

Analysis of different prebiotic combination with isolated fish probiotics to enhance their potential

Abdul Baseer Mir and Rahul Singh

School of Bioengineering and Biosciences, Lovely professional University, Phagwara Punjab

Abstract

Prebiotics and Probiotics act as natural biological control agents against the Pathogenic micro-organisms in any culture systems. The present study was conducted to examine the effect of Synbiotic prepared feed on the Growth performances of *Channa striata*. Prebiotics used were: Guar gum, Banana peel powder and Water hyacinth powder while Probiotic strains used were isolated from the fish gut which includes: BDK2, S3, BDK7, FIH4 and BDK9. Prebiotics concentration used was 6% with bacterial colonies 10^4 . Results of the study revealed that fish fed with Banana peel powder showed increase in length by (0.3%), followed by Guar gum (0.25%) and water hyacinth powder (0.25%) as compared to control diet (0.03%). In case of weight, Guar gum showed maximum increase by (1.2%), followed by banana peel powder (1%) and then by water hyacinth (0.9%) as compared to control (0.8%). Therefore these results indicated that synbiotic feed decreases the mortality rate in treatment groups (0%) as compared to control group (12%) and have beneficial effect on the Growth performances of *Channa striata*.

Keywords: Prebiotics, Probiotics, Synbiotics, Pathogenic micro-organisms, *Channa striata*

Introduction

Aquaculture production is enormously increasing with the growing population. In 2003, aquaculture food fish production was 127 million metric tons but, now it is increased up to 175 million metric tons (World Bank). Due to diseases and deterioration of environment conditions, causes serious economic loss to aquaculture production. Fish disease causes major economic loss up to USD 6 billion annually (World Bank 2014). Different methods are used to increase the aquaculture productions and make the aquaculture disease free. Antibiotics are used from the past decades to cure fish diseases and to improve their growth efficiency, but due to the adverse effects of chemicals and antibiotics there is a chance of spread of resistant antimicrobial pathogens from the aquaculture to the terrestrial environment (human's environment,) which can cause serious effect to human health. Considering these elements in view, researchers developed novel dietary supplement tactics, which are beneficial and provide the confrontation against diseases and also enhance the growth of fish. These compounds include prebiotics, probiotics and Synbiotics. These enhancements are eco-friendly and have no side effect on the fish. Other benefits of such supplements include improved sustenance value, contributes to enzymatic digestion, inhibits the pathogen microorganisms, anti-mutagenic and anti-carcinogenic activity [1].

Probiotics

Probiotics are the beneficial bacteria that are having beneficial effect on the host. According to joint world health organization/ food and agriculture organization (WHO/FAO), probiotics are live microorganisms which when consumed in adequate amounts confer health benefits to the host [2]. Probiotics may be Gram-negative bacteria, Gram- positive bacteria and several other organisms like unicellular algae, yeast and bacteriophage. Bacteria like *Lactobacillus*, *Bacillus*, *Bifidobacterium*, *streptococcus*, and *Enterococcus* follow the gram positive category of probiotics and are present in the gastrointestinal tract of vertebrates. Bacteria like *Bacillus sp.* helps in the improvement of water quality, because they have the quality of converting organic matter into CO₂ [3]. Probiotics can be added to water directly, which are useful to reduce the water contamination and organic pollutants. It has been found that probiotic bacteria reduces the amount of heavy metal pollutants such as (Pb, Hg, Cd, Ni) [4].

Prebiotics:

Prebiotics are the feed supplements which are beneficial in the aquaculture to improve the growth of fish probiotics as well as the development of the host. Based on definition of Gibson and Roberforoid [5] prebiotics are the ingredients of food, that are non-digestible in the host but stimulate the health of the host by enhancing the progress of the bacteria in the colon region. Prebiotics like inulin, fructo-oligosaccharides, Mano-oligosaccharides galacto-oligosaccharides are considered as active prebiotics in aquaculture [6]. Both probiotic and prebiotic play an important role in the aquaculture, Use of prebiotic and probiotic shows many beneficial effect on the fish, which includes growth improvement, disease resistance against pathogens ,utilization of feed, vigorous health, gut morphology and Microbiota modulation [7]; [8].

Synbiotics:

Synbiotics are the combination of prebiotics and probiotics feed supplements in the form of synergism, thus enhancing the advantageous effect of probionts. Synergism occurs when the effect of the two combined products is significant and more than that of each product administered individually. Synbiotic are used to decrease the existence of the opportunistic pathogens and stimulate the host immune system, also used to enhance the growth parameters, utilization of feed, nutrition improvement, facilitate stress response, improve morphology of gut, survival of larva, disease resistance, reduce the risk of cancers (bladder, colon) [9].

Methodology

Culture of isolated bacteria: De –Mann Rogosa Sharpe (MRS agar) was prepared in flask (33.5g in 500ml of distilled water) then media and petriplates were autoclaved. Media was poured on petriplates under sterile condition in the laminar. After that Red hot loop was taken and dipped into the bacterial broth of the isolated

probiotic bacteria, and was streaked on the solidified media. Streaked plates were incubated for 24 hours at 37°C. After 24 hours of incubation, bacterial colonies were seen on petri plates.

Nutrient broth culture: Nutrient broth was prepared (6.5gm in 500ml of distilled water). Then under sterile conditions in laminar airflow; bacterial strain was inoculated with the help of loop. After that incubation was done for 24 hours at 37°C in shaking incubator at 110rpm.

Prebiotic collection and its feed preparation: Banana peels, yellow in colour were collected from the fruit seller from Deep Nagar Jalandhar. The peels were washed with tap water followed with double distilled water and acetone to remove the contamination. Banana peels were cut into small pieces with the help of knife. Then the small pieces were oven dried for 24 hours at 50°C. Banana peels were grinded into small fine powder with the help of grinder. Then autoclaved the fine powder of banana peels and stores it at -15 to -20°C unit use. Same procedure was applied for water hyacinth leaves to get fine powder. But water hyacinth leaves were collected from the pond which is situated in the Chehru area of Jalandhar and guar gum powder was brought through online mode AMAZON.

Bacterial dilution to count number of colonies (CFU) of bacteria: Phosphate buffer saline (PBS 4.29g) was dissolved in 500ml of distilled water and then autoclaved. Equal concentration of PBS 9ml was added to the each test tube under the sterile condition in the laminar. Bacterial strains were centrifuged at 3000rpm for 10 minutes to obtain the pellets and then washed with PBS. PBS added to the pellets and again centrifuged at 3000rpm for 10 minutes and then pellets dissolved in the PBS by shaking the centrifuge tube. After that 1ml dilution was added to the 1st test tube to make its volume to 10ml and then 1ml from test tube one was added to the 2 test tube up to 10⁻⁴ dilution. MRS agar (33.5g) and 6% prebiotic concentration was prepared as early mentioned and equal concentration of MRS agar with prebiotic was spread on the petri plates in the sterile condition under the laminar airflow. After that 10 microliter of diluted bacteria was spread on the petri plates and with the help of spreader followed by incubation for 24 hours at 37°C, and then bacterial colonies were counted in the colony forming unit.

Synbiotic feed preparation: Commercial diet (Hopper Grow) was crushed in to fine powder with the help of grinder and appropriate concentration of prebiotic 6% was added along with water and mixed properly in the well maintained condition. Reshaped it into pellet again and dried the feed pellets for 18 hours at 45°C and stored at 4°C. After that probiotic bacteria were inoculated in the nutrient broth culture and incubated for 24 hours at 37°C in the shaker incubator. Bacterial culture was centrifuged at 3000rpm for 10 minutes to obtain the pellets and with the help of phosphate buffer saline (PBS pH7.2) pellets were washed three times and later pellets were resuspended in the phosphate buffer saline. Bacterial concentration was standardized by using UV-Vis spectrophotometer and the absorbance were taken at 600nm and adjusted at 0.3 to standardize the number of bacteria (10⁸ CFU/ml). Probiotic bacteria having CFU10⁸/ml was later sprayed properly on the prepared pellet diet to achieve the proper dose under sterile condition and the prepared diet was dried at room

temperature under sterile condition for 3-4 hours and stored in air tight bottles at 4°C. Control diet was prepared by grinding the commercial diet and water and made it in to pellet form again. Control diet was sprayed with PBS only.

Table 1: Nutritional value of commercial diet used for synbiotic feed preparation.

Hopper Grow fish feed	Nutritional value
Crude protein (Min.)	30%
Crude fat (Min.)	3%
Crude fibre (Max.)	4%
Moisture (Max.)	10%
Crude ash (Max.)	10%

Fish were collected from Baba khel basti Jalandhar market and kept in different transparent buckets with water and continuous aeration was supplied. In each set 10 fish were kept and first five days they were fed with normal control diet and 70% water was daily changed. Each day fish were fed at 9am and pH of the water was continuously checked and it was 7.4 pH. The initial weight of the fish was taken before they were fed with synbiotic diet. After five days fish were fed with synbiotic feed and in the control bucket fish was fed continuously with normal control diet. The final weight of the fish were taken after 20 days.

$$\text{Growth rate} = \frac{\text{weight at the end of study} - \text{weight at the start of study}}{\text{Time interval in days}}$$

Results and Discussion

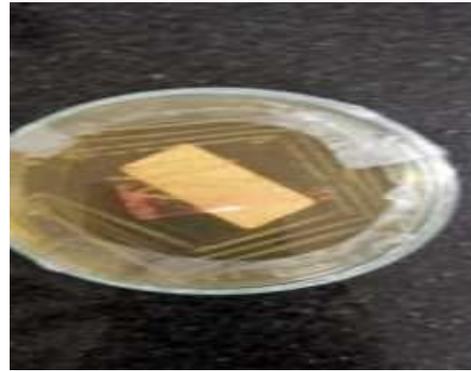
Isolated bacteria were streaked on the MRS agar plates and incubated for 24 hours at 37°C to obtain the colonies. These colonies were used as a mother colony for broth culture.



Sample 1(BDK2)



Sample 2 (S3)



Sample 3 (BDK)



Sample 4 (FIH4)

Sample 5 (BDK9)

Fig 1: Subculture of isolated bacteria

Effect of guar gum powder: By Analyzing different concentration of Guar gum powder with MRS agar such as 0%, 2%, 4%, 6%, and 8%, it was found that 6% of Guar gum powder concentration shows the concentrated number of colonies on the MRS agar plate. This 6% concentration of guar gum powder was then used for synbiotic feed preparation.

Sample 1(BDK2)



Sample 2(S3)



Sample 3 (BDK7)



Sample 4 (FIH4)

Sample 5 (BDK9)

Fig 2: Number of bacterial colonies when guar gum powder added with MRS agar

Different concentration of Guar gum powder was mixed in the MRS agar to check the potential of different probiotics. When 6% concentration of Guar gum powder was added with MRS agar, it showed 1403.33 mean numbers of colonies in (BDK2) strain, 1240 in (BDK7), 1241.66 in (BDK9), 1430 in (S3) and 954.66 in (FIH4) when incubated at 37°C for 24 hours. Furthermore when 2, 4, 6% Guar gum powder administered there is increase in the CFU value but the value declines when 8% guar gum powder was administered, except (BDK9) which showed 1054 number of colonies at 8% concentration as compared to 2 and 4%. S3 shows 1430 number of colonies at 6% as compared to other strains then followed by BDK2, BDK9, and BDK7 whereas least number of colonies at 6% was shown by FIH4.

Table 2: Effect of Guar gum powder on probiotic strains

STRAIN	CONCENTRATION OF GUAR GUM POWDER	MEAN NUMBER OF COLONIES
BDK2	0%	521.33±38.27
	2%	766.66±43.68
	4%	1256±20.18
	6%	1403.33*±30.55
	8%	557.33±31.64
BDK7	0%	537.66±38.55
	2%	938.33±43.68
	4%	934±38.93
	6%	1240*±70
	8%	533.33±22.03
BDK9	0%	523.33±41.63
	2%	980.66±49.56
	4%	925±15
	6%	1241.66*±93.85
	8%	1054±42.50
S3	0%	575.33±75.2
	2%	861±44.50
	4%	1270.60±54.34
	6%	1430±67.26
	8%	947*±45.17
FIH4	0%	581.66±32.53
	2%	905±22.91
	4%	880±95.39
	6%	954.66*±19.73
	8%	784.66±73.05

Note:*shows mean number of colonies at 6%.

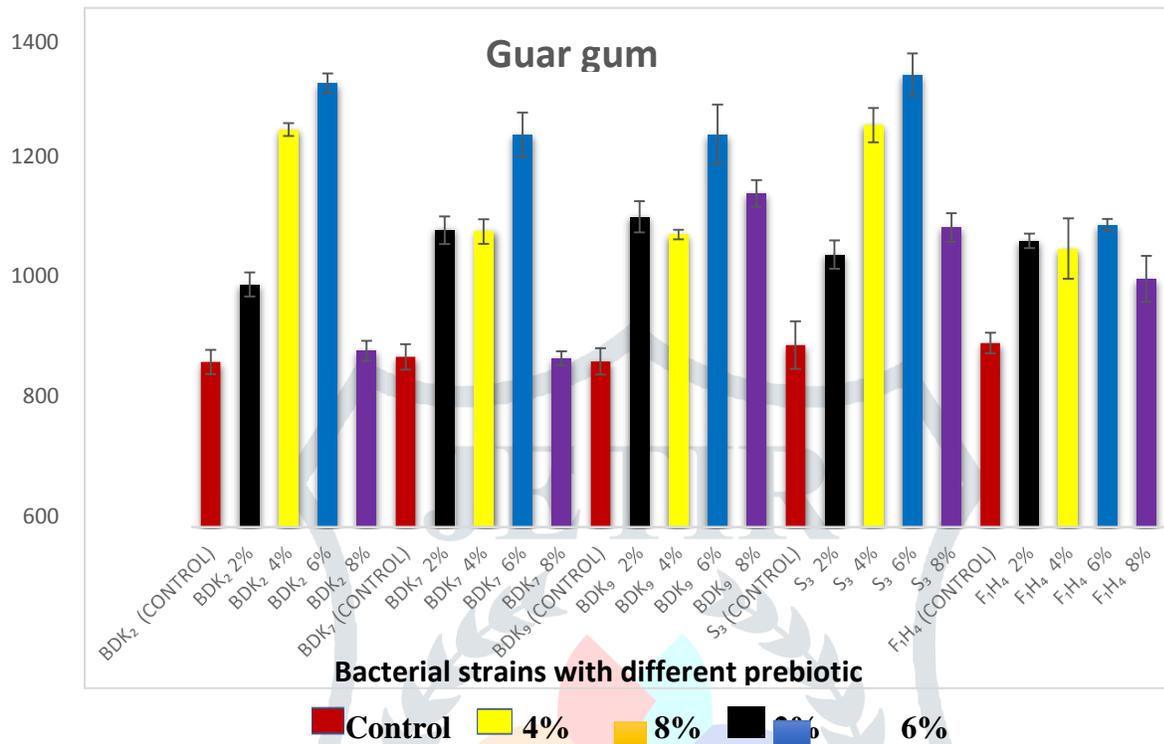


Figure 3: Effect of Guar gum powder on different bacterial strains

Banana peels powder: Banana peel powder mixed with MRS agar media and different probiotic strains were grown on Plates

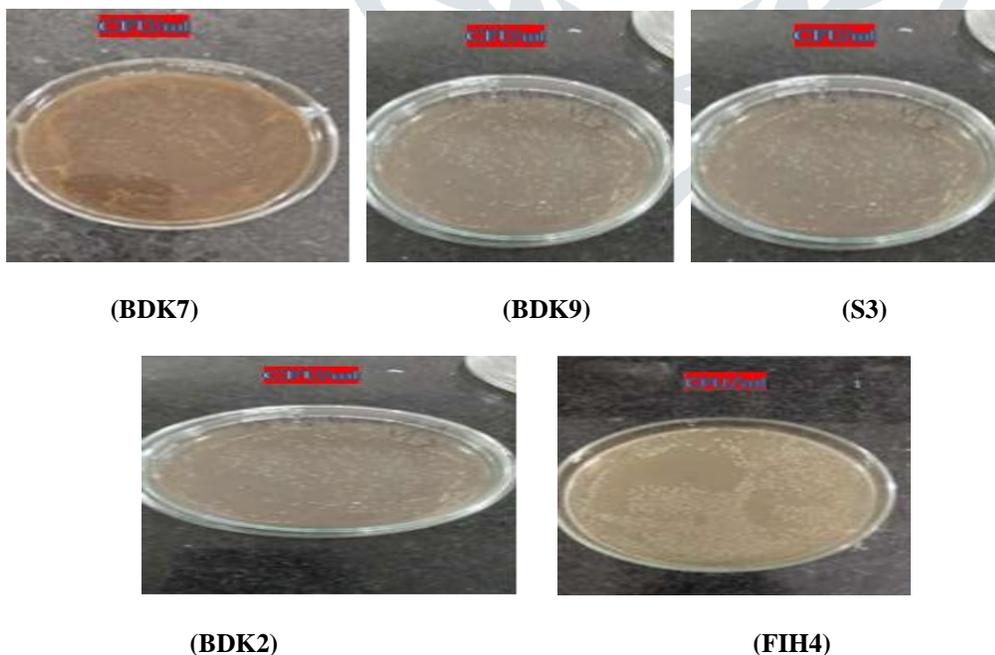


Fig 4: Number of colonies when Banana peels powder mixed with MRS agar.

Banana peels powder was used as a prebiotic ingredient to enhance the growth of probiotic. Different concentrations of banana peels powder was mixed with MRS agar and different strains of probiotic were spread on the agar plate under sterile condition. There was an increase in the number of colonies of probiotic bacteria as compared to those which spread only on MRS agar plate. Different concentration used like 2%, 4%, 6%, 8% with 10^{-4} dilution. 6% prebiotic concentration shows 1560 mean number of colonies in (BDK2), 1405.33 (BDK7), 1300 (BDK9), 995.60 (S3), and 636.66 (FIH4) at 10^{-4} dilution as compared to other concentration. 6% is the optimum concentration for the growth of probiotic. BDK2 at 6% shows 1560 mean number of colonies then followed by BDK7, BDK2, S3, and FIH4.

Table 3: Banana peels powder with different probiotic strains.

PROBIOTIC STRAIN	CONCENTRATION/BANANA PEELS POWDER	MEAN NUMBER OF COLONIES
BDK2	0%	569.33±73.91
	2%	882.66±102.78
	4%	903.33±25.65
	6%	1560*±52.91
	8%	861±116.92
BDK7	0%	501±37.07
	2%	532±37.64
	4%	605.66±37.63
	6%	1405.33*±52.16
	8%	565.33±50.80
BDK9	0%	502.66±38.21
	2%	585±34.04
	4%	620±26.45
	6%	1300*±50
	8%	791.33±32.51
S3	0%	554.66±98.10
	2%	606±33.04
	4%	658.33±38.83
	6%	995.60*±47.26
	8%	784.33±77.51
FIH4	0%	517.33±8.02
	2%	541.66±17.55
	4%	620.66±60.5
	6%	636.66*±75.71
	8%	578.66±26.63

Note:*shows mean number of colonies at 6%

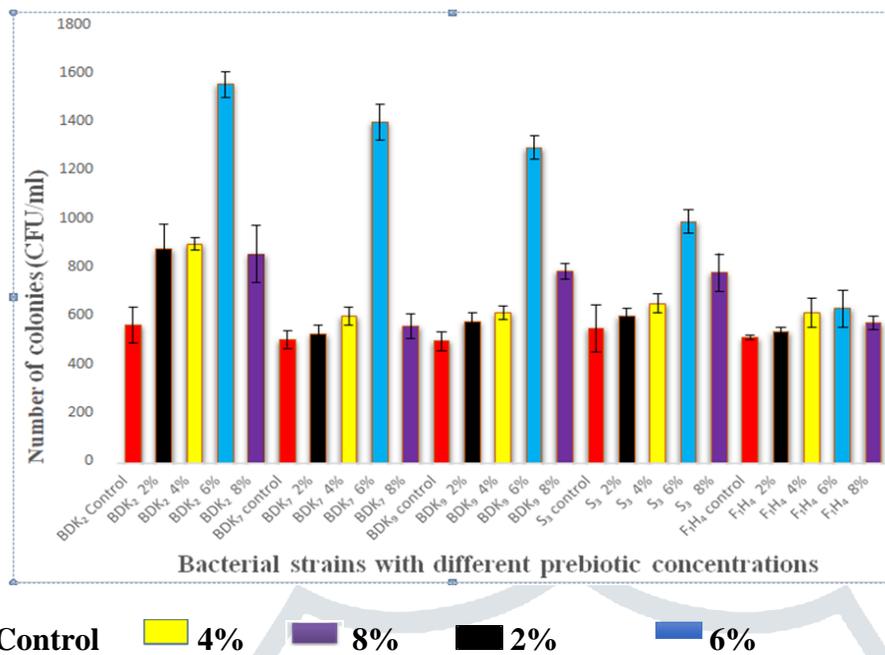


Fig 5: Banana peels powder with MRS agar

Water hyacinth powder: as a prebiotic was mixed with MRS agar to enhance the potential of probiotic. Different concentrations were checked to increase the growth of probiotic.

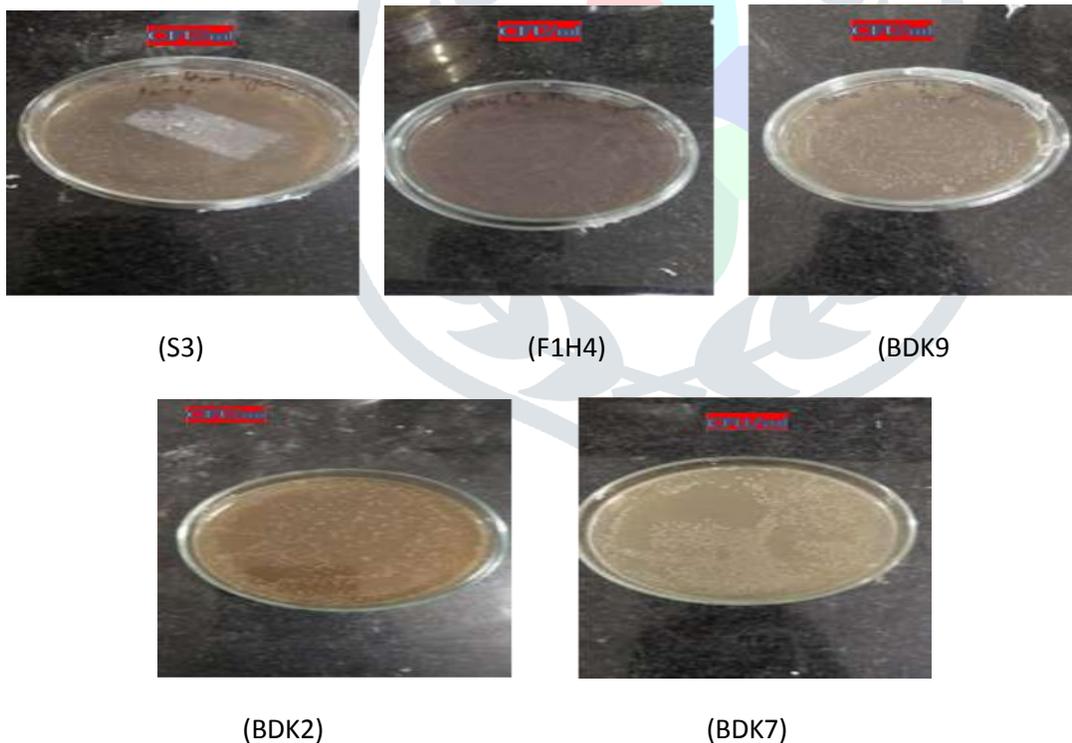


Fig 6: Number of colonies when 6% water hyacinth powder mixed with MRS agar.

Water hyacinth powder showed the beneficial effect when mixed with MRS agar media. Different probiotic strains were spread on different concentrations of water hyacinth powder under sterile condition and incubated for 24hrs at 37°C. It was found that concentrated number of colonies was seen on 6% concentration. While as F1H4, Showed 847.66 number of colonies at 6% concentration followed by BDK2, S3, BDK9, and BDK7.

These strains were also checked with only MRS agar media but the number of colonies on MRS agar was 511 in (F1H4) less than the 6% concentration of water hyacinth leaves powder.

Table 4: Number of probiotic colonies with water hyacinth powder

PROBIOTIC STRAIN	CONCENTRATION/WATER HYCANTH	MEAN NUMBER OF COLONIES
BDK2	0%	573.33±114.28
	2%	594.66±56.01
	4%	597.33±39.55
	6%	830*±8.14
	8	516±59.22
BDK7	0%	524.33±54.92
	2%	551.66±32.53
	4%	569.66±38.97
	6%	641.66*±37.85
	8%	480±19.07
BDK9	0%	598±91.99
	2%	682.33±33.53
	4%	698±20.29
	6%	734*±32.44
	8%	555.66±36.11
S3	0%	530±53.16
	2%	555±30.11
	4%	651.66±21.73
	6%	710*±32.78
	8%	576±10.58
F1H4	0%	511.33±4.16
	2%	590±86.74
	4%	632±52.91
	6%	847.66*±28.91
	8%	675.33±7.02SS

Note:*shows mean number of colonies at 6%.

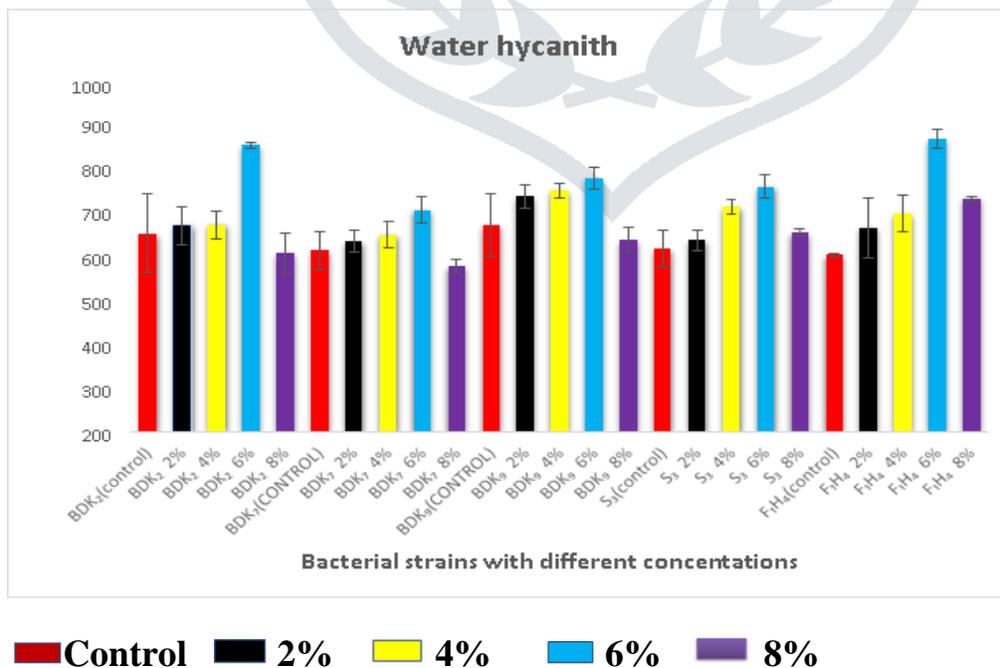


Fig 7: Effect of water hyacinth leaves on the growth of probiotic bacteria.

After checking the potential of banana peel powder, water hyacinth and guar gum powder it was found that all of these are beneficial for probiotic growth as compared to control media MRS agar. Different concentrations were checked to enhance the growth of probiotic like 2%, 4%, 6%, and 8% along with control. But it was found that at 6% concentration the growth of probiotic bacterial colonies are 20-30% more as compared to other concentrations. All of these diets having high content of fibre and all other qualities which are required for the probiotic growth. The number of colonies of probiotic bacteria was counted with the help of colony counter. Dilution of 10^{-4} was used for the counting of colonies. All of these strains are isolated from the fish gut and having high potential and provides resistance against diseases. After checking the potential of all the five stains against these supplements can used as an additive in future.

After testing the potential of probiotic bacteria against diet supplements such as Banana peel powder, guar gum powder, water hyacinth powder. Then synbiotic feed was prepared in which 6% concentration of supplement was taken and fed to fish (*Channa striata*). Trail of 20 days was conducted on the *Channa striata* fish to check the potential of synbiotic feed on growth performance and survival rate of fish.

Table 5: weight and length of fish after 20 days of synbiotic feed

Parameters	Guar gum diet fed to group of fishes	Banana peels diet fed to group of fishes	Water hyacinth leaves diet fed to group of fishes	Control diet fed to group of fishes
Initial weight/g	10.8±4.82	8.4±3.50	7±3.01	8.2±4.04
Final weight/g	12±4.70	9.4±3.06	7.9±3.14	9±4.65
Increase in weight/g	1.2	1	0.9	0.8
Initial length/cm	9.2±2.21	7.95±1.42	7.45±2.52	7.6±2.50
Final length/cm	9.45±2.45	8.25±1.78	7.7±2.57	7.85±2.13
Increase in length/cm	0.25	0.3	0.25	0.25
Mortality	0	0	0	3

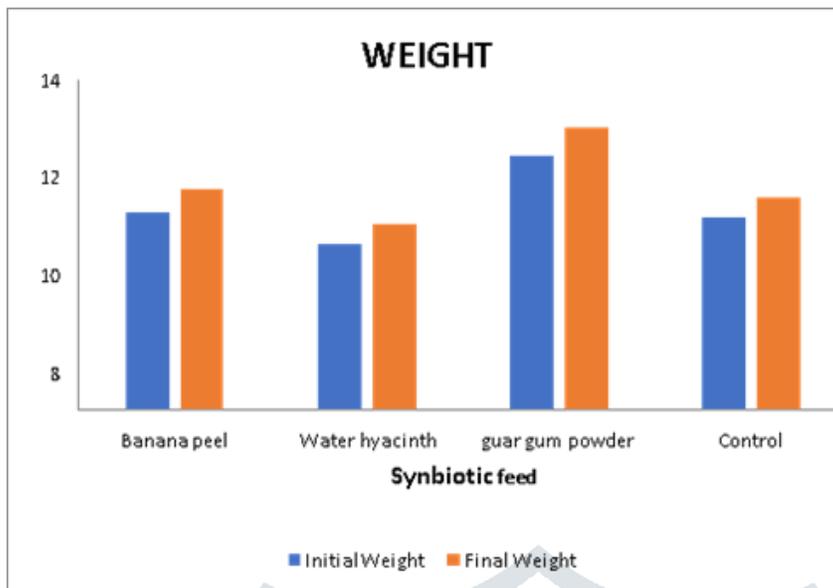


Fig 8: Effect of synbiotic feed on fish weight

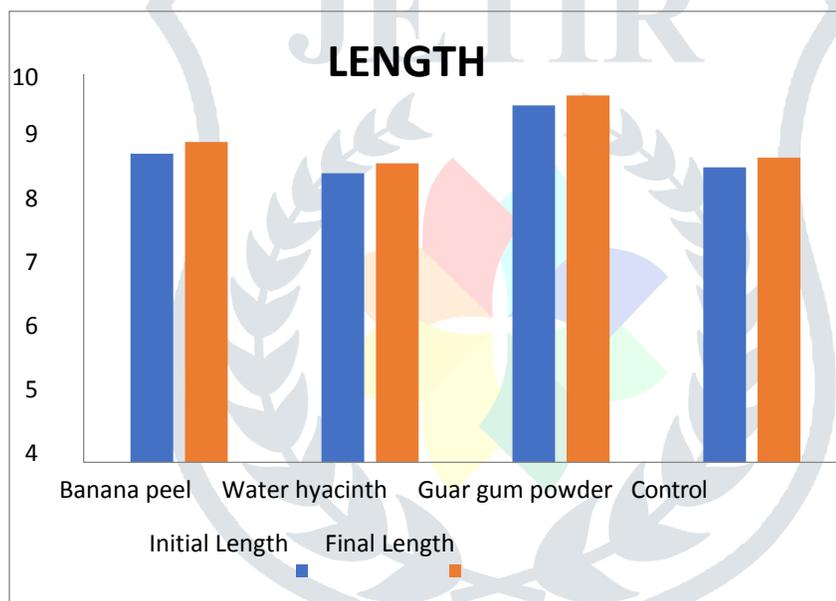


Fig 9: Effect of synbiotic feed on fish length

Discussion

The experimental data obtained after 20 days trial stated that after the consumption of synbiotic diet, there was an increase in length and weight of fish supplemented with synbiotic feed. Results revealed that fish fed with banana peel powder showed increase in length by 0.3% then followed by guar gum powder (0.25%), and water hyacinth powder (0.25%) as compared to control diet which shows less increase in length (0.03%). However in case of weight, guar gum powder shows maximum increase in weight by (1.2%) then followed by banana peels powder(1%), water hyacinth powder (0.9%) as compared to control diet (0.8%). According to [10] fish fed with banana peel diet along with *bacillus sp* showed good positive result at end of the study. There was increase in the Weight of the fish (*E-suratensis*) in the group fed with banana peel diet as compared

to control diet. Supplementation of 0.3% guar gum to Rainbow trout increases the overall growth performance [11].

Mortality is the net effect of this study to check whether the synbiotic feed is efficient to fish host or not. The data obtained after 20 days trail showed that there is 12% mortality in control fish fed with normal diet. Therefore it may be concluded that certain supplements present in prebiotic and potential of probiotic bacteria may decrease the mortality rate in fish fed with synbiotic feed. Fish mortality mostly occurs during larval stage to fingerlings, because during this period there are physiological and ecological changes in the development of fish. During this period larva shifts from endogenous nutrition to exogenous. At this stage old functions are quickly replaced by new functions inside the body [12]. This could be the reason of mortality (12%) in the control diet. While as others fishes fed with probiotic and prebiotic shows 100% survival rate.

Conclusion

Probiotics in aquaculture plays an important role to improve the health status of aquatic organisms by enhancing immune system and prevents gastrointestinal tract disorders. Prebiotic are applied as a feedstuff supplements for achieving fruitful performance of probiotics in the system. In this work different prebiotics were added as nutritional supplements for probiotics to enhance the growth. From this study it can be concluded that the prepared synbiotic feed is effective when fed to fish as compared to control diet. Probiotic bacteria are beneficial in aquaculture and play an important role in the growth, digestion, survival of the fish. The potential of probiotic strains can be increased by adding ingredients having high fibre content. All the five strains of probiotic used in this work shows improved growth when prebiotic added as nutrition for them. Probiotic bacteria can be used as an alternative to antibiotics in the aquaculture to control the diseases.

References

- [1] M. S. Pandiyan P, Balaraman D, Thirunavukkarasu R, George EG, Subaramaniyan K, "Probiotics in aquaculture. *Drug Invent Today* 5 (1): 55–59," 2013.
- [2] J. Luis Balcázar, O. Decamp, D. Vendrell, I. De Blas, and I. Ruiz-Zarzuela, "Health and nutritional properties of probiotics in fish and shellfish," *Microb. Ecol. Health Dis.*, vol. 18, no. 2, pp. 65–70, Jan. 2006, doi: 10.1080/08910600600799497.
- [3] G. Dalmin, K. Kathiresan, and A. Purushothaman, "Effect of probiotics on bacterial population and health status of shrimp in culture pond ecosystem," 2001.
- [4] G. Banerjee, A. Nandi, S. K. Dan, P. Ghosh, and A. K. Ray, "Mode of Association, Enzyme Producing Ability and Identification of Autochthonous Bacteria in the Gastrointestinal Tract of Two Indian Air-Breathing Fish, Murrel (*Channa punctatus*) and Stinging Catfish (*Heteropneustes fossilis*)," *Proc. Zool. Soc.*, vol. 70, no. 2, pp. 132–140, Dec. 2017, doi: 10.1007/s12595-016-0167-x.

- [5] G. Gibson, M. R.-T. J. of nutrition, and undefined 1995, “Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics,” *academic.oup.com*.
- [6] I. Guerreiro, A. Couto, M. Machado, ... C. C.-F. & shellfish, and undefined 2016, “Prebiotics effect on immune and hepatic oxidative status and gut morphology of white sea bream (*Diplodus sargus*),” *Elsevier*.
- [7] O. Carnevali *et al.*, “Microbial manipulations to improve fish health and production-A Mediterranean perspective Microbial manipulations to improve fish health and production e A Mediterranean perspective,” *Fish Shellfish Immunol.*, vol. 30, pp. 1–16, 2011, doi: 10.1016/j.fsi.2010.08.009.
- [8] D. L. Merrifield, G. M. Harper, A. Dimitroglou, E. Ringø, and S. J. Davies, “Possible influence of probiotic adhesion to intestinal mucosa on the activity and morphology of rainbow trout (*Oncorhynchus mykiss*) enterocytes,” *Aquac. Res.*, vol. 41, no. 8, pp. 1268–1272, Jul. 2010, doi: 10.1111/j.1365-2109.2009.02397.x.
- [9] R. Cerezuela, ... J. M.-J. of A., and undefined 2011, “Current knowledge in synbiotic use for fish aquaculture: a review,” *pdfs.semanticscholar.org*.
- [10] S. Sreeja, A. Tamilselvi, A. P.-I. J. A. R. B. Sci, and undefined 2016, “Effect of probionts with different feed additives on growth in pearl spot, *Eetroplus suratensis*.”
- [11] A. Brinker, R. R.- Aquaculture, and undefined 2011, “Fish meal replacement by plant protein substitution and guar gum addition in trout feed, Part I: Effects on feed utilization and fish quality,” *Elsevier*.
- [12] L. Sifa and J. A. Mathias, “The critical period of high mortality of larvae fish -A discussion based on current research,” *Chinese J. Oceanol. Limnol.*, vol. 5, no. 1, pp. 80–96, Mar. 1987, doi: 10.1007/BF02848526.