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Studies on the effect of commercial and formulated probiotic application in the growth performance and intestinal microbial flora of the cat fish (*Pangasianodon hypopthalmus*)

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Abstract
Probiotic bacteria confer beneficial effect to the host animal. Probiotic diets are being incorporated together with prebiotics in recent years. Probiotics with synbiotics elicit synergistic and more favorable actions. Administration of probiotic feed *Lactococcus spp, Saccharomyces cerevisiae* in the diets kept the histo-architectural structure intact and promoted regeneration in the intestine of fish to increase the functionality of fish. This increased advantage of formulated probiotic and its benefits in maintaining good water quality and rapid increase in growth of *Pangasianodon hypopthalmus* were evaluated in this study. Feeds with commericial probiotic feed obtained were evaluated with the isolated probiotic feed. The adherence of isolated probiotic feed in the intestine of *Pangasianodon hypopthalmus*, alter the enzymes, microbial metabolism and improve the weight gain and survival rate.

Keywords: probiotic, *Pangasianodon hypopthalmus*, fish feed, water quality

1. Introduction
The term 'probiotic' was first used by Lilly and Stillwell in 1965 to describe the 'substances secreted by one microorganism that stimulate the growth of another' [1]. A powerful evolution of this definition was coined by Parker in 1974. He proposed that probiotics are 'organisms and substances, which contribute to intestinal microbial balance' [2]. These microorganisms contribute to intestinal microbial balance and play a role in maintaining health. The probiotic microorganisms consist mostly of the strains of the genera *Lactobacillus* and *Bifidobacterium*, but strains of *Bacillus*, *Pediococcus* and some yeasts have also been found. Together they play an significant role in the protection of the organism against harmful microrganisms and also strengthen the host's immune system. Probiotics can be found in dairy and non dairy products. They are usually consumed after the antibiotic therapy (for some illnesses), which destroys the microbial flora present in the digestive tract (both the useful and the targeted harmful microbes). Regular consumption of food containing probiotic microorganisms is recommended to establish a positive balance of the population of useful or beneficial microbes in the intestinal flora [3].
Currently, Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) [4] endorsed by the International Scientific Association for Probiotics and Prebiotics [5], define probiotics as "live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host".
The use of probiotics addresses the concerns on administration strategies, multifaceted benefits and bacterial viability. To keep up with the rapid growth of aquafarming, which grows annually at 11% increase the price of feed. Ingredients together with their limited supply play a tremendous role to meet the demand. While remaining cost effective and competitive, the formulation of alternative feed ingredients generally meets the nutritional requirements of the target species [6]. Fish which are poikilothermic vertebrates inhabit aquatic ecosystem and are most susceptible to seasonal and diurnal variations in water temperature. Temperature regulated the growth and other physiological and biochemical functions of fish [7-8]. Alarming rise in temperature caused by global warming is thought to negatively affect the wild capture fisheries as well as the world aquaculture production [9]. Among alternative feed, probiotic feed provide protein source with high digestible protein and energy contents and good amino acid profile. Several studies have been conducted to evaluate the nutritional value [10-11].
In this concept the present study comparative aspects was carried out the impact of supplementation of diets with Probiotic *Lactobacillus* on the growth, length and intestinal microbial flora of fish, *Pangasianodon hypopthalmus* in a laboratory condition.

2. Materials and Methods
2.1 Isolation of *Lactobacillus spp.*
Idli is the best source for *lactobacillus spp.* Among the other dairy products such as milk, buttermilk etc. Idli is taken in sterilized flask. Under the aseptic conditions idli was serially diluted from 10^{-2} to 10^{-5} from this dilutions 10^{-4}, are selected. Spread plate technique further with streak plate technique is done on MRS medium. They are incubated in incubator 37^0C which is optimum temperature for *Lactobacillus* broth. Incubation at 37^0C for 24 hrs. Broth after 24-48 hrs shown *Lactobacillus* species growth and these species for 24 hours. After the period of incubation the specific isolated colonies were grown. Colony characterization is done for this colonies found to be *Lactobacillus* species. One colony shows 100% resemblance with *Lactobacillus plantarum*. The culture was

kept in MRS agar slant and stored at 4 ^{0}C for long term storage [12].

2.2 Identifications of the organisms

The isolated probiotic bacterium was identified by following morphological and biochemical characterization methods.

2.3 Morphological characterization

2.3.1 Studies on Nutrient Agar

Morphological and cultural characteristics such as abundance of growth pigmentation, optical characteristics, size, form, margin and elevation were studied on nutrient agar plates.

2.3.2 Gram Staining

A thin smear of the isolate was made on a clean glass slide and heat fixed. Then the smear was stained with crystal violet for 1 minute and then washed with water, gram's iodine was added for 1 minute and decolorized with alcohol. After decolourization the smear was counter stained with safranin for 1 minute. Finally the smear was washed with water and air- dried. Then the slide was observed under the microscope.

2.4 Biochemical Characteristics

The following biochemical tests were carried out according to the method described by Cappuccino and Sherman [13].

2.4.1 Catalase Test

A clean glass slide was taken and a drop of culture suspension was placed on the glide. To the culture few drops of hydrogen peroxide was added. A positive reaction indicates the release of air bubbles from the suspension.

2.4.2 Starch Hydrolysis Test

Starch agar medium was prepared and transferred aseptically into sterile petridish. The isolated colonies were streaked on starch agar plates and incubated at 37ºC for 48 hours. The plates were flooded with Gram's Iodine. Amylase production was indicated by colorless zone surrounded by bacteria and rest of the plate appeared purple.

2.4.3 Casein Hydrolysis

Skim milk agar medium was prepared and transferred aseptically into sterile Petri plates. The medium was allowed to set and the isolated colonies were streaked on skim milk agar plate and incubated at 37ºC for 48 hours. The opaque zones surrounding the microbial growth consist of casein milk powder, indicating protease activity.

2.4.4 Indole Production Test

One percentage peptone broth was prepared, sterilized and incubated with the isolated colonies and incubated at 37 ºC for 48 hours. After incubation 1 ml of Kovac's regent was added and gently shaken. The results were observed after allowing the tubes to stand. A cherry red ring indicates the positive reaction.

2.4.5 Methyl Red Test

MR-VP broth was prepared, sterilized and incubated with the isolates, 5 drops of methyl red indicator was added and the tubes were observed for a color to red that indicates a positive reaction.

2.4.6 Voges Proskauer Test

MR-VP broth was prepared sterilized and incubated with the isolated, incubated at 37ºC for 48 hours. After, incubation few drops of Baritt's regent B and A were added and the results noted. Development of crimson to pink color indicates a positive reaction.incubated with the isolates, incubated at 37ºC for 24-48 hours. Presence of nitrate was tested by adding a few drops of sulfanilic acid and napthalamine regent to each of the tubes. Results were observed without shaking the tubes. A distinct red colour that may turn brown indicates reduction of nitrate.

2.4.7 Citrate Utilization Test

Simmon's citrate agar medium was prepared, sterilized and transferred aseptically to the test tubes and slant was prepared. The isolated colonies were streaked on the surface of the slant and incubated at 37ºC for 24 hours. A change in green colour to Prussian blue indicates the positive results.

2.4.8 Tolerance of NACL and Phenol

Observing the tolerance of Nacl of the culture of the 1% fresh one night culture of the bacteria incubate into MRS broth with 4% Nacl Con^c for 24 hours and then observe their turbidity. Similar experiments were performed using 0.4% phenol as inhibitory substance [14].

2.4.9 Milk Coagulation Assay

For milk coagulation test, 100% fresh one night culture of the bacteria was added into 10% sterile skim milk and incubate 37ºC for 48 hours in incubator [14].

2.4.10 Lactose Utilization

For this experiment media was prepared using Peptone- 10 gms, Nacl-15 gms, Phenol Red 0.018 gm, Lactose 5 gm in 1 litter distilled water, controlling P^H 7, kept it 35ºC, for 48 hours in rotary incubator. Change of colour, red from yellow, concluded as positive result [15].

2.5 Determination of the growth

The minimal broth was prepared in the fish growing water and supplemented with various nutrient supplements such as oil cake, Black gram, Green gram and wheat bran (Treatment I: Fish feed(Control), Treatment II: Fish feed with Commericial probiotic feed, Treatment III: Fish feed with Formulated feed). The length and weight of the control and experimental fishes were measured using scale and weighing machine [16].

2.6 Histological assessment of intestine

To determine the effect of probiotics diets on intestine, *Pangasianodon hypopthalmus* in each treatment were sampled for histological sections at the end of experimental period. Fish samples were stored in formalin solution (4%) and then eviscerated to remove their digestive tract. Paraffined blocks of fish intestine were prepared and then sliced by microtom to give sections of 4 to 5 µ. Sections were stained by the coloration methods of hematoxilin and eosin and then studied under phasecontrast microscope [17].

3. Results and Discussion

3.1 Identification of *Lactobacillus spp.*

The isolated bacteria were observed by phase contrast microscope. It is clear that the bacteria was gram positive, rod shaped occurring single or in chains forms. The gram staining results indicated that the isolated bacteria could be identified as *Lactobacillus plantarum.* Hanging drop wet method showed that the isolated bacteria were non motile. Thus, the results obtained coincided with *L. plantarum* strain characteristics. Their distinguishing features are shown in (Table 1 and Figure 1). Their biochemical characters are also shown in the (Table 2).

3.2 Determination of length and weight of fish

Control and experimental fishes were exposed to various nutrient supplements such as oil cake, Black gram, Green gram and wheat bran along with supplemented probiotics (*Lactococcus plantarum*). Increased length and weight were observed at the end of 10, 20 and 30 days from various sampling groups than control (Table 3 and Figure 2) as reported by [18].

3.3 Histology of intestine

In the present study, no difference among various treatments was observed in histological assessment of intestine (Figure 3). Similar findings reported by [19]. Probiotic feed obtained commercially were feeded with normal fish feed and compared with the formulated isolated feed. The isolated probiotic, strains are more efficient in converting organic matter, large polymer into smaller units and adhere to the intestine of *Pangasianodon hypopthalmus.*

3.4 Water quality

As a result of probiotic activity, water quality improved and reduced the organic matter load. Nitrifying bacteria also reduced, which lead to good water quality (Table 4). The probiotic bacteria adhesion increased and altered certain enzymes, microbial metabolisms which are beneficial.

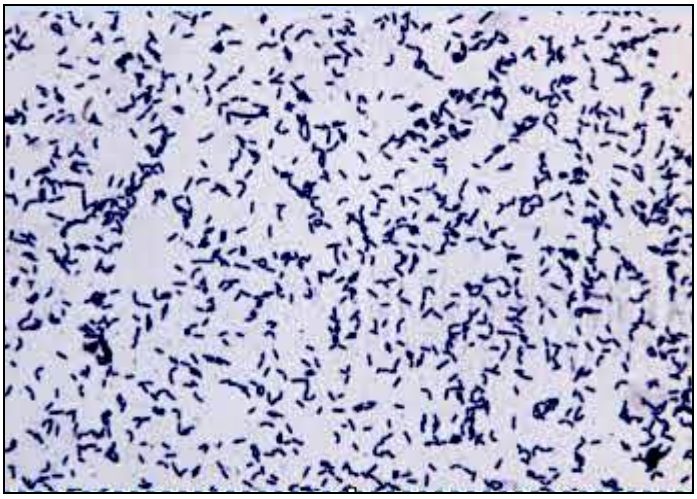

Fig 1: Phase contrast microscopy images of *Lactobacillus sp*(Magnification 10X).

Table 1: Morphological and physiological characterization of the isolated bacterial strain.

Configuration	Round
Margin	Wavy
Elevation	Flat
Surface	Mucoid
Texture	Dry
Gram's Reaction	+ve
Spore(s)	-Ve
Capsul	-Ve
Motility	Non motile

Table 2: Biochemical characterization of the isolated bacterial strain.

Tests	Results
Catalase test	-ve
Indole	-ve
Methyl red	-ve
Voges-Proskauer	-ve
Citrate Utilization Test	+ve
Starch Hydrolysis	- ve
Casein Hydrolysis	-ve
Lactose Utilization	-ve
Phenol (0.4%) test	+ ve
4% Nacl test	+ ve
Milk Coagulation Assay	+ve

Table 3: Length and weight of fishes to various nutrient supplement groups.

Feed	Parameters	0th Day	10th Day	20th Day	30th Day
Treatment -I	Length(cm)	10.5	10.6	10.7	10.8
	Weight(gm)	8.2	8.6	8.8	9.2
Treatment -II	Length(cm)	10.6	10.8	10.9	11.3
	Weight(gm)	9.3	9.4	10.2	10.4
Treatment -III	Length(cm)	9.8	10.0	10.4	10.6
	Weight(gm)	8.4	8.6	8.8	9.2

Treatment –I: Fish feed(Control
Treatment –II: Fish feed with Commericial probiotic feed
Treatment –III: Fish feed with Formulated feed.

Fig 2: Experimental design of study the growth parameters of *Pangasianodon hypopthalmus.*

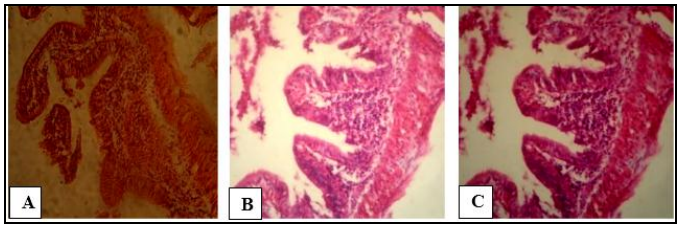

Fig 3: A) Histology of intestine microvilli in control fish *Pangasianodon hypopthalmus* B) Histology of intestine microvilli of commercial probiotic feed fish, *Pangasianodon hypopthalmus* C) Histology of intestine microvilli of formulated probiotic feed fish, *Pangasianodon hypopthalmus*.

Table 4: Physico-chemical properties of water

Sl. No.	Parameters	Treatment I	Treatment II	Treatment III
1.	P^H	7.51	7.35	7.46
2.	Turbidity	0.29	0.17	0.19
3.	Biological Oxygen Demand mg/L	4.21	4.01	3.92
4.	Dissolved Oxygen mg/L	8.39	8.12	7.18
5.	Chemical Oxygen Demand mg/L	33.0	27.0	21.0

4. Conclusion

A mixture of isolated bacterial strains positively influenced growth and survival of *Pangasianodon hypopthalmus.* This evaluation study showed the nutritional value of probiotic feed and its utilization. The appropriate feed selected for feeding fish *Pangasianodon hypopthalmus* regarded as a very promising feed and novel strategy to be used in aquaculture from this research study.

As a result formulated isolates were used in the *Pangasianodon hypopthalmus* fish culture. The selection of probiotics for aquaculture for growth, attachment to the intestinal mucus and production of beneficial compounds can be considered for improving *Pangasianodon hypopthalmus* fish growth.

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