ROLE OF PROBIOTICS IN CONTROLLING PROMINENT DISEASE OF CATFISHES

BY

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Thesis submitted to the Chaudhary Charan Singh Haryana Agricultural University in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

in

ZOOLOGY

COLLEGE OF BASIC SCIENCES AND HUMANITIES
CCS HARYANA AGRICULTURAL UNIVERSITY
HISAR-125004

2009

CERTIFICATE - I

This is to certify that this dissertation entitled, **"ROLE OF PROBIOTICS IN CONTROLLING PROMINENT DISEASE IN CATFISHES",** submitted in partial fulfilment for the degree of **Doctor of Philosophy** in the subject of **Zoology,** to the Chaudhary Charan Singh Haryana Agricultural University, Hisar, is a bonafide research work carried out by **Mr. Tejpal Dahiya** under my supervision and that no part of this dissertation has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

(Prof. R. C. Sihag)
Major Advisor

CERTIFICATE - II

This is to certify that this dissertation entitled, "**ROLE OF PROBIOTICS IN CONTROLLING PROMINENT DISEASE IN CATFISHES** ", submitted by **Mr. Tejpal Dahiya** to the CCS Haryana Agricultural University, Hisar in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in the subject of **Zoology**, has been approved by the Student's Advisory Committee after an oral examination on the same.

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ACKNOWLEDGEMENT

It gives me immense pleasure to embellish my heartiest feelings with deepest sense of gratitude to my Major Advisor, Prof. R.C. Sihag, Department of Zoology and Aquaculture, for his keen interest, continual encouragement, valuable suggestions and kind advice. His insightful encouragement, valuable guidance and constructive suggestions had always been an inspiration to me during the entire course of my studies.

It is my proud privilege to express my gratitude and best regard to Prof. S.K. Gahlawat, Department of Biotechnology, CDL University, Sirsa for his unstinted guidance, diligent help, constant supervision and timely help throughout this investigation.

I express my sincere thanks to the member of my advisory committee, Prof. and Head V.P. Sabhlok, (Zoology, and Aquaculture), Dr. R. K. Gupta, (Zoology, and Aquaculture), Dr. Rajender Panwar, (Mathematics & Statistics) and Prof. Mohender Singh, (Soil Science) for their valuable suggestions and critical appraisal of this manuscript for the improvement of quality of work and its presentation.

I am also grateful to Dr. V.P. Sabhlok, Prof. and Head, Department of Zoology and Aquaculture, CCS HAU, Hisar for kind help and providing of laboratory facilities during these investigations.

I owe sincere appreciation and gratitude to my parents and brothers " Chandkanwar and Balinder Singh Dahiya" who have silently borne with me the ups and downs during the course of this study and with their affection, encouragement, valuable suggestions and help; I was able to complete this study.

I sincerely appreciate the moral support, inspiration and affection received from my friends Ravi Kant, Vijayanti, Shashank and Nitish Bansal during the critical phase of this work. Special thanks are also conveyed to Dr. S. S. Sindhu (Dept. of Microbiology), Dr. Anil Choudhary (Plant Pathology) and Dr. Manoj Verma (Microbiology) for their constant help, during the present study.

I personally offer my sincere thanks to Sh. Balraj Sehrawat, Fisheries Officer, Govt. of Haryana, for his help and cooperation during my research work.

I also extend my heartfull thanks to the members of non-teaching staff viz. Mrs. Raj Leekha, Rakesh Tondon, Ram Gopal, Dinesh, Balbir Singh and Mr. Panday who always helped me during the course of this study.

The financial help in the form of Rajiv Gandhi National fellowship from UGC, New Delhi is gratefully acknowledged.

Above all, without the blessing of the Almighty God, this effort would have remained a far-fetched dream.

Hisar **Tejpal Dahiya**
December, 2009

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CHAPTER 1

INTRODUCTION

Fish is a vital source of food for people to provide important proteins in regions where livestock is relatively scarce. It is man's most important single source of high-quality protein, providing approximately 16 per cent of the animal protein consumed by the world's population; 26.2 per cent in Asia, 17.4 per cent in Africa, 9.2 per cent in Europe, 7.4 per cent in North and Central America and 7.2 per cent in South America (Fisheries and Food Security, 2006).

Fisheries sector plays an important role in the socio-economic development of the country due to its potential contribution to national income due to export, and contribute significantly towards the Indian agricultural economy. In India there was an annual export earning of Rs. 7296 crore in 2006-07 and the share of fisheries sector was 1.4 per cent in the national gross domestic production (GDP) and 4.5 per cent in the agricultural GDP (FAO, 2006). Total fisheries production in India during 2006-07 was 68.69 lakh tonnes in which inland fisheries contributed 30.24 Lakh tonnes and marine fisheries did 38.45 lakh tonnes while total seed production was 31,668 million fry. Out of total world fish production, India shares 4.3 per cent (National Fisheries Development Board, 2009). In Haryana, the area under fish culture in 2008-09 was 12,885 hectares and total fish production was 67,236 metric tonnes and out of 30748 peoples 9466 were fish farmers and 21282 engaged in other activities thus gaining total income of 1862.85 million rupees. In district Hisar in 2007-08, the area under aquaculture was 1158 hectares and the state marketed 5710 tonnes of fishes, fetching an income of 171.3 million rupees (Statistical Abstract of Haryana, 2009)

The most important species used for fish culture in India are the Indian major carps (*Catla catla*, *Labeo rohita* and *Cirrhinus mrigala*), common carp (*Cyprinus carpio*), grass carp (*Ctenopharyngodon idella*), silver carp (*Hypophthalmichthys molitrix*) and giant freshwater prawn (*Macrobrachium rosenbergii*). Fish species such as snakehead murrel (*Channa punctatus*), magur (*Clarius batrachus*), North African catfish (*C. gariepinus*), stinging (*Heteropneustes fossilis*) and sutchi (*Pangasius hypophthalmus*) catfishes are also farmed, but to a lesser extent. Cold-water fish such as rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta fario*), snow trout (*Schizothorax richardsonii*) and golden mahseer (*Tor putitora*) are cultured in the uplands of northern India, mainly for sport fisheries (Jhingran, 1991; Nayak, 1993; lqbal *et al.,* 1999; Cerro *et al*., 2002), out of these Indian major carps (*C. catla*, *L. rohita* and *C. mrigala*), common carp (*C. carpio*), grass carp (*C. idella*), silver carp (*H. molitrix*), magur (*C. batrachus*), North African catfish (*C. gariepinus*), stinging (*H. fossilis*) and giant freshwater prawn (*M. rosenbergii*) are cultured in Haryana

(Jhingran, 1991; Roberts, 1997; Raj *et al*., 2008; Jayaprakash *et al*., 2006; Dahiya and Sihag, 2009).

Disease outbreak is being increasingly recognized as a significant constraint to aquaculture production, affecting trade and economic returns of this sector. According to recent reports, global losses due to shrimp disease alone are more than Rs. 18000 crores and the World Bank is investing of Rs. 1240 crores in fish disease research. In fishes, the annual loss is more than 1.5 million metric tonnes which costs multi crores of rupees. The above figures provide an indication of the overall economic significance of aquatic animal diseases (FAO, 2007).

With the increasing fish culture activities, several bacterial diseases, causing morbidity and mortality in fish have been reported word over in fresh water aquaculture. Bacterial diseases and infections are very common in fishes (Kumar, 1989). Fishes require optimal hydrobiological parameters for growth and survival. When the environmental conditions are not stable, like sudden change in water salinity, temperature, dissolved oxygen, pH or electrical conductivity, such changes lead growth and proliferation of disease causing organisms (Dey, 1989). Poor pond management practices and higher stocking rate often result into outbreaks of diseases which lead to mass mortality in fishes (Harikrishnan *et al*., 2005 and Mohanty and Sahoo, 2007).

Innumerable diseases like epizootic ulcerative syndrome, hemorrhagic septicemia, vibriosis, Edwardsiellosis, fin and tail rot, gill rot and dropsy etc. are caused in fishes due to bacterial pathogens, and several of them are reported in Indian literature (Kumar, 1989; Mukherjee *et al*., 1991). Some of the important bacterial pathogens are *Flavobacterium sp*., *Photobacterium damsela* subspecies *pisicida* (Aoki *et al*., 1995, 1996); *Vibrio damsela*, *V. alginolyticus* (Jayaprakash *et al*., 2006), *V. cholerae*, *V. vulnificus*, *Pasturella piscida*, *Providencia rettgeri*, *Aeromonas hydrophila*, *A. salmonicida*, *Pseudomonas fluorescens*, *P. aeruginosa*, *Flexibacter columnaris*, *Edwarsiella tarda* (Yeh *et al*., 2005; Dhanraj *et al*., 2008), *Enterococcus*, *Staphylococcus aureus* and *Micrococcus* sp. These have been identified as the most commonly occurring bacterial agents of fish diseases (Boon *et al*., 1996; Savan *et al*., 2004; Selvaraj *et al.,* 2005; Sahoo *et al*., 2007; Sugita *et al.*, 2008).

To control the incidences and spread of fish diseases, farm management techniques are employed which are diverse, specific to farm type and species farmed (Boyd, 1979). However, many preventative measures used to exclude pathogens are common to most pond systems (Tucker and Lloyd, 1985). These include exclusion of all wild fish from the farm site, collection of seed stock and brood stock from disease free areas, treatment of contaminated water, improving water quality and sterilization of equipments (Panigrahi and Azad, 2007).

Earlier the control of fish diseases was focused on chemical compounds like formalin (Klontz, 1979), organophosphates (Burridge and Nava, 1995), malachite green, copper

sulphate (Gomez- Gil *et al.,* 2000; Ghosh *et al*., 2003), quaternary ammonium compound (Snieszkd, 1981), furazolidane (Samuelson *et al.,* 1991), sulphadiazine (Capone *et al.,* 1996), oxytetracycline (Smith*,* 1996), di-n-butyl tin oxide (Burridge and Nava, 1995), and also the use of chemotherapeutants, such as erythromycin and deoxycycline (Munday, 1994). But now it is increasingly based on vaccination (Eldar *et al.,* 1997; Klesius *et al.,* 2000; Evans *et al.,* 2004). The use of antibiotics have problem of contamination of aquaculture facilities and livestock. The indiscriminate worldwide use of antibiotics in aquaculture has led to the development of drug-resistant in pathogenic bacteria (Thomson *et al.*, 2007; Midilli *et al.*, 2008) which are becoming increasingly difficult to control and eradicate (Sahul and Balasubramanian, 2000; van der Waij and Nord, 2000; Brun *et al.,* 2000; Miranda and Zemelman, 2002). Besides development of drug resistant bacteria and pathogens, the adverse effect of antibiotics is also on the beneficial aquatic microflora. The retention of harmful residues in aquatic animals finds their entry in to the human food chain causing health problem in human beings (Fuller, 1989; Gatesoupe, 1999; Robertson *et al*., 2000; Esiobu *et al.,* 2002; Sarter *et al.,* 2007).

Recently probiotics have found their use as alternative agents to control the fish diseases. Probiotics, which are micro-organisms or their products, are used for the health welfare of the host. These have found use in aquaculture as a means of disease control, supplementing or even in some cases replacing the use of antimicrobial compounds (Irianto and Austin, 2002). A wide range of microalgae, yeasts and gram-positive (*Bacillus subtilis* (Kumar *et al*., 2008; Rajesh *et al*., 2007)*, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus* (Rengipipat *et al*., 2008), *Micrococcus, Streptococcus* and *Weissella*) and gram-negative (*Aeromonas, Altermonas, Photorhodobacterium, Pseudomonas* and *Vibrio*) bacteria have been isolated from the aquatic medium. Probiotics actively inhibit colonization of potential pathogens in the digestive tract by antibiosis or by competition for nutrients or space or by alteration of microbial metabolism, and also by the stimulation of host immunity (Moustafa, 2004; Balcazar *et al*., 2006; Zizhong *et al.*, 2008). Aerobic gram-positive endospore-forming bacteria i.e. *Bacillus* spp. have been evaluated as probiotics for the improvement of water quality with vitamin C (Nayak *et al.*, 2007) by influencing the composition of water borne microbial population and by reducing the number of pathogens in the vicinity of farmed species (Stanier, 1993; Wang *et al.,* 1999). An isolate of *Micrococcus luteus* was found to have potential in combating *Aeromonas salmonicida* infections in rainbow trout (*Oncorhynchus mykiss*) (Irianto and Austin, 2003). Gatesoupe (1991, 1999) reported the benefit of using *Lactobacillus plantarum* and *L. helveticus* in turbot (*Scophthalmus maximus*) leading to enhanced growth. It was reported that the administration of *Carnobacterium divergens* to Atlantic cod (*Gadus morhua* L.) fry resulted in development of resistance against *Vibro anguillarum* (Gildberg *et al.,* 1997). Chang and Liu (2002) used

Bacillus toyoi and *Enterococcus fascium* from commercial product to reduce Edwardsiellosis in European eel (*Anguilla anguilla*). *Pseudomonas flourescens* had been reported to inhibit *Saprolegnia* and *A. salmonicida* in finfish culture (Smith and Davey, 1993; Bly *et al.,* 1997). Irianto and Austin (2002, 2003), however, tested the culture of *A. hydrophila* and V. *fluvialis* and found these bacteria to be effective in controlling the infection by A. *salmonicida* in rainbow trout. Park *et al.* (2000) found that oral administration (in feed) of bacteriophages protected the cultured fish against bacterial infection of *P. plecoglossicida.* Bacteria and yeasts were used as probiotics in *Catla catla* to enhance its survival and body weight (Mohanty *et al.,* 1996). A unicellular alga (*Tetraselmis cuecia*) was used as a feed for panaeids and as a feed additive for salmonids, and resulted in to the reduction of level of bacterial disease (Austin *et al.,* 1992; Vasudevan, 2000; Karunasagar, 2001; Azad *et al*., 2005; Panigrahi *et al*., 2005, 2007; Sahu *et al*., 2008). Several reports suggest that dietary yeast or nucleotides act as probiotics in fishes enhancing their immune function and disease resistance (Ramadan *et al.,* 1994; Burrells *et al.,* 2001), besides improving the growth performance (Adamek *et al.,* 1996; Kubitza *et al.,* 1997). It has been proposed that *de novo* synthesis and several pathways of nucleotides is a costly metabolic process, a dietary supply of nucleotides or precursors may have a protein sparing effect (Sanderson and He, 1994). Probiotics protect their host against neighboring or invading pathogens by interfering their cellular functions by producing metabolites (Ouwehand *et al.,* 2001; Mukai *et al.,* 2002; Fiorillo *et al.,* 2002) which inhibit the colonization, growth of other microorganisms or by competing for resources such as nutrients or space (Servin and Coconnier, 2003 and Vine *et al.,* 2004). The addition of potentially probiotic microorganisms to culture water in larval fish systems is a means of biocontrol (Moriarty, 1998; Verschuere *et al*., 1999; Douillet, 2000). It is possible that some of these may be ingested and has a probiotic effect on the host animal (Ruiz-Ponte *et al*., 1999; Chythanya *et al*., 2002).

It is clearly revealed from the forgoing paragraphs that catfishes are an integral part of aquaculture and an important component of sustainable food security in the country but these are infected with wide variety of diseases viz. hemorrhagic septicemia, epizootic ulcerative syndrome and vibriosis; the Indian Magur (*C. batrachus*) is also not the exception.. To take the advantage of these new generations of curative bioagents (probiotics) for the protection of environment and amelioration of ill effects of antibiotics, the present investigations were proposed with the following objectives:

1) Isolation and characterization of bacterial pathogens associated with catfish diseases.
2) Pathogenicity test of the causative organisms.
3) Role of probiotics in controlling the prominent bacterial pathogens.

CHAPTER-2

REVIEW OF LITERATURE

Fish is a vital source of food for people. It is an important protein source in regions where livestock is relatively scarce. It is man's most important single source of high-quality protein. It provides approximately 16.7 per cent of the animal protein consumed by the world's population, and contribute 26.2 per cent in Asia, 17.4 per cent in Africa, 9.2 per cent in Europe, 7.4 per cent in North and Central America and 7.2 per cent in South America, fish as a percentage of total animal protein intake to human diet (Fisheries and Food Security, 2006). The FAO (2009) estimates reveal that about 520 million people (8% of world population) world-wide depend upon fish as their primary source of animal protein. Due to its extremely high nutritional quality i.e. the proper balance of amino acids, fatty acids and extremely high digestibility, the use of fishmeal in the diet has increased. New fish products such as fish cakes, squid rings, extruded sticks and fish balls have been developed for sale in local urban supermarkets. According to the FAO (2007) the value of fish traded internationally is estimated to be US $ 92 billion per annum. Over 36 million people are employed directly through fishing and aquaculture and as many as 200 million people derive direct and indirect income from fisheries. Consumption of fish as food and non food uses were 110.4 and 33.3 million tonnes respectively in 2006 (FAO, 2009). In some countries, the foreign exchange earnings increased by fisheries and aquaculture are also making a growing contribution to the economy. Exports of fish and fishery products, although are relatively small particularly in India, Pakistan and Bangladesh, yet these contribute a quarter of the nation's gross domestic production (FAO, 2009). In India, aquaculture has evolved as the fastest growing food-producing sector and has been developed as an important component in food security. In real terms, food security would mean that food is available at all times; all persons have access to it; it is nutritionally adequate in terms of quantity, quality and variety and it is acceptable within a given culture. When all these conditions are present, a population can be considered to be "food secure". It is significant that in majority of Asian countries, supplies of food fish for domestic consumption have been boosted only by recourse to large and growing imports, and domestic requirements through aquaculture are still far off. In 2007-08, area under aquaculture in Haryana was 12885 hectares and the state marketed 67235 tonnes of fishes, generating a total income of Rs. 18.6285 crores. The state attained second position in the country after Punjab in the total annual fish production. In Hisar district, the area occupied under aquaculture was 1158 hectares in 2007-08, marketing 5710 tonnes of fishes, worth Rs. 17.13 crores (Anonymous, 2009).

Disease outbreaks are being increasingly recognized as a significant constraint on aquaculture in many countries (Riquelme *et al.,* 1997). It constitutes a limiting factor in the

development of aquaculture production. Commercial fish farming creates favorable conditions for the development of infectious diseases in fish. With the increasing fish culture activities, several bacterial diseases causing morbidity and mortality in fish have been reported in freshwater aquaculture. Pathogenic microorganisms (bacteria, fungi, viruses and protozoan) generally enter the fish through the gills, the skin or gastrointestinal tract (Birkbeck and Ringo, 2005). These three routes of entry represent physical and immunological barriers against pathogens. Thus, their integrity, both at the cell and tissue levels, is vital for the later outcome of the host-pathogen interaction.

Bacterial diseases of fish and their pathogens

Bacterial diseases in fishes are broadly classified as surface ulcerative, acute systemic and chronic granulomatous types. Surface ulcerative types of diseases are characterized by hemorrhagic surface ulcers and are caused by species of *Aeromonas, Pseudomonas, Vibrios, Flexibacter* and *Myxobacter.* These infections lead to acute systemic diseases with passage of time (Kumar *et al.,* 1986). Systemic disease is characterized by the presence and proliferation of bacteria in internal organs like kidneys, heart, spleen, liver and even blood. These diseases produce necrotic changes like bacterial hemorrhagic septicemia in all affected organs and cause mass mortality. Chronic granulomatous type of disease conditions are characterized by formation of granulomas with the initiating bacteria in the centre of glands (Dey, 1989).

Bacterial fish diseases like dropsy, hemorrhagic septicemia, edwardsiellosis /edwardsiella septicemia, bacterial kidney disease, bacterial gill disease, pop eye, vibriosis, epizootic ulcerative syndrome, ulcerative disease, and fin and tail rot were reported by different workers from different parts of the country in fresh water fishes like catla (*Catla catla*) , rohu (*Labeo rohita*), mrigal (*Cirrihinus mrigala*), tilapia (*Oreochromis niloticus*), grass carp (*Ctenopharyngodon idella*), common carp (*Cyprinus carpio*) and silver carp (*Hypophthalmichthys molitrix*) and (Kumar *et al.,* 1986; Dey, 1989; Karunasagar *et al.,* 1989; Shankar and Mohan, 2002); catfishes (*Clarius batrachus* and *Clarius garipienus*) and *Macrobrachium roesenbergii* (Dahiya and Sihag, 2009). Bacterial pathogens were also reported to cause heavy mortality in both cultured and wild fish species in different parts of the world (Joseph and Clark, 2002; Bader *et al.,* 2003), may be either obligate or facultative bacteria. The facultative bacterial pathogens proved a potential threat when fish were under environmental and physiological stress (Wedemeyer, 1970). Six gram negative rods (*Aeromonas, Proteus, Citrobacter, Pseudomonas, Flavobacterium* and *Chromobacterium*) and three gram positive cocci (*Micrococcus, Streptococcus* and *Staphylococcus*) genera of bacteria, potentially pathogenic, were identified from *Aristichthys nobilis* and *C. idella* (grass carp) fingerlings (Shamsudin, 1986; Li, *et al.,* 1996; Welker *et al.,* 2005; Rollo *et al.,* 2006).

Traditional methods of disease control:

Hygiene maintenance: In traditional methods, pond hygiene used to be the first priority of

pond management. Pond must be periodically dried and then refilled with abundant water of good quality. It should be well maintained to avoid silting and weeds should be controlled. High stocking densities, over feeding and pollution are avoided. Entry of wild fish or restocking of ponds with unhealthy or sub quality eggs and young fish are prevented. Too frequent handling, transfer or transport of pond fish is avoided. The condition of gills must be periodically checked in random samples of the selected fishes (Gomez-Gil *et al.,* 2000). As soon as the outbreak of a disease is noticed, the seriously affected, moribund or the dead fish must be removed from ponds and buried with quicklime. Prophylactic measures are taken to disinfect the pond and the gears. The nets and other tools are routinely disinfected with benzalkonium chloride solution. Ponds having fish and threatened with disease outbreak are disinfected with either potassium permanganate (0.5g /100 litres) or benzalkonium chloride solution (600 ppm) or calcium cyanamide (Wood, 1974).

Treatment by chemicals and use of antibiotics: Formalin (40 per cent formaldehyde) is a traditionally tried chemical. This is very effective when applied @ 167 to 250 mg/litre against parasitic infections of protozoan ectoparasites (Costia) and trematodes like Discocotyle (Klontz, 1979). Organophosphates are useful for such ectoparasites where formalin fails. These are effective against crustacean parasites like *Lernaea* and *Argulus* and such trematodes as *Gyrodactylus* (Burridge and Haya, 1995). Malachite green is the next most tried chemical, especially effective against fungal infections including *Saprolegnia.* It is used at a concentration of 1-2 mg/l. Copper sulphate is effective against bacterial infections, but it is not favored for reasons of its toxic effects (Gomez-Gil *et al.,* 2000). Quaternary ammonium compounds are very effective against bacterial infections. These are generally used at concentrations of 1-4 mg/l (Snieszkd, 1981). Furazolidane, a nitrofuran, popular in veterinary therapy, is used in U.S.A. for incorporation in fish food to control/prevent bacterial infection. The recommended dosage is 11 g/100 kg fish/day for about a week (Samuelson *et al.,* 1991). Sulphadiazine, a sulphonamide, mixed with trimethoprim, is also found to be equally effective against bacterial diseases when administered through diet. The recommended dosage is 5.5 gm/ 100 kg fish/day for a week (Capone *et al.,* 1996). Oxytetracycline an antibiotic used in veterinary therapy is commonly used for bacterial therapy of vibriosis and ulcer disease in fishes. The recommended dosage is 7.5 g/100 kg fish/day for one to two weeks (Smith, 1996). Epson salt (magnesium sulphate) mixed with diet when given is found very useful in treatment of protozoan parasites of the gut. Di-n-butyl tin oxide also administered via diet at a dosage of 25 g/100 kg fish/ day for three days is effective against most parasites of the gut other than protozoans (Burridge and Nava, 1995). Earlier, the control of fish diseases was focused on the use of chemotherapeutants, such as erythromycin and deoxycycline (Munday, 1994), but now it is increasingly being shifted to vaccination (Klesius *et al.,* 2000; Evans *et al*., 2004).

Ill effects of antibiotics: Combined with the problem of antibiotic contamination of aquaculture facilities and livestock, the indiscriminate worldwide use of antibiotics in aquaculture has led to the development of drug-resistant bacteria which are becoming increasingly difficult to control and eradicate (Sahul and Balasubramanian, 2000; van der Waij and Nord, 2000; Brun *et al.,* 2000; Miranda and Zemelman, 2002). To keep a sustainable growth pattern, health management strategies must go beyond antibiotics and chemotherapeutics, which create resistance bacteria and irnmunosuppression in the host. Besides development of drug resistant in bacteria and pathogens, the adverse effect of antibiotics on the non target aquatic microflora, and the retention of harmful residues in aquatic animals (Fuller, 1989; Esiobu *et al.,* 2002; Sarter *et al,* 2007). Subsequently, certain antibiotics such as chloramphenicol have been banned in many countries (FAO, 2000).

Use of probiotics as an alternative method of disease control: A growing concern for the high consumption of antibiotics in aquaculture has initiated a search for alternative methods of disease control (Gatesoupe, 1999). One of the methods gaining recognition for controlling pathogens within the aquaculture industry is the use of beneficial or probiotic bacteria (Ringo and Birkbeck, 1999; Verschuere *et al.,* 2000). Improved resistance against infectious diseases can be achieved by the use of probiotics (Irianto and Austin, 2002). Probiotics are live microorganisms administered in adequate amounts as feed or food supplements which have beneficial effects on the intestinal microbial balance of the host. These are emerging as significant microbial food supplements in the field of prophylaxis (Geovanny *et al.,* 2007). In aquaculture, the term "probiotics" is often loosely used to describe a microbial formulation responsible for biocontrol or bioremediation of pathogens. The term probiotics comes from the Greek word "pro bios" meaning "for life". The original definition of probiotics given by Lilly and Stillwell (1965) "substances produced by one protozoan that stimulated the growth of another" was expanded from an agricultural perspective and redefined as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller, 1989). Probiotics are commonly defined as mono or mixed cultures of live microbes that, when applied to animal or human, generate a beneficial effect on health of the host. These beneficial effects include disease treatment and prevention as well as improvement of digestion and absorption in the host (Havenaar and Veld, 1992). However, Salminen *et al.* (1999) suggested that it includes "microbial cell preparations or components of microbial cells". Gatesoupe (1999) redefined probiotics for aquaculture as "microbial cells that are administered in such a way so as to enter the gastrointestinal tract of aquatic animals, with the aim of keeping it alive and improving health". According to Verschuere *et al.* (2000) the ability of a probiotic is modify the "host-associated or ambient microbial community" to improve the quality of its surroundings.

A large number of probiotics (bacteria, fungi, bacteriophages and algae) have now been discovered and their use in aquaculture is well established (Toma *et al*., 2006) which be given single or mixture (Anukam *et al*., 2006; Uomo, 2008). Bacteria are (Gram-positive bacteria—*Bacillus* sp. S11, *Bacillus* sp. (Yu *et al*., 2009a), *Bacillus coagulans* (Meltem, 2009) *Carnobacterium* sp. BA211, *Carnobacterium inhibens* K1, *Carnobacterium divergens*, *Enterococcus faecium* SF 68 (Gugolek *et al*., 2004), *Lactobacillus* sp., *Lactobacillus helveticus*, *Lactobacillus lactis* AR21, *Lactobacillus plantarum*, *Lactobacillus rhamnosus* ATCC 53103 (Ramiah *et al*., 2009), *Micrococcus luteus* A1-6, *Streptococcus thermophilus* (Khali, 2009), *Weissella helenica* DS-12 and unnamed lactic acid bacteria (Simon, 2005) and Gram-negative bacteria- *Aeromonas hydrophila* A3-51, *Aeromonas media*, *Alteromonas* sp. CA2, *Pseudoaltrompnas* sp. (Hjelm *et al.*, 2004), *Photorhodobacterium* sp., *Pseudomonas fluorescens*, *Pseudomonas fluorescens* AH2, *Roseobacter* sp. BS 107, *Vibrio alginolyticus* and *Vibrio fluvialis* (Moustafa, 2004). Bacteriophages used as probiotics are representative of Myoviridae, coticovirdae, Tectiviridae, Leviviridae, Cystoviridae, Inoviridae (Inovirus and Plectovirus) and podoviridae families. These infect pathogenic bacteria, filtered easily through the kidney and harmless to human and animals (Niels-Goreng *et al.*, 2004; Dock *et al*., 2004; Alberda *et al*., 2007). Yeasts (*Saccharomyces cerevisiae*, *S. exiguous*, *Phaffia rhodozoma*; *Debaryomyces hansenii*) and one Microalgae named *Tetraselmis cuecia* are also used as probiotics (Zizhong *et al.*, 2008). Bacteria produce bacteriocins (Carine *et al*., 2009) and yeasts produce many bioactive substances, like protein, amino acids, vitamin, polysaccharide, fatty acid, phospholipid, polyamine, astaxanthin, 13-carotenoid, trehalose, glutathione, superoxide dismutase, chitinase, amylase, phytase, protease, killer toxin which promote better health of animals (Zhenming *et al*., 2006; Yousefi and Karkoodi, 2007; Bari *et al*., 2009). Various bacteria commercially used as probiotics are: *Lactobacillus* species *viz*. *L. acidophilus*, *L. casei*, *L. fermentum*, *L. gasseri*, *L. johnsonii*, *L. lactis*, *L. paracasei*, *L. plantarum*, *L. reuteri*, *L. rhamnosus*, *L. salivarius*; Bifidobacterium species *viz*. *B. bifidum*, *B. breve*, *B. lactis*, *B. longum*, and *Streptococcus* species *viz*. *S. thermophilus*. Likewise, non-bacterial sources of probiotics are yeast (*Saccharomyces boulardii* and *Debaryomyces hansenii*), which has also been commonly identified as probiotic (Kahan *et al.,* 2003; Burrells *et al.,* 2001). Nucleotides also act as probiotics, enhancing immune function and disease resistant (Wang *et al.*, 1999; Ouwehand *et al*., 2001; Vine *et al.*, 2004b).

Probiotics selection criteria:

***In vitro* antagonism tests:** A common way to select a probiotic is to perform *in vitro* antagonism tests, in which pathogens are exposed to the probiotics or their extracellular products in liquid (Gram *et al.,* 1999) or solid medium (Austin *et al.,* 1992). Depending on the exact arrangement of the tests, candidate probiotics can be selected based on the production of inhibitory compounds (Gram *et al.,* 1999) or siderophores, or on the basis of competition for

nutrients (Dopazo *et al.,* 1998). The pre-selection of candidate probionts based on these *in vitro* antagonism tests has often led to the finding of effective probiotics.

A probiotic should either be supplied on a regular basis or be able to colonize and persist in the host or in its ambient environment. The ability of a strain to colonize in the gut or an external surface of the host and adhere to the mucus layer may be a good criterion for pre-selection among the putative probiotics (Shi and walter, 2004). This involves the viability of the potential probiotic within the host and/or within its culture environment, adherence to host surfaces, and the ability to prevent the establishment of potentially pathogenic bacteria (Verschuere *et al*., 2000 and Panigrahi and Azad, 2007; Yu *et al*., 2009b).

***In vivo* evaluation of effects of potential probiotic on the host:** The effect of candidate probiotics should be tested *in vivo* as well. When the probiotic effect is supposed to be nutritional, the candidate probiotics could be added to the culture of the aquatic species and their effect on growth and survival parameters could be assessed. However, when biological control of the microbiota is desired, representative *in vivo* challenge tests seem to be the appropriate tool to evaluate the potential effect of the candidate probiotic on the host (Gildberg *et al.,* 1995). The approach outlined above should result in formulating a set of strains with a well-established probiotic effect on the target organism without affecting other possibly involved trophic levels. Comparative pilot experiments under hatchery or grow out conditions in the farms should be performed to estimate the economic consequences of the probiotic application. An important factor in the economic evaluation is the mass production of the probiont. Also, effective legislation, if any, should be taken into account before commercial application is begun. Finally, a cost-benefit analysis will determine whether the probiotics could be applied in practice or not (Verschuere *et al.,* 1999; Ruiz-Ponce *et al.,* 1999; Douillet, 2000). In general, the selection criteria for a bacterium to be used as a `probiotic' should be followed. It should be non-pathogenic; withstand incorporation into a delivery vehicle at high cell counts, and remain viable throughout the shelf-life of the product; withstand transit through the gastrointestinal tract, show acid and bile tolerance; be able to adhere to cells of the intestinal epithelium and/or colonize the lumen of the tract; show antagonistic activity towards enteric pathogens and/or provide demonstrated health benefits (Chythanya *et al.,* 2002). A probiotic must possess certain properties in order to aid in correct establishment of new, effective and safe products. It should not be harmful and be acceptable to the host through ingestion, potential colonization and replication within the host. It should reach the location where the effect is required to take place. It should actually work in *in vivo* as well as *in vitro* findings. It should preferably not contain virulence resistance or antibiotic resistance genes (Verschuere *et al.,* 2000).

Possible modes of action

Production of antagonistic compounds: Antagonistic compounds are described as chemical

substances produced by bacteria which are toxic or inhibitory towards other micro organisms (Ramirez and Dixon, 2003). These substances may be produced as either primary or secondary metabolites and therefore, have different modes of inhibitory action. Microbial populations may release chemical substances that have a bactericidal or bacteriostatic effect on other microbial populations, which can alter interpopulation relationships by influencing the outcome of competition for chemicals or available energy. The presence of bacteria producing inhibitory substances in the intestine of the host, on its surface, or in its culture medium is thought to constitute a barrier against the proliferation of opportunistic pathogens (Fredrickson and Stephanopoulos, 1981, Balcazar *et al*., 2006).

In general, inhibitory effect of bacteria is due to many factors, either singly or in combination with others. These include production of antibiotics (Williams and Vickers, 1986), bacteriocins (Bruno and Montville, 1993), siderophores, lysozymes, proteases, and hydrogen peroxide, and also the alteration of pH values by the production of organic acids (Sugita *et al.,* 1997). Lactic acid bacteria are known to produce compounds such as bacteriocins that inhibit the growth of other microorganisms (Vandenbergh, 1993). There are many reports of the inhibitory activity of lactic acid bacteria mediated by bacteriocins. Nair *et al.* (1985) showed that a large proportion of bacteria produced bacteriolytic enzymes against *Vibrio parahaemolyticus*. Bacteriocins are proteinacious agents produced by bacteria to inhibit or kill other bacteria (Vijaybasker and Somasundram 2008)). Other inhibitory compounds produced by bacteria include organic acids, hydrogen peroxide, carbon dioxide and siderophores. Siderophores are iron-complexing chemicals secreted by bacteria and fungi. Siderophore-producing bacteria can survive in nutrient-poor environments (Vine *et al.,* 2004a).

Competition for chemicals or available energy: Competition for chemicals or available energy may determine how different microbial populations co-exist in the same ecosystem (Fredrickson and Stephanopoulos, 1981). The microbial ecosystem in aquaculture environments is generally dominated by heterotrophs competing for organic substrates as both carbon and energy sources. Specific knowledge of the factors governing the composition of the microbiota in aquaculture systems is required to manipulate it. Verschuere *et al.* (1999) selected several strains of bacteria with a positive effect on the survival and growth of *Artemia* juveniles. It was suggested that the selected bacteria exerted their protective action by competing with the pathogen for chemicals and available energy.

Competition for iron: Virtually all microorganisms require iron for growth (Reid *et al,,* 1993). Siderophores are low-molecular-weight (<1,500 Daltons), ferric ion-specific chelating agents which can dissolve precipitated iron and make it available for microbial growth. The ecological significance of siderophores resides in their capacity to scavenge an essential nutrient from the environment and deprive competitors of it. Successful bacterial pathogens

are able to compete successfully for iron in the highly iron-stressed environment of the tissues and body fluids of the host. The ecological significance of siderophores in soils as important tools for iron acquisition by microorganisms and plants and their involvement in suppression of plant root pathogens have been established (Wang *et al*., 1999). Harmless bacteria which can produce siderophores could be used as probiotics to compete with pathogens whose pathogenicity is known to be due to siderophore production and competition for iron or to out compete all kind of organisms requiring ferric iron from solution (Dalmin *et al.,* 2001; Gullian *et al.,* 2004).

Competition for adhesion sites: One possible mechanism for preventing colonization by pathogens is competition for adhesion sites on gut or other tissue surfaces. It is known that the ability to adhere to enteric mucus and wall surfaces is necessary for bacteria to become established in fish intestines (Westerdahl *et al.,* 1991). Adhesion can be non specific, based on physicochemical factors, or specific, involving adhesin molecules on the surface of adherent bacteria and receptor molecules on epithelial cells. Adhesion capacity and growth on or in intestinal or external mucus has been demonstrated *in vitro* for fish pathogens like *Vibrio anguillarum* and *Aeromonas hydrophila* (Krovacek *et al.,* 1999), and for candidate probiotics such as *Carnobacterium* strain K1, and also for several isolates inhibitory to *Vibrio anguillarum*. In one of these studies, the aim was to measure the *in vitro* capacity of the strains to adhere to and grow in turbot intestinal mucus in order to investigate their potential to colonize the intestine of farmed turbot as a means of protecting the host from infection by *V. anguillarum*. The intestinal isolates generally adhered much better to a film of turbot intestinal mucus, skin mucus, and bovine serum albumin than did *V. anguillarum,* indicating that they could compete effectively with the pathogen for adhesion sites on the mucosal intestinal surface (Chabrillon *et al*., 2006*;* Watson *et al*., 2008).

Enhancement of the immune response: Immunostimulants are chemical compounds that activate the immune systems of animals and provide them more resistance to infections by viruses, bacteria, fungi, and parasites (Raa, 1996). Fish larvae, shrimps, and other invertebrates have less developed immune systems than adult fish, and are dependent primarily on nonspecific immune responses for their resistance to infection (Sakai *et al.,* 1995; Balcazar, *et al.*, 2006) Observations obtained in various experiments with warm-blooded animals indicate that probiotic (lactic acid) bacteria administered orally may induce increased resistance to enteric infections (Nikoskelainen *et al.,* 2003). It has also been suggested that ingestion of bacteria and subsequent endocytosis in Cod and Herring larvae are involved in stimulation of the developing immune system (Brunt and Austin, 2005; Selvaraj *et al.,* 2005 and Rajesh *et al.,* 2008).

Improvement of water quality: In several studies, water quality has been recorded to be improved during the addition of the probiotics as these are more efficient in converting

organic matter back to CO_2 and convert a greater percentage of organic carbon to bacterial biomass or slime (Stanier *et al.*, 1993). It is reasoned that by maintaining higher levels of these probiotic bacteria in the production pond, farmers can minimize the buildup of dissolved and particulate organic carbon during the culture cycle while promoting more stable phytoplankton blooms through the increased production of CO_2 (Verschuere *et al.,* 1999).

Interaction with Phytoplankton: Many bacterial strains may have a significant algicidal effect on many species of micro algae, particularly on red tide plankton (Fukami *et al.,* 1997). Some bacterial strains inhibited the growth of the unicellular alga (*Pavlova lutheri*) to various degrees, but bacteria antagonistic towards algae would be undesirable in larval rearing pond where unicellular algae are added (e.g. the green-water technique), but would be advantageous only when undesired algal species develop in the culture pond (Munro *et al.*, 1995; Ramirez and Dixon, 2003). It is conceivable that bacteria can indirectly influence the health or the zoo-technical performance of the cultured aquatic animals through their effect on the micro algae used as food. Positive effects of bacteria on cultured micro algae have also been observed (Fukami *et al*., 1997; Huys *et al.,* 2005).

Vitamin production: All probiotics bacteria produce small amounts of certain B vitamins, including foliates and vitamin B (Kumari and sahoo, 2005 and McFarland, 2006). Microbial synthesis of vitamin K and vitamin C in the intestine have nutritional significance in most animal species (Conway, 2001; Nayak *et al.,* 2007). Bifidobacteria, Streptococci, and Enterococci have been shown to produce vitamin K (Bentley and Meganathan, 2007).

Reduction of cholesterol: Studies have shown that some probiotics can lower total serum cholesterol and low density lipoprotein cholesterol (Dambekodi and Gilliland, 1998). *In vitro* studies have shown that *Lactobacillus casei* and *L. acidophilus* effectively remove cholesterol from culture media (Anderson and Gilliland, 1999). Researchers postulated that lactic acid bacteria assimilate cholesterol in the gut or deconjugate bile acids disrupting the intestines-to-liver circulation of cholesterol and reduce the chances of hypertension (Sanders, 2000).

Short-chain fatty acid (SCFA) production: Probiotics especially the bifidobacteria (*Bifidibacterium bifidum*, *B. breve*, *B. lactis* and *B. longum*) are able to break down and metabolize non-digestible carbohydrates such as fiber (Topping and Clifton, 2001). The major by-products of these are short-chain fatty acids (SCFA) such as lactate, acetate, propionate, and butyrate. SCFA lower down intestinal pH and create an environment inhospitable to pathogenic bacteria such as *E. coli* and *Salmonella* species. SCFA nourish mucosal cells of colon supplying 60-70% of colonocyte energy needs. Butyrate is the preferred energy source for colonocytes (Chi *et al.,* 2006). Studies in animals and humans have found that SCFA directly stimulated colonic calcium, magnesium, and potassium absorption, increased colonic blood flow, enhanced tissue oxygenation and transport of nutrients, and might be of therapeutic value for various intestinal disorders (Jha *et al.,* 2007; Zhou *et al.*, 2007).

Mode of application of the probiotics: The probiotics can be added to the host or its ambient environment in different ways e.g. i) addition to the artificial diet, ii) addition to the culture water, iii) bathing and addition through live food (Verschuere *et al.,* 2000). Inoculation of the culture system and adding to live food via *Artimia* is advisable when the small volumes are used (Gomez-Gil *et al.,* 2000). The inclusion in the artificial diet is more adapted when the greater volumes are used; however, this requires sufficient number of probiotics to last when they reach the gastrointestinal tract and capacity to adhere to the epithelial mucosa.

Use of Probiotic in Aquaculture: Probiotics are used in crustacean (Maeda and Liao, 1994), shrimp- *Penaeus monodon*, *M. rosenbergii* (Ajitha *et al.,* 2004; Hong *et al.,* 2005; Ghosh *et al.,* 2008); freshwater eggs, hatchlings and of larvae (*Scophthalmus maximus*) (Mata *et al.,* 2008); juveniles of goldfish, *Carassius auratus* (Ahilan 2004; Ringo *et al.,* 1995, 1997), fish-gilthead seabream (*Sparus aurata*) (Chabrillo *et al.*, 2006; Sugita and Shibuga, 1996;

Hematological studies: Hematological parameters of fish are used as indicators of their physiological state, and their study has become widespread from the last three decades, in the control of pathogens and manipulation of stress in fish (Barisal *et al.,* 1979). The total erythrocyte count (Irianto and Austin 2002; Ranzani-Paiva *et al.*, 2004), total leucocyte count (Selvaraj *et al.*, 2005), haemoglobin, MCV, MHC and MCHC counts, total protein, albumin, globulin, albumin-globulin ratio, alkaline phosphatase activity, alanine and aspartate aminotransferase activities, creatinine, sodium, cortisol, insulin, glucose, per cent survival and weight gain were reported to increase in *Labeo rohita* (Siwicki *et al*., 2003; Das *et al.*, 2006; Rajesh *et al.,* 2008), *Catla catla* (Rengpipat *et al.*, 2000), *Tilapia nilotica* (Mona *et al.*, 2008) and *Cyprinus carpio* (Palikova *et al.*, 2004; Aubin *et al.*, 2005; Olafsen, 2001).

The massive use of antibiotics for the control of diseases has been questioned by acquisition of antibiotic resistance in disease causing agents, and the need of alternative control measures is of prime importance. Recently, probiotics are being used as alternative disease control measures. Probiotics have been used by people for millennia since the time humans first consumed fermented milk products. Probiotics can be essential for the normal digestive, endocrine and immunological functions of the bowel. They inhibit pathogenic microorganisms and have been used therapeutically to a variety of gastrointestinal and even systemic disorders. Probiotics transiently colonize the bowel and, except when used to treat an acute disorder, must be regularly consumed to maintain benefit. Use of microbial probiotics to promote health maintenance and disease prevention is now widely accepted. Traditional probiotic dairy strains such as lactic acid bacteria are considered as major sources due to their long history of safe use in foods. However, with a broad range of applications, several probiotic strains are now being investigated and proposed for an alternative medication. It is therefore necessary to ensure that novel isolated probiotics should be screened and subjected to appropriate field tests with animals or humans.

CHAPTER -3

MATERIAL AND METHODS

3.1 Survey of fish farms and characterization of catfish diseases.

Three fish farms around Hisar practicing polyculture of catfishes were selected for the periodical survey of their diseases. These were Muklan fish farm (Hisar), Nagalpur fish farm, Narwana (Jind) and CCSHAU fish farm (Hisar) (Figure 1, 2, 3). Surveys of these fish farms were carried out from April, 2007 to June, 2009 at monthly intervals to monitor the incidence of any disease in the cultured catfishes. On each fish farm, 20 samples of the catfishes were drawn through netting and each sample consisted of 25 - 35 catfishes, and percentage of diseased fishes of each species in the samples was determined.

The samples of diseased catfishes from the fish farms were brought to the Fish Biotechnology Laboratory, Department of Zoology and Aquaculture, Chaudhary Charan Singh Haryana Agricultural University, Hisar. The external symptoms of the diseased fishes were recorded and compared with those enlisted by Gopal Krishan (1999), Jhingran and Das (1990) and Jhingran (1991). Accordingly, the diseases were characterized. The hydrological parameters of the three fish farms were maintained as per local recommendations of this region (Table 1). The minor differences were due to local conditions.

Table 1: Hydrobiological parameters managed at different fish farms surveyed around Hisar.

Catfish farms	Ranges of hydrobiological parameters				
	DO (mg/ml)	pH	Salinity (ppt.)	Hardness (ppm)	EC (MS/CM)
Muklan catfish farm (Hisar)	5.4-6.1	6.8-7.5	0.49-0.56	80-110	0.9-1.2
CCSHAU catfish farm, Hisar,	5.3-6.2	7.1-7.5	0.51-0.57	70-100	1.0-1.3
Nagalpur (Narwana) Jind	5.5-6.0	6.9-7.4	0.43-0.49	70-100	0.9-1.3

The physiochemical parameters of water during the experiments were maintained. The freshwater was supplied from dechlorinated tap water and fish were fed a normal recommended commercial diet.

3.2 Isolation and characterization of catfish pathogens

3.2.1 Culture and isolation of bacteria

For the culture and isolation of the pathogenic bacteria, method suggested by OIE

Figure 1 Muklan fish farm (Hisar)

Figure 2 Nagalpur fish farm (Jind)

Figure 3 CCSHAU fish farm (Hisar)

(2006) was followed. The specimens of diseased catfish were dissected, the affected tissue from kidney, liver, skin lesions or muscles were taken in a test tube then, homogenized it into a homogenizer and spread over the nutrient agar medium in petri plates under aseptic conditions. These plates were incubated in B.O.D at 30 ± 1^0C for 24 h. Bacterial growth on the nutrient agar plate was observed after 24 h. Pure colonies of bacteria were isolated and obtained further by reculturing of the single colonies on nutrient agar by proper streaking method (OIE, 2006). The inoculation loop used for streaking was flamed before and after streaking for its proper sterilization. The culture plate of pure bacterial colony was labeled on the agar side with (i) type of specimen, (ii) date of inoculation, and (iii) reference number. A water proof marker pen was used for this purpose. These pure cultures were stored at -20^0C for further investigations/tests.

3.2.2 Identification of bacteria

Isolated pure cultures of bacteria were subjected to a number of important biochemical tests (primary and secondary) for identification as reported by Krieg and Holt (1984) and OIE (2006).

A. Primary tests

The primary tests were carried out for the identification of pathogenic bacteria up to generic level. These included:-

ii) **Gram reaction**

Gram-stain smear was prepared from a pure culture for recording the gram reaction as gram - positive or gram - negative, and also the cellular morphology of the bacterium (coccus, rod or bacillus). The Gram staining was done following the method of Hans Christian Gram (1884).

Reagents

Crystal violet, gram's iodine solution, acetone/ethanol, safranine and distilled water.

Procedure

1. Preparation of bacterial slide smear

a) A drop of water was taken on the slide and a minute amount of a bacterial colony was transferred aseptically on to it with the help of inoculation loop.

b) The culture was spread with this loop to an even thin film over a circle of 1.5 cm in diameter.

c) The culture was air dried and heat fixed over a gentle flame.

2. Staining procedure

a) The heat fixed smear was flooded with crystal violet (the primary stain) for 1 min. Then the stain was poured off and the excess stain was gently rinsed with a stream of water.

b) Then, iodine was added for 1 min which acted as a mordant resulting into

formation of a crystal violet-iodine complex. Iodine fixed the primary dye to the bacterial cells. The iodine solution was poured off with running water.

c) Then, alcohol was added for about 30 seconds which decolorized the cells, and the solution was trickled down the slide. The slide was again rinsed off with tap water.

d) Subsequently, safranine was added to the slide for 10-30 sec and the solution was washed off with water. The slide was blotted with filter paper to remove the excess water. After staining the bacterial slides, a drop of immersion oil was added on the slide and the latter was examined under microscope.

If the microorganisms showed pink colour, these were classified as Gram negative and if purple colour, then as Gram-positive.

ii) **Aerobic/ anaerobic/ facultative nature**

To check whether the bacterium was aerobic, anaerobic, or facultative in nature, the pure culture was grown in nutrient broth, two test tubes with screw cap were taken. One test tube was half filled and another was fully filled with nutrient broth, and bacterium was inoculated in each tube. The growth was observed after 24 h and the results were interpreted as:

a) If there was turbidity in half filled tube it was due to the aerobic nature of the bacterium,

b) If there was turbidity in fully filled tube, it was due to the anaerobic nature of the bacterium, and

c) If there was turbidity in both half and fully filled tubes, it was due to the facultative nature of the bacterium**.**

iii) **Motility test:**

This test detected the motile and non-motile nature of the bacterium as motility of the bacterium in question proves an identification character (Figure 4). If bacterium does not spread in the medium, it is non motile (A), if spreads in the medium in motile (B and C).

iv) **Catalase test**

This test detected the catalase enzyme reaction that converted hydrogen peroxide into water and gaseous oxygen. The bacterial cells were placed on a clean microscope slide and a drop of three per cent hydrogen peroxide was added. An effervescence of oxygen gas, within a few seconds, indicated a positive reaction (Figure 5).

v) **Oxidase test**

This test showed the presence of cytochrome - C oxidase in a bacterial cell. Equal volume of 1 per cent α - napthol in 95 per cent ethanol and 1 per cent aqueous solution of p-aminodimethylaniline oxalate were mixed. A drop of mixture was placed on the surface of few colonies of bacterium on an agar plate. The blue colour developed by bacterial colonies

within 10-30 seconds was interpreted as a positive reaction (Figure 6).

B. Secondary tests

Once the bacterium was identified up to a generic level, further series of tests were carried out by the method of OIE (2006) to tentatively identify the particular bacteria up to species level. The different secondary biochemical tests performed were**:**

i) **Nutrient agar**

This culture medium was used for less fastidious microorganisms as well as for permanent culture. The pure culture of bacterium was inoculated into nutrient agar and incubated the plates at 35^0C for 24 h. The growth of colony indicated a positive test.

ii) **Arginine hydrolysis**

A pure culture of bacterial colony was inoculated into the bottom of a tube containing, arginine test medium and incubated at 30 $\pm 1^0$C, and examined daily for a maximum of 5 days. Then few drops of phenol red was added, the change in colour of the phenol red indicator from red to another was interpreted as a positive reaction (Figure 7).

iii) **Nitrate reduction test**

Many facultative bacteria use inorganic nitrate as final electron acceptor in the absence of oxygen thus convert nitrate to nitrite. The reaction is catalyzed by the enzyme, nitrate - reductase. The bacterial culture were inoculated in to nitrate broth in a tube and incubated at 35^0C for 48 h. After this, 5 drops of Nitrate reagent-A (di-methyl-α-naphthylamine) and 5 drops of nitrate reagent-B (Sulphanilic acid) were added to the tube inoculated with bacterial culture. The appearance of red colour within 30 seconds was an indication of positive test (Figure 8).

iv) **Malonate utilization**

Many bacteria utilize the malonate (0.3% Sodium malonate) as sole source of carbon. A pure culture of bacterial colony was inoculated into the bottom of a tube containing malonate test medium. The bacterial culture tubes were incubated at 35^0C for 24-48 h. Bromothymol blue was used as an indicator which was green at neutral pH. The change in colour of the bromothymol blue from green to deep blue was interpreted as a positive reaction and no change in colour indicated the negative test (Figure 9).

v) **Urea broth**

A pure culture of bacterial colony was inoculated into the bottom of a tube containing urea broth which was yellow- orange in colour. The bacterial culture tubes were incubated at 32^0C for 8 h. The broth turned into bright pink colour after 8 h. This was interpreted as a positive reaction and no change of broth in colour indicated the negative test (Figure 10).

vi) **Vogus-Proskauer (VP) Test**

In Voges-Proskauer test, the same methyl red/Vogus-Proskauer broth was used. The pure bacterial culture was inoculated into this broth. The bacterial culture tubes were

Figure 4 Motility test

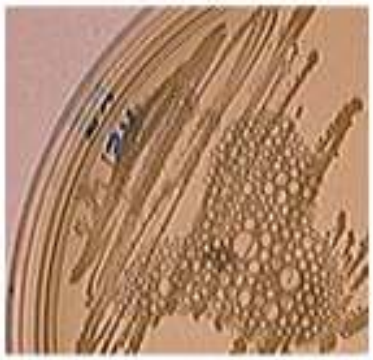

Figure 5 Catalase test

Figure 6 Oxidase test

Figure 7 Arginine hydrolysis test

Figure 8 Nitrate reduction test

Figure 9 Malonte utilization test

incubated at 31^0C for 48 h. The chief end products of glucose metabolism was acetone and 2, 3-butanediol. After 48 h of incubation, a drop of Barritt's Reagent-A (α-napthol) and Barritt's Reagent-B (Potassium hydroxide) were added to the sample and the tubes were gently shaken for aeration. The formation of red colour indicated a positive reaction. No change in colour or a copper colour indicated a negative result.

vii) **Sucrose broth**

This test was done for checking whether the bacterium was able to ferment sucrose. The pure bacterial culture was inoculated into the sucrose broth. The culture tubes were incubated at 35^0C for 24h aerobically. The turbidity in the cultured tubes indicated a positive reaction, and no turbidity a negative reaction.

viii) **Glucose broth**

This test was done for anaerobic fermentation of the bacteria. A pure culture of bacterial colony was inoculated into the bottom of a tube containing glucose broth. The culture tubes were incubated at 35^0C for 24h anaerobically. The turbidity in the cultured tubes indicated a positive reaction, and no turbidity a negative reaction.

ix) **Methyl red (MR) test**

This test was used to identify bacteria that produce stable acid end products by means of mixed acid fermentation of glucose. The combination medium used for the test was methyl red/Vogus-Proskauer broth or glucose broth. A pure culture of bacterial colony was inoculated into the bottom of a tube containing broth. The culture tubes were incubated at 35-37^0C for 48h. The pH indicator, methyl red was added in the cultured medium after 48 h. A red colour indicated a positive test, a yellow colour a negative test and an orange colour indicated that the culture required further incubation.

x) **Indole production**

Indole, a Nitrogen- containing compound, was formed by the degradation of an amino acid, tryptophan, by certain bacteria. This test was performed by inoculating the bacterium into tryptone by inoculating broth and detected indole by the addition of dimethylaminobenzaldehyde (Kovac's reagent) after 24-48 h of culture at 32^0C. After 48 h of incubation, Kovac's reagent was added into the reagent and allowed to stand for sometime. Kovac's reagent arose to the top and the presence of indole was indicated by a cherry or deep red colour in the reagent layer (Figure 11).

xi) **Fermentation of carbohydrates**

Many bacteria ferment monosaccharides (glucose, fructose, ribose and xylose), disaccharides (sucrose, maltose and lactose), polysaccharides (starch, inuline and cellulose), alcohols (adonitol, mannitol and sorbitol) and amino acids (arginine) and produced organic acids, hydrogen and carbon dioxide gas. A fermentation tube containing fermentation medium and desired carbohydrate was used to detect fermentation of carbohydrates. The phenol red

indicator was red at neutral pH and changed to yellow colour in the presence of acids. The gas produced was trapped in inverted Durham's tube. The bacterial cultures were inoculated into fermentation broths (glucose, lactose and sucrose) and Incubated at 35^0C for 24 - 48 h. The change in colour from red to yellow indicated that the organism was capable of metabolizing the sugar in the tube with production of acid. Some bacteria produced gas bubbles in addition to acid. The fermentation test was negative if:

(a) There was no change in colour indicating that the sugar was not utilized by the organism; or

(b) There was pinkish-red colour (pinkish-red) indicating an alkaline or basic metabolic product has been produced which was due to the utilization of the peptone rather than the sugar (Figure 12).

xii) **Hydrogen sulphide (H_2S) production**

Degradation of sulphur containing amino acids frequently resulted in the liberation of H_2S gas. The bacterial cultures were grown on media containing lead or bismuth or iron salts. The bacterial culture was incubated at 37^0C for 48 h. The colour change from brawn to black indicated the production of H_2S and was interpreted as a positive reaction.

xiii) **Lactose broth**

The pure bacterial culture was inoculated into lactose broth and tubes were incubated at 35^0C for 24-48 h. The bacterial culture showed turbidity in tubes indicating a positive reaction.

xiv) **MacConkey agar**

MacConkey agar is widely-used as selective and differential culture medium for gram negative bacteria which inhibit the growth of gram positive bacteria. In addition to the nutrient agar, base of bile salts and crystal violet also inhibited the growth of gram positive bacteria and made the MacConkey agar selective. The pure culture of bacteria was inoculated into MacConkey agar and incubated the plates at 35^0C for 24 h aerobically. The bacterial culture turned from colourless to pink colour indicating a positive reaction.

xv) **Peptone water**

Peptone water was used for the detection of indole and carbohydrate fermentation. The peptone was prepared from casein peptone, yeast extract and distilled water. The casein peptone and yeast extract provided nitrogen, vitamins and minerals to growing bacteria. Sodium chloride was for maintaining the osmotic balance. The pure culture of bacteria was inoculated into peptone water and incubated the plates at 35 ^{0}C for 24h. The fermentation ability of different carbohydrates was detected by addition of 0.5 per cent carbohydrates (like dextrose, mannose, saccharose etc.) and phenol red (0.02 gm) as indicator. The change in colour from red to yellow showed the indole and carbohydrate fermentation**.**

xvi) **Simmon's citrate utilization**

Simmon's citrate agar medium containing sodium citrate as the sole carbon source and the ammonium ion as the sole nitrogen source was used for bacterial identification. Citrate was utilized by action of the bacteria. The pure culture of bacteria was inoculated into Simmon's citrate medium. The bacterial culture plates were incubated at 35 ^{0}C for 48 h. The bacterial culture showed black precipitate indicating a positive reaction.

xvii) **Starch hydrolysis test**

This test was used to differentiate bacteria based on their ability to hydrolyze starch using the exoenzyme amylase. Starch was hydrolyzed into smaller fragments or individual glucose molecules by extracellular enzymes, amylase and oligo-1, 6- glucosidase. These smaller molecules then entered the bacterial cells. The pure culture of bacteria was inoculated into starch broth and the bacterial culture plates were incubated at 35 ^{0}C for 48 h. Iodine was used as a starch indicator and it turned blue in colour in the presence of starch. The bacterial culture turned from colourless to blue colour indicating a positive reaction.

xviii) **Gelatin hydrolysis**

The pure bacterial culture was inoculated into medium contains gelatin and tubes were incubated at 35^{0}C for 24-48 h, if he bacterial culture showed liquidity in tubes indicating a positive reaction (Figure 13).

xix) **Triple sugar iron agar (TSI)**

This test differentiated the bacteria on the basis of their ability to ferment glucose, lactose and sucrose, and to reduce sulphur to hydrogen sulfide. TSI agar contained peptone, glucose, sucrose, lactose, and thiosulphate. The pure culture of bacteria was inoculated into this medium. Phenol red was used as a pH indicator (yellow at pH <6.8 and red >6.8). The bacterial culture was incubated at 35 ^{0}C for 24-48 h. The growth of bacteria in the form of turbidity on that medium indicated a positive reaction (Figure 14).

xx) **Trypticase Soya agar**

This enriched medium contained 1.5% trypticase peptone, 0.5% phytone peptones, 0.5% NaCl and 1.5% agar. The pure culture of bacteria was inoculated into this medium. The culture plates were incubated at 35 ^{0}C for 24 h. The growth of bacteria in the form of turbidity on the medium was indicated a positive reaction.

Tentative identification of causative bacterium

After performing various secondary tests, results were subjected to a computer software programme PIBWin (Website: *http//www.soton.ac.in.uk/pibwin*) for the tentative identification of bacteria. On the basis of this programme ID scores were allotted to each bacterial isolate. These scores were then matched/ compared with the standard scores of the reference bacterium, and on the basis of similarity of the scores, the bacterial isolates were tentatively identified.

Figure 10 Urea test

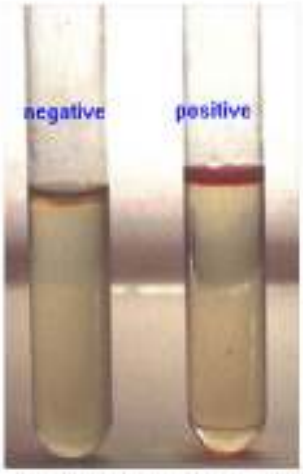

Figure 11 Indole production test

Figure 12 Fermentation of carbohydrates

Figure 13 Gelatin hydrolysis test

Figure 14 Triple sugar iron agar test

C. Tertiary test for the confirmation of bacteria

The pure cultures of bacteria were prepared as described under point 3.2.1. The confirmation test of these bacteria was done with the help of selective media used for culturing that particular bacterium. The following media were used when tentative identification of the pathogenic bacteria became known.

i). **Hugh Leifson glucose medium**

This medium was used for the isolation and identification of *Micrococcus* sp. on the basis of anaerobic fermentation of glucose. The culture plates were incubated at 35 ^{0}C for 24 h. The yellow colour pigment in the developed colony shown by the bacterial culture on this medium confirmed the presence of *Micrococcus* sp. (Hugh and Leifson, 1953).

ii). **Rimler-Shott medium base (RS Medium Base)**

This medium was used for selective isolation, cultivation and presumptive identification of *A. hydrophila*. The pure bacterial culture was streaked on the medium. The culture plates were incubated at 35 ^{0}C for 24 h. The green colour pigment in the developed colony showed by the bacterial culture on this medium confirmed the presence of *A. hydrophila* (Claus Jeppesen, 2000).

iii). **Eosin- methylene blue (EMB) agar**

This agar is a selective and differential medium used for isolation and differentiation among members of the Enterobacteriaceae. EMB agar contained methylene blue and eosin dyes to inhibit the growth of gram positive bacteria (Yavankar *et al*., 2007). The pure bacterial culture was streaked on the medium. The culture plates were incubated at 35 ^{0}C for 24 h. The blue colour colony shown by the bacterial culture on this medium confirmed the presence of *Klebsiella oxytoca***.**

iv). **Blood agar base**

This agar is a selective and differential medium was used for differentiation of *Streptococcus faecalis* this was the confirmative test for *S. faecalis* (Snyder and Lichstein, 1940). The culture plates were incubated at 35 ^{0}C for 24 h. The red colour colony showed by the bacterial culture on that medium confirmed the presence of *S. faecalis*.

v). **Vogel-Johnson agar base W/O Tellurite (VJ Agar)**

This agar base medium was used for selective isolation of coagulase positive mannitol fermenting *Staphylococcus aureus* from heavily contaminated foods and clinical specimens.

vi). **Mannitol salt agar**

This medium is used for selective isolation of coagulase positive and mannitol fermenting *Staphylococcus*. Formation of yellow colour colony indicated that mannitol fermentation has been carried out by pathogenic bacteria. The bacterial plates were incubated at 30 ±1 ^{0}C for 24 h. The bacterial culture had metabolized mannitol was indicated by a colour change on the mannitol salt agar plate.

3.2.3. Identification of pathogenic fungus

a) **Culture of fungus**

From the samples collected during survey of catfish farms at CCSHAU, eggs (fertilized and unfertilized) became dump and almost all hatchlings died (almost 100 per cent mortality).

The dump eggs and died hatchling picked up from the water aseptically with the help of a sterile brush and homogenized in a homogenizer. The homogenized mixture was spread over the Czepacks medium in plates (Table 3), each containing about 35 ml of this medium mixed with broad spectrum streptomycin (75µg/ml) under aseptic conditions. The plates were sealed, incubated in B.O.D. at 28 ± 2^0C for 4-7 days and examined daily. Emerging hypha tips were repeatedly transferred on to fresh plates of Czepacks medium (Table 2) until cultures were free from contamination. The brush was dipped before and after in 100 per cent alcohol for sterilization. The culture plate was labelled, a) on the medium side, b) with the type specimen, c) date of inoculation, and d) reference number. A water proof marker pen was used.

Table 2: Composition of Czepacks medium (Czapek. 1902-1903**)**

Ingredients*	Quantity
Sodium	2.0 gm
Potassium	1.0 gm
Phosphorous	0.5 gm
Ferrous	0.1gm
Magnesium	0.5 gm
Sucrose	30 gm
Agar	20 gm
Distilled water	1.0 litre

*All the contents of medium were put into the boiled water through proper shaking of the flask. After that, the medium was autoclaved. At the time of pouring and plating at 60-62 ^{0}C, antibiotic Streptomycin was added @.75g/100ml medium to inhibit the growth of the bacteria.

b) **Procedure for the characterization of fungus (0IE, 2003)**

i). A drop of Lacto-phenol was taken on the slide and fungal hyphae from pure culture were transferred aseptically on to it with the help of inoculation loop.

ii). The culture was spread with an inoculation loop to an even thin film on the slide.

iii). The fungal hyphae were stained with cotton blue, and a permanent slide was

prepared followed by observing the slide under a microscope**.**

Identification and characterization of fungus was got done by the pathology laboratory of the Department of Plant Pathology, College of Agriculture, CCS HAU, Hisar (Table). One fungus sample (No. EF-5) was identified and confirmed by the Indian Type Culture Collection (ITCC), Identification/Culture Supply Services, Division of Plant Pathology, Indian Agricultural Research Institute at New Dehli-110012.

3.3 Pathogenicity test for causative organisms

3.3.1 *In vitro* pathogenicity test

In vitro pathogenicity test of different bacteria was done by streaking pure culture of isolated bacteria on blood agar plate. The plates were incubated in B.O.D. at 30 ± 1^0C for 24 hours. The pathogenicity of bacteria was confirmed by determining α and β-zone of growing bacteria on the plates (Ryan and Ray, 2004).

3.2.2 *In vivo* pathogenicity tests

The healthy individuals of Magur catfish of average weight 12-25g were brought from the catfish farms of this study to the laboratory and were acclimated at 25^0C for one week in flat bottomed cuboidal 30 glass tubs with 50 litres water capacity. The tubs were filled with dechlorinated tap water which was removed on alternate days and was also properly aerated. The catfish were fed a normal recommended prepared diet (Table 3). Only the healthy fishes showing normal activities were selected for further experimentation. *In vivo* pathogenicity test was carried out following Keskin *et al.* (2004).

Preparation of fish feed

Dietary ingredients were cleaned, milled and mixed in given proportion (Table 3). Thereafter, thick dough was made using lukewarm water and 0.5 mm thick pellets were obtained with a mechanical pellatizer. The pellets were dried at $60\text{-}62^0$C temperature in oven, before using as feed. The feed was given to the catfishes @ 2 % body weight, out of which 40 % was given in the morning and 60 % in the evening (Figure 28, 29).

Table 3: Composition of the normal recommended diet

Components	Amount in grams
Soya Flour	570
Ground nut oil cake (GNOC)	1200
Rice Bran	110
Wheat Flour	100
MPA	20

Hydrobiological parameters

Water parameters were regulated during the experiment and maintained in the optimal values under laboratory conditions (Table 4).

Table 4: Average hydrobiological parameters during the *in vivo* challenge experiments.

Average hydrobiological parameters					
Months	DO (mg/L)	Temperature (^{0}C)	pH	CO_2 (mg/L)	Salinity (ppt)
October, 2008	6.4	21.05	7.6	8.7	0.25
November, 2008	6.4	20.8	7.3	8.6	0.28
December, 2008	6.3	19.6	7.4	8.8	0.27
January, 2009	6.3	19.4	7.4	8.6	0.26

(i) Experimental design

Ten catfishes were kept in 30 litre flat bottom cuboidal tubs filled with a well aerated and dechlorinated tap water. Fresh tap water was also stored in big rectangular tanks for 24 hours, and was well aerated in order to dechlorinate it. Each tub was cleaned on alternate days by siphoning catfish's fecal matter and food remains, and 70 per cent of its water was then refilled to ensure clean water in the tubs.

In this experiment, pure culture of the isolated and identified bacteria was inoculated @ 200 µl of bacterial suspension into the intraperitoneal cavity of magur catfish each weighing 25g with known viable counts (Table 4). The control fish were inoculated only 200 µl of sterilized physiological buffer saline. In each treatment only one bacterial isolate was injected. The fungi were not tested for *in vivo* pathogenicity due to lack of specific media and other facility.

Table 5: Viable counts of different pathogenic bacteria administrated to the experimental magur fish.

Bacterial Isolates	Mean
A. hydrophila	1.45×10^{11}
Kl. oxytoca	4.95×10^{11}
Staphylococcus aureus	2.96×10^{12}
S. haemolyticus	4.6×10^{9}
S. faecalis	7.1×10^{9}
Micrococcus sp.	1.64×10^{10}

The symptoms of disease appearance were examined and incubation period of different bacteria in fishes and longevity of the catfish, inoculated/ diseased were recorded.

To re-confirm/ cross-check that the disease appearance was due to the inoculated bacterium, the infected tissues of these experimental fish were re-subjected to isolation and

characterization tests as described under point 3.2. The experiment was continued up to eight weeks and data were recorded to find out the pathogenicity of isolated and identified bacteria.

(ii) Procedure followed for preparation of doses of viable counts

One fish from each treatment was sacrificed; the bacterial flora was isolated and identified from each treatment and replication. The viable, counts of the bacterial pathogens were worked out at weekly interval.

The number of bacteria in a given sample is usually too great to be counted directly. Therefore, the bacterial samples were serially diluted and then plated out on an agar surface in such a manner that single isolated bacteria form visible isolated colonies. Serial dilution involved repeatedly mixing of known amounts of source culture with (sterilized) liquid. When 1 ml was added to 9 ml, it gave a 10-fold dilution; 1 ml added to 99 ml gave a 100-fold dilution. The numbers of colonies on the plate were used as a measure of the number of viable count in that known dilution.

a) The serial steps taken were:

i) Fresh culture of bacteria was taken in the nutrient broth.
ii) Ten tubes and petri dishes were labeled.
iii) Nine ml of phosphate saline buffer (PBS) was taken in each tube.
iv) One ml of fresh pure culture of bacteria from the flask was transferred into the first tube and than mixed well by shaking.
v) One ml of liquid from the first tube was transferred into second tube after proper pippetting and this procedure was followed up to tenth tube.
vi) Then, 1 ml of liquid was transferred from first tube to first plate (already prepared with nutrient agar). The pipette was then discarded.
vii) The edge of tube 1 was flamed. The contents were sealed and mixed gently for further experimentation.
viii) Then 5-10 ml of melted nutrient agar (62^0C) was poured over the bacterial suspension at Sr. no. (6).
ix) The plate was covered and then mixed thoroughly by gentle swirling.
x) The plate was placed on a flat surface undisturbed for about 10 minutes to allow the agar to get settled.
xi) The lid of second tube was flamed and loosens and one ml of liquid to the second plate was transferred with sterile pipette handled aseptically; the pipette was then discarded.
xii) The same process was repeated up to tenth tube and plate.
xiii) The procedure was repeated three times.
xiv) The plates were incubated in B.O.D. at $30 \pm 2\ ^0$C for 24 h.
xv) After incubation, the plates having 30-300 colonies were chosen for counting the

bacterial colonies.

xvi) The bacterial colonies of these plates were then counted, and the average counts of bacterial colonies were taken. Now using a sterile pipette handled aseptically.

Then, the colony forming unit was derived by utilizing following formula:

$N_{cfu} = N_C \times D_f$

Where,

N_{cfu} = number of colony forming units per ml of sample

N_c = number of colonies (33-330 colonies per plate)

D_f = Dilution factor of the plate counted.

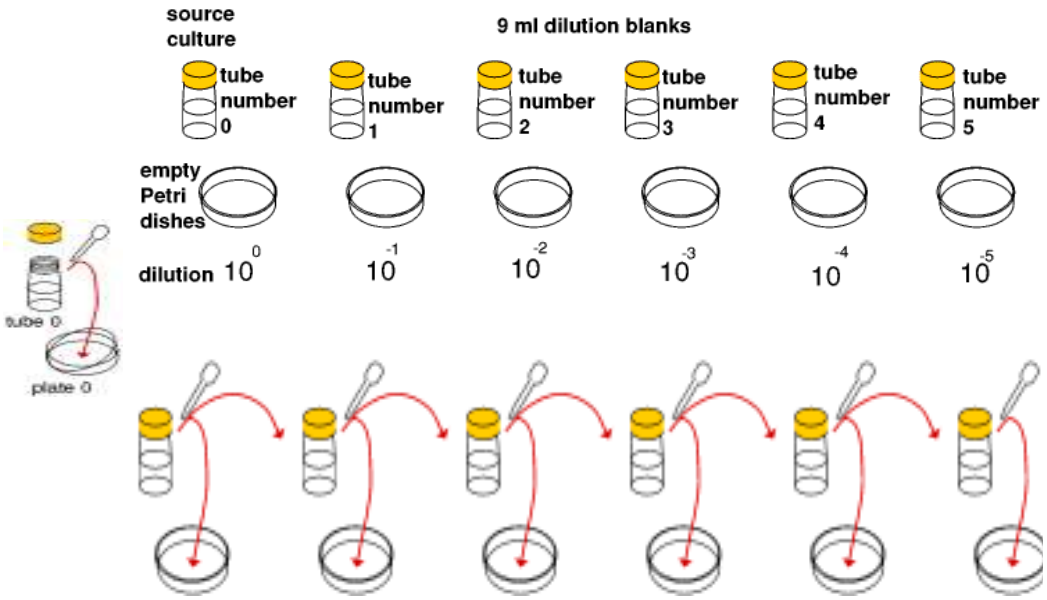

Figure 15: Serial dilution method to determine the bacterial viable counts

b) **Procedure followed for preparations of bacterial injection of known counts of colony forming unit (cfu):**

i) Fresh culture of bacteria was taken in nutrient broth with known cfu.

ii) One ml of bacteria from that culture was poured into ten ml of PBS for washing purpose.

iii) The suspension was centrifuged at 5000 rpm for 30 minutes and pellet was formed.

iv) The supernatant was discarded and the suspension was again dissolved in ten ml of PBS for washing again.

v) Again, the suspension was centrifuged at 5000 rpm for 30 minutes and pellet was formed.

vi) The supernatant was discarded and the pellet was dissolved in 10 ml PBS. The pellet dissolved in PBS was used for inoculation of fish.

iii) Production of Antibiotic Resistant pathogenic bacteria

Antibiotic resistant bacteria were produced for pathogenicity tests by incubating bacteria on antibiotic supplemented nutrient agar plates for about 5-7 days in B.O.D. at 30 ± 2^0C. The bacteria were grown for 4-7 times on increased concentration antibiotics in culture medium and two pathogenic bacteria were made antibiotics resistant at 75mg/ml of the culturing medium. *A. hydrophila* was made Chloroamphinicol resistant while *Micrococcus* sp. by using Oxytetracycline. Both were used for the further experiment.

3.4 Role of Probiotics in controlling the prominent bacterial pathogens.

Three probiotics were obtained and used to control the infections caused by pathogenic bacteria. These probiotics were:

a) **Composition of probiotics 1 (P1)**

Probiotics 1 contained only single bacterium named lactic acid bacteria (*Lactobacillus sporogenes*).

b) **Composition of probiotics 2 (P2)**

Probiotics 2 contained only single fungus - the yeast named *Saccharomyces boulardii*.

c) **Composition of probiotics 3 (P3)**

Probiotics 3 contained a mixture of many bacteria viz. *Nitromonas*, *Rhodococcus*, *Bacilus megaterium*, *Lecheni formis*, *Desulphovibrio sulphuricum*, *Psuedomonas*, *Chromatium*, *Chlorobium*, *Thiobacillus*, *Thioxidants*, *Thiobacilus ferroxidant*, *Methylomonas metyhanica*, *Glucon acetobactor*, *Azospirillum*, *Trichoderma*, *Shizophyllum commune* and *Sclertium gluconicum*. These bacteria are generally present in the soil (for nitrogen fixation), pond bottom and water.

3.4.1 *In vitro* test of probiotics against the pathogenic bacteria.

In vitro test of available probiotics for their antagonistic potential against bacteria (only two pathogenic bacteria) was done by using agar well diffusion method (Gram *et al*., 2004). The basic principle of this technique was to poison the culture medium with pathogen and then allow the test probiotic to grow on such medium.

(a) Inhibition zone of probiotics against bacteria.

To observe measure the zone of inhibition by agar well diffusion method the following procedure was followed step by step:

i) All pathogenic bacterial isolates were added in to the petri plates containing melted nutrient agar medium at the temperature of 60 to 62 ^{0}C and then solidified by placing in the laminar flow with ultraviolet light remained off for 15-20 minutes.

ii) Three well were bored in the nutrient agar plates having pathogenic bacteria by the well borer. Every time the borer pipe was sterilized on the flame.

iii) Then the melted water agar (5-10 µl) was added at the bottom of the each well. The water agar was added to prevent the seepage of the probiotic bacteria to the bottom of the petri plates.

iv) Then the probiotics (50 µl) were added to each well.

v) Plates were incubated in B.O.D. at temperature 35 to 37 ^{0}C for 18-24 h.

vi) The Zone of Inhibition (in millimeters) was measured by using simple scale and recorded.

3.4.2 *In vivo* tests of probiotics

Experimental design was the same as described under point 3.2.2. Antibiotic resistant bacterial isolates of *A. hydrophila* (Chloramphenicol resistant) and *Micrococcus* sp. (Oxytetracycline resistant) were taken as pathogenic organisms for inoculation in catfishes.

The following treatments were given to the magur catfishes.

i) **Control:** In this treatment, 200µl of physiological buffer saline (PBS) was given into the intraperitoneal cavity of each acclimated catfish.

ii) **Control + bacterium 1 (*A. hydrophila*):** Here, 200 µl bacterial suspension in PBS with 5 x 10^{11} cfu per ml of bacteria was inoculated into intraperitoneal cavity of each catfish.

iii) **Control + bacterium 2 (*Micrococcus* sp.):** Here, 200 µl bacterial suspension in PBS with 5 x 10^{11} cfu per ml of bacteria was inoculated into intraperitoneal cavity of each catfishes.

iv) **Control + probiotic 1 (*Lactobacillus sporogenes*):** Here, 0.1 gm probiotic l dissolved in PBS, with 5 x 10^{11} cfu per ml of bacteria was injected into the intraperitoneal cavity of each catfish.

v) **Control + probiotic 2 (*Saccharomyces boulardii*):** Here, 0.1 gm probiotic 2 dissolved in PBS, with 5 x 10^{11} cfu per ml of bacteria was injected into the intraperitoneal cavity of each catfish.

vi) **Control + probiotic 3 (Mixture of many bacteria):** Here, 0.1 gm probiotic 3 dissolved in PBS, with 5 x 10^{11} cfu per ml of bacteria was injected into the intraperitoneal cavity of each catfish.

vii) **Control + bacteria 1 + probiotic 1:** Here, 0.1 gm probiotic l dissolved in PBS; along with 250 µl of bacterial suspension with 5 x 10^{11} cfu per ml of bacteria was injected into the intraperitoneal cavity of each catfish.

viii) **Control + bacteria 1 + probiotic 2:** Here, 0.1 gm probiotic 2 dissolved in PBS; along with 250 µl of bacterial suspension with 5 x 10^{11} cfu per ml of bacteria was injected into the intraperitoneal cavity of each catfish.

ix) **Control + bacteria 1 + probiotic 3**: Here, 0.1 gm probiotic 3 dissolved in PBS; along with 250 µl of bacterial suspension with 5 x 10^{11} cfu per ml of bacteria was

injected into the intraperitoneal cavity of each catfish.

x) **Control + bacteria 2 + probiotic 1:** Here, 0.1 gm probiotic l dissolved in PBS; along with 250 µl of bacterial suspension with 5 x 10^{11} cfu per ml of bacteria was injected into the intraperitoneal cavity of each catfish.

xi) **Control + bacteria 2 + probiotic 2:** Here, 0.1 gm probiotic 2 dissolved in PBS; along with 250 µl of bacterial suspension with 5 x 10^{11} cfu per ml of bacteria was injected into the intraperitoneal cavity of each catfish**.**

xii) **Control + bacteria 2 + probiotic 3:** Here, 0.1 gm probiotic 3 dissolved in PBS; along with 250 µl of bacterial suspension with 5 x 10^{11} cfu per ml of bacteria was injected into the intraperitoneal cavity of each catfish.

One catfish from each treatment was sacrificed at weekly interval, the bacterial flora from each treatment and replication was isolated and identified by following method as described under point 3.2. The viable counts of the bacterial pathogens were worked out as described under point 3.4.2.ii.

The following parameters were recorded from the treated fish:

3.6 Hematological, growth and survivality parameter studies

Different blood parameters *viz*. level of hemoglobin (Hb), Total Erythrocyte Count (TEC), Total Leucocytes Count (TLC), Hemotocrit /Packed Cell Volume (PCV) were determined with help of a haemocytometer and calculated from the equations given by Anderson and Klontz (1965).

3.6.1 Collection of blood from the magur (*C. batrachus*) under different treatments.

Blood samples of treated fish were taken at weekly interval after initiation of treatments. Sampling was also done at the same time from the control group. Blood was drawn from the caudal peduncle region using a sterile syringe of 2 ml rinsed with 2.7% Ethylene dimethyl tetra amine (EDTA) solution. Blood was collected in small glass vials after drying the vials in hot air oven.

a) Hemoglobin estimation in the blood of magur (*C. batrachus*) under different treatments.

The hemoglobin contents of blood were analyzed following the Cyanmethemoglobin methods using Darbkin's Fluid. Twenty micro litre of blood was mixed with 5 ml Darbkin's working solution. The absorbance was measured using a spectrophotometer at wavelength of 540 nm. Hemoglobin contents were expressed as g/dl.

b) Total erythrocyte count estimation in the blood of magur (*C. batrachus*) under different treatments.

The blood was drawn from the caudal vein and EDTA was used as an anticoagulant to prevent the blood cells from lysis and clotting. The blood was diluted to 1:200, with RBC counting pipette. The mixture was shaken well to suspend the cells uniformly in the solution.

Then the cells were counted using a haemocytometer as follows (Anderson and Klontz, 1965):

Number of RBC/mm^3 = N x 10000

where,

N= total number of red blood cells counted in 5 squares of the haemocytometer slide and 10,000 is the dilution factor.

c) Total leucocyte count estimation in the blood of magur (*C. batrachus*) under different treatments.

The blood was drawn from the caudal vein and EDTA was used as an anticoagulant. Blood was diluted 1:20 with WBC diluting fluids using WBC counting pipette. The mixture was shaken well to suspend the cells uniformly in the solution. Then the cells were counted using a haemocytometer as follows:

Number of WBC/mm^3 = N x 50

where,

N = total number of white blood cells counted in 4 squares of the haematocytometer slide and 50 is dilution factor.

d) Hemotocrit (Packed Cell Volume) estimation in the blood of magur (*C. batrachus*) under different treatments

This method was used to determine the volume of packed cells volume in the blood. A heparinised capillary tube was filled with blood up to mark 100 and sealed with plasticize sealant. The capillary tube was centrifuged for five minutes at 12,000 rpm for 30 minutes in a microhaematocrit centrifuge. The reading of packed cells volume in percent was recorded i.e. mass of erythrocytes settled down in tube.

3.6.2 Effect of probiotics on the viable counts of pathogenic bacteria in the treated catfishes

Viable counts of the two antibiotic resistant pathogenic bacteria (*Micrococcus* sp. and *A. hydrophila*) from each catfish treatments (bacteria + probiotics) for every week were evaluated as suggested in 3.2.2.ii.a section.

3.7 Survival rate of inoculated catfish

Survival rate of inoculated fish was measured in terms of differences between total number of catfishes and mortality of catfishes. Mortality of catfishes was observed routinely.

S= {(N-M)/N} x 100

where,

N = Total number of catfishes

M = Mortality of catfishes

3.8 Effect of probiotics on growth performance in probiotics treated fishes.

i) **Effects of probiotics on gain in catfish length**

Per cent increase in length of fish were observed with the help of initial length and final length

$L_g = L_2 – L_1$ and,

$\% L_g = [\{L_2 – L_1\}/L_1] x100$

where,

L_1 = Initial length

L_2 = Final length

L_g = Length gain (cm)

ii) **Effects of probiotics on gain in catfish weight**

Gain in weight was determined in terms of difference between final weight and initial weight.

Weight gain = $W_g = W_2 - W_1$

Growth percent in body weight was measured by the following formula:

$\%W_g = [\{W_2-W_1\}/W_1] x 100$

where,

W_1 = Initial weight

W_2 = Final weight

W_g = Weight gain

3.9 Effect of probiotics on the intestinal histopathology of Indian magur (*C. batrachus*)

The histology of the intestine was studied under compound microscope to know the effects of probiotics on the intestinal cells by preparing slides of the small intestinal tissue. The slides were prepared by fixing in Bouin's fluid and then material was embedded in paraffin wax and thin sections were cut by a microtome.

Hematoxylin and eosin stain; the most widely used stain in medical diagnosis was used for this purpose. Mayer's hematoxylin was used because it eliminates the necessity for differentiation and bluring of the section. It can be considered a progressive stain which, produces a stained section with clearly defined nuclei while the background is completely colorless (Bancroft and Gamble 2002).

Staining Procedure (Pluske *et al.,* 1997) was done in the following steps:

1. Deparaffinized the slides and hydrated to water.
2. Applied Mayer's hematoxylin for 15 minutes
3. Washed the sections in running tap water for 20 minutes
4. Counter stain with eosin from 15 seconds to 2 minutes depending on the age of the eosin, and the depth of the counter stain desired.
5. Dehydrated in 30%, 50%, 70%, 95% and absolute alcohols, two changes of 2 minutes each or until excess eosin was removed. Checkd every time under microscope.
6. Cleared the sections in xylene, two changes of 2 minutes each were made.

7. Mounted the sections in DPX.
8. Again, the slides were viewed under microscope and photographs were taken.

3.10 Statistical analysis:

The obtained results were analyzed statistically using completely randomized design (CRD) to evaluate differences among different treatments means at 0.05 significant levels following Snedecor and Cochran (1989).

CHAPTER-4

RESULTS

The results of the present investigations are presented under the following heads:-

4.1 Survey of fish farms and characterization of the fish diseases

The results of survey on incidence of catfish disease are presented in table 6 and 7.

In the fish farm studied for this study, the catfishes like Indian magur (*Clarius batrachus*), African magur (*C. garipienus*), stinging catfish (*Heteropnuestus fossilis*), freshwater shark (*Wallago attu*) and pangas (*Pangasius pangasius*) were used for fish culture. Among these, only Indian magur catfish (*C. batrachus*) was found to be infected with diseases; all other catfishes were free from any disease. Therefore, results of this study concentrate only on Indian magur. The diseases in this catfish appeared in rainy (July-August) and post-rainy (August and September); and in winter (January - February) seasons (Table 6).

Table 6. Incidence of diseases in Indian magur (*C. batrachus*) in different fish farms around Hisar.

Survey Month	Fish diseases in the fish farm		
	Muklan	Nagalpur	CCSHAU
March, April, 2007	+	+	+
May, June, July, 2007	-	-	-
August, September, 2007	+	+	+
October, November, December, 2007	-	-	-
January, February, 2008	-	-	-
March, April, 2008	+	+	+
May, June, July, 2008	-	-	-
August, September, 2008	+	+	+
October, November, December,	-	-	-
January, February, 2009	-	-	-
March, April, 2009	+	+	+
May, June, 2009	-	-	-

+: Presence of disease; the diseases were found only in Indian magur; in remaining fishes the diseases were absent.

-: Absence of diseases in all fishes.

When the disease characters of these catfishes were compared with standard keys, it was revealed that the disease identified were hemorrhagic septicemia with fin and tail rot ulcerative disease, having symptoms *viz*. hemorrhages particularly on snout, head and lateral side of the body, skin erosions developing ulcers, rottening of gill lamella with large amount of mucus, bending of the body and inflammations at Muklan fish pond, Hisar (Figure 16) and

Figure 16 Diseased catfish from Muklan fish farm (Hisar)

Figure 17 Diseased catfish from Nagalpur fish farm (Jind)

Figure 18 Diseased catfish from Muklan fish farm (Hisar)

Nagalpur fish pond, Jind (Figure 17). Saprolegniasis having symptoms of dumping of fertilized and unfertilized eggs as well as death hatchlings and fishes (Figure 18) were present at fish pond at CCSHAU, Hisar (Table 7). The range of level of infestation was variable at the three fish farms during the survey. It was 7.52 to 32.44 per cent in March, April and August, September (post rainy season) at Muklan catfish farm (Hisar); 18.35 to 23.78 per cent in at Nagalpur catfish (Narwana) (Table 8); 20.17 to 36.72 per cent in rainy season at CCSHAU, farm (Hisar) (Table 8). Over all, if the catfish diseases are not controlled, then from 20 to 35 per cent of the catfishes were at a risk of mortality due to disease, especially the hemorrhagic septicemia, fin and tail rot and ulcerative disease and; about 20-36 per cent eggs and hatchings of catfishes are at risk of dumping of eggs as well as mortality. Infact, this is a great loss from the catfish farmer's point of view. The hydrobiological parameters of different fish farms were kept in optimum ranges during the period of survey (Table 1).

Table 7. Symptoms of diseases identified in the Indian Magur catfishes (*C. batrachus*) sampled from three catfish farms/ponds around Hisar.

Catfish farm	Symptoms of catfish disease	Disease identified
Muklan Catfish pond, Hisar	a Hemorrhages on body surface b Skin erosions c Inflammations d Irregular swimming e Fin and tail rot	Hemorrhagic Septicemia, Fin and tail rot
Nagalpur Catfish pond, Jind	a Hemorrhages b Skin erosions c Inflammations d Developing ulcers	Hemorrhagic Septicemia, Ulcerative disease
CCSHAU, freshwater catfish pond	a Hatchling had fungal hyphae b Dumping of eggs	Saprolegniasis

Table 8. Proportions of diseased catfishes in different catfish farms around Hisar.

Survey Month	Proportion of disease fishes (%)*		
	Muklan	Nagalpur	CCSHAU
March, April, 2007	7.52	20.86	20.17
May, June, July, 2007	-	-	-
August, September, 2007	19.48	18.35	25.70
October, November, December, 2007 January, February, 2008	-	-	-
March, April, 2008	11.50	23.78	32.14
May, June, July, 2008	-	-	-
August, September, 2008	28.16	21.85	36.72
October, November, December,	-	-	-
January, February, 2009	-	-	-
March, April, 2009	32.44	19.25	28.30
May, June, 2009	-	-	-

*After the appearance of disease, the farmers treated the ponds with control package as per recommended dose.

4.2.1 Isolation and characterization of pathogens associated with Indian magur (*C. batrachus*).

The results of different tests are presented in Table 9. These are as follows:

Isolate no. 1(a)

The results of primary tests of this isolate revealed that this bacterium was gram positive, aerobic cocci, catalase positive, oxidase negative and had white colour of colony. The results of secondary tests revealed that the bacterium in this isolate was positive for sucrose, galactose, maltose, galactose, sorbitol, starch, glycerol, Simmon's citrate, nitrate reduction, and arginine dehydrogenase. However, the bacterium was negative for mannitol, sodium malonate, inositol, fructose, lactose, motility test, indole, adonitol, urea and xylose. The bacterium present in to the isolate ferment glucose from Triple Sugar Iron (TSI) medium as well as alone and it did not produce H_2S gas from TSI.

Based on these tests, identification score assigned to this isolate by the PIBWin Programme was 0.99985. On the basis of this identification score, the bacterium species identified in isolate 1(a) was *Streptococcus haemolyticus.*

The growth of this isolate on *S. haemolyticus* specific Mannitol Salt Agar base (Table 12) confirmed that "isolate 1(a)" represented *S. haemolyticus* (Figure 19).

Isolate no. 1(b)

The results of primary tests of this isolate revealed that this bacterium was gram negative, aerobic rod, catalase positive, oxidase negative and had white colour of colony. The results of secondary tests revealed that the bacterium in this isolate was positive for sucrose, mannitol, galactose, inositol, adonitol, maltose, fructose, lactose, sorbitol, indole, starch, D (+) xylose, Erhlich indole, urea, and glycerol. However, the bacterium was negative for arginine dehydrogenase, Simmons citrate, nitrate reduction, motility test, H/L (oxidative and alkaline). The bacterium had showed good growth in methyl red and did not grow on Vogus-Proskauer broth at 37 ^{0}C temperatures.

Based on these tests, identification score assigned to this isolate by the PIBWin Programme was 0.999636. On the basis of this identification score, the bacterium species identified in isolate 1(b) was *Klebseilla oxytoca*.

The growth of this isolate on Eosin methylene blue medium/agar base (Table 12) confirmed that "isolate 1(b)" represented *Kl. Oxytoca* (Figure 20).

Isolate no. 1(c)

The results of primary tests of this isolate revealed that this bacterium was gram positive rod, catalase, and oxidase - positive and had yellow colour of colony. The results of

secondary tests revealed that the bacterium in this isolate was positive for sucrose, mannitol, galactose, inositol, maltose, fructose, lactose, sorbitol, starch, glycerol and arginine dehydrogenase. However, the bacterium was negative for Simmons citrate, nitrate reduction, motility test, indole, adonitol, urea, and xylose.

Based on these tests, identification score assigned to this isolate by the PIBWin Programme was 0.99148. On the basis of this identification score, the bacterium species identified in isolate 1(c) was *Streptococcus faecalis*.

The growth of this isolate on *Streptococcus faecalis* specific Vogel-Johnson agar base (Table 12) confirmed that "isolate 1(c)" represented *Streptococcus faecalis* (Figure 21).

Table 9. Physical characteristics and biochemical response of different bacterial isolates taken from diseased Indian magur (*C. batrachus*) from Muklan fish farm (Hisar).

Biochemical tests	**Bacterial isolates**		
	1(a)	1(b)	1(c)
Gram reaction	+	-	+
Shape	Cocci	Rods	Rods
Colour of colony	White	White	White
Aerobic	Facultative	+	+
Anaerobic	-	-	-
Catalase	+	+	+
Oxidase	-	-	+
Glucose Fermentation	+	+	+
Urease	-	+	-
Simmons citrate	+	+	-
Starch hydrolysis	-	+	-
Ehrlich indole	-	+	+
Triple Sugar Iron	Ferment Glucose only	Ferment Glucose, Lactose or Sucrose	
H_2S Production, Gelatin liquefication, D(+) Xylose, H/L (oxidative & alkaline), Vogues Proskauer 37^0C	-	-	-
Nitrate-Nitrite	+	+	+
Adonitol	-	+	-
Fructose	-	+	+
OF aerobic	-	+	+
Sodium Malonate	-	+	+
Arginine dihydrolase	+	-	-
Lactose	-	+	+
Mannitol	-	+	+
Galactose, Glycerol, Maltose, Sucrose, Sorbitol, Starch	+	+	+
Inositol	-	+	+
H/L (fermentive)	-	+	+
Methyl red 37^0C; Growth at 37^0C	+	+	+
ID score	0.99985	0.989636	0.99148
Bacteria identified	*S. haemolyticus*	*Klebseilla oxytoca*	*Streptococcus faecalis*

+ : For good growth; - : for no growth

The results of different tests are presented in Table 10. These are as follows:

Isolate no. 2(a)

The results of primary tests of this isolate revealed that this bacterium was gram positive, aerobic cocci present in groups and positive for catalase, negative for oxidase and had creamish-white colour of the colonies. The results of secondary tests revealed that the bacterium in this isolate was positive for starch, sucrose, sorbitol, glucose, fructose, Ehrlich Indole, Simmon's citrate, Vogus- Proskauer, glycerol, and galactose and arginine dihydrolase. However, the bacterium was negative for maltose, mannitol, adonitol, xylose, urease, lactose, nitrate, and inositol.

The bacterium present in this isolate had showed good growth in Vogus-Proskauer medium but did not grow in methyl red at 37^0C temperature. It did not liquefied gelatin and did not produce H_2S gas from the Triple Sugar Iron medium/agar.

Based on these tests, identification score assigned to this isolate by the PIBWin Programme was 0.99533. On the basis of this identification score, the bacterium species identified in isolate 2(a) was *Staphylococcus aureus*.

The growth of this isolate on *Staphylococcus aureus* specific Blood agar base w/low pH (Table 12) confirmed that "isolate 2(a)" represented *Staphylococcus aureus* (Figure 22).

Isolate no. 2(b)

The results of primary tests of this isolate revealed that this bacterium was gram negative, single short rod/bacillus shaped, aerobic, positive for catalase and oxidase; and had white colour of colony. The results of secondary tests revealed that the bacterium in this isolate was positive for glucose fermentation, nitrate reduction, glucose acid, maltose, mannitol, glycerol, fructose, galactose, arginine dihydrolase, sucrose**,** lactose**,** sorbitol**,** starch, H/L (fermentive) and showed well growth at 37^0C. However, the bacterium was negative for urease, Simmons citrate, H/L (oxidative), H/L (alkaline), inositol, TSI and H_2S production. The bacterium in this isolate had liquefied gelatin and showed good growth in Vogus-Proskauer medium at 37^0C.

Based on these tests, identification score assigned to this isolate by the PIBWin Programme was 0.99954. On the basis of this identification score, the bacterium species identified in isolate 2(b) was *Aeromonas hydrophila***.**

The growth of this isolate on *Aeromonas hydrophila* specific Rilmer-Sholts Medium Base (Table 12) confirmed that "isolate 2(b)" represented *Aeromonas hydrophila* (Figure 23).

Table 10. Physical characteristics and biochemical response of different bacterial isolates taken from diseased Indian magur (*C. batrachus*) from Nagalpur fish farm (Jind).

Biochemical tests	Bacterial isolates	
	2(a)	2(b)
Gram reaction	+	-
Shape	Cocci in groups	Single short rod
Colour of colony	White cream	White
Aerobic	+	+
Anaerobic	-	-
Catalase	+	+
Oxidase	-	+
Glucose fermentation	+	+
Triple Sugar Iron	-	-
H_2S Production	-	-
H/L (Oxidative & Fermentative)	-	+
Urease	-	-
Simmons citrate	+	-
Starch hydrolysis	-	+
Ehrlich indole	+	-
Nitrate-Nitrite	-	+
Adonitol	-	+
D(+) Xylose	-	+
Sucrose, Galactose, Fructose, Glucose, Sorbitol	+	+
Arginine dihydrolase	+	+
Lactose	-	+
Maltose	-	+
Mannitol	-	+
Gelatin liquefication	-	+
Inositol	-	-
OF aerobic	-	+
Voges-Proskauer 37^0C	+	+
Methyl Red	-	-
ID score	0.98678	0.98406
Bacteria identified	*Staphylococcus aureus*	*A. hydrophila*

+ : For good growth; - : for no growth

The results of different tests are presented in Table 11. These are as follows:

Isolate no. 3(a)

The results of primary tests of this isolate 3(a) revealed that this bacterium was gram positive, cocci, motile, aerobic, positive for catalase and oxidase; and had white colour of colony. The results of secondary tests revealed that the bacterium in this isolate was positive for starch hydrolysis, Ehrlich indole, sorbitol, arginine dihydrolase, MacConkey, cetrimide,

sodium malonate, mannitol, glycerol, inuline, nitrate reduction, sucrose, fructose, maltose, Simon citrate, glucose acid and 10% bile salt. However, the bacterium was negative for urease, adonitol, galactose, inositol, lactose, 0/2 % maltose, 10 % glucose, 10 % lactose, 40 % bile salt, methyl red and Vogus-Proskauer. The bacterium present in to the isolate did not grow at low (5^0C) and high 42^0C of temperatures. It hydrolyzed the casein proteins and liquefied gelatin. The bacterium present in this isolate fermented glucose, lactose and sucrose from TSI medium but did not produce H_2S gas.

Based on these tests, identification score assigned to this isolate by the PIBWin Programme was 0.98777**.** On the basis of this identification score, the bacterium species identified in isolate 3(a) was *Micrococcus* sp.

The growth of this isolate on *Micrococcus* sp. specific Hugh Leifson glucose medium and Fermentation medium (Table 12) confirmed that "isolate 3(a)" represented *Micrococcus* sp. (Figure 24).

Isolate no. 3(b)

The results of primary tests of this isolate revealed that this bacterium was gram negative, short rods, motile, aerobic, fermentive, and negative for catalase and; positive for oxidase, had red colour of colony. The results of secondary tests revealed that the bacterium in this isolate was positive for glucose fermentation from TSI and did not produce H_2S gas, nitrate reduction to nitrite, adonitol, galactose, glucose, mannitol, fructose, maltose, 0.2 % maltose, 10 % glucose, 4% NaCl, 10% bile salt, MacConkey, methyl red, arginine dihydrolase, sucrose, starch, sorbitol, H/L (fermentive) and growth at 37^0C. However, the bacterium was negative for urease, lactose, Simmons citrate, H/L (oxidative), H/L (alkaline), casein hydrolysis, 2% lactose, 10 % lactose, 40% bile salt, D(+) xylose, Ehrlich indole and inositol. The bacterium in this isolate also did not grow at low (5^0C) and high 42^0C temperatures.

Based on these tests, identification score assigned to this isolate by the PIBWin Programme was 0.99760**.** On the basis of this identification score, the bacterium species identified in isolate 3(b) was *Aeromonas hydrophila.*

The growth of this isolate on *Aeromonas hydrophila* specific Rilmer-Sholts Medium Base confirmed that "isolate 3(b)" represented *Aeromonas hydrophila* (Figure 23).

Figure 17 *Streptococcus faecalis* on Blood Agar Base Medium

Figure 18 *Klebsiella oxytoca* on Eosin-methylene blue agar

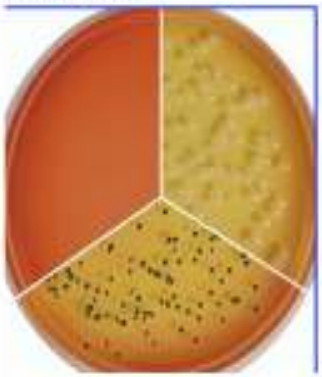

Figure 19 *Staphylococcus haemolyticus* on Mannitol salt agar

Figure 20 *Staphylococcus aureus* on Vogel-Johnson agar base

Figure 21 *Aeromonas hydrophila* on Rilmer-Shott agar

Figure 22 *Micrococcus* sp. on Hugh-Leifson glucose base medium

Table 11: Physical characteristics and biochemical response of different bacterial isolates taken from diseased Indian magur (*C. batrachus*) from CCSHAU freshwater fish farm (Hisar).

Biochemical tests	Bacterial isolates	
	3(a)	3(b)
Gram reaction	+	-
Shape	Cocci	Short Rods
Colour of colony	White	Red
Motility	+	+
Aerobic	+	+
Anaerobic	-	-
Catalase	+	-
Oxidase	+	+
Glucose fermentation	+	-
Triple Sugar Iron	Glucose, Lactose, Sucrose fermented	Glucose Fermented
H_2S Production	-	-
Urease	-	-
Simmons citrate	+	-
Starch hydrolysis	+	+
Ehrlich indole	+	-
Nitrate-Nitrite	+	+
Adonitol	-	+
Galactose	-	+
D(+) Xylose	-	-
Glucose, Fructose, Mannitol, Glycerol, Sorbitol	+	+
Sucrose	+	-
Inositol	-	-
Arginine dihydrolase	+	+
Lactose	-	-
Maltose (0.2 %)	-	+
10% Glucose	-	+
10% Lactose	-	-
4% NaCl	+	+
10% Bile salt	+	+
40% Bile salt	-	-
Sodium malonate	+	-
Casein hydrolysis	+	-
Oxidative Fermentative	Fermentative	Oxidative
Cetrimide	+	+
Gelatin Liquefication	+	+
Vogus Proskauer	-	-
Growth at 37^0C	+	+
Growth at 5^0C and 42^0C	-	-
Methyl Red	-	+
Mac Conkey Agar	+	+
ID score	0.98777	0.99760
Bacteria identified	*Micrococcus* sp.	*A. hydrophila*

+ : For good growth; - : for no growth

These results, on the basis of confirmative tests, revealed that six bacteria inhabited the affected tissues of diseased samples of Magur catfish in three catfish farms surveyed for this study. The fish samples of Muklan catfish farms were infected with *S. haemolyticus*, *Kl. oxytoca*, and *Streptococcus faecalis* where as samples of Nagalpur catfish farm were infected with *Staphylococcus aureus* and *Aeromonas hydrophila*. Like wise, the eggs and hatchlings of catfishes of catfish farm at CCSHAU, Hisar were infected with *Micrococcus* sp. and *Aeromonas hydrophila* (section 3.3).

Table 12. Selective media used for the confirmation of presence of a particular bacterium in the lesions of diseased Indian magur (*C. batrachus*).

Selective medium used	Isolate which developed the colonies	Characteristics of the colony of bacterium developed on the medium	Confirmation of bacterium
Hugh Leifson glucose medium	3(a)	Yellow	*Micrococcus* sp.
Mannitol salt agar	1(a)	White	*Staphylococcus haemolyticus*
Blood agar base w/low pH	1(c)	Red color colony	*Streptococcus faecalis*
Vogel-Johnson agar base (Tellurite)	2(a)	Black	*Staphylococcus aureus* NCTC 6571
Rimler-Shott Medium	3 (b), 2 (b)	Green color colony	*Aeromonas hydrophila*
Eosin- Methylene Blue agar medium	1(b)	Dark blue color colony	*Klebsiella oxytoca*

4.2.2 Isolation and Identification of fungi isolated from catfishes in the CCSHAU fish farm.

From the catfishes sampled from CCSHAU fish farm, three fungi were isolated (Figure 25, 26. 27) and identified from the eggs and hatchlings (Table 13). All these three isolated fungi showed same symptoms in *in vivo* pathogenicity tests by injecting pathogen intraperitoneally as well mixed with the diet (Figure 28, 29).

Table 13: Pathogenic fungi isolated from the catfish in the CCSHAU fish farm.

Isolate No.	Class	Identified Fungi
EF1 and EF3	Fungi imperfecti	*Penicillin* sp.
EF2 and EF6	Phycomycetes	*Saprolegnia* sp.
EF5	Fungi imperfecti	*Aspargillus ochracaeus*

4.3 Pathogenicity tests for the causative organisms

To further confirm/ascertain and for cross- checking of the earlier results, *in vitro* and *in vivo* pathogenicity tests were performed.

Figure 25 *Penicillin* sp. on Czepack's medium

Figure 26 *Saprolegnia* sp. on Czepack's medium

Figure 27 *Aspergillus ochraceaus* on Czepack's medium

Figure 28 Feed given to the catfishes

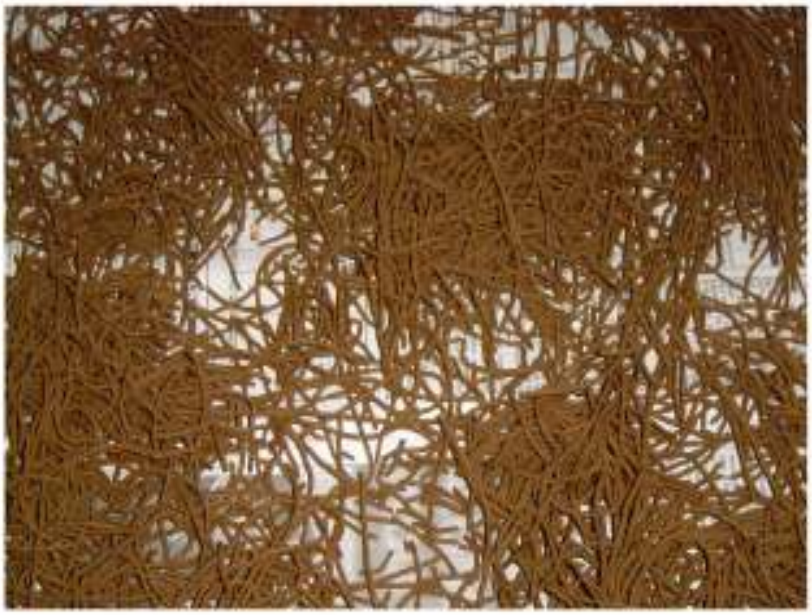

Figure 29 Dried feed given to the catfishes

4.3.1 *In vitro* pathogenicity test

The results of *in vitro* test revealed that all six bacteria showed growth and α-β zone of haemolysis on blood agar plate (Figure 30, 31, 32). These were i) *Aeromonas hydrophila*, ii) *Klebsiella oxytoca* iii) *S. faecalis*, iv) *Micrococcus* sp., v) *Staphylococcus aureus* and vi) *S. haemolyticus*. These bacteria seemed to be pathogenic.

4.3.2 *In vivo* pathogenicity tests

ii). **Incubation period of the inoculated bacterial pathogens**

The catfishes inoculated with *A. hydrophila* showed symptoms after 7 days post infection. The catfishes inoculated with *Kl. oxytoca* and *Micrococcus* sp. showed symptoms approximately after 10-11 days post inoculation, whereas the catfishes inoculated with *Staphylococcus aureus*, *S. haemolyticus* and *S. faecalis* showed symptoms after 8, 12, and 13 days post infection, respectively (Table 14). These results revealed that the fish inoculated with *A. hydrophila* showed symptoms earlier as compared to other pathogenic bacteria. The lesions were observed at the site of the injection in all the pathogenic bacteria. Later on these lesions were transformed into red ulcers; fin and tail rot, hemorrhages all over the body surface. The control inoculated with the sterilized phosphate buffer saline (PBS) showed only slightly pale colouration on 14 days of post infection but no lesions on the general body surface and at the site of injection was seen. The difference among bacteria was significant (C.D., $P \leq 0.05$, Table 14).

ii). **Effect of inoculated pathogenic bacterium on fish longevity**

The catfishes inoculated with *A. hydrophila*, *Micrococcus* sp. and *Staphylococcus aureus* remained alive for 12 days post inoculation, whereas, the catfishes inoculated with *S. haemolyticus* and *S. faecalis* remained alive for 13-14 days post inoculation. On the other hand, the catfishes inoculated with *Klebsiella oxytoca* do so for 17 days post inoculation while, in control, the catfishes survived till the end of the experiment i.e. 60 days (Table 15). These results revealed that the fish inoculated with *A. hydrophila* showed minimal longevity period as compared to those inoculated with other remaining bacterial pathogens. The per cent mortality of catfishes inoculated with *A. hydrophila* was 92.33 and of those inoculated with *Micrococcus* sp., *Staphylococcus aureus*, *Streptococcus haemolyticus*, *S. faecalis* and *Klebseilla oxytoca* the corresponding values were 85.33, 82.00, 73.67, 64.33 and 64.33; respectively (Table 16). The difference among bacteria was significant (C.D., $P \leq 0.05$, Table 15, 16).

Figure 30 Non-haemolytic bacteria on Sheep blood agar medium

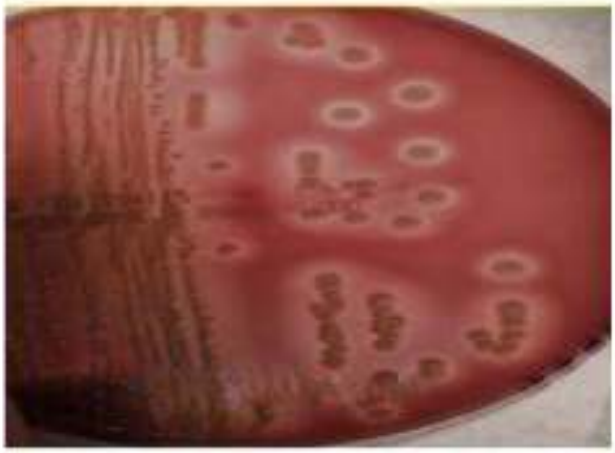

Figure 31 Pathogenic bacteria showing alpha-haemolysis on Sheep's blood agar

Figure 32 Pathogenic bacteria showing beta-haemolysis on Sheep's blood agar

Table 14. Incubation period of different pathogenic bacteria for the appearance of disease symptoms in Indian Magur (*C. batrachus*).

	Treatment	Disease symptoms	Incubation period (in days)[a] of bacteria for the appearance of disease symptoms
1.	*A. hydrophila*	Hemorrhages, reddening of pectoral fins, anemia, hypoxia	7.33 ± 0.33
2.	*Kl. oxytoca*	Hemorrhages on the mouth, head and body, ulcers	11.33 ± 0.33
3.	*Staphylococcus aureus*	Pale in colour and passive, Depigmentation and hemorrhages	8.33 ± 0.33
4.	*S. haemolyticus*	Hemorrhages, surfacing, gulping of air	12.33 ± 0.33
5.	*S. faecalis*	Hemorrhages all over the body surfaces	13.67 ± 0.33
6.	*Micrococcus* sp.	Hemorrhages, Red ulcers, Bleeding from ulcer, anemia, hypoxia	10.00 ± 0.58
7.	Phosphate Buffer Saline (PBS)	Normal	Fishes survive till the termination of the experiment
C.D. Value (P ≤ 0.05)			1.158

a = Mean ± S.D.; N=30 (10 catfishes x 3 replications)

Table 15. Longevity of Indian magur (*C. batrachus*) inoculated with different pathogenic bacteria.

Sr. no.	Treatment	Longevity (in days)[a]
1.	*Aeromonas hydrophila*	12.00 ± 0.58
2.	*Klebseilla oxytoca*	17.33 ± 0.33
3.	*Staphylococcus aureus*	12.00 ± 0.58
4.	*Staphylococcus haemolyticus*	13.67 ± 0.33
5.	*Streptococcus faecalis*	14.33 ± 0.33
6.	*Micrococcus* sp.	12.33 ± 0.33
7.	Phosphate Buffer Saline (PBS)	60.00 ± 0.00[b]
C.D. Value (P ≤ 0.05)		0.39

a = Mean ± S.D.; N=30 (10 catfishes x 3 replications). The experiment was terminated after 60 days.
b = All fishes survive during the period

Table 16. Per cent mortality of Indian magur (*C. batrachus*) inoculated with different pathogenic bacteria.

Sr. no.	Treatment	Mortality (in per cent)[a]
1.	*Aeromonas hydrophila*	92.33 ± 0.58
2.	*Klebseilla oxytoca*	61.33 ± 0.33
3.	*Staphylococcus aureus*	82.00 ± 0.58
4.	*Staphylococcus haemolyticus*	73.67 ± 0.33
5.	*Streptococcus faecalis*	64.33 ± 0.33
6.	*Micrococcus* sp.	85.33 ± 0.33
7.	Phosphate Buffer Saline (PBS)	00.00 ± 0.58
	C.D. Value ($P \leq 0.05$)	0.39

a = Mean ± S.D.; N=30 (10 catfishes x 3 replications)

4.4 Role of probiotics in controlling the prominent bacterial pathogens.

4.4.1 *In vitro* test of probiotics

Inhibition zone of probiotic against each bacterium was found to be different (Table 17). Probiotic 1 (Figure 33, 34) showed bigger inhibition zone as compared to probiotic 3 (Figure 35, 36) and probiotic 2 ((Figure 37, 38) against each bacterium. From these results, it seemed that probiotic was better than probiotic 3 and probiotic 3 was better than the probiotic 2, in gushing out the pathogenic bacteria from diseased fish. The zone of inhibition by all the three probiotics against *A. hydrophila* revealed that it was inhibited lesser than *Micrococcus* sp.

Table 17: Inhibition zones of three probiotics against different pathogenic bacteria.

Sr. no.	Inoculated bacterium	Inhibition zone (mm)			C.D. Value
		Probiotic 1	Probiotic 2	Probiotic 3	
1.	*A. hydrophila*	19.67 ± 0.67	17.00 ± 0.58	19.33 ± 0.33	1.92
2.	*Kl. oxytoca*	24.33 ± 0.33	16.67 ± 0.67	17.67 ± 0.58	1.84
3.	*Staphylococcus aureus*	20.67± 0.33	17.33 ± 0.33	18.67 ± 0.33	1.67
4.	*S. haemolyticus*	22.33± 0.33	18.33 ± 0.67	19.67 ± 0.67	1.54
5.	*S. faecalis*	22.67± 0.33	19.00 ± 0.00	19.33 ± 0.67	1.81
6.	*Micrococcus* sp.	23.00 ±0 .58	20.00 ± 0.00	22.00 ± 0.58	1.66

a = Mean ± S.D.; N=30 (10 catfishes x 3 replications)

Figure 33 Probiotic 1 showing zone of inhibition against *A. hydrophila*

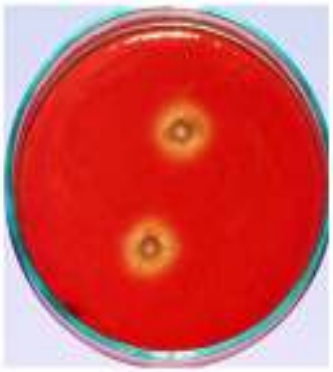

Figure 34 Probiotic 1 showing zone of inhibition against *Micrococcus* sp.

Figure 35 Probiotic 2 showing zone of inhibition against *A. hydrophila*

Figure 36 Probiotic 2 showing zone of inhibition against *Micrococcus* sp.

Figure 37 Probiotic 3 showing zone of inhibition against *A. hydrophila*

Figure 38 Probiotic 3 showing zone of inhibition against *Micrococcus* sp.

4.4.2 *In vivo* tests of probiotics

The results of *in vivo* tests revealing the effect of probiotics on the hematological parameters of Indian magur (*C. batrachus*) over a period of eight weeks are presented in tables 18, 19, 20, and 21. Blood contains mobile cells viz. red blood cell (erythrocytes), white blood cells (leucocytes) and platelets. The cells are made in bone marrow – the spongy tissues filled the centre of bones of fishes, bone marrow in skull, sternum, ribs, vertebral column, and pelvic bones. Each type of cells play an important role in the body's normal functioning.

a) Level of hemoglobin in the blood of Indian magur (*C. batrachus*) under different treatments

The results of hemoglobin levels in the blood of Indian magur (*C. batrachus*) under different treatments over a period of eight weeks are presented in Table 18. The hemoglobin level of normal catfish (T1) remained in the range of 7.32 ± 0.17 to 7.80 ± 0.01g/100ml. However, in catfishes inoculated with pathogenic bacterium, *A. hydrophila* (T2) and *Micrococcus* sp. (T3) respectively; the level of hemoglobin fell drastically and remained in the range of 5.76 ± 0.02 to 7.63 ± 0.01 g/100ml and 5.62 ± 0.03 to 7.35 ± 0.06 g/100m1, respectively. The decrease was more in T2 (1.83 g/100ml) than T3 (1.73 g/100ml); indicating that *A. hydrophila* was more pathogenic than *Micrococcus* sp. and caused anemic conditions in catfishes.

The hemoglobin level increased in the range of 6.97 ± 0.06 to 7.96 ± 0.04, 7.03 ± 0.07 to 7.79 ± 0.03 and 6.99 ± 0.07 to 7.85 ± 0.04 g/100ml in catfish inoculated with *A. hydrophila* + probiotic 1 (T7), *A. hydrophila* + probiotic 2 (T8) and *A. hydrophila* + probiotic 3 (T9) in treatment, respectively. The increase in the levels of hemoglobin in *A. hydrophila* + probiotic 1 (0.99 g/100ml), *A. hydrophila* + probiotic 2 (0.76g/100ml) and *A. hydrophila* + probiotic 3 (0.86 g/100ml) indicated that probiotic 1 was more antagonistic and stimulated higher hemoglobin production than probiotic 3 followed by probiotic 2.

The hemoglobin level increased in the range of 6.56 ± 0.0 to 7.47 ± 0.02, 6.91 ± 0.01 to 7.60 ± 0.01 and 6.88 ± 0.08 to 7.81 ± 0.02 g/100ml in catfishes inoculated with *Micrococcus* sp. + probiotic 1 (T10), *Micrococcus* sp. + probiotic 2 (T11) and *Micrococcus* sp. + probiotic 3 (T12) treatments, respectively. The increase in the levels of hemoglobin in *Micrococcus* sp. + probiotic 1 (T10) (1.11 g/100ml), *Micrococcus* sp. + probiotic 2 (T11), (0.69 g/100ml) and *Micrococcus* sp. + probiotic 3 (T12), (0.93 g/100ml) indicated that probiotic 1was more antagonistic and stimulated higher hemoglobin production than probiotic 3 followed by probiotic 2

On the other hand, the catfishes given the treatment of probiotics only i.e. probiotic 1 (T4), probiotic 2 (T5) and probiotic 3 (T6) showed maximal value of hemoglobin level as compared to all other treatments including control. The hemoglobin level was in the range of 4.79 ± 0.07 to 6.41 ± 0.10, 6.78 ± 0.08 to 7.75 ± 0.05 and 5.30 ± 0.00 to 6.68 ± 0.03 g/100ml

in catfishes administrated with probiotic 1, probiotic 2 and probiotic 3. The increase in the levels of hemoglobin in T4, T5 and T6 were 1.62, 0.97 and 1.38; respectively. These results revealed that probiotic 1 gives better results in increasing the hemoglobin level of catfishes and stimulated more production of hemoglobin as compared to probiotic 3 and probiotic 1.

Table 18: Effects of probiotics on the hemoglobin levels of Indian magur (*C. batrachus*) under *in vivo* induced pathogenicity over a period of eight weeks.

Treatments	Hemoglobin (g/100ml) level over a period of 8 weeks							
	1	2	3	4	5	6	7	8
Normal Diet (T1)	7.59 ±0.00	7.32 ±0.17	7.63 ±0.00	7.69 ±0.00	7.75 ±0.01	7.79 ±0.01	7.79 ±0.01	7.80 ±0.01
A. hydrophila (T2)	7.63 ±0.01	6.83 ±0.02	6.13 ±0.02	5.76 ±0.02	-	-	-	-
Micrococcus sp. (T3)	7.35 ±0.06	6.87 ±0.03	6.23 ±0.23	6.05 ±0.23	5.62 ±0.03	-	-	-
Probiotic 1 (T4)	4.79 ±0.07	4.91 ±0.08	4.93 ±0.05	4.95 ±0.07	5.23 ±0.17	5.34 ±0.07	5.97 ±0.09	6.41 ±0.10
Probiotic 2 (T5)	6.78 ±0.08	6.89 ±0.02	6.92 ±0.07	7.16 ±0.03	7.32 ±0.07	7.45 ±0.06	7.70 ±0.04	7.75 ±0.05
Probiotic 3 (T6)	5.30 ±0.00	5.55 ±0.05	5.79 ±0.05	5.58 ±0.07	5.94 ±0.02	6.11 ±0.12	6.40 ±0.02	6.68 ±0.03
A. hydrophila + Probiotic 1 (T7)	6.97 ±0.06	7.29 ±0.05	7.43 ±0.00	7.49 ±0.03	7.62 ±0.04	7.82 ±0.04	7.90 ±0.02	7.96 ±0.04
A. hydrophila + Probiotic 2 (T8)	7.03 ±0.07	7.27 ±0.05	7.49 ±0.04	7.56 ±0.12	7.62 ±0.04	7.66 ±0.03	7.77 ±0.02	7.79 ±0.03
A. hydrophila + Probiotic 3 (T9)	6.99 ±0.07	7.46 ±0.09	7.34 ±0.10	7.40 ±0.07	7.47 ±0.07	7.71 ±0.02	7.77 ±0.08	7.85 ±0.04
Micrococcus sp. + Probiotic 1 (T10)	6.56 ±0.08	6.69 ±0.07	6.88 ±0.03	6.90 ±0.09	6.97 ±0.04	7.23 ±0.05	7.34 ±0.05	7.47 ±0.02
Micrococcus sp. + Probiotic 2 (T11)	6.91 ±0.01	6.98 ±0.01	7.05 ±0.04	7.28 ±0.01	7.39 ±0.01	7.47 ±0.01	7.51 ±0.01	7.60 ±0.01
Micrococcus sp. + Probiotic 3 (T12)	6.88 ±0.08	7.28 ±0.04	7.41 ±0.11	7.51 ±0.04	7.57 ±0.07	7.81 ±0.02	7.67 ±0.11	7.76 ±0.08
CD value ($p \leq 0.01$)	0.27	0.15	0.26	0.26	0.15	0.15	0.17	0.17

a = Mean ± S.D.; N=30 (10 catfishes x 3 replications)

- = Catfishes died after four and five week

b) **Level of total erythrocyte count in the blood of Indian magur (*C. batrachus*) under different treatments**

The results of erythrocyte counts in the blood of Indian Magur (*C. batrachus*) under different treatments over a period of eight weeks are presented in Table 19. The erythrocyte count of normal catfish remained in the range of 1.80 ± 0.01 to 1.89 ± 0.01. However, in catfishes inoculated with pathogenic bacterium, *A. hydrophila,* (T2) and *Micrococcus* sp. (T3) respectively; the counts of erythrocyte fell drastically and remained in the range of 1.73 ±

0.02 to 1.81 ± 0.00 and 1.75 ± 0.00 to 1.82 ± 0.01, respectively. The counts of erythrocyte fell drastically seemed to be due to the haemolysis caused by pathogenic bacteria. The decrease was more in the *A. hydrophila* 0.08 than *Micrococcus* sp. 0.07; indicated that *A. hydrophila* was more pathogenic than the *Micrococcus* sp. bacteria and caused anemic condition. The erythrocyte count increased and remained in the range of 1.76 ± 0.01 to 1.87 ± 0.00, 1.84 ± 0.00 to 1.92 ± 0.01 and 1.76 ± 0.01 to 1.86 ± 0.00 in the catfish inoculated with *A. hydrophila* + probiotic 1 (T7), *A. hydrophila* + probiotic 2 (T8) and *A. hydrophila* + probiotic 3 (T9), respectively. The increase in the counts of eryrthrocyte in T7 (0.11), T8 (0.08) and T9 (0.10) indicated that probiotic 1was more antagonistic and stimulate higher production of eryrthrocyte than probiotic 3 followed by probiotic 2.

The erythrocyte count increased and remained in the range of 1.76 ± 0.01 to 1.90 ± 0.00, 1.80 ± 0.00 to 1.86 ± 0.00 and 1.78 ± 0.01 to 1.91 ± 0.01 in the catfish inoculated with *Micrococcus* sp. + probiotic 1 (T10), *Micrococcus* sp. + probiotic 2 (T11) and *Micrococcus* sp. + probiotic 3 (T12), respectively. The increase in the counts of erythrocyte in T10 (0.14), T8 (0.06) and T9 (0.13) indicated that probiotic 1 was more antagonistic and stimulate higher production of erythrocyte than probiotic 3 followed by probiotic 2.

On the other hand, the catfishes given the treatment of probiotics in treatments probiotic 1 (T4), probiotic 2 (T5) and probiotic 3 (T6) showed maximal value of erythrocyte counts as compared to all other treatments including control. The erythrocyte counts were remained in the range of 1.53 ± 0.01 to 1.76 ± 0.00, 1.72 ± 0.00 to 1.87 ± 0.01 and 1.60 ± 0.01 to 1.78 ± 0.00 in catfishes administrated with probiotic 1, probiotic 2 and probiotic 3. The increase in the counts of erythrocyte in T4, T5 and T6 were 1.62, 0.97 and 1.38; respectively. These results revealed that probiotic 1 gives better results in increasing the erythrocyte counts of catfishes and stimulated more production of erythrocytes or increased the rate of erythropoesis as compared to probiotic 3 and probiotic 1 and reduced the chances of anemia in the catfishes.

Table 19: Effects of probiotics on the total erythrocyte counts of Indian magur (*C. batrachus*) under *in vivo* induced pathogenicity over a period of eight weeks.

Treatments	Total erythrocyte counts (10^6 cells/ml) level over a period of 8 weeks							
	1	2	3	4	5	6	7	8
Normal Diet (T1)	1.80 ± 0.01	1.81 ± 0.01	1.83 ± 0.01	1.84 ± 0.00	1.85 ± 0.00	1.87 ± 0.00	1.89 ± 0.00	1.89 ± 0.01
A. hydrophila (T2)	1.80 ± 0.00	1.77 ± 0.01	1.75 ± 0.01	1.74 ± 0.02	-	-	-	-
Micrococcus sp. (T3)	1.82 ± 0.01	1.78 ± 0.01	1.76 ± 0.01	1.74 ± 0.00	1.72 ± 0.00	-	-	-
Probiotic 1 (T4)	1.60 ± 0.01	1.63 ± 0.01	1.63 ± 0.01	1.64 ± 0.01	1.64 ± 0.01	1.67 ± 0.01	1.76 ± 0.00	1.70 ± 0.01
Probiotic 2 (T5)	1.72 ± 0.00	1.75 ± 0.01	1.79 ± 0.01	1.81 ± 0.01	1.84 ± 0.00	1.85 ± 0.01	1.85 ± 0.01	1.87 ± 0.01
Probiotic 3 (T6)	1.62 ± 0.01	1.60 ± 0.01	1.63 ± 0.01	1.68 ± 0.01	1.70 ± 0.01	1.74 ± 0.01	1.69 ± 0.01	1.78 ± 0.00
A. hydrophila + Probiotic 1 (T7)	1.76 ± 0.01	1.74 ± 0.00	1.78 ± 0.03	1.83 ± 0.00	1.85 ± 0.00	1.87 ± 0.00	1.89 ± 0.01	1.87 ± 0.00
A. hydrophila + Probiotic 2 (T8)	1.84 ± 0.00	1.82 ± 0.01	1.87 ± 0.00	1.88 ± 0.01	1.89 ± 0.00	1.89 ± 0.02	1.92 ± 0.01	1.91 ± 0.01
A. hydrophila + Probiotic 3 (T9)	1.76 ± 0.01	1.76 ± 0.01	1.74 ± 0.01	1.77 ± 0.02	1.80 ± 0.01	1.85 ± 0.01	1.86 ± 0.01	1.86 ± 0.00
Micrococcus sp.+ Probiotic 1 (T10)	1.76 ± 0.01	1.81 ± 0.01	1.84 ± 0.01	1.85 ± 0.00	1.87 ± 0.01	1.87 ± 0.00	1.89 ± 0.01	1.90 ± 0.00
Micrococcus sp.+ Probiotic 2 (T11)	1.80 ± 0.01	1.81 ± 0.02	1.80 ± 0.01	1.80 ± 0.01	1.82 ± 0.00	1.86 ± 0.00	1.85 ± 0.00	1.84 ± 0.00
Micrococcus sp.+ Probiotic 3 (T12)	1.78 ± 0.01	1.80 ± 0.01	1.81 ± 0.01	1.85 ± 0.00	1.87 ± 0.01	1.90 ± 0.01	1.91 ± 0.01	1.90 ± 0.01
CD value ($p \leq 0.01$)	0.03	0.03	0.03	0.02	0.02	0.03	0.02	0.02

a = Mean ± S.D.; N=30 (10 catfishes x 3 replications)

- = Catfishes died after four and five week

c) Level of total leukocyte count (TLC in 10^3cells/ml) in the blood of Indian magur (*C. batrachus*) under different treatments

The results on leukocyte counts in the blood of Indian magur (*C. batrachus*) under different treatments over a period of eight weeks are presented in Table 20. The leukocyte counts of normal diet (T1) fed catfish remained in the range of 6.61 ± 0.03 to 7.68 ± 0.06. However, in the catfishes inoculated with, *A. hydrophila* (T2) and *Micrococcus* sp. (T3); leukocytes counts increased and remained in the range of 6.53 ± 0.12 to 7.72 ± 0.05 and 6.67 ± 0.03 to 7.81 ± 0.09 respectively. The counts of leukocyte increased steeply seemed to be due to the increased production simulated by *A. hydrophila* and *Micrococcus* sp. as well as stimulation of immune system of catfishes and increased phagocytes. The increase was more

in the T2 (1.19) than T3 (1.14); indicated that *A. hydrophila* was more pathogenic than the *Micrococcus* sp. bacteria.

The leukocyte count increased and remained in the range of 5.70 ± 0.03 to 6.48 ± 0.11, 6.69 ± 0.11 to 7.69 ± 0.08 and 6.65 ± 0.06 to 7.46 ± 0.03 in catfish inoculated with the *A. hydrophila* + probiotic 1 (T7), *A. hydrophila* + probiotic 2 (T8) and *A. hydrophila* + probiotic 3 (T9), respectively and the increase in the counts of leukocytes in T7, T8 and T9 were 0.78, 1.00 and 0.81 respectively.

The erythrocyte count increased and remained in the range of 6.34 ± 0.06 to 7.35 ± 0.04, 6.22 ± 0.11 to 7.11 ± 0.10 and 6.89 ± 0.09 to 7.80 ± 0.03 in catfish inoculated with *Micrococcus* sp. + probiotic 1 (T10), *Micrococcus* sp. + probiotic 2 (T11) and *Micrococcus* sp. + probiotic 3 (T12), respectively and the increase in the counts of leukocytes in T10, T11 and T12 were 1.01, 0.89 and 0.91 respectively.

On the other hand, the catfishes given probiotics in treatments probiotic 1 (T4), probiotic 2 (T5) and probiotic 3 (T6) showed maximal value of leukocyte counts as compared to all other treatments including control. The counts were remained in the range of 6.40 ± 0.05 to 7.72 ± 0.05, 5.20 ± 0.04 to 6.46 ± 0.15 and 6.59 ± 0.08 to 7.80 ± 0.01 in catfishes administrated with probiotic 1, probiotic 2 and probiotic 3, respectively. The decrease in the counts of leukocyte in T4, T5 and T6 were 0.87, 0.63 and 0.81; respectively.

These results revealed that probiotic 1 gives better results in decreasing the leukocyte counts of catfishes and stimulated less production of leukocyte or decreased the rate of leucopoiesis as compared to probiotic 3 and probiotic 2.

Table 20. An effect of probiotics on the total leucocytes counts of Indian magur (*C. batrachus*) under *in vivo* induced pathogenicity over a period of eight weeks

Treatments	Total leucocytes counts (10^3 cells/ml) level over a period of 8 weeks							
	1	2	3	4	5	6	7	8
Normal Diet (T1)	6.61 ±0.03	6.85 ±0.13	7.11 ±0.08	7.37 ± 0.15	7.54 ± 0.06	7.58 ± 0.03	7.68 ± 0.13	7.68 ± 0.06
A. hydrophila (T2)	6.53 ±0.12	6.57 ±0.07	7.28 ±0.09	7.72 ± 0.05	-	-	-	-
Micrococcus sp. (T3)	6.67 ±0.03	6.78 ±0.04	6.74 ±0.01	6.68 ± 0.04	7.81 ± 0.09	-	-	-
Probiotic 1 (T4)	6.40 ±0.05	6.38 ±0.09	6.36 ±0.06	6.30 ± 0.04	6.18 ± 0.03	6.06 ± 0.04	5.82 ± 0.05	5.53 ± 0.04
Probiotic 2 (T5)	6.46 ±0.04	6.42 ±0.04	6.39 ±0.07	6.35 ± 0.07	6.23 ± 0.06	6.08 ± 0.10	5.86 ± 0.10	5.83 ± 0.15
Probiotic 3 (T6)	6.59 ±0.08	6.51 ±0.10	6.43 ±0.00	6.38 ± 0.07	6.26 ± 0.12	6.12 ± 0.06	6.01 ± 0.02	5.88 ± 0.01
A. hydrophila + Probiotic 1 (T7)	5.70 ±0.03	5.34 ±0.04	5.81 ±0.01	6.15 ± 0.05	6.37 ± 0.07	6.49 ± 0.03	6.48 ± 0.11	6.44 ± 0.10
A. hydrophila + Probiotic 2 (T8)	6.69 ±0.11	6.86 ±0.03	6.85 ±0.09	7.04 ± 0.07	7.19 ± 0.15	7.36 ± 0.04	7.48 ± 0.07	7.69 ± 0.08
A. hydrophila + Probiotic 3 (T9)	6.65 ±0.06	6.86 ±0.03	7.12 ±0.05	7.26 ± 0.02	7.22 ± 0.05	7.30 ± 0.01	7.46 ± 0.03	7.46 ± 0.03
Micrococcus sp. + Probiotic 1 (T10)	6.44 ±0.12	6.34 ±0.06	6.58 ±0.14	6.63 ± 0.11	6.75 ± 0.04	6.97 ± 0.05	7.26 ± 0.12	7.35 ± 0.04
Micrococcus sp. + Probiotic 2 (T11)	6.22 ±0.11	6.32 ±0.05	6.50 ±0.03	6.58 ± 0.03	6.65 ± 0.05	6.84 ± 0.05	7.11 ± 0.10	6.93 ± 0.00
Micrococcus sp. + Probiotic 3 (T12)	7.13 ±0.03	6.89 ±0.09	7.35 ±0.06	7.41 ± 0.01	7.52 ± 0.08	7.63 ± 0.05	7.71 ± 0.04	7.80 ± 0.03
CD value ($p \leq 0.01$)	0.22	0.22	0.21	0.21	0.27	0.20	0.24	0.23

a = Mean ± S.D.; N=30 (10 catfishes x 3 replications)

- = Catfishes died after four and five week

d) **Level of packed cell volume (PCV) in the blood of Indian magur (*C. batrachus*) under different treatments**

The results of packed cell volume in the blood of Indian magur (*C. batrachus*) under different treatments over a period of eight weeks are presented in Table 21. The packed cell volume of normal diet (T1) catfishes remained in the range of 38.29 ± 0.99 to 48.60 ± 0.67. However, in catfishes inoculated with *A. hydrophila* (T2), and *Micrococcus* sp. (T3) respectively; the packed cell volume fell drastically and remained in the range of 28.79 ± 0.96 to 36.51 ± 2.34 and 24.79 ± 0.22 to 31.64 ± 0.64, respectively. The drastic fall in packed cell volume seemed to be due to the haemolysis due to *A. hydrophila* and *Micrococcus* sp. The

decrease was more in T2 (7.72) than T3 (6.85); indicated that *A. hydrophila* was more pathogenic than the *Micrococcus* sp. bacteria caused anemic situation.

The packed cell volume increased and remained in the range of 22.79 ± 0.61 to 36.52 ± 0.50, 34.83 ± 1.35 to 45.32 ± 0.29 and 32.77 ± 0.17 to 44.86 ± 1.12 in the catfish inoculated with *A. hydrophila* + probiotic 1 (T7), *A. hydrophila* + probiotic 2 (T8) and probiotic 3 (T9), respectively. The increase in the levels of packed cell volume in T7 (13.73), T8 (10.49) and T9 (12.09) indicated that probiotic 1was more antagonistic and stimulated increased production blood cells than probiotic 3 followed by probiotic 2.

Table 21. Effects of probiotics on the packed cell volume of Indian magur (*C. batrachus*) under *in vivo* induced pathogenicity over a period of eight weeks

Treatments	Packed cell volume level over a period of 8 weeks							
	1	2	3	4	5	6	7	8
Normal Diet (T1)	38.29 ±0.99	39.15 ±1.21	40.13 ±0.86	40.87 ±0.93	42.43 ±0.55	43.96 ±0.38	45.88 ±0.66	48.60 ±0.67
A. hydrophila (T2)	36.51 ±2.34	34.02 ±1.04	3.36 ±2.45	28.79 ±0.96	-	-	-	-
Micrococcus sp. (T3)	31.64 ±0.60	31.63 ±0.59	26.83 ±0.19	25.69 ±0.40	24.79 ±0.22	-	-	-
Probiotic 1 (T4)	33.25 ±0.23	33.99 ±0.54	35.49 ±0.74	39.25 ±1.99	44.74 ±1.01	47.24 ±1.79	51.35 ±0.65	53.70 ±1.21
Probiotic 2 T5)	35.02 ±0.27	35.02 ±0.30	43.29 ±0.39	45.54 ±0.98	47.72 ±0.66	48.26 ±0.23	49.62 ±0.37	51.55 ±0.39
Probiotic 3 (T6)	41.70 ±0.75	44.04 ±0.90	46.53 ±0.33	49.79 ±0.39	52.51 ±1.32	56.65 ±0.50	58.49 ±0.35	59.98 ±0.88
A. hydrophila + Probiotic 1 (T7)	29.10 ±0.32	25.00 ±1.21	22.79 ±0.61	24.26 ±0.39	29.10 ±0.39	32.10 ±0.50	34.19 ±1.08	36.52 ±0.50
A. hydrophila + Probiotic 2 (T8)	34.83 ±1.35	34.83 ±1.36	40.99 ±0.57	39.31 ±0.97	42.58 ±0.28	41.84 ±0.75	45.27 ±0.26	45.32 ±0.29
A. hydrophila + Probiotic 3 (T9)	32.77 ±0.17	33.88 ±0.59	36.16 ±0.28	38.29 ±1.05	41.39 ±0.63	44.86 ±1.12	42.64 ±0.62	42.08 ±0.37
Micrococcus sp. + Probiotic 1 (T10)	40.55 ±1.00	42.19 ±0.97	44.13 ±1.10	45.13 ±0.90	47.07 ±0.99	48.77 ±0.85	51.35 ±0.87	54.92 ±1.27
Micrococcus sp. + Probiotic 2 (T11)	34.83 ±0.47	34.81 ±0.46	38.00 ±1.31	38.60 ±0.95	40.11 ±0.35	42.26 ±0.51	46.71 ±1.53	43.28 ±0.48
Micrococcus sp. + Probiotic 3 (T12)	32.76 ±0.46	33.51 ±0.34	34.36 ±0.48	37.49 ±2.11	39.41 ±0.37	41.39 ±0.54	43.55 ±1.05	43.68 ±1.45
CD value ($p \leq 0.01$)	2.80	2.81	2.87	3.33	2.16	2.60	2.31	2.83

a = Mean ± S.D.; N=30 (10 catfishes x 3 replications)

- = Catfishes died after four and five week

The packed cell volume increased and remained in the range of 40.55 ± 1.00 to 54.92 ± 1.27, 34.81 ± 0.46 to 46.71 ± 1.53 and 32.76 ± 0.46 to 43.68 ± 1.45 in the catfish inoculated

with *Micrococcus* sp. + probiotic 1 (T10), *Micrococcus* sp. + probiotic 2 (T11) and probiotic 3 (T12), respectively. The increase in the volume of packed cells in T10 (14.37), T11 (11.90) and T12 (10.92) indicated that probiotic 1was more antagonistic and stimulated increased production blood cells than probiotic 3 followed by probiotic 2.

On the other hand, the catfishes given probiotics in treatments probiotic 1 (T4), probiotic 2 (T5) and probiotic 3 (T6) showed maximal value of packed cell volume as compared to all other treatments including control. The packed cell volume were remained in the range of 33.25 ± 0.23 to 53.70 ± 1.21, 35.02 ± 0.27 to 51.55 ± 0.39 and 41.70 ± 0.75 to 59.98 ± 0.88 in catfishes administrated with probiotic 1, probiotic 2 and probiotic 3; respectively. The increase in the volumes of packed cells in T4, T5 and T6 were 20.45, 16.53 and 18.28; respectively.

These results revealed that probiotic 1 gives better results in increasing the packed cell volumes of the blood of catfishes and stimulated more production of erythrocytes and leukocyte as compared to probiotic 3 and probiotic 1.

4.5 Effect of probiotics on the survival rate of Indian magur (*C. batrachus*) under *in vivo* induced pathogenicity

The results of survival of magur catfish (*C. batrachus*) under different treatments over a period of eight weeks are presented in Table 22. The survival of normal catfish during this period was 83.33 per cent. But in the catfishes inoculated with *A. hydrophila* (T2), the survival was only 52.67 per cent. At the end of fourth week all the catfishes died. In the catfishes inoculated with *Micrococcus* sp. (T3), the survival was only 67.67 per cent and at the end of fifth week all the catfishes died.

The per cent survival in catfishes inoculated with *A. hydrophila* + probiotic 1 (T7), *A. hydrophila* + probiotic 2 (T8) and probiotic 3 (T9), were 93.33, 83.33 and 86.67; respectively. The per cent survival in catfishes inoculated with *Micrococcus* sp. + probiotic 1 (T10), *A. hydrophila* + probiotic 2 (T11) and *A. hydrophila* + probiotic 3 were 96.67, 93.33 and 96.67, respectively

Other groups of catfishes administered with probiotics only showed 100 per cent survival were probiotic 1 (T4), probiotic 2 (T5) and probiotic 3 (T6),

Table 22. Effects of probiotics on per cent survival of Indian magur (*C. batrachus*) under *in vivo* induced pathogenicity over a period of eight weeks.

Treatments	% Survivality
Normal Diet (T1)	83.33 ± 8.82
A. hydrophila (T2)	52.67 ± 3.33
Micrococcus sp. (T3)	66.67 ± 3.33
Probiotic 1 (T4)	100.00 ± 0.00
Probiotic 2 (T5)	96.67 ± 3.33
Probiotic 3 (T6)	100.00 ± 0.00
A. hydrophila + Probiotic 1 (T7)	93.33 ± 3.33
A. hydrophila + Probiotic 2 (T8)	83.33 ± 3.33
A. hydrophila + Probiotic 3 (T9)	86.67 ± 3.33
Micrococcus sp. + Probiotic 1 (T10)	93.33 ± 3.33
Micrococcus sp. + Probiotic 2 (T11)	83.33 ± 3.33
Micrococcus sp. + Probiotic 3 (T12)	100.00 ± 0.00
CD value ($p \leq 0.01$)	11.30

a = Mean ± S.D.; N=30 (10 catfishes x 3 replications)

* = Catfishes died after four and five weeks

4.6 Effect of probiotics on the growth performance of Indian magur (*C. batrachus*)

i) **Length gain of Indian magur (*C. batrachus*) under different treatments**

The results of length of Indian Magur (*C. batrachus*) under different treatments over a period of eight weeks are presented in table 23. The catfishes under normal condition (T1) showed 6.17 ± 0.16 increases in length; the increase in length of the fish inoculated with *A. hydrophila* (T2) and *Micrococcus* sp. (T3) were only 3.67 ± 0.17 and 4.17 ± 0.16 cms, respectively. The catfishes inoculated with pathogenic bacteria showed less increase in length indicating that catfish's growth is severely affected by the diseases and infections.

The increase in lengths, were 5.51 ± 0.29, 4.67 ± 0.17 and 5.50 ± 0.00 in catfishes inoculated with *A. hydrophila* + probiotic 1 (T7), *A. hydrophila* + probiotic 2 (T8) and probiotic 3 (T9), respectively. The increase in the body lengths in these three treatments indicated that probiotic 1 was more antagonistic and had growth promoting effect than probiotic 3 followed by probiotic 2.

The increase in lengths, were 6.00 ± 0.29, 4.67 ± 0.17 and 5.83 ± 0.17 in catfishes inoculated with *Micrococcus* sp. + probiotic 1 (T10), *Micrococcus* sp. + probiotic 2 (T11) and probiotic 3 (T12), respectively. The increase in the body lengths in these three treatments indicated that probiotic 1 was more antagonistic and had growth promoting effect than probiotic 3 followed by probiotic 2.

The increase in lengths, were 9.17 ± 0.17, 6.67 ± 0.33 and 7.67 ± 0.33 in catfishes provided with only probiotic 1 (T4), probiotic 2 (T5) and probiotic 3 (T6), respectively. The increase in the body lengths in these three treatments indicated that probiotic 1 was having growth promoting than probiotic 3 followed by probiotic 2.

Table 23. Effects of probiotics on length gain of Indian magur (*C. batrachus*) under *in vivo* induced pathogenicity over a period of eight weeks.

Treatments	Initial lt (cm)	Final lt (cm)	Length gain (cm)
Normal Diet (T1)	10.17 ± 0.17	16.33 ± 0.17	6.17 ± 0.17
A. hydrophila (T2)	10.33 ± 0.17	14.00 ± 0.00	3.67 ± 0.17
Micrococcus sp. (T3)	11.00 ± 0.33	15.17 ± 0.17	4.17 ± 0.17
Probiotic 1 (T4)	9.67 ± 0.17	18.83 ± 0.17	9.17 ± 0.17
Probiotic 2 (T5)	9.67 ± 0.17	16.33 ± 0.17	6.67 ± 0.33
Probiotic 3 (T6)	9.67 ± 0.17	17.33 ± 0.17	7.67 ± 0.33
A. hydrophila + Probiotic 1 (T7)	9.83 ± 0.17	15.33 ± 0.17	5.51 ± 0.29
A. hydrophila + Probiotic 2 (T8)	10.50 ± 0.00	15.17 ± 0.17	4.67 ± 0.17
A. hydrophila + Probiotic 3 (T9)	10.67 ± 0.17	16.17 ± 0.17	5.50 ± 0.00
Micrococcus sp. + Probiotic 1 (10)	10.17 ± 0.17	16.17 ± 0.17	6.00 ± 0.29
Micrococcus sp. + Probiotic 2 (11)	11.17 ± 0.17	15.83 ± 0.17	4.67 ± 0.17
Micrococcus sp. + Probiotic 3 (12)	9.83 ± 0.17	15.67 ± 0.17	5.83 ± 0.17
CD value ($p \leq 0.01$)	0.44	0.47	0.65

a = Mean ± S.D.; N=30 (10 catfishes x 3 replications)

* = Catfishes died after four and five weeks

ii). **Weight gain of Indian magur (*C. batrachus*) under different treatments**

The results of weights of Indian magur (*C. batrachus*) under different treatments over a period of eight weeks are presented in Table 24. The increase in weight of catfishes under normal condition (T1) showed 93.36 ± 0.73 and; the increase in weights of the catfishes inoculated with *A. hydrophila* (T2) and *Micrococcus* sp. (T3) were 90.05 ± 0.18 and 91.41 ± 0.19 gms, respectively. The catfishes inoculated with *A. hydrophila* and *Micrococcus* sp. showed less increase in body weight indicating that catfish's growth is severely affected by the diseases and infections.

The increase in weights, were 128.04 ± 0.18, 118.20 ± 0.14 and 123.86 ± 0.20 in catfishes inoculated with *A. hydrophila* + probiotic 1 (T7), *A. hydrophila* + probiotic 2 (T8) and *A. hydrophila* + probiotic 3 (T9), respectively. The increase in the body weight in these three treatments (T7, T8 and T9) indicated that probiotic 1 was more growth promoting than probiotic 3 followed by probiotic 2.

The increase in weights, were 132.99 ± 0.10, 121.27 ± 0.10 and 123 ± 0.06 in catfishes inoculated with *Micrococcus* sp. + probiotic 1 (T10), *Micrococcus* sp. + probiotic 2 (T11) and probiotic 3 (T12), respectively. The gain in the body weight in these three

treatments (T10, T11 and T12) indicated that probiotic 1 was more growth promoting than probiotic 3 followed by probiotic 2.

The increase in weights, were 139.42 ± 0.41, 127.71 ± 0.44 and 132.87 ± 0.13 in catfishes provided with only probiotic 1 (T4), probiotic 2 (T5) and probiotic 3 (T6), respectively. The gain in the live body weights in these three treatments (T4, T5 and T6) indicated that probiotic 1 was more growth promoting than probiotic 3 followed by probiotic 2.

Table 24. Effects of probiotics on weight gain of Indian magur (*C. batrachus*) under *in vivo* induced pathogenicity over a period of eight weeks.

Treatments	Initial wt (g)	final wt (g)	live wt
Normal Diet (T1)	5.96 ± 0.03	98.33 ± 0.70	93.36 ± 0.73
A. hydrophila (T2)	6.01± 0.01	96.06 ± 0.19	90.05 ± 0.18
Micrococcus sp. (T3)	4.93 ± 0.03	96.34 ± 0.18	91.41 ± 0.19
Probiotic 1 (T4)	5.41 ± 0.01	144.83 ± 0.42	139.42 ± 0.41
Probiotic 2 (T5)	5.67± 0.01	133.38 ± 0.43	127.71 ± 0.44
Probiotic 3 (T6)	5.74 ± 0.02	138.61 ± 0.15	132.87 ± 0.13
A. hydrophila + Probiotic 1 (T7)	6.36 ± 0.28	134.40 ± 0.10	128.04 ± 0.18
A. hydrophila + Probiotic 2 (T8)	5.90 ± 0.02	124.10 ± 0.13	118.20 ± 0.14
A. hydrophila + Probiotic 3 (T9)	6.31 ± 0.03	130.17 ± 0.18	123.86 ± 0.20
Micrococcus sp. + Probiotic 1 (T10)	6.06 ± 0.04	139.05 ± 0.05	132.99 ± 0.10
Micrococcus sp. + Probiotic 2 (T11)	6.79 ± 0.01	128.06 ± 0.09	121.27 ± 0.10
Micrococcus sp. + Probiotic 3 (T12)	5.67 ± 0.02	128.99± 0.07	123.31 ± 0.06
CD value (p≤0.01)	0.25	0.86	0.89

a = Mean ± S.D.; N=30 (10 catfishes x 3 replications)

* = Catfishes died after four and five weeks

4.7 Effect of probiotics on the viable count of Indian magur (*C. batrachus*) under *in vivo* induced pathogenicity

a). **Elimination of *A. hydrophila***

The results of viable counts of antibiotic (chloramphenicol) resistant pathogenic bacteria (*A. hydrophila*) under different treatments over a period of eight weeks are presented in Table 25. The catfishes inoculated with *A. hydrophila* showed progressive increase in the number of viable counts till the fishes died after four weeks. Viable counts of bacteria in fish inoculated with *A. hydrophila* increased from 1.54 x 10^{11} in first week to 2.10 x10^{12} cells/ml in four week. The viable counts of *A. hydrophila* became so high in fourth week that the catfishes could not tolerate this and subsequently showed mortality. But the catfishes inoculated with *A. hydrophila* + three probiotics, showed progressive decline in the viable counts of *A. hydrophila*. The viable counts of *A. hydrophila* + probiotic 1 declined from 1.54

x 10^{11} cells/ml in the first week to 1.90 x 10^{1} in fourth week; those of *A. hydrophila* + probiotic 2 declined from 1.54 x 10^{11} cells/ml in the first week to 6.33 x 10^{1} cells/ml in fourth week while those of *A. hydrophila* + probiotic 3 viable counts declined from 1.54 x 10^{11} cells/ml in the first week to 2.30 x10^{1} in fourth week. *A. hydrophila* was eliminated by all the three probiotics (probiotic1- *Lactobacillus sporogenes*; probiotic 2- *Saccharomyces boulardii* and probiotic 3- (a mixture of many soil bacteria) successfully**.**

Table 25: Effects of probiotics on the viable counts of *A. hydrophila* (chloramphenicol resistant) bacteria in Indian Magur (*C. batrachus*) under *in vivo* induced pathogenicity over a period of eight weeks.

Treatment	Viable counts of *A. hydrophila* (chloramphenicol resistant) bacteria in different weeks				
	0	1	2	3	4
A. hydrophila	1.54 x 10^{11}	6.07 x 10^{11}	3.15 x 10^{12}	3.10 x 10^{12}	2.10 x 10^{12}
A. hydrophila + probiotic 1	1.54 x 10^{11}	3.64 x 10^{4}	1.96 x 10^{3}	8.60 x 10^{1}	1.90 x 10^{1}
A. hydrophila+ probiotic 2	1.54 x 10^{11}	6.53 x 10^{5}	6.07 x 10^{4}	5.75 x 10^{2}	6.33 x 10^{1}
A. hydrophila+ probiotic 3	1.54 x 10^{11}	4.50 x 10^{6}	8.27 x 10^{4}	9.27 x 10^{2}	2.30 x 10^{1}

***** = Catfishes died after four weeks

b). Elimination of *Micrococcus* sp.

The results of viable counts of antibiotic (oxytetracycline resistant) resistant pathogenic bacteria (*Micrococcus* sp.) under different treatments over a period of eight weeks are presented in Table 26. The catfishes infected with *Micrococcus* sp. showed progressive increase in the number of viable counts till the fishes died after four weeks. Viable counts of bacteria in fish inoculated with *Micrococcus* sp. increased from 1.68 x 10^{10} in first week to 4.7 x10^{12} cells/ml in four week. The viable counts of *Micrococcus* sp. became so high in fourth week that the catfishes could not tolerate this and subsequently showed mortality. But the catfishes inoculated with *Micrococcus* sp. and along with the three probiotics showed progressive decline in the viable counts of *Micrococcus* sp. The viable counts of *Micrococcus* sp. along with probiotic 1 in the treatment declined from 1.6 x 10^{10} cells/ml in first week to 2.80 x 10^{1} in fourth week; those of *Micrococcus* sp. + probiotic 2 in the treatment declined from 1.6 x 10^{11} cells/ml in first week to 1.90 x 10^{1} cells/ml in fourth week while in *Micrococcus* sp. + probiotic 3 treatment viable counts declined from 1.6 x 10^{10} cells/ml in first week to 3.5 x 10^{1} bacterial cells/ml in fourth week. *Micrococcus* sp. was eliminated by the all three probiotics (probiotic 1- *Lactobacillus sporogenes*, probiotic 2- *Saccharomyces boulardii* and probiotic 3- (a mixture of many soil bacteria) successfully.

Table 26: Effects of probiotics on the viable counts of *Micrococcus* sp. (oxytetracycline resistant) bacteria in Indian Magur (*C. batrachus*) under *in vivo* induced pathogenicity over a period of eight weeks.

Treatment	Viable counts of *Micrococcus* sp. (oxytetracycline resistant) bacteria in different weeks					
	0	1	2	3	4	5
Micrococcus sp.	1.6×10^{10}	7.0×10^{10}	1.8×10^{11}	7.3×10^{12}	4.7×10^{12}	4.8×10^{12}
Micrococcus sp.+ probiotic 1	1.6×10^{10}	7.3×10^{4}	9.3×10^{3}	6.8×10^{1}	2.8×10^{1}	2.5×10^{1}
Micrococcus sp.+ probiotic 2	1.6×10^{10}	8.6×10^{5}	6.8×10^{4}	5.8×10^{2}	1.9×10^{1}	2.0×10^{1}
Micrococcus sp.+ probiotic 3	1.6×10^{10}	2.6×10^{6}	7.6×10^{4}	7.3×10^{3}	3.5×10^{1}	3.3×10^{1}

* = Catfishes died after five weeks

The above results revealed that the incidence of catfish diseases *viz*. Hemorrhagic septicemia, fin and tail rot, ulcerative and saprolegniasis in Indian Magur (*C. batrachus*) during July-August-September October and January – February. Six bacteria were found to be pathogenic in catfishes. In the experimental catfishes, these pathogenic bacteria were inoculated alone and along with probiotics. Various hematological and growth parameters were recorded over a period of eight weeks. Three commericial probiotics (probiotic 1 probiotic 2 and probiotic 3) having different composition were used in the experiments and all the results revealed that probiotic gave better results as compared to probiotic 3 and then 2.

3.9 Effect of probiotics on the intestinal histopathology of Indian magur (*C. batrachus*)

The results of histological studies of Indian magur (*C. batrachus*) under different treatments over a period of eight weeks showed normal inner lining of intestine of catfishes under normal condition (T1) with no degeneration and desquamation (Figure 39). But the intestinal epithelium of catfishes inoculated with *A. hydrophila* (T2) and *Micrococcus* sp. (T3) were very severely degenerated and desquamated as compared to the control. This severe degeneration indicated severe enteritis and enhanced counts of mononuclear cells were also observed. The catfishes inoculated with *A. hydrophila* and *Micrococcus* sp. showed less number of villi (Figure 40, 41) than the *A. hydrophila* + probiotic 1, *A. hydrophila* + probiotic 2 and *A. hydrophila* + probiotic 3 (Figure 42) and *Micrococcus* sp. + probiotic 1, *Micrococcus* sp.+ probiotic 2 and *Micrococcus* sp. + probiotic 3 (Figure 43), and catfishes trated with probiotic alone (Figure 44). The catfishes inoculated with the probiotic 1 (T4), probiotic 2 (T5) and probiotic 3 (T6) showed more number of villi which indicated the increased absorptive intestinal surface areas (Figure 44). In probiotic 1 (T4) treated group,

Figure 39 Histology of intestine of catfish in control treatment (T1) (100X)

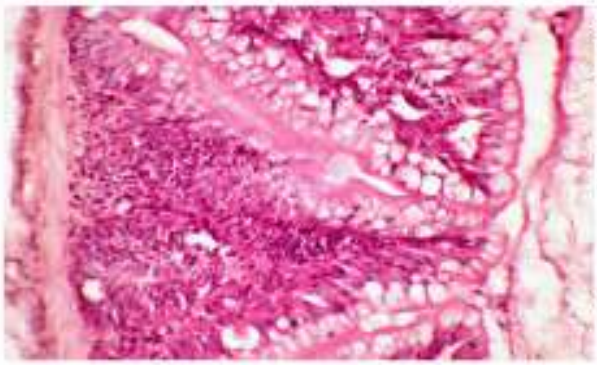

Figure 40 Histology of intestine in *Aeromonas hydrophila* treated catfishes (T2) (100X)

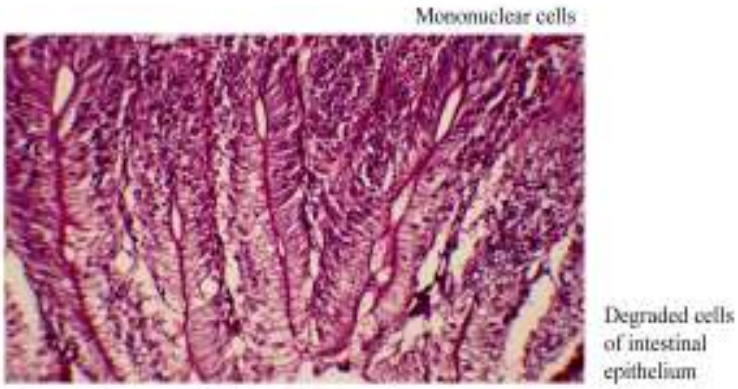

Figure 41 Histology of intestine in *Micrococcus* sp. treated catfishes (T3) (100X)

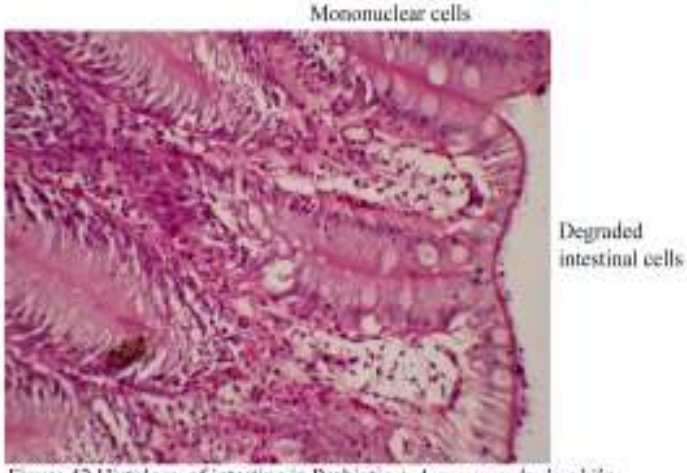

Figure 42 Histology of intestine in Probiotic + *Aeromonas hydrophila* treated catfishes (100X)

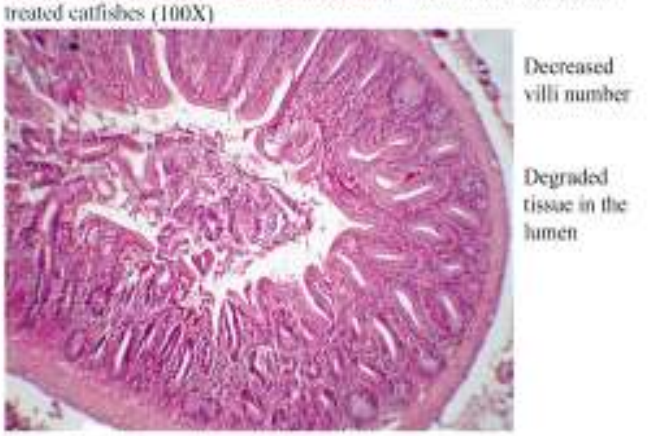

Figure 43 Histology of intestine in Probiotic + *Micrococcus* sp. treated catfishes (100X)

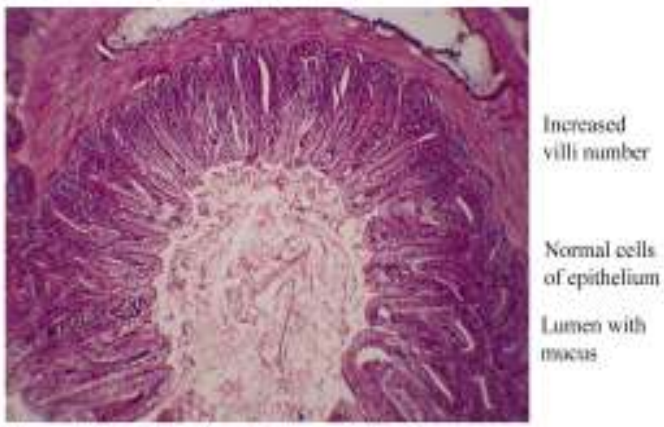

Figure 44 Histology of intestine in Probiotic treated catfishes (100X)

increase in intestinal folds were observed with slight mononuclear cell infiltration indicating presence of normal intestinal microflora (Figure 42 and Figure 43). The increase in number of folds caused by *Lactobacillus sporogenes* is definitely a beneficial effect rendered by the probiotic on the host.

In treatment where fishes were inoculated with pathogenic bacterium (*A.hydrophila)* and probiotic 1, probiotic 2 and probiotic 3, only slight peeling up of intestinal epithelium was seen as compared to the treatment *A. hydrophila* (T2) and *micrococcus* sp. (T3), in which fishes were inoculated with pathogenic bacteria only. Severity of enteritis was very less when probiotics were given along with *A. hydrophila* as characterized by slight degeneration and desquamation as well as peeling up of intestinal epithelium into lumen of the intestine. The same pattern was observed in case of *Micrococcus* sp. and three probiotics treatments.

The results clearly indicate that probiotics have beneficial effects on health of catfishes and aid in their disease abatement. The above results indicated that the three probiotics strains *L. sporogenes*, *Saccharomyces boulardii* and the mixture of soil bacteria had considerable role in the control of bacterial diseases in Indian magur. *L. sporogenes* however gave better results than *Saccharomyces boulardii* and a mixture of bacteria in all the experimental studies.

CHAPTER 5

DISCUSSION

Aquaculture continues to be the fastest growing animal food-producing sector to outpace population growth, with per capita supply from aquaculture increasing from 0.7 kg in 1970 to 7.8 kg in 2006, an average annual growth rate of 6.9 percent. It is set to overtake capture fisheries as a source of food fish. From a production of less than 1 million tonnes per year in the early 1950s, production in 2006 was reported to be 51.7 million tonnes with a value of US $ 78.8 billion, representing an annual growth rate of nearly 7 percent. World aquaculture is heavily dominated by the Asia–Pacific region, which accounts for 89 percent of production in terms of quantity and 77 percent in terms of value (FAO, 2009).

Fisheries and aquaculture, directly or indirectly, play an essential role in the livelihoods of millions of people around the world. In 2006, an estimated 43.5 million people were directly engaged, part time or full time, in primary production of fish either in capture from the wild or in aquaculture, and a further 4 million people were engaged on an occasional basis, out of which 2.5 million from India. In the last three decades, employment in the primary fisheries and aquaculture sector has grown faster than the world's population and employment in traditional agriculture. Eighty six per cent of fishermen and fish farmers worldwide live in Asia, with China having the greatest numbers (8.1 million fishermen and 4.5 million fish farmers). In 2006, other countries with a significant number of fishers and fish farmers were India, Indonesia, the Philippines and Vietnam. Most fishers and fish farmers are small-scale, artisanal fishers, operating on coastal and inland fishery resources. In 2006, the estimated number of fish farmers was nearly 9 million people, with 94 per cent operating in Asia. It is estimated that for every person employed in the primary sector i.e. fish capturing activities and fish farming, another four persons would be employed/required in the secondary sector i.e. fish processing, marketing and service industries, indicating employment of about 170 million in the whole industry. Taking account of dependants, about 520 million people could be dependent on the sector, or nearly 8 per cent of the world population (FAO, 2009).

The fisheries sector, including capture fisheries and aquaculture, contributes significantly to the Indian agricultural economy. Freshwater aquaculture is the largest contributor to aquaculture production and has grown at an average rate of 6.6 per cent per year. More than 7 million fishermen and fish farmers depend on fisheries and aquaculture for their livelihood and a further 6 million people are engaged directly or indirectly in the related industrial activities. Total fish production in India has crossed 6.4 million tonnes per annum to which aquaculture contribute about 2.37 million tonnes (37.0 per cent). The share of fisheries in the national GDP is 1.4 per cent, and in the agricultural GDP its share is 4.5 per

cent (Ayyappan *et al.,* 2006).

In Haryana, the total water area is 85,900 hectares which is about 2 per cent of the total land area of the state, and out of which the total area under aquaculture is 12,885 ha. Haryana ranks second in the country in per hectare fish production after Punjab. In 2007-08, the total fish production from the ponds was 61,000 tonnes with the productivity of 4,800 kg per ha per year. The total income to the state due to fish production is about Rs. 18,300 Lakh. In district Hisar (2007-08), the area occupied under aquaculture is around 1158 ha resulting into 5710 tonnes of fishes production, worth Rs. 1713 Lakh (Annonymous, 2009).

Losses due to fish diseases

At present the world population is more than 6 billion, if it increases at the same rate, it may reach 12 billion by the end of the 21st century. To sustain an ever-increasing population, adequate food sources of ever-increasing protein must be found, placing increasing demands on aquatic animals. Fishes are one of the best protein sources in this category. To suffice the need of fast growing population, aquaculture has shown significant growth in the last two decades and has transformed itself into an industry, both in terms of quantity and value (FAO, 2009). However, disease outbreaks are the major constraint to this venture thereby affecting both economic development and socio-economic status of the local people in many countries of Asia-Pacific region. Disease control in aquaculture industry has been achieved through different means using traditional methods, synthetic chemicals and antibiotics (FAO, 2000). However, each one of these has restricted application due to negative impacts like ineffectiveness when used repeatedly, accumulation of residues, development of drug resistance in pathogens especially bacteria and immunosuppression in the host.

The world's total annual potential for fish production is estimated to be 8.9 million metric tonnes, but total annual actual fish production is 7.4 million metric tonnes; there is a loss of 1.5 million metric tonnes. This loss occurs due to mismanagement, malpractices, unhygienic conditions, and diseases outbreaks (FAO, 2009). Disease outbreaks are recognized as a significant constraint on aquaculture production in all countries (Requelme *et al.,* 1997) and become a limiting factor in the aquaculture production. This is because; fishes are in intimate contact with their aquatic environment, which can contain very high concentrations of viruses, bacteria, and fungi. Many of these are saprophytic, some are pathogenic, and both are very capable of digesting and degrading the fish tissues. Commercial fish farming creates favorable conditions for the development of infectious diseases in fishes. Pathogenic microorganisms are generally accepted to enter the fish through the gills, skin, or gastrointestinal tract. These three routes of entry provide physical and immunological barriers against pathogens, and thus, their integrity, both at the cell and tissue levels, is vital for the later outcome of the host-pathogen interaction (Birkbeck and Ringo, 2005).

The available figures on direct economic losses due to fish diseases indicate the significance of the problem, although social and other related impacts, such as trade and employment issues, drug use and environmental costs have never been properly quantified. Estimates of economic losses due to fish diseases are available since 1990s which suggest that developing countries in Asia lost at least Rs. 7000 crores due to diseases in 1990 alone. Since then, losses have increased. Reports from China suggest that losses due to fungal disease outbreaks were Rs. 4500 crores in 1993. A 1995 estimate suggests that aquatic animal disease and environment-related problems may cause annual losses to aquaculture production in Asian countries more than Rs. 13,500 crores per year (FAO, 1997). According to recent reports, global losses due to fish disease are more than Rs. 18,000 crores and the World Bank is investing Rs. 1240 crores in fish disease research. The above figures provide an indication of the overall economic significance of aquatic animal diseases. So, fish diseases are now considered to be the most limiting factors in the fisheries production sector (FAO, 2003).

In Haryana, average loss to fisheries due to diseases is more than 40 per cent which is estimated approximately to 1920 tonnes fishes per annum that amount to more than Rs. 9600 lakh per year (Annonymous, 2009). The present study also suggests approximately 10 - 40 per cent impending losses to the fish farmers due to fish diseases (Table 9). This is a great economic loss to the state as well as farmers and need immediate remedial measures.

Incidence of fish diseases

Natural infections in channel catfish (*Ictalurus punctatus*), brown bullhead (*Ictalurus nebulosus*), blue catfish (*I. furcatus*), danio (*Danio devario*), green knifefish (*Eigemannia virens*), walking catfish (*Clarias batrachus*) and white catfish (*Ictalurus catus*) have been reported. Factors including the disease and pollution both cause massive mortality of aquatic animals in the many aquaculture countries leading to major loss to the fish farmers (Smith, 2006). Crumlish *et al.* (2002) noticed Edwardsiellosis disease with typically necrosis lesions in freshwater catfish, *Pangasius hypophthalmus* cultured in the Mekong delta, Vietnam caused by *Edwardsiella tarda*. Danley *et al.* (1999) noticed enteric red mouth disease which exhibited extraordinary hemorrhagic rings around the eyes and raised hemorrhaged areas overlying the frontal foramen. The symptoms caused by the infection of *Yersinia ruckeri* in two separate channel catfish farms in Arkansas, USA included abnormal swimming, lethargy, loss of equilibrium and exophthalmia and the same types of symptoms were observed by injecting pathogen intraperitoneally. Epizootic Ulcerative Syndrome (EUS) in catfishes like walking catfish or Indian Magur (*Clarius batrachus* and *C. gariepinus*) and *Ictalurus punctatus* caused by fungi and bacterial pathogens inflict a big loss to fish farmers every year (OIE 2006).

Fungal diseases viz. saprolegniasis and branchiomycosis caused major economic losses in aquaculture and are second to bacterial diseases in terms of economic loss (Ramaiah, 2006, Bangyeekhun *et al.* 2001). Bruno and Wood (1994) and Hatai and Hoshiai (1994) observed 50% mortality in Coho salmon (*Oncorhynchus mykiss* Walbaum) and *Anguilla anguilla* due to the infection of *Saprolegnia parasitica* Coker in Miyagi Prefecture, Japan. In Southeastern United States, major financial losses occur in channel catfish farming due to a condition called "winter kill." Some catfish farmers have reported losses to fish production up to over 50% leading to an economic loss of $40 million (Li 1996; Doriga and Martinez, 1998; Goodwin 2002; Lakshmanaperumalsamy *et al.,* 2005). Fungal infections are generally restricted to chronic yet steady losses.

In the present study, the magur (Clarius *batrachus*) was found to be infected with hemorrhagic septicemia, ulcerative disease, fin and tail rot and saprolegniasis (Table 7) in the surveyed catfish farms during the month of March, April and August, September (post winter and post rainy seasons)**;** the disease appeared in a cyclic manner year after year during these periods (Table 6). The rainfall and low temperatures seemed to be a stimulus for the appearance of catfish disease, when catfishes are considered to have low immunity to the pathogens (Catap and Barry, 1988). The level of infection ranged from 7.52 to 32.44 per cent in fingerlings and adult catfishes but 20.17-36.72 per cent in eggs and hatchlings of catfishes in the month of July and August (Table 8). Ahmed and Rab (1995) also reported that fish ponds were worst affected during September and October when, as high as, 27 per cent fishes were found to be infected with fish pathogens. These diseases, therefore, seemed to inflict heavy economic losses to the farmers.

In Haryana, Indian major carps (*Catla catla*, *Labeo rohita*, and *Cirrhinus mrigala*), common carp (*Cyprinus carpio*), silver carp (*Hypophthalmichthys molitrix*) and catfishes (*Clarius batrachus*, and *C. gariepinus*) are cultured in village ponds and fish farms. The state government has already started a project in 2006-07 to introduce and culture Indian magur (*C. batrachus*) on large scale although culturing of catfishes in the village ponds of the state were banned before 2006-2007. The traditional aquaculture, in recent years, has entered into commercial activity involving heavy inputs. Simultaneously, the diseases of all kinds are also now known to occur on an increasingly large scale. The mortality is not the only criterion to evaluate the effects of catfish diseases, even the morbidity, which leads to weight losses and poor growth in surviving catfishes, contributes substantially to the farm losses.

Characterization of fish disease

Proper characterization and identification of fish diseases is very important before applying any treatment/measure to control. The preliminary characterization is often based on the study of symptomology or pathology of the diseases and the characters of the lesions

occurring there from. In the same practices and sequel to, the symptoms of the diseased catfishes collected from different catfish farms in the present study were recorded. These included the rottening of gill lamellae, hemorrhages particularly on head, gills and lateral side, ulcers on lateral side of the body, fin and tail rot and secretion of the mucus from the wounds (Table 7). The symptoms of diseased fishes were same as that reported by Karunasagar, 1989. On the basis of these symptoms, the diseases identified were hemorrhagic septicemia, ulcerative disease, fin and tail rot and saprolegniasis.

These diseases in catfishes were reported earlier also by many other workers from India (Das *et al.,* 1990; Kumar *et al.,*1991; Mukherjee *et al.,*1991; Das, 1992; Nayak, 1993) and different parts of the world (Dey, 1989; Roberts, 1997; Cerro *et al.,*2002). The iteology of the fish revealed that surviving fish typically had lesions of varying degrees of severity (Ducarme, and Micha, 2003). These appeared as red-spots, hemorrhages particularly on head and lateral side, blackish burn-like marks, or deeper ulcers with red centres and white rims (Gopalkrishan, 1961; Jhingran and Das, 1990; Jhingran, 1991). The ultimate result was occurrence of mass mortality of the fishes.

The fish pathogens

It is important to identify the bacterial fish pathogens which are causing diseases in Haryana. The detection and treatment of disease in fish is an important area which has long been neglected. The most effective way of controlling a disease is to find out the cause and then ascertain its treatment.

Chaudhary (1998) reported the involvement of aeromonads and pseudomonads in the ulcer type disease of freshwater fishes. The involvement of A. *hydrophila, Micrococcus* sp*., Streptococcus* sp. and *Shigella* spp. might be the cause of highly significant secondary infection (ulcer formation) agents in the fish (Lilly *et al.,* 1992). Dhanraj *et al.* (2008) identified and reported 17 isolates from the liver, gills, intestine and muscle of diseased *Clarius striatus*; among these 17 isolates, dominant bacterial and fungal species were *Aeromonas hydrophila*, *Flavobacterium* sp., *Aspergillus flavus* and *Aphanomyces invadans*. *Aeromonas hydrophila*, *Aeromonas* sp., *Flavobacterium* sp., *Vibrio vulnificus*, *Staphylococcus* sp., *Yersinia enterocolotica*, *Shigella* sp., *A. salmonicida*, *Aspergillus flavus*, *Aspergillus* sp. and *Aphanomyces invadans* constituted the most frequently isolated bacterial and mycotic flora with a prevalence of greater than 10%. From 25 microbial colonies in gill samples, 11 colonies of *Aeromonas hydrophila* were identified predominantly. *A. hydrophila* remains in the center stage so far as Indian major carps are concerned. The disease due to *A. hydrophila* was classified under two categories namely hemorrhagic septicemia and chronic ulcerative form (Gopalkrishan, 1961). *A. hydrophila* is also suspected to be the principal causative agent of ulcerative disease noticed in cultured fish in indo-pacific region

(Tonguthai, 1985) and Southeast Asian countries (Robert, 1989) like Thailand (Liobrera and Gacutan, 1987) and Malaysia (Torres *et al.,* 1993). In the present study, *Penicillin* sp. and *Aspergillus ochracaeus* were detected as very common fungal pathogens, and these were present in the diseased catfishes sampled. These results are in agreement with the findings reported by Sarker *et al.* (1999) who reported that the fungus (*Saprolegnia* sp.) was variably found in the infected fishes. Among the investigated fishes, *Clarius batrachus* was found to be severely affected by this disease.

Bacterial pathogens cause heavy mortality in both cultured and wild fish species in different parts of the world (Hambal, 1985). These are either obligate or facultative pathogens. Facultative bacterial pathogens become a potential threat when fish are under environmental and physiological stress (Wedemeyer, 1970). Six gram negative rods (*Aeromonas, Proteus, Citrobacter, Pseudomonas, Flavobacterium* and Chromobacterium**)** and three gram positive cocci **(***Micrococcus*, *Streptococcus* and *Staphylococcus*) genera of bacteria which were potentially pathogenic were identified from *Aristichthys nobilis* and grass carp (*Ctenopharydon idella*) fingerlings (Shamsudin, 1986). Welker *et al.* (2005) isolated and identified *Flavobacterium columnare* from the culturing tank and tissues of channel catfish (*Ictalurus punctatus*) by PCR (polymerase chain reaction), and observed the similarity between the symptoms of natural infection by injecting the bacterial pathogen intraperitoneally into fingerlings (*in vivo* pathogenicity) and giving bacterial bath.

Kozinska (2007) isolated and identified *Aeromonas* isolates viz. *A. hydrophila*, *A. bestiarum*, *A. salmonicida* (motile biogroup), *A. caviae*, *A. sobria*, *A. veronii*, *A. jandaei*, *A. encheleia* and *A. allosaccharophila* from cultured common carp and rainbow trout fishes. The pathogenicity of all isolates was assayed on the basis of hemolytic and proteolytic reactions and challenge tests; and observed that all isolates of *A. hydrophila*, *A. bestiarum*, *A. salmonicida* and *A. veronii* were pathogenic and virulent; and found to present in all diseases and healthy common carp and rainbow trout fishes. Eggs are badly damaged by *Saprolegnia* when infected (Willoughby, 1978) during artificial incubation in cold water in salmonids as well as in warm water in cyprinids, cichlids and clariids (Alderman and Polglase, 1984). Invasion is promoted by existing necrotic substances such as unfertilized and damaged eggs (Neish and Hughes, 1980).

Saprolegnia fungi are opportunistic facultative parasites. They are necrotrophs when they grow on living sources and saprotrophs when they derive their nutrition from non-living sources. *Saprolegnia* often acts as a 'wound parasite' and handling fish often predisposes them to infection (Willoughby 1978). However, there is good evidence to suggest that *Saprolegnia* fungi can act as primary invaders, in physiologically debilitated (example-decline in mucus production) and immunologically compromised fish or in "stress" situations (Neish and Hughes, 1980).

In the present, study for the confirmation of the causative organism, three series of tests were performed. These included primary, secondary and tertiary. Identification of bacteria was done with the help of Bergey's manual of microbiology (Krieg and Holt, 1984) in old method. All the characteristics of obtained colony were matched with different colony characteristics present in this manual. Under the primary test, the bacteria were differentiated with gram reaction into gram positive or gram negative. Under the secondary tests, pure colonies of bacteria were isolated and each bacterial isolate was subjected to many biochemical tests.

The results of these tests were then subjected to a computer software programme, 'PIBwin' (Website: *http//www.soton.ac.uk*). It is an advanced and very sensitive programme in which results of different tests are put together and within few seconds, tentative identification of bacterium is done. It is very easy and time saving. Based on this programme, PIBwin Identification scores were allotted to each bacterial isolate. These scores were then matched/ compared with the standard scores of the reference bacterium, and on the basis of similarity of the scores, the bacterial isolates were tentatively identified. The tertiary tests were done for the confirmation of these bacteria with the help of selective media used for culturing that particular bacterium. The growth of bacterium on the selective medium confirmed the presence of reference bacterium.

In the present study, in the recovered bacterial isolates from the diseased fish samples collected from different fish farms, *Aeromonas hydrophila* was present in all the catfish samples collected from the two catfish farms (Nagalpur and CCS HAU catfish farm); other species like *Micrococcus* sp*, Streptococcus faecalis, Streptococcus haemolyticus, Staphylococcus aureus,* and *Klebseilla oxytoca* were present selectively (Table 8-11).

Pathogenicity test

To confirm whether the particular bacterium/fungus were pathogenic or not; this test is carried out. Ryan and Ray (2004) reported the -α zone of haemolysis on blood agar plate of pathogenic bacteria. The Alpha haemolysis is the reduction of hemoglobin to methemoglobin in the medium surrounding the colony, causing a greenish decolorization of the medium where as beta haemolysis is defined as the lysis of red blood cells, resulting in a clear zone surrounding the colony.

In the present studies, six bacterial isolates of this study were found to be pathogenic to the magur catfish as compared by inoculation method, where *Aeromonas hydrophila* was found to be the worst, followed by *Micrococcus* spp., *Staphylococcus aureus, Streptococcus faecalis, Streptococcus haemolyticus* and *Klebseilla oxytoca* (Table 14). When these bacteria were inoculated into the catfish's, thier longevity period of the latter decreased (Table 15). This indicated that the incubation period of bacteria slowed down the immune response of the

catfishes. Multiple infections of bacteria increased the severity of disease, and the longevity period of the fish became only seven days (Table 15). These results directly correlate with the findings of Sarker *et al.* (1999) who reported that different bacterial mixtures found in the pond water caused earlier mortality in fishes. The *in vivo* pathogenicity test of all the bacteria produced characteristic lesions which were very similar to the ulcers present in hemorrhagic septicemia and ulcerative disease. These results support the findings of Lilley and Roberts (1997) who reported that the hemorrhages and ulcers were formed on the body of fish when infected with bacteria.

Effect of pathogenic bacteria on hematological parameter

Being poikilothermic animals, fishes are subjected to change in the body functions in response to changes in environment in which they live (Hambal, 1985). Hematological indices are very important parameters for the evaluation of physiological status of the fishes. Hematological parameters reflect the poor condition of fish more quickly than other commonly measured parameters and reflect the gravity of these changes. That's why these are considered to be patho-physiological indicators of whole body and, therefore, are very important in diagnosing the functional and structural status of fishes invaded by the pathogens (Golovina, 1996). Values of hematological parameters of fishes can be affected by environmental and biological factors such as age, weight, sex, food, bacteria, viruses, fungi and water quality parameters (Das and Das, 1993). A number of hematological indices such as hemoglobin, red blood cells, and white blood cells, packed cell volume and so on are used to assess the functional status of oxygen carrying capacity and defense system of the blood stream which enhance the immune system of the fishes (Chinabut *et al.,* 1995). However, very scanty work has been done on the blood parameters. Palikova *et al.* (2004) and Ishikawa (1998) observed decrease in the level of blood in the common carp after exposure to cyanobacteria extract. Ranzani-Paiva *et al.* (2004) showed that the decrease in erythrocytes count and haematocrit/PCV of Nile tilapia when inoculated with *Mycobacterium marinum* may lead to a tendency to anemia. Mona *et al.* (2008) and Zaki *et al.* (2008) noticed a significant decrease in red blood cell, hemoglobin, packed cell volume in *Tilapia nilotica* and amount of iron in water and significant increase in aspartate aminotransferase, urea, creatinine, sodium, cortisol, insulin, and glucose in this fish. A massive fungal growth in eyes, gills, fins and in localized areas of the skin and marked degenerative, necrotic, and inflammatory reactions were also seen in the infected fishes.

Results of all these studies resemble those of the present investigation showing that fish inoculated with pathogenic bacteria showed a decrease in its blood parameters. Hemoglobin level reduced approximately to 50 per cent in its value (Table 18); erythrocytes count reduced approximately to 40 per cent in its value (Table 19); leukocyte count increased

to approximately 30 to 45 per cent in its value (Table 20), and packed cell volume reduced approximately to 40 per cent in its value (Table 22) in three weeks. This clearly indicated a marked changes in the hematological parameters of diseased fishes.

Use of probiotics as an alternate method of disease control

In large aquaculture systems, traditional methods are ineffective against controlling new diseases (FAO, 2000). Therefore, alternative methods need to be developed to maintain a healthy microbial environment in the aquaculture systems and the health of the cultured organisms. There is reduced consumer preference for aqua - products treated with antibiotics. Therefore, use of probiotics is one of such methods that are gaining importance in controlling potential pathogens.

Effect of probiotic on hematological parameter

Probiotics are commonly defined as mono- or mixed cultures of live microbes that, when applied to animal or human, generate a beneficial effect on health of the host. These beneficial effects include disease treatment and prevention as well as improvement of digestion and absorption in the host (Havenaar and Huis in't Veld, 1992). Hematological parameters of fish are used as indicators of their physiological state, and their study has become widespread in the control of pathogens and manipulation of stress in fish (Barisal *et al.,* 1979). Rajesh *et al.* (2008) noticed increased amount or number of total erythrocyte count, total leucocytes count (TLC), hemoglobin, total protein, albumin, globulin, albumin-globulin ratio, alkaline phosphatase activity, alanine aminotransferase activity and aspartate aminotransferase activity in *Labeo rohita* provided with *Bacillus subtilis* as probiotics. Irianto and Austin (2002) observed an increase in erythrocyte count in fish fed on probiotic bacteria. Selvaraj *et al.* (2005) investigated increase in total leukocyte counts and an increase in proportion of neutrophills and monocytes when the fish fed with *Saccharomyces cerviase*.

Irianto and Austin (2002) used dead probiotic cells to control disease in *Onchorhynchus mykiss* (Walbaum) and observed higher number of leucocytes, erythrocytes, and macrophages. Rengpipat *et al.* (2000), Siwicki *et al.* (2003) and Brunt *et al.* (2007) found the development of disease resistance in due to the use of probiotics *Bacillus* sp. As probiont and also reported an increase in the level of selected hematological parameters in the blood (red blood cell count (RBC), haematocrit (Ht), hemoglobin (Hb), various leukocyte counts, the total leukocyte level and mean corpuscular volume (MCV), mean hemoglobin concentration (MCH) and mean corpuscular hemoglobin concentration (MCHC). Das *et al.* (2006) and Nayak *et al.* (2007) studied the hematological parameters in the three species of Indian major carps viz. catla (*Catla catla*), rohu (*Labeo rohita*) and mrigal (*Cirrhinus mrigal*) for 21 days by subjecting fingerlings to acidic (pH 5.5, 6.5) and alkaline (pH 8.0, 8.5 and 9.0) waters and control groups were maintained at neutral pH. A change in water pH either to

acidic or alkaline conditions exerted stress in fish characterized by incrased size of erythrocytes, production of immature erythrocytes and reductions in the total erythrocyte counts, hemoglobin and serum protein content. There were also increases in total leukocyte counts and blood glucose. However, the degree of these responses varied among the three species. Rohu was found to be least affected followed by mrigal to the stress of altered water pH, while catla was the most vulnerable to pH changes. Jha *et al.,* (2007) noticed significantly higher respiratory burst activity, hemoglobin content, total erythrocyte count, blood serum, total leukocyte count, serum globulin content, serum lysozymes activity and relative survival percentage in *C. catla* juveniles when fed with omega-3 fatty acid (3%) in the diet.

When the probiotic effect is supposed to be nutritional, the candidate probiotics could be added to the culture of the aquatic species and their effect on growth and survival parameters could be assessed. However, when biological control of the microbiota is desired, representative *in vivo* challenge tests seem to be the appropriate tool to evaluate the potential effect of the candidate probiotic on the host (Gildberg *et al.,* 1995). The approach outlined above should result in formulating a set of strains with a well-established probiotic effect on the target organism without affecting other possibly involved trophic levels. Comparative pilot experiments under hatchery or grow out conditions in the farms should be performed to estimate the economic consequences of the probiotic application. An important factor in the economic evaluation is the mass production of the probiont. Also, effective legislation, if any, should be taken into account before commercial application is begun. Finally, a cost-benefit analysis will determine whether the probiotics could be applied in practice or not (Verschuere *et al.,* 1999; Ruiz-Ponce *et al.,* 1999; Douillet, 2000). In general, the selection criteria for a bacterium to be used as a `probiont' should be followed. It should be non-pathogenic; withstand incorporation into a delivery vehicle at high cell counts, and remain viable throughout the shelf-life of the product; withstand transit through the gastrointestinal tract, show acid and bile tolerance (Rollo *et al*., 2006); be able to adhere to cells of the intestinal epithelium and/or colonize the lumen of the tract; show antagonistic activity towards enteric pathogens and/or provide demonstrated health benefits (Chythanya *et al.,* 2002).

The results of present study revealed that probiotic had a positive effect on haemoglobin level which increased approximately to 24 per cent in its value (Table 18), erythrocytes count which increased approximately to 10 per cent in its value (Table 19), leucocyte count which decreased ased approximately to 8 per cent in its value (Table 20), and packed cell volume increased approximately to 20 per cent in its value (Table 21). This clearly indicated that there was positive response in the values of haematological parameters of fishes.

Effect of probiotics on survival and growth of treated fishes

In the probiotic treated fishes, growth rate and survival are significantly greater than the control group (Rengpipat *et al.,* 2008; Prabhu *et al.,* 1999; Ahilan *et al.,* 2004). This has been reported in many fish species. Chabrillon *et al.* (2006) in *Saparus aurta* and Fazeli and Takami (2006) in nauplii (*Artemia urmiana*) observed the maximum survival rate in probiotic fed fishes. Rajesh *et al.* (2008) noticed higher survival in probiotics fed fry of Indian major carps *Catla catla* during packing, transportation, and post transportation. Ghosh *et al.* (2008) noticed that the addition of bacterial cells (*B. subtilis*) in the rearing water resulted in greater survival, faster growth rate, greater length and weight increments of the livebearers (*Poecilia reticulate* Peters, *Poecilia sphenops* Valenciennes, *Xiphophorus helleri* Heckel and *Xiphophorus maculatus* Gunther; and also observed significantly lower concentrations of dissolved organic matter and total ammonium nitrogen; counts of motile aeromonads and total coliforms in the pond water. Tovar-Ramirez *et al.* (2004) noticed that the survival of larvae of sea bass was significantly higher than the control when fed 1.1 per cent live yeast as a probiotic. Kennedy *et al.* (1998) observed increased survival, phagocytosis and antibacterial activity in juveniles of white shrimp, after administration of a mixture of bacterial strains (*Bacillus* sp. and *Vibrio* sp.) and a protective effect against the pathogens *Vibrio harveyi* and white spot syndrome virus. Selvaraj *et al.* (2005) also observed the same results by administration of β-glucan (extracted and purified from *Saccharomyces cerviaseae)* in *C. carpio* through different routes viz. administrated intraperitoneally, bathing and orally. They also reported significantly enhanced per cent survival when 500 pg of β-glucan was injected intraperitoneally, but bathing and oral administration did not influence. Likewise, *Streptococcus faecium* improved the growth and feed efficiency of carp (Noh *et al.,* 1994; Bogut *et al.,* 1998; Irianto and Austin, 2002) by stimulating appetite and improve nutrition by the production of vitamins, detoxification of compounds in the diet, and by the breakdown of indigestible components. Several probiotic bacterial species including *Lactobacillus sp.* (Jonsoon, 1986) and mixed cultures of different bacteria (Lessard and Brinson, 1987) were used to improve the nutrition level and immunity of aquacultural animals against pathogenic microorganisms. In addition, the use of antibiotics can be reduced and frequent outbreaks of diseases can be prevented. Lactic acid bacteria had an effect as growth promotor on the growth rate in carps (Noh *et al.,* 1994). Also, *Enterococcus faecium* has been used as probiotic to improve growth when fed to sheath fish, *Silurus glanis* L. (Bogut *et al.,* 2000). Riquelme *et al.* (1997) studied the naturally occurring bacteria which are able to promote the growth and survival of oyster *(Argopecten purpuratus)* larvae by inhibiting the activity of other bacteria that flourish in hatchery cultures.

In the present study, a significant difference in growth was observed between the probiotic treated catfishes and the control. The survival rate of fish increased and reached up

to 100 per cent in the probiotic treated fishes. Over all, length and weight gain and survival were found to increase in magur (*C. batrachus*) fed a diet containing probiotic. The probiotics used in present study seemed to act as growth promoters as these caused significant increase in growth in *C. batrachus.* The effect of probiotic 1 was better than probiotic 2 and 3 the length gain on the 60th day of the experiment was found to be maximal in catfishes fed with probiotic 1 which was 91 per cent. On the other hand the fishes fed with probiotic 2 showed 66 per cent increase in length. The length gain was 36 per cent in the fishes fed with control feed (Table 23). Likewise, the weight gain on the 60th day of the experiment was found to be maximal in magur fed with probiotic 1 which was 91 per cent. On the other hand, the fishes fed with probiotic 3 showed 76 per cent a per cent increase in weight. The weight gain was 93 gms in the control catfishes (Table 25).

This contention has been supported by the finding of Noh *et al.* (1994) who reported that probiotics acted as growth promoters in carps. Metaillier and Hollocou (1993) in Atlantic salmon and Gildberg *et al.* (1995; 1997) in Atlantic cod also made the same observations. Gatesoupe (1991) also stated that the probiotic incorporated feed had a definite role in enhancing the growth of channel catfish and turbot larvae. The present study also confirmed the findings of Maeda and Liao (1992), Garriques and Arevalo (1995), and Ringpipat *et al.* (1998) who reported a significant increment in growth of *Penaeus monodon* fed with probiotic incorporated feeds.

In the present study, the effect of probiotic 1 was better than probiotic 2 than 3. The former contained *Lactobacillus sporogenes* bacteria. The purpose of parallel use of three probiotics was to check the relative effectiveness of these probiotics to control the disease and to ascertain the role of other components in the probiotics responsible for that.

The mode of action of the probiotics is rarely investigated, but possibilities include competitive exclusion principle i.e. the probiotics actively inhibit the colonization of potential pathogens in the digestive tract by antibiosis or by competition for nutrients and space, alteration of microbial metabolism, and by the stimulation of host immunity (Irianto and Austin, 2002). The major taxonomic groups contributing to the healthy intestinal flora of fish species include *Vibrio, Lactobacillus, Acinetobacter,* and *Achromobacter,* followed by *Micrococcus, Bacillus* and representatives from the family Enterobacteriaceae (Liston, 1957; Colwell, 1962; Newman *et al.,* 1972; Ringo and Strom, 1994). In aquaculture, non-pathogenic strains of identified bacteria have been successfully used as probiotics to control the diseases in fish (Austin *et al.,* 1995; Gomez-Gil *et al.,* 2002; Chythanya *et al.,* 2002). These probiotic bacteria suppress proliferation of pathogenic and opportunistic bacteria in the mucus in intestine as well as ambient environment of the fishes simultaneously, (Skjermo and Vadstien, 1999). Consequently the probionts reduce the incidence of diseases. There are several modes

of probiotic action in the aquatic environment. These include improved feed conversion efficiency and feed utilization, higher adhesion capacity to the intestinal mucosa and reduction of adherence of pathogenic bacteria, production of extra-cellular antibiotics or iron chelating agents (siderophores) which prevent the growth of almost all pathogenic bacteria (Verschuere, *et al.*, 2000) and improvement of water quality (bioremediation) especially in the pond and reducing the problem of red tide planktons (Watson *et al.,* 2008). This ultimately improved feed conversion efficiency and feed utilization by the cultures fishes.

Effect of probiotics on immune system

In intensive aquaculture conditions, fish are badly affected and are often infected by different microbial pathogens that have been treated with chemotherapeutic substances of which antibiotics are intensively used. These curative substances produce the problem against the action of bacterial drug on one hand and the public health hazards on the other hand (Robertson *et al.,* 2000). These awaited drawbacks enforced the fish pathologists to seek for other alternatives. The use of natural immunostimulants in fish culture for the prevention of diseases is a promising new development and could solve the problems of massive antibiotic use. Natural immunostimulants are biocompatible, biodegradable, and safe for both the environment and human health. Moreover, they possess an added nutritional value. The use of biological products namely the probiotic is recently the goal of the disease biocontrol strategy in aquaculture as they improve the fish health and modify the fish associated microbial community (Ellis, 2001).

The intestines are the primary immune organ in the body. The bowel- associated immune system contains the largest mass of lymphoid tissue in the human body, a vitally important component of total host immunological capacity (Isolauri *et al.,* 2001). Bowel mucosa and lymphoid tissue are closely linked immunologically with gastrointestinal microflora. Substantial evidence associates probiotic bacteria with modulation of host-mediated immune responses. Probiotic bacteria boost both innate and acquired immune responses. These include increases in circulating lymphocytes, stimulation of phagocytosis and antigen specific antibody secretion, and increased production of interferon-g and other cytokines. Immunological enhancement properties are best documented for *Lactobacillus casei, L. rhamnosus, L. plantarum, L. bulgaricus, L. acidophilus, B. bifidum,* and *Bacillus breve.* While these species are almost certainly not the only probiotics that modulate immune function, they should definitely be part of any therapeutic probiotic which supports the immune system (Isolauri *et al.,* 2001; Bourlioux *et. al.,* 2002; Rautava *et al.,* 2005). Selvaraj *et al.* (2005) investigated the enhanced immune response of P-glucan (extracted and purified from *Saccharomyces cerviaseae*) administered in *C. carpio* on day 1, 3 and 5 through different route (intraperitoneally, bathing and orally). Sakai *et al.* (1995) stimulated non-

specific immune system by administrating probiotics bacteria *Clostridium butyricum* to rainbow trout orally and reported enhanced resistance of fish to vibriosis by increased phagocytic activity of leucocytes. The production of immunoglobulin A is also stimulated that helps protecting the mice against *Salmonella typhimurium* (Perdigon *et al.,* 1990). Nikoskelainen *et al.* (2003) reported that the selected probiotic bacteria may have an impact on the specific and innate immunity of fish. They recorded that immunoglobin (1g) level increased significantly with the probiotic feed group. However, much less work has been done on the immunological enhancement of defense mechanisms of fish by probiotic bacteria or the protective mechanisms of probiotic bacteria in fish (Nikoskelainen *et al.,* 2003). Brunt and Austin (2005) used probiotics *A. sobria* GC2 to control lactococcosis and streptococcosis caused by *L. garvieae* and *S. iniae* respectively, in rainbow trout, *Oncorhynchus mykiss*, and carp, *Cyprinus* sp. and observed increased number of leucocytes and enhanced phagocytic and respiratory burst activity.

This study therefore, clearly reveals that probiotics are very effective in controlling the catfish diseases in order to improve their health status. The present study further shows that catfishes treated with probiotics showed increase in the level of different hematological parameters viz. hemoglobin, erythrocytes count, leucocytes count and packed cell volume; growth rate in the form of length and weight gain, and increase in the survival of catfishes significantly.

Effects of probiotics on intestinal histopathology

The intestine is the main immunological organ: it contains 50% of all reticulo-endothelial and other immune cells, and produces the greatest amount of secretory IgA (Hulsewe *et al*., 1999). The gut-associated lymphoid tissue (GALT) represents the largest mass of lymphoid tissue in the human body (Isolauri *et al*., 2001). The stimulation of host immunity is related to the ability of microorganisms to adhere to the mucosa and interact with the gut-associated lymphoid tissue (McGhee *et al*., 1992). The ability of probiotics to adhere to the intestinal cells reduces pathogenic bacterial colonization and thus, contributes to stop pathogenic bacterial translocation. In this context, probiotics, currently defined as live microflora feed supplement that beneficially affects the host animal by improving its intestinal microbial balance (Fuller, 1989), and may enable valuable modifications of the immune system (Isolauri, 2001).

The overall results of histo-morphometric study demonstrated a recovery from the gut atrophy status associated with pathogenic bacteria after the addition of probiotic 1 (*L. sporogenes*), probiotic 2 (*S. boulardii*) and probiotic 3 (mixture of many soil bacteria) in the diet of catfishes. Moreover, when the three probiotics groups were compared with groups containing pathogenic bacteria (*A. hydrophila* and *Micrococcus* sp.), the probiotic diet was

associated with normal mucosa living in the small intestine. Earlier also, enhanced mucosal trophism was found with the addition of *Streptococcus* and *Lactobacillus in* the fish diet as probiotic bacteria (Ishikawa *et al*., 1999; Allori *et al*., 2000). These results are most probably due to enhanced short chain fatty acis (SCFA) formation as induced by probiotics. Short chain fatty acids (SCFA) are the best fuel for the colonocytes and act directly as trophic source for the colonic mucosa (Roediger, 1980; Sakata, 1990; Gildberg and Mikkelsen 1998). The findings of best recovery of atrophy at the colon were also probably due to short chain fatty acids production.

The overall results of this study showed that the addition of the three probiotic positively influenced a more rapid restoration of the gut atrophy associated with pathogenic bacteria. Although the findings of an experimental study should be transposed to the clinical setting with caution, it could be concluded that probiotic 1 (*L. sporogenes*), probiotic 2 (*S. boulardii*) and probiotic 3 (mixture of many soil bacteria) if added to a diet enhanced the recovery of the gut atrophy induced by pathogens.

From the foregoing account it is evident that hemorrhagic septicemia appeared in the Indian magur in cyclic pattern in post winter and post rainy seasons, when the ambient temperature was low. Six pathogenic bacteria were found to infect this fish. Three probiotics were used to control these bacteria. These probiotics enhanced the blood haemoglobin, red blood counts (RBC), packed cell volume (PCV) and decreased the counts of white blood cells. These probiotics also helped in recovery of atrophy of intestinal epithelial mucosa. Out of these three probiotics tested, probiotic 1 was found to be better than probiotic 2 and probitoic 3 in its efficacy.

CHAPTER 6

SUMMARY AND CONCLUSIONS

The world's total annual potential for fish production is estimated to be 8.9 million metric tonnes but total annual actual fish production is 7.4 million metric tonnes; there is a loss of 1.5 million metric tonnes. This loss occurs due to mismanagement, malpractices, unhygienic conditions and diseases outbreaks. The available figures on direct economic losses due to fish diseases indicate the significance of this problem. Estimates of economic losses due to fish diseases are available since 1990s which suggest that developing countries in Asia lost at least Rs. 7000 crores due to diseases in 1990 alone. Since then, losses have increased. A 1995 estimate suggests that aquatic animal disease and environment-related problems may cause annual losses to aquaculture production in Asian countries more than Rs. 13,500 crores per year. According to recent reports, global losses due to fish disease are more than Rs. 18,000 crores and the World Bank is investing Rs. 1240 crores in fish disease research. The above figures provide an indication of the overall economic significance of aquatic animal diseases. So, Fish diseases are now considered to be the most limiting factors in the fisheries production sector.

In Haryana average loss to fisheries due to diseases is more than 40 per cent which is estimated approximately 1920 tonnes fishes per annum that amount to more than Rs. 9600 Lakh per year. The present study also suggests approximately, 10 - 40 per cent impending loses to the fish farmers due to fish diseases. This is a great economic loss to the state as well as farmers and need immediate remedial measures.

In large aquaculture systems, traditional methods are ineffective against controlling new diseases. Therefore, alternative methods need to be developed to maintain a healthy microbial environment in the aquaculture systems and the health of the cultured organisms. There is reduced consumer preference for aquacultural products treated with antibiotics. Therefore, use of probiotics is one of such methods that are gaining importance in controlling potential pathogens.

In the present investigations, surveys were carried out to collect the samples of diseased fish in three fish farms of district Hisar from, April, 2007 to June, 2009 at monthly intervals. The Indian magur fish (*Clarius batrachus*) was found to be infected with disease in each fish farm. The level of infection in diseased fish ranged from 7.52 to 36.72 per cent of the total stocking density during the survey. Characterization of disease was done on the basis of symptoms present on the body of fish. The bacterial pathogens were isolated and identified from affected tissue samples (skin lesions/ muscle) of infected fishes by culturing on nutrient agar plate under aseptic conditions and the cultured plates were incubated in B.O.D. at 30 ±

1^0C for 24 hours. The bacteria were subjected to a number of important biochemical tests reported by Krieg and Holt (1984) in "Bergey's Manual of Determinative Bacteriology" and Quinn *et al*., (1994) in "Clinical Veterinary Microbiology" and tentatively identified with the help of Computer Programme, PIBWin. The confirmation of these bacteria was done with the help of growth of pure culture of these on selective media. The other associated pathogens were the fungii. The fungus was cultured on the Czepacks medium and stained by following the method of OIE, 2003. The physico-chemical parameters like temperature, salinity, dissolved oxygen (DO) and pH (Hydrogen ion concentration) were recorded from each fish farm.

Six bacterial types were isolated and identified from the diseased fish. These were: i) *Aeromonas hydrophila,* ii) *Micrococcus* sp., iii) *Streptococcus faecalis,* iv) *Klebsiella oxytoca,* v) *Streptococcus haemolyticus* and vi) *Staphylococcus aureus.* The pathogenicity of these bacteria was checked through *in vitro* and *in vivo* tests. *In vitro* pathogenicity test of different bacteria was done by streaking pure culture of isolated bacteria on sheep blood agar plate. The pathogenicity of bacteria was confirmed by determining alpha and beta pattern of haemolysis of all bacterial isolates on the Sheep blood agar plates (Ryan and Ray, 2004). All six bacteria were found to be pathogenic. In *in vivo* pathogenicity test, the catfish fish weighing 20g were taken and acclimated for one week in flat bottomed cubical tubs of 40 litres of water capacity, in the laboratory. In this experiment, pure culture of an isolated and identified bacterium was inoculated and bacterial suspension with known viable counts into the intra peritoneal cavity of magur. The symptoms of disease appearance were examined and the pathogenicity of isolated and identified bacteriawas again tested. Incubation period of different bacteria in fishes and longevity of the fish were recorded up to eight weeks. On the basis of symptoms of the disease appeared *Aeromonas hydrophilla* was more pathogenic as compared to other bacteria. Fish showed symptoms after seven days of post-inoculation when administered with A. *hydrophilla.* The fish inoculated with this bacterium remained alive for 13 days after post-inoculation. These organisms acted synergistically with fungii in quick expression of the pathogenicity in the experimental fishes.

The three probiotics used to control the infections caused by pathogenic bacteria and fungi were commercially available. The composition of both the probiotics were different. The antagonistic potential of available probiotics against bacteria and fungus was done by using poisoned food technique in *'in vitro'* test. The zone of Inhibition of probiotic was detected by using fresh culture of bacteria in nutrient broth. A spot of 10 µl of probiotic was put as measure of anti- pathogenic activity of probiotic against the pathogenic bacteria that was inoculated in nutrient agar plate. These plates were incubated at 32^0C for 48h. The probiotics showed different inhibition zone against each pathogenic bacterium. Probiotic 1 showed bigger inhibition zone as compared to probiotics 3 followed by probiotic 2 against

each bacterium. All the three probiotic showed small inhibition zone in *A. hydrophila* as compared to *Micrococcus* sp., which had larger inhibition zone.

The role of probiotic in controlling the disease was also checked by *in vivo* experiments. The treatments given to the fishes in this experiment were i) control, ii) control + bacteria (*A. hydrophila*), iii) control + Bacteria, iv) control + probiotics 1, v) control + probiotic 2, vi) control + probiotic 3, vii) control + bacteria (*A. hydrophila*) + probiotic 1, viii) control + bacteria (*A. hydrophila*) + probiotic 2, ix) control + bacteria (*A. hydrophila*) + probiotic 3, x) control + bacteria (*Micrococcus* sp.) + probiotic 1, xi) control + bacteria (*Micrococcus* sp.) + probiotic 2, xi) control + bacteria (*Micrococcus* sp.) + probiotic 3.

The various life parameters were recorded to note down the effect of probiotics on diseased fish compared with control and the probiotic treated fish over a period of 8 weeks. These were: hematological parameters, growth parameters and survival rate. Hematological parameters included, level of haemoglobin, total erythrocyte count, total leucocytes count hemotocrit/ packed cell volume. These were determined with the help of a hemocytometer and calculated from the equations given by Anderson and Klontz (1965).

The level of hemoglobin decreased in the blood of fish administrated with pathogenic organisms as compared with control and probiotic treated groups of fish. The fish administrated with pathogenic organisms died after three weeks. The level of hemoglobin increased in the blood fish of treated with probiotics alone and also in fish administered with administered pathogenic organisms and probiotics. The increase in level of hemoglobin in the blood of probiotic administrated fish was more than that of control. In a comparison of between three probiotics used, probiotic 1 increased the level of hemoglobin significantly more than probiotic 3 and followed by probiotic 2. The same patterns were followed in case of erythrocyte count, leucocytes count and packed cell volume

The survival of fish was also recorded over a period of 8 weeks. The control fishes which were inoculated with phosphate buffer saline (PBS) only showed 100 per cent survival rate over a period of eight weeks. The fishes inoculated with pathogenic organisms showed survival only up to three weeks. The survival of fishes decreased from first to third week. The probiotic treated fish also showed 100 per cent survival rate. Both the probiotics were effective to increase the survival of fishes. In growth parameters, body length and weight of fish under different treatments were measured. The length of the fish inoculated with pathogenic organisms (bacteria and fungus) increased less as compared to control as well as probiotic treated groups. The increase in the length of probiotic treated fish was significantly more than that of control. However, the increase in the length of fish was significantly more under probiotic 1 as compared to probiotic 3 and probiotic 2. The same pattern was followed by the fish weight.

The viable counts of bacteria were maximal in the fish inoculated with pathogenic

organisms. However, these counts decreased in number in probiotic treated fish. The numbers of viable counts decreased more in probiotic 1 as compare to probiotic 3 followed by probiotic 2 over a period of eight weeks.

The results of the histo-morphometric study demonstrated a faster recovery from the gut atrophy status associated with addition of probiotic 1 (*L. sporogenes*), probiotic 2 (*S. boulardii*) and probiotic 3 (mixture of many soil bacteria) in the diet of catfishes. Moreover, when the three probiotics groups were compared with groups containing pathogenic bacteria (*A. hydrophila* and *Micrococcus* sp.), the probiotic diet was associated with better mucosa liningin the small intestine. These results are most probably due to enhanced short chain fatty acid (SCFA) formation induced by probiotics. SCFA is the best fuel for the colonocytes and directly trophic for the colonic mucosa.

The overall results of this study showed that the addition of the three probiotic positively influenced a more rapid restoration of the gut atrophy associated with pathogenic bacteria.

In conclusion probiotics are the new generation of alternative measures for the prevention of bacterial and fungal diseases in fish which should be used for maintaining the fish health. Probiotic 1 should be preferred over Probiotic 3 and Probiotic 2 for preventing catfish diseases and maintenance of fish health in the magur.

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ANNEXURE –I

MEDIA COMPOSITION

The composition of used media during the present investigation is given below.

The ingredients were suspended in double distilled water and heated with agitation to dissolve. Autoclaved for 15 – 20 minutes at 121°C. Agar medium consists of agar agar into it but broth consists of the same ingredients without the agar.

1) Decarboxylase basal medium (Arginine, Lysine, Ornithine)

Base Peptone or gelysate	5 g
Yeast extract	3 g
Glucose	1 g
Bromcresol purple	0.02 g
Distilled water	1 litre
Final pH	6.5 ± 0.2.

For arginine broth, 5 g L-arginine was added to 1liter base; for lysine (Falkow) broth, 5 g L-lysine was added to 1 liter base; for Ornithine, 5 g L-Ornithine was added to 1 liter base.

2) Nitrate broth (SRL, NM014)

Peptone	5 gm
Beef extract	3 gm
Potassium nitrate	1 gm
Distilled water	1 litre
Final pH,	7.0 ± 0.2.

3) Malonate broth

Yeast extract	1 g
$(NH_4)_2SO_4$	2 g
K_2HPO_4	0.6 g
KH_2PO_4	0.4 g
NaCl	2 g
Sodium malonate	3 g
Glucose	0.25 g
Bromthymol blue	0.025 g
Distilled water	1 litre
Final pH,	6.7 ± 0.2.

4) Urea broth

Urea	20 g
Yeast extract	0.1 g
Na_2HPO_4	9.5 g
K_2HPO_4	9.1 g
Phenol red	0.01 g
Distilled water	1 liter

5) Urea agar

Agar	12.0 g
Distilled water	1000 ml
Final pH,	6.8 ± 0.2.

All ingredients used in urea broth were dissolved except urea in 900 ml water (basal medium). Autoclaved 15 min at 121°C and then cooled it to 50-55°C. Urea was dissolved in 100 ml water.
Precaution: DO NOT HEAT.

6) Pseudomonas agar for Fluorescein (SRL, PM017)

Proteose peptone	10 gm
Agar	15.0 gm
Glycerol Pancreatic	10 gm

Digest of casein	
K_2HPO_4	1.50 gm
$MgSO_4$	1.50 gm
Glycerol	10 ml
Distilled water	1 liter
Final pH,	7.0 $\pm$ 0.2

7) Pseudomonas agar, base (SRL, PM028)

Agar	11 gm
Geletin peptone	16 gm
Tryptone	10 gm
Potassium sulphate	10 gm
Magnesium chloride	1.40gm
Glycerol	10 ml
Distilled water	1 liter

8) Pseudomonas isolation agar, base (SRL, PM019)

Peptone	20 gm
Agar	13.60 gm
K_2SO_4	10 gm
$MgCl_2.6H_2O$	1.40 gm
Irgasan (Triclosan)	0.025 gm
Glycerol	10 ml
Distilled water	1 liter

9) Cetrimide agar

Peptone	20 g
Magnesium chloride	1.40 g
Potassium sulphate	10 g
Cetrimide	0.30 g
Agar	13.60 g
Glycerol	10 g
Distilled water	1 litre

10) King's B medium

Proteose peptone No. 3	20 g
Glycerol	10 ml
K_2HPO_4	1.5 g
Mg SO_4	1.5 g
Agar	15 g
Distilled water	1 litre

All the ingredients were added except Mg SO_4. Heated with agitation to dissolve agar. Adjust pH to 7.2 ± 0.2. Mg SO_4 was added slowly and mixed.

11) Voges-Proskauer medium

Proteose peptone	7g
NaCl	5g
Dextrose	5g
Distilled water	1 litre
Final pH,	6.5 ± 0.2.

12) Lactose / Sucrose broth

Beef extract	3 g
Peptone	5 g
Lactose	5 g
Distilled water	1 litre
Final pH,	6.9 ± 0.2.

Autoclaved just before use.

13) Indole medium

L-Tryptophan	1 g
NaCl	1 g
K_2HPO_4	3.13 g
KH_2PO_4	200 ml
Distilled water	1 liter
Final pH,	7.2 ± 0.2.

14) Oxidative-fermentative test medium

Base peptone	2 g
NaCl	5 g
K_2HPO_4	0.3 g
Bromothymol blue	0.03 g
Agar	2.5 g
Distilled water	1 liter
Final pH	7.1.

For carbohydrate stock solution 10 g carbohydrate was dissolved in 90 ml distilled water. 0.3 ml stock solution was added to 2.7 ml base in tube. Mixed gently and cooled at room temperature.

15) LB agar (Luria Bertani agar)

Tryptone	10 g
Yeast extracts	10 g
NaCl	5 g
Agar	10 g
Distilled water	1 liter

16) Mannitol salt agar

Beef extract	1 g
Polypeptone	10 g
NaCl	75 g
Mannitol	10 g
Phenol red	0.025 g
Agar	15 g
Distilled water	1 liter
Final pH,	7.4 ± 0.2.

17) Buffered glucose broth

Proteose Peptone	5.0 g
D-glucose	5.0 g
Distilled water	1 liter

Ingredients were added to water, heated gently with stirring to dissolve completely.

18) Nutrient agar

Beef extract	3 g
Peptone	5 g
Agar	15 g
Distilled water	1 liter

19) Nutrient broth

Beef extract	3 g
Peptone	5 g
Distilled water	1 liter
Final pH,	6.8 ± 0.2.

20) Simmons's citrate agar

Sodium citrate	2 g
NaCl	5 g
K_2HPO_4	1 g
$NH_4H_2PO_4$	1 g
$MgSO_4$	0.2 g

Bromothymol blue	0.08 g
Agar	15 g
Distilled water	1 liter
Final pH,	6.8 ± 0.2.

21) Starch agar

Nutrient agar	23 g
Potato starch	10 g
Distilled water	1 liter

Heated to dissolve agar in 500 ml water. Starch was dissolved in 250 ml water. Combined and diluted to 1 liter. Autoclaved 15 min at 121°C. Distilled water 1 litre.

22) Koser citrate medium (SRL, KM011)

Sodium citrate	3 gm
$NaNH_4HPO_4.4H_2O$	1.50 gm
KH_2PO_4	1.00 gm
$MgSO_4.7H_2O$	0.20 gm
Distilled water	1 litre
Final pH	6.7± 0.2

23) Hugh Leifson glucose medium (HIMEDIA, REF- M871)

Peptic digest of animal tissue	2 gm
Yeast extracts	0.50 gm
NaCl	30.00 gm
Glucose	10 gm
Bromo cresol purple	0.015 gm
Agar	3 gm
Distilled water	1 liter
Final pH	7.4 ± 0.2

24) Fermentation medium for *Staphylococcus* and *Micrococcus* (HIMEDIA, REF- M827)

Casein enzymatic hydrolysate	10 gm
Yeast extracts	1 gm
Glucose	16 gm
Bromo cresol purple	0.04 gm
Agar	2.20 gm
Distilled water	1 litre
Final pH	7.0 ± 0.2

25) Rimler-Shott medium base (RS medium base) (HIMEDIA, REF- M576)

Yeast extracts	3 gm
Maltase	3.50 gm
L-Cysteine hydrochloride	0.30 gm
L-lysine hydrochloride	5.00 gm
L-Ornithine hydrochloride	6.50 gm
Sodium thiosulphate	6.80 gm
Ferric ammonium citrate	0.80 gm
Sodium deoxycholate	5 gm
Bromothymol blue	0.03 gm
Agar	13.50 gm
Distilled water	1 litre
Final pH	7.0 ± 0.2.

Precaution: DO NOT AUTOCLAVE.

26) Antibiotic assay medium L-AOAC (HIMEDIA, REF- M991)

Dipotassium hydrogen phosphate	0.69 gm
Monopotassium phosphate	0.45 gm
Yeast extracts	2.5 gm
Glucose, anhydrous	10 gm
Agar	15 gm

Distilled water	1 litre
Final pH	6.0 ± 0.2

27) Antibiotic assay medium No. 38 (HIMEDIA, REF- M799)

Peptone	15 gm
Peptonic digest of Soybeans	5 gm
NaCl	4 gm
Sodium Sulphite	0.20 gm
Dextrose	5.50 gm
Agar	15.00 gm
Distilled water	1 litre
Final pH	7.0 ± 0.1

28) Vogel and Johnson Agar

Tryptone	10.0 g
Yeast Extract	5.0 g
Mannitol	10.0 g
Dipotassium Phosphate	5.0 g
Lithium Chloride	5.0 g
Glycine	10.0 g
Agar	15.0 g
Phenol Red	25.0 mg
Distilled water	1 liter

Autoclave at 121°C for 15 minutes. Cool to 45-50°C.

29) *Staphylococcus* Medium

Pancreatic Digest of Casein	10.0 g
Yeast Extract	2.5 g
Gelatin	30.0 g
Lactose	2.0 g
D-Mannitol	10.0 g
Sodium Chloride	75.0 g
Dipotassium Phosphate	5.0 g
Agar	15.0 g
Distilled water	1 litre

30) Water agar

Agar	15 g
Distilled water	1 litre

31) Stock Culture Agar

Beef Heart, Infusion from 500	10.0 g
Proteose Peptone	10.0 g
Gelatin	10.0 g
Isoelectric Casein	5.0 g
Dextrose	0.5 g
Disodium phosphate	4.0 g
Sodium citrate	3.0 g
Agar	7.5 g
Distilled water	1 litre

32) Potato Dextrose Agar

Potato Starch	4.0 g
Dextrose	20.0 g
Agar	15.0 g
Distilled water	1 litre

33) Motility Test Medium

Beef Extract	3.0 g
Pancreatic Digest of Casein	10.0 g
Sodium Chloride	5.0 g

Agar	4.0 g
Distilled water	1 litre

34) MacConkey Agars

Peptone	17.0 g
Proteose Peptone	3.0 g
Lactose	10.0 g
Bile Salts No. 3	15 g
Sodium Chloride	5.0 g
Agar	13.5 g
Neutral Red	0.03 g
Crystal Violet	1.0 mg
Distilled water	1 litre
Final pH	7.4 ± 0.2

35) Infusion Broth

Heart Muscle, Infusion from (solids)	2.0 g
Pancreatic Digest of Casein	13.0 g
Yeast Extract	5.0 g
Sodium Chloride	5.0 g
Distilled water	1 litre
Final pH	7.4 ± 0.2

36) Buffered Peptone Water

Peptone	10.0 g
Sodium chloride	5.0 g
Disodium Phosphate	3.5 g
Monopotassium Phosphate	1.5 g
Distilled water	1 litre
Final pH	7.4 ± 0.2

37) Brain Heart Infusion, Porcine

Pork Brains, Infusion from 200 g	7.7 g
Pork Heart, Infusion from 250 g	9.8 g
Pork Peptone No. 2	10.0 g
Dextrose	2.0 g
Sodium chloride	5.0 g
Disodium Phosphate	2.5 g
Distilled water	1 litre
Final pH	7.4 ± 0.2

38) **Yeast Extract Glucose Chloroamphinicol Agar**

Yeast Extract	5.0 g
Glucose	20.0 g
Chloroamphinicol	0.1 g
Agar	13.0 g
Distilled water	1 litre
Final pH	6.6 ± 0.2

39) Aspergillus Differential Agar

Aspergillus Differential Agar contains an enzymatic digest of casein to provide amino acids and other nitrogenous substances. Yeast extract primarily supplies the B-complex vitamins. Ferric citrate is essential for the production of a bright, yellow orange pigment that differentiates *Aspergillus* sp. from most other clinically significant *Aspergillus* species.

40) Azide Blood Agar Base

Proteose Peptone No. 3	4.0 g
Pancreatic Digest of Casein	5.8 g
Beef Extract	3.0 g
Sodium Chloride	5.0 g

Sodium Azide	0.2 g
Agar	15.0 g
Distilled water	1 litre
Final pH	7.4 ± 0.2

41) Anaerobic Agar

Pancreatic Digest of Casein	20.0 g
Sodium Chloride	5.0 g
Dextrose	10.0 g
Agar	20.0 g
Sodium thioglycollate	2.0 g
Sodium Formaldehyde sulphoxylate	1.0 g
Methylene Blue	2.0 mg
Distilled water	1 litre
Final pH	7.4 ± 0.2

42) Eosin Methylene Blue Agar, Levine

Pancreatic Digest of Gelatin	10.0 g
Lactose	10.0 g
Dipotassium Phosphate	2.0 g
Eosin Y	0.4 g
Methylene Blue	65.0 mg
Agar	15.0 g
Distilled water	1 litre
Final pH	7.4 ± 0.2

43) Esculin Iron Agar

Proteose Peptone No. 3	7.5 g
Pancreatic Digest of Casein	7.5 g
Soy Peptone	5.0 g
Dextrose	5.5 g
L-Cystine	0.7 g
Sodium Chloride	4.0 g
Sodium Sulfite	0.2 g
Agar	15.0 g
Distilled water	1 litre
Final pH	7.4 ± 0.2

44) Inhibitory Mold Agar with Gentamicin

Pancreatic Digest of Casein	3.0 g
Peptic Digest of Animal Tissue	2.0 g
Yeast Extract	5.0 g
Dextrose	5.0 g
Starch	2.0 g
Dextrin	1.0 g
Chloroamphinicol	125.0 mg
Sodium Phosphate	2.0 g
Magnesium Sulfate	0.8 g
Ferrous Sulfate	0.04 g
Sodium Chloride	0.04 g
Manganese Sulfate	0.16 g
Agar	15.0 g
Distilled water	1 litre
Final pH	7.4 ± 0.2

Inhibitory Mold Agar, which contains Chloroamphinicol, is a moderately selective medium used for the isolation of pathogenic fungi. Prepared plates of Inhibitory Mold Agar and Inhibitory Mold Agar with Gentamicin are deep filled to reduce the effects of drying during prolonged incubation.

45) KF *Streptococcus* Broth

Proteose Peptone No. 3	10.0 g

Yeast Extract	10.0 g
Sodium Chloride	5.0 g
Sodium Glycerophosphate	10.0 g
Maltose	20.0 g
Lactose	1.0 g
Sodium Azide	0.4 g
Bromcresol Purple	15.0 mg
Distilled water	1 litre
Final pH	7.4 ± 0.2

K.F. (Kenner Fecal, 1961) *Streptococcus* Broth is used for isolating fecal *streptococci*.

46) Lactobacilli MRS Agar

Proteose Peptone No. 3	10.0 g
Beef Extract	10.0 g
Yeast Extract	5.0 g
Dextrose	20.0 g
Polysorbate 80	1.0 g
Ammonium Citrate	2.0 g
Sodium Acetate	5.0 g
Magnesium Sulfate	0.1 g
Manganese Sulfate	0.05 g
Dipotassium Phosphate	2.0 g
Agar	15.0 g

Lactobacilli MRS Agar and Lactobacilli MRS Broth are recommended for use in the isolation, enumeration, and cultivation of *Lactobacillus* species.

47) OF Basal Medium

Pancreatic Digest of Casein	2.0 g
Sodium Chloride	5.0 g
Dipotassium Phosphate	0.3 g
Bromothymol Blue	0.08 g
Agar	2.0 g
Distilled water	1 litre
Final pH	6.8 ± 0.2

OF (Oxidation/Fermentation) media are used for the determination of oxidative and fermentative metabolism of carbohydrates by gram-negative rods on the basis of acid reaction in either the open or closed system.

48) Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS Agar)

Yeast Extract	5.0 g
Proteose Peptone No. 3	10.0 g
Sodium Citrate	10.0 g
Sodium Thiosulfate	10.0 g
Oxgall	8.0 g
Saccharose	20.0 g
Sodium Chloride	10.0 g
Ferric Ammonium Citrate	1.0 g
Bromothymol Blue	0.04 g
Thymol Blue	0.04 g
Agar	15.0 g
Distilled water	1 litre
Final pH	8.6 ± 0.2

TCBS Agar is used for the selective isolation of cholera *vibrios* and *Vibrio parahaemolyticus* from a variety of clinical and non-clinical specimens.

ANNEXURE –II

REAGENTS COMPOSITION

The composition of used reagents during the present investigation is given below.

R1. Catalase test

One ml 3% hydrogen peroxide was poured over growth on slant culture. Gas bubbles indicate positive test. Alternatively, colony was emulsified in 1 drop 3% hydrogen peroxide on glass slide. Immediate bubbling is positive catalase test.

R2. Gram Stain

Hucker's crystal violet

Solution A

Crystal violet (90% dye content)	2 g
Ethanol, 95%	20 ml

Solution B

Ammonium oxalate	0.8 g
Distilled water	80 ml

Solutions A and B were mixed. Stored 24 h and filtered through coarse filter paper.

R3. Gram's iodine

Iodine	1 g
Potassium iodide (KI)	2 g
Distilled water	300 ml

KI was placed in mortar, added iodine, and grinded with pestle for 5-10 s. 1 ml water was added and grinded; then 5 ml of water was added and grinded, then 10 ml and grinded. This solution was poured into reagent bottle. Rinsed mortar and pestle with amount of water needed to bring total volume to 300 ml.

R4. Hucker's counter stain (stock solution)

Safranine O (certified)	2.5
Ethanol, 95%	100 ml

Working solution: 10 ml stock solution was added to 90 ml distilled water.

R5. Kovac's reagent

p-Dimethylaminobenzaldehyde	5 g
Amyl alcohol (normal only)	75 ml
HCl (concentrated)	25 ml

p-dimethylaminobenzaldehyde was dissolved in normal amyl alcohol. HCl was slowly added. Stored at 4°C. To test for indole, 0.2-0.3 ml reagent was added to 5 ml of 24 h bacteria culture in tryptone broth. Dark red color in surface layer was positive test for indole.

R4. Lugol's Iodine solution

Potassium iodide (KI)	10 g
Iodine	5 g
Distilled water	100 ml

KI was dissolved in about 20-30 ml of distilled water. Iodine was added and heated gently with constant mixing until iodine was dissolved. Diluted to 100 ml with distilled water. Stored in amber glass-stoppered bottle in the dark.

R5. Nitrite detection reagents

A. Sulphanilic acid reagent

Sulphanilic acid	1 g
5 N acetic acid	125 ml

B. N-(l-naphthyl) ethylenediamine reagent

N-(l-naphthyl) ethylenediamine dihydrochloride	0.25 g
5 N acetic acid	200 ml

C. alpha-Naphthol reagent

alpha-Naphthol	1 g
5 N acetic acid	200 ml

For preparation of 5 N acetic acid, 28.75 ml glacial acetic acid was added to 71.25 ml distilled water.

R6. Oxidase reagent

N, N, N', N'-Tetramethyl-p-Phenylenediamine 2HCl	1 g
Distilled water	100 ml

This is the preferred reagent. Freshly prepared reagent was used. However, reagent can be used up to 7 days if stored in a dark glass bottle under refrigeration.

R7. Potassium hydroxide (40%) solution

KOH	40 g
Distilled water to make	100 ml

R8. Voges-Proskauer (VP) test reagents

Solution 1

alpha-Naphthol	5 g
Alcohol (absolute)	100 ml

Solution 2

Potassium hydroxide	40 g
Distilled water to make	100 ml

R9. Acetone-Alcohol solution

Absolute Ethanol	75 ml
Acetone	25 ml

R10. 3% Hydrogen peroxide

Hydrogen peroxide	3ml
Distilled water	97 ml

R10. 1% alpha napthol (w/v) in 95 % ethanol

Alpha Napthol	1g
Ethanol	95ml
Distilled water	4.5 ml

R11. Phosphate Buffered Saline

Potassium dihydrogen phosphate	26.22 g
Sodium Carbonate	7.78 g
Distilled water	1Liter
Final pH	7.2± 0.5

Inoculate Phosphate Buffered Saline with approximately 1 g of stool specimen and refrigerate at 2-8°C for up to 3 weeks. Subculture may be done at 3, 7, 14 and, for maximal recovery, 21 days to MacConkey Agar.

R12. Saline (0.45% w/v).

Sodium chloride	0.45g
Distilled water	100 ml

R13. Bouin's Solution

Picric acid, saturated aqueous solution	75.0 ml
Formaldehyde, 37% to 40%	25.0 ml
Glacial acetic acid	5.0 ml

R14. Gomori's Trichrome Stain

Chromotrope 2R (Baker No. 5-F703)	0.6 g
Fast green FCF, light green or aniline blue	0.3 g
Phosphotungstic acid	0.8 g

Glacial acetic acid	1.0 ml
Distilled water	100.0 ml

Store this solution in the refrigerator.

R15. 0.5% Acetic Acid Solution

Glacial acetic acid	0.5 ml
Distilled water	99.5 ml

ABSTRACT

Title of thesis	**:**	**ROLE OF PROBIOTICS IN CONTROLLING PROMINENT DISEASE OF CATFISHES**
Full name of degree holder	:	Tejpal Dahiya
Admission No.	:	2005BS16D
Title of degree	:	Doctor of Philosophy in Zoology
Name of Advisor	:	Prof. R. C. Sihag, Deptt. of Zoology and Aquaculture, CCS Haryana Agricultural University, Hisar – 125 004 (India)
Degree awarding University	:	CCS Haryana Agricultural University, Hisar – 125 004 (India)
Year of award of degree	:	2009
Major subject	:	Zoology
Total number of pages in thesis	:	89+xiv
Number of words in abstract	:	310 words
Key words	:	catfish diseases, hemorrhagic septicemia, pathogens, hematological parameters, probiotics

In the present investigations, surveys were carried out to collect the samples of diseased fish in three fish farms of district Hisar from April, 2007 to June, 2009 at monthly intervals. The Indian magur fish *(Clarias batrachus*) was found to be infected with disease in each fish farm. All six bacteria viz. *Aeromonas hydrophila, Micrococcus* sp., *Streptococcus faecalis, Klebsiella oxytoca, Streptococcus haemolyticus* and *Staphylococcus aureus* isolated and identified bacteria were found to be pathogenic. The other associated pathogens were three fungii viz. *Penicillin* sp., *Saprolegnia* sp. and *Aspargillus ochracaeus.* Three probiotics were used to control the infections caused by pathogenic bacteria and fungi. All the three probiotics showed smaller zone of inhibition against *A. hydrophila* as compared to *Micrococcus* sp.. In comparison, probiotic 1 increased the level of hemoglobin, erythrocyte counts and packed cell volume and decreased leucocytes counts significantly more than probiotic 3 followed by probiotic 2. The survival of fish was also recorded over a period of 8 weeks. The probiotic treated fish showed 100 per cent survival rate. All the three probiotics were effective to increase the survival of fishes.. The increase in weight and length of probiotic treated fishes was significantly more than that of control. However, this increase in the length and weight of fish was significantly more under probiotic 1 as compared to probiotic 3 and probiotic 2. The numbers of viable counts were decreased more in probiotic 1 as compare to probiotic 3 followed by probiotic 2 over a period of eight weeks. The addition of the three probiotic positively influenced a more rapid restoration of the gut atrophy associated with pathogenic bacteria.

In short, probiotics are the new generation of alternative measures for the prevention of bacterial and fungal diseases in fish which should be used for maintaining the fish health; Probiotic 1 should be preferred over Probiotic 3 and Probiotic 2.

DEGREE HOLDER

MAJOR ADVISOR **HEAD OF DEPARTMENT**

UNDERTAKING OF COPY RIGHT

"I **Tejpal Dahiya** admission no. **2005BS16D** undertake that I give copy right to the CCS HAU, Hisar of my thesis entitled "**ROLE OF PROBIOTICS IN CONTROLLING PROMINENT DISEASE OF CATFISHES**".

I also undertake that patent, if any, arising out the research work conducted during the programme shall be filed by me only with due permission of the competent authority of CCS HAU, Hisar.

Signature of the Student

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