Project ID 493

Competitive Research Grant

Sub-Project Completion Report

on

Characterization of Commercial Probiotics being Used for Fish and Shrimp Culture in Bangladesh

Project Duration

May 2017 to September 2018

Department of Fisheries Biology and Aquatic Environment, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur – 1706

Project Implementation Unit-BARC, NATP 2

Bangladesh Agricultural Research Council

Farmgate, Dhaka-1215

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Project Implementation Unit
National Agricultural Technology Program-Phase II Project (NATP-2)
Bangladesh Agricultural Research Council (BARC)
New Airport Road, Farmgate, Dhaka – 1215
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Acronyms

BARC	: Bangladesh Agricultural Research Council
NATP	: National Agricultural Technology Project
GOB	: Government of Bangladesh
MRS	: de-Man Rogosa agar
LB	: Luria Broth
NB	:Nutrient Broth
PDA	: Potato Dextrose Agar
BHI	: Brain Heart Infusion
TSB	: Tryptone Soya Broth
ASP	: Aspergillus Differentiated Media
AAG	: Auto-aggregation
MT	: Metric Ton
OD	: Optical Density
H_2O_2	:Hydrogen Peroxide
NaOH	: Sodium Hydroxide
hr	: Hour
µg	: Microgram
nm	: bNano-meter
mm	: Millimeter
ml	: Milliliter
α	: Alpha
β	: Beta
Y	: Gamma
EDTA	: Ethylene-Diamine-tetra-Acetic Acid
DNA	: Deoxyribo-nucleic Acid
CFU	: Colony Forming Unit
BD	: Bangladesh
UV	: Ultra-violate
LAB	: Lactic Acid Bacteria

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Executive Summary

Probiotics are living beneficial microorganisms which confer positive health benefits to the host that has been used in aquaculture in Bangladesh since last decades. However, no comprehensive information on the commercial probiotic products has been found in the literature. Therefore, this study was carried out to isolate and characterize the commercial probiotics being used for fish and shrimp culture in Bangladesh. A total of 31 probiotic products were collected from aqua product sellers and stored of which 27 products were in powder form and 4 in liquid state. All the products were imported from foreign countries like Japan, Thailand, Vietnam and India and in most cases no details label information were found. For isolating the microorganisms, eight different types of selective media such as MRS, LB, NB, PDA, BHI, TSB, Aspergillus differentiated, antibiotic resistance etc. were used. A total of 122 isolates were isolated from twenty-two companies' commercial probiotics and stored in 30% glycerol at -22^{0}C. For molecular identification of isolates, DNA sequence was performed. The results revealed that diversified genus including 59% *Enterobacter*, 15% *Aeromona*s, 13% *Enterococcus*, 8% *Streptococcus* and 2% *Bacillus, Rhodobacter, Moraxella* were identified. The preliminary identification of the selected 122 bacterial isolates were carried out based on morphological and biochemical tests such as gram staining, catalase and oxidase test. During biochemical characterization, 80/122 isolates were found gram positive and 42/122 gram negative, 56/122 oxidase positive and 66/122 oxidase negative, 115/122 catalase positive and 7/122 catalase negative. To determine the probiotic properties, different in-vitro assay such as pH tolerance, bile salt tolerance and auto-aggregation were performed. Most of the tested isolates showed moderate tolerance in acidic condition whereas high resistance in bile salt. In case of auto-aggregation, most of the isolates showed low auto-aggregation capacity. In addition to that antibiotic resistance and hemolytic assay of different isolates were tested as well. A total of 56 isolates were screened for antibiotic resistance and showed different level of resistance in response to different antibiotics. To determine the pathogenicity, a total of 122 isolates were screened out by hemolytic assay and 48 isolates found to show β hemolysis indicating complete lysis of blood cell. Based on the findings it can be concluded that imported probiotic products are in question in terms of label information, contamination with pathogenic microbes, antibiotic susceptibilities as well as other probiotic properties which ultimately hamper the food safety and quality. More comprehensive study is needed to assess these properties of commercial probiotic products and emphasis should be given on the evaluation of efficacy of different commercial probiotic products according to their instructions of uses.

CRG Sub-Project Completion Report (PCR)

A. Sub-project Description

1. **Title of the CRG sub-project:** Characterization of commercial probiotics being used for fish and shrimp culture in Bangladesh.

2. **Implementing organization:**Department of Fisheries Biology and Aquatic Environment, Faculty of Fisheries, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur-1706

3. **Name and full address with phone, cell and E-mail of PI/Co-PI (s):**
   Principle Investigator: Dr. S. M. Rafiquzzaman, Associate Professor, Department of Fisheries Biology and Aquatic Environment, Faculty of Fisheries, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur-1706. Mobile: +8801754041311, e-mail: bokul44@yahoo.com
   Co- Principle Investigator: Dr. Mohammad ShafiqulAlam, Assistant Professor, Department of Genetics and Fish Breeding, Faculty of Fisheries, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur-1706. Mobile: +8801712718483 e-mail: msalamjp@gmail.com

4. **Sub-project budget (Tk):**
   4.1 Total: 34,72,768.00
   4.2. Revised (if any): Not Applicable

5. **Duration of the sub-project:**
   5.1. Start date (based on LoA signed): April, 2017
   5.2. End date: 30 September 2018

6. **Justification of undertaking the sub-project:**

Fisheries and aquaculture in Bangladesh have changed dramatically since last two decades. Fish production of Bangladesh is increasing every year and the yearly rate of increment is 5.35% during last five years. Total fish production of Bangladesh is 3.68 million MT in 2014-15 which has a value of 60 thousand crore taka. Aquaculture has increasingly been playing a major role in total fish production of the country and the production has been achieved from dominantly aquaculture in inland closed waters. In spite of that still there is scope of increasing of fish production through intensification of aquaculture in the country. Various emerging diseases in fish and shrimp farming are identified as major barriers for sustaining and enhancing the productivity. To combat these diseases, farmers are using globally banned antibiotics those causes locally health hazard and internationally rejection of fish and shrimpproducts in the international market causes reduction of foreign exchange earnings. Although probiotic has been used as effective alternative for controlling diseases and enhancing the aquaculture production throughout the world. In Bangladesh use of probiotics in aquaculture has been started since last decades. But all the probiotics using in shrimp and fish culture imported from other countries. Where there is question about their efficacy, survivability, contamination with pathogenic strain of imported probiotic products. Till now no qualitative analysis-basedinformation is available on commercial probiotics being used by Bangladeshi farmers. Additionally, concerns have also been raised about the information on probiotic product labels provided by sellers. In some instances, probiotic bacteria may show antimicrobial resistance and contaminated with pathogenic strain. Therefore, this research project had been undertakento identify and characterizethe commercially available probiotic products used for fish and shrimp culture, and evaluate the probiotic properties, antimicrobial susceptibilities and efficacy of commercial probiotics at farmer's level.

7. **Sub-project goal:**To develop comprehensive microbial database of probiotics used for fish and shrimp culture in Bangladesh for ensuring food safety and quality

8. **Sub-project objective (s):**
   i. To enlist probiotics being used for aquaculture in Bangladesh.
   ii. To isolate and characterize bacteria from commercial probiotics being used for aquaculture
   iii. To determine the probiotic properties and antibiotic susceptibilities of different bacterial isolates.

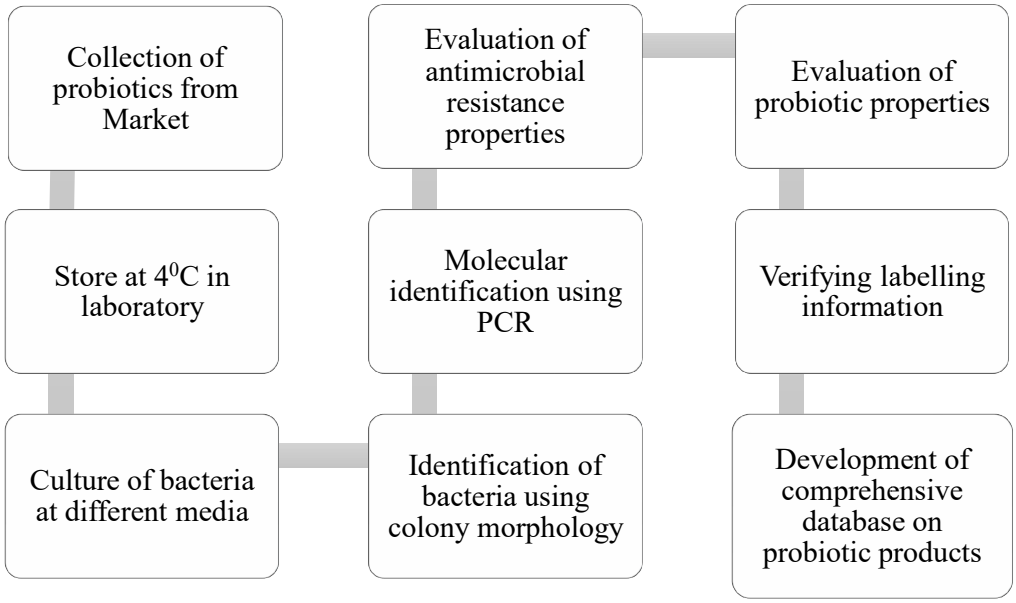
9. **Implementing location (s):** Department of Fisheries Biology and Aquatic Environment

10. **Methodology in brief:**

Approaches/Methodology:

Approaches:

The following flow chart reflecting the sequential approaches of the research starting from probiotic sample collection to development of comprehensives data base on probiotic products used for shrimp and fish culture in Bangladesh.



10.1 Collection of Probiotics Products

[A Total of 31 probiotic products were collected mainly from the shops that deal with aqua feed and aqua chemicals and also from some farmers who weredirectly involved in the application of those probiotics. After collection, samples were stored aseptically in air tight jars to protect them from contamination and deterioration for further study.

10.2 Sterilization and Disinfection

All the glass wares and equipment related to this work were sterilized and disinfected by autoclaving at 121^0C temperature for 15 minutes. Before starting work in clean bench the UV light was switched on for 10-15 minutes. Just before entering into the clean bench 70% ethanol was sprayed over the hands as well as on the working top of the clean bench.

10.3 Media Preparation

Eight different types of selective media were prepared according to the instructions given on the media. Those were de-Man Rogosa Sharpe (MRS) media, Aspergillus differentiated media, Potato Dextrose Agar (PDA) media, Nutrient broth media (NB), Antibiotic resistance media, Brain Heart Infusion (BHI) media, Luria broth (LB) media and Trypton Soya broth (TSB) media. These media were autoclaved at 121^0 C temperature for 15 minutes. Then plating of each media was done in the clean bench and plates were kept in the clean bench for solidifying. These plates were labeled properly according to the media name and preparation date. These plates were used for culture of different microbes from different commercial probiotics. These plates were stored at 4^0C temperature.

10.4 Dilution of Probiotic Sample

One gram of each probiotic was measured aseptically with electronic balance and diluted in 10-fold by autoclaved distilled water in clean bench. All the dilutes were kept in incubator at 30^0C temperature overnight for the activation of microbes.

10.5 Isolation of Microbial Strains

Initially the probiotics were isolated on new cultured plates by their visual morphological characteristics. Basically, pure colonies were isolated by their colony characteristics: color, opacity, surface, border structures and diameter size. These plates (newly streaked plates from old mixed plates) were kept in incubation at previously described temperature. After pure colony streaking, each isolates were labeled properly as different number to avoid any kind of misleading activities. Continuous observation was done and incubation time required for proper growth wasnoted properly. These plates are kept in 4^0 C temperature for short time until the final stocking is done.

10.6 Liquid Culture

At first different liquid media like MRS broth, Potato Dextrose broth, Nutrient broth etc. were prepared and autoclaved and poured into tubes. Each tube contained 10 ml of liquid media. The isolates were cultured in appropriate media by pipette tips. After that all the culture tubes were placed in incubation in their suitable temperature as previously described for overnight.
In the following day, from these liquid cultured tubes, further liquid cultures were done. For this new culture 9ml fresh liquid media and 1ml of existing liquid culture were placed in different incubation temperature asbefore for 4.30-5.00 hours(OD=1).

10.7 Stocking

30 % of 50 % glycerol solutions were kept in autoclaved eppendorf tubes. Then they were labeled according to probiotic particulars. After that 700µl from 2^{nd} liquid culture was taken and placed in 30 % glycerol containing eppendorftube. Single microbial strain was stocked in total 10 eppendrop tube. Finally, 10 tube of a single microbial strain were labeled collectively and kept in -22^0 C.

10.8 Biochemical characterization of the isolates:
10.8.1 Gram staining

According to Cappuccino & Sherman (2005), Gram staining was performed to observe the morphological characteristics of the organisms by taking inoculum from broth culture and pure agar medium culture. A grease free glass slide was taken on which a thin smear of the selected bacteria was prepared and left it for air dry. Then the slide with the specimen was heat fixed by passing it over a heat source, such as a flame, several times using a forceps. The slide was passed very quickly through the flame and not to be heated excessively. The fixed smear was flooded with crystal violet solution and allowed to remain for 1 minute and then the crystal violet was rinsed off with distilled water. Then the slide was flooded with iodine solution and allowed to remain for 1 minute. The iodine solution was washed with distilled water and then it was decolorized with few drops of 95% ethanol for 5-15 seconds. The decolorizer was washed thoroughly with distilled water. After that the slide was stained with safranin which was allowed to remain for 30 seconds. The safranin was rinsed off with distilled water and then it was air dried and placed in an upright position. Finally, the slide was examined with emersion oil in light microscope under 100X.

10.8.2 Catalase test

Microorganisms capable of producing catalase rapidly degrade H_2O_2 to oxygen and water. This test is used to determine the ability of microorganisms to degrade H_2O_2 by producing the enzyme catalase. Two round circles were made in the glass slide and one drop of distilled water was placed in each of circled area. Overnight bacterial cultures were taken in the marked area and pea shaped smear formed. One to two drop of H_2O_2were added over the test smear and in the control H_2O_2 was not added. Then the changes in the test smear and bubble formation were noted down.

10.8.3 Oxidase test

The oxidase test is a test used in microbiology to determine if a bacterium produces certain cytochrome c oxidase. A filter paper was taken and was soaked with the substrate N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride. Then the paper was moistened with sterile distilled water. After that the test organism colony was picked with a platinum loop and smeared in the filter paper. Finally, the inoculated area of the paper was observed for a color change to deep blue or purple within 10-30 seconds.

10.8 Identification of Probiotic Properties:

Probiotics properties were identified by colony morphology, physiological and as well as some biochemical characteristics. Different probiotic properties like tolerance against bile, resistance to low pH, physiological and biochemical characterization were identified through *in vitro* testing

10.9.1 Bile salt tolerance test

Bile salt tolerance was further tested in different selective broth (MRS, NB, PDA, TS, ASP, LB and BHI) which included 0.0, 0.15, 0.3 and 0.5% (w/v) Bile salt (Sigma Chemical Co.St. Louis, MO, USA). Duplicate bottles of these broth containing filtered different concentrations of bile salt were inoculated by 100 µl of cultured strain and incubated at 25°C, 30°C, and 37°C based on media. Growth rate was assessed by measuring the optical density by spectrophotometer at 600 nm after 1, 2, 3 and 4 h incubation (Kim & Austin, 2008; Balcázar*et al*., 2008). The viability of the isolates was also controlled by duplicate inoculation on MRS, NB, PDA, TS, ASP, LB and BHI agar (Balcázar*et al*., 2008; Kim & Austin, 2008).

10.9.2 pH tolerance test

Acid and alkalinity tolerance of the selected bacterium was investigated at different pH. First, different selective broths with different pH including 3, 4, 5, 6, 7,8, 9 and 10 were prepared using HCl 1% and NaOH 1N and divided in universal bottles (Samelis*et al*., 1994). The broths media along with control bottles were autoclaved at 121°C for 15 min and then inoculated with 4 h sub-culture broth with an OD value ranging 0.8 to 1.0 of the selected strain in different selective broth followed by incubation at 25, 30 and 37°C. Optical density (OD) as growth rate of bacteria was measured by spectrophotometer at 600 nm after 1, 2, 3 and 4 h incubation. The viability of the isolates was also controlled by duplicate inoculation on MRS, NB, PDA, TS, ASP, LB and BHI agar (Balcázar*et al*., 2008; Kim & Austin, 2008).

10.9.3 Auto aggregation assay

This assay was performed according to Del Re *et al*., (2000) with some modifications. Frozen stock of the isolates was reactivated by two consecutive sub-cultures in MRS and LB broth at 37^0C for 12-72 hrs NB, BHI and PDA broth at 30^0C for 24-48 hrs and ASP and TS broth in 25^0C for 24h under anaerobic condition. After reactivating, the overnight culture of the respective isolate was inoculated at 1% (v/v) in a test tube containing 10 ml of MRS, LB, BHI, NB, PDA, ASP and TS media and incubated at 37^0C, 30^0C and 25^0C for 24 h under anaerobic condition. After growing the 30 reactivated isolates in all selective broth, bacterial cells were harvested by centrifugation at 14000g for 5 minutes, washed twice with 50 Mm K_2HPO_4 (pH 7.00) and then resuspended in the same buffer to an absorbance of about 0.2 at 600 nm (bacterial suspension). 5 milliliters of bacterial suspension were mixed well by vertexing for 15 s and then allowed to stand at room temperature.

After 5 h interval, changes in absorbance of the top portion of the cultured media due to precipitation were monitored at 600 nm using a spectrophotometer. Auto aggregation ability was expressed as auto aggregation percentage (AAg %) and calculated using the formula as follows:

$AAg\ \% = [(A0 – A)/ A0] \times 100$

Where A0 and A are the absorbances of cultured media at 0 h and after 4 h intervals, respectively. From AAg %, isolate could be classified into three groups: high auto aggregation (H AAg: > 70 % AAg), medium auto aggregation (AAg: 20 – 70 % AAg), and low auto aggregation (L AAg: < 20 %

10.10 Antibiotic resistance test

Antibiotic sensitivity test was carried out for selected strain on the most common antibiotics in aquaculture by disc diffusion technique. They included Ciprofloxacin (5 µg), Cephalexin (30 µg), Tetracycline(30 µg), Amoxycillin (10 µg) and Doxycycline(30 µg). 50 µl of the 4 h sub-culture broth with an OD value ranging 0.8 to 1.0 of the strain were spread on different selective agar and, antibiotic Bio-discs were subsequently placed on plates. Finally, the plates were incubated at 25°C, 30°C and 37°C for 24 h to observe and measure the inhibition zone (Kim & Austin, 2008). The interpretations and zone sizes were illustrated based on table of Kirby-Bauer test (Bauer *et al*., 1966).

10.11 Hemolytic activity test

This test was performed to determine either the organism was pathogenic or non- pathogenic. At 18-24 hour grown colony of the selected bacteria was streaked on Blood agar plate with 5% sheep blood. Then the plate was incubated. Result was examined after 24 hours incubation. The result was interpreted as follows.

Alpha-hemolysis: This was indicated by presence of brown-green discoloration under the bacterial growth.

Beta- hemolysis: This was observed from the clearing of red color from the agar around the bacteria due to breakdown of RBC in the agar.
Gamma-hemolysis: The lack of discoloration or clearing of medium indicated gamma- hemolytic bacteria (negative for hemolysis).

10.12 Extraction of genomic DNA

The genomic DNA was extracted by using the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. At first, 1ml purified overnight culture was added to a 1.5ml microcentrifuge tube and centrifuged at 13,000–16,000 × *g* for 2 minutes to pellet the cells. Supernatants were removed from the tubes. For Gram Positive Bacteria, proceed to Step 3. For Gram Negative Bacteria proceeded directly to Step 6.The cells were resuspended thoroughly in 480µl of 50mM EDTA.Then added the appropriate lytic enzyme(s) to the resuspended cell pellet in a total volume of 120µl, and gently pipetted to mix. The purpose of this pretreatment was to weaken the cell wall so that efficient cell lysis can take place. For example, certain *Staphylococcus* species, a mixture of 60µl of 10mg/ml lysozyme and 60µl of 10mg/ml lysostaphin is required for efficient lysis. However, many Gram-Positive Bacterial Strains (e.g., *Bacillus subtilis*, *Micrococcus luteus*, *Nocardiaotitidiscaviarum*, *Rhodococcusrhodochrous*, and *Brevibacteriumalbidium*) lyse efficiently using lysozyme alone. The samples were incubated at 37°C for 30–60 minutes and centrifuged for 2 minutes at 13,000–16,000 × *g* and supernatants were removed. Then 600µl of Nuclei Lysis Solution was added and gently pipetted until the cells are resuspended. Incubated at 80°C for 5 minutes to lyse the cells and cooledat room temperature. Added 3µl of RNase Solution to the cell lysate and invertedthe tubes 2–5 times to mix. Incubated at 37°C for 15–60 minutes and cooled the samples to room temperature. Then, added 200µl of Protein Precipitation Solution to the RNase-treated cell lysate and vortexedvigorously at high speed for 20 seconds to mix the protein precipitation solution with the cell lysate and incubated on ice for 5 minutes. Centrifuged at 13,000–16,000 × *g* for 3 minutes.Supernatant containing the DNA was transferred to a clean 1.5ml microcentrifuge tube containing 600µl isopropanol in room temperature. Note:Some supernatant might remain in the original tube containing the protein pellet. Residual liquid was left in the tube to avoid contaminating the DNA solution with the precipitated protein. Gently mixed by inversion until the thread-like strands of DNA formed a visible mass.Centrifuged at 13,000–16,000 × *g* for 2 minutes. Carefully pourd off the supernatant and drained the tube on clean absorbent paper. Added 600µl of room temperature 70% ethanol and gently inverted tubes several times to wash the DNA pellet. Centrifuged at 13,000–16,000 × *g* for 2 minutes and carefully aspirated the ethanol. Drained the tubes on clean absorbent paper and allowed the pellet to air-dry for 10–15 minutes. Added 100µl of DNA Rehydration Solution to the tube and rehydrated the DNA by incubating at 65°C for 1 hour. Mixed the solutions by gentle tapping periodically. Alternatively, rehydrated the DNA by incubating the solution overnight at room temperature or at 4°C and stored at 2–8°C.

10.12.1 Quantification of extracted DNA

The extracted DNA products were analyzed using a nanodrop to check the purity of DNA. For nucleic acids the 260/ 280 ratio was taken into consideration. A value of ~1.8 was accepted as pure for DNA. The obtained DNA was stored at -20^{0}C in the freezer until further use.

10.12.2 PCR and Sequencing

PCR was performed using purified DNA and *16S rDNA* gene was used for primers. Then PCR product was used for sequencing which were compared using BLASTN on the whole NCBI refseqgenomic database.

11. Results and discussion:

The whole research project was implemented by carrying out different experiment set up based on the objective of the project. The result of the different experiments is given below:

11.1 Objectve-1: To enlist probiotics used for aquaculture in Bangladesh

Thirty-one probiotic products were collected from different aqua product selling companies. All the products were imported from different foreign countries like Thailand, Vietnam, Japan and India. Most of the probiotic products were powder (85%) and liquid (15%) form. In this probiotic, the available microorganisms were *Bacillus sp*, *Lactobacillus sp*, *Aspergillussp*and *Saccharomyces sp.* These probiotics are used for water and soil quality, enhancement of digestibility, immunostimulant of fish. Reported cell number is 10^8-10^{10}CFU/kg for most of the probiotic products**.**However, many of the company's product did not mention label information properly**.** Thesummary and detail information of probiotic products are given below in the table 1 & table 2.

Table-1. Summary information for collected commercial probiotics using for aquaculture

Criteria	**Information**
Number of commercial probiotics collected	Thirty One
Microorganisms available	*Bacillus sp*, *Lactobacillus sp*, *Aspergillussp*and *Saccharomyces sp*
Form of Product	Powder (85%) and Liquid (15%)
Origin of the product	Thailand, Vietnam, Japan and India
Recommended use of Product	Water and Soil quality, enhancement of digestibility, immunostimulant
Reported CFU/kg	10^8-10^{10}(in many cases not reported)

Table-2: Detail list of collected probiotic products along with their trade name, marketing company, origin of the products, CFU/g, function of the products and their product form which are given below:

SL NO	Trade Name	Marketing Company in BD	Source of Origin	Declared Content	Conc (CFU/g)	Function of the Product	Product Form
1	Pond Care	SK+F	India	Not Mentioned	22×10^{9} /g	Control the pH of Water and sediment	Powder
2	ACP Prob	ACP Agri Science Ltd	Japan	1. *Bacillus amyloquefaciens strain D203* *2.Bacillus pumilus Strain d1728* *3.Bacillus licheniformis Strain D3270* *4.Bacillus subtilis* *5.Bacillus megaterium* *6.Lactobacillus acidophilis* *7.Lactobacillus plantarum* *8.Saccharomyces cerevisiae*	1×10^{10} /kg 1×10^{10} /kg 1×10^{11} /kg 1×10^{9} /kg 1×10^{9} /kg 1×10^{9} /kg 1×10^{9} /kg 1×10^{9} /kg	Effectively digest organic waste, increase enzymatic activity in fish, prevent growth of pathogenic microbes and enhance plankton growth.	Powder
3	Nutri Grow	Nutri Foerte Limited	India	Not specified	Not Mentioned	Promote appetite and growth, increase immunity of fish and shrimp	Powder
4	Biotics	AnovaPharma Limited	Vietnam	*1. Saccharomyces cerevisiae* *2. Lactobacillus acidophilis* *3. Bacillus subtilis* *4. Aspergillusoryzae*	1.75×10^{11} /kg 1.5×10^{9} /kg 2.1×10^{9} /kg 1.5×10^{9} /kg	Effectively digest organic waste and reduce organic load of soil and water	Powder

5	Navio plus	ACI	Thailand	*1. Bacillus subtilis* *2. Bacillus licheniformis* *3. Bacillus megaterium* *4. Lactobacillus acidophilus* *5. Lactobacillus plantarum* *6. Saccharomyces cerevisiae*	1×10^{9} /kg 1.0×10^{9} /kg 1.0×10^{9} /kg 1.0×10^{5} /kg 1.0×10^{5} /kg 1.0×10^{5} /kg	Control pathogen bacteria, increase growth rate, enhace immune system , improve water quality process, Used for fish and shrimp	Powder
6	Sahin			Not mentioned	Not specified	Not specified	
7	Pro -2	Sanolife	Thailand	*Bacillus subtilis* *2. Bacillus licheniformis* *3. Bacillus pumilus*	Not specified	Applied mainly for shrimp	Powder
8	MIC-S	Sanolife	Thailand	Not Specified	Not specified	Not mentioned	Powder
9	PRO-W	Sanolife	Thailand	Not Specified	Not specified	Not mentioned	
10	Sentinel BMO		Thailand	Not Specified	Not specified	Not mentioned	
11	Super-biotics	CPF Private Limited	India	Not Specified	Not specified	Not mentioned	
12	Ecotoxnil	Fishtechbd	Bangladesh	Not Specified	Not specified	Not mentioned	
13	ProAct-95	Pischina Biotech	India	Not specified	5×10^{9} CFU/G	Applied for Fish, Crab, Shrimp. It contains beneficial bacteria that inhibit intestinal pathogen, Enhances immune system,	Powder

14	VernoBac	Naphavet	Vietnam	*Saccharomyces cerevisiae* *Bacillus subtilis* *Bacillus licheniformis*	2.0 X 10^{10}/KG	Reduces Ammonia Nitrite, Hydrogen sulfide and other toxic gases. Stimulates appetite, fast growth and reduce FCR;	Powder
15	Promax-Aqua	Rims Bangladesh	India	*Bacillus subtilis* *Bacillus lincheniformis* *Bacillus polymyxa* *Bacillus megaterium* *Bacillus pimilus* *Pseudomonas putida* *Pseudomonas denitrificans* *Aspergillusoryzae* *Aspergillusniger* *Nitrosomonas* *Nitrobacter* *Saccharomyces cervisae* *Bactovacilluslactis* *Lactobacillus helvecticus* *Cellulomonasuda* *Thiobacillusthiooxidans*	Not mentioned	Not Mentioned	Powder
16	Uni.Light	Uni President Vietnam Co. LTD	Vietnam	Not specified	Not mentioned	Not specified	Liquid
17	Ariake-3	Kyushu Medical Co. Japan	Japan	*Bacillus amyloquefaciens s.D203* *Bacillus pumilus S.D1728* *Bacillus licheniformis S. D3270*	1 X 10^{10} / KG 1 X 10^{10} / KG 1 X 10^{11} / KG	Not specified	Powder
18	PowerLac	Kyushu Medical Co. Japan	Japan	*Lactobacillus lactis S. D1813*	1X 10^{11}/Kg	Not specified	Powder

19	Aqua Photo	ACI limited		*Rhodopseudomonas sp.* *Bacillus subtilis*	Not mentioned	Not specified	Liquid
20	Red Cap	Red Cap	Thailand	Not specified	Not mentioned	Not specified	Liquid
21	Green Cap	Green Cap	Thailand	Not specified		Not specified	Liquid
22	Yellow Cap	Yellow Cap	Thailand	Not specified		Not specified	Liquid
23	Super Ps Probiotics	CPF Private Limited	India	Not specified		Not specified	Liquid
24	Viva Pond	Fish Tech bd					Powder
25	Viva Soil	Fishtechbd					Powder
26	Biosol Aqua PS	International Health Care India					Powder
27	pH Fixer	CP india					Powder
28	Pond Cure	Anova Joint Venture Company Limited	Vietnam	*Bacillus licheniformis* *Bacillus megaterium* *Bacillus mensentericus* *Nitrosomonas* *Nitrobacter*	Each one 3.00x 10^{10} CFU	Modulates water quality by decomposing organic matter which causes turbidity and control toxic gases	Powder
29	Gasone x + Y	Growel Formulations PVT. LTD	India	*Pseudomonas florecium* *Bacillus subtilis* *Rodospirillum* *Bacillus megaterium* *Thiothrix* *Nitrococcus*	1.00x 10^{9} CFU 1.00x 10^{9} CFU 4.00x 10^{8} CFU 4.00x 10^{8}	Clean the pond bottom and absorbs ammonia, hydrogen sulphide and Sulphur di oxide present in the culture	Powder

					CFU 6.00x 10^8 CFU 4.00x 10^8 CFU	pond	
30	PRO-B	PVF Agro Ltd	Thailand	*Bacillus subtilis* *Bacillus licheniformis* *Bacillus pumilus*	Each one 1.00x 10^{10} CFU/kg	Improve digestion and nutrient absorption, control Enteropatho genic bacteria , reduce manure odor	Powder
31	Aqua Magic Plus	Bio pharmache mie-Vietnam	Vietnam	*Bacillus subtilis* *Lactobacillus acidophilus* *Saccharomyces cerevisiae* *Asperzillusoryzae*	Each one - 1.50x 10^9 CFU/kg	Prevent infectious disesses, absorb toxic gases and heavy metal , improve growth of algae and plankton	Powder

11.2 Objective-2: To isolate and characterize bacteria from commercial probiotics using for aquaculture

11.2.1 Isolation of bacteria from commercial probiotics:

Bacteria were isolated from commercial probiotics using different selective media. Different media were used as per label information of the products. To enlist the number of. The trade name and number of isolated bacteria were given below which were isolated from different selective media such as MRS, TS, PDA, LB, ASP, NB and BHI (Table 3 to 9).

Table-3. Number of isolated bacteria using MRS media from commercial probiotic products

SL NO	Trade Name	No of isolates
1	Pond Care	3
2	ACP Prob	2
3	Nutri Grow	2
4	Biotics	2
6	Super Biotics	2
7	Navio Plus	2

8	PRO 2	2
9	MIC-S	1
10	PRO-W	2
11	Sentinel BMO	1
12	Ecotoxnil	1
13	ProAct 95	1
14	VarnoBac	3
15	Uni. Light	2
16	Promax- Aqua	2
17	Green Cap	1
18	Yellow Cap	1

Table-4. Number of isolated bacteria using trypton soya from commercial probiotic products

Serial No	Product Name	No of Isolated Bacteria
1	Sahin	2
2	Pro -2	2
3	MIC-S	2
4	Sentinel BMO	1
5	Super- biotics	1
6	PowerLac	2
7	Red Cap	1
8	Yellow Cap	1

Table-5. Number of isolated bacteria using Nutrient broth media from commercial probiotic products

Serial No	Product Name	No of Isolated Bacteria
1	Pond care	3
2	ACP Prob	6
3	Nutri Grow	1
4	Biotics	2
5	Navio plus	2
6	Pro b	2
7	ProAct-95	4
8	VarnoBac	3
9	Ariake-3	4
10	Aqua Photo	3
11	Red Cap	3

12	Green Cap	1
13	Yellow Cap	2
14	Super Ps Probiotics	1
15	PRO-2	2
16	MIC-S	2
17	Pro-w	2
18	Super Biotics	2
19	Sentinel-BMO	4
20	Ecotoxnil	2

Table-6: Number of isolated bacteria using potato dextrose agar from commercial probiotic products

SL NO	Product Name	No of Isolated Bacteria
1	Biotics	1
2	Navio plus	1
3	Sahin	1
4	Pro-2	1
5	MIC-S	1
6	Super Biotics	1
7	Sentinel-BMO	2
8	Ecotoxnil	2
9	ProAct-95	2
10	Promax-Aqua	1
11	Pro-W	2
12	Uni.Light	1
13	PowerLac	1
14	Red Cap^{Tm}	1
15	Green Cap ^{Tm}	1

Table-7: Number of isolated bacteria using Luria Broth media from commercial probiotic products

SL NO	Product Name	No of Isolated Bacteria
1	Biotics	1
2	Navio plus	1
3	Sahin	1
4	ProAct-95	2
5	Promax-Aqua	1
6	Pro-2	1
7	MIC-S	1
8	Pro-W	2

9	Super Biotics	1
10	Sentinel-BMO	2
11	Ecotoxnil	1
12	Uni.Light	1
13	PowerLac	1
14	Baxel Company Limited	1
15	Green Cap ™	1

Table-8: Number of isolated bacteria using Aspergillus differentiating media from commercial probiotic products

SL NO	Product Name	No of Isolated Bacteria
1	Pond Care	1
2	ACP Prob	1
3	Nutri Grow	2
4	ProAct-95	2
5	Promax-Aqua	1
6	Pro-2	1
7	Pro-W	1
8	Super Biotics	2
9	Sentinel-BMO	1
10	Ecotoxnil	1
11	Super Ps Probiotics	1
12	PowerLac	1
13	Red Cap™	1

Table-9: Number of isolated bacteria using Brain Heart Infusion media from commercial probiotic products

SL NO	Product Name	No of Isolated Bacteria
1	ACP Prob	1
2	Biotics	1
3	Navio plus	2
4	Sahin	2
5	ProACt-95	3
6	Promax Aqua	1
7	MIC-S	1
8	Pro-W	2
9	Super Biotics	2
10	Sentinel-BMO	1
11	Ecotoxnil	2
12	Unilight	3

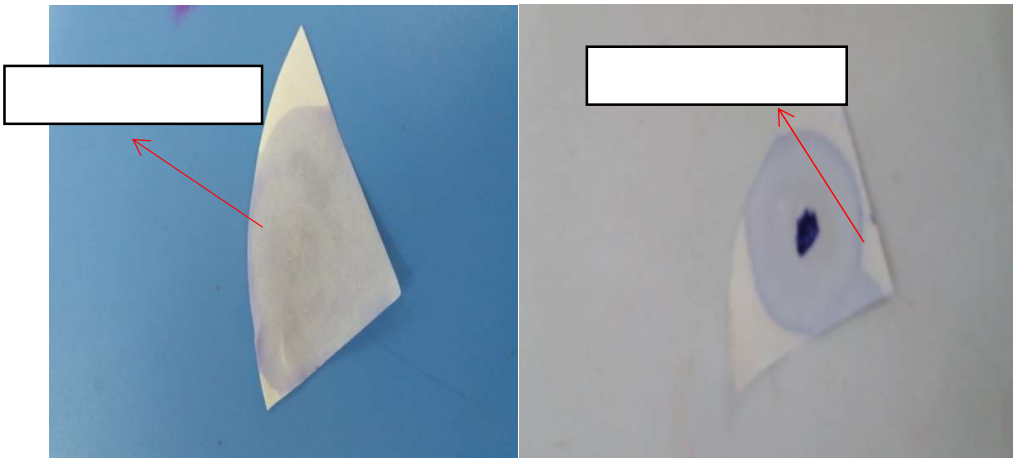
13	Super Ps Probiotics	2
14	Yellow cap ^{Tm}	1
15	Red Cap ^{Tm}	1
16	Green Cap^{Tm}	1
17	Aquaphoto	1

11.2.2: Characterization of bacteria from commercial probiotics:

A total of 173 isolates were isolated from twenty-two commercial probiotics using different selective media (MRS, BHI, PDA, TS, ASP, LB, NB). About 122 isolates were tested for biochemical characterization. Regarding this biochemical characterization 80/122 isolates were shown gram (+) and 37/122 gram (-), 56/122 oxidase (+) and 66/122 oxidase (-), 115/122 catalase (+) and 7/122 catalase negative. The biochemical characterization (Gram test, oxidase test and catalase test) of these isolates were given below in the **table-10, plate-1, plate-2, plate -3, plate-4:**

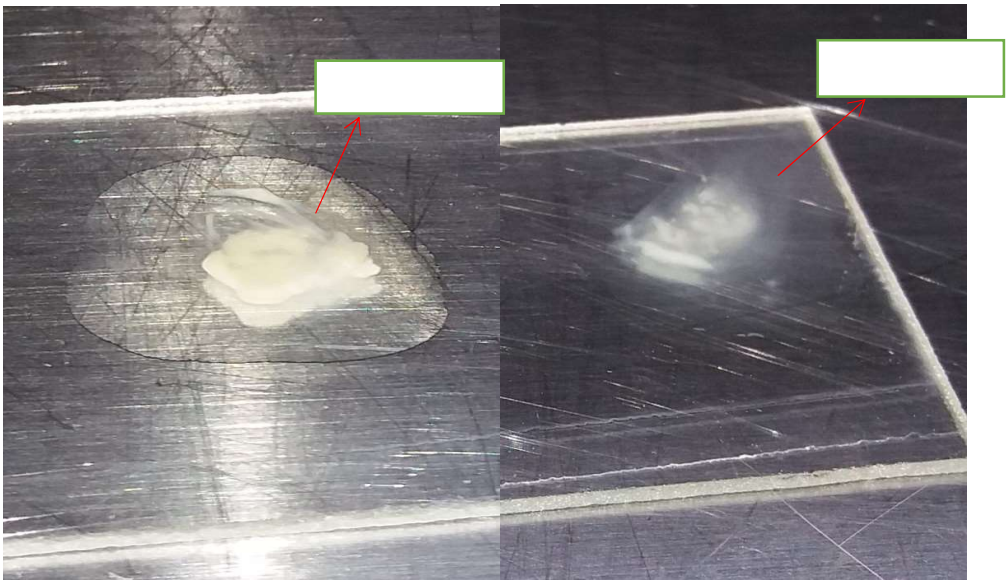
Table:10 Biochemical Characterization of different isolates

ID	Product Name	No of Isolates	Gram Test	Oxidase Test	Catalase Test
1	Pond care	6	Positive(+)	3 Positive(+) 3 Negative (-)	4 Positive(+) 2 Negative
2	ACP Prob	8	Positive(+)	3 Positive(+) 5 Negative (-)	7 Positive(+) 1 Negative
3	Nutri Grow	3	2 Positive(+) 1 Negative (-)	2 Positive(+) 1 Negative (-)	Positive(+)
4	Biotics	6	4 Positive (+) 2 Negative (-)	2 Positive(+) 4 Negative (-)	4 Positive(+) 2 Negative
5	Navio plus	6	Positive(+)	1 Positive(+) 5 Negative (-)	3 Positive(+) 2 Negative
6	Sahin	3	Positive (+) Negative (-)	1 Positive(+) 2 Negative (-)	Positive(+)
7	Pro -2	6	4 Positive (+) 2 Negative (-)	1 Positive(+) 5 Negative (-)	Positive(+)
8	MIC-S	6	4 Positive (+) 2 Negative (-)	Negative (-)	Positive(+)
9	Pro-w	7	6 Positive(+) 1 Negative (-)	Positive (+) Negative (-)	Positive(+)
10	Sentinel BMO	11	8 Positive(+) 3 Negative (-)	1 Positive (+) 10 Negative (-)	Positive(+)
11	Super- biotics	7	5 Positive(+) 2 Negative (-)	2 Positive (+) 5 Negative (-)	Positive(+)
12	Ecotoxnil	6	5 Positive(+) 1 Negative (-)	3 Positive(+) 3 Negative (-)	Positive(+)



(a) **(b)**

Plate 1: (a) Oxidase negative (b) Oxidase positive



(a) (b)

Plate 2: (a) Gram negative (b) Gram positive

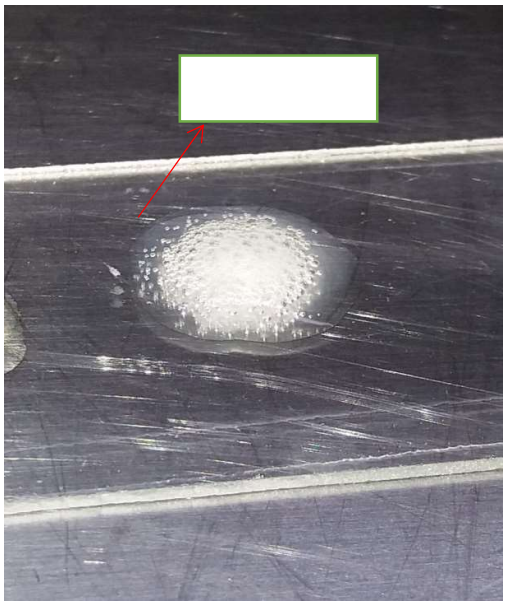


Plate3: Catalase positive (+)

Most of colonies grown in MRS media were creamy to white. The strains were phenotypically characterized on the basis of their colony morphology and biochemical characteristics. The gram test results indicated that the isolated bacteria was gram positive could be identified as *lactobacillus*. The catalase test is one of the most useful diagnostic tests for the recognition of bacteria due to their simplicity. Catalase test showed that the test organisms were not able to produce bubbling when mixed with 3% H_2O_2. This showed that there was absence of catalase enzyme. It is well known that *Lactobacillus* is catalase negative.Thus, the results obtained coincided with *Lactobacillus* strain characteristics. The bacteria uses peroxidase to detoxify H_2O_2, an enzyme that does not evolve O_2. For confirmation, PCR identification was done.

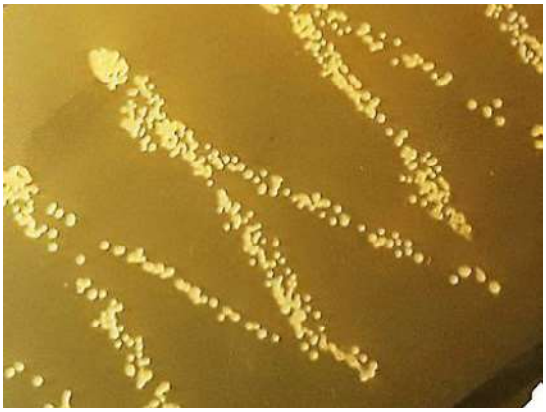


Plate4: Bacterial growth in MRS media

11.3. Objective-3: To determine the probiotic properties and antibiotic susceptibilities of different bacterial isolates.

11.3.1. Bile salt tolerance test

According to Fuller (1992), bile, even at low concentrations, can inhibit the in vitro growth of microorganisms. Gilliland *et al*., (1984) reported that 0.3% is considered to be a critical concentration for screening for resistant strains. Bile salt tolerance is required for probiotic bacteria to grow and survive in fish intestine (Salminen*et al*., 2004). Tolerance to detrimental action of bile salts (0.0 and 0.3 % at 5 hour incubation period) was recorded. Optical density was high in control. As bile salt concentration increased, the growth rate of LABS decreased a bit. In the recent study, the bile salt could not affect the growth rate of isolated bacteria. In our study, most of the tested isolates showed high resistance in bile salt (figure 1 & table 11).This results indicated that many of the isolates showed probiotic properties which is useful for aquaculture uses.

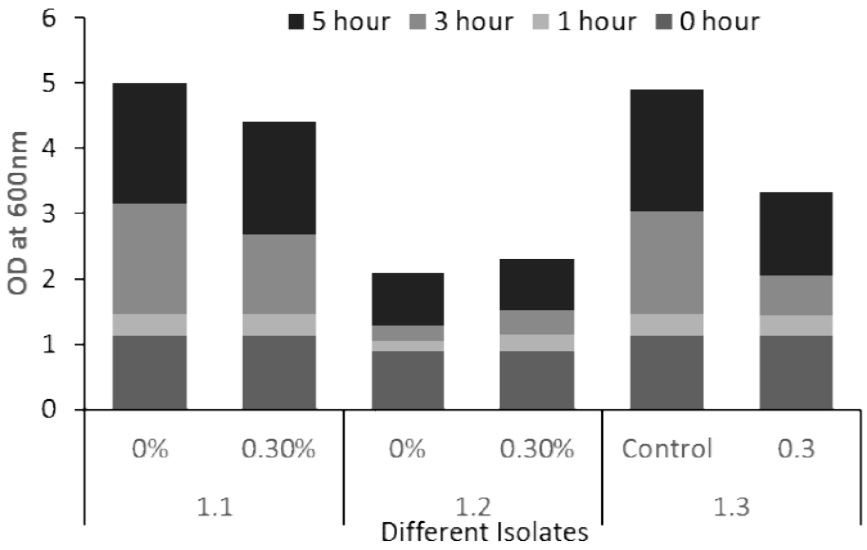


Figure-1: Bile salt tolerance test

Table 11. Bile salt tolerance of different isolates

Sl No	Trade Name	Isolate No	Bile (%)	Time			
				0 hours	1 hours	2 hours	3 hours
1	Pond care	4	Control	.8	.619	.799	.813
			0.3	.8	.328	.398	.500
			Control	.9	.591	.847	.811
			0.3	.9	.709	.396	.491
			Control	.8	.502	.543	.534
			0.3	.8	.340	.400	.509
			Control	0.8	0.101	0.109	0.135
			0 .3	0.8	0.245	0.342	0.477
2	ACP-02	7	Control	0.8	0.091	0.093	0.101
			0 .3	0.8	0.226	0.245	0.267
			Control	.9	.385	.380	.408
			0.3	.9	.317	.390	.475
			Control	1.1	.465	.588	.649
			0.3	1.1	.316	.380	.495
			Control	.8	.593	.894	.860
			0.3	.8	.683	.391	.486
			Control	.8	.575	.721	.678
			0.3	.8	.335	.422	.545
			Control	1.02	.095	.123	.181
			0.3	1.02	.258	.276	.327
			Control	.9	.569	.746	.714
			0.3	.9	.326	.388	.529
3	Nutri Grow	3	Control	1	0.132	0.162	0.195
			0 .3	1	0.306	0.378	0.509
			Control	1.2	0.117	0.154	0.178
			0 .3	1.2	0.266	0.346	0.609
			Control	.8	.596	.833	.765
			0.3	.8	.328	.398	.517
4	Biotics	4	Control	.8	.592	.838	.805
			0.3	.8	.326	.390	.528
			Control	.8	.527	.676	.707
			0.3	.8	.340	.413	.532
			Control	0.9	0.352	0.659	0.831
			0.3	0.9	0.332	0.428	0.658
			Control	1	.125	.166	.321
			0 .3	1	.250	.317	.589

Sl No	Trade Name	Isolate No	Bile (%)	Time			
				0 hours	1 hours	2 hours	3 hours
5	Navio plus	7	Control	1.1	.195	.477	.748
			0.3	1.1	.210	.285	.569
			0.5	1.1	.384	.510	.848
			Control	1.2	.176	.424	.699
			0.3	1.2	.210	.296	.601
			0.5	1.2	.391	.518	.835
			Control	.8	.408	.523	.532
			0.3	.8	.357	.393	.534
			Control	1	.532	.728	.651
			0.3	1	.357	.452	.538
			Control	0.8	0.11	0.23	0.492
			0.3	0.8	0.258	0.412	1.202
			Control	1.1	.151	.180	.332
			0 .3	1.1	.251	.316	.586
			Control	1	.117	.182	.332
			0 .3	1	.252	.317	.569
6	Sahin	3	Control	1.1	.194	.463	.726
			0.3	1.1	.214	.296	.639
			0.5	1.1	.329	.502	.854
			Control	1	.233	.559	.786
			0.3	1	.222	.319	.658
			0.5	1	.347	.516	.886
			Control	0.8	0.363	0.594	0.806
			0.3	0.8	0.359	0.735	1.056
			Control	1	.111	.143	.242
			0 .3	1	.227	.269	.464
7	PRO-2	3	Control	1.1	.219	.511	.779
			0.3	1.1	.228	.308	.645
			0.5	1.1	.437	.625	1.04
			Control	0.8	0.349	0.632	0.828
			0.3	0.8	0.343	0.641	1.065
			Control	0.8	0.099	0.121	0.159
			0 .3	0.8	0.267	0.385	0.542
8	MIC-S	5	Control	.9	.379	.387	.440
			0.3	.9	.450	.462	.491
			Control	.8	.481	.524	.589
			0.3	.8	.319	.366	.431
			Control	.8	.502	.573	.809
			0.3	.8	.328	.403	.511
			Control	.8	.429	.416	.456
			0.3	.8	.334	.418	.506
			Control	0.8	0.1	0.179	0.133
			0.3	.8	0.218	0.23	0.404

Sl No	Trade Name	Isolate No	Bile (%)	Time			
				0 hours	1 hours	2 hours	3 hours
9	PRO-W	6	Control	.9	.378	.386	.475
			0.3	.9	.461	.478	.494
			Control	1	0.112	0.138	0.205
			0.3	1	0.302	0.37	1.11
			Control	1	0.112	0.128	0.132
			0.3	1	0.279	0.314	0.483
			Control	1.1	0.123	0.149	0.185
			0 .3	1.1	0.297	0.357	0.516
			Control	0.8	0.098	0.189	0.323
			0.3	0.8	0.445	1.426	0.262
			Control	1.1	.119	.139	.107
			0 .3	1.1	.238	.289	.281
10	Sentinel -BMO	1	Control	0.8	0.11	0.24	0.298
			0.3	0.8	0.449	0.744	1.28
11	Super- biotics	8	Control	.8	.103	.128	.239
			0.3	.8	.247	.295	.405
			Control	1	.094	.132	.176
			0.3	1	.238	.263	.305
			Control	0.8	0.117	0.128	0.174
			0.3	0.8	0.283	0.318	0.528
			Control	0.9	0.125	0.157	0.192
			0 .3	0.9	0.284	0.349	0.758
			Control	1.2	0.127	0.148	0.183
			0 .3	1.2	0.286	0.332	0.45
			Control	0.9	0.466	0.775	0.278
			0.3	.9	0.266	0.546	1.091
			Control	0.9	0.249	0.545	0.816
			.3	0.9	0.291	0.523	1.406
			Control	1.1	.116	.145	.116
			0 .3	1.1	.243	.292	.348
12	Ecotoxnil	4	Control	1.03	.093	.135	.171
			0.3	1.03	.243	.275	.315
			Control	.8	.097	.117	.231
			0.3	.8	.246	.285	.394
			Control	1.2	0.115	0.141	0.179
			0 .3	1.2	0.252	0.308	0.431
			Control	1	.113	.142	.118
			0 .3	1	.228	.276	.316

Sl No	Trade Name	Isolate No	Bile (%)	Time			
				0 hours	1 hours	2 hours	3 hours
13	ProAct-95	10	Control	1.1	.220	.526	.757
			0.3	1.1	.201	.271	.560
			0.5	1.1	.341	.520	.865
			Control	1.1	.228	.542	.763
			0.3	1.1	.214	.293	.631
			0.5	1.1	.375	.567	.879
			Control	.8	.098	.123	.267
			0.3	.8	.248	.289	.397
			Control	1.02	.090	.125	.172
			0.3	1.02	.245	.272	.318
			Control	1	0.128	0.153	0.207
			0.3	1	0.309	0.392	1.052
			Control	0.8	0.119	0.149	0.205
			0.3	0.8	0.292	0.38	1.024
			Control	0.8	0.209	0.501	0.811
			0.3	.8	0.095	0.088	0.087
			Control	1.2	0.152	0.168	0.206
			0 .3	1.2	0.29	0.378	0.596
			Control	0.8	0.127	0.156	0.193
			0 .3	0.8	0.264	0.341	0.582
			Control	1.1	.111	.140	.110
			0 .3	1.1	.239	.297	.312
14	Promax Aqua	9	Control	1.1	.165	.360	.656
			0.3	1.1	.232	.322	.693
			0.5	1.1	.345	.552	.954
			Control	.8	.103	.118	.263
			0.3	.8	.247	.289	.393
			Control	.9	.116	.152	.192
			0.3	.9	.238	.270	.319
			Control	.8	.093	.081	.082
			0.3	.8	.243	.293	.398
			Control	.9	.099	.120	.224
			0.3	.9	.243	.281	.388
			Control	0.8	0.122	0.142	0.185
			0.3	0.8	0.276	0.318	0.543
			Control	0.9	0.119	0.272	0.352
			0.3	.9	0.093	0.102	0.103
			Control	0.8	0.085	0.115	0.253
			0.3	.8	0.215	0.269	0.548
			Control	1.2	0.139	0.158	0.187
			0 .3	1.2	0.313	0.361	0.622

Sl No	Trade Name	Isolate No	Bile (%)	Time			
				0 hours	1 hours	2 hours	3 hours
15	VernoBac	3	Control	1	.097	.131	.172
			0.3	1	.250	.277	.318
			Control	.9	.096	.121	.239
			0.3	.9	.254	.296	.401
			Control	0.8	0.083	0.113	0.127
			0 .3	0.8	0.237	0.295	0.401
16	Uni.Light	6	Control	.8	.097	.125	.237
			0.3	.8	.249	.297	.410
			Control	1.02	.089	.123	.169
			0.3	1.02	.246	.283	.320
			Control	0.8	0.117	0.14	0.199
			0.3	0.8	0.286	0.337	0.88
			Control	.8	0.166	0.391	0.762
			0.3	.8	0.252	0.379	1.087
			Control	1	.157	.187	.342
			0 .3	1	.262	.342	.701
			Control	1.1	.160	.192	.389
			0 .3	1.1	.260	.341	.964
17	Ariake-3	4	Control	.8	.371	.387	.421
			0.3	.8	.459	.466	.495
			Control	.9	.367	.388	.414
			0.3	.9	.485	.492	.518
			Control	.8	.358	.370	.416
			0.3	.8	.443	.473	.492
			Control	1.03	.359	.367	.397
			0.3	1.03	.442	.465	.490
18	Power lac	4	Control	1.1	.284	.606	.798
			0.3	1.1	.236	.329	.667
			Control	0.8	0.106	0.122	0.172
			0.3	0.8	0.278	0.319	0.496
			Control	0.8	0.333	0.607	0.786
			0.3	0.8	0.328	0.884	1.27
			Control	0.8	0.102	0.131	0.157
			0 .3	0.8	0.25	0.339	0.46
19	Aqua Photo	1	Control	.8	.387	.391	.420
			0.3	.8	.479	.489	.505

Sl No	Trade Name	Isolate No	Bile (%)	Time			
				0 hours	1 hours	2 hours	3 hours
20	Red Cap TM	5	Control	1.1	.204	.450	.630
			0.3	1.1	241	.344	.734
			0.5	1.1	.393	.649	1.54
			Control	0.9	0.087	0.177	0.282
			0.3	0.9	0.416	1.36	0.254
			Control	.8	.369	.373	.407
			0.3	.8	.467	.477	.506
			Control	0.8	0.208	0.101	0.112
			0 .3	0.8	0.297	0.401	0.506
			Control	0.9	.126	.163	.291
			0 .3	0.9	.238	.297	.588
21	Green Cap TM	3	Control	0.8	0.117	0.126	0.163
			0.3	0.8	0.291	0.335	0.654
			Control	0.8	0.089	0.131	0.343
			0.3	0.8	0.531	1.151	0.255
			Control	0.8	0.111	0.232	0.387
			0.3	0.8	0.554	1.217	0.306
22	Yellow Cap IM	2	Control	0.8	0.109	0.125	0.189
			0.3	0.8	0.285	0.324	0.561
			Control	0.9	0.105	0.241	0.431
			0.3	0.9	0.296	0.584	1.201
23	Super Ps Probiotics	5	Control	.8	0.09	0.18	0.476
			0.3	0.8	0.248	0.588	1.111
			Control	0.8	0.153	0.159	0.195
			0 .3	0.8	0.302	0.405	0.616
			Control	1.1	.165	.228	.414
			0 .3	1.1	.321	.435	.916
			Control	1	.190	.406	.701
			0.3	1	.229	.269	.408
			0.5	1	.325	.393	.631
			Control	1	.207	.437	.731
			0.3	1	.221	.261	.401
			0.5	1	.342	.402	.672
24	Pro b	2	Control	.8	.580	.615	.710
			0.3	.8	.310	.450	.508
			Control	.8	.599	.668	.717
			0.3	.8	.332	.452	.500

11.3.2. pH tolerance test:

Wide range of pH tolerance has been considered as an important criterion for determining the probiotic properties. Considering this, isolates were grown in different selective broth at pH 3 and 4 pH of media which was adjusted by adding HCl (1 mol/L). Bacterial cultures were incubated for 24-48 h. In this study, pH tolerance test was performed to evaluate the tolerance and growth of different isolates at 3 and 4 pH level. Optical density was high in control pH. In our study, most of tested isolates showed moderate tolerance in acidic condition (figure 2 and table 12).This results showed that most of the isolates has moderate pH tolerance indicating that isolates have moderate probiotic properties.

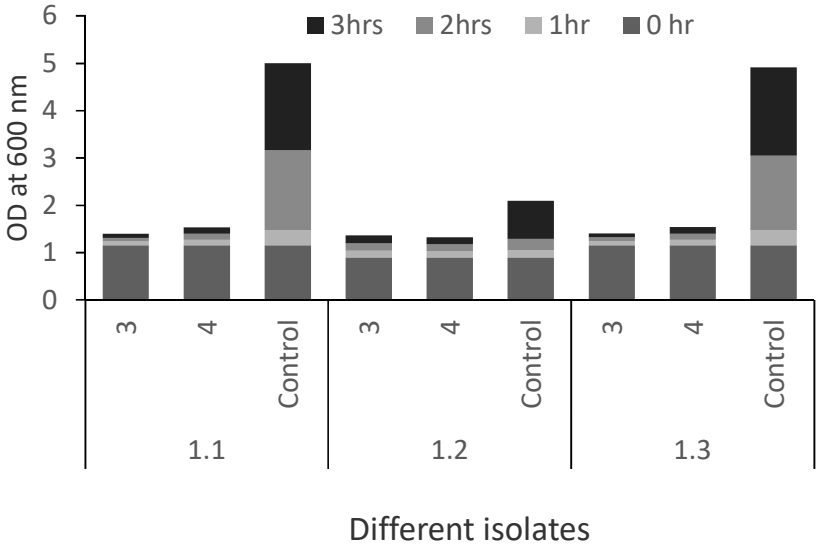


Figure 2: pH tolerance test at different time intervals

Table 12: pH tolerance test of different isolates

Serial no	Product Name	No of Isolated Bacteria	PH	Time			
				0 hour	1 hour	2 hour	3 hour
1	Pond care	4	Control	.8	.619	.799	.813
			3	.8	.438	.446	.421
			4	.8	0.089	0.075	0.095
			Control	0.9	0.591	0.847	0.811
			3	.9	0.411	0.421	0.38
			4	.9	0.111	0.12	0.127
			Control	.8	0.502	0.543	0.534
			3	.8	0.411	0.414	0.393
			4	.8	0.094	0.08	0.099
			Control	0.8	0.101	0.109	0.135
			3	0.8	0.12	0.111	0.12
			4	0.8	0.096	0.09	0.104
2	ACP Prob	7	Control	.9	0.385	0.38	0.408
			3	.9	0.412	0.413	0.446
			4	.9	0.09	0.077	0.087
			Control	1.2	0.465	0.588	0.649
			3	1.2	0.415	0.417	0.399
			4	1.2	0.083	0.518	0.085
			Control	.8	0.593	0.894	0.86
			3	.8	0.594	0.406	0.389
			4	.8	0.094	0.073	0.091
			Control	.8	0.575	0.721	0.678
			3	.8	0.419	0.414	0.378
			4	.8	0.089	0.088	0.089
			Control	1.02	.95	1.23	.181
			3	1.02	.082	.089	.085
			4	1.02	.073	0.089	.103
			Control	.9	0.569	0.796	0.714
			3	.9	0.422	0.42	0.391
			4	.9	0.326	0.38	0.529
			Control	0.8	0.091	0.093	0.101
			3	0.8	0.115	0.114	0.116
			4	0.8	0.096	0.093	0.097

Serial no	Product Name	No of Isolated Bacteria	PH	Time			
				0 hour	1 hour	2 hour	3 hour
3	Nutri Grow	3	Control	.8	0.596	0.833	0.765
			3	.8	0.419	0.418	0.385
			4	.8	0.096	0.102	0.105
			Control	1	0.132	0.162	0.195
			3	1	0.161	0.155	0.154
			4	1	0.114	0.141	0.157
			Control	1.2	0.117	0.154	0.178
			3	1.2	0.13	0.129	0.128
			4	1.2	0.117	0.147	0.179
4	Biotics	4	Control	.8	0.838	0.805	0.592
			3	.8	0.407	0.39	0.406
			4	.8	0.087	0.095	0.074
			Control	.8	0.676	0.707	0.527
			3	.8	0.405	0.377	0.653
			4	.8	0.083	0.089	0.087
			Control	0.9	0.352	0.659	0.831
			3	.9	0.095	0.087	0.09
			4	.9	0.83	0.073	0.078
			Control	1	.125	.166	.321
			3	1	.130	.121	.116
			4	1	.105	.103	.104
5	Navio plus	7	Control	0.8	0.532	0.728	0.651
			3	0.8	0.412	0.397	0.389
			4	0.8	.091	0.087	0.097
			Control	0.8	0.532	0.728	0.651
			3	0.8	0.412	0.397	0.389
			4	0.8	0.085	0.088	0.088
			Control	0.8	0.11	0.23	0.492
			3	.8	0.089	0.094	0.097
			4	.8	0.087	0.082	0.086
			Control	1.1	.151	.180	.332
			3	1.1	.117	.116	.118
			4	1.1	.104	.104	.109
			Control	1	.117	.182	.332
			3	1	.121	.098	.117
			4	1	.099	.102	.103
			Control	1.1	.195	.477	.748
			3	1.1	.184	.169	.153
			4	1.1	.098	.078	.096
			Control	1.2	.176	.424	.699
			3	1.2	.168	.162	.168
			4	1.2	.106	.084	.094

Serial no	Product Name	No of Isolated Bacteria	PH	Time			
				0 hour	1 hour	2 hour	3 hour
6	Sahin	5	Control	0.8	0.599	0.668	0.717
			3	0.8	0.398	0.404	0.381
			4	0.8	0.079	0.083	0.087
			Control	0.8	0.363	0.594	0.806
			3	.8	0.093	0.85	0.084
			4	.8	0.085	0.082	0.088
			Control	1	.111	.143	.242
			3	1	.117	.116	.114
			4	1	.098	.098	.108
			Control	1.1	.194	.463	.726
			3	1.1	.176	.176	.158
			4	1.1	.104	.093	.088
			Control	1	.233	.559	.786
			3	1	.166	.157	.152
			4	1	.098	.086	.097
7	Pro-2	5	Control	0.8	0.103	0.128	0.239
			3	.8	0.088	0.086	0.085
			4	.8	0.086	0.085	0.082
			Control	1	0.094	0.132	0.176
			3	1	0.081	0.088	0.083
			4	1	0.074	0.083	0.084
			Control	0.8	0.349	0.632	0.828
			3	.8	0.097	0.088	0.086
			4	.8	0.078	0.75	0.085
			Control	0.8	0.099	0.121	0.159
			3	0.8	0.133	0.125	0.127
			4	0.8	0.094	0.112	0.119
			Control	1.1	.219	.511	.779
			3	1.1	.165	.172	.155
			4	1.1	.113	.097	.090
8	MIC-S	**3**	Control	1	*0.093*	0.135	0.171
			3	1	0.086	0.087	0.089
			4	1	0.075	0.086	0.084
			Control		0.088	0.084	0.085
			3		0.083	0.082	0.085
			4		0.083	0.081	0.081
			Control	0.8	0.1	0.179	0.133
			3	.8	0.09	0.094	0.095
			4	.8	0.082	0.077	0.074
9	Sentinel-BMO	1	Control	0.8	0.11	0.24	0.298
			3	.8	0.093	0.089	0.093
			4	0.8	0.084	0.081	0.077

Serial no	Product Name	No of Isolated Bacteria	PH	Time			
				0 hour	1 hour	2 hour	3 hour
10	Pro-W	7	Control	0.8	0.098	0.123	0.267
			3	0.8	0.088	0.084	0.085
			4	0.8	0.083	0.082	0.085
			Control	0.8	0.098	0.123	0.267
			3	0.8	0.088	0.084	0.085
			4	0.8	0.083	0.082	0.085
			Control	1	0.112	0.138	0.205
			3	1	0.131	0.132	0.124
			4	1	0.097	0.107	0.129
			Control	1	0.112	0.128	0.132
			3	1	0.138	0.138	0.132
			4	1	0.097	0.104	0.127
			Control	0.8	0.098	0.189	0.323
			3	.8	0.093	0.089	0.082
			4	.8	0.086	0.081	0.075
			Control	1.2	0.123	0.149	0.185
			3	1.2	0.135	0.151	0.146
			4	1.2	0.108	0.127	0.119
			Control	1.1	.119	.139	.107
			3	1.1	.117	.112	.113
			4	1.1	.096	.094	.103
11	Super Biotis	8	Control	1	0.097	0.131	0.172
			3	1	0.084	0.089	0.085
			4	1	0.083	0.086	0.088
			Control	0.9	0.096	0.121	0.239
			3	0.9	0.089	0.085	0.085
			4	0.9	0.084	0.093	0.083
			Control	0.8	0.117	0.128	0.174
			3	0.8	0.13	0.135	0.128
			4	0.8	0.094	0.101	0.12
			Control	0.8	0.466	0.775	0.278
			3	0.8	0.086	0.084	0.093
			4	0.8	0.085	0.091	0.093
			Control	0.9	0.249	0.545	0.816
			3	.9	0.079	0.082	0.087
			4	.9	0.078	0.091	0.086
			Control	0.9	0.125	0.157	0.192
			3	0.9	0.131	0.154	0.141
			4	0.9	0.114	0.133	0.147
			Control	1.2	0.127	0.148	0.183
			3	1.2	0.131	0.143	0.145
			4	1.2	0.108	0.118	0.133
			Control	1.1	.116	.145	.116
			3	1.1	.132	.124	.123
			4	1.1	.102	.096	.094

Serial no	Product Name	No of Isolated Bacteria	PH	Time			
				0 hour	1 hour	2 hour	3 hour
12	Ecotoxnil	4	Control	0.8	0.097	0.125	0.237
			3	0.8	0.084	0.084	0.08
			4	0.8	0.08	0.08	0.078
			Control	1	0.089	0.123	0.169
			3	1	0.088	0.087	0.085
			4	1	0.092	0.093	0.094
			Control	1.2	0.115	0.141	0.179
			3	1.2	0.142	0.143	0.144
			4	1.2	0.11	0.135	0.135
			Control	1	.113	.142	.118
			3	1	.122	.119	.119
			4	1	.101	.100	.103
13	ProAct-95	11	Control	1.1	.111	.140	.110
			3	1.1	.125	.115	.117
			4	1.1	.094	.097	.101
			Control	0.8	0.481	0.524	0.589
			3	0.8	0.406	0.412	0.386
			4	0.8	0.089	0.089	0.093
			Control	0.8	0.481	0.524	0.589
			3	0.8	0.406	0.412	0.386
			4	0.8	0.089	0.089	0.093
			Control	0.8	0.429	0.416	0.456
			3	0.8	0.403	0.424	0.423
			4	0.8	0.091	0.08	0.09
			Control	1	0.128	0.153	0.207
			3	1	0.136	0.131	0.124
			4	1	0.104	0.117	0.139
			Control	0.8	0.119	0.149	0.205
			3	0.8	0.133	0.132	0.126
			4	0.8	0.098	0.105	0.121
			Control	0.8	0.209	0.501	0.811
			3	.8	0.095	0.088	0.087
			4	.8	0.081	0.085	0.086
			Control	1.2	0.152	0.168	0.206
			3	1.2	0.156	0.159	0.149
			4	1.2	0.119	0.131	0.143
			Control	0.8	0.127	0.156	0.193
			3	0.8	0.141	0.135	0.142
			4	0.8	0.115	0.133	0.157
			Control	1.1	.220	.526	.757
			3	1.1	.172	.160	.151
			4	1.1	.105	.089	.088
			Control	1.1	.228	.542	.763
			3	1.1	.168	.155	.162
			4	1.1	.089	.094	.092

Serial no	Product Name	No of Isolated Bacteria	PH	Time			
				0 hour	1 hour	2 hour	3 hour
14	VernoBac	1	Control	0.8	0.083	0.113	0.127
			3	0.8	0.121	0.128	0.124
			4	0.8	0.091	0.095	0.101
15	Promax-Aqua	9	Control	0.8	0.103	0.118	0.263
			3		0.088	0.086	0.086
			4		0.084	0.085	0.082
			Control	0.8	0.116	0.152	0.192
			3	0.8	0.086	0.083	0.087
			4	0.8	0.083	0.084	0.085
			Control	0.9	0.093	0.121	0.24
			3	0.9	0.083	0.081	0.08
			4	.9	0.082	0.079	0.077
			Control	0.8	0.099	0.121	0.224
			3	0.8	0.086	0.081	0.084
			4	0.8	0.076	0.074	0.075
			Control	0.8	0.122	0.142	0.185
			3	0.8	0.133	0.13	0.126
			4	0.8	0.098	0.105	0.13
			Control	1.2	0.139	0.158	0.187
			3	1.2	0.151	0.175	0.144
			4	1.2	0.115	0.126	0.143
			Control	1.1	.165	.360	.656
			3	1.1	.174	.170	.150
			4	1.1	.118	.099	.100
			Control	0.9	0.119	0.272	0.352
			3	. 9	0.093	0.102	0.103
			4	.9	0.084	0.075	0.071
			Control	0.8	0.085	0.115	0.253
			3	.8	0.095	0.095	0.099
			4	.8	0.079	0.075	0.072
16	Uni.Light	6	Control	0.8	0.117	0.14	0.199
			3	0.8	0.128	0.139	0.13
			4	0.8	0.101	0.11	0.138
			Control	.8	0.166	0.391	0.762
			3	.8	0.093	0.094	0.092
			4	0.8	0.081	0.091	0.081
			Control	.8	0.09	0.18	0.476
			3	.8	0.085	0.083	0.081
			4	.8	0.082	0.074	0.071
			Control	1	.157	.187	.342
			3	1	.121	.114	.112
			4	1	.098	.096	.103
			Control	1.1	.160	.192	.389
			3	1.1	.121	.119	.120
			4	1.1	.097	.118	.098

Serial no	Product Name	No of Isolated Bacteria	PH	Time			
				0 hour	1 hour	2 hour	3 hour
17	PowerLac	4	Control	0.8	0.106	0.122	0.172
			3	0.8	0.128	0.134	0.135
			4	0.8	0.108	0.108	0.13
			Control	0.8	0.333	0.607	0.786
			3	.8	0.088	0.084	0.096
			4	.8	0.081	0.08	0.074
			Control	0.8	0.102	0.131	0.157
			3	0.8	0.121	0.115	0.117
			4	0.8	0.092	0.095	0.097
			Control	1.1	.284	.606	.798
			3	1.1	.165	.097	.100
			4	1.1	.119	.606	.798
18	Red Cap ™	4	Control	0.9	0.087	0.177	0.282
			3	.9	0.088	0.09	0.086
			4	.9	0.081	0.061	0.059
			Control	0.8	0.208	0.101	0.112
			3	0.8	0.12	0.142	0.132
			4	0.8	0.1	0.1	0.107
			Control	0.9	.126	.163	.291
			3	0.9	.118	.114	.113
			4	0.9	.096	.095	.098
			Control	1.1	.204	.450	.630
			3	1.1	160	.100	.112
			4	1.1	123	.512	.613
19	Green Cap ™	3	Control	0.8	0.117	0.126	0.163
			3	0.8	0.141	0.14	0.136
			4	0.8	0.101	0.11	0.129
			Control	0.8	0.089	0.131	0.343
			3	.8	0.089	0.09	0.095
			4	.8	0.083	0.069	0.083
			Control	0.8	0.111	0.232	0.387
			3	.8	0.092	0.088	0.095
			4	.8	0.094	0.084	0.08
20	Yellow Cap IM	2	Control	0.8	0.109	0.125	0.189
			3	0.8	0.126	0.126	0.12
			4	0.8	0.115	0.106	0.123
			Control	0.9	0.105	0.241	0.431
			3	.9	0.093	0.088	0.087
			4	.9	0.08	0.083	0.085

Serial no	Product Name	No of Isolated Bacteria	PH	Time			
				0 hour	1 hour	2 hour	3 hour
21	Super ps probiotic	4	Control	0.8	0.153	0.159	0.195
			3	0.8	0.13	0.152	0.141
			4	0.8	0.103	0.118	0.141
			Control	1.1	.165	.228	.414
			3	1.1	.135	.120	.135
			4	1.1	.111	.111	.113
			Control	1	.190	.406	.701
			3	1	.166	.134	.153
			4	1	.116	.103	.091
			Control	1	.207	.437	.731
			3	1	.157	.148	.140
			4	1	.124	.101	.102

11.3.3. Antibiotic resistance test

Antibiotic susceptibility assay was also carried out in this study to investigate the suitability of these bacterial isolates. The interpretations of inhibition zone were determined according to zone size of chart of Kirby-Bauer test results (Bauer *et al*., 1966).

Table 13: Standard diameter of clear zone for different antibiotics for susceptibilities test

Name of Antibiotics	Diameter of Clear Zone (mm)		
	Susceptible (S)	Intermediate (I)	Resistance (R)
Ciprofloxacin	≥ 21	15-20	≤ 14
Cephalexin	≥ 26	23-25	≤ 22
Tetracycline	≥ 31	30-24	≤ 23
Amoxycillin	≥ 28	22-27	≤ 21
Doxycycline	≥ 19	-	≤ 18

In our study, antibiotic susceptibilities were completed by using five antibiotics including ciprofloxacin, cephalexin, amoxicillin, tetracycline and doxycycline. Most of the isolates showed diverse range of susceptibilities against the antibiotics. Total 56 isolates were tested for antibiotic resistance and about 56 isolates were resistant to cephalexin, 56 were resistance to amoxicillin, 9 were intermediate and 47 susceptible to ciprofloxacin, 36 were resistant, 11 intermediate and 9 susceptible to tetracycline, 30 were resistant, 6 intermediate and 20 susceptible to doxycycline antibiotics.Many of the isolates showed resistance to antibiotics which is not desirable for probiotics.

Table 14: Antibiotics Susceptibilities of different isolates

SL NO	Product Name	Isolate No	Antibiotics Susceptibilities				
			CIP	CL	TE	AX	DO
1	Pond Care	1	I	R	R	R	R
2	ACP Prob	2	I	R	R	R	I
			S	R	I	R	S
3	NutriGrow	2	S	R	R	R	R
			S	R	I	R	R
4	Biotics	2	S	R	I	R	S
			S	R	S	R	S
5	Navio plus	2	S	R	S	R	S
			S	R	I	R	S
6	Sahin	2	S	R	S	R	S
			S	R	R	R	R
7	Pro-2	6	S	R	I	R	S
			S	R	S	R	S
			I	R	R	R	S
			S	R	S	R	S
			I	R	R	R	I
			S	R	S	R	S
8	Pro-W	3	I	R	R	R	I
			S	R	I	R	S
			S	R	I	R	S
9	Sentinel-BMO	3	I	R	R	R	I
			R	R	R	R	S
			S	R	S	R	S
10	Super Biotics	3	I	R	R	R	I
			R	R	R	R	R
			S	R	I	R	S
11	Ecotoxnil	3	S	R	R	R	R
			S	R	I	R	S
			S	R	S	R	S
12	ProAct-95	2	I	R	R	R	R
			I	R	R	R	I

SL NO	Product Name	Isolate No	Antibiotics Susceptibilities				
			CIP	CL	TE	AX	DO
13	Vernobac	3	S	R	R	R	R
			S	R	R	R	R
			S	R	R	R	R
14	Promax-Aqua	1	I	R	R	R	R
15	Uni.Light	4	S	R	R	R	R
			S	R	R	R	R
			S	R	R	R	R
			S	R	R	R	S
16	Ariake-3	3	S	R	R	R	R
			S	R	S	R	R
			S	R	I	R	R
17	PowerLac	4	S	R	R	R	R
			S	R	R	R	R
			S	R	R	R	R
			I	R	R	R	R
18	Red Cap	3	I	R	R	R	R
			S	R	R	R	R
			S	R	R	R	R
19	Green cap	1	S	R	R	R	S
20	Yellow Cap	1	S	R	R	R	S
21	Super Ps Probiotics	1	R	R	R	R	I

Table 15: Percentage of antibiotic resistance test for different isolates

Name of Antibiotics	Percentage(%)		
	Susceptible	Intermediate	Resistance
Ciprofloxacin	47 (3.92%)	9 (16.07%)	0
Cephalexin	0	0	56 (100%)
Tetracycline	9 (6.07%)	11 (19.6%)	36 (64.3%)
Amoxycillin	0	0	56 (100%)
Doxycycline	20 (35.7%)	6 (10.71%)	30 (53.5%)

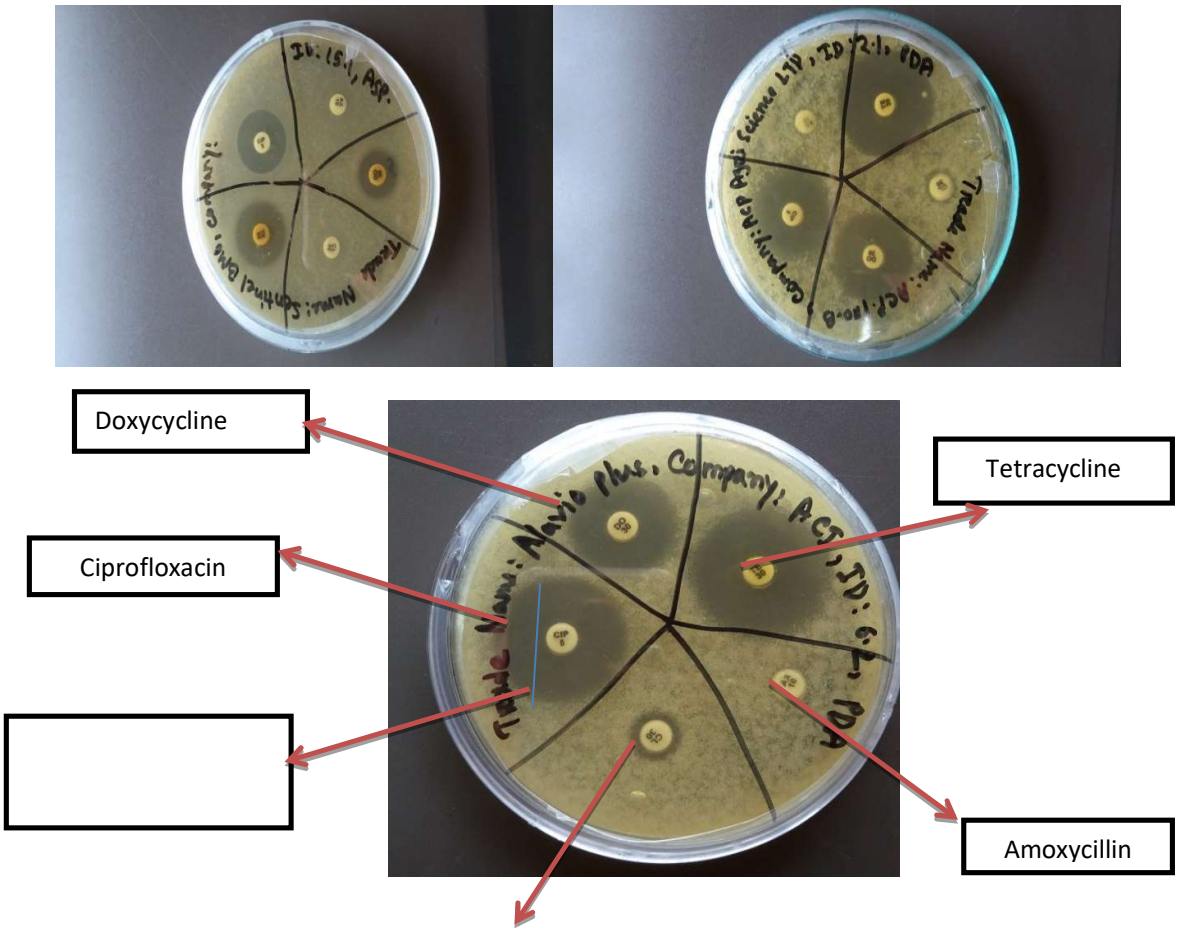


Plate 5: Antibiotic resistance test

11.3.4. Auto aggregation assay:

Many bacteria, both environmental and pathogenic, exhibit the property of autoaggregation. In autoaggregation (sometimes also called autoagglutination or flocculation), bacteria of the same type form multicellular clumps that eventually settle at the bottom of culture tubes. This aggregation helps to protect the inner layer of intestine from pathogenic strain. In our study, total 133 isolates were tested and most of the isolates about 103 were shown low, 3 with medium and 27 absence of auto aggregation assay (table 16). From the above studies it can be concluded that isolated strains are not potential for probiotics especially as gut probiotics.

Table 16: Auto aggregation capacity of different isolates

Sl NO	Trade Name	Isolate No	AAg%	AAg Rank
1	Pond care	6	3	Low
			7	
			17	
			2.89	
			.05	
			.6.2	
2	ACP Prob	10	-12.93	1 Absent 9 low
			1	
			4	
			9.7	
			.7	
			2.3	
			4	
			1.6	
			2.8	
			4.6	
3	Nutri Grow	3	2.3	Low
			9.5	
			1.4	
4	Biotics	6	29.43	2 Absent 1 medium 3 low
			-13.44	
			.6	
			-1.2	
			.79	
			2.5	
5	Navio plus	7	-1.9	1 Absent 5 low 1 medium
			5.3	
			26.8	
			3	
			3.3	
			0	
			1.2	
6	Sahin	6	-.1	3 low 3 Absent
			-.35	
			3.1	
			3	
			0	
			-6.9	

Sl NO	Trade Name	Isolate No	AAg%	AAg Rank
7	PRO-2	6	2	Low
			3.5	
			4.8	
			4	
			2.02	
			2.5	
8	MIC-S	5	2.3	4 low 1 absent
			.35	
			5	
			-1.7	
			1.08	
9	Pro-W	7	1.51	4 low 3 absent
			6.7	
			11	
			-2.37	
			-.33	
			-2.01	
			2.08	
10	Sentinel-BMO	8	1.6	7 low 1 absent
			1.2	
			14.1	
			4	
			.2	
			2	
			-7.0	
			.35	
11	Super Biotics	7	1.5	4 low 3 absent
			2.6	
			-.06	
			-.17	
			0	
			.92	
			-2.07	
12	Ecotoxnil	3	2.6	3 low 1 absent
			3.6	
			4.8	

Sl NO	Trade Name	Isolate No	AAg%	AAg Rank
13	Pro Act-95	10	-.5	6 Absent 4 low
			-.1	
			4.9	
			.06	
			-1.83	
			-.5	
			5.8	
			-38.39	
			-.95	
			2.7	
14	VernoBac	6	3	5 low 1 medium
			21.4	
			1.1	
			1.8	
			4.13	
			2.3	
15	Promax Aqua	5	-1.12	2 Absent 3 low
			7	
			15	
			3.2	
			-4.12	
16	Uni-light	9	4.5	8 low 1 absent
			3.8	
			7.5	
			5	
			-1.36	
			3.07	
			2.7	
			1.7	
			4.7	
17	Ariake	2	4.5	low absent
			-25.66	
18	PowerLac	5	15.3	4 low 1 absent
			-27.5	
			0	
			3.3	
			4	

Sl NO	Trade Name	Isolate No	AAg%	AAg Rank
19	Red Cap	6	.94	
			7.6	Low
			4.2	
			3	
			.48	
			.9	
20	Aquaphoto	1	1.5	low
21	Green Cap	5	2.7	low
			2.7	
			11	
			12	
			5.8	
22	Yellow Cap	4	3.4	low
			9	
			-.72	
			.15	
23	Super Ps Probiotics	6	2.5	low
			19.59	
			14.36	
			15	
			.43	
			.68	

11.3.5. Hemolytic activity test:

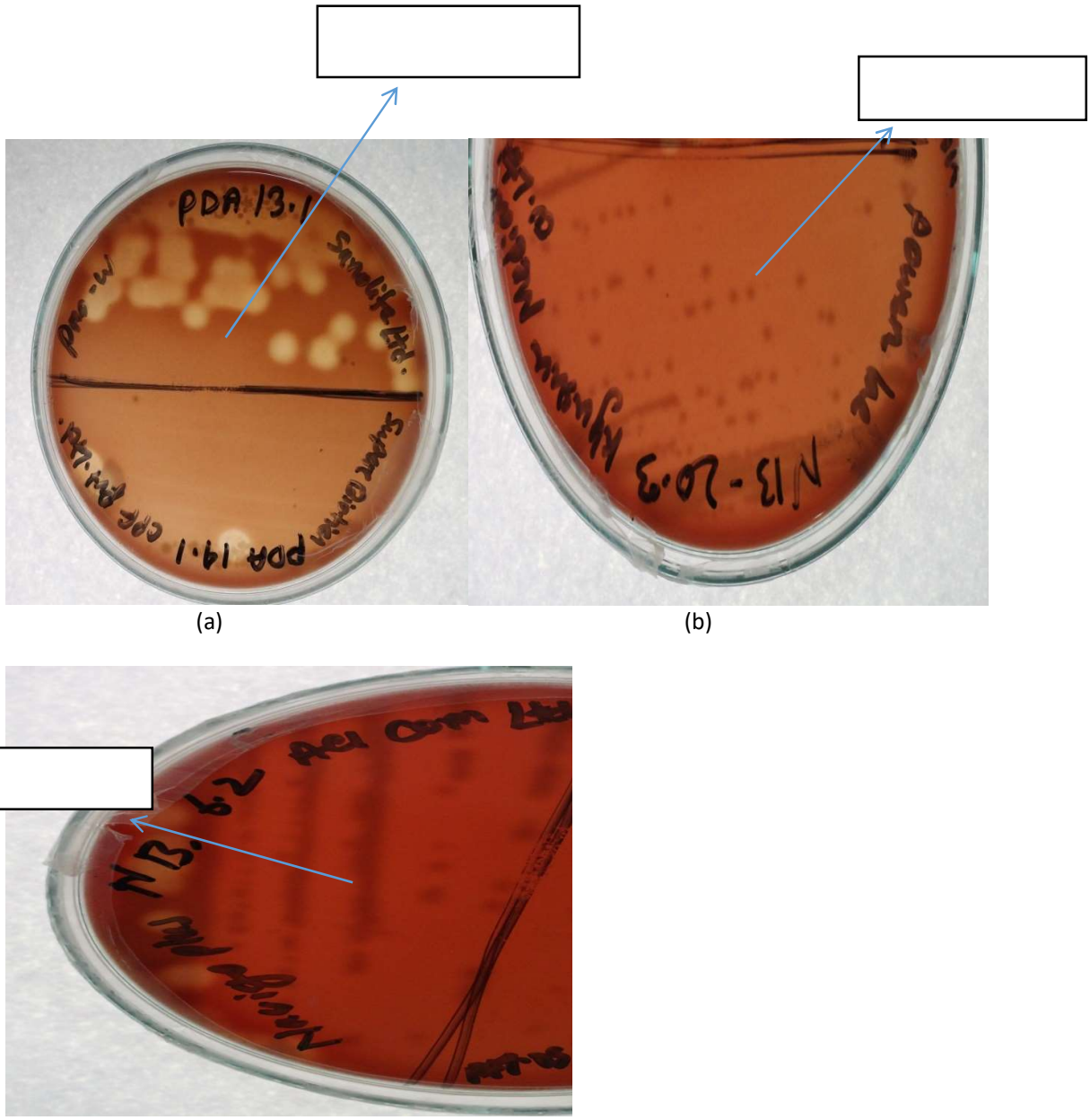
As the isolate, LABS showed positive antagonistic activity, it was subjected to hemolytic activity assay to determine either pathogenic or non-pathogenic bacteria. LABS showed γ-hemolysis that means no breakdown of the red blood cell. The hemolytic assay further revealed the suitability of intestinal micro flora for further antibiotic susceptibility assay. The γ-hemolytic isolate (LABS) revealed no red blood cell lysis activity on the blood agar. This safety precaution is relatively important in the characterization process, as hemolytic bacteria would break down the epithelial layer of host cells and cause malfunction in the defense system. Failure of this defense mechanism would cause invasive diseases in the host (Nurhidayu*et al*., 2012).

In this study, total 174 isolates were tested for hemolytic activity. Here, most of the isolates about 90 isolates were shown γ hemolysis, 48 were β hemolysis and 36 were α hemolysis. Most of the isolates which grown on MRS media were shown γ hemolysis and which grown on BHI media were shown β hemolysis.These findings indicated that there is contamination with the pathogenic strains which alarming for aquaculture and food safety. Results of hemolytic activity test were given below in table 17 and plate 6, as follows:

Table 17: Hemolytic activity of different isolates

Sl NO	**Trade Name**	**Isolate No**	**Hemolytic activity**
1	Pond Care	9	2α 3β 4γ
2	ACP Prob	11	3α 3 β 5γ
3	Nutri Grow	7	3α β 3γ
4	Biotics	6	α 3β 2γ
5	Navio plus	10	3α 4β 3γ
6	Sahin	7	α 6γ
7	PRO-2	8	γ
8	MIC-S	7	α 2β 4γ

Sl NO	Trade Name	Isolate No	Hemolytic activity
9	Pro-W	10	2α 4β 4γ
10	Sentinel -BMO	7	2α β 4γ
11	Super Biotics	11	5α 6 γ
12	Ecotoxinil	8	α 3β 4γ
13	ProAct-95	14	3α 7β 4γ
14	VernoBac	5	α 4γ
15	Promax Aqua	8	2β 6γ
16	Uni.Light	10	α 3β 6γ
17	Ariake-3	3	2α B
18	PowerLac	8	2α β 5γ
19	Aqua photo	2	B
20	Red Cap ™	4	β 3γ
21	Green Cap ™	5	β 4γ
22	Yellow Cap IM	3	α β γ
23	Super Ps Probiotics	7	5β 2γ
24	Sanolife	2	Γ
25	Baxel Company Limited	3	2α B



(a) (b)

(c)

Plate 6: Hemolytic activity test (a) β hemolysis (complete hemolysis), (b) γ hemolysis (no hemolysis) and (c) α hemolysis (partial hemolysis)

11.3.6 Molecular identification of Bacterial Strain:

Molecular identification of different isolates showed that diversified genus (Table 18) including 59% *Enterobacter*, 15% *Aeromonas*, 13% *Enterococcus*, 8% *Streptococcus* and 2% *Bacillus, Rhodobacter, Moraxella* were found (Plate 7).

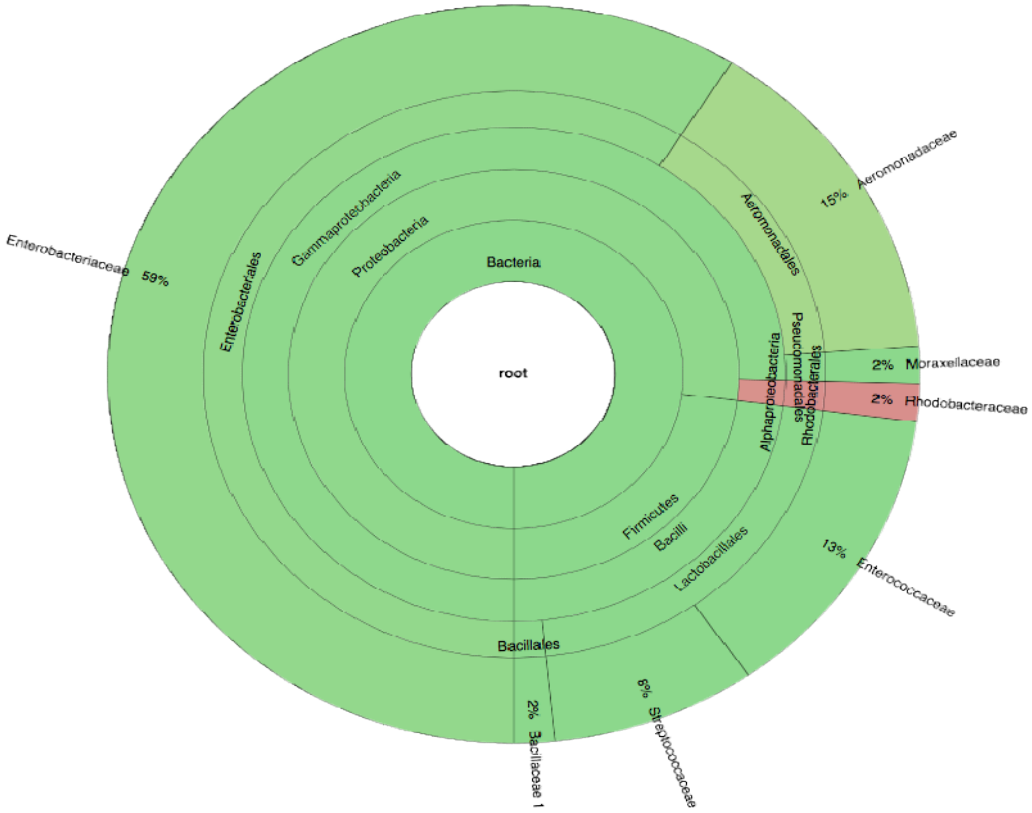


Plate 7: Contribution of different genus of isolates identified through Sequencing

Table 18: Molecular identification of different isolates

SL No.	No. of total isolates	No. of sequence isolates	Trade Name	Marketing Company in BD	Source of Origin	Declared Content	Observed Genus Name	Pathogenic/ Non-pathogenic	Hemolysis
1	9	2	Pond Care	SK+F	India	Not Mentioned	*Lactococcus*	Non-pathogenic	γ
							Zobellella	Partial-pathogenic	α
2	11	6	ACP Prob	ACP Agri Science Ltd	Japan	1. *Bacillus amyloquefaciens strain D203* 2.*Bacillus pumilus Strain d1728* 3.*Bacillus licheniformis Strain D3270* 4.*Bacillus subtilis* 5.*Bacillus megaterium* 6.*Lactobacillus acidophilis* 7.*Lactobacillus plantarum* 8.*Saccharomyces cerevisiae*	*Enterobacter*	Non-pathogenic	γ
							Enterobacter	Non-pathogenic	γ
							Enterobacter	Pathogenic	β
							Aeromonas	Non-pathogenic	γ
							Enterococcus	Partial-pathogenic	α
							Pseudocitrobacter	Partial-pathogenic	α
3	7	2	Nutri Grow	Nutri Foerte Limited	India	Not specified	*Enterobacter*	Pathogenic	β
							Enterococcus	Partial-pathogenic	α
4	6	5	Biotics	AnovaPharma Limited	Vietnam	*1.Saccharomyces cerevisiae* 2. *Lactobacillus acidophilis* 3. *Bacillus subtilis* 4. *Aspergillusoryzae*	*Aeromonas*	Partial-pathogenic	α
							Enterococcus	Non-pathogenic	γ
							Samsonia	Pathogenic	β
							Enterobacter	Pathogenic	β
5	10	3	Navio plus	ACI	Thailand	*1. Bacillus subtilis* 2. *Bacillus*	*Enterobacter*	Pathogenic	β
							Enterobacter	Non-pathogenic	γ

						licheniformis *3. Bacillus megaterium* *4.Lactobacillus acidophilus* *5.Lactobacillus plantarum* *6.Saccharomyces cerevisiae*	*Enterococcus*	Non-pathogenic	γ
6	7	1	Sahin			Not mentioned	*Pantoea*	Non-pathogenic	γ
7	8	5	Pro -2	Sanolife	Thailand	*Bacillus subtilis* *2. Bacillus licheniformis* *3. Bacillus pumilus*	*Enterobacter*	Non-pathogenic	γ
							Lactococcus	Non-pathogenic	γ
							Klebsiella	Pathogenic	γ
							Cedecea	Pathogenic	γ
							Thiobacimonas	Non-pathogenic	γ
8	7	2	MIC-S	Sanolife	Thailand	Not Specified	*Zobellella*	Non-pathogenic	γ
							Pragia	Non-pathogenic	γ
9	10	3	PRO-W	Sanolife	Thailand	Not Specified	*Enterobacter*	Pathogenic	β
							Enterobacter	Partial-pathogenic	α
							Lactococcus	Non-pathogenic	γ
10	7	3	Sentinel BMO			Not Specified	*Enterobacter*	Non-pathogenic	γ
							Enterobacter	Non-pathogenic	γ
							Enterococcus	Non-pathogenic	γ
11	11	4	Super-biotics	CPF Private Limited	India	Not Specified	*Enterobacter*	Partial - pathogenic	α

							Enterobacter	Non-pathogenic	γ
							Enterobacter	Non-pathogenic	γ
							Enterococcus	Non-pathogenic	γ
12	8	2	Ecotoxnil	Fishtechbd	Bangladesh	Not Specified	*Enterococcus*	Partial-pathogenic	α
							Zobellella	Pathogenic	β
13	14	2	ProAct-95	Pischina Biotech	India	Not specified	*Klebsiella*	Pathogenic	β
							Aeromonas	Pathogenic	β
14	5	2	Verno Bac	Naphavet	Vietam	*Saccharomyces cerevisiae* *Bacillus subtilis* *Bacillus licheniformis*	*Enterococcus*	Non-pathogenic	γ
							Aeromonas	Pathogenic	β
15	8	4	Promax-Aqua	Rims Bangladesh	India	*Bacillus subtilis* *Bacillus lincheniformis* *Bacillus polymyxa* *Bacillus megaterium* *Bacillus pimilus* *Pseudomonas*	*Aeromonas*	Pathogenic	β
							Lactococcus	Non-pathogenic	γ
							Kosakonia	Pathogenic	β
							Escherichia/Shigella	Non-pathogenic	γ

						putida *Pseudomonas denitrificans* *Aspergillusoryzae* *Aspergillusniger* *Nitrosomonas* *Nitrobacter* *Saccharomyces cervisae* *Bactovacilluslactis* *Lactobacillus helvecticus* *Cellulomonasuda* *Thiobacillusthiooxidans*			
16	10	3	Uni. Light	Uni President Vietnam Co. LTD	Vietnam	Not specified	Pseudocitrobacter	Non-pathogenic	γ
							Lactococcus	Non-pathogenic	γ
							Bacillus	Pathogenic	β
17	3	1	Ariake-3	Kyushu Medical Co. Japan	Japan	*Bacillus amyloquefaciens s.D203* *Bacillus pumilus S.D1728* *Bacillus licheniformis S. D3270*	*Aeromonas*	Partial-pathogenic	α
18	8	1	Power Lac	Kyushu Medical Co. Japan	Japan	*Lactobacillus lactis S. D1813*	*Klebsiella*	Pathogenic	β
19	2	1	Aqua	ACI limited		*Rhodopseudomonas sp.*		Pathogenic	β

			Photo			*Bacillus subtilis*	*Rahnella*		
20	4	2	Red Cap	Baxel Company Ltd	Thailand	Not specified	*Klebsiella*	Non-pathogenic	γ
							Enterobacter	Pathogenic	β
21	5	1	Green Cap	Baxel Company Limited			*Klebsiella*	Non-pathogenic	γ
22	3	3	Yellow Cap	Baxel Company Limited	Thailand	Not specified	*Enterobacter*	Partial-pathogenic	α
							Acinetobacter	Non-pathogenic	γ
23	7	1	Super psprob io tics	CPF private Ltd.			*Enterobacter*	Pathogenic	β

12. Research highlight/findings (Bullet point – max 10 nos.):

- Thirty one commercial aqua probiotics were collected and enlisted with the product label information.
- A total of 173 isolates were isolated from different commercial probiotics using selective media and tested for hemolytic activity where 48 isolates showed hemolytic activity.
- Biochemical characterization of some isolates including gram staining, catalase and oxidase were completed.
- Most of the tested isolates showed moderate tolerance in acidic condition whereas highly resistant in bile salt and showed diverse range of susceptibilities in different kind of antibiotics.
- Molecular identification of different isolates revealed diversified genus including 59% *Enterobacter*, 15% *Aeromona*s, 13% *Enterococcus*, 8% *Streptococcus* and 2% *Bacillus, Rhodobacter, Moraxella*.

B. Implementation Position

1. Procurement:

Description of equipment and capital items	PP Target		Achievement		Remarks
	Phy (#)	Fin (Tk)	Phy (#)	Fin (Tk)	
(a) Office equipment 1. Laptop 2. Desktop	 01 01	90000	 01 01	100%	Completed as per PP provision
(b) Lab &field equipment 1. Laminar flow 2. PCR machine	 01 01	810000	 01 01	100%	
(c) Other capital items					

2. Establishment/renovation facilities: Not applicable

Description of facilities	Newly established		Upgraded/refurbished		Remarks
	PP Target	Achievement	PP Target	Achievement	

3. Training/study tour/ seminar/workshop/conference organized: Not Applicable

Description	Number of participant			Duration (Days/weeks/ months)	Remarks
	Male	Female	Total		
(a) Training					
(b) Workshop					

C. Financial and physical progress

Items of expenditure/activities	Total approved budget	Fund received	Actual expenditure	Balance/ unspent	Physical progress (%)	Reasons for deviation
A. Contractual staff salary	578400	542150	578400	0	100	Pending honorarium for PI
B. Field research/lab expenses and supplies	1604607	1533056	1584371	20236	98.74	Amount to remain during for RFQ processing
C. Operating expenses	251931	237662	261125	-9194	103	For Vat, Tax and others purchases
D. Vehicle hire and fuel, oil & maintenance	0	0	0	0	0	
E. Training/workshop/seminar etc.	0	0	0	0	0	
F. Publications and printing	105000	29567	29567	75433	28.15	"PCR printing cost remain with PIU, BARC".
G. Miscellaneous	40210	37606	37606	2604	93.52	
H. Capital expenses	892600	904143	892600	0	100	
Total	**3472748**	**3284184**	**3383669**	**89079**	**97.43**	

D. Achievement of Sub-project by objectives: (Tangible form)

Specific objectives of the sub-project	Major technical activities performed in respect of the set objectives	Output(i.e. product obtained, visible, measurable)	Outcome(short term effect of the research)
1.To enlist probiotics using for aquaculture in Bangladesh.	i) Collection and preparation of list of commercial probiotic product with details label information	i) List of available probiotic product	i) Information on probiotic product has been generated
2. To isolate and characterize bacteria from commercial probiotics using for aquaculture	i) Probiotic sample incubated within different specific media ii) Pure colony was isolated based on colony morphology and biochemical characterization. iii) Pure colony was stocked at 30% glycerol for further studies. iv) DNA was isolated for molecular studies	i) 122 pure isolates were preserved in the laboratory ii) Pure isolates were biochemically and morphologically characterized	i) A good number of pure strains of probiotically important microorganism were reposited.
3. To determine the probiotic properties and antibiotic susceptibilities of different bacterial isolates.	i) pH tolerance, bile tolerance and autoaggregation assay of isolated strains were performed. ii) Antibiotic susceptibilities of isolated strains were carried out iii) Hemolytic assay for determining pathogenicity was done.	i) Scientific information on probiotic properties of isolated strains were documented ii)Antibiotic susceptibilities of pure strain was identified iii) Hemolytic activity of isolated strains were identified	i) Increased use of better quality probiotic will contribute in higher production of fish & shrimp.

E. Materials Development/Publication made under the Sub-project:

Publication	Number of publication		Remarks (e.g. paper title, name of journal, conference name, etc.)
	Under preparation	Completed and published	
Technology bulletin/ booklet/leaflet/flyer etc.			
Journal publication	√	Two MS thesis	**Title and name of journal**
Information development	-		-
Other publications, if any	-		-

F. Technology/Knowledge generation/Policy Support (as applied):

i. **Generation of technology (Commodity & Non-commodity):** Not Applicable

ii. **Generation of new knowledge that help in developing more technology in future**

Imported commercial probiotics are in question in terms of label information, contamination, pathogenicity and antibiotic susceptibilities

iii. Technology transferred that help increased agricultural productivity and farmers' income: Not Applicable

iv. Policy Support

- Government should adopt appropriate policy which can ensure the quality of probiotics for importing the commercial probiotics for sustainable aquaculture.
- Research and development for screening suitable indigenous probiotic products should be strengthen and executed.

G. Information regarding Desk and Field Monitoring

i) Desk Monitoring [description & output of consultation meeting, monitoring workshops/seminars etc.):

Desk and laboratory monitoring was completed during project executing period.

No. of Visit	Date	Team Members	Outputs	Remarks
01	01.03.2018	Dr Md Saleh Uddin	Some points for executing the project were discussed.	Suggestions and comments were taken into consideration during the project period.

ii) Field Monitoring (time& No. of visit, Team visit and output): Not Applicable

H. Lesson Learned/Challenges (if any)

i) Imported probiotics are not properly labelled
ii)Pure isolates were biochemically and molecularly characterized
iii) Some pathogenic strains were found in the commercial probiotic products due to contamination.

I. Challenges (if any)

i.Procurement of specific high-quality bacterial media and other necessary chemicals.
ii. Project period was too short.

Signature of the Principal Investigator
Date ……
Seal

Counter signature of the Head of the organization/authorized representative
Date ……
Seal

J. References:

Balcázar, J. L., Vendrell, D., de Blas, I., Ruiz-Zarzuela, I., Muzquiz, J. L., &Girones, O. (2008). Characterization of probiotic properties of lactic acid bacteria isolated from intestinal microbiota of fish. *Aquaculture*, *278*(1-4), 188-191.

Bauer, A. W., Kirby, W. M. M., Sherris, J. C., &Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *American journal of clinical pathology*, *45*(4_ts), 493-496.

Cappuccino, J. G., & Sherman, N. (2005). Microbiology: a laboratory manual.

Del Re, B., Sgorbati, B., Miglioli, M., &Palenzona, D. (2000). Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. *Letters in applied microbiology*, *31*(6), 438-442.

Fuller, R. (1992). History and development of probiotics. In *Probiotics* (pp. 1-8). Springer, Dordrecht.

Gilliland, S. E., Staley, T. E., & Bush, L. J. (1984). Importance of bile tolerance of *Lactobacillus acidophilus* used as a dietary adjunct. *Journal of dairy science*, *67*(12), 3045-3051.

Kim, D. H., & Austin, B. (2008). Characterization of probiotic carnobacteria isolated from rainbow trout (*Oncorhynchus mykiss*) intestine. *Letters in applied microbiology*, *47*(3), 141-147.

Nurhidayu, A., Ina-Salwany, M. Y., Daud, H. M., &Harmin, S. A. (2012). Isolation, screening and characterization of potential probiotics from farmed tiger grouper (*Epinephelusfuscoguttatus*). *African Journal of Microbiology Research*, *6*(9), 1924-193.

Salminen, S., Wright, A. V., & Ouwehand, A. (2004). Lactic acid bacteria (Vol. 1).

Samelis, J., Maurogenakis, F., &Metaxopoulos, J. (1994). Characterisation of lactic acid bacteria isolated from naturally fermented Greek dry salami. *International Journal of Food Microbiology*, *23*(2), 179-196.