

**DIETARY ADMINISTRATION OF PROBIOTICS TO MODULATE
NON-SPECIFIC IMMUNITY AND GUT MICROBIOTA OF NILE
TILAPIA (*Oreochromis niloticus*)**

Submitted in partial fulfilment of the requirements for the award of Bachelor of
Technology degree in Biotechnology

By

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**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO AND CHEMICAL ENGINEERING**

SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

Accredited with Grade “A” by NAAC

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DEPARTMENT OF BIOTECHNOLOGY

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the Bonafide work of Harshni S (37230026) who carried out the project entitled "Dietary administration of probiotics to modulate non-specific immunity and gut microbiota of Nile tilapia (*Oreochromis niloticus*)" under my supervision from January 2021 to April 2021.

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Submitted for Viva voce Examination held on 15/04/2021

Internal Examiner

External Examiner

DECLARATION

I HARSHNI S (37230026) hereby declare that the Project Report entitled “**Dietary administration of probiotics to modulate non-specific immunity and gut microbiota of Nile tilapia (*Oreochromis niloticus*)**” done by me under the guidance of **Dr. S. Kumaran** at Centre for Drug Discovery and Development, *Col. Dr. Jeppiaar Research Park*, Sathyabama institute of science and technology, Chennai has submitted in partial fulfilment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

DATE: 10/04/2021

PLACE: Chennai

A handwritten signature in black ink, appearing to read 'S. Harshni', is positioned above the signature line.

SIGNATURE OF THE CANDIDATE

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ABSTRACT

Probiotics are live beneficial microbe which are used as feed supplement for the improvement of gut microbiome. Fish highly susceptible to infectious pathogens which lead to diseases and mortalities are found, yet the effects of probiotics on immunity, gut microbiota and microbiological quality of fish in low input ponds are unknown. Nile tilapia, *Oreochromis niloticus* fingerlings (25 g) are randomly stocked at 100 fish m⁻³ in tanks. The fish were fed on diets supplemented with either *probiotic bacteria* (1×10^{10} CFU g⁻¹) (1×10^9 CFU g⁻¹) at three levels: No probiotic (Diet 0); *Probiotic* at 2 g kg⁻¹ (Diet 1); 4 commercial probiotics at 5 g kg⁻¹ (Diet 3). The results were analysed that the morphological parameters such as weight and size of the fish which are found significantly higher in fish fed on probiotic treated diets compared to the control ($P < 0.05$). In conclusion, *Bacterial probiotic* supplementation in diets of Nile tilapia reared in low input ponds improves immunity, manipulates gut microbiota and enhances fish flesh quality.

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LIST OF ABBREVIATIONS

1.	LAB	Lactic acid bacteria
2.	GI	Gastrointestinal tract
3.	SR	Survival rate
4.	MRS agar	De Man, Rogosa and Sharpe agar
5.	OD	Optical density
6.	HCl	Hydrochloric acid
7.	NaOH	Sodium hydroxide
8.	NaCl	Sodium Chloride
9.	rpm	Revolutions per minute
10.	NCBI	National Centre for Biotechnology Information
11.	mL	Milli liter
12.	μL	Micro liter
13.	nm	Nano meter

CHAPTER-1

INTRODUCTION

1.1 AQUACULTURE:

Aquaculture is fast developing food industry sector in various parts of the world. Most of the forms of food production like other human activity affect the environment by some other means. One-fourth of the fishes that are farmed are consumed by humans. But then the increase in population and its dependency on fishes that are farmed are the major source of nutrition.

Aquaculture is the practice of raising aquatic species under regulated conditions. It entails the cultivation of plants and animals for human consumption, recreational fishing, the enhancement of commercially valuable stocks, the conservation of endangered species, and the processing of bait and ornamental species. Ponds, raceways, cages, and net pens are used in aquaculture. Suspending mollusks from rafts or chains, rearing them in baskets or trays, or dropping them on the bottom in the natural setting are all possibilities. The highest level of technology is used in combination with recirculating systems, which has the highest level of technology. (Banerjee & Ray, 2017).

Culturists try to keep water quality, fertility, and disease under control. They often use feeds that are tailored to the animals being raised. Throughout their lives, most animals are kept in captivity. Selective breeding is often used to improve cultured organism stocks. Diseases can be a major headache for aqua culturists. Although avoiding stress decreases disease occurrence, treatments for at least some aquatic animal diseases, such as drugs and vaccines, are available. The final job of aqua culturist is to make sure the product gets to the processor in the best possible shape. Product consistency is improved by careful handling during harvest.

The development of aquaculture helps to maintain the sustainability of ocean fisheries. Some types of aquaculture which includes shrimp and salmon farming, creates a latent damage to the coastal and ocean resources by destruction in habitat. The diversity in aquaculture is the possible way as a solution and also contributes to the collapse of fisheries stock all over the world. (Bandyopadhyay *et al.*, 2015; Wu *et al.*, 2015)

According to FAO research, aquaculture production reached 106 million tonnes in 2017, with an average cost of USD 163 \$ and a growth rate of 6.6 percent. Some issues such as high stocking density, water pollution, improper or unscientific fish feeding increase the risk of pathogenic infections in cultured animals. This type of disease outbreak causes major problems by reducing food industry income and obstructing the country's socioeconomic situation. Pathogens such as *Aeromonas* sp., *Escherichia tarda*, *Escherichia coli*, *Vibrio vulnificus*, *Vibrio parahaemolyticus*, and *Vibrio cholerae* have evolved and spread, resulting in the use of antibiotics. Thereby the use of antibiotics in aquaculture had confined in many countries which has led to the development of probiotics as a healthy alternative with the dietary supplementation has become a constructive strategy for the resistance of pathogenic micro-organisms affecting the growth of cultured organisms (FAO, 2020).

1.2 PROBIOTICS IN AQUACULTURE

Probiotics are usually defined as live beneficial microbe which can be used as feed supplement for the improvement of gut microbiome in an organism. In the recent days Lazaro had stated that probiotics are not only the live organism it also includes the dead and even the bacterial component that take part in various activities which are beneficial to the host organism and also to the environment. These beneficial microbes help the fish to sustain during the stressful situation by improving the immune system and by regulating the gut microbiota with the presence of probiotic which synthesize antibiotics, lysozyme, acid phosphatase etc., (Mohapathra *et al.*, 2012).

Some of the common probiotic organism that are used for the benefit of aquaculture industry are *Lactobacillus* spp., *Bacillus* spp., *Saccharomyces cerevisiae*, and *Lactococcus* spp. The main concern of the commercial feed manufacturers are the quality and cost effectiveness.

In the field of aquaculture, the definition of probiotics is radically different from those used in terrestrial species, depending on a number of important influencing factors. It is now widely accepted that probiotics play a significant role in gut health maintenance by modulating the function of the microbial population (Nayak, 2010).

In response to diseases, the microbes multiply independently of the host animal. Kozasa made the first experimental attempt at probiotic application in aquaculture, based on the positive effects of probiotics on humans and poultry. The adverse effects of commonly used antibiotics and broad-spectrum chemicals, which destroy most of the beneficial bacteria along with the pathogenic bacteria, have sped up the evolution of probiotics in aquaculture (Lakshmi *et al.*, 2013).

As a result of the introduction of 'bioremediation' and 'biocontrol' when dealing with environmental issues, probiotics often operate through various processes in aquaculture systems to remove organic wastes and contaminants. Probiotics can play an important role in aquaculture development by providing increased non-specific disease protection as well as providing pollution-free water sources. With the goal of lowering culture costs, work has been done to reduce the cost of fish feed formulation by replacing costly ingredients in a more cost-effective manner. (Nandi, Banerjee, Dan, Ghosh, & Ray, 2018)

Because of its healthy essential aminoalkanoic acid (EAA) profile, high protein content, and excellent source of essential fatty acids (EFAs), digestible energy, vitamins, and minerals, organic fish meal has traditionally been used as a main protein in economical aqua feeds. Feed formulations have moved to rising levels of other protein sources while utilising small quantities of organic or animal proteins due to high cost and limited availability. Probiotics are used to resolve questions about administration methods, advantages, and bacterial viability. (Panigrahi, Kiron, Satoh, & Watanabe, 2010)

To keep up with the rapid growth of aquafarming, which grows annually at 11% increase the price of feed. Ingredients, in combination with their restricted availability, play a critical role in meeting demand. The formulation of other feed ingredients typically meets the nutritional requirements of the target species while remaining cost efficient and competitive. Incorporation of probiotics stimulates hemopoiesis and induces non-specific immunity in fish, according to recent reports. Poikilothermic vertebrates such as fish live in aquatic ecosystems and are particularly susceptible to seasonal and diurnal changes in water temperature. Temperature regulated fish expansion as well as other physiological and biochemical functions. The alarming increase in temperature

caused by global warming is expected to have a negative impact on wild catch fisheries as well as global aquaculture production. Probiotic feed is an alternative feed that offers a protein source with a high digestible protein and energy content as well as a balanced amino acid profile. Several studies have been carried out to determine the nutritional value. The aim of this study was to determine the quality of fish-growing water and, as a result, the benefits of improving fish-growing water quality. (Lazado *et al.*, 2014)

Dietary supplementation of various feed additives, such as immunostimulants, probiotics, and prebiotics, in small amounts to fortify feed with certain nutrients has been found to benefit crustaceans and finfish immune status, feed quality, and growth performance. The use of prebiotics and supplementary enzymes in fish feed, on the other hand, is gaining popularity in commercial aquaculture. The effects of prebiotics on live body weight gain, dressing percentage, weight of important organs and muscles, and mean villus lengths in fish alimentary canals, as well as their use as growth promoters in commercial aquafeed. (Nayak, 2010; Gomez *et al.*, 2009)

1.2.1 SOURCE FOR PROBIOTICS

Various probiotic microorganisms have been identified and characterized in the last three decades. These beneficial organisms are of two different origins host and non-host. The commonly used probiotics are obtained from intestine of aquatic animals and also some terrestrial animals. Bacterial organisms isolated from marine fishes, such as *Vibrio* and *Pseudomonas* sp., are said to be probiotics. The advantages of different probiotic organisms used in aquaculture are discussed. There is no agreement on which source of probiotics is best for use in fish. Natural probiotics have been shown to benefit cultivated animals in a number of ways. Probiotics from the host, on the other hand, have a variety of benefits, including a leg up on certain biotechnical issues (such as temperature, salinity, and familiarity with the environment (Lazado *et al.* 2015).

In aquaculture, different characteristics were used, such as the type of probiotic (bacteria, fungi, or algae) and the host from which they were derived, single-strain probiotic vs. multi-strain probiotic, use of viable or non-viable species as probiotic, and spore formers vs. non-spore formers (i.e., host or non-host). These are some

of the reasons why probiotics that are commonly applicable are a pipe dream. (Nayak, 2010)

1.2.2 PROBIOTIC ORGANISMS:

In recent times application of probiotics has become a practice in aquaculture sectors which are particularly isolated from fish gut. Lactic acid bacteria (LAB), Bifidobacterium, and Streptococcus are among the most common bacterial species (Giri *et al.*, 2013). In spite of the fact that the introduction of probiotics is a new approach which is also gaining attention towards its potential activity for the control of various physiological activity of aquatic organisms. Thereby, some of the significantly effective probiotic organisms include *Aeromonas media*, *Bacillus subtilis*, *Lactobacillus helveticus*, *Enterococcus faecium*, *Carnobacterium inhibens*, etc. Gram-negative facultative symbiotic anaerobes including Vibrio, Pseudomonas, Plesiomonas, and Aeromonas have also been found in the gastro-intestinal tract (GIT) of fish and shellfish as possible probiotic candidates. (Lakshmi *et al.*, 2013)

1.2.3 PROBIOTICS AND MICROBIAL COMMUNITY OF THE FISH GUT

The intestine provides favourable environment which includes the space, attachment sites and nutrient for the indigenous microorganisms. The health of the gut highly relies on microbial communities that are well balanced. The normal microbial populations in the gut are disturbed during illness, resulting in a variety of health issues. Fish exist in a state where they are surrounded by a large number of pathogenic bacteria, fungi, and deadly viruses. (Banerjee & Ray, 2017)

The use of dietary probiotic supplementation to restore gut microbial communities is an efficient way to improve fish health. However, in order to maintain a healthy good-to-bad bacteria ratio in the gut mucosal surface, the preference of probiotics varies greatly from one fish species to the next. A number of external/environmental (water quality, temperature, and pH) and internal (fish age, probiotic binding power, duration of probiotic supplement diet, delivery system, and so on) factors influence a probiotic's ability to manipulate bacteria on the gut mucosal surface. All of these variables may have an impact on the effectiveness of

probiotic. The complex phenomenon of host-microbe cross talk on the gut epithelial surface is responsible for keeping the environment clean. In humans, using faecal microbial therapy (microbiota acquired from healthy individuals) to treat a range of diseases, it is common practise to recover gut microbiota in patients. In mammals, including humans, probiotic research is at an all-time high; however, in aquaculture, such research is still lacking. *Bacillus* sp., *Micrococcus* sp., *Enterococcus* sp., *Shewanella* sp., lactic acid bacteria, and *Pseudomonas* sp. have all been studied for their probiotic ability. (Hasan and Banerjee., 2020)

1.2.4 PROBIOTICS ON DISEASE RESISTANCE

Several researches on probiotics have shown that they have beneficial effects in the gut of the host, which are crucial in the prevention of disease and the treatment of digestive tract inflammation. Probiotic microorganisms, such as the lactic acid bacteria *Brevibacillus brevis* and *Vibrio harveyi*, adhere to the mucosal epithelium of the gastrointestinal tract and help to resist pathogens, in addition to immune modulation. (Hoseinifar *et al.*, 2018; Modanloo *et al.*, 2017)

On the other way, probiotics increase the digestion process by elevating different digestive enzymes like alginate lyases, amylases, and proteases. They also aid in the development of organic acids like fatty acids, biotin, and vitamin B12, as well as hydrogen peroxide, antibiotics, bacteriocins, siderophores, and lysozyme, all of which are beneficial to the host's health. Other studies have shown that introducing probiotics to aquatic species such as Japanese flounder, black tiger prawns, *Penaeus monodon*, and white leg prawns will improve their health. (Zokaeifar *et al.*, 2012)

1.3 NILE TILAPIA

Nile tilapia (*Oreochromis niloticus*) is an African freshwater cichlid that is one of the most popular food fishes in the world. It has been widely introduced for aquaculture, augmentation of capture fisheries, and sport fishing due to its hardy nature and wide range of tropic and ecological adaptations, and can now be found in any tropical region. Since it thrives in disturbed habitats and migrates and reproduces opportunistically, the Nile tilapia is often referred to as a "pioneer"

species. These characteristics mean that in areas where it has been introduced, the Nile tilapia sometimes outcompetes native species. (FAO, 2020)

1.3.1 SIGNIFICANCE OF NILE TILAPIA – PROBIOTICS

In China and many parts of South India, tilapia is one of the most common farmed fish species for freshwater aquaculture. *Streptococcus agalactiae* disease has become a major threat for tilapia culture in recent years, resulting in huge losses for tilapia farmers all over the world. Probiotics (especially antagonistic probiotics) can reduce pathogenic bacteria through competitive exclusion, provide nutrients and enzymes to promote host growth, boost the immune response through immune stimulation, and are free of secondary contamination concerns. As a result, receiving *S. agalactiae* antagonising probiotics appropriate for tilapia culture is critical for improving tilapia tolerance and reducing antibiotic use. By modulating the intestinal microbial composition, probiotics are an effective and appealing way to protect and promote host health. (Komen and Trong, 2014).

Some of the key mechanisms of action of probiotics include improved epithelial barrier function, stronger adhesion to intestinal cells, pathogen inhibition by occupying adhesion sites, production of antibacterial substances, and immune function regulation. The mechanisms mentioned above achieve the goal of regulating intestinal microbes and inhibiting pathogen development. (Almada *et al.*, 2015).

The use of beneficial bacteria in the form of probiotics has recently been shown to be beneficial in aquaculture. Lactic acid bacteria (LAB) and *Bacillus* spp. are the most commonly used probiotic microorganisms in aquaculture. According to previous research, certain *Bacillus* spp. (including *Bacillus subtilis*) have been widely used as probiotics in aquaculture due to their beneficial properties of enhancing the immune system, competitive exclusion, and the development of antibacterial substances. *Bacillus subtilis* probiotic candidates improved Nile tilapia growth efficiency and disease tolerance by stimulating immune responses both locally and systemically in the fish. (Galagarza *et al.*, 2018)

In addition, both *B. subtilis* and *Bacillus cereus* promote intestinal colonisation and increase tilapia survival rates (SR) without affecting feed consumption, total biomass, gross revenue, partial operating costs, or net revenue. *Bacillus* spp. are

used as a feed additive to promote the skin's beneficial bacterial composition, which can outcompete intestinal pathogenic bacteria. In the intestines of the hosts, *B. subtilis* increased the growth and vitality of beneficial LAB. Streptococcosis infections can be prevented and treated with *Bacillus*. *B. subtilis* was isolated from a mixed species *Bacillus* spp. probiotic used in our lab to control aquaculture water quality, and *B. cereus* was isolated from stable Nile tilapia faeces in this study. According to a previous study, both strains were active in antagonising *S. agalactiae* in vitro, but it is unclear if they have a probiotic effect in vivo. This study looked into the effects of potential probiotic strains with particular antagonistic activity in Nile tilapia. The effect on growth, survival, immunity, disease resistance, and the intestinal microbiota provides a solid theoretical foundation for future probiotic strain commercialization and implementation. (Liu *et al.*, 2015)

CHAPTER-2

LITERATURE SURVEY

Mohapathra and research team had explained that Probiotics are historically described as "a live microbial feed supplement that improves the microbial balance of the host animals." This concept of probiotics has changed over time, particularly in terms of its applicability in aquaculture. "Live or dead, or maybe a part of the bacteria that function under different modes of action in conferring beneficial effects to the host or to its environment," according to Lazaro's concept of probiotics. (Mohapathra *et al.*, 2012)

Gomez and his fellow members had concluded that fish are poikilothermic vertebrates that live in aquatic environments and are highly sensitive to seasonal and diurnal temperature changes. Fish development, as well as other physiological and biochemical functions, were influenced by temperature. Global warming is predicted to have a detrimental impact on both wild catch fisheries and global aquaculture development. Probiotic feed is a high-digestible protein and energy source with a high aminoalkanoic acid profile. Several tests are performed to determine the nutritional value. (Gomez *et al.*, 2009)

Opiyo and her research team had reported that *Saccharomyces cerevisiae* and *Bacillus subtilis* are two of the most commonly used probiotics in aquaculture to reinforce growth and improve feed utilisation due to their low cost, viability, ability to live in the digestive tract of fish, and ability to colonise the gut of fish. While probiotics are widely used in Kenya's livestock industry, their use in aquaculture is often unintentional. This may be due to a lack of understanding about their importance, efficacy, and level of implementation. Dietary probiotics are critical for keeping fish safe and promoting growth in low-input ponds by enhancing nutrient utilisation and growth. (Opiyo, *et al.*, 2019)

Mamun and team has explained that probiotic research has become a commonly accepted and well-recognized part of veterinary health management. The subject of probiotic research in aquaculture has gotten a lot of attention in the last two decades. Several probiotic products are currently on the Indian market, and farmers often use them in fish and shrimp farms. As a result, probiotics have emerged as a viable

alternative to chemotherapeutics, particularly antibiotics, which are notorious for harming living organisms. However, there is still much work to be done in the aquaculture industry. Since several empirical studies failed to produce sufficient results. It's likely that it's due to improvements in environmental conditions. Since the use of probiotics is likely to raise assembly costs, research must be prioritised and stressed. (Mamun, *et al.*, 2019)

Abareethan and Amsath had stated that *L. rohita*'s growth and survival were affected positively by a mixture of isolated bacterial strains. The nutritional value of probiotic feed, as well as how it is used. From this research review, the acceptable feed selected for feeding fish *L. rohita* was regarded as a very promising feed and novel strategy to be used in aquaculture. As a result, formulated isolates were used in the culture of *L. rohita* fish. Probiotics for aquaculture are frequently considered for improving *L. rohita* fish development, attachment to intestinal mucus, and production of beneficial compounds. (Abareethan and Amsath, 2015)

Jatoba and research team has stated that In Nile tilapia and shrimp, dietary supplementation with *L. plantarum* strengthened the gut bacterial microbiota and increased the number of circulating thrombocytes, total leukocytes, and lymphocytes. In Nile tilapia, adding *Lactobacillus plantarum* to the diet resulted in increased weight gain and feed production. If it works on all animals, a diet supplemented with only one probiotic strain can be used for tilapia in polyculture systems with marine shrimp as well as systems with multiple organisms. (Jatoba *et al.*, 2011)

Lakshmi and her colleagues had explained that since probiotics are natural immune enhancers that induce disease resistance in shrimp farms, they were chosen as the best antimicrobial alternatives. Viruses are the primary constraint in shrimp farms, resulting in significant production losses. Probiotics are helpful bacteria that can have antiviral properties. The application of these probiotics in shrimp aquaculture for viral therapeutic applications is a novel and beneficial process. (Lakshmi *et al.*, 2013)

Muthukumar and Kandeepan had concluded their research that it is possible that the isolates have a strong affinity for fish mucus. Furthermore, these isolated species, especially *A. hydrophila*, have a high ability to inhibit pathogen growth in

freshwater fish. As a result, isolated species tend to have a high probiotic capacity, and molecular characterization is being performed on these organisms. (Muthukumar and Kandeepan., 2015)

Yasmin and his team had reported their study that the researcher attempts to isolate *Bifidobacterium* strains from raw camel milk to see if they had any probiotic properties. Gram-positive, catalase-negative, non-spore forming, non-motile, and V or Y shaped rods were found in eight of the 35 isolates. Antioxidant properties, antagonistic activities, nitrite reduction, and cholesterol absorption were all investigated. Out of all the isolates tested, one was selected because it had the most probiotic properties. *Bifidobacterium longum* B-11 was classified as a member of the *Bifidobacterium longum* group by 16S rRNA gene sequencing. For in vivo attachment, the *B. longum* B-11 strain was selected. (Yasmin *et al.*, 2020)

Keysami, Mohammadpour and Saad observed the apparent probiotic properties of *Bacillus subtilis* and the best ways to incorporate it into commercial feed in *Macrobrachium rosenbergii* juveniles *Bacillus subtilis* was isolated from the intestine of a juvenile *M. rosenbergii* and used as a probiotic in commercial prawn feed. *B. subtilis* at a concentration of 10^8 cells g⁻¹ was combined with commercial feed in five separate ways using mixing, soaking, spraying, and bathing. *M. rosenbergii* juveniles grew and survived much better on diets containing *B. subtilis*. The tested strain was found to be a promising probiotic for *M. rosenbergii* after soaking. (Keysami, Mohammadpour and Saad., 2012)

CHAPTER-3

AIM AND SCOPE

Cultured or aqua farmed fish are easily susceptible to a variety of infectious diseases, which may result in death. Probiotics has become an effective alternative to antibiotics exhibiting potential development in the immunity and improve gut microbiota. Therefore, the main aim of the project is that the development of cost-effective probiotic fish feed which enhances non-specific immunity and gut microbiota. As probiotic bacteria are beneficial and do not harm the human body, studies on its antimicrobial properties provide a useful field in order to replace the results of this investigation to control and treat fish pathogenic bacterial infections. Thereby this study on probiotic bacteria in fish feed are of high commercial and economical value for human and aquatic welfare, by reducing the mortality rate and enhancing the flesh quality which meets the demands of the consumers not affecting the aquatic ecosystem.

CHAPTER-4

MATERIALS AND METHODS

4.1 SELECTION OF FISH:

Preferred Scientific Name: *Oreochromis niloticus*

Preferred Common Name: Nile tilapia

Taxonomic Tree

Domain: Eukaryota

Kingdom: Metazoa

Phylum: Chordata

Subphylum: Vertebrata

Class: Actinopterygii

Order: Perciformes

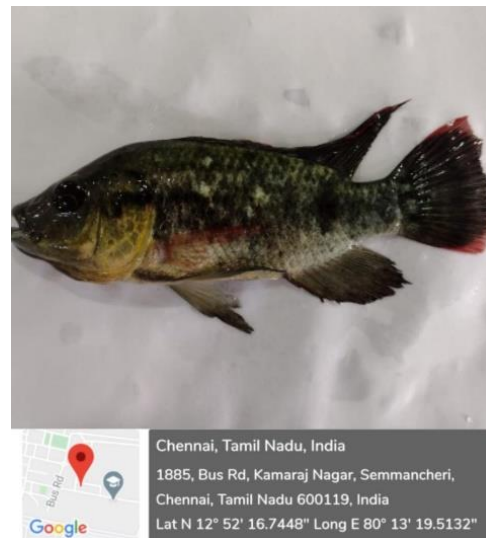
Family: Cichlidae

Genus: *Oreochromis*

Species: *Oreochromis niloticus*



(A)



(B)

Fig 4.1 Nile Tilapia

Nile tilapia (*Oreochromis niloticus*) are known as food fish from ancient period where the fish was reared from man-made ponds. The Nile tilapia is said to be India's most famous fish, and it's most commonly found in South Indian reservoirs that are open all year. In a short period of time, *O. niloticus* grows faster and reaches larger sizes. Nile tilapia nests abound in the littoral areas of Kelavarappalli Reservoir, and they breed during the south-west monsoon (July–September). The fish eat mostly detritus. Zooplankton, phytoplankton, and macrophytes have been found in the guts of Nile tilapia on rare occasions. Because this fish is affordable to the lowest income group in this region, demand is high, especially from local poor people. However, illness causes a certain amount of mortality, leading to the production of probiotic feed administration, which modifies non-specific immunity and intestinal microbiota. (Feroz Khan *et al.*, 2009)

4.2 SAMPLING OF FISH GUT:

The fish under investigation, Nile Tilapia was transversely dissected towards its abdomen and the gut was removed. It was then washed with saline water to remove the unwanted parts surrounding the gut. The separated gut was rinsed thoroughly and ground using mortar and pestle.

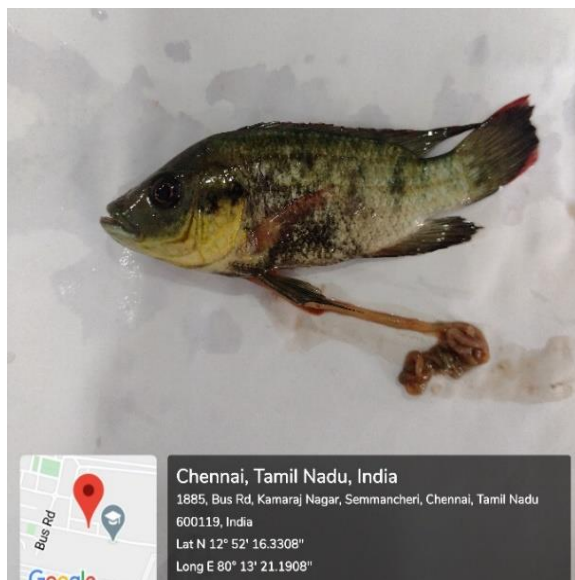


Fig 4.2 Dissected gut



Fig 4.3 Gut of Nile Tilapia (A)



Fig 4.4 Gut of Nile Tilapia (B)

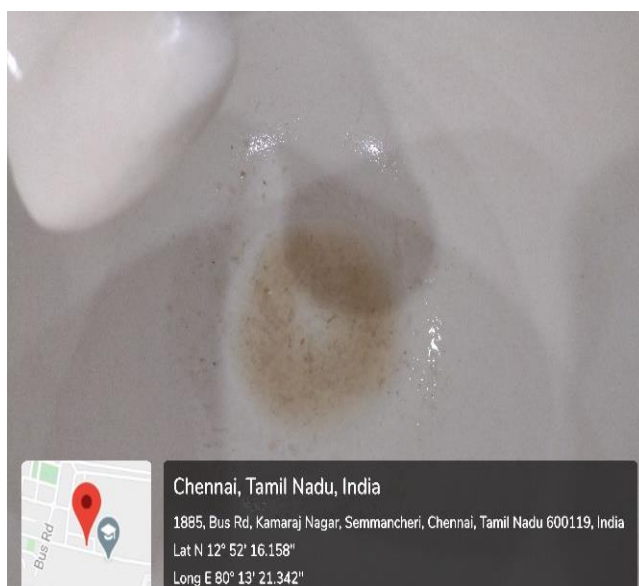


Fig 4.5 Minced gut

4.3 ISOLATION OF BACTERIAL SAMPLE

4.3.1 MRS (De Man, Rogosa and Sharpe) AGAR:

MRS agar, a medium for the cultivation of Lactobacilli from various sources, was created by De Man, Rogosa, and Sharpe with the aim of producing a given medium as an alternative to tomato juice agar. It is used to cultivate the whole family of lactic acid bacteria. Reduce the pH to 5.7 and add 0.14 percent sorbic acid to form MRS-S agar, which has shown good productivity for the growth of all types of lactic acid bacteria. Some strains obtained from dairy sources grew at a slower pace than others.

The composition of MRS agar are as follows:

Composition	g/L
Peptone	10
Beef Extract	10
Yeast extract	5
Dextrose	20
Polysorbate 80 (Tween 80)	1
Ammonium citrate	2
Sodium acetate	5
Magnesium Sulphate	0.10

Manganese Sulphate	0.05
Di-potassium hydrogen phosphate	2
Agar	5

Preparation of MRS agar:

The composition was suspended in water and dissolved completely. This was then sterilized at 121°C. (De man *et al.*, 1960)

4.3.2 SPREAD PLATE METHOD:

The crude sample that was homogenized with 1 ml saline was serially diluted to different decimal dilutions of 10^{-1} to 10^{-4} and the samples were spread using L-rod on the MRS agar plates which as sterilized, inoculated and incubated at 37°C for 24 hours. After 24 hours, the culture was then isolated for colonies by quadrant streaking and simple streaking method. (Chu *et al.*, 2011)

10 colonies were isolated and sub cultured by the inoculation of colonies in nutrient broth.

4.4 CHARACTERIZATION OF PROBIOTICS:

4.4.1 GRAM'S STAINING METHOD:

This technique is used characterize the isolated colonies morphologically by smearing the isolated colonies on a glass slide and heat fixed. Then after, it is stained with crystal violet and left to sit for a minute before being rinsed with water. The iodine solution is then applied and left on for one minute before being rinsed off. The residual stain was rinsed with water after being washed with decolorizer for 5 seconds. After that, the slide was flooded with safranin for 30–60 seconds before being rinsed with water. This was then dried and examined under a microscope. (Sharifuzzaman *et al.*, 2018)

4.4.2 ANTIMICROBIAL ACTIVITY:

The agar well diffusion method was used to test the antimicrobial activity of the isolated bacteria strains against some of the most common pathogens. *Streptococcus sp.*, *Vibrio parahaemolyticus*, *Vibrio harvei*, *Salmonella sp.*, and *Flavobacterium columnare* were the bacterial test strains used, which were provided by the Centre of Drug Discovery and Development, Sathyabama Institute of Science

and Technology, Chennai, India. The bacterial strains isolated from the GI tract of *O. niloticus* were cultured in MRS broth medium (pH 7.0) and incubated in a thermal shaker for 48 hours at 37 °C. The supernatant was then centrifuged and tested for antimicrobial activity. Plates containing 15 mL nutrient agar were spread with sterile cotton swabs to develop a bacterial lawn with test bacterial cultures, and wells (6 mm diameter) were made with a 6 mm diameter cork borer and filled with samples from the fish intestinal microbiota. After a 24-hour incubation period at 37°C, the plates were tested for the zone of inhibition. (Alonso *et al.*, 2019)

4.4.3 SAFETY ANALYSIS OF PROBIOTIC ISOLATES

Hemolytic activity:

The hemolytic activity of bacterial samples was evaluated as a biosafety indicator. The culture was streaked on blood agar plate and incubated for 24 hours at 37°C after being inoculated and incubated for 24 hours at 37°C. Positive activity is indicated by the clear zone. The plates were tested for α -hemolysis, β -hemolysis, and non-hemolytic or gamma hemolytic activities after incubation. (Mangia *et al.*, 2019)

Antioxidant assay (Hydroxyl radical scavenging activity):

The antioxidant assay was carried out according to Wang et al (2009). 1.0 mL (0.75 mmol⁻¹) 1,10-phenanthroline 2.0 mL sodium phosphate buffer (pH 7.4) and 1.0 mL ferrous sulphate (FeSO₄) (0.75 mmol⁻¹) were combined, and 1.0 mL of 109 CFU/mL culture was added; 1.0 mL hydrogen peroxide (H₂O₂) (0.01% v/v) was added to start the reaction, and the mixture was incubated at 37°C for 90 minutes. After incubation, the sample was centrifuged at 9000 rpm for 10 minutes, and the absorbance was measured with the supernatant at 536 nm. Hydroxyl radical scavenging percentage was calculated with the formula:

$$\text{Hydroxyl radical scavenging activity (\%)} = (A_s - A_c) / (A_B - A_c) \times 100$$

Where,

A_s is the absorbance of the test sample

A_c is the absorbance of the control including 1,10-phenanthroline, FeSO₄, and H₂O₂

A_B is absorbance of the blank including 1,10-phenanthroline and FeSO_4 . (Amaretti et al., 2013).

4.4.4 CATALYSE TEST:

Culture was smeared on a clean glass slide and one or two drops of hydrogen peroxide was added to it. Release of air bubbles indicates positive reaction. (Abareethan and Amsath, 2015)

4.4.5 pH TOLERANCE TEST:

MRS broth was chosen as the medium for the culture in which the broth was altered with the pH with the Addition of 1% HCl and NaOH. The pH was maintained from 3 to 9 and sterilized at 121°C . This was inoculated with the bacterial cultures and incubated for 2 hours and OD values were measured using spectrophotometer at 600nm. (Allamesh et al., 2012)

4.4.6 SODIUM CHLORIDE TOLERANCE TEST:

Sodium Chloride was added in 4 different concentrations of 2%, 6%, 10% and 15% to MRS broth. This was then sterilized and inoculated with the culture samples. The culture was incubated for 24 hours at 37°C for which OD values are measured at 600 nm using spectrophotometer. (Hoque et al., 2012)

4.4.7 SALINITY TEST:

Sea water of 28 ppt salinity was collected from Centre for Ocean Research, Col. Jeppiaar Research Park, Sathyabama Institute of science and Technology, Chennai. The sea water was taken in different concentrations of 0%, 15%, 30%, 50% and 70% which was made up with the addition of MRS broth. This was then sterilized at 121°C for 15 minutes and then inoculated with the selected culture samples Tp11 and Tp6. It was then incubated for 24 hours and optical density was observed at 550 nm using spectrophotometer. (He et al., 2021)

4.4.8 KINETICS OF BACTERIAL GROWTH:

100 ml of MRS broth was made up. This was added with 2.5% NaCl and sterilized at 121°C and inoculated with the culture samples. This was then incubated and OD

values are noted at an interval of one hour from the time of inoculation. (Jiang *et al.*, 2020)

4.5 ISOLATION OF DNA

The samples that exhibited better characteristics was proceeded for the isolation of DNA. The DNA was isolated from the culture samples of fish gut. The commercial Medox-Bio™ Genomic DNA extraction kit was used for the isolation of DNA. The instructions used with the above DNA isolation kit were strictly followed for all samples.

The bacterial cells that were cultured in nutrient broth was spun at 8000rpm for 10 minutes and the supernatant was discarded and the pellet was resuspended in 200µl cold TE buffer. 400 µL digestion solution was added to the sample of 200 µL and mixed well. This was then added with Proteinase K solution and incubated at 55°C for 5 minutes. This was then centrifuged for 2 minutes at 8000rpm and 2 mL of supernatant was taken in a collection tube and added with 260µL absolute ethanol. 2 mL collection mixture was added to the column and centrifuged at 8000 rpm for 1 minute. The flow through in collection tube was discarded and 500 µL of wash solution was added and centrifuged at 8000 rpm for 1 minute. This is then repeated again. The flow through was discarded and again spun at 1000 rpm for one minute to remove the left-out solution. The MX-10 column was placed in a clean 1.5 mL microfuge tube and added with 30-50 µL elution buffer to the centre part of the membrane in the column. This is then incubated at 37-50°C for 2 minutes. It was centrifuged at 10000 rpm for 1 minute and the DNA was measured in UV absorption at A₂₆₀. The quality of DNA was assessed by gel electrophoresis method. (Talwar *et al.*, 2018)

4.6 SEQUENCING:

Preliminary identification of the strain was carried out in which the isolates were morphologically and biochemically characterized. For further molecular analysis isolated samples was extracted and culture. The 16s rRNA gene of the isolated bacterial samples was amplified in a thermal cycler under certain conditions, where initial denaturation at 95°C for 5 minutes which was followed by 34 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute and extension at

72°C for 3 minutes and the final extension was for 7 minutes at 72°C. The obtained product of PCR was separated by agarose gel electrophoresis. The eluted amplicons were eluted, purified and sequenced (Rajiv Gandhi Centre for Aquaculture, Nagapattinam). The chromatograms were compared with the nucleotide sequence of *Lactobacillus* sp., available in the National Centre for Biotechnology Information (NCBI) database. The gene sequences of Tp11 and Tp6 were identified and submitted to NCBI. (Thankappan *et al.*, 2015)

4.7 PRODUCTION OF BACTERIOCIN:

The probiotic bacterial culture was inoculated in 200 mL of MRS broth which was maintained at 35-37°C in thermal shaker for continuous shaking for 3 days. (Banerjee *et al.*, 2017)

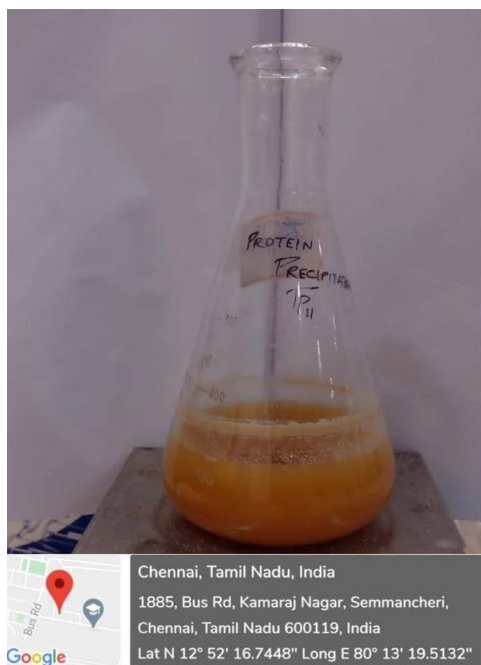


Fig 4.6 Probiotic production

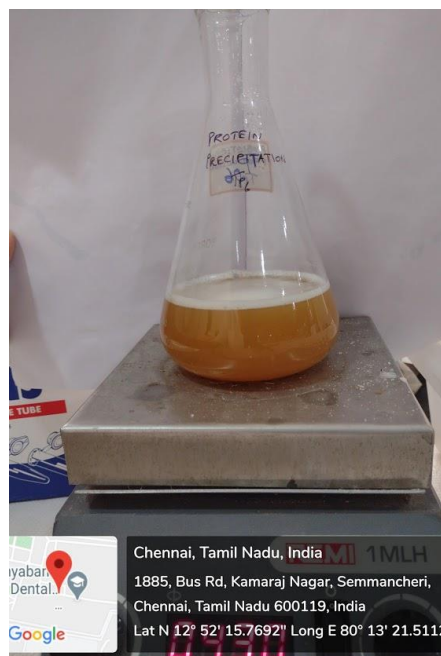
4.8 PROTEIN PRECIPITATION AND DIALYSIS

The bacterial strain Tp11 and Tp6 was cultured in 200 mL MRS broth. This culture was added with solid ammonium sulphate of 70% with simultaneous stirring in magnetic stirrer. Ammonium sulphate was added to its saturation level and centrifuged at 4000 rpm for 30 minutes for the precipitation of protein. To extract the excess ammonium sulphate, the supernatant was collected and resuspended in Tris HCl buffer (20mM, pH 8) upon being dialyzed overnight at 4-6°C using a

Spectra/PorR, VWR 2003 dialysis membrane against the same buffer. The antimicrobial protein was purified using a DEAE-cellulose column (12×0.6 cm) with an increasing Tris-HCl buffer gradient. The fractions were collected and used for further studies.



Tp11



Tp6

Fig 4.7 Protein precipitation



Fig 4.8 Dialysis

CHAPTER-5

RESULTS AND DISCUSSION

5.1 ISOLATION OF BACTERIAL STRAINS

By culturing in MRS agar, a selective growth medium for Lactobacilli and Lactic acid bacteria, aerobic culturable bacteria were isolated from the gut of Nile Tilapia (*Oreochromis niloticus*). Bacteria were included in all of the samples, and a total of 10 different strains were chosen and obtained from two different fish.

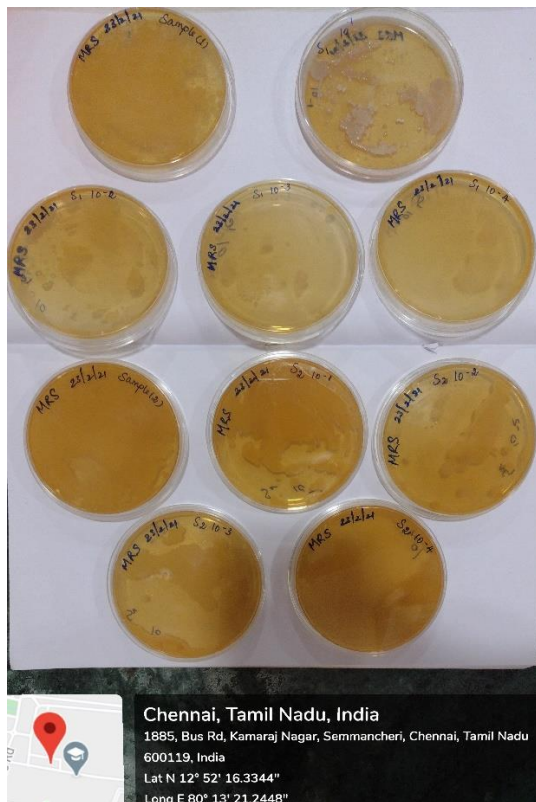


Fig 5.1 Spread Plated samples



Fig 5.2 Control



Fig 5.3 Quadrant streaking

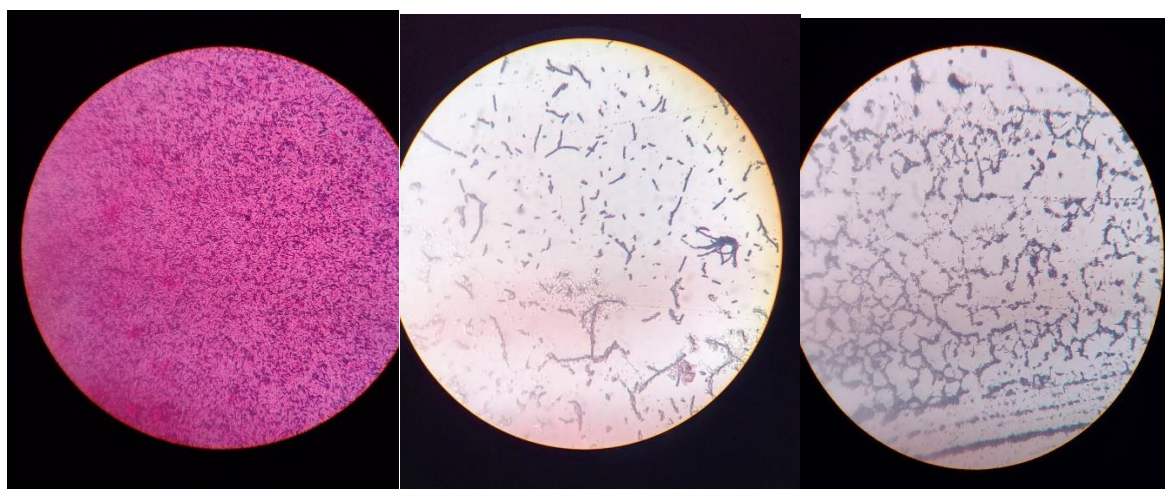


Fig 5.4 Simple streaking

5.2 CHARACTERIZATION OF PROBIOTICS:

5.2.1 GRAM'S STAINING:

The 10 samples selected was subjected for characterization studies and staining was performed. The results were observed for Gram positive bacteria in which 6 samples exhibited Gram positive characteristics.



Sample Tp11

Sample Tp33

Sample Tp1

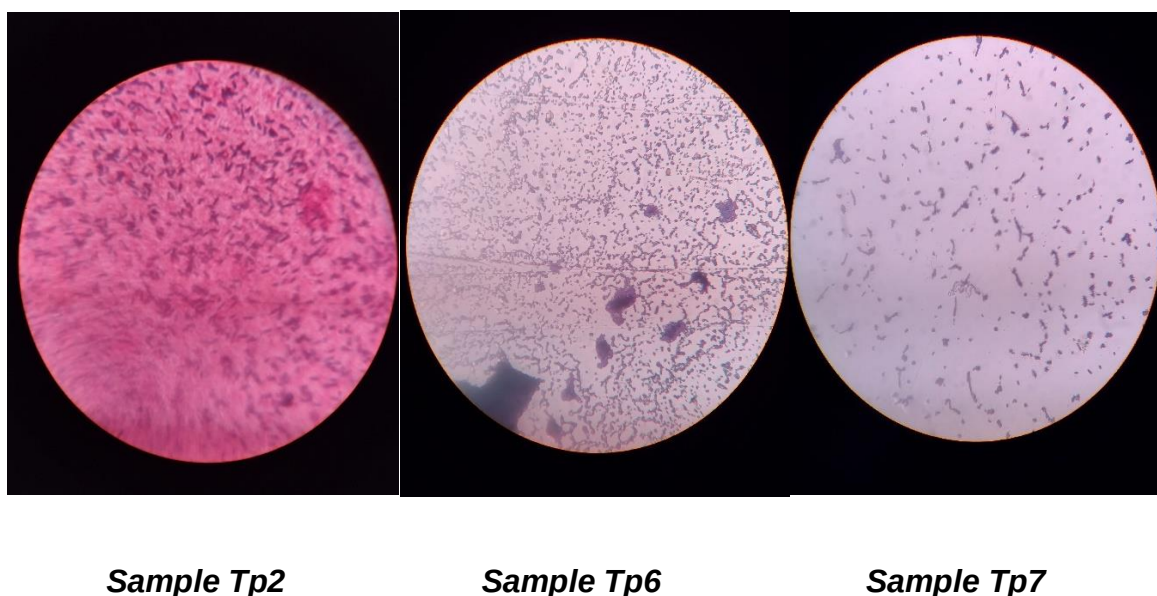


Fig 5.5 Gram's staining

5.2.2 ANTIMICROBIAL ACTIVITY:

Antimicrobial activity for the isolated bacterial samples was observed against the given fish pathogens *Streptococcus sp.*, *Vibrio parahaemolyticus*, *Vibrio harvei*, *Salmonella sp.*, and *Flavobacterium columnare* and the zone of inhibition was measured. Here the isolated probiotic bacteria have the antimicrobial activity. The results were reported in (Table 5.1).

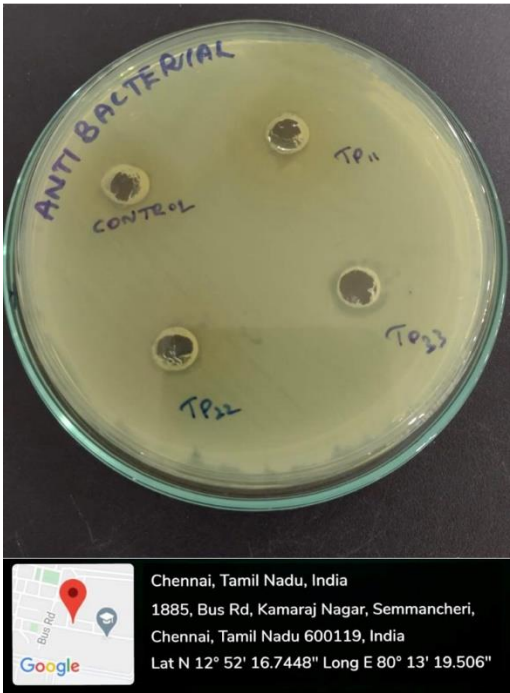
Microbes	Tp11	Tp22	Tp33
<i>Streptococcus sp.</i> ,	10 mm	6 mm	4 mm
<i>Vibrio parahaemolyticus</i>	2 mm	-	-
<i>Vibrio harvei</i>	8 mm	6 mm	6 mm
<i>Salmonella sp.</i> ,	4 mm	4 mm	2 mm
<i>Flavobacterium columnare</i>	6 mm	4 mm	4 mm

Table 5.1 Zone of inhibition

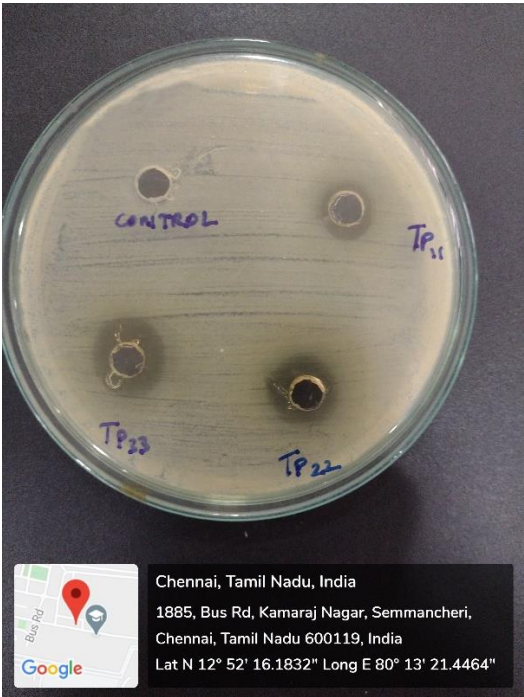
This showed that the culture sample Tp11 exhibited better antimicrobial activity and it was selected for further studies.



Streptococcus sp.,



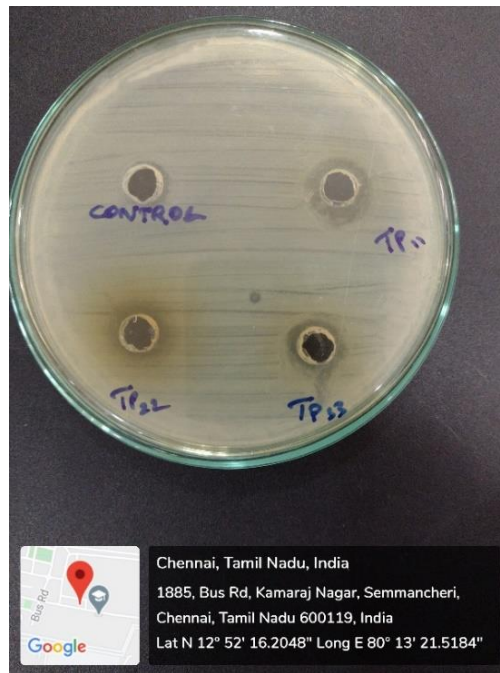
Vibrio paraheamolyticus



Vibrio harvei



Salmonella sp.,

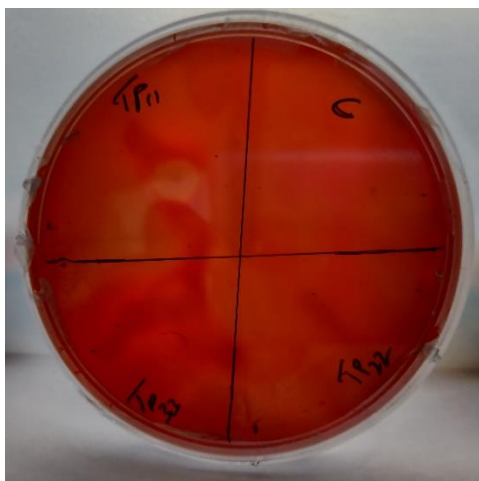


Flavobacterium columnare

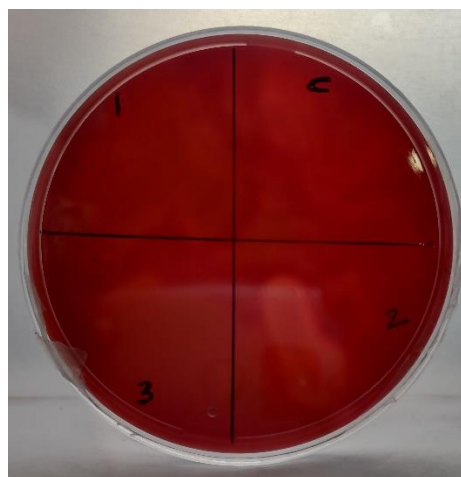
Fig 5.6 Anti-bacterial activity using Well diffusion method

5.2.3 HEMOLYSIS TEST:

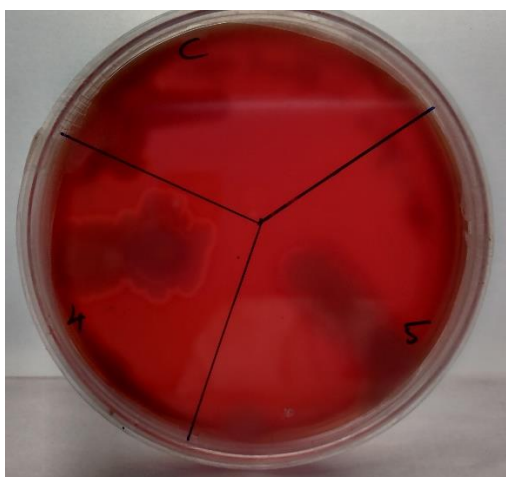
The alpha, beta, and gamma haemolysis cultures streaked on the blood agar revealed three forms of haemolysis. The partial or green haemolysis in which the blood cells are partially lysed is known as alpha haemolysis. Beta haemolysis is the complete haemolysis and gamma haemolysis is that there is no lysing of blood cells. Samples Tp5 and Tp7 exhibited alpha haemolysis and samples Tp2 and Tp4 exhibited beta haemolysis and samples Tp11, Tp22, Tp33, Tp1, Tp3 and Tp6 exhibited gamma haemolysis which are considered safe for the intake of fishes and selected for further studies.



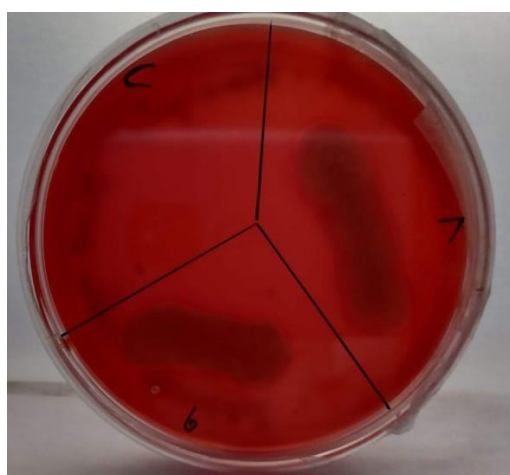
Samples Tp11, Tp22, Tp33



Samples Tp1, Tp2, Tp3



Samples Tp4, Tp5



Samples Tp6, Tp7

Fig 5.7 Haemolysis test

5.2.4 ANTIOXIDANT ASSAY:

Hydroxyl radical scavenging percentage was calculated with the formula:

$$\text{Hydroxyl radical scavenging activity (\%)} = (A_s - A_c) / (A_B - A_c) \times 100$$

The absorbance of the control A_c is 0.251

The absorbance of the blank A_B is 0.991

The absorbance of the test sample, A_s

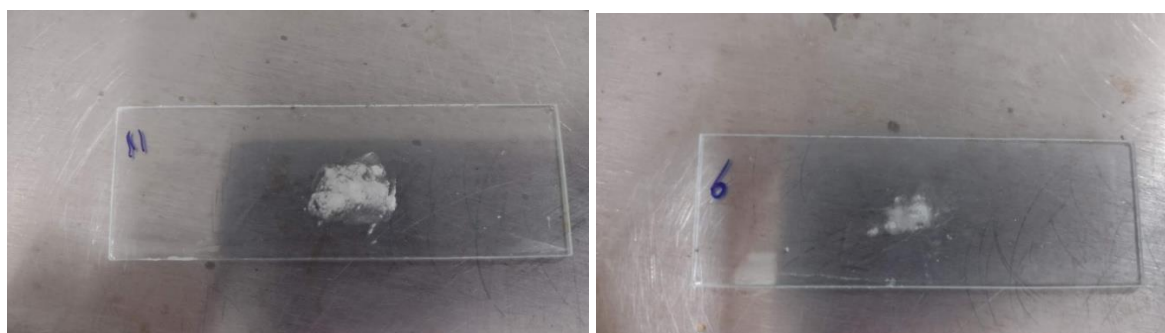
Test samples	Absorbance	Hydroxyl radical scavenging percentage
Tp11	0.971	97.29%
Tp22	0.899	87.56%
Tp33	0.962	83.91%
Tp1	0.915	89.72%
Tp3	0.863	82.7%
Tp6	0.962	96.081%

Table 5.2 Hydroxyl radical scavenging activity

The percentage of hydroxyl scavenging activity is higher in samples Tp11 and Tp6 thereby, these two samples are isolated for its DNA and sequenced and also used for further studies.

5.2.5 CATALASE TEST:

There was no release of air bubbles. It was observed that the culture smeared on the glass slide did not produce air bubbles in two samples Tp11 and Tp6 among the six samples, which is said to be the characteristic of probiotic bacteria.



Sample Tp11

Sample Tp6

Fig 5.8 Catalase test

5.2.6 pH TOLERANCE TEST:

The pH of the isolates was determined. Using 1% HCl and 1% NaOH in MRS broth, different pH of 3, 4, 5, 6, 7, 8, and 9 were developed. The pH-varying media were autoclaved for 15 minutes at 121°C before being inoculated and incubated at 30°C for 24 hours. After 2 hours of incubation, the optical density values were obtained at

600 nm. The culture Tp11 exhibited better growth at the pH of 7 and the sample Tp6 exhibited better growth at pH 8. (Muthukumar & Kandeepan.,2015)

pH	3	4	5	6	7	8	9
Tp11	0.312	0.315	0.324	0.313	1.495	1.486	1.312
Tp6	0.342	0.335	0.731	0.358	0.970	2.154	1.398

Table 5.3 pH tolerance test

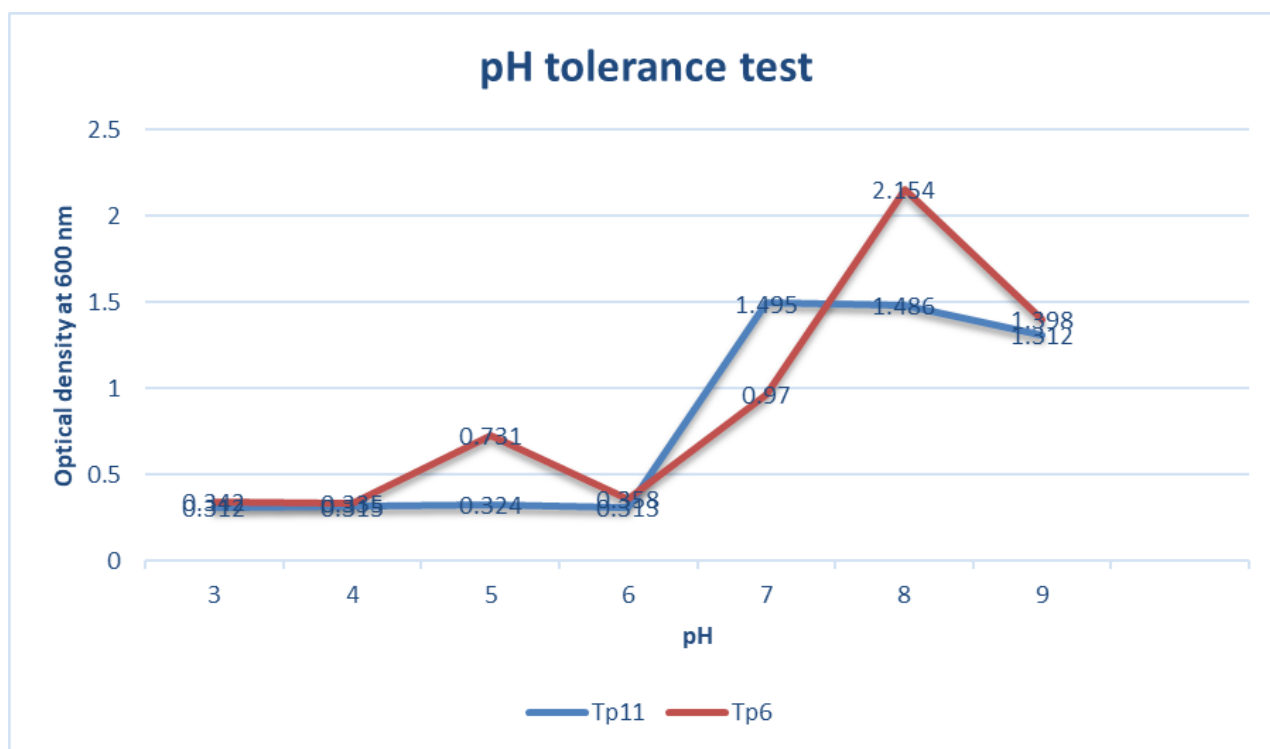


Fig 5.9 Graphical representation of pH tolerance test

5.2.7 SODIUM CHLORIDE TOLERANCE TEST:

The OD values are measured at 600 nm using spectrophotometer and the values are represented in graph. This shows that the growth of bacteria at 2% sodium chloride revealed better growth.



Fig 5.10 NaCl tolerance test

Control	0.245			
Sample	2%	6%	10%	15%
Tp11	0.362	0.261	0.241	0.241
Tp6	1.304	0.254	0.244	0.237

Table 5.4 NaCl tolerance test

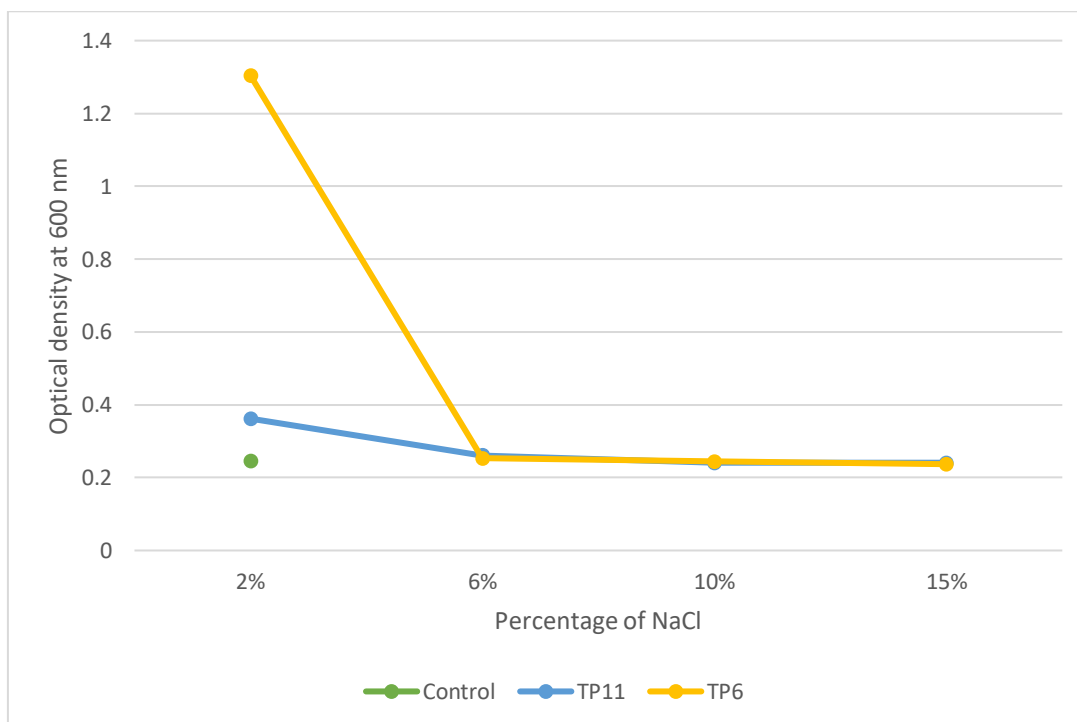


Fig 5.11 Graphical representation of NaCl tolerance test

5.2.8 SALINITY TOLERANCE TEST:

The optical density was observed at 550 nm after the incubation of 24 hours. The culture was able to tolerate the salinity up to 70%.

	0%	15%	30%	50%	70%
Tp11	0.631	1.262	1.432	1.435	1.444
Tp6	0.621	1.330	1.536	1.550	1.584

Table 5.5 Salinity tolerance test

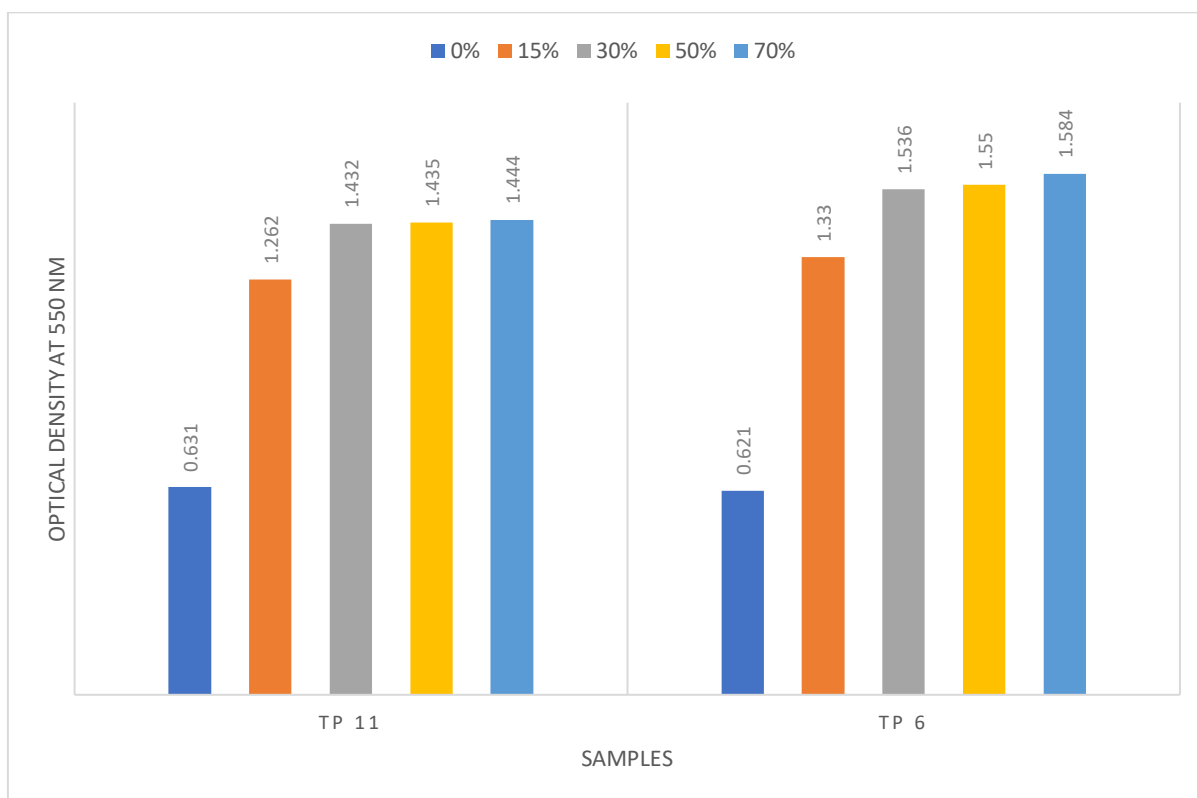


Fig 5.12 Graphical representation of salinity tolerance test

5.2.9 KINETICS OF BACTERIAL GROWTH:

The optical density of the culture was observed at an interval of 0, 1, 2, 3 and 24 hours from the time of inoculation and the values were tabulated and graphically represented which shows the highest growth rate after 24 hours of inoculation.

	0 hour	1 hour	2 hours	3 hours	24 hours
Control	0.385	0.379	0.245	0.237	0.249
Tp11	0.238	0.388	0.389	0.404	0.498
Tp6	0.198	0.340	0.343	0.345	0.804

Table 5.6 Kinetics of bacterial growth

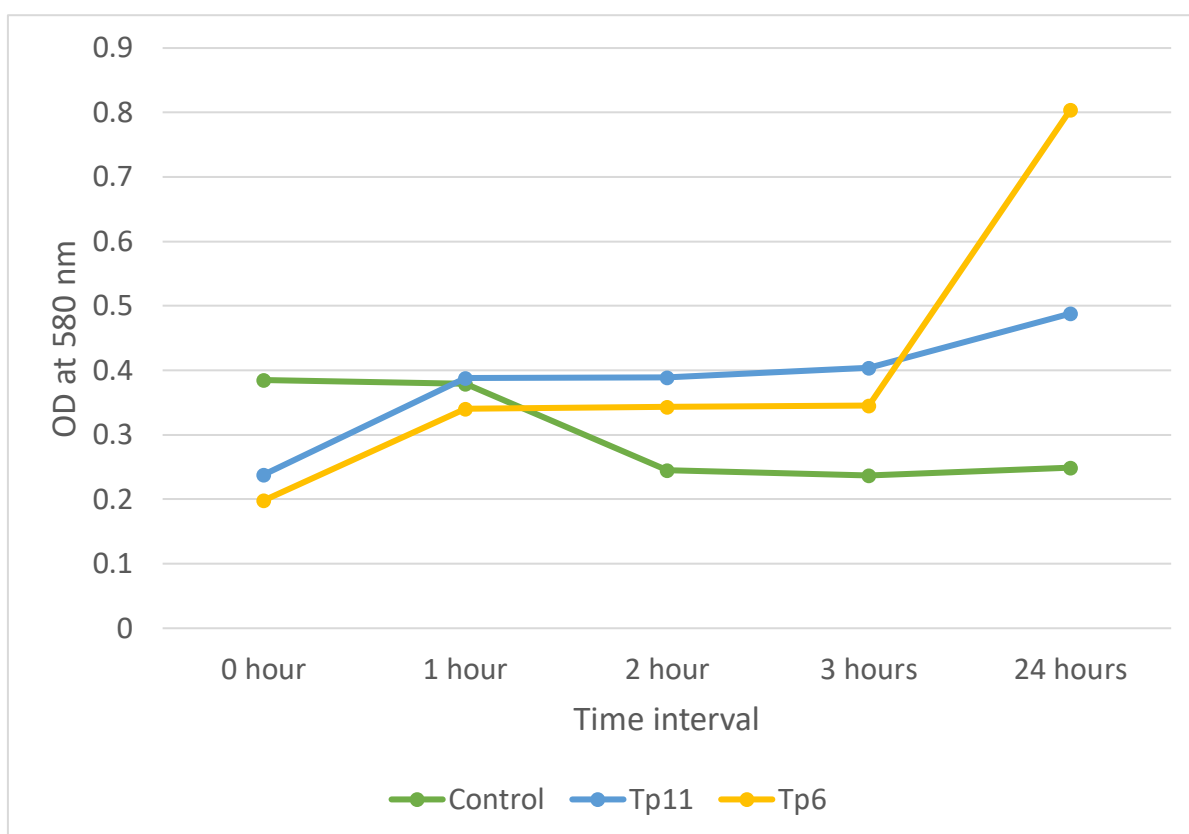


Fig 5.13 Graphical representation of growth kinetics

5.2.10 MORPHOLOGICAL, PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF THE ISOLATES

Tests	Tp11	Tp6
Morphology	Rod	Rod
Gram's staining	+	+
Catalase test	—	—
Growth at different pH		
4	—	—
6	—	—
7	+	+
8	+	+
9	—	—
Growth at different NaCl concentration		
2%	+	+
6%	+	+
10%	—	—
15%	—	—
Growth at different saline concentration		
30%	+	+
50%	+	+
70%	+	+
Motility test	—	—

Endospore test	—	—
Carbohydrate fermentation		
Glucose	+	+
Maltose	+	—
Lactose	+	+
Galactose	+	+

Table 5.7 Morphological, Physiological and Biochemical characterization

5.3 ISOLATION OF DNA

DNA of the bacterial culture was isolated with the procedure followed and observed under UV transilluminator. It was observed that there was a formation of band in the well 6 which consisted the sample Tp6.



Fig 5.14 Gel electrophoresis

5.4 SEQUENCING:

The sequences obtained from the samples were aligned and edited using MEGA software version 7. Similarity search was carried out using the aligned sequence against the sequences submitted in NCBI using NCBI-BLAST. The submitted sample TP6 and sample TP11 are showing 99% similarity with ***Lactobacillus sp.***

Nucleotide sequences of 16SrRNA genes of the purified PCR products of lactic acid bacteria isolates Tp11 and Tp6 are as follows

>Bact16S TP6

GTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACT
GGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGG
GCTCAACCTGGGAACTGCATTCGAACTGGCAGGCTAGAGTCTTGTAGAGGG
GGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACC
GGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGC
GTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGT
CGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAA
GTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAACTCAAATGAATTGAC
GGGGGCCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAA
GAACCTTACCTACTCTTGACATCCAGAGAACTTACCAGAGATGCTTTGGTGCC
TTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGA
AATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT

>Bact16S TP11

GTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACT
GGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGG
GCTCAACCTGGGAACTGCATTCGAACTGGCAGGCTAGAGTCTTGTAGAGGG
GGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACC
GGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGC
GTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGT
CGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAA
GTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAACTCAAATGAATTGAC
GGGGGCCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAA
GAACCTTACCTACTCTTGACATCCAGAGATCTTAGCAGAGATGCTTTGGTGCC
TTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGA
AATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT

5.5 EXPERIMENTAL TRIALS

5.5.1 FEED FORMULATION:

The feed was formulated using biological waste and probiotic culture. It contains 35% fed protein, 25% of nutrients and minerals which includes corn, groundnut, fishmeal, bloodmeal, sunflower oil, vitamins, salt, soyabean meal, casein and probiotic were present in the fish feed. It was resistant to the bacterial infection containing antibacterial or antimicrobial agents, vitamins, minerals, fat, protein and energy.

5.5.2 FISH TRIAL:

The experiment took place over the course of seven days. In this analysis, tilapia fingerlings were weighed individually, and each fish tank was stocked with twelve fish weighing 6-10 g on average. A fully randomised design was used to distribute thirty tilapia fingerlings into three experimental groups of ten replicates each. All of the tanks were aerated 24 hours a day, and a manual water exchange was performed every day.



Feed from biowaste



Probiotic feed



Commercial feed

Fig 5.15 Fish tank setup

5.5.3 GROWTH STUDY:

To assess their development the fish was bulk-weighed every two days. The output of fingerlings in terms of growth was measured. Fishes fed by feed from bio waste showed a growth of ± 0.7 g, probiotic feed exhibited growth interval of ± 0.10 g and commercial feed had a growth rate of ± 0.3 g.

Experimental tanks	Day 1	Day 3	Day 5	Day 7
Tank 1 - Feed from biowaste	11.37 g	11.82 g (WG-0.45g)	12.5 g (WG-0.68g)	13.01 g (WG-0.51g)
Tank 2 – Probiotic feed	10.09 g	10.81 g (WG-0.72g)	11.67 g (WG-0.86g)	12.69 g (WG-1.02g)
Tank 3 – Commercial feed	10.79 g	11.02 g (WG-0.23g)	11.36 g (WG-0.34g)	11.77 g (WG-0.41g)

Table 5.8 Weight records of Nile tilapia

WG is given as Weight Gained by the fish

DISCUSSION

This project represents a method for studying fish intestinal microbiota in the search for potential probiotics. The microbial world has been the subject of numerous studies. Probiotics, a non-dominant community of natural intestinal microbiota with the ability to combat pathogens, are being studied in particular in the gut composition of a wide range of marine organisms. Many lactic acid bacteria participants are non-dominant, as well as a minority group with nutritional needs that necessitate the use of specialised foods. As a result, MRS agar was used to isolate and select bacteria for this report, as it has been shown to be a good medium for recovering probiotic bacteria from the gut microbiota of fish. In addition to LAB, we were able to acquire other Gram-positive bacteria with inhibitory potential against common aquaculture pathogens by using MRS agar as a selective media. (Elayaraja *et al.*, 2014)

This study isolated 6 Gram-positive bacteria was subjected to safety analysis like haemolysis and antioxidant assay and the characteristics to confirm it as probiotic bacteria were analysed. Some of the assays such as catalase test, pH tolerance test, Sodium chloride tolerance test, salinity tolerance test, Kinetics of bacterial growth and were performed to study characterization of the isolates that meet the characteristics of a probiotic bacteria.

The isolate Tp11 was chosen for further testing because of its broad spectral activity against *Streptococcus sp.*, *Vibrio parahaemolyticus*, *Vibrio harvei*, *Salmonella sp.*, and *Flavobacterium columnare*. The inhibitory spectrum of antimicrobial-affecting strains is diverse: 3 isolates (30%) showed strain-specific inhibition, indicating that probiotic strain selectivity has been observed in other studies. Two isolates were selected which resulted in the favour of probiotic characteristics. The work approach of Alonso and his colleagues represented the selection of wide range of bacterial species from fish gut. A total of 45 Gram-positive bacteria were isolated in this sample, all of which inhibit the growth of aquatic pathogens. Antimicrobial-affecting strains have a broad inhibitory spectrum: Thirteen strains (29%) were found to be inhibited in a strain-specific manner. The result ratio was consequent with the results obtained by Alonso *et al.*, 2019.

These isolated species have a high capacity to inhibit the growth of pathogens in freshwater fish. As a result, isolated species seem to have a high probiotic ability, and these organisms are being studied further through molecular characterization. Among the ten bacterial samples Tp11 and Tp6 were sequenced and found to have 99% similarity with *Lactobacillus* sp., wherein the results of Yasmin *et al.*, 2020 obtained Bifidobacterium sp., among eight isolated strains.

The safety analysis of the obtained 10 isolates was performed which is the haemolytic activity in which 6 isolated exhibited gamma haemolysis or no haemolysis such that these six isolated were assessed for antioxidant activity which is hydroxyl radical scavenging activity. From this assay the isolates Tp11 and Tp6 exhibited higher percentage of inhibition and selected for characterization studies such as catalase test, pH tolerance test, Sodium chloride tolerance test, salinity tolerance test and Kinetics of bacterial growth. These assays revealed positive results for both the strains (Tp11 and Tp6) with respect to probiotic bacteria.

The selected strains were tested for catalase which showed catalase negative. The isolates were checked for the tolerance of pH in which the strains developed better microbial growth at the pH of 7 and 8 compared to the other. In sodium chloride tolerance test the samples had better growth under the least sodium chloride concentration of 2%. When salinity tolerance test was considered, the isolates were able to tolerate the highest level of salinity of 70% saline solution had good microbial growth in it. The graphical line of growth kinetics showed a regular growth of log phase at 24 hours. These results made to conclude that the selected isolates had the characteristics of a probiotic bacteria. This was then potentially characterized to the molecular level, wherein 16s RNA sequencing was performed which confirmed that the isolated bacteria are *Lactobacillus* sp., The culture was then inoculated to produce a probiotic biomass when was then precipitated for its protein and dialysed. Further studies can be done by the separation of bacteriocin at different fractions using column chromatography technique.

The isolated and characterized strains were further used in the experimental feed trials, where the fishes were stocked and comparative study of no probiotic, probiotic and commercial probiotic feed was fed and the physiological changes like weight

and growth of the Nile tilapia fishes were noted. The fishes that was fed with probiotic feed exhibited better growth rate comparatively.

The growth and survival of *O. niloticus* were positively affected by a mixture of isolated bacterial strains. This research looked at the nutritional value of probiotic feed and how it was used. From this research study, the acceptable feed selected for feeding fish *O. niloticus* was regarded as a very promising feed and novel technique to be used in aquaculture. As a result, in the fish community, formulated isolates were used. Probiotics for aquaculture can be chosen for growth, attachment to intestinal mucus, and development of beneficial compounds, all of which can help *O. niloticus* fish grow faster.

CHAPTER-6

SUMMARY AND CONCLUSION

Probiotic bacteria are essential for a positive impact on an organism's health and nutrition, as well as for proper gastrointestinal function. Intestinal flora has a number of important functions, including pathogen resistance and immune system development. First and foremost, probiotic microorganisms must be non-pathogenic and non-toxic; second, they must be able to endure harsh external and internal conditions, such as gastric acid and bile salts. Furthermore, the ability of LAB to adhere to surfaces is an important factor to consider when choosing probiotics.

Another significant probiotic property is antioxidant activity of LAB, which would be linked to a variety of functional properties. Probiotics have also been shown to effectively treat a variety of chronic diseases. But apart from probiotic properties, the starter characteristics of potential probiotics, such as fermentability and sensory properties, are also important. (Saad *et al*, 2013)

The current research aims to improve and optimise probiotic use in the aquaculture industry. Because of the growing demand for probiotics among aqua cultured animals, the future application appears to be promising. While fish in low-input ponds are highly susceptible to infectious pathogens that cause diseases and deaths, the effects of probiotics on immunity, gut microbiota, and microbiological quality are unknown. The morphological parameters of the fish, such as weight and height, were found to be significantly higher in fish fed on probiotic-treated diets than in fish fed on control diets. Finally, adding bacterial probiotics to the diets of Nile tilapia raised in low-input ponds increases immunity, manipulates gut microbiota, and improves the consistency of the fish flesh.

More study will be done to see how to screen host specific probiotic strains from aquaculture rearing systems to enhance the system's efficiency and functionality. In addition, research should concentrate on the effects and mechanisms of the effects of probiotics on reproductive health and gonadal function. On a large scale, the growth of marine species and system of hatcheries are increasing but then certain amount of mortality occurring which is caused by the pathogens. Probiotic bacteria

have a wide range of benefits. There is a wide range of host-beneficial effects, but there are still some restrictions. Antimicrobial compounds or bacteriocins made by probiotic candidates to fight pathogenic bacteria, for example, are not species specific. As a result, strain improvement is needed to improve the efficiency of probiotic bacteria. Molecular biology techniques such as recombinant technology, mutagenesis, and others are available to improve the genetic makeup of the probiotic strain. To resolve these serious issues and develop effective probiotics, further research is required. (Hasan and Banerjee., 2020)

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