment of partial sequence of 16S rDNA gene with the existing sequences available in the database. In the present experiment 8 different samples were used to amplify their 16S rDNA and were preceded for further analysis. PCR amplified DNA of the isolates was gel purified using phenol freeze method and sent for automated sequencing. The sequence generated from automated sequencing of PCR products were analyzed through NCBI-BLAST database (http://blast.ncbi.nlm.nih.gov/) and rDNA BLAST

(http://bioinformatics.psb.ugent.be/cgi-bin/rDNA/blastform.cgi) programs to find out possible similar organism through alignment of homologous sequences.

2.16.1 Bioinformatics tools

Different bioinformatics tools were used to analyze the sequences. These tools are given below-

Finch TV version 1.4

To view DNA sequence Geospiza's Finch TV version 1.4 was used. And it leads the way with raw data views, BLAST searching and the ability to reverse complement sequences and traces.

Nucleotide BLAST

BLAST for Basic Local Alignment Search Tool was used for comparing primary sequence information. The statistical significance of matches was used to infer functionaland evolutionary relationships between sequences as well as helpsto identify members ofgene families.

CLUSTALW

CLUSTALW a program which was used for multiple sequence alignment (MSA). This helps to find out the similarity, dissimilarity or identity between different sequences.

Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 software

MEGAwas used for the comparative analysis of molecular sequence data was used for reconstructing the evolutionary histories of species and inferring the nature and extent of selective forces shaping the evolution of genes and species.

2.17Antibiotic susceptibility test (Hudzicki 2009)

The Kirby-Bauer disc diffusion test was done to determine the sensitivity or resistance of pathogenic bacteria for 14 antimicrobial compounds. The pathogenic bacteria were grown on Muller Hinton agar in the presence of 14 antimicrobial impregnated filter paper disks. The 14 antimicrobial-impregnated disks are represented in a table 2.4

Table 2.4. List of 14 different antibiotics used for Antibiogram and their potency.

Name of the Antibiotics	Disk Code	Potency
Erythromycin	E	15 µg
Nalidixic acid	NA	30 µg
Tetracycline	TE	30 µg
Polymyxin B	PB	300 µg
Nitrofurantoin	F	300 µg
Streptomycin	S	10 µg
Ciprofloxacine	CIP	5 µg
Amoxycillin	AML	10 µg
Amikacin	AK	30 µg
Gentamicin	CN	10 µg
Sulphamethoxazole	RL	25 µg
Chloramphenicol	C	30 µg
Ampicillin	AMP	10 µg
Kanamycin	K	30 µg

The steps of antibiotic susceptibility test is given below-

17 different isolates were grown in 5ml of Luria broth (LB) for overnight at 37º C.The Muller-Hinton agar plate was inoculated with the test organism by streaking the swab in a back and forth motion very close together as the plate was moved across down.The plate was rotated 60° and the action was repeated and the lid was slightly jarred allowing the plate to sit at room temperature for the surface of the agar plate to dry.Selected 14 different antibiotic discs were placed on the surface of the agar using sterilized forceps to dispense each disc at a time.The plates were placed inverted position at 37°C for 24 h.After 24 h, diameter of the zone of inhibition was measured using millimeter scale and the results were compared with the standard zones of inhibition for each antibiotic and the sensitivity, resistance or intermediary relationship of each of the selected bacteria was determined.

Plate 2.8. Required materials for antibiotic susceptibility test

2.18 Statistical Analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS) v. 20.0 for windows (SPSS, SAS Institute Inc. Cary, USA). The data were analyzed to determine the descriptive statistics such as Standard Deviation (SD), Statistic Mean, Minimum and Maximum value and Ranges of variables. One way and two way ANOVA was performed to test the significance using 5% level of significance. Microsoft excel (2013) was used to plot graph.

Chapter 3

Results

In the present study, Indigenous Rohu (*Labeo rohita*) and Invasive Silver carp (*Hypophthalmichthys molitrix*) examined microbiologically and biochemically. The molecular identification and characterization was also performed to ensure the knowledge about gastrointestinal pathogenic bacteria of Indigenous and Invasive carps.

3.1 Length and weight of the collected samples

For the current study, the range of length and weight of Rohu was 27.2 to 40.6 cm and 288.21 to 830.81 g. For Silver carp, they were 18.5 to 51.6 cm and 104.02 to 1303 g. Details of length and weight showed in table 3.1 (Appendix A).

3.2 Quantitative enumeration

3.2.1A Enumerated Total bacterial density (cfu/g) (mean ± SD) from gut sample of Rohu and Silver carp on Luria agar (LA) media

The average total bacterial density of Rohu samples on LA plate was found 5.27 ± 2.01× 10^7 cfu/g. (Table 3.2 shows in Appendix A).For Silver carp, enumerated average total bacterial load on LA plate was 3.02 ± 1.42 × 10^7 cfu/g. (Table 3.2 shows in Appendix A).

Total bacterial density of Rohu and Silver carp showed significant difference.

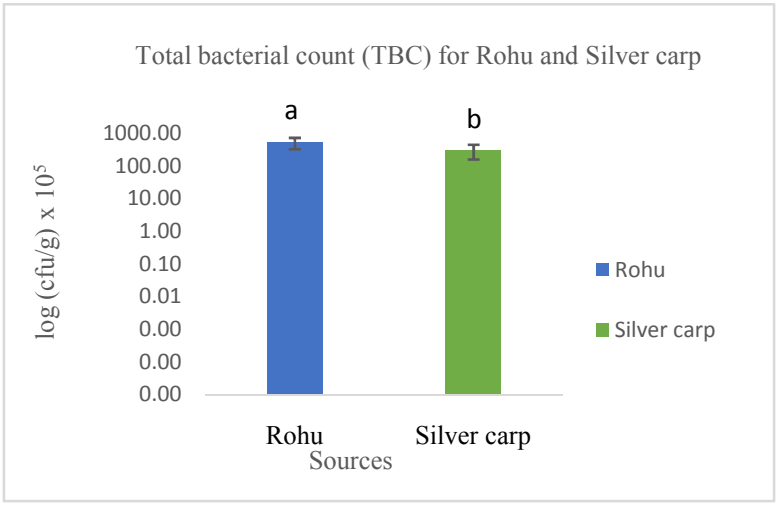


Fig 3.1A. Total Bacterial density displayed in logarithmic scale and counted in LA plate from the gut samples of the corresponding Rohu and Silver carp sample. Bars (mean ± SD) with letters represented significant difference (ANOVA, HSD; P<0.05).

3.2.1B Enumerated Total *Vibrio* count (cfu/g) (TVC)(mean ± SD) from gut sample of Rohu and Silver carp on TCBS media

On TCBS agar Plate, the enumerated average total *Vibrio* count from Rohu sample was 1.58 ± 3.51 × 10^6 cfu/g. However, from Silver carp, average total *Vibrio* density was found2.38 ± 3.63 × 10^3 cfu/g.

No significant difference was found between the total *Vibrio* count of Rohu and Silver carp. Total *Vibrio* count (cfu/g) (mean ± SD) of gut sample of Rohu and Silver carp on TCBS agar plate showed in table 3.2 (Appendix A).

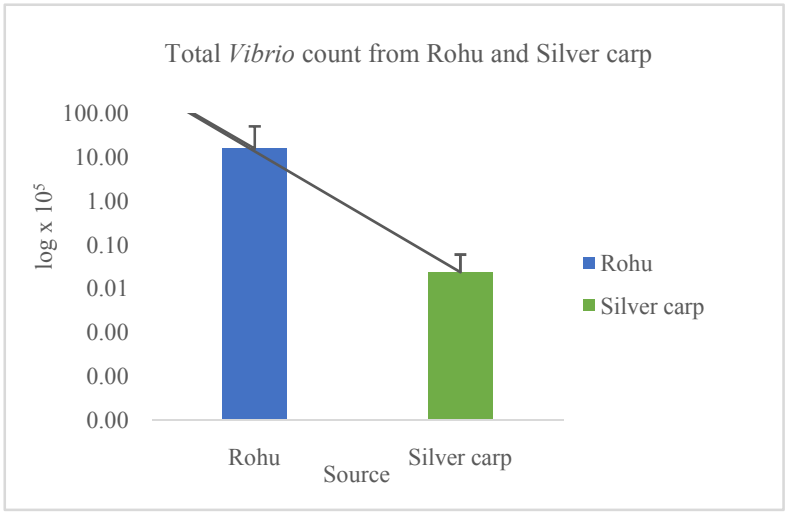


Fig 3.1B. Bacterial density displayed in logarithmic scale and counted in TCBS plate from the gut samples of the corresponding Rohu and Silver carp sample. Bars (mean ± SD) with no letters represented no significant difference (ANOVA, HSD; P<0.05).

3.2.1C Enumerated Total *Salmonella- Shigella* count (TSSC) (cfu/g) (mean ± SD) from gut sample of Rohu and Silver carp on *Salmonella* and *Shigella* agar (SS) media

On SS agar plate, counted average Total *Salmonella* and *Shigella* was 6.94 ± 7.15 × 10^6cfu/g from Rohu. Therefore,determined average total *Salmonella* and *Shigella* on SS agar plate was 1.11 ± 0.97 × 10^6 cfu/g from Silver carp samples. Similar to LA plates

total *Salmonella* and *Shigella* count showed significant difference between Rohu and Silver carp (table 3.2, Appendix A).

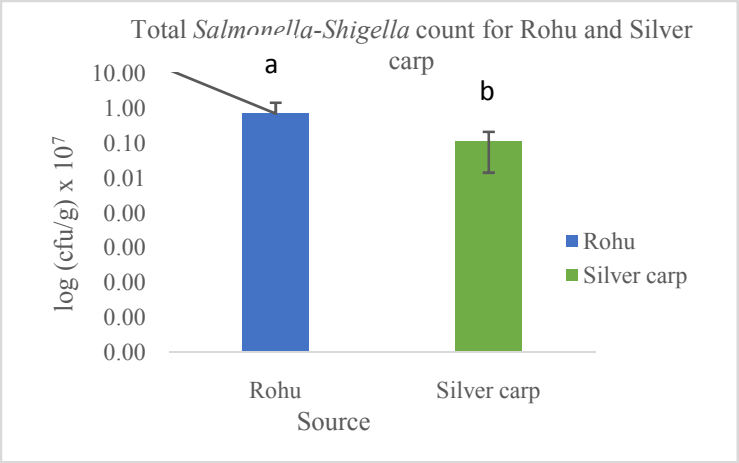


Figure 3.1C. Bacterial density displayed in logarithmic scale and counted in SS plate from the gut samples of the corresponding Rohu and Silver carp sample. Bars (mean ± SD) with different letters represented significant difference (ANOVA, HSD; P<0.05).

3.2.1D Enumerated Total *Aeromonas* count (TAC) (cfu/g) (mean ± SD) from gut sample of Rohu and Silver carp on *Aeromonas* agar (AA) media

In Rohu sample, averagetotal *Aeromonas* build up found on *Aeromonas* agar platewas 1.31 ± 1.06 × 10^7cfu/g. For Silver carp sample,average density was 6.09 ± 4.61 × 10^6 cfu/g.

On *Aeromonas* agar plate, bacterial density detected in Rohu and Silver carp were not showed significant difference (Table 3.2, Appendix A).

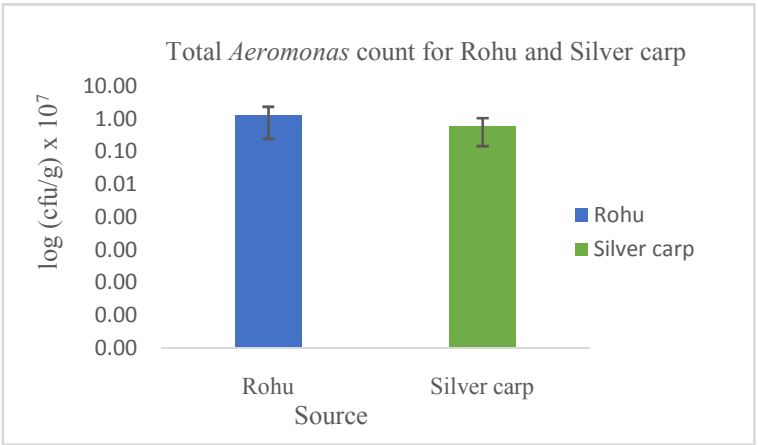


Fig 3.1D. Bacterial density displayed in logarithmic scale and counted in AA plate from the gut samples of the corresponding Rohu and Silver carp fish sample. Bars (mean ± SD) with no letters represented no significant difference (ANOVA, HSD; P<0.05).

3.2.1E Enumerated Total *Staphylococcal* count (TSC) (cfu/g) (mean ± SD) from gut sample of Rohu and Silver carp on MSAmedia

In Rohu,average total *Staphylococcal* count on Mannitol salt agar (MSA) plate, the bacterial load was 1.03 ± 0.52 × 10^7cfu/g. However, for Silver carp,average total Staphylococcal count on Mannitol salt agar (MSA) plate was5.48 ± 3.98 × 10^6 cfu/g. On MSA plate, significant difference was found on total *Staphylococcal* count between these 2 specimens.

Total *Staphylococcal* count(cfu/g) (mean ± SD) of gut sample of Rohu and Silver carp on Mannitol salt agar plate showed inTable 3.2 (Appendix A).

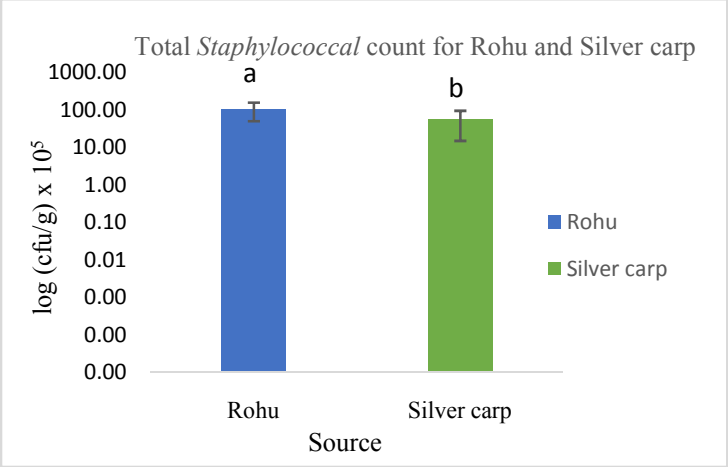


Fig. 3.1E. Bacterial density displayed in logarithmic scale and counted in MSA plate from the gut samples of the corresponding Rohu and Silver carp fish sample. Bars (mean ± SD) with different letters illustrated significant difference of bacterial load between Rohu and Silver carp (ANOVA, HSD; P<0.05).

3.2.1F Enumerated Total coliform count (cfu/g) (TCC)(mean ± SD) from gut sample of Rohu and Silver carp on EMBmedia

On EMB plate, For Rohu sample, average enteric bacteria with special reference to coliform count was 1.68 ± 0.981 × 10^7 cfu/g and in Silver carp was 1.39 ± 2.35 × 10^7cfu/g. No significant difference was observed on total coliform count between Rohu and Silver carp.

Table 3.2 shows (Appendix A) total coliform count(cfu/g)(mean ± SD) of gut sample of Rohu and Silver carp on EMB agar plate.

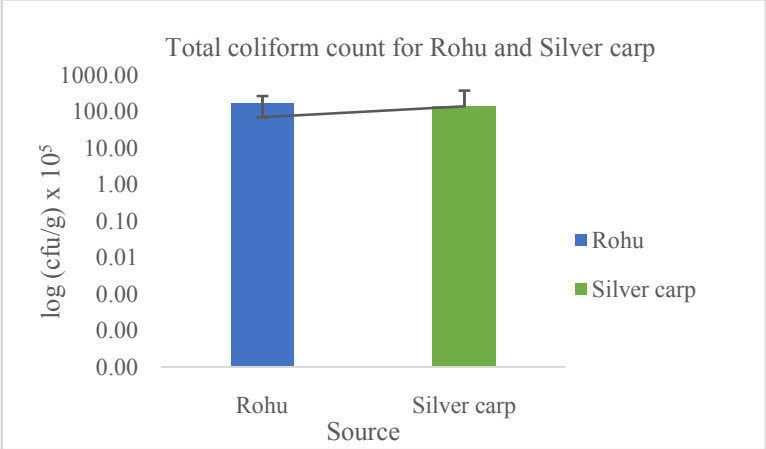


Fig 3.1F Bacterial density displayed in logarithmic scale and counted in EMB plate from the gut samples of the corresponding Rohu fish and Silver carp sample. Bars (mean ± SD) with no letters represented no significant difference (ANOVA, HSD; $P<0.05$).

3.2.2A Enumerated Total bacterial density (cfu/g) (mean ± SD) from gut sample of Rohu and Silver carp of 3 different markets of Dhaka city on LA media

The bacterial density of Rohu samples on LA plate ranged from $4.26 \pm 1.8 \times 10^7$ to $6.6 \pm 1.6 \times 10^7$ cfu/g. The highest count of TBC was sampled from Palashi Bazar and lowest was observed in Nobabgonj Bazar(Table 3.3 shows in Appendix A).

For Silver carp, enumerated total bacterial load on LA plate was $2.5 \pm 1.96 \times 10^7$ to $3.8 \pm .36 \times 10^7$ cfu/g. The highest count was found from Anando Bazar and lowest TBC count was $2.5 \pm 1.96 \times 10^7$ cfu/g sample in Nobabgonj Bazar.

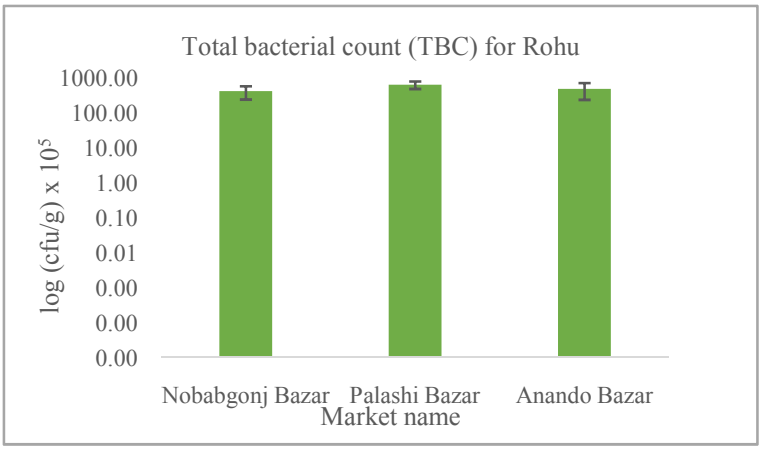


Fig 3.2A. Bacterial density displayed in logarithmic scale and counted in LA plate from the gut samples of the corresponding Rohu fish sample collected from Nobabgonj, Palashi and Anando bazar, Dhaka. Bars (mean ± SD) with no letters represented no significant difference (ANOVA, HSD; P<0.05).

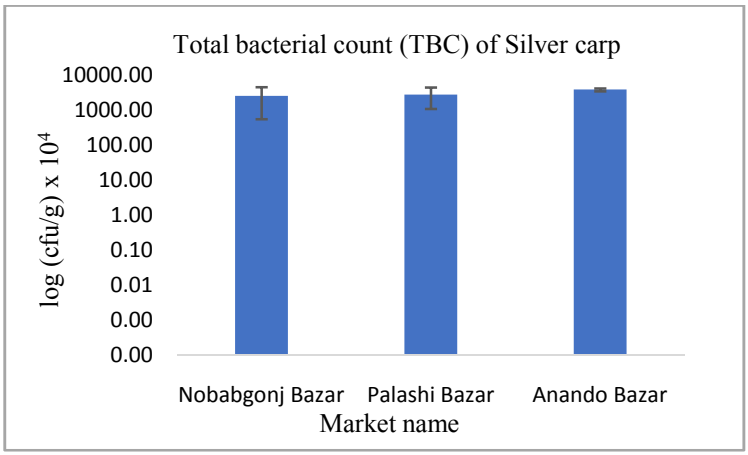


Fig 3.2B. Bacterial density displayed in logarithmic scale and counted in LA plate from the gut samples of the corresponding Silver carp fish sample collected from Nobabgonj, Palashi and Anando bazar, Dhaka. Bars (mean ± SD) with no letters represented no significant difference (ANOVA, HSD; P<0.05).

3.2.2B Enumerated Total *Vibrio* count (cfu/g) (mean ± SD) from gut sample of Rohu and Silver carp of 3 different markets of Dhaka city on TCBS media

The enumerated total *Vibrio* count from Rohu sample was ranged from $8.04 \pm 12.97 \times 10^4$ to $3.63 \pm 6.06 \times 10^6$ cfu/g. In this study, the highest count was $3.63 \pm 6.06 \times 10^6$ cfu/g (Anando Bazar) and lowest was $8.04 \pm 12.97 \times 10^4$ cfu/g (Nobabgonj Bazar).

On TCBS agar Plate, The counted total *Vibrio* density ranged between $2.63 \pm 1.24 \times 10^2$ to $4.97 \pm 3.36 \times 10^3$ cfu/g from Silver carp samples. The highest total *Vibrio* like colonies count was $4.97 \pm 3.36 \times 10^3$cfu/g (Anando Bazar) and lowest was $2.63 \pm 1.24 \times 10^2$ (Palashi Bazar).

The table 3.4 represents Total *Vibrio* count (cfu/g) (mean ± SD) of gut sample of Rohu and Silver carp from 3 different markets of Dhaka city on TCBS agar plate attached in Appendix A.

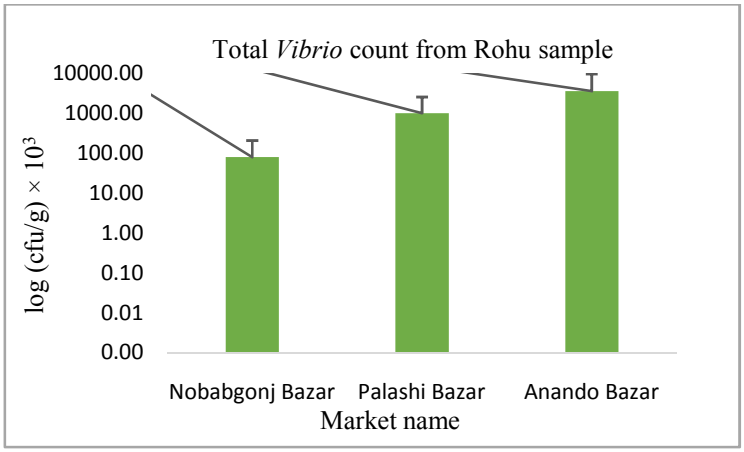


Fig 3.2C. Bacterial density displayed in logarithmic scale and counted in TCBS plate from the gut samples of the corresponding Rohu fish sample collected from Nobabgonj, Palashi and Anando bazar, Dhaka. Bars (mean ± SD) with no letters represented no significant difference (ANOVA, HSD; P<0.05).

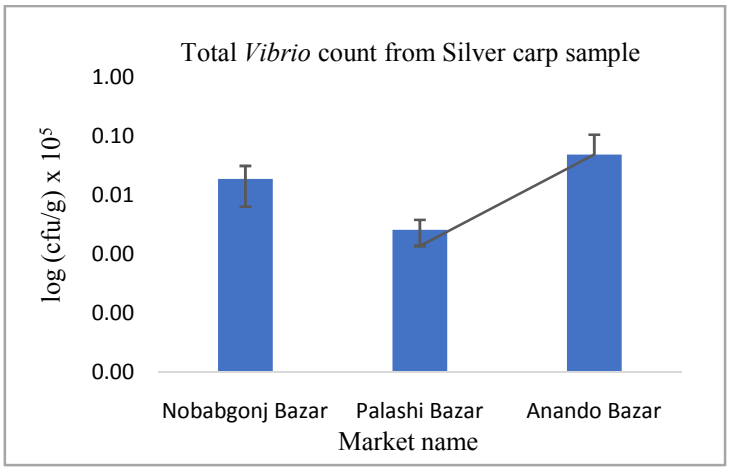


Figure 3.2D. Bacterial density displayed in logarithmic scale and counted in TCBS plate from the gut samples of the corresponding Silver carp fish sample collected from Nobabgonj, Palashi and Anando bazar, Dhaka. Bars (mean ± SD) with no letters represented no significant difference (ANOVA, HSD; P<0.05).

3.2.2C Enumerated Total *Salmonella- Shigella* count (cfu/g) (mean ± SD) from gut sample of Rohu and Silver carp of 3 different markets of Dhaka city on SS media

On SS agar plate, counted Total *Salmonella* and *Shigella* was 3.21 ± 3.0 × 10^6 to 1.2 ± 1.2 × 10^7cfu/g from Rohu. The highest *Salmonella-Shigella* count was found in 1.2 ± 1.2 × 10^7 cfu/g (Anando Bazar) and the counted lowest *Salmonella- Shigella* was 3.21 ± 3.0 × 10^6 cfu/g (Nobabgonj Bazar). Determined total *Salmonella* and *Shigella* on SS agar plate was 5.5 ± 3.3 × 10^5 to 1.69 ± 1.56 × 10^6 cfu/g from Silver carp samples. Among the three different markets, highest *Salmonella-Shigella* count was 1.69 ± 1.56 × 10^6 cfu/g collected from Palashi Bazar and lowest *Salmonella- Shigella* count was 5.5 ± 3.3 × 10^5 sampled from Anando Bazar.

The table 3.4 represents Total *Salmonella-Shigella* count (cfu/g) (mean ± SD) of gut sample of Rohu (*Labeo rohita*) and Silver carp (*Hypophthalmichthys molitrix*) from 3 different markets of Dhaka city on SS agar plate attached in Appendix A.

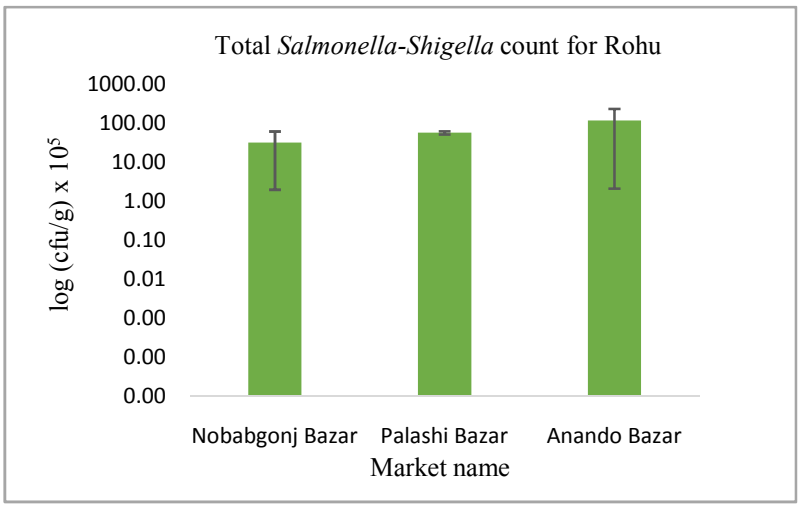


Fig 3.2E. Bacterial density displayed in logarithmic scale and counted in SS plate from the gut samples of the corresponding Rohu fish sample collected from Nobabgonj, Palashi and Anando bazar, Dhaka. Bars (mean ± SD) with no letters represented no significant difference (ANOVA, HSD; $P<0.05$).

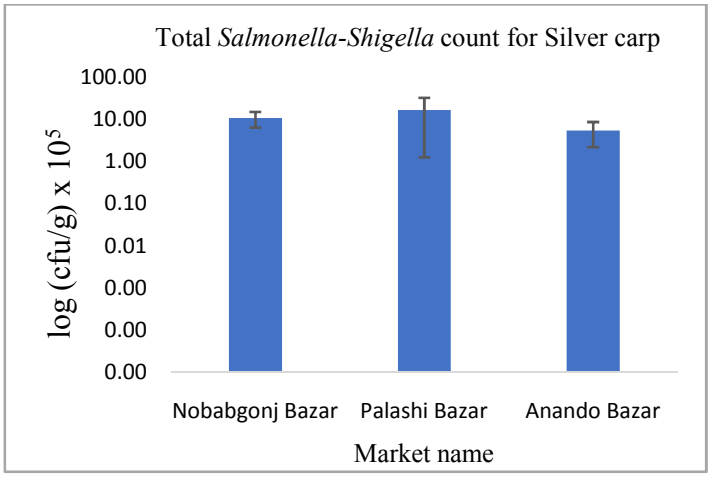


Fig 3.2F. Bacterial density displayed in logarithmic scale and counted in SS plate from the gut samples of the corresponding Silver carp fish sample collected from Nobabgonj, Palashi and Anando bazar, Dhaka. Bars (mean ± SD) with no letters represented no significant difference (ANOVA, HSD; $P<0.05$).

3.2.2D Enumerated Total *Aeromonas* count (cfu/g) (mean ± SD) from gut sample of Rohu and Silver carp of 3 different markets of Dhaka city on *Aeromonas* agar media

In Rohu sample, total *Aeromonas* build up found between $6.89 \pm 7.03 \times 10^6$ to $2.09 \pm 0.99 \times 10^7$ cfu/g. Among the three different markets, highest *Aeromonas* count was $2.09 \pm 0.99 \times 10^7$ cfu/g sampled from Palashi Bazar and lowest count was $6.89 \pm 7.03 \times 10^6$cfu/g collected from Nobabgonj Bazar.

For Silver carp sample, Total *Aeromonas* density was $4.01 \pm 3.6 \times 10^6$ to $9.96 \pm 4.2 \times 10^6$ cfu/g. among the three different markets, highest *Aeromonas* count was $9.96 \pm 4.2 \times 10^6$ cfu/g found from Anando Bazar and lowest count was $4.01 \pm 3.6 \times 10^6$ cfu/gcollected from Palashi Bazar.

Table 3.6 Total *Aeromonas* count (cfu/g) (mean ± SD) of gut sample of Rohu and Silver carp from 3 different markets of Dhaka city on *Aeromonas* agar plate (Appendix A)

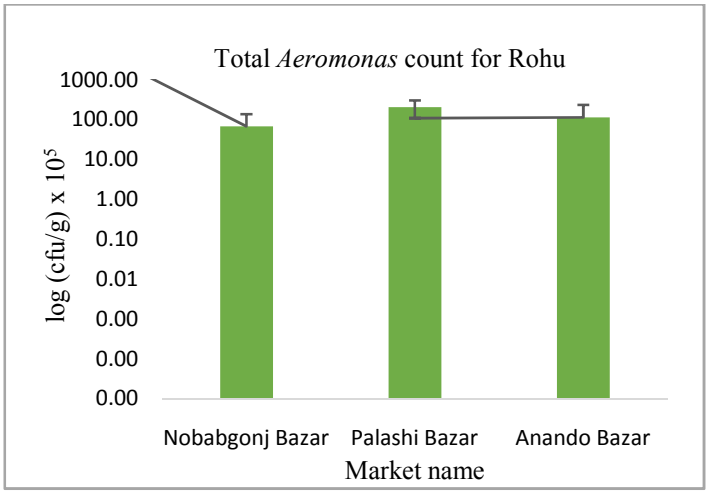


Fig 3.2.G. Bacterial density displayed in logarithmic scale and counted in AA plate from the gut samples of the corresponding Rohu fish sample collected from Nobabgonj, Palashi and Anando bazar, Dhaka. Bars (mean ± SD) with no letters represented no significant difference (ANOVA, HSD; P<0.05).

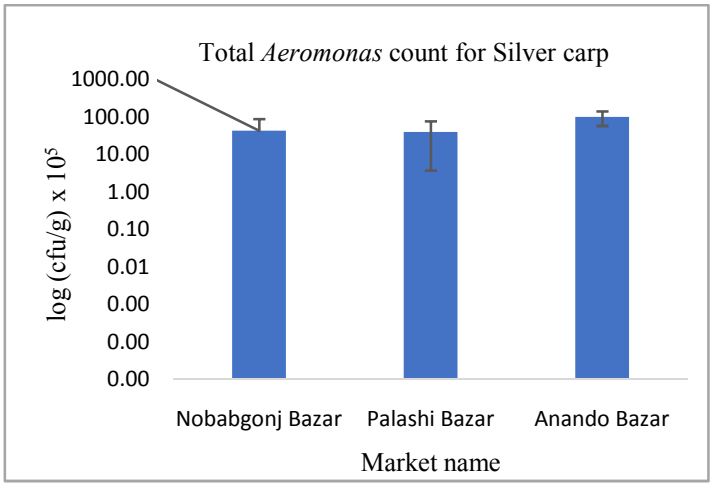


Fig 3.2H. Bacterial density displayed in logarithmic scale and counted in AA plate from the gut samples of the corresponding Silver carp fish sample collected from Nobabgonj, Palashi and Anando bazar, Dhaka. Bars (mean ± SD) with no letters represented no significant difference (ANOVA, HSD; P<0.05).

3.2.2E Enumerated Total *Staphylococcal* count (cfu/g) (mean ± SD) from gut sample of Rohu and Silver carp of 3 different markets of Dhaka city on MSAmedia

To assess total *Staphylococcal* count on Mannitol salt agar plate, the bacterial load ranged from $9.2 \pm 7.4 \times 10^6$ to $1.23 \pm 0.38 \times 10^7$ cfu/g. In the present study, highest Total *Staphylococcal* count was $1.23 \pm 0.38 \times 10^7$ cfu/g found from Rohu sample of Anando

Bazar and lowest count was 9.2 ± 7.4 × 10^6 cfu/g, sample collected from Nobabgonj Bazar.

For Silver carp, small density difference ranged from 5.4 ± 4.4 × 10^6 to 5.5 ± 4.4 × 10^6 cfu/g, found on total *Staphylococcal* count. In the present study, highest total *Staphylococcal* count was 5.5 ± 4.4 × 10^6 cfu/g found from Nobabgonj Bazar Silver carp sample and lowest count was 5.4 ± 4.4 × 10^6 found fromsamples collected from Palashi Bazar.

Total *Staphylococcal* count(cfu/g) (mean ± SD) of gut sample of Rohu and Silver carp from 3 different markets of Dhaka city on Mannitol salt agar plate is shown in Table3.7(Appendix A).

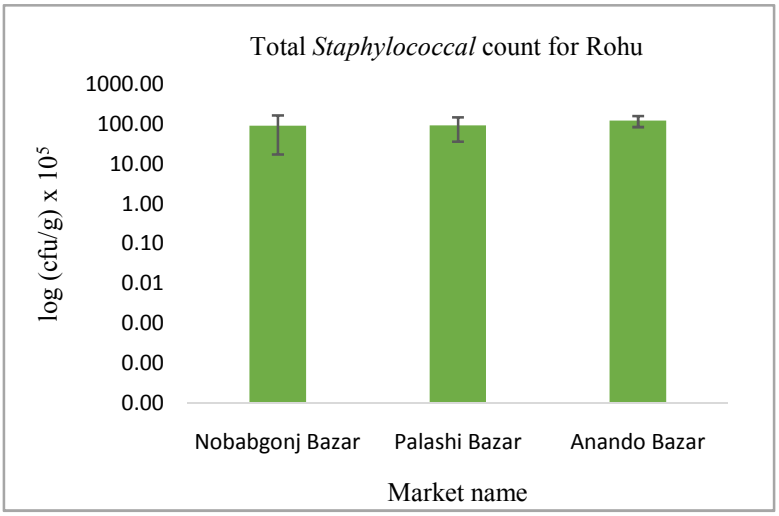


Fig 3.2I. Bacterial density displayed in logarithmic scale and counted in MSA plate from the gut samples of the corresponding Rohu fish sample collected from Nobabgonj, Palashi and Anando bazar, Dhaka. Bars (mean ± SD) with no letters represented no significant difference (ANOVA, HSD; $P<0.05$).

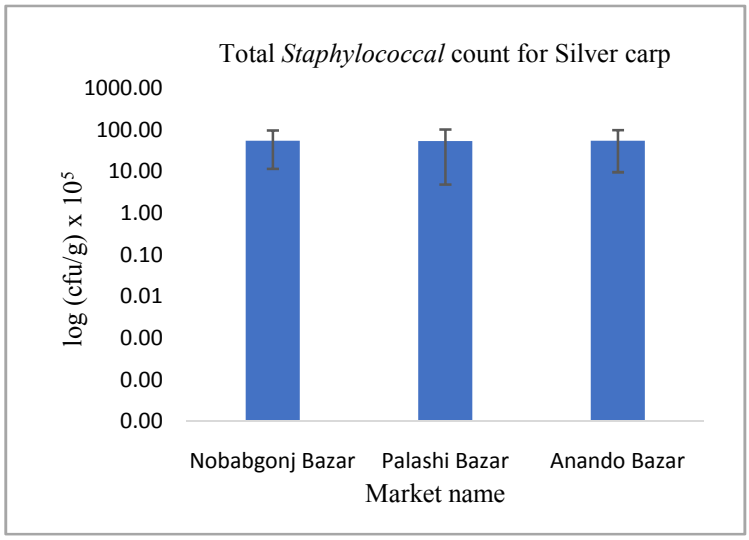


Fig 3.2J. Bacterial density displayed in logarithmic scale and counted in MSA plate from the gut samples of the corresponding Silver carp fish sample collected from Nobabgonj, Palashi and Anando bazar, Dhaka. Bars (mean ± SD) with no letters represented no significant difference (ANOVA, HSD; $P<0.05$).

3.2.2F Enumerated Total coliform count (cfu/g) (mean ± SD) from gut sample of Rohu and Silver carp of 3 different markets of Dhaka city on EMBmedia

For Rohu sample, Enteric bacteria with special reference to coliform count on EMB agar plate ranged between $1.5 \pm 0.71 \times 10^7$ to $1.98 \pm 1.7 \times 10^7$ cfu/g. In the present study, highest total coliform count was $1.98 \pm 1.7 \times 10^7$ cfu/g measured from samples of Palashi Bazar and lowest count was $1.5 \pm 0.71 \times 10^7$ cfu/g estimated from samples of Anando Bazar.

Enteric and related bacterial count for Silver carp on EMB agar plate was $4.53 \pm 2.85 \times 10^6$ to $3.1 \pm 3.9 \times 10^7$ cfu/g. In the present study, highest total coliform count was $3.1 \pm 3.9 \times 10^7$ cfu/g found from Silver carp sample of Nobabgonj Bazar and lowest count was $4.53 \pm 2.85 \times 10^6$ cfu/g investigated from samples of Anando Bazar.

Table 3.8 Total coliform count(cfu/g)(mean ± SD) of gut sample of Rohu and Silver carp from 3 different markets of Dhaka city on EMB agar plate (Appendix A).

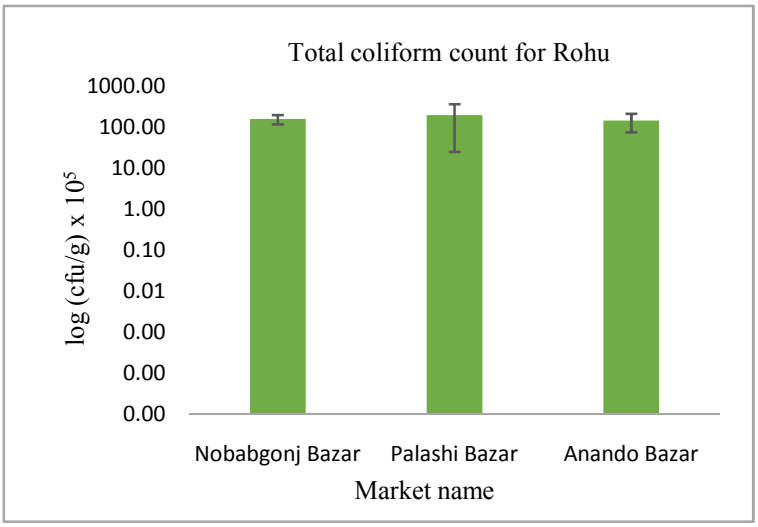


Fig 3.2K. Bacterial density displayed in logarithmic scale and counted in EMB plate from the gut samples of the corresponding Rohu fish sample collected from Nobabgonj, Palashi and Anando bazar, Dhaka. Bars (mean ± SD) with no letters represented no significant difference (ANOVA, HSD; $P<0.05$).

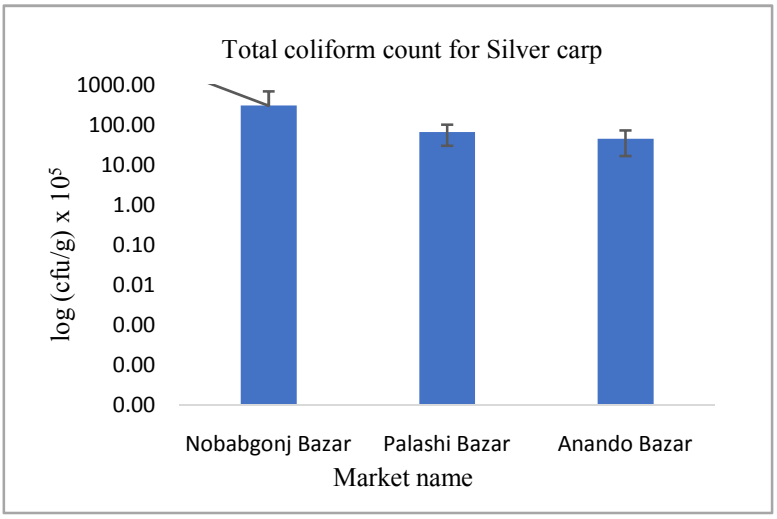
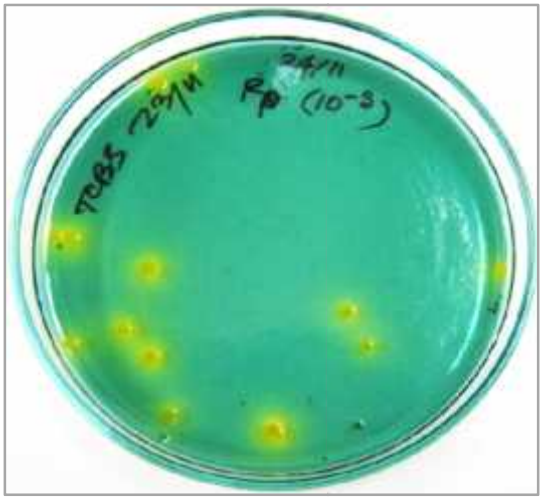


Fig 3.2L. Bacterial density displayed in logarithmic scale and counted in EMB plate from the gut samples of the corresponding Silver carp fish sample collected from Nobabgonj, Palashi and Anando bazar, Dhaka. Bars (mean ± SD) with no letters represented no significant difference (ANOVA, HSD; $P<0.05$).

3.3 Isolation and purification of the selected bacterial isolates

In the present study, a total of 216 colonies were primarily selected from different selective medium. 216 colonies were purified and among them 18 isolates were selected and purified for detail study towards identification. These 18 colonies were selected on the basis of different morphology of the colonies and from different selective media plate. Out of these 18 isolates 3 were Gram positive bacteria and remaining 15 were Gram negative bacteria. Bacterial colonies developed on the spread plate and streak plate techniques were shown in Plate 3.1, 3.2 (A, B).

A. Luria Agar Plate



B. TCBS Agar Plate

C. SS Agar Plate

D. Aeromonas Agar Plate

E. Mannitol Salt Agar Plate

F. EMB Agar Plate

Plate 3.1. Photographs showing bacterial colonies in different selective media

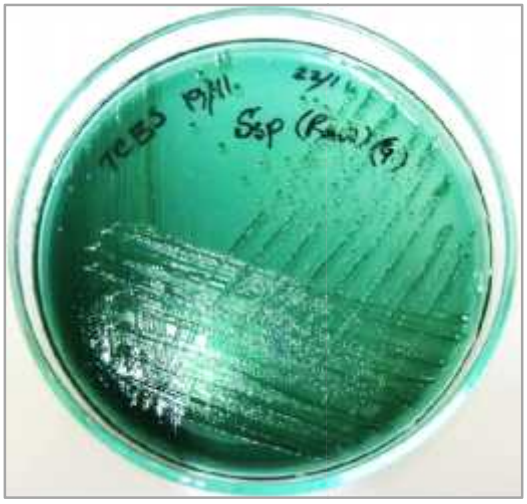
3.4 Colonial morphology of the selected isolates

Colonies of the selected isolates were found to be different in their form, elevation, margin, surface and color. The dissimilarities of colonial morphology of the selected 18 isolates were presented in Table 3.10. Most of the colonies of the isolated bacteria were entire, round and smooth in nature.

Table 3.9. Colonial morphology of the 18 selected isolates

Isolate number	Size	Shape	Pigmentation	Elevation	Margin	Surface
Rp´1/2	Small	Round	Yellow	Convex	Entire	Smooth
RA´2/1	Medium	Round	Colorless	Convex	Entire	Smooth
RN´´2/5	Small	Round	Colorless with black Centre	Convex	Entire	Smooth
RN 3/1	Small	Round	Colorless	Convex	Entire	Smooth
RN´3/2	Small	Irregular	Pink	Flat	Not entire	Smooth
Rp´4/1	Small	Irregular	Colorless	Flat	Entire	Smooth
RA 4/2	Small	Round	Pink	Convex	Entire	Smooth
RN´ 5/2	Small	Round	Green metallic sheen	Convex	Entire	Smooth
Rp´´5/5	Small	Round	Pink creamy	Convex	Entire	Smooth
Sp´1/4	Small	Round	Green	Convex	Entire	Smooth
SA´2/3	Small	Round	Black with pink	Convex	Entire	Smooth
Sp´2/12	Medium	Round	Colorless	Convex	Entire	Smooth
SA´´3/3	Small	Irregular	Pink	Flat	Not entire	Smooth
SA´3/4	Small	Round	Colorless	Convex	Entire	Smooth
SA´4/1	Small	Irregular	Colorless	Flat	Entire	Smooth
SA´4/2	Small	Round	Pink	Convex	Entire	Smooth
SA´´5/3	Small	Round	Green metallic sheen	Convex	Entire	Smooth
SA´5/4	Small	Round	Creamy Pink	Convex	Entire	Smooth

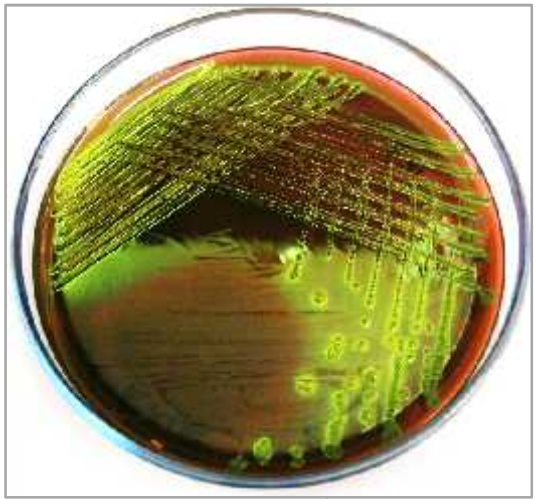
A. TCBS Agar Plate (Yellow colony)



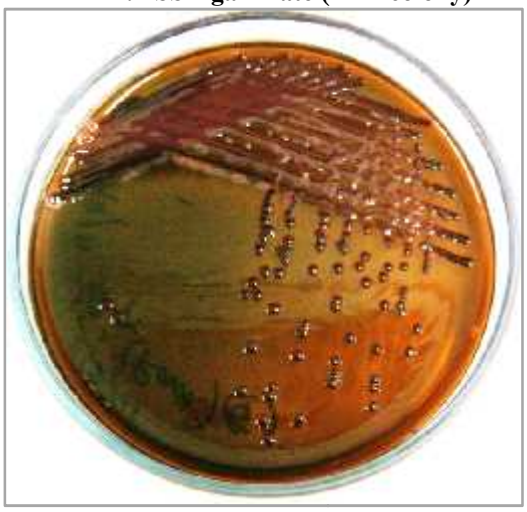
B. TCBS Agar Plate (Green colony)

C. SS Agar Plate (Colorless colony)

D. SS Agar Plate (Pink colony)

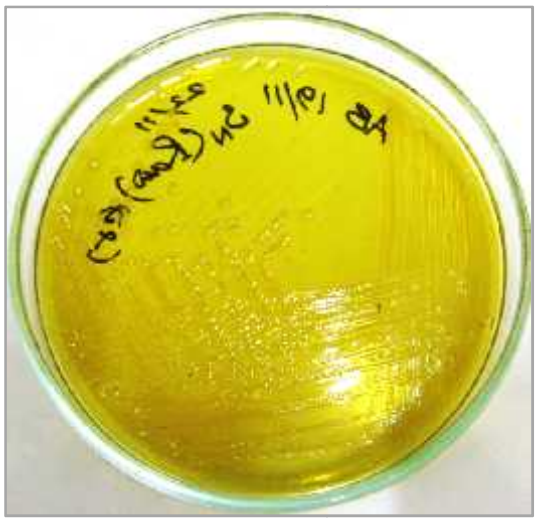


E. EMB Agar Plate (Metallic green colony)



F. EMB Agar Plate (Creamy pink colony)

Plate 3.2A. Purification of selected isolates by streak plate technique



A. Aeromonas Agar Plate (Colorless colony)

B. Aeromonas Agar Plate (Purple colony)

C. Mannitol Salt Agar Plate (Pink colony)

D. Mannitol Salt Agar Plate (Colorless colony)

E. MacConkey Agar Plate (Colorless colony)

F. MacConkey Agar Plate (Pink colony)

Plate 3.2B. Purification of selected isolates by streak plate technique

3.5 Morphological characteristics and Gram reaction of bacterial isolates

3.5.1 Gram staining of bacterial isolates

In this current study both gram-positive (cells have a thick peptidoglycan layer and stained purple). Andgram-negative (cells have a thin peptidoglycan layer and stained pink) bacteria was found (Plate 3.3). 3 gram positive bacterial isolates was found among 18 isolates.

Table 3.10. Gram reaction of bacterial isolates of Rohu and Silver carp

Bacterial isolates of *Labeo rohita*	Gram reaction	Bacterial isolates of *Hypophthalmichthys molitrix*	Gram reaction
Rp´1/2	-	Sp´1/4	-
RA´2/1	-	SA´2/3	-
RN´´2/5	-	Sp´2/12	-
RN 3/1	-	SA´´3/3	-
RN´3/2	-	SA´3/4	-
Rp´4/1	+	SA´4/1	+
RA 4/2	-	SA´4/2	+
RN´ 5/2	-	SA´´5/3	-
Rp´´5/5	-	SA´5/4	-

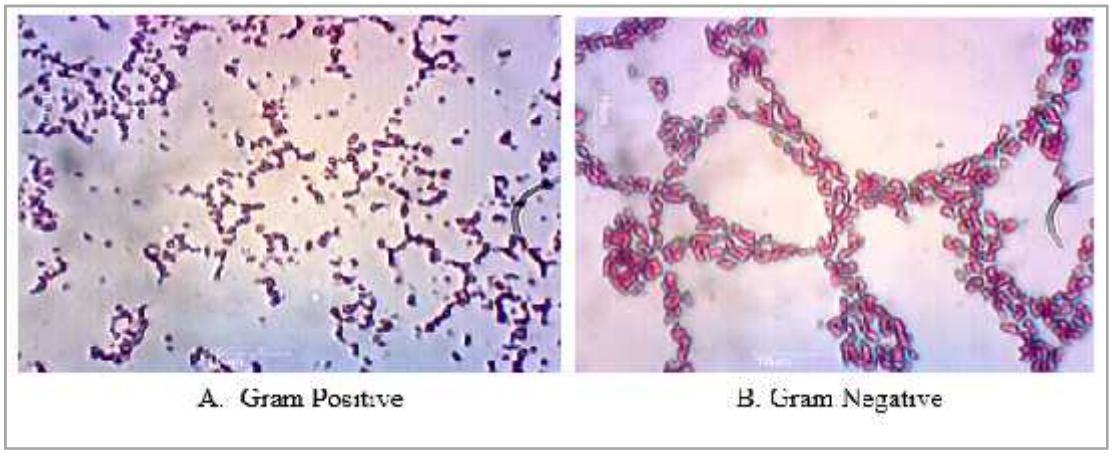


Plate 3.3. Gram staining of Bacterial isolates

3.6 Physiological and biochemical characteristics of bacterial isolates

Different physiological and biochemical tests were exhibited here in Table 3.11 and 3.12. The results are shown here individually for Rohu and Silver carp isolates.

3.6.1 Physiological and biochemical characteristics of bacterial isolates of Rohu (*Labeo rohita*)

9 selected isolates of Rohu samples were studied for their biochemical characteristics. The results of biochemical tests are presented in (Table 3.11). Among the 9 isolates, all isolates were non motile except 1 (R_N 3/1) isolate was motile. However, 3 isolates were Catalase negative whereas 4 isolates (R_A´2/1, R_N 3/1, R_N´3/2 and Rp´4/1) were Oxidase positive. In Gelatin hydrolysis test only 2 isolates (R_N 3/1, Rp´´5/5) were shown negative result. All the isolates 3 (R_A´2/1, Rp´4/1, Rp´´5/5) shown Indole positive test. 4 isolates shown MR negative test and 6 isolates shown VP positive test. 2 isolates (R_A´2/1, R_N´3/2) could utilize Citrate whereas 3 of the selected isolates (R_A´2/1, R_N 3/1 and R_N´ 5/2) could hydrolyze Urease.

For the Starch Hydrolysis, 4 isolates showed positive result whereas 5 isolates showed negative results.

All the tested isolates 3 were fermented Glucose but 2 isolates couldn't ferment Glucose, Lactose and Sucrose. All the isolates produced Gas. Among the isolates, 3 isolates formed H_2S. Plate 3.4 and 3.5 showed different physiological and biochemical tests.

Table 3.11. Physiological and biochemical characteristics of the *Labeo rohita* isolates

Bacterial isolates	Motility test	Catalase test	Oxidase test	Gelatin test	Indole test	Methyl red test	Voges-Proskauer test	Citrate test	Urease test	Starch hydrolysis test	Triple Sugar Iron test				Provisional identification
											Butt	Slant	Gas	H_2S	
Rp´1/2	-	-	-	+	-	+	+	-	+	-	A	A	+	-	*Vibrio metschnikovil*
R_A´2/1	-	-	+	+	+	+	+	+	-	+	A	K	+	-	*Aeromonas sp.*
R_N´´2/5	-	+	-	+	-	+	-	-	+	-	A	A	+	+	*Proteus mirabilis*
R_N 3/1	+	+	+	-	-	-	-	-	-	+	K	K	+	-	*Pseudomonas citronellolis*
R_N´3/2	-	+	+	+	-	-	-	+	+	+	K	K	+	+	*Aeromonas salmonicida*
Rp´4/1	-	-	+	+	+	+	+	-	+	-	A	K	+	-	*Staphylococcus caprae*
R_A 4/2	-	+	-	+	-	-	+	-	+	-	A	A	+	-	*Pseudomonas sp.*
R_N´ 5/2	-	+	-	+	-	+	+	-	-	-	A	A	+	-	*Enterobacter nimipressuralis*
Rp´´5/5	-	+	-	-	+	-	+	-	+	+	A	A	+	+	*Klebsiella oxytoca*

'+' sign indicates catalase, oxidase, Voges- Proskauer test positive; Methyl red produced; Indole produced; Gelatin, Urease and Starch hydrolyzed; Citrate utilized; H_2S and gas produced and motile.

'-' sign indicates catalase, oxidase, Voges- Proskauer test negative; Methyl red and Indole not produced; Gelatin, Urease and Starch un hydrolyzed; Citrate unutilized; H_2S and gas not produced and non-motile.

A=Acid (yellow), K= Alkaline (red) reaction

3.6.2 Physiological and biochemical characteristics of bacterial isolates of Silver carp (*Hypophthalmichthys molitrix*)

9 selected isolates of Silver carp samples were studied further for their biochemical characteristics. The results of biochemical tests are presented in Table 3.12. Among the 9 isolates, 4 isolates were non motile and 5 isolates were motile. However, 3 isolates were Catalase negative whereas 4 isolates (S_P´1/4, S_P´2/12, S_A´3/4 and S_A´4/2) were Oxidase positive. In Gelatin hydrolysis test only 1 isolate (S_A´2/3) was shown negative result.

Out of all the isolates 4 (S_P´2/12, S_A´3/4, S_A´4/2 and S_A´´5/3) shown Indole positive test. 2 isolates shown MR negative test and 4 isolates shown VP positive test. 2 isolates (S_P´1/4, S_A´5/4) could utilize Citrate whereas 6 of the selected isolates (S_P´1/4, S_A´2/3, S_P´2/12, S_A´3/4, S_A´4/1 and S_A´5/4) could hydrolyze Urease.

For the Starch Hydrolysis, 4 isolates showed positive result whereas 5 isolates showed negative results.

All of the tested isolates 3 were fermented Glucose but 2 isolates couldn't ferment Glucose, Lactose and Sucrose. All the isolates produced Gas except S_P´2/12. Among the isolates, 3 isolates produced H_2S as black color in slant or butt. Plate 3.5 and 3.6 shows different physiological and biochemical test.

Table 3.12. Physiological and biochemical characteristics of the *Hypophthalmichthys molitrix*isolates

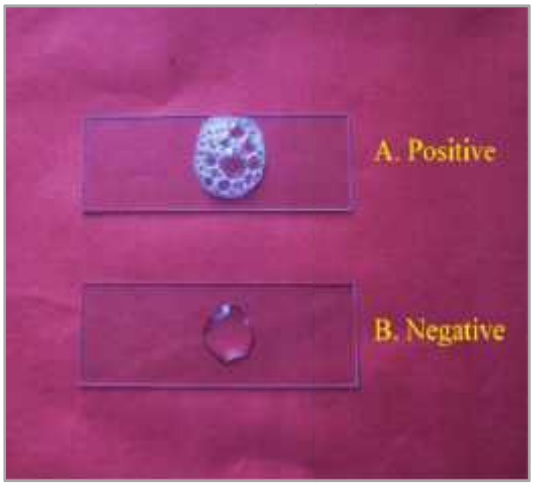
Bacterial isolates	Motility test	Catalase test	Oxidase test	Gelatin test	Indole test	Methyl red test	Voges-Proskaur test	Citrate test	Urease test	Starch hydrolysis test	Triple Sugar Iron Test				Provisional identification
											Butt	Slant	Gas	H_2S	
S_P´1/4	+	-	+	+	-	+	-	+	+	+	A	K	+	-	*Vibrio furnissii*
S_A´2/3	+	+	-	-	-	+	-	-	+	-	A	K	+	+	*Salmonella sp.*
S_P´2/12	+	-	+	+	+	+	+	-	+	+	K	K	-	-	*Aeromonas sp.*
S_A´´3/3	+	+	-	+	-	+	-	-	-	-	K	K	+	+	*Pseudomonas graminis*
S_A´3/4	-	+	+	+	+	+	+	-	+	+	A	A	+	-	*Aeromonas salmonicida*
S_A´4/1	-	-	-	+	-	-	+	-	+	+	A	A	+	-	*Staphylococcus aureus*
S_A´4/2	+	+	+	+	+	+	-	-	-	-	A	K	+	+	*Staphylococcus carnosus*
S_A´´5/3	-	+	-	+	+	+	-	-	-	-	A	A	+	-	*Escherichia coli*
S_A´5/4	-	+	-	+	-	-	+	+	+	-	A	A	+	-	*Klebsiella pneumoniae*

'+' sign indicates catalase, oxidase, Voges- Proskauer test positive; Methyl red produced; Indole produced; Gelatin, Urease and Starch hydrolyzed; Citrate utilized; H_2S and gas produced and motile.

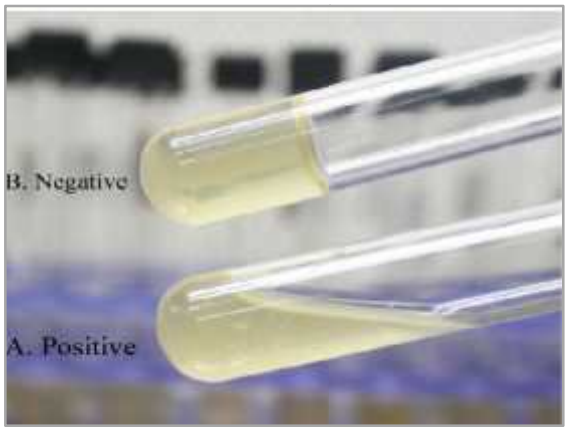
'-' sign indicates catalase, oxidase, Voges- Proskauer test negative; Methyl red and Indole not produced; Gelatin, Urease and Starch unhydrolyzed; Citrate unutilized; H_2S and gas not produced and non-motile.

A= Acid (yellow), K= Alkaline (red) reaction

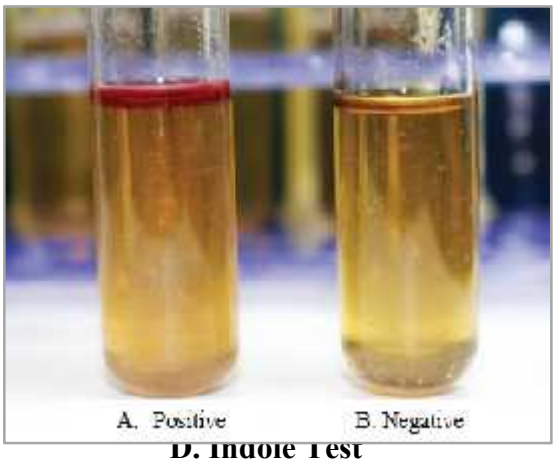
A. Motility Test



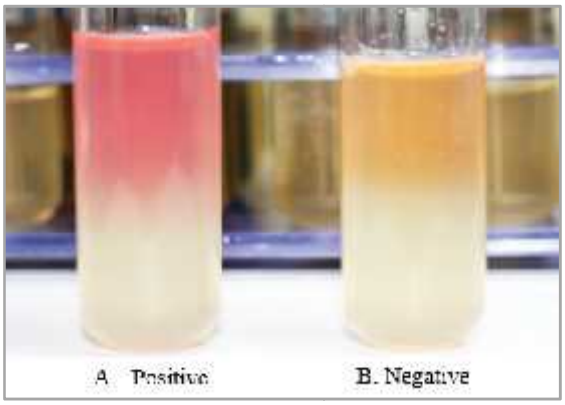
B. Catalase test (ref. image)



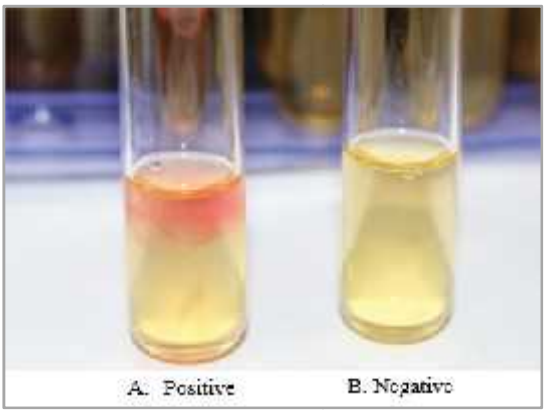
C. Gelatin Test



D. Indole Test



C. Methyl red Test



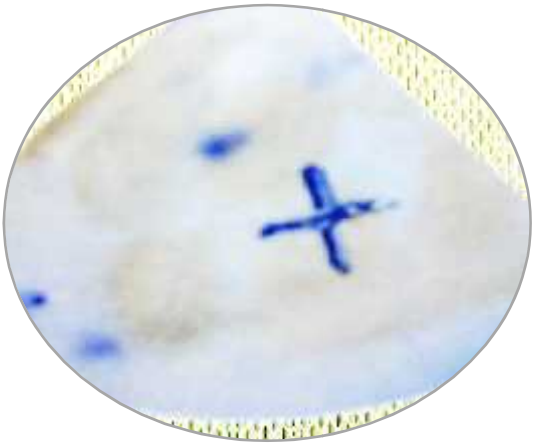
D. Voges-Proskauer Test

Plate 3.4. Photographs of different biochemical tests

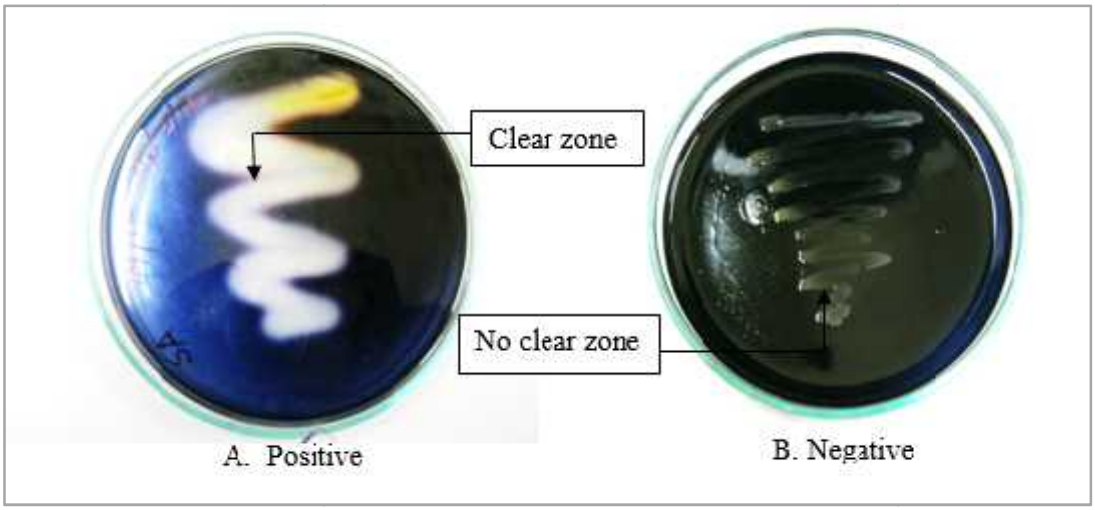
E. Citrate Test

F. Urease Test

G. Triple sugar Test



H. Oxidase Test



I. Starch Hydrolysis Test

Plate 3.5. Photographs (A-J) of different biochemical tests

3.7 Growth kinetics of different bacterial isolates

Growth curves of different bacterial isolates of Rohu were studied to know the growth performance of these bacterial isolates at 37°C temperature up to 95 hour. Result showed that the growth was increased up to 20 hr. This indicates that isolates were in that time in log phase. Although after that time period the growth rate of bacterial isolates was slower than the log phase. This indicates the stationary stage or phase of these particular bacterial isolates. After 48 hr isolates shows the gradual fall like death phase. The following curves represent the growth kinetics of 3 different Rohu samples.(Fig 3.3A) (Table 3.13, Appendix A).

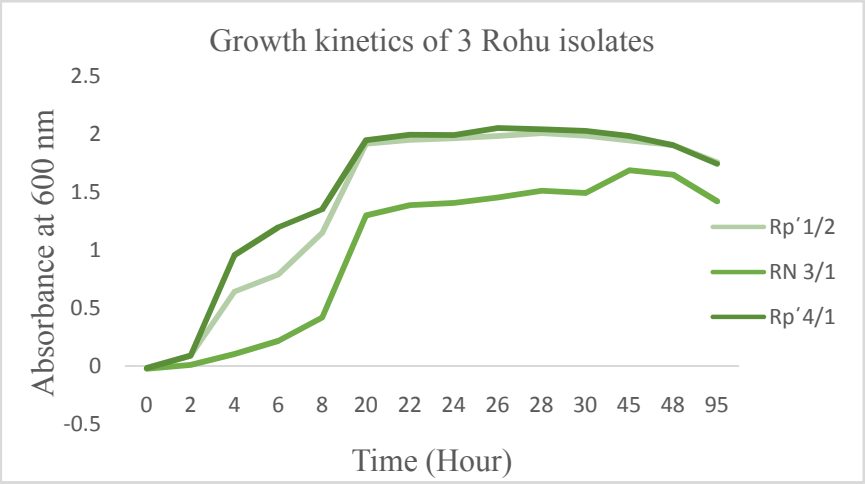


Fig 3.3A. Growth kinetics of bacterial isolates from Rohu sample at absorbance 600 nm under different time period (Rp´1/2, RN 3/1, Rp´4/1 indicates 3 isolates of Rohu).

Growth curves of 3 different bacterial isolates of Silver carp were studied to know the growth performance of these bacterial isolates at 37°C temperature up to 95 hour. Result showed that the growth was increased up to 20 hr. This indicates that isolates were in that time in log phase. Although after that time period the growth rate of bacterial isolates was slower than the log phase. This indicates the stationary stage or phase of these particular bacterial isolates. After 48 hr isolates shows the gradual fall like death phase. The following curves represent the growth kinetics of 3 different Silver carp samples.(Fig 3.3B) (Table 3.13, Appendix A).

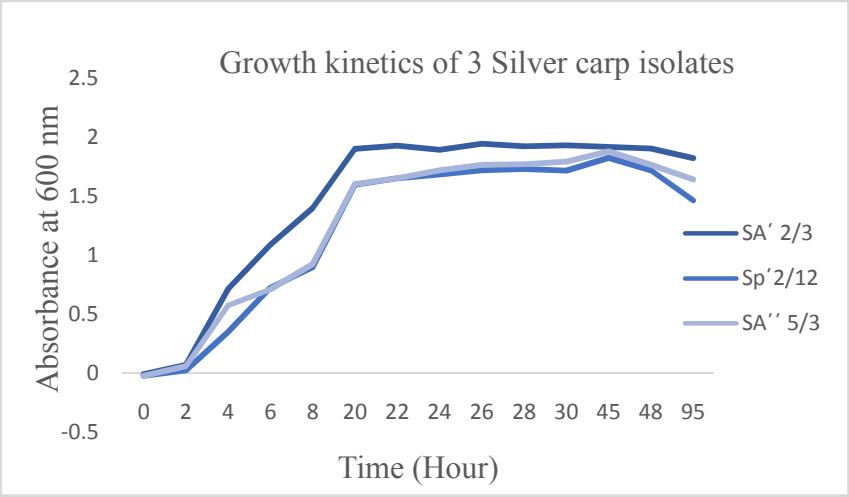


Fig 3.3B Growth performance of bacterial isolates from Silver carp sample at absorbance 600 nm under different time period.(SA′2/3, Sp′2/12, SA′′5/3 indicates 3 isolates of Silver carp).

3.8 Molecular Identification of bacterial isolates

The final concentration and purity of the 10 groups measured before 16S rRNA sequencing with their corresponding colony ID and sample ID are summarized in Table 3.14 (Appendix A). The gel autoradiograph (Plate 3.6) shows that the size of the PCR product was 1.5 kb.

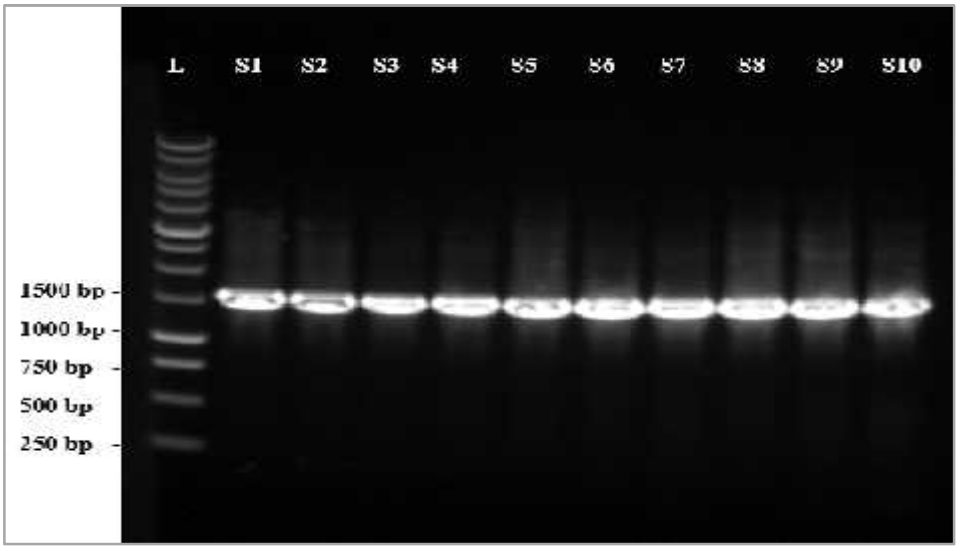


Plate 3.6. PCR Product profiles of 16S rDNA primer generated from 10 different Samples: S1, S2, S3, S4, S5, S6, S7, S8, S9, S10 and L denotes DNA ladder, 1kb (Marker).

3.8.1 16S rRNA sequence based identification

The identification of the 16S rDNA gene sequences of ten representative isolates (S1, S2, S3, S4, S5, S6, S7, S8, S9 and S10) of 10 groups (njp1, njp2, njp3, njp4, njp5, njp6, njp7, njp8, njp9, njp10) through nucleotide BLAST of NCBI is summarized in table 3.15 and 3.16.

It is clear that, from table 3.17, the sequences of all the groups of isolates were matched with the strains presented in their respective following column. The table also provides information about scoring *viz*. maximum score, total score, percentage of query cover, E value and percentage of identification of the identified sequences with NCBI Gene Bank Accession number of the matched sequences.

As group njp1, njp4, njp7 was identified as *Aeromonas hydrophila* subsp. *dhakensis, Aeromonas caviae, Aeromonas sp.*Among 3 groups njp7 was isolated from Silver carp whereas njp1 and njp4 from Rohu sample.

In table 3.18, a total summary of identification of all the isolates has shown with their corresponding isolates name that were collected from three different markets of Dhaka city.

Two of ten of our representative isolates that were njp9 and njp10 were identified as *Klebsiella pneumonia* and groups are sampled from Silver carp. Other isolates like njp2, njp5 and njp8 were identified as *Proteus penneri*, *Enterobacter* sp. and *Citrobacter freundii* respectively. Among 3 groups njp2 and njp5 were isolated from Rohu, therefore, njp8 was isolated from Silver carp.

Overall, of the total ten representative groups, 2 groups named by njp3 and njp6 were identified as *Pseudomonas plecoglossicida* and *Pseudomonas aeruginosa* species, both of them were isolated from Rohu sample.

Table 3.15. 16S rRNA sequence (1341 bp) based identification of 10 representative bacterial isolates from Rohu and Silver carp sample

Isolates no.	Identified sp.	Max score	Total score	Query cover	E value	Identity	Gen Bank Accession no.
njp1	*Aeromonas hydrophila* subsp. *dhakensis* strain SSE50	2477	2477	100%	0.0	100%	KF769535
njp 2	*Proteus penneri* strain wf-3	2477	2477	100%	0.0	100%	KT029132
njp 3	*Pseudomonas plecoglossicida* strain Pp20	2471	2471	100%	0.0	99%	KU321233
njp 4	*Aeromonas caviae* strain J5	2471	2471	100%	0.0	99%	KP262417
njp 5	*Enterobacter* sp. UIWRF1185	2470	2470	100%	0.0	99%	KR189394
njp 6	*Pseudomonas aeruginosa* strainR4	2470	2470	100%	0.0	99%	KU321274
njp 7	*Aeromonas* sp. ID1	2477	2477	100%	0.0	100%	KT695849
njp 8	*Citrobacter freundii* strain BCD12	2470	2470	100%	0.0	99%	KT156814
njp 9	*Klebsiella pneumoniae* subsp. *rhinoscleromatis* strain HE17	2464	2464	100%	0.0	99%	LN624806
njp 10	*Klebsiella pneumoniae* subsp. *rhinoscleromatis* strain HE17	2464	2464	100%	0.0	99%	LN624806

Table 3.16. Summary of molecular identification of 10 representative bacterial isolates for 16S rRNA sequencing of Rohu and Silver carp

Group ID	Identified species	Colony ID	Sample ID	Sample details
njp1	*Aeromonas hydrophila* subsp. *dhakensis* strain SSE50	Rp´1/2	S1	Rohu, Palashi Bazar
njp2	*Proteus penneri* strain wf-3	RN´´2/5	S2	Rohu, Nobabgonj Bazar
njp3	*Pseudomonas plecoglossicida* strain Pp20	R_N 3/1	S3	Rohu, Nobabgonj Bazar
njp4	*Aeromonas caviae* strain J5	Rp´4/1	S4	Rohu, Palashi Bazar
njp5	*Enterobacter* sp. UIWRF1185	R_A 4/2	S5	Rohu, Anando Bazar
njp6	*Pseudomonas aeruginosa* strainR4	R_N´ 5/2	S6	Rohu, Nobabgonj Bazar
njp7	*Aeromonas* sp. ID1	Sp´2/12	S7	Silver carp, Palashi Bazar
njp8	*Citrobacter freundii* strain BCD12	S_A´´3/3	S8	Silver carp, Anando Bazar
njp9	*Klebsiella pneumoniae* subsp. *rhinoscleromatis* strain HE17	S_A´4/1	S9	Silver carp, Anando Bazar
njp10	*Klebsiella pneumoniae* subsp. *rhinoscleromatis* strain HE17	S_A´5/4	S10	Silver carp, Anando Bazar

3.8.2 Multiple Sequence Alignment

A multiple sequence alignment is shown by view CLUSTALW to compare the sequences. After comparing the obtained sequences of 3 *Aeromonas* sp. group (njp1, njp4 and njp7), total 20 sites were found polymorphic. Therefore, the dissimilarity was 1.49% (20/1341=0.0149). Among 3 sequences njp1 was closely related to njp4 while less relation was found with njp1 and njp4 to njp7. All of them njp7 was more dissimilar in

15 (33, 35, 36, 58, 59, 60, 70, 71, 134, 135, 136, 168,1213, 1234) sites with other 2 sequences.

Meanwhile, njp7 was the strain which isolated from Silver carp whereas others 2 (njp1 and njp4) were isolated from Rohu sample, respectively showed in fig 3.4A and 3.4B.

Similarly, in 2 *Pseudomonas* sp. (njp3 and njp6) group, while compared, 52 polymorphic positions were found, showed in fig 3.6.Thus the variation in the base pair was 3.88% (52/1341=0.0388).Both sequence was isolated from Rohu sample.

All of 10 sequences, 2 sequences showed the similar identity. But in multiple sequence alignment (MSA) these 2 group (njp9 and njp10) of *Klebsiella pneumoniae* subsp. *rhinoscleromatis* were absolutely similar in all positions of the sequence to each other except in 3 positions (338, 361 and 1038) were polymorphic (figure 3.5).

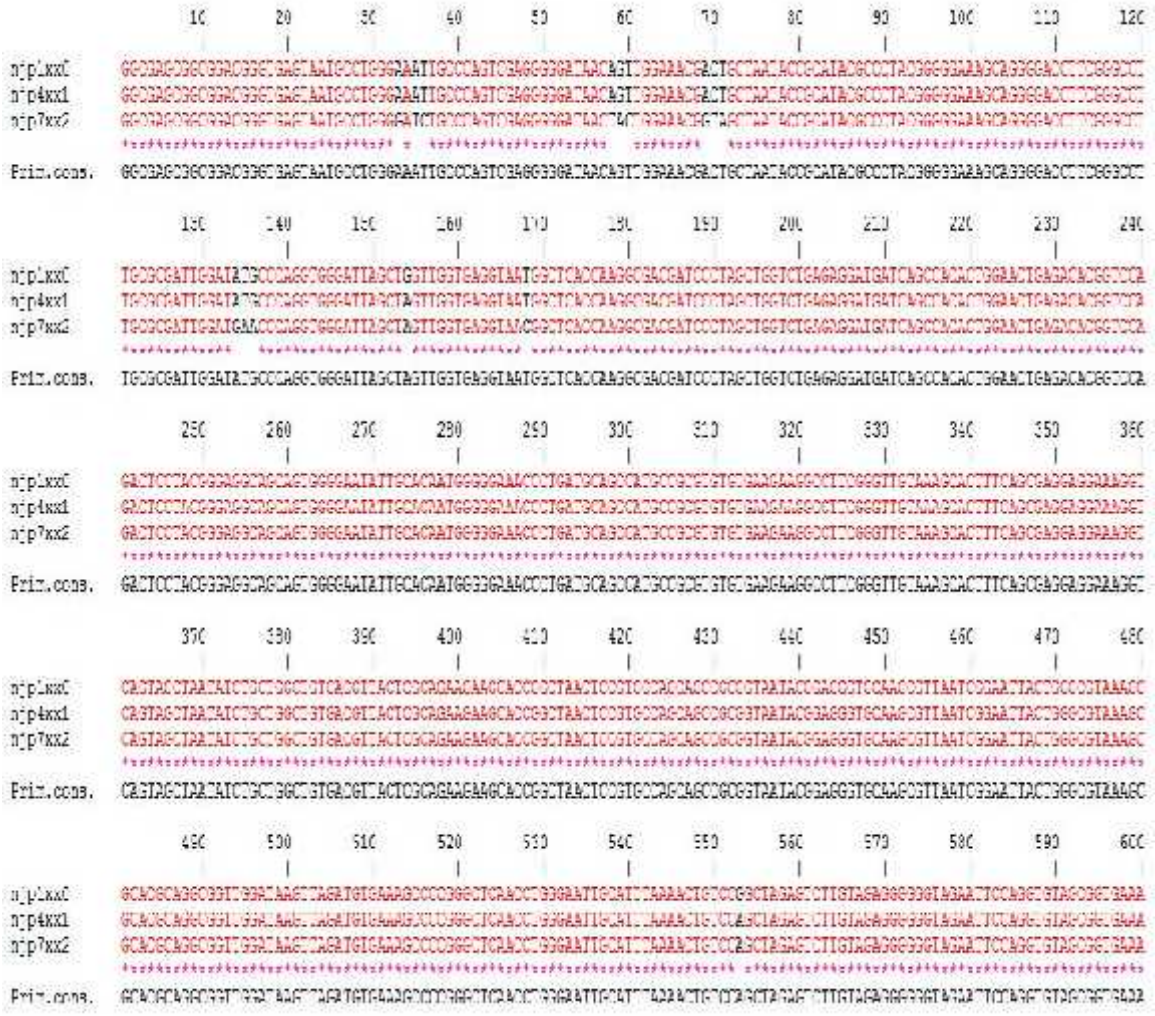


Fig 3.4A. Multiple sequence alignment of 16S rRNA gene fragments of the closely related group njp1, njp4, njp7 where black among the red indicates polymorphic

sites. (View CLUSTALW, alignment width 120).

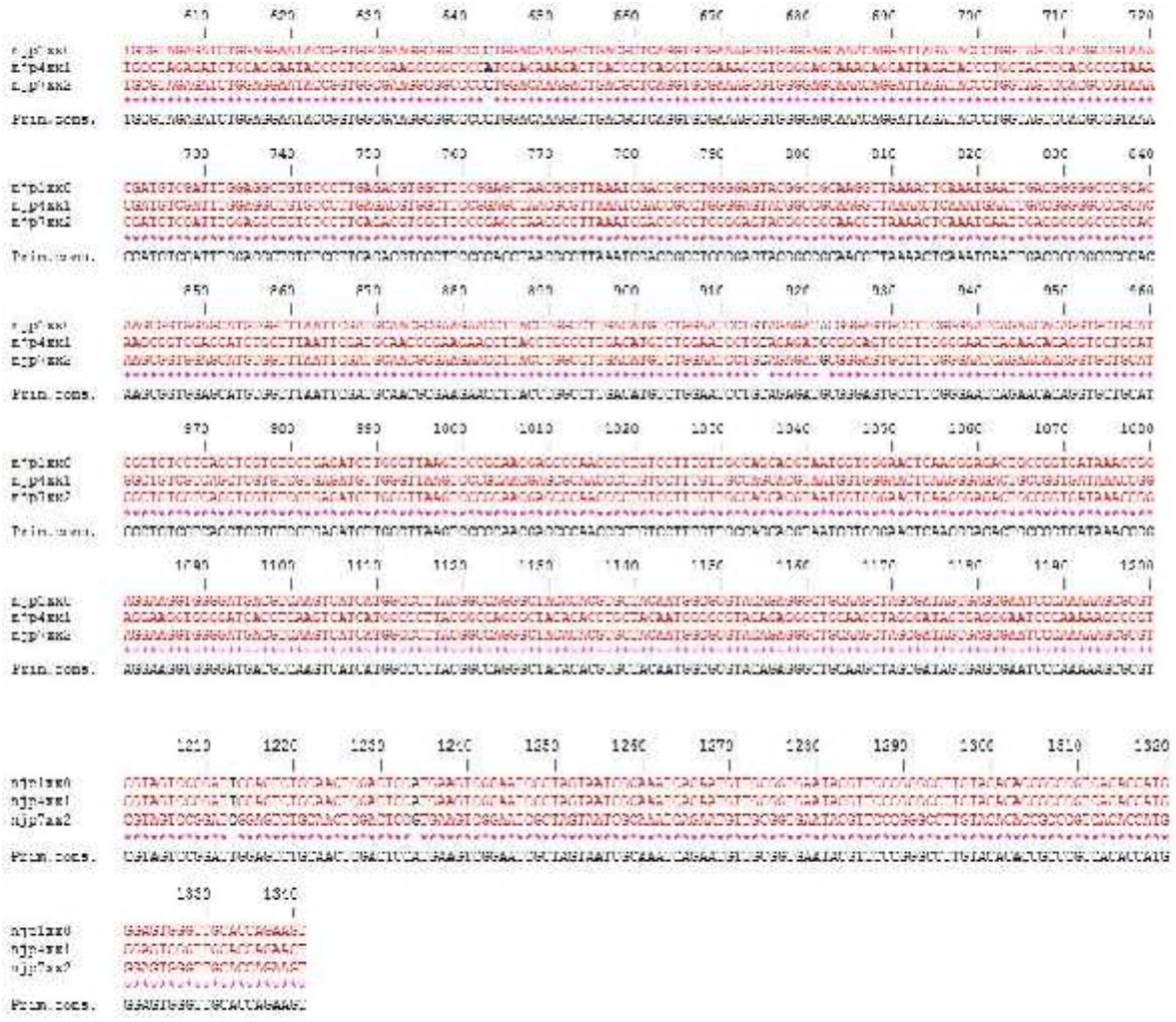


Fig 3.4B. Multiple sequence alignment of 16SrRNA gene fragments of the closely related group njp1, njp4, njp7 where black among the red indicates polymorphic sites. (View CLUSTALW, alignment width 120).

Fig 3.5. Multiple sequence alignment of 16SrRNA gene fragments of the closely

related group njp9 and njp10 where black boxes among the red indicates polymorphic sites. (View CLUSTALW, alignment width 120).

Fig 3.6. Multiple sequence alignment of 16SrRNA gene fragments of the closely related group njp3 and njp6 where black color among the red indicates polymorphic sites. (View CLUSTALW, alignment width 120).

3.8.3 Phylogenetic Analysis

Phylogenetic analysis by constructing the Phylogenetic tree (Figure 3.7) based on the partial 16S rDNA gene sequences of the representative 10 isolates using neighbor-join and

BioNJ algorithms confirmed the taxonomic position of the isolates (njp1, njp2, njp3, njp4, njp5, njp6, njp7, njp8, njp9 and njp10) and the comparison of these bacterial strain sequences with other homologous bacterial sequences.

The phylogenetic tree indicates that njp1 and njp4 found closely related to njp7 which supporting their similarity with the allocated strains *Aeromonas hydrophila* subsp. *dhakensis* strain SSE50, *Aeromonas caviae* strain J5 and *Aeromonas* sp. ID1 and *Aeromonas enteropelogenes* AB 25, *Aeromonas caviae* strain J3 and Aeromonadaceae bacterium BFE9B.

It is clear that from the tree njp9, njp10 are closely related to *Klebsiella pneumoniae* subsp. *rhinoscleromatis* strain HE17 and *Klebsiella* sp. KB 52.

The Phylogenetic tree also confirms the taxonomic position of njp3 and njp6 in the genus *Pseudomonas* supporting their similarity with the allocated strains *Pseudomonas plecoglossicida* strain Pp20, *Pseudomonas aeruginosa* strainR4 and *Pseudomonas putida* Y-4.

The tree also supports the taxonomic position of njp2, njp5 and njp8 as the genus *Proteus, Enterobacter* and *Citrobacter* those resemblance with the allocated strains of *Proteus penneri* strain wf-3 and *Proteus vulgaris* KL22, *Enterobacter* sp. UIWRF1185 and *Citrobacter freundii* strain BCD12 and *Citrobacter freundii* strain XW722. These 3 groups closely related to njp9 and njp10 (genus *Klebsiella*) than genus *Aeromonas* (njp1, njp4, njp7).

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura*et al.* 1993). The tree with the highest log likelihood (-4013.7604) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The

analysis involved 26 nucleotide sequences with 1000 bootstrap replications. Evolutionary analyses were conducted in MEGA6 (Tamura*et al*. 2013).

Phylogeny Test

Test of Phylogeny: Bootstrap method
No. of Bootstrap Replications: 1000
Substitutions Type: Nucleotide
Model/Method: Tamura-Nei model
Rates among Sites: Uniform rates
ML Heuristic Method: Nearest-Neighbor-Interchange (NNI)
No. of Sites: 1339

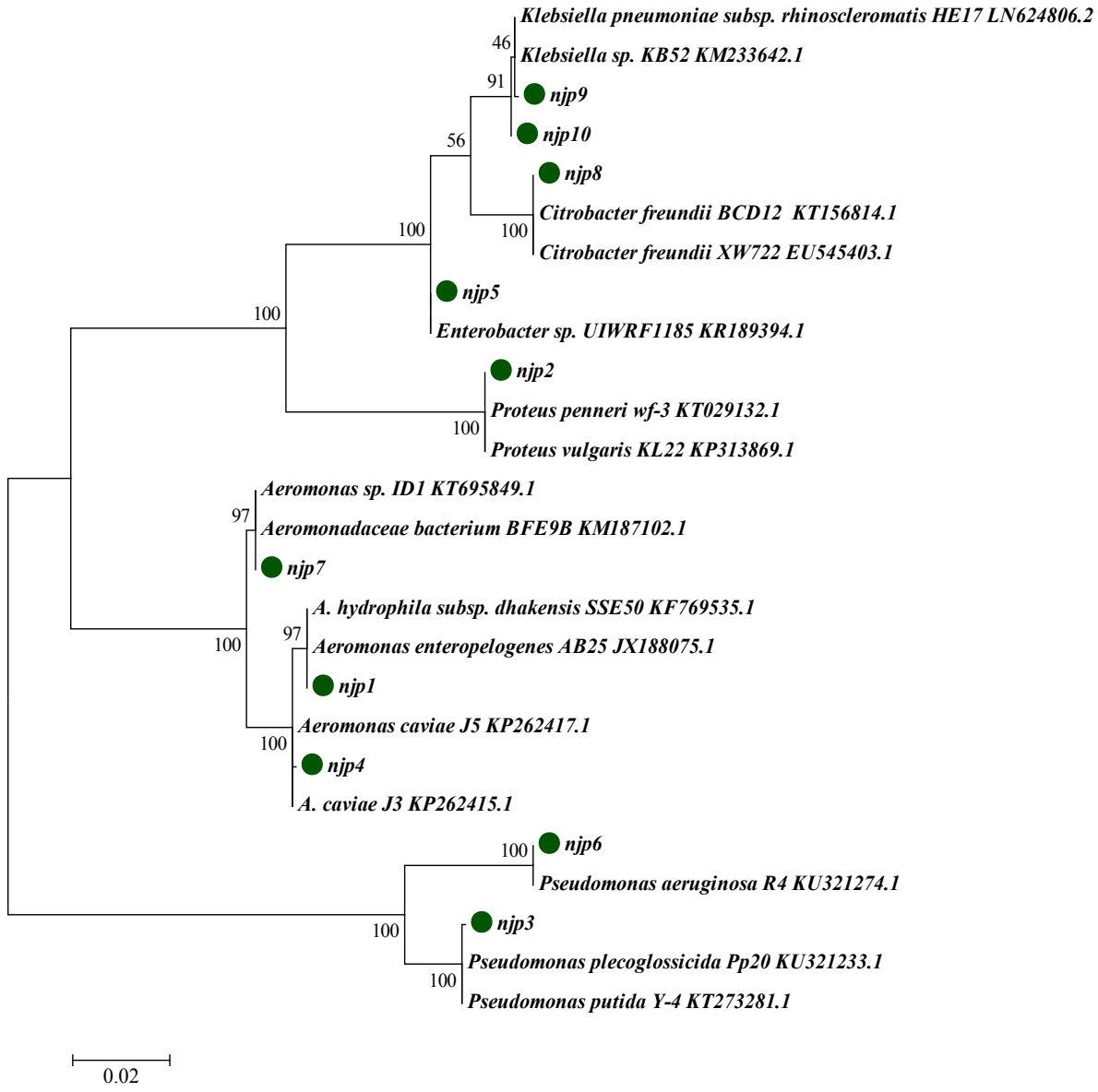


Fig 3.7. The neighbor-joining (NJ) phylogenetic tree based on partial 16S rRNA gene sequences. The evolutionary distances were compared by Maximum Likelihood method. Numbers in tree are bootstrap values. Green circles indicates position of the studied strains.

3.9 Provisional and Molecular identification of the bacterial isolates

Confabulating all morphological, biochemical and molecular characters of the isolated organisms identification were performed. Out of 18 provisionally identified sp. 10 isolates were further used for molecular identification. Among 10 identified species, 6 identified isolates were found similar up to genus both provisionally and molecularly presented with cream color in table 3.17.Therefore, among of 6 identified species 4 species were isolated from Rohu sample and 2 species were isolated from Silver carp.

Table 3.17. List of provisionally identified species and molecularly identified species

Group name	Isolates name	Provisionally identified sp.	Molecularly Identified sp.
njp1	**Rp´1/2**	*Vibrio metschnikovil*	*Aeromonas hydrophila* subsp. *dhakensis*
njp2	**RN´´2/5**	***Proteus mirabilis***	***Proteus penneri***
njp3	**R_N 3/1**	***Pseudomonas citronellolis***	***Pseudomonas plecoglossicida***
njp4	**Rp´4/1**	*Staphylococcus caprae*	*Aeromonas caviae*
njp5	**R_A 4/2**	***Enterobacter nimipressuralis***	***Enterobacter* sp.**
njp6	**R_N´ 5/2**	***Pseudomonas sp.***	***Pseudomonas aeruginosa***
njp7	**Sp´2/12**	***Aeromonas sp.***	***Aeromonas* sp.**
njp8	**S_A´´3/3**	*Pseudomonas graminis*	*Citrobacter freundii*
njp9	**S_A´4/1**	*Staphylococcus aureus*	*Klebsiella pneumoniae* subsp. *rhinoscleromatis*
njp10	**S_A´5/4**	***Klebsiella pneumoniae***	***Klebsiella pneumoniae* subsp. *rhinoscleromatis***

3.10 Antibiotic susceptibility Test of Bacterial isolates

During the present study 9 bacteria isolates of Rohu and 9 bacterial isolates of Silver carp including 1 reference strain (*E. coli* DH5α) were tested against 14 common antibiotics viz. Amikacin (30µg), Amoxycillin (10µg), Ampicillin (10µg), Chloramphenicol (30µg), Ciprofloxacin (5µg), Erythromycin (15µg), Gentamycin (10µg), Kanamycin (30µg), Nalidixic Acid (30µg), Nitrofurantoin (300µg), Polymyxin B (300 unit), Streptomycin (10µg), Sulphamethoxazole (25µg) and Tetracycline (30µg). Results of antibiotic susceptibility test are shown in Table 3.18, Table 3.19 and Plate 3.7.

All isolates of Rohu and Silver carp including reference strain were resistant to Sulphamethoxazole whereas all isolates of Rohu and Silver carp showed sensitivity to Ciprofloxacin.

For all isolates of Silver carp, Ampicillin, Amoxycillin, Erythromycin and Polymyxin B showed resistance but for Rohu isolates, 8 isolates showed resistance to both Ampicillin and Amoxycillin, not resistant for Erythromycin and 6 isolates showed resistance to Polymyxin B.

Both Rohu and Silver carp isolates were not resistant to Gentamycin. In Erythromycin no resistance found in Rohu isolates but all Silver carp isolates showed resistance.

Amikacin, Nitrofurantoin, Tetracycline, Chloramphenicol and Kanamycin were sensitive, intermediate and resistant for all isolates of Rohu and Silver carp. Both Rohu and Silver carp isolates were intermediate to Nalidixic Acid but not intermediate to Ampicillin, Amoxycillin and Polymyxin B.

There were no sensivity observed for Streptomycin in Rohu isolates but sensitivity found in Silver carp isolates.

The reference strain (*E. coli* DH5α) showed sensitivity for all 11 antibiotics except intermediate to Erythromycin and Nalidixic Acid. Only resistant for Sulphamethoxazole.

Table 3.18. Results of Antibiotic Susceptibility Test against 14 antibiotics for 18 bacterial isolates (9 isolates of Rohu and 9 isolates of Silver carp) with reference strain (*E. coli* DH5α)

Isolate name	Amikacin (30µg)	Nitrofuran (300µg)	Gentamicin (10µg)	Erythromycin (15µg)	Tetracyclin (30µg)	Ampicillin (10 µg)	Polymixin (300 unit)	Chloramphenicol (30 µg)	Sulphmethoxazole (25 µg)	Streptomycin (10 µg)	Amoxycillin (10µg)	Kanamycin (30µg)	Ciprofloxacin (5µg)	Nalidixic acid (30µg)
Control	S	S	S	I	S	S	S	S	R	S	S	S	S	I
Rp´1/2	S	S	S	R	S	S	S	R	R	I	S	S	S	S
R_A´2/1	S	S	S	I	S	R	S	S	R	I	R	I	S	S
R_N´´2/5	S	R	S	R	S	R	R	I	R	I	R	I	S	S
R_N 3/1	S	R	S	R	S	R	R	R	R	I	R	S	S	R
R_N´3/2	I	R	I	R	R	R	S	I	R	I	R	R	S	R
Rp´4/1	S	S	S	I	S	R	R	S	R	R	R	I	S	S
R_A 4/2	S	R	S	R	I	R	R	S	R	I	R	S	S	S
R_N´ 5/2	S	I	I	R	S	R	R	S	R	I	R	I	S	S
Rp´´5/5	R	S	S	R	S	R	R	S	R	I	R	I	S	S
Sp´1/4	S	I	S	R	R	R	R	S	R	I	R	I	S	S
S_A´2/3	S	I	S	R	R	R	R	S	R	I	R	R	S	S
Sp´2/12	S	R	I	R	I	R	R	S	R	R	R	R	S	R
S_A´´3/3	S	S	S	R	S	R	R	S	R	I	R	S	S	S
S_A´3/4	S	S	S	R	S	R	R	S	R	R	R	I	S	S
S_A´4/1	S	I	S	R	S	R	R	S	R	S	R	S	S	S
S_A´4/2	R	S	S	R	S	R	R	R	R	R	R	I	S	S
S_A´´5/3	S	S	S	R	I	R	R	R	R	I	R	I	S	S
S_A´5/4	I	R	I	R	S	R	R	I	R	R	R	S	S	S

*S= Sensitive, I= Intermediate, R= Resistant
*Control= Reference strain

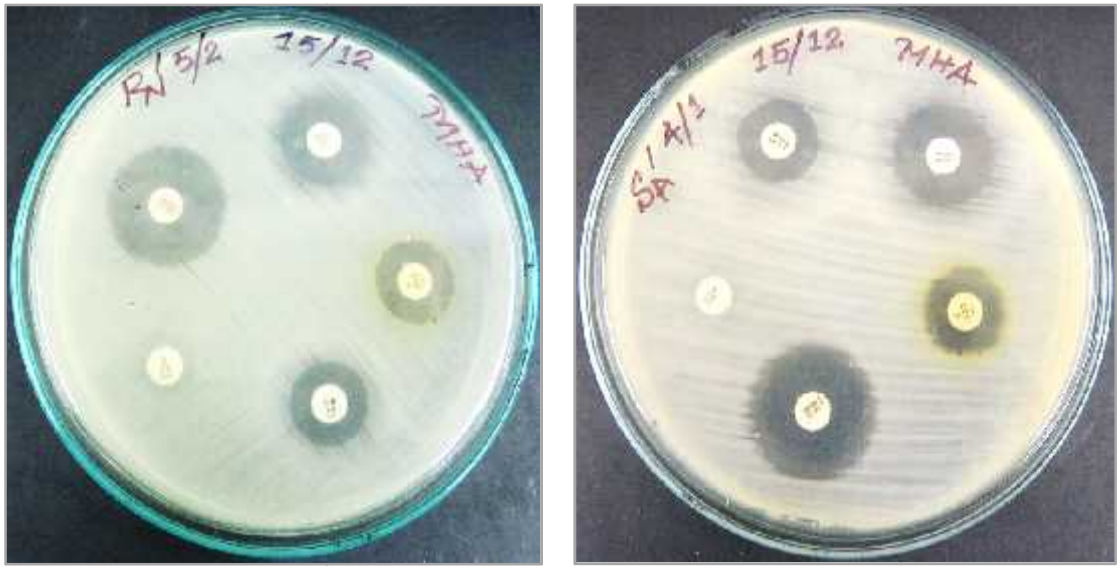
Name of Antibiotics	Isolates of *Labeo rohita* (N=9)	Isolates of *Hypophthalmichthys molitrix* (N=9)

Table 3.19. Percentage of antibiotic sensitivity, intermediate and resistance for 18 isolates against 14 antibiotics

	R%	I%	S%	R%	I%	S%
Amikacin	1 (11.11%)	1 (11.11%)	7 (77.78%)	1 (11.11%)	1 (11.11%)	7 (77.78%)
Nitrofurantoin	4 (44.44%)	1 (11.11%)	4 (44.44%)	2 (22.22%)	3 (33.33%)	4 (44.44%)
Gentamicin	0 (0%)	2 (22.22%)	7 (77.78%)	0 (0%)	2 (22.22%)	7 (77.78%)
Erythromycin	0 (0%)	2 (22.22%)	7 (77.78%)	9 (100%)	0 (0%)	0 (0%)
Tetramycin	1 (11.11%)	1 (11.11%)	7 (77.78%)	2 (22.22%)	2 (22.22%)	5 (55.56%)
Amplicillin	8 (88.89%)	0 (0%)	1 (11.11%)	9 (100%)	0 (0%)	0 (0%)
Polymixin B	6 (66.67%)	0 (0%)	3 (33.33%)	9 (100%)	0 (0%)	0 (0%)
Chloramphenicol	2 (22.22%)	2 (22.22%)	5 (55.56%)	2 (22.22%)	1 (11.11%)	6 (66.67%)
Sulphmethoxazole	9 (100%)	0 (0%)	0 (0%)	9 (100%)	0 (0%)	0 (0%)
Streptomycin	1 (11.11%)	8 (88.89%)	0 (0%)	4 (44.44%)	4 (44.44%)	1 (11.11%)
Amoxycillin	8 (88.88%)	0 (0%)	1 (11.11%)	9 (100%)	0 (0%)	0 (0%)
Kanamycin	1 (11.11%)	5 (55.56%)	3 (33.33%)	2 (22.22%)	4 (44.44%)	3 (33.33%)
Ciprofloxacin	0 (0%)	0 (0%)	9 (100%)	0 (0%)	0 (0%)	9 (100%)
Nalidixic acid	2 (22.22%)	0 (0%)	7 (77.78%)	1 (11.11%)	0 (0%)	8 (88.89%)

*S= Sensitive, I= Intermediate, R= Resistant

*N= Number of isolate



A. Antibiotic susceptibility of Rohu sample

B. Antibiotic susceptibility of Silver carp sample

Plate 3.7. Antibiotic Susceptibility of Rohu and Silver carp sample where clear zone indicates antibiotic sensitivity and unclear zone.

Chapter 4

Discussion

The gastrointestinal tract of Rohu and Silver carp act as a reservoir of pathogenic bacteria. These bacteria has impact on health of human and fish. Microbial abundance in gut might be found in these fish due to the contamination by fishing vessel, source of water, poor hygiene and sanitation condition of processing (Wahab *et al.* 2003, Hatha *et al*. 2003). But on newly caught fish it depends on the environment from where it was caught rather markets (Shewan 1961). Bacterial and chemical composition reported to be the major factors contributing to the rapid deterioration of fish quality and stated the shelf life of fishery products (Laycock 1971 and Morten *et al.* 2002). Bligh (1971)explained freshwater fish have a different type of spoilage microflora that cause fish borne disease results from the ingestion of bacteria and the toxins produced by microorganisms present in the marketed food.

The present study was carried out to investigate the pathogenic gut microbial quality assessment of the locally available indigenous and invasive fresh water fish in relation to environmental condition of fish markets with a view to provide potential approaches for food safety with regard to food borne pathogens.

4.1 Quantitative enumeration of Bacterial density

In this study, comparative analysis of TBC, TVC, TSSC, TAC, TSC and TCC showed great variation from species to species as well as market to market. For both Rohu and silver carp, the highest TBC was found from Anando Bazar ($4.95 \pm 2.5 \times 10^7$cfu/g and $3.8 \pm 0.36 \times 10^7$ cfu/g, respectively). According to Adams and Moses (2008), the normal bacterial load of the surface slime of fish can range from $10^2 – 10^7$ cfu/cm^2 and the Gills and Intestines can range up to 10^3 and 10^7 cfu/g respectively. According to Huss (1995), the proportion of microorganisms commensal the fish on the surface and gills/ guts of fish is 10^2-10^7 (cfu/cm^2) and between 10^3 and 10^9 cfu/g respectively. The processed food or food products are considered as spoiled when the TBC values reach to 10^6 cfu/g or more in food items (Shewan 1970). So, these findings agree with the present study. The quantitative and qualitative aspects of intestinal bacteria of Rohu fish *(Labeo rohita)* showed that total viable count of bacteria ranged from 9.9×10^6 to 1.4×10^7 cfu/g of intestine in different age groups of fish (Hossain *et al.* 1999). This time the present count

exceed the limit for Rohu. For Silver carp, total bacterial count of gut of Silver carp was found $3.53 \pm 2.85 \times 10^8$ cfu/g (Rahman *et al*. 2010).

According to the International Commission on the Microbiological Specification of Foods (ICMSF 1986) guideline, acceptable limit of total bacterial counts for giant prawns and white fish are 10^6 and 5×10^5 cfu per gram, respectively. In this study, total bacterial count was maximum $4.14 \pm 0.48 \times 10^7$ cfu/g which exceeds the acceptable limit. So this study clarified that the collected Rohu and Silver carp samples from different fish market were unacceptable for the consumption.

On total *Vibrio* like colony count, enumerated bacterial count was ranged from $8.04 \pm 12.97 \times 10^4$ to $3.63 \pm 6.06 \times 10^6$ cfu/g for Rohu. On TCBS agar Plate, counted total *Vibrio* density for Silver carp ranged between $2.63 \pm 1.24 \times 10^2$ to $4.97 \pm 3.36 \times 10^3$ cfu/g. The highest TVC was found in Rohu ($3.63 \pm 6.06 \times 10^6$ cfu/g) of Anando Bazar whereas for Silver carp, enumerated highest total *Vibrio* load was $4.97 \pm 3.36 \times 10^3$ cfu/g which sampled also from Anando Bazar. According to Rahman *et al.* (2010), the TBC, TC, FC, FS and total *Vibrio* counts ranged from $1.72 \pm 0.68 \times 10^8$ to $7.00 \pm 3.39 \times 10^8$, $2.49 \pm 1.72 \times 10^6$ to $6.55 \pm 3.00 \times 10^6$, $1.58 \pm 1.29 \times 10^6$ to $2.76 \pm 1.42 \times 10^6$, $4.83 \pm 2.09 \times 10^4$ to $1.19 \pm 0.46 \times 10^5$ and $2.06 \pm 0.67 \times 10^3$ to $3.68 \pm 2.02 \times 10^5$, respectively among various feeding groups of fish.*Vibrio* was found in the gastrointestinal tract of *Labeo rohita* (Hossain *et al.* 1999). The densities of total *Vibrio*-like colonies on TCBS also found in the gut sample of Silver carp (Rahman *et al.* 2010; Muniruzzaman 1993).

According to the recommendation of International Association of Microbiological Societies (IAMS), fresh and frozen fish should be free of *Vibrio* (0 cfu/g). The present study revealed that microbial quality of Rohu and Silver carp of local markets were not acceptable in order of presence of *Vibrio.* Hadin *et al*. (2004), detected the presence of eight potentially pathogenic *Vibrio* species. According to the guideline of ICMSF (1986), acceptable limit of *Vibrio cholerae* counts for giant prawns and white fish are 0 cfu/g. The findings of the present study were beyond the standard value suggested by IAMS and ICMSF, which indicate the unacceptability as food for human consumption.

On SS agar plate, counted total *Salmonella* and *Shigella* was $3.21 \pm 3.0 \times 10^6$ to $1.2 \pm 1.2 \times 10^7$cfu/g on Rohu whereas for Silver carp, determined total *Salmonella* and *Shigella* on SS agar plate was $5.5 \pm 3.3 \times 10^5$ to $1.69 \pm 1.56 \times 10^6$cfu/g. The highest *Salmonella-Shigella* count was found in $1.2 \pm 1.2 \times 10^7$ Rohu sample collected from Anando Bazar

and highest total *Salmonella-Shigella* count was $5.5 \pm 3.3 \times 10^5$ cfu/g found from Silver carp sample collected from Palashi Bazar. The limit of harmful and pathogenic microorganisms, such as *Salmonella* spp., *Shigella* spp. is 0 cfu/g and 1.0×10^2 cfu/g (ICMSF 1986), revealed the unacceptability of the studied fish. *Salmonella, Shigella* are not usually found as these are not the normal flora of fishes or of their environment (FAO 1979). This result proves that the processing, handling, storage condition, hygiene and sanitary maintenance of the local markets was not good enough to maintain the infection of *Salmonella, Shigella* of the sampled fish. Besides, the relatively long storage period with low ice quality until sold and improper storage condition due to handling disruption might be the reasons for the SS infection in local markets fish (Nilla *et al.* 2012).

Total *Aeromonas* build up found on Rohu was $6.89 \pm 7.03 \times 10^6$ to $2.09 \pm 0.99 \times 10^7$ cfu/g. In the present study, total *Aeromonas* density was $4.01 \pm 3.6 \times 10^6$ to $9.96 \pm 4.2 \times 10^6$ cfu/g on Silver carp sample. Among the three different markets, highest *Aeromonas* spp. count was found $2.09 \pm 0.99 \times 10^7$ cfu/g in Rohu sample collected from Palashi Bazar and highest count of Silver carp was $9.96 \pm 4.2 \times 10^6$ cfu/g found from Anando Bazar. Presence of *Aeromonas, Pseudomonas* were ensured in the marketedRohu by Sinha *et al.* (1991) and Ye *et al.*(2014) found *Aeromonas* in the gastrointestinal tract of Silver carp.

Aeromonas hydrophila has been recovered from a wide range of freshwater fish species worldwide (Austin and Adams 1996) and also revealed their opportunistic pathogenicity (Ottaviani *et al*. 2011). So, there presence in both kind of experimental fish could be the cause of improper handling or their presence in the water body or environment (Hess 1932 and Ito *et al*.1993).

A considerable numbers of *Staphylococcus* spp. found in both type of experimented sample. To assess total *Staphylococcal* count on Mannitol salt agar plate, the bacterial load ranged from $9.2 \pm 7.4 \times 10^6$ to $1.23 \pm 0.38 \times 10^7$ cfu/g. Small density difference ranged from $5.4 \pm 4.4 \times 10^6$ to $5.5 \pm 4.4 \times 10^6$ cfu/g, found on total Staphylococcalcount of Mannitol salt agar plate. In the present study, highest Total Staphylococcal count was $1.23 \pm 0.38 \times 10^7$ found from Rohu sample of Anando Bazar and lowest count was $5.5 \pm 4.4 \times 10^6$ found from Silver carp sample collected from Nobabgonj Bazar. *Staphylococcus* spp. was reported in Rohu by Sinha *et al*. (1991) and according to Mukherjee *et al*. (1992)*Staphylococcusaureus* was isolated from Silver carp. According

to ICMSF (1986) the suggested limit for *Staphylococcus* spp. is $>10^3$ cfu/g which confirms the contagion of the experimental samples with *Staphylococcus* via infected food handlers or from the environment. The infected individual with an infection on hands or with a cold or sore throat more often acts as the contamination source in food. According to WHO the presence of *Staphylococcus aureus* in large number in raw fish is harmful because of possible bacterial food poisoning. Presence of *Staphylococcus* sp. suggests higher level of environmental contamination and its presence indicates possible risk of food poisoning.

Enteric bacteria with special reference to coliform count on EMB agar plate ranged between $1.5 \pm 0.71 \times 10^7$ to $1.98 \pm 1.7 \times 10^7$ cfu/g in Rohu. Enteric and related bacterial count on EMB agar plate was $4.53 \pm 2.85 \times 10^6$ to $3.1 \pm 3.9 \times 10^7$ cfu/g in Silver carp. In the present study, highest count $1.98 \pm 1.7 \times 10^7$ cfu/g was found from Rohu sample collected from Palashi Bazar whereas highest total coliform count was $3.1 \pm 3.9 \times 10^7$ found from Silver carp sample of Nobabgonj Bazar.Presence of total coliform in gut of Rohu was reported $9.98 \pm 4.69 \times 10^5$cfu/g and in Silver carp $4.34 \pm 2.21 \times 10^5$cfu/g found from the marketed sample (Rahman *et al.* 2010). The present count for both Rohu and Silver carp beyond this value could be indicated the presence of these bacteria in the environment. Because in general, the presence of coliform and faecal coliform is not the normal flora of bacteria in fish (Mandal *et al*. 2009). The origins of faecal coliforms are more specific than the origins of the total coliform group, faecal coliforms are considered as more accurate indication of animal or human waste than the total coliforms. According to ICMSF (1986), the suggested limit for total coliform count in Raw fish is 100 cfu/g and for E. coli 0 cfu/g. In this study, for both specimen (Rohu and Silver carp) cross the acceptable limit for consumption.

4.2 Provisional identification of bacteria by biochemical tests

In the present study, higher number of gram negative pathogenic bacteria was found. Out of 18 isolates 15 bacterial isolates were found as gram negative and at the end of the biochemical assay only 3 bacterial isolates was provisionally identified as gram positive *Staphylococcus* sp. Huss *et al.*(1995) reported that gram negative bacteria is more dominant over Gram positive bacteria in fish. However this depends on many factors such as fish species, location of fish body from where sample is taken, storage time after dying, habitat of fish etc.

12 different biochemical tests was performed and these tests confirms 1 *Vibrio* sp., 2 *Aeromonas* sp. and *Pseudomonas* sp., 1 *Proteus* sp., 1 *Staphylococcus* sp., 1 *Enterobacter* sp. and 1 *Klebsiella* sp. was provisionally identified from Rohu sample. Therefore, in Silver carp, 1 *Vibrio* sp., 1 *Salmonella* sp., 1 *Pseudomonas* sp., 1 *Escherichia* sp., 1 *Klebsiella* sp. and 2 *Aeromonas* sp. and *Staphylococcus* sp. were provisionally identified.

According to Hossain (2002) stated that *Salmonella* when allowed to ferment the five sugarsto ferment dextrose, maltose and mannitol with production of acid and gas but no fermentation was observed in lactose and sucrose. For Silver carp sample in the current study 1 *Salmonella* sp. was isolated which showed triple sugar test and was allowed to ferment glucose with production of acid and gas but no fermentation was observed in lactose and sucrose. This also found similarity with the previous studies of Dhruba *et al.* (1999) and Hossain (2002).

Perez-Guzzi *et al.* (2000)concluded *E. coli* strains (75% of the sample) in biochemical tests were sorbol non-fermenting. In this study isolated *E. coli* from invasive Silver carp was not fermented lactose and/or sucrose. Ali *et al.* (1998) reported the fermentation tests of *E. coli* four sugars was found fermented (Dextrose, lactose, sucrose, and mannitol).

2 *Aeromonas* spp. was isolated from Rohu sample non motile but shown the citrate positive test whereas from Silver carp isolated 1 *Aeromonas* spp. was motile and another one is non-motile also both could not utilize citrate. These results agreed with the findings ofIqbal *et al.* (1998)**.** They were isolated motile *Aeromonas spp* that showed esculin hydrolysis, acetate utilization, Christensen's citrate, Jordan's tartrate and acid from sucrose and salicin.

Shetty and Ravishankar (1992) reported biochemically identified *Pseudomonas* spp. shown glucose fermentation produce acid and gas, oxidase and catalase positive. 2 isolates of *Pseudomonas* spp. of Rohu and 1 isolates of *Pseudomonas* spp. was not fermented glucose but produce acid and gas and also found catalase positive. But 1 isolate of *Pseudomonas* spp. of Rohu found both oxidase and catalase positive which agreed with the previous studies.

Manikandan *et al.* (2012) examined the microbial population of intestine of *Channapunctatus* and found *Proteus vulgaris* (Gram –ve), *Pseudomonas aeruginosa* (Gram –ve) and *Klebsiella pneumonia* (Gram – ve) were isolated. This is also correlate with the current study findings.

In the current study, 2 isolates of *Aeromonas sp. and* 1isolates of *Klebsiella* sp. of Rohushowed positive result in starch hydrolysis whereas in Silver carp 1 isolates of *Vibrio* sp., 1 isolates of *Aeromonas* sp. and 1 isolates of*Staphylococcus* showed the similar result. Vignesh *et al*. (2012) isolated *Vibrio, Staphylococcus, Salmonella* and *Klebsiella* species and primarily screened for amylase production by starch plate method.

4.3 Molecular identification of bacterial isolates by 16S rRNA sequencing

The use of 16S rRNA gene sequences in the present study to investigate bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons (Ferris *et al.* 1996, Weller *et al.* 1991, Nubel *et al*. 1997). The 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Patel 2001). In this study, the 16S rDNA gene sequencing confirmed the identity of the 10 representative bacterial isolates from gut of *Labeo rohita* and *Hypophthalmichthys molitrix*by using 1341 bp of 16S rDNA gene.Before the sequencing genomic DNA were amplified by using 2 universal primers, 27F and 1492R, which allowed for the identification of bacteria up to the species level (Jiang *et al*. 2006).

Current investigation strongly suggest that gut microorganism (10 representative isolates) like *Aeromonas hydrophila* subsp. *dhakensis*, *Proteus penneri, Pseudomonas plecoglossicida, Aeromonas caviae, Enterobacter* sp., *Pseudomonas aeruginosa* were identified from *Labeo rohita.* Whereas*Aeromonas* sp., *Citrobacter freundii*, *Klebsiella pneumoniae* subsp. *rhinoscleromatis* were identified from *Hypophthalmichthys molitrix.*

Aeromonas hydrophila subsp. *dhakensis* strain SSE50 (accession no. KF769535) and *Aeromonas caviae* strainJ5 (accession no. KP262417) were found similar in the present study from two isolates (njp1 and njp4) of *Labeo rohita.* Presence of *Aeromonas* sp. in Rohu also agreed by Ghosh *et al*. (2010).

Pseudomonas spp. found in the digestive tract of Rohu (Kumari *et al*. 2001). In the presently reported study two isolates (njp3 and njp6) showed high similarity to *Pseudomonas plecoglossicida* strain Pp20 and *Pseudomonas aeruginosa* strainR4,(accession no. KU321233 and KU321274) found 99% identity. Trakroo and Agarwal (2011) isolated *Proteus* sp., *Aeromonas hydrophila,Pseudomonas aeruginosa*, *Flavobacter* sp*., Vibrio* sp. and *Corynebacterium* sp. from pond water of farm raised Rohu, *Labeo rohita*.

*Enterobacte*r sp. UIWRF1185(accession no. KR189394) (njp5) and *Proteus penneri* strain wf-3(accession no. KT029132) (njp2) (Trakroo and Agarwal 2011) were also found similar to the isolated bacteria in current study. Their presence in fish gut may possibly correlated to its feeding habit.

Presence of *Salmonella, Staphylococcus, Aeromonas, Pseudomonas, E. coli, Micrococcus, Streptococcus, Proteus, Klebsiella* and molds was also observed in the marketedRohu by Sinha *et al.* (1991).Only one *Aeromonas* sp. ID1(accession no. KT695849) and isolate no. njp7 found in *Hypophthalmichthys molitrix* sample. *Aeromonas* sp. considered as the enzyme producer strains in gut of fresh water fishes (Sugita *et al*. 1997; Ray *et al*. 2012). So, there findings agreed with the result of present study. However, the highly abundant presence of *Aeromonas* in the mucosa may agree with the findings of Hiney *et al.* (1994), Lodemel *et al.* (2001) and Yang (2010) that the intestine might be the primary location for *Aeromonas* colonization under stress-induced infections.Other species (njp8) *Citrobacter freundii* strain BCD12 (accession no. KT156814) was also investigated. *Citrobacter* is also cellulose degrading bacteria of Silver carp (Li *et al*. 2003; Saha *et al*. 2006; Wu *et al.*2012).The query sequence of 2 isolates (njp9 and njp10) were similar to *Klebsiella pneumoniae* subsp. *rhinoscleromatis* strain HE17 (accession no. LN624806).

Bacillus species like *E. coli, Pseudomonas* and *Klebsiella* spp. were isolated from the epidermis of Silver carp *(Hypohthalmichthys molitrix*) andRohu (*Labeorohita).* Harvesting, handling, transportation and storage of local market fish should be performed hygienically to reduce the risk of spoilage. *Bacillus, Proteus, Pseudomonas, Klebsiella, Streptococcus, Salmonella, Staphylococcus,Micrococcus, Serratia* and *Escherichia* are found in the skin and intestine of fish.

Gut microorganisms can be involved in cellulose degradation (Henebry *et al.* 1988). This possibility was further supported by the observation of microbial populations of Silver carp (for example, *Vibrio, Aeromonas, Bacillus, Enterobacter, Anoxybacillus, Leuconostoc, Clostridium, Actinomyces and Citrobacter*) (Li *et al.* 2003; Saha *et al.* 2006; Wu *et al.* 2012). So, the presence of pathogenic gut microbiota in Rohu and Silver carp might be the cause of cellulose degradation.

Multiple sequence alignment has been performed for the 3 identified *Aeromonas* sp. (njp1, njp4, njp7) to find out the dissimilarity among the isolates found from Rohu and

Silver carp. Among 3 sequences njp1 was closely related to njp4. All of them njp7 was more dissimilar in 15 (33, 35, 36, 58, 59, 60, 70, 71, 134, 135, 136, 168, 1213, 1234) sites with other 2 sequences. Mainly, njp7 was the strain which isolated from Silver carp whereas others 2 (njp1 and njp4) were isolated from Rohu sample, respectively. Similarly, in 2 *Pseudomonas* sp. (njp3 and njp6) group, while compared, 52 polymorphic positions were found. Both sequence was gained from the bacteria isolated from Rohu sample. No *Pseudomonas* sp. was observed from Silver carp representative isolates.

Among the 10 sequences, 2 query sequences of njp9 and njp10 showed the similar identity. But in multiple sequence alignment (MSA) these 2 group (njp9 and njp10) were absolutely similar in all positions of the sequence to each other except in 3 positions (338, 361 and 1038) were polymorphic. Both isolates were obtained from the Silver carp sample.

Construction of phylogenetic tree, involved by a total of 26 (10 of experimented sequences with 16 downloaded sequences from NCBI GenBank) nucleotide sequences that supports the findings of multiple sequence alignment. To strong the taxonomic position 6 more related sequences were downloaded. By using neighbor-join and BioNJ algorithms confirmed the taxonomic position of the isolates (njp1, njp2, njp3, njp4, njp5, njp6, njp7, njp8, njp9 and njp10) and the comparison of these bacterial strain sequences with other homologous bacterial sequences. The percentage of replicate trees in which the associated taxa clustered together in bootstrap test where 1000 replications was used. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura*et al.* 1993). The tree with the highest log likelihood (-4013.7604) was found. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

From molecular identification, 10 identified species were found similar up to genus with the 6 provisionally identified isolates of biochemical test. All isolates were representative from both Indigenous Rohu and Invasive Silver carp.

Interestingly, from the isolates of Rohu 1 isolate of *Aeromonas* sp., 1 isolate of *Pseudomonas* sp. and both 1 isolate of *Aeromonas* sp. and *Pseudomonas* sp. were identified by 16S rRNA sequencing where all isolates were isolated from TCBS, SS and MSA agar plate, respectively. From Silver carp sample, 1 isolate of *Aeromonas* sp., 1 isolate of *Citrobacter* sp. and 1 isolate of *Klebsiella* sp. were found from SS, *Aeromonas*

agar and MSA agar plate, respectively whose identified by 16S rRNA sequencing. TCBS is a selective agar for *Vibrio* species, is inhibitory to *Aeromonas* species and should not be used when *Aeromonas* gastrointestinal infections are suspected because of their partial inhibitory growth on TCBS plate (Public Health England 2015). Morphology of bacterial strains of *Salmonella* cannot differ from *Proteus* on SS agar, so identification of *Salmonella* from *Proteus* only by growth on SS agar is not recommended (https://catalog.hardydiagnostics.com). MSA agar plate is selective for salt loving *Staphylococcus* sp. *Most other bacteria are inhibited, with the exception of a few halophilic species.* This media is recommended for the detection and enumeration of coagulase-positive or negative *Staphylococci* (http://www.oxoid.com). In this experiment coagulase test was not performed.

4.4 Antibiotic susceptibility of bacterial isolates

In this study, all 18 isolates showed multiple antibiotic resistance for 14 different antibiotics where all 18 isolates of Rohu and Silver carp including reference strain were resistant to Sulphamethoxazole and sensitive to Ciprofloxacin. Ghosh and Mandal(2010) reported that Multiple antibiotic resistance (MAR) have been found in fish pathogen and bacteria from aquaculture environment with a variety of drug or an uncertain antibiotic usage history. High level of water contamination with industrial effluents and agricultural pollutants may magnify this condition. Widespread use of antibiotics in the aquaculture systems and agricultural sectors in Bangladesh may act as the source of antibiotics diffusion into the sediment (Nilla *et al*. 2012). According to Sorum (2006), the uncontrolled antibiotics will remain in the sediment and an alternation of micro flora composition of the sediment and antibiotic-resistant bacteria may occur with exerting of selective pressure. So the result of antibiotic susceptibility test can be the reason of uncontrolled and irregular use of antibacterial agents in aquaculture systems and agricultural sectors. This irregular use may responsible for the occurrence of the multiple antibiotic resistance traits among the isolated pathogenic bacteria and also these isolates can carry drug resistant factor (Keys *et al.* 1986). Besides this the studied fishes could act as the reservoir of multiple antibiotic resistance and also facilitate the dissemination of the antibiotic resistant bacteria (Ryu *et al.* 2012).

Chapter 5

Conclusion and Recommendations

5.1 Conclusion

Quality and safety of food have the prime concern of consumer and indicate the grade or degree of excellence of food. This study confirms the existence of pathogenic bacteria in gut of *Labeo rohita* and *Hypophthalmichthys molitrix* which are of public health significance. The counted bacterial load which always exceed the acceptable limit of ICMSF (1986) is due to poor water or feed quality, improper handling, unhygienic condition of markets as well as habitat (Olufemi, 1998) or source from where the fish samples were collected for selling in market. Cross contamination could be another cause of it. Normally the gut is removed before cooking in our country yet it will require a careful handling to prevent contamination to other parts during processing, hence good hygiene and sanitation is essential. This study also provide a clear perspective of the bacterial profusion in marketed Rohu and Silver carp. This experiment also suggests the abundance of pathogenic gastrointestinal bacteria as well as the presence of antibiotic resistant bacteria into Rohu and Silver carp sold in the fish markets of Dhaka city.

5.2 Recommendations

1. Proper training for retailers and handlers should be taken on the aspect to avoid the health risks and cross contamination.
2. The present study emphasizes the necessity of increasing awareness about the use of antibiotics in aquaculture sector to ensure the absence of antibiotic resistant pathogenic bacteria in fish to achieve the food safety.
3. Current study was conducted in the Dhaka city; so further field studies are needed in the other important city of Bangladesh to find out the variability of gut microbiota of Rohu and Silver carp.
4. This study was limited for the identification of some selective gastrointestinal pathogenic bacteria by 16S rRNA sequencing with culture dependent method. More accurate result can be determined by multi locus gene and next generation sequencing to know the whole community.

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Appendices

Appendix A

Table: 3.1 Length and weight of the collected samples

Sampling sites	Sampling types	Length (cm)	Weight (g)
Nobabgonj Bazar	Rohu	29.2	290.01
	Silver carp	51.6	1303.00
Palashi Bazar	Rohu	32.5	335.43
	Silver carp	49.6	1230.00
Ananda Bazar	Rohu	40.6	830.81
	Silver carp	37.2	649.50
Nobabgonj Bazar	Rohu	30.5	410.10
	Silver carp	18.5	104.02
Palashi Bazar	Rohu	31.7	402.12
	Silver carp	35.1	568.02
Anando Bazar	Rohu	32.2	448.98
	Silver carp	38.9	679.21
Nobabgonj Bazar	Rohu	31.2	407.15
	Silver carp	45.5	1103.02
Palashi Bazar	Rohu	34.1	452.10
	Silver carp	39.5	611.12
Anando Bazar	Rohu	27.2	288.21
	Silver carp	38.4	574.90

Table 3.2Bacterial density (cfu/g) variation between Rohu and Silver carp measured on LA and TCBS, SS, Aeromonas, MSA, EMB Agar media.

Bacterial density (cfu/g)	Sample name	
	Rohu	Silver carp
Total bacterial count	$5.27\pm2.01\times10^{7a}$	$3.02\pm1.42\times10^{7b}$
Total Vibrio count	$1.58\pm3.51\times10^{6}$	$2.38\pm3.63\times10^{3}$
Total *Salmonella- Shigella* count	$6.94\pm7.15\times10^{6a}$	$1.11\pm0.97\times10^{6b}$
Total Aeromonas count	$1.31\pm1.06\times10^{7}$	$6.09\pm4.61\times10^{6}$
Total staphylococcal count	$1.03\pm0.52\times10^{7a}$	$5.48\pm3.98\times10^{6b}$
Total coliform count	$1.68\pm0.98\times10^{7}$	$1.39\pm2.35\times10^{7}$

Table: 3.3 Total bacterial count(cfu/g) (mean ± SD) of gut sample of Rohu (*Labeo rohita*) and Silver carp (*Hypophthalmichthys molitrix*) from 3 different markets of Dhaka city on Luria agar plate

Market name	Sample name	
	Rohu (*Labeo rohita*)	Silver carp (*Hypophthalmichthys molitrix*)
Nobabgonj Bazar	$4.26 \pm 1.8 \times 10^{7}$	$2.5 \pm 1.96 \times 10^{7}$
Palashi Bazar	$6.6 \pm 1.6 \times 10^{7}$	$2.7 \pm 1.7 \times 10^{7}$
Anando Bazar	$4.95 \pm 2.5 \times 10^{7}$	$3.8 \pm .36 \times 10^{7}$

Table 3.4. Total *Vibrio* count(cfu/g) (mean ± SD) of gut sample of Rohu (*Labeo rohita*) and Silver carp (*Hypophthalmichthys molitrix*) from 3 different markets of Dhaka city on TCBS agar plate

Market name	Sample name	
	Rohu (*Labeo rohita*)	Silver carp (*Hypophthalmichthys molitrix*)
Nobabgonj Bazar	$8.04 \pm 12.97 \times 10^{4}$	$1.9 \pm 1.27 \times 10^{3}$
Palashi Bazar	$1.02 \pm 1.6 \times 10^{6}$	$2.63 \pm 1.24 \times 10^{2}$
Anando Bazar	$3.63 \pm 6.06 \times 10^{6}$	$4.97 \pm 3.36 \times 10^{3}$

Table 3.5.Total *Salmonella-Shigella*count (cfu/g)(mean ± SD)of gut sample of Rohu (*Labeo rohita*) and Silver carp (*Hypophthalmichthys molitrix*) from 3 different markets of Dhaka city on SS agar plate

Market name	Sample name	
	Rohu (*Labeo rohita*)	Silver carp (*Hypophthalmichthys molitrix*)
Nobabgonj Bazar	$3.21 \pm 3.0 \times 10^6$	$1.08 \pm 0.44 \times 10^6$
Palashi Bazar	$5.74 \pm 0.56 \times 10^6$	$1.69 \pm 1.56 \times 10^6$
Anando Bazar	$1.2 \pm 1.2 \times 10^7$	$5.5 \pm 3.3 \times 10^5$

Table 3.6 Total *Aeromonas* count(cfu/g) (mean ± SD) of gut sample of Rohu (*Labeo rohita*) and Silver carp (*Hypophthalmichthys molitrix*) from 3 different markets of Dhaka city on Aeromonas Lab agar plate

Market name	Sample name	
	Rohu (*Labeo rohita*)	Silver carp (*Hypophthalmichthys molitrix*)
Nobabgonj Bazar	$6.89 \pm 7.03 \times 10^6$	$4.31 \pm 4.5 \times 10^6$
Palashi Bazar	$2.09 \pm 0.99 \times 10^7$	$4.01 \pm 3.6 \times 10^6$
Anando Bazar	$1.15 \pm 1.2 \times 10^7$	$9.96 \pm 4.2 \times 10^6$

Table 3.7 Total Staphylococcal count(cfu/g)(mean ± SD) of gut sample of Rohu (*Labeo rohita*) and Silver carp (*Hypophthalmichthys molitrix*) from 3 different markets of Dhaka city on Mannitol salt agar plate

Market name	Sample name	
	Rohu (*Labeo rohita*)	Silver carp (*Hypophthalmichthys molitrix*)
Nobabgonj Bazar	$9.2 \pm 7.4 \times 10^6$	$5.5 \pm 4.4 \times 10^6$
Palashi Bazar	$9.3 \pm 5.7 \times 10^6$	$5.4 \pm 4.4 \times 10^6$
Anando Bazar	$1.23 \pm 0.38 \times 10^7$	$5.47 \pm 4.49 \times 10^6$

Table 3.8 Total coliform count(cfu/g)(mean ± SD) of gut sample of Rohu (*Labeo rohita*) and Silver carp (*Hypophthalmichthys molitrix*) from 3 different markets of Dhaka city on EMB agar plate

Market name	Sample name	
	Rohu (*Labeo rohita*)	Silver carp (*Hypophthalmichthys molitrix*)
Nobabgonj Bazar	$1.6 \pm 0.4 \times 10^7$	$3.1 \pm 3.9 \times 10^7$
Palashi Bazar	$1.98 \pm 1.7 \times 10^7$	$6.64 \pm 3.6 \times 10^6$
Anando Bazar	$1.5 \pm 0.71 \times 10^7$	$4.53 \pm 2.85 \times 10^6$

Table 3.14. Summary of the 10 representative isolates for 16S rRNA sequencing

Group ID	Concentration (ng/µL)	Purity	Colony ID	Sample ID
njp1	40.2	2.04	Rp′1/2	S1
njp2	116	2.08	RN′′2/5	S2
njp3	82.3	2.08	RN 3/1	S3
njp4	59.1	2.02	Rp′4/1	S4
njp5	47.7	1.92	RA 4/2	S5
njp6	64.6	2.0	RN′ 5/2	S6
njp7	79.5	1.96	Sp′2/12	S7
njp8	45.1	1.94	SA′′3/3	S8
njp9	41.3	1.9	SA′4/1	S9
njp10	39	1.91	SA′5/4	S10

Table 3.13. Growth kinetics of bacterial isolates of Rohu and Silver carp at absorbance of 600nm under different time period

Sample no.	0 hr	2 hr	4 hr	6 hr	8 hr	20 hr	22 hr	24 hr	26 hr	28 hr	30 hr	45 hr	48 hr	95 hr
Rp´1/2	-0.016	0.089	0.641	0.789	1.148	1.92	1.951	1.965	1.984	2.01	1.987	1.947	1.909	1.759
R_N 3/1	-0.025	0.009	0.103	0.215	0.418	1.299	1.388	1.406	1.455	1.511	1.494	1.689	1.65	1.72
Rp´4/1	-0.02	0.088	0.957	1.196	1.352	1.95	1.995	1.993	2.054	2.044	2.029	1.985	1.906	1.902
S_A´ 2/3	-0.013	0.066	0.71	1.084	1.396	1.9	1.928	1.891	1.943	1.921	1.929	1.915	1.902	1.822
Sp´2/12	-0.025	0.022	0.347	0.723	0.895	1.597	1.651	1.684	1.719	1.73	1.716	1.824	1.718	1.462
S_A´´ 5/3	-0.025	0.057	0.572	0.709	0.923	1.6	1.651	1.717	1.763	1.77	1.791	1.878	1.763	1.638

Appendix B

Microbiological media

Media used were prepared by standard methods using appropriate compositions. All media were sterilized by autoclaving for 15 minutes. The composition of the media and reagents used in this study are as follows-

Ammonium Crystal violet solution (SAB 1957)

Ingredients	Amount
Solution A	
Crystal violet (85% dye content)	2.0 g
Ethyl alcohol (95%)	20 ml
Solution B	
Ammonium oxalate	0.8 g
Distilled water	80 ml

Solution A and B were mixed and stored for 24 hrs before use.

Aeromonas Agar Media

Ingredients	Amount
Agar powder	45.5 g
Distilled water	1000 ml
pH	7.4 ±0.2

Chistensen's Urea Agar Media

Ingredients	Amount
Peptone	1 g
Dextrose	1 g
NaCl	5 g
Potassium phosphate (mono basic)	2 g
Urea	20 g
Phenol red	0.012
Agar	20 g
pH	7.0±0.2
Distilled water	1000 ml

EMB Agar Media

Ingredients	Amount
Agar powder	37.5 g
Distilled water	1000 ml
pH	7.2 ± 0.2

Iodine solution (SAB 1957)

Ingredients	Amount
Iodine	0.33 g
Potassium Iodide	0.66 g
Distilled water	100 ml

Triple Sugar Iron Agar (TSI) Medium

Ingredients	Amount
Beef extract	3 g
Yeast extract	3 g
Peptone	20 g
Glucose	1 g
Lactose	10 g
Sucrose	10 g
$Na_2S_2O_3$	0.3 g
$FeSO_4$	0.2 g
NaCl	5 g
Phenol red	0.024 g
Agar	13 g
Distilled water	1000 ml
pH	7.4 ± 0.2

Methyl Red/Voges-Proskauer broth medium

Ingredients	Amount
Peptone	7 g
Dextrose	5 g
Dipotassium phosphate	5 g
pH	6.9 ± 0.2
Distilled water	1000 ml

Methyl red solution (Bryan 1950)

Ingredients	Amount
Methyl red	0.1 gm
Ethyl alcohol (95%)	300 ml
Distilled water	200 ml

Motility Test Medium

Ingredients	Amount
Beef extract	3 g
Tryptone	10 g
NaCl	5 g
Agar	4 g
Distilled water	1000 ml

α -Napthol solution (Bryan 1950)

Ingredients	Amount
α –Napthol	15 g
Ethyl alcohol (95%)	100 ml

Nutrient gelatin medium

Ingredients	Amount
Peptone	5 g
Beef extract	3 g
Gelatin	120 g
pH	6.8 ± 0.2

Distilled water	1000ml

Luria Agar Medium

Ingredients	**Amount**
Peptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar	15 g
Distilled water	1000ml
pH	7.0 ± 0.2

Luria Broth Medium

Ingredients	**Amount**
Peptone	10 g
Yeast extract	5 g
NaCl	10 g
Distilled water	1000ml
pH	7.0 ± 0.2

Oxidase test reagent (Claus 1995)

Ingredients	**Amount**
Tetramethyl-p-phenylene-diamine dihydro-chloride	1 g
Ethyl alcohol (95%)	100 ml
Distilled water	100 ml

Physiological saline

Ingredients	**Amount**
Sodium chloride	0.85 g
Distilled water	100 ml

Saffranin solution

Ingredients	Amount
Safranin	2.5 g
Ethyl alcohol (95%)	100 ml

Simmon's citrate agar

Ingredients	Amount
$MgSO_4$. $7H_2O$	0.2 g
Ammonium dihydogen phosphate	1 g
Dipotasssium phosphate	1 g
Na-citrate	2 g
NaCl	5 g
Bromo-thymol-blue	0.08 g
Agar	15 g
Distilled water	1000 ml
pH	6.9 ± 0.2

Thiosulphate Citrate Bile salts Sucrose Agar Medium

Ingredients	Amount
Agar powder	88 g
Distilled water	1000 ml
pH	8.6 ± 0.2

Tryptophan peptone broth

Ingredients	Amount
Peptone	10 g
NaCl	5 g
Tryptone	10 g
Distilled water	1000 ml

Starch Agar Medium

Ingredients	Amount
Beef extract	3 g
Soluble starch	10 g
Agar	12 g
Distilled water	1000 ml
pH	7.5 ± 0.2

SS Agar Medium

Ingredients	Amount
Agar powder	63.02 g
Distilled water	1000 ml
pH	7.0 ± 0.2

Mannitol salt Agar Medium

Ingredients	Amount
Agar powder	111 g
Distilled water	1000 ml
pH	7.4 ± 0.2

Appendix C

SPSS Output

One way ANOVA to determine the variation between species to species

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
TBC	Rui fish	9	52697888.89	20124416.764	6708138.921	37228892.80	68166884.98	20580000	78950000
	Silver Carp Fish	9	30154876.67	14264084.270	4754694.757	19190530.90	41119222.44	6480000	45510540
	Total	18	41426382.78	20514691.008	4835359.042	31224666.95	51628098.61	6480000	78950000
TCBS	Rui fish	9	1576788.89	3511551.160	1170517.053	-1122428.28	4276006.05	0	10620000
	Silver Carp Fish	9	2381.11	3632.449	1210.816	-411.04	5173.26	120	11680
	Total	18	789585.00	2541449.995	599025.508	-474248.35	2053418.35	0	10620000
SS	Rui fish	9	6936666.67	7147592.252	2382530.751	1442540.90	12430792.43	590000	23850000
	Silver Carp Fish	9	1105555.56	965389.962	321796.654	363491.14	1847619.97	320000	3480000
	Total	18	4021111.11	5786235.075	1363828.686	1143684.10	6898538.12	320000	23850000
ALA	Rui fish	9	13092222.22	10592880.602	3530960.201	4949813.40	21234631.05	2540000	32000000
	Silver Carp Fish	9	6093333.33	4606677.219	1535559.073	2552327.76	9634338.91	320000	13510000
	Total	18	9592777.78	8703869.191	2051521.642	5264445.46	13921110.10	320000	32000000
MSA	Rui fish	9	10286666.67	5280629.697	1760209.899	6227615.36	14345717.97	880000	15600000
	Silver Carp Fish	9	5476666.67	3985570.850	1328523.617	2413085.71	8540247.62	300000	10150000
	Total	18	7881666.67	5169315.861	1218419.433	5311026.37	10452306.97	300000	15600000
EMB	Rui fish	9	16827777.78	9789260.414	3263086.805	9303086.11	24352469.44	4640000	38510000
	Silver Carp Fish	9	13900000.00	23483526.673	7827842.224	-4151036.54	31951036.54	1270000	75900000
	Total	18	15363888.89	17518089.185	4129053.219	6652348.09	24075429.69	1270000	75900000

ANOVA						
		Sum of Squares	df	Mean Square	F	Sig.
TBC	Between Groups	2286843300230672.000	1	2286843300230672.000	7.517	.014
	Within Groups	4867650001325689.000	16	304228125082855.560		
	Total	7154493301556361.000	17			
TCBS	Between Groups	11154419328272.223	1	11154419328272.223	1.809	.197
	Within Groups	98648037966377.780	16	6165502372898.611		
	Total	109802457294650.000	17			
SS	Between Groups	153008355555555.560	1	153008355555555.560	5.883	.027
	Within Groups	416160422222222.250	16	26010026388888.890		
	Total	569168777777777.800	17			
ALA	Between Groups	220430005555555.560	1	220430005555555.560	3.304	.088
	Within Groups	1067444755555555.500	16	66715297222222.220		
	Total	1287874761111111.000	17			
MSA	Between Groups	104112449999999.970	1	104112449999999.970	4.757	.044
	Within Groups	350158600000000.000	16	21884912500000.000		
	Total	454271050000000.000	17			
EMB	Between Groups	38573472222222.180	1	38573472222222.180	.119	.734
	Within Groups	5178445155555556.000	16	323652822222222.250		
	Total	5217018627777778.000	17			

Mean bacterial load (cfu/g) with SD and two way ANOVA variable detected in the LA plates of the samples sampled from both Rohu and Silver carp from 3 different markets

Two way ANOVA

Descriptive Statistics

Dependent Variable: TBC

Species	Market	Mean	Std. Deviation	N
Rui fish	Nobabgonj Bazar	42597000.00	17554975.278	3
	Palashi Bazar	66030000.00	15796116.611	3
	Anando Bazar	49466666.67	25039034.193	3
	Total	52697888.89	20124416.764	9
Silver Carp Fish	Nobabgonj Bazar	25096846.67	19577212.688	3
	Palashi Bazar	27353373.33	16584050.667	3
	Anando Bazar	38014410.00	3570977.193	3
	Total	30154876.67	14264084.270	9
Total	Nobabgonj Bazar	33846923.33	19195165.419	6
	Palashi Bazar	46691686.67	25662884.672	6
	Anando Bazar	43740538.33	17182208.805	6
	Total	41426382.78	20514691.008	18

Tests of Between-Subjects Effects

Dependent Variable: TBC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[b]
Corrected Model	34430981783289 59.000[a]	5	688619635665791 .800	2.227	.119	.481	11.133	.518
Intercept	30890613420917 332.000	1	308906134209173 32.000	99.878	.000	.893	99.878	1.000
Species	22868433002306 72.500	1	228684330023067 2.500	7.394	.019	.381	7.394	.704
Market	54316167868581 1.000	2	271580839342905 .500	.878	.441	.128	1.756	.167
Species * Market	61309319941247 7.900	2	306546599706238 .940	.991	.400	.142	1.982	.183
Error	37113951232274 00.000	12	309282926935616 .700					
Total	38045106722473 696.000	18						
Corrected Total	71544933015563 59.000	17						

a. R Squared = .481 (Adjusted R Squared = .265)

b. Computed using alpha = .05

Post Hoc Tests

Multiple Comparisons						
Dependent Variable: TBC Tukey HSD						
(I) Market	(J) Market	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Nobabgonj Bazar	Palashi Bazar	-12844763.33	10153536.772	.440	-39933015.10	14243488.43
	Anando Bazar	-9893615.00	10153536.772	.606	-36981866.77	17194636.77
Palashi Bazar	Nobabgonj Bazar	12844763.33	10153536.772	.440	-14243488.43	39933015.10
	Anando Bazar	2951148.33	10153536.772	.955	-24137103.43	30039400.10
Anando Bazar	Nobabgonj Bazar	9893615.00	10153536.772	.606	-17194636.77	36981866.77
	Palashi Bazar	-2951148.33	10153536.772	.955	-30039400.10	24137103.43
Based on observed means. The error term is Mean Square (Error) = 309282926935616.700.						

Mean bacterial load (cfu/g) with SD and two way ANOVA variable detected in the TCBS, SS, AA and MSA, EMB plates of the samples sampled from both Rohu and Silver carp from 3 different markets

2 way ANOVA

Descriptive Statistics				
Dependent Variable: TCBS				
Species	Market	Mean	Std. Deviation	N
Rui fish	Nobabgonj Bazar	80366.67	129662.652	3
	Palashi Bazar	1020000.00	1580000.000	3
	Anando Bazar	3630000.00	6055022.708	3
	Total	1576788.89	3511551.160	9
Silver Carp Fish	Nobabgonj Bazar	1910.00	1267.162	3
	Palashi Bazar	263.33	124.231	3
	Anando Bazar	4970.00	5834.698	3
	Total	2381.11	3632.449	9
Total	Nobabgonj Bazar	41138.33	92586.382	6
	Palashi Bazar	510131.67	1144778.960	6
	Anando Bazar	1817485.00	4313651.164	6
	Total	789585.00	2541449.995	18

Tests of Between-Subjects Effects

Dependent Variable: TCBS

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[b]
Corrected Model	31449361158316.688[a]	5	6289872231663.338	.963	.477	.286	4.817	.235
Intercept	11222000500049.994	1	11222000500049.994	1.719	.214	.125	1.719	.227
Species	11154419328272.217	1	11154419328272.217	1.708	.216	.125	1.708	.226
Market	10169069930133.336	2	5084534965066.668	.779	.481	.115	1.557	.153
Species * Market	10125871899911.113	2	5062935949955.557	.775	.482	.114	1.551	.152
Error	78353096136333.310	12	6529424678027.776					
Total	12102445779470000.000	18						
Corrected Total	10980245729465000.000	17						

a. R Squared = .286 (Adjusted R Squared = -.011)

b. Computed using alpha = .05

Post Hoc Tests

Multiple Comparisons

Dependent Variable: TCBS

Tukey HSD

(I) Market	(J) Market	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Nobabgonj Bazar	Palashi Bazar	-468993.33	1475288.071	.946	-4404860.76	3466874.10
	Anando Bazar	-1776346.67	1475288.071	.473	-5712214.10	2159520.76
Palashi Bazar	Nobabgonj Bazar	468993.33	1475288.071	.946	-3466874.10	4404860.76
	Anando Bazar	-1307353.33	1475288.071	.659	-5243220.76	2628514.10
Anando Bazar	Nobabgonj Bazar	1776346.67	1475288.071	.473	-2159520.76	5712214.10
	Palashi Bazar	1307353.33	1475288.071	.659	-2628514.10	5243220.76

Based on observed means.

The error term is Mean Square (Error) = 6529424678027.776.

Dependent Variable: SS

Species	Market	Mean	Std. Deviation	N
Rui fish	Nobabgonj Bazar	3210000.00	3009800.658	3
	Palashi Bazar	5740000.00	555697.760	3
	Anando Bazar	11860000.00	11646703.396	3
	Total	6936666.67	7147592.252	9
Silver Carp Fish	Nobabgonj Bazar	1080000.00	441361.530	3
	Palashi Bazar	1690000.00	1563681.553	3
	Anando Bazar	546666.67	325781.113	3
	Total	1105555.56	965389.962	9
Total	Nobabgonj Bazar	2145000.00	2250011.111	6
	Palashi Bazar	3715000.00	2454039.527	6
	Anando Bazar	6203333.33	9627989.752	6
	Total	4021111.11	5786235.075	18

Tests of Between-Subjects Effects

Dependent Variable: SS

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power [b]
Corrected Model	273649911111111.120 [a]	5	54729982222222.230	2.222	.119	.481	11.112	.517
Intercept	291048022222222.100	1	291048022222222.100	11.818	.005	.496	11.818	.883
Species	153008355555555.470	1	153008355555555.470	6.213	.028	.341	6.213	.629
Market	50253544444444.440	2	25126772222222.220	1.020	.390	.145	2.041	.187
Species * Market	70388011111111.100	2	35194005555555.550	1.429	.278	.192	2.858	.248
Error	295518866666666.600	12	24626572222222.220					
Total	860216800000000.000	18						
Corrected Total	569168777777777.800	17						

a. R Squared = .481 (Adjusted R Squared = .264)

b. Computed using alpha = .05

Multiple Comparisons

Dependent Variable: SS

Tukey HSD

(I) Market	(J) Market	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Nobabgonj Bazar	Palashi Bazar	-1570000.00	2865110.366	.849	-9213723.82	6073723.82
	Anando Bazar	-4058333.33	2865110.366	.364	-11702057.16	3585390.49
Palashi Bazar	Nobabgonj Bazar	1570000.00	2865110.366	.849	-6073723.82	9213723.82
	Anando Bazar	-2488333.33	2865110.366	.669	-10132057.16	5155390.49
Anando Bazar	Nobabgonj Bazar	4058333.33	2865110.366	.364	-3585390.49	11702057.16
	Palashi Bazar	2488333.33	2865110.366	.669	-5155390.49	10132057.16

Based on observed means.

The error term is Mean Square (Error) = 24626572222222.220.

Descriptive Statistics

Dependent Variable: AA

Species	Market	Mean	Std. Deviation	N
Rui fish	Nobabgonj Bazar	6890000.00	7029658.598	3
	Palashi Bazar	20876666.67	9884464.241	3
	Anando Bazar	11510000.00	12221493.362	3
	Total	13092222.22	10592880.602	9
Silver Carp Fish	Nobabgonj Bazar	4313333.33	4513317.331	3
	Palashi Bazar	4006666.67	3633515.286	3
	Anando Bazar	9960000.00	4195581.009	3
	Total	6093333.33	4606677.219	9
Total	Nobabgonj Bazar	5601666.67	5468657.666	6
	Palashi Bazar	12441666.67	11390394.930	6
	Anando Bazar	10735000.00	8216317.302	6
	Total	9592777.78	8703869.191	18

Tests of Between-Subjects Effects

Dependent Variable: AA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[b]
Corrected Model	592556761111111.500[a]	5	118511352222222.300	2.045	.144	.460	10.227	.479
Intercept	1656384938888889.500	1	165638493888 8889.500	28.586	.000	.704	28.586	.998
Species	220430005555555.660	1	220430005555555.660	3.804	.075	.241	3.804	.434
Market	152098844444444.470	2	76049422222222.230	1.312	.305	.179	2.625	.230
Species * Market	220027911111111.220	2	110013955555555.610	1.899	.192	.240	3.797	.318
Error	695318000000000.000	12	57943166666666.664					
Total	2944259700000000.000	18						
Corrected Total	1287874761111111.500	17						

a. R Squared = .460 (Adjusted R Squared = .235)

b. Computed using alpha = .05

Multiple Comparisons

Dependent Variable: AA

Tukey HSD

(I) Market	(J) Market	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Nobabgonj Bazar	Palashi Bazar	-6840000.00	4394813.863	.301	-18564764.19	4884764.19
	Anando Bazar	-5133333.33	4394813.863	.493	-16858097.53	6591430.86
Palashi Bazar	Nobabgonj Bazar	6840000.00	4394813.863	.301	-4884764.19	18564764.19
	Anando Bazar	1706666.67	4394813.863	.921	-10018097.53	13431430.86
Anando Bazar	Nobabgonj Bazar	5133333.33	4394813.863	.493	-6591430.86	16858097.53

Palashi Bazar	-1706666.67	4394813.863	.921	-13431430.86	10018097.53

Based on observed means.

The error term is Mean Square (Error) = 57943166666666.660.

Descriptive Statistics

Dependent Variable: MSA count of bacterial density

Species	Market	Mean	Std. Deviation	N
Rui fish	Nobabgonj Bazar	9200000.00	7444037.883	3
	Palashi Bazar	9336666.67	5656238.444	3
	Anando Bazar	12323333.33	3845313.165	3
	Total	10286666.67	5280629.697	9
Silver Carp Fish	Nobabgonj Bazar	5530000.00	4352321.220	3
	Palashi Bazar	5430000.00	4937782.903	3
	Anando Bazar	5470000.00	4495230.806	3
	Total	5476666.67	3985570.850	9
Total	Nobabgonj Bazar	7365000.00	5812334.299	6
	Palashi Bazar	7383333.33	5208503.304	6
	Anando Bazar	8896666.67	5299798.738	6
	Total	7881666.67	5169315.861	18

Tests of Between-Subjects Effects

Dependent Variable: MSA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[b]
Corrected Model	122821716666666.690[a]	5	2456434333333.336	.889	.518	.270	4.447	.219
Intercept	1118172049999999.500	1	1118172049999999.500	40.483	.000	.771	40.483	1.000
Species	1041124499999999.970	1	1041124499999999.970	3.769	.076	.239	3.769	.431
Market	9273033333333.318	2	4636516666666.659	.168	.847	.027	.336	.070
Species * Market	9436233333333.332	2	4718116666666.666	.171	.845	.028	.342	.071
Error	331449333333333.300	12	27620777777777.777					
Total	1572443100000000.000	18						

Corrected Total	45427105000 0000.000	17						

a. R Squared = .270 (Adjusted R Squared = -.034)

b. Computed using alpha = .05

Multiple Comparisons

Dependent Variable: MSA

Tukey HSD

(I) Market	(J) Market	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Nobabgonj Bazar	Palashi Bazar	-18333.33	3034291.668	1.000	-8113409.81	8076743.14
	Anando Bazar	-1531666.67	3034291.668	.870	-9626743.14	6563409.81
Palashi Bazar	Nobabgonj Bazar	18333.33	3034291.668	1.000	-8076743.14	8113409.81
	Anando Bazar	-1513333.33	3034291.668	.873	-9608409.81	6581743.14
Anando Bazar	Nobabgonj Bazar	1531666.67	3034291.668	.870	-6563409.81	9626743.14
	Palashi Bazar	1513333.33	3034291.668	.873	-6581743.14	9608409.81

Based on observed means.

The error term is Mean Square (Error) = 27620777777777.770.

Descriptive Statistics

Dependent Variable: EMB

Species	Market	Mean	Std. Deviation	N
Rui fish	Nobabgonj Bazar	16020000.00	4010935.053	3
	Palashi Bazar	19766666.67	17222207.563	3
	Anando Bazar	14696666.67	7062551.475	3
	Total	16827777.78	9789260.414	9
Silver Carp Fish	Nobabgonj Bazar	30526666.67	39489504.091	3
	Palashi Bazar	6643333.33	3605444.956	3
	Anando Bazar	4530000.00	2851438.234	3
	Total	13900000.00	23483526.673	9
Total	Nobabgonj Bazar	23273333.33	26331282.283	6
	Palashi Bazar	13205000.00	13247944.369	6
	Anando Bazar	9613333.33	7362911.562	6
	Total	15363888.89	17518089.185	18

Tests of Between-Subjects Effects

Dependent Variable: EMB

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[b]
Corrected Model	1330773561111111.000[a]	5	266154712222222.200	.822	.557	.255	4.109	.204
Intercept	4248883472222220.500	1	4248883472222220.500	13.120	.004	.522	13.120	.913
Species	3857347222222.195	1	3857347222222.195	.119	.736	.010	.119	.062
Market	6017340111111111.500	2	3008670055555555.750	.929	.422	.134	1.858	.174
Species * Market	6904660777777777.900	2	3452330388888888.940	1.066	.375	.151	2.132	.194
Error	3886245066666667.000	12	323853755555555.560					
Total	9465902100000000.000	18						
Corrected Total	5217018627777778.000	17						

a. R Squared = .255 (Adjusted R Squared = -.055)

b. Computed using alpha = .05

Multiple Comparisons

Dependent Variable: EMB

Tukey HSD

(I) Market	(J) Market	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Nobabgonj Bazar	Palashi Bazar	10068333.33	10389959.184	.609	-17650661.19	37787327.86
	Anando Bazar	13660000.00	10389959.184	.414	-14058994.53	41378994.53
Palashi Bazar	Nobabgonj Bazar	-10068333.33	10389959.184	.609	-37787327.86	17650661.19
	Anando Bazar	3591666.67	10389959.184	.937	-24127327.86	31310661.19
Anando Bazar	Nobabgonj Bazar	-13660000.00	10389959.184	.414	-41378994.53	14058994.53
	Palashi Bazar	-3591666.67	10389959.184	.937	-31310661.19	24127327.86

Based on observed means.

The error term is Mean Square (Error) = 323853755555555.600.

Appendix D

16S rRNA Sequences of 10 representative strains isolated in this study (1341 bp)

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