Review of Literature
2.0. REVIEW OF LITERATURE

Aquaculture is one of the most economically important applied strategies all over the world and fishes are one of the most beneficial and nutritional resources to human beings. Aquaculture has grown rapidly over the last decades and there are about 600 species in total, that are being farmed worldwide (FAO, 2012). Although most of the finfish production comes from the extensive production of carps, the industrialization of finfish farming is expanded for both high and low value species (Brudeseth et al., 2013). The most important prerequisite of fish production is availability of healthy fish fingerlings of carp. It is evident from the available literature that parasitic diseases caused significant damage in nursery system of carp mostly affecting the fry and fingerlings (Gopalakrishnan, 1961). Fish fingerlings become more susceptible to infection because of their immature immune system (Anderson, 1974). Indian major carps are the most important freshwater species cultured in India and it is essential that they are protected against infections of Edwardsiella tarda and Pseudomonas fluorescens, a scourge of freshwater fish worldwide.

2.1 CIRRHINUS MRIGALA

Cirrhinus mrigala is one of the most preferred Indian major carp (IMC), constitutes about 20-25% of the total IMCs production in India. In India, with the emergence of large-scale commercial carp culture, diseases of varied aetiology are being increasingly recognised as a major hurdle to successful and sustainable farming (Raa et al., 1992). The use of chemotherapeutants for controlling diseases has been widely criticized for their negative impacts like accumulation of tissue residues, development of drug resistance and immunosuppression (Van Muiswinkel et al., 1985; Ellis, 1988). Hence, there is an urgent need to look for ecofriendly disease preventative measures to promote sustainable culture of Indian major carps.
In order to reduce the risk of disease, the level of resistance to infection in the cultured organisms should be increased by the use of better feeds, vaccines, and immunostimulants or by selective breeding for higher disease resistance (Raa et al., 1992). Earlier reports on this study were reviewed and presented below.

Karunasagar et al., (1991), have investigated the immunological response of the Indian major carps to *Aeromonas hydrophila* vaccine. Fingerlings of the three types of Indian major carp, *C. catla*, *L. rohita* and *C. mrigala* were immunized using a haemolysin-negative mutant of *A. hydrophila*. Result indicated that, very high titters of antibodies were induced in *Catla catla*, followed by *C. mrigala* and *Labeo rohita* and good protection against homologous challenge which was higher compared to heterologous Challenge.

Chandran et al., (2002), studied that the Indian major carp’s *C. catla*; *L. rohita* and *C. mrigala* were immunized against the potent bacterial fish pathogen, *A. hydrophila* by the intraperitoneal route, in field conditions. Two different polyvalent antigen preparations namely, whole cell and Extracellular Products (ECP) were used. Immunisation schedule consisting of a single booster dose given 28 days after priming resulted in a good agglutinating antibody response in the carps. Kinetics of the response was similar in the three carp species with both whole cell and ECP. Upon challenge with virulent strains, relative percent survival as high as 80–90% was recorded.

Sobhana et al., (2002), investigated the effect of dietary vitamin C (at 1000 mg vitamin C/kg diet) on the disease susceptibility of mrigal, *C. mrigala* (Hamilton) to experimental infection of *A. hydrophila*. At the end of the feeding period, fishes were challenged by virulent strain of *A. hydrophila*. Mortality curves were clearly distinct and the vitamin C non-supplemented (VNS) group showed
significantly higher mortality rates compared to the vitamin C supplemented (VS) group.

Kalita et al., (2006), studied the humoral and protective response of Indian major carps to immersion vaccination with *Aeromonas hydrophila*. Fry of the Indian major carps, *Catla catla* (Ham.), *L. rohita* (Ham.) and *C. mrigala* (Ham.) were immunized at 4 and 8 weeks post hatching (wph) by direct immersion in a suspension (108 cells /m1) of heat inactivated *A. hydrophila*. Antibodies as well as protective response produced in both the groups after the first and the booster immersion were different and significant (P<0.05). No significant difference was found between the species in the two age groups. The specimens immunized 8 wph showed higher antibody titres and protection than the 4 wph group.

Sivagurunathan et al., (2011), reported the feed incorporated with *Zingiber officinale* and *Curcuma longa* in *Cirrhinus mrigala* Challenged with *P. aeruginosa*. Result revealed that *Zingiber officinale* and *C. longa* enhanced the non-specific immune responses in *Cirrhinus mrigala* against the pathogen of *P. Aeruginosa*.

Abdul Kadhar et al., (2012), conducted an experiment to determine the growth performance of two Indian Major Carp *C. catla* and *C. mrigala* fingerlings for a period of 40 days. Nine experimental groups fed commercial pellet diet incorporated with three types of nutritional supplements (Gram positive lactobacil probiotic, Parry's Spirulina and Vitamin C-Ascorbic acid) at different concentrations (2 %, 4 % and 8 %). The results revealed that the Catla fingerlings showed maximum increase in length (28.66±0.70mm), weight gain (353.25mg), FCR (1.01) and SGR (0.88) were observed in 4% probiotic and similar growth parameters were observed with 4% spirulina and 2% vitamin C. In mrigal fingerlings fed with 4%
probiotic significant increase in length, weight gain, FCR, SGR were observed (32.55±1.94mm), 447.78 mg, 0.80 and 1.11 respectively.

Hazrat Ali et al., (2014), conducted an experiment to isolate and identify *E. tarda* from diseased fish, evaluated their antibiotic sensitivity pattern and screened the antibacterial activity of some medicinal plant extracts against the isolates. Pathogenicity of the isolates was assessed experimentally by using various fish models. The isolates exhibited strong virulence to mrigal fish (*C. mrigala*) with LD 50 ranging from 1.3×103 to 1.8×108 CFU/fish. Seven isolates were found highly virulent exhibiting high (100%) mortality in experimental fish. *In vitro* antibiotic sensitivity pattern of the *E. tarda* isolates was found to be sensitive against ciprofloxacin, streptomycin, chloramphenicol and gentamycin. Conversely, majority of the isolates were resistant to oxytetracycline (75%), ampicillin (66%) and nalidixic acid (50%). A total of 82 plant extracts were screened for their antibacterial activity against the *Edwardsiella tarda* isolates where 12 plant extracts were able to show antibacterial activity. Among the plant species tested, *Tamarindus indica*, *Citrus aurantifolia*, *Terminalia bellirica*, *Terminalia chebula*, *Spondius pinnata* showed the most promising result against all of the *E. tarda* isolates.

Aderolu and Sahu (2015), carried out a 45-days experiment to evaluate the growth performance, digestive enzymes activities along with their gene expression in mrigal fish (*C. mrigala*) fed with graded levels of carbohydrate. Three isonitrogenous and isolipidic diets containing either 30, 40 or 50% carbohydrate were formulated using purified ingredients. Weight Gain (%), SGR, FCR and hepato-somatic index (HSI) was evaluated and found significantly (P<0.05) different in relation to the level of carbohydrate in the experimental diets. No significant difference (P>0.05) was found in % weight gain, SGR and FCR between the 30 and
50% carbohydrate fed groups but the HSI increased significantly across the inclusion levels.

Kumar et al., (2015), carried out 60 days feeding trial to study the effects of dietary supplementation of anthroquinone extract on growth, metabolic and haemato-immunological responses in C. mrigala fingerlings challenged with A. hydrophila infection. Five diets were prepared with graded level of AE (anthroquinone extract). Result reveals that dietary AE at 1% incorporation level augments growth, metabolic and hematological-immunological responses and protects the animal against A. hydrophila infection in C. mrigala fingerlings.

Susmita and Umesh (2016), evaluated the Vitamin A content, Nutritional Value and Seasonal Variation of Proximate Composition of Indian Major Carps (L. rohita, C. catla and C. mrigala) were collected from local fish market and fishing sites of rive Brahmputra. Results have revealed that there were no significant seasonal variations in body composition of Indian major carp and ratio of retinol: dehydroretinol. The lipid content was found to be comparatively low compared to L. rohita. Protein content was found to be high in C. mrigala compared to the other two species.

Sujatha (2016), demonstrated an effect of immunostimulant viz. vitamin C along with formalin inactivated polyvalent vaccine on fingerlings of Labeo rohita (rohu). The statistical significance of difference in the number of survivals in four vaccines and vitamin C groups, for before and after challenge was obtained using Fisher’s exact test. The RPS was found to be higher for the group treated with immune stimulant vitamin C supplement; while lower for group treated with monovalent and polyvalent without vitamin C. The difference in the proportion of
survivals in vaccine and control group after challenge was found statistically significant at 36th day.

2.2 **EDWARDSIELLA TARDA**

*Edwardsiella tarda* is an intracellular Gram-negative bacterium of the *Enterobacteriaceae*, first isolated from pond-cultured eel by Hoshina in 1962. It can infect (edwardsiellosis) both marine and fresh water fishes which leads to extensive losses in many fresh water and marine water fish worldwide. The use of synthetic chemicals and antibiotics (DePaola *et al*., 1995) for the control of fish disease may result with the emergence of antibiotic-resistant microbes, drug residues and environmental impacts. *E. tarda*, antibiotic resistance has been reported widely in the world (Aoki *et al*., 1989). To limit the use of chemicals and antibiotics, vaccination is highly recommended (Chinabut and Puttinaowarat, 2005). Over the last decade vaccination has become increasingly important for the prevention of infectious diseases in farmed marine and freshwater fish (Gudding *et al*. 1999). To induce protection against edwardsiellosis, including formalin-killed *Edwardsiella tarda* bacterin (Gutierrez and Miyazaki, 1994), cellular lipid (Salati and Kusuda, 1986) and lipopolysaccharides (Salati *et al*., 1987), several studies have been reported.

Ellis (1988), suggested that potency of vaccines was tested in controlled laboratory conditions as the reason for mortality is not known in the pond environment. The vaccinated and control groups were challenged intraperitoneally 14 days after booster vaccination with 106 CFU/ml of *E. tarda*.

Ashida *et al*., (1999), has stated that, the Japanese flounder, formalin-killed *E. tarda* cells were administered to fish by feeding in the absence or presence
of curdlan or curdlan together with Quil A saponin. Although the incorporation of curdlan gave higher survival rates, only the group in which the vaccine was administered with both curdlan and Quil A showed significantly better survival when compared to unvaccinated fishes.

Tu and Kawai (1999), studied the Antigenic Profile and Protective Role of a 37 kDa Major OMP of *E. tarda* (EF-1 strain). The protein had a glycoprotein profile and showed immunogenicity to Japanese Eel Anguilla japonica. Immunoblot analysis by using an Eel antiserum raised against this MOMP revealed no antigenic cross-reaction with other MOMPs of this bacterium. Eel antisera against 37 kDa MOMP extracted from EF-1 and V1 strains showed cross agglutination to formalin-killed cells and heated cells between the two strains. The results indicate that the heat-resistant antigen, 37 kDa, MOMP is located on the cell surface and has a common antigenic determinant. Immunization with the purified 37 kDa MOMP of EF-1 and V1 strains increased resistance in eel against challenge by intraperitoneal injection with live EF-1 strain. The results have shown that 37 kDa MOMP is one of the protective antigens of *E. tarda*.

Darvish *et al.*, (2001), studied the effect of incubation temperature and salinity on expression of the Outer Membrane Protein of *Edwardsiella tarda*. Outer Membrane Proteins of 10 isolates of *E. tarda* were compared by SDS-PAGE. The protein profile of the type strain *E. tarda* ATCC 15947 cultured at 25° C had 5 major protein bands of 40, 36.5, 34, 28.5, and 25 kDa and a large number of minor proteins ranging in size from approximately 10 to 120 kDa. There was no difference in the OMP profiles of 9 out of 10 isolates of *E. tarda* incubated at a temperature of 25° C compared with those at 35° C. The OMP profile differences and the different reactions to salinity levels suggest that the isolates are heterogeneous.
Liu et al., (2007), evaluated the *E. tarda* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) may be an effective vaccine candidate against infection by *E. tarda* in Japanese flounder *Paralichthys olivaceus*. The GAPDH of *E. tarda* is highly homologous to that of *Vibrio cholerae* (91%) and therefore *E. tarda* GAPDH have protective antigenicity against *Vibrio* species. Immunized the Japanese flounder with GAPDH of *E. tarda* and infected the fish with *V. anguillarum*. The result showed that GAPDH prepared from *E. tarda* protected Japanese flounder effectively in a challenge of *V. anguillarum*. Therefore, *E. tarda* GAPDH should be considered as a multi-purpose vaccine candidate against several kinds of pathogenic bacteria.

Castro et al., (2008), suggested that since 2004, *E. tarda* is known to be emerging pathogenic bacteria that have been known to affect the fish species. The aim of the study was to develop an effective *Scophthalmus maximus* vaccine strategy against *E. tarda*. The author has developed two different vaccines with different formulations that are with aqueous bacterin and adjuvanted vaccine. These vaccines were administered by immersion method or by intraperitonial injection. The RPS and antibody levels were measured after the admistrations. At the end of the study the results reveal that adjuvant vaccine through intraperitonial injection was found to be the most effective vaccination stragy as the RPS level was found to be 90% after 6 months of vaccination.

El-Jakee et al., (2008), stated that, formalized inactivated bacterin, OMP and LPS vaccines were prepared from this *E. tarda* and injected intraperitoneally in three groups of *Clarias gariepinus* fish. Survival rate was analysed by using Immune responses of vaccinated groups that were estimated by microagglutination and ELISA methods. The agglutinating and ELISA antibody titers of fish vaccinated with OMP showed a value of 2560 and 2570 at 4 weeks post vaccination,
respectively, followed by LPS (1280 and 2132) and formalin-inactivated vaccine (1040 and 1382), respectively. The laboratory study have showed that the protection rates of OMP, formalin-inactivated and LPS vaccines were found to be 100%, 96% and 92%, respectively.

Mohanty and Sahoo (2010), investigated the immune responses and expression profiles of some immune-related genes in Indian major carp, *L. rohita* to *E. tarda* infection. Results have revealed that a significant decrease in the superoxide production, alternative complementary activity, total protein levels and anti-protease activity of serum was observed in the infected fish. Similarly, significant increase in specific antibody titres was noticed on and after 10 days post challenge. This study also elucidates the changes in the relative expression of some immune related genes during the infection and 48 h post challenge.

Kumar et al., (2010), investigated the Monoclonal antibodies (MAbs) were produced against OMP of *E. tarda* ET-7, isolated from Snakehead (*Ophiocephalus punctatus*). Two stable hybridoma clones, designated as 3F10 and 2C3 MAbs were found to be potentially specific for *E. tarda* by indirect ELISA. These MAbs strongly reacted with 17 isolates of *Edwardsiella tarda* by indirect ELISA and Western blotting. These MAbs recognized major immunogenic OMP band at 44 kDa in Western blotting and it could be used for specific detection of *E. tarda* infection in fish by immunoassays.

Maiti et al., (2011), studied the recombinant OMP A of *E. tarda* was used as a vaccine candidate in common carp. The recombinant OMP A containing His6 residues was estimated to have a molecular weight of 38 kDa. In western blot the native protein showed expression of 36 kDa within the range of major OMP (36-44 kDa) was observed in this study. The OMP A protein characterized in this
study was observed to be highly immunogenic in both rabbit and fish. Common carp vaccinated with recombinant OMP A protein elicited high antibody production and immunized fish showed a RPS of 54.3 on challenge. Similar results obtained with the OMP of both bacterial pathogens which showed high efficiency of immunization in comparison to WC vaccines.

Jiao et al., (2010), studied that the Japanese flounder were i.p. injected with a vaccine containing a major antigenic protein of *E. tarda* in the absence or presence of FIA. Protection against the experimental challenge of *E. tarda* achieved by the vaccine without adjuvant resulted in a relative per cent survival (RPS) of 34% that was increased to 81% in the presence of FIA. The above results were similar to our findings, wherein, the vaccine with immunoadjuvant produced significant RPS rate when compared to nonadjuvanated vaccines in all experimental groups.

Khushiramani et al., (2012), characterized and immunized the fish with recombinant OMP of *A. hydrophila* and challenged with virulent *A. hydrophila* and another fish pathogen *E. tarda*. The protein was revealed to have a good immunogenic property and also gives protection to Rohu when challenged with the organism and showed that RPS was 69% and 60% respectively. Results have suggested that OMP of *A. hydrophila* could be used as a potential vaccine candidate for protection against *A. hydrophila* infection, but also against the fish pathogen *E. tarda*. In our studies with OMP and WC vaccines prepared from *E. tarda*, the OMP vaccination showed significant protection against *E. tarda* than WC vaccines.

Hossain et al., (2012), studied the efficacy of inactivated *E. tarda* have evaluated and compared by intraperitoneal injection-immunization or challenge against Japanese eel (*A. japonica*). Formalin, formalin with heat, citric acid, pressure and electric current were used for inactivation of the bacteria and the RPS values of
pressure (600 psi for 5 min) killed cells was determined. Protection of the different-inactivated vaccines was evaluated at different time post immunization, and the peak of protection was observed at 9 days post-challenge. Fish immunized with PKC vaccine showed higher protection (89-93), significantly (P<0.05) higher serum and mucus antibody titers elicit both systemic and mucosal adaptive immune responses, and induce specific humoral immune responses in eel.

Yang et al., (2015), studied the immune response of flounder (Paralicthyes olivaceus) was associated with the concentration of inactivated E. tarda vaccine and immersion time for example, inactivated E. tarda vaccine can be used by immersing the fishes at different concentration such as 10^9 cfu/ml, 10^8 cfu/ml, 10^7 cfu/ml immersed for a time period of 30, 60, 90 and 90 mim respectively. At the 6th week post vaccination, the flounders were challenged with E. tarda and the relative percent survival was analysed. The results have revealed that the flounders that are were immersed at 10^8 cfu /ml for 60 min were found to have elevated antibody titers and increased RPS rate (78%) when compared to other experimental groups and control groups.

2.3 **PSEUDOMONAS FLUORESCENS**

*Pseudomonas fluorescens* is a Gram-negative bacterium of the family of Pseudomonadaceae, among the recognized bacterial pathogens that commonly associated with reared aquaculture species (Wang et al., 2009). *Pseudomonas fluorescens* is a pathogen for a wide range of fish species including Indian major carps (Labeo rohita, Catla catla, and Cirrhinus mrigala) (Swain et al., 2007), grass carp (Ctenopharyngodon idellus) (Geng et al., 2006). Although vaccines have provided varying degrees of protection in fish, still until now no commercial vaccine available for *P. fluorescens* (Wang et al., 2009). The antigenic diversity of
P. fluroscens presents a major problem in vaccine development. Perusal of literature showed that a variety of vaccination methods and vaccine types have been tried for the immunization of different group of fishes against P. fluroscens.

Hora and Pillay (1962), have reported finrot in both in young and adult fishes. Furthermore, they have suggested that the bacterial disease to be more contagious and it is capable of causing immense damage. Scientists at CIFA have reported that the Fin and Tail rot in young fish are due to a mixed infection of A. hydrophila and P. fluorescence.

Austin and Austin (1987), have reported that stress from low dissolved oxygen, high stocking density; physical trauma and poor nutrition are pre disposing factors in the development of Pseudomonas Septicemias. Therefore, avoidance of these conditions is necessary to prevent epizootic outbreaks suggested bath treatment during the early stage of the disease.

Abdel-Hady et al., (2009), studied the Monovalent, killed and live attenuated vaccines of A. hydrophila and P. putida were used in the immunization of red tilapia against Motile Aeromonad and Pseudomonad septicemias. The 4 treatments included Heat killed vaccine of A. hydrophila, live-attenuated vaccine of A. hydrophila (using herbs), Heat-killed vaccine of P. putida and live-attenuated vaccine of P. putida. Vaccination was conducted via the Intra Peritoneal route as an initial dose followed by 2 booster doses every 2 weeks. The last dose was applied via the immersion route. The evaluation of vaccination was carried out through periodical antibody titration of the serum of the examined fish (every 2 weeks) using direct agglutination method as well as by the experimental challenge 3 months after the initial immunization. Results revealed that there were a significant difference between the vaccinated and unvaccinated fish of the control group regarding
antibody titers and RPS of the challenge test. Differences in immunity levels within the vaccinated groups were also demonstrated. Similar results were obtained in our studies with both monovalent and bivalent vaccines showing a significant RPS rate in vaccinated group.

Pratheepa and Sukumaran (2011), conducted an experiment on the pathogen \((Pseudomonas fluorescens)\) infected \(C. carpio\) Linn. \((Cyprinidae)\), using \(E. hirta\) Linn. \((Euphorbiaceae)\) plant leaves as immunostimulants. The aqueous extract of the leaves was prepared and the immunostimulant action was recorded by giving different concentrations of plant extract supplemented diet. The results obtained that the higher concentration of the extract (50g/kg diet) provided significant immune response (specific and nonspecific) on the fish. The 50g/kg leaf extract of \(E. hirta\) enhanced the phagocytic ratio on 10th and 15th day after the infection. The results of the specific and nonspecific immunostimulation studies were found to be statistically significant.

Attia et al., (2012), studied four different prepared \(P. fluorescence\) antigens to develop the best adequate strategy to control such infection in Nile tilapia. Fish in groups 15 were injected intraperitonial with 0.2 ml from each of sterilized saline, Formalin killed bacterin, Extracellular product (ECP) suspension, Sonicated cells (SC) suspension and mixture of ECP and SC suspension respectively. At 1, 2, 4, 6, and 8 weeks post vaccination, whole blood was tested for nitro blue tetrazolium (NBT), neutrophil adherence tests, lysozyme activity and the serum bactericidal test. The NBT, Neutrophil adherence, lysozyme activity, Serum bactericidal activity, antibody titer, and RPS of vaccinated fish showed significant increases in all immunized groups in comparison with control at 1, 2 and 4 weeks post vaccination. The higher values of the RPS was found to be mixed with
sonicated and extracellular product antigen followed by formalin killed antigen, sonicated cell antigen then extracellular product antigen.

Mastan (2013), evaluated the Pseudomonads Septicemia in *Labeo rohita* and *Cyprinus carpio* in Andhra Pradesh (Natural occurrence and artificial challenge). Diseased fish samples were collected from fish farms and fish markets (Bhimavaram, Andhra pradesh) and microbiological methods were used to analyse and characterized the pathogens. Result showed that the occurrence of 4 species of *Pseudomonads* namely *P. anguilliseptica*, *Pseudomonas fluorescence*, *Pseudomonas aeruginosa* and *Pseudomonads* sps. The artificial challenge studies have indicated that *P. anguilliseptica*; *P. fluorescence* was highly pathogenic towards *L. rohita* and *C. carpio*. With reference to the above literature *Pseudomonas fluorescence* was selected to prepare a potential vaccine candidate for this pathogen.

Younes *et al.*, (2014), *Pseudomonas fluorescens* is one of serious fish diseases responsible for severe economic losses. Four *Pseudomonas fluorescens* isolates were previously isolated from outbreaks in Al-Abbassa and Al-Fayoum farms were used in this study. The plasmid profile of all isolates showed the pattern of two bands. Disc diffusion method was used to test the potency of some natural plant extracts in controlling *Pseudomonas fluorescens* infections. The most effective methanolic extracts of plants and substances were Propolis, Thyme (*Thymus vulgaris*) whole plant, Juniper (*Juniperus communis*) fruits, Myrrh (*Commiphora molmol*) oleoresin, Aloe (*Aloe vera*) dried gel, Tamarind (*Tamarindus indica*) paste and Tea (*Camellia sinensis*) leaves. Other extracts did not show any inhibition zone and presumptively was found to be not effective.

Mahmoud *et al.*, (2014), have conducted a study to evaluate effects of Turmaric (*Curcuma longa*) supplementation (3 months) on growth performance,
feed utilization and resistance of Nile tilapia (*Oreochromis niloticus*) to *P. fluorescens* Challenge. Result revealed that the group has received 0.50% turmeric supplemented diet significantly improved the growth performance, crude protein content and survival rate.

Dhondiraj and Ravi (2015), carried out a detailed analysis to evaluate the association of various bacterial pathogens with *C. catla* from Marathwada region of Maharashtra. The freshwater fishes were collected from different water bodies and fish culturing centre of eight districts of Marathwada region and analysed the pathogens. The bacterial strains were identified based on colony morphology, cell morphology and biochemical characters. Result revealed thirteen pathogenic bacteria from the fish samples that included *Micrococcus* sp., *Bacillus* sp., *Lactobacillus* sp., *Vibrio* sp., *Aeromonas* sp., *Streptococcus* sp., *Flavobacterium* sp., *Vibrio* sp., *Proteus* sp., *Staphylococcus* sp., *Enterobacteria* sp., *E. coli*, *Pseudomonas* sp. The dominant bacterial pathogen was *Pseudomonas* sp. The *Pseudomonas* sp associated with *Catla catla* could survive on host as well as in water. So we found that it is the need of the hour to prevent this highly pathogenic organism causing severe damage in aquaculture industries and took up this major work which was fruitful.

### 2.4 FISH VACCINATION

In recent years, a new technique for the prevention of fish disease is rapidly emerging as a result of research into the development of fish vaccine. Fish vaccination in the aquaculture industry has been considered to be very important in reducing economics losses caused by disease (Ellis *et al.*, 1988; Rahman and Kawai, 2000 and Ebanks *et al.*, 2004). During the last few years, the application of vaccines for preventing the bacterial diseases in the field of aquaculture have been developed
both in regard to the number of fish species and the number of diseases. At present there are many bacterial vaccines against various bacterial diseases that affect the production in the fish farming industries. Vaccination is the best method to increase survival rate and profitability in aquaculture when used in combination with several factors such as good nutrition, high-quality fingerlings, good farming and husbandry practices and health management. Vaccine can be administered in several ways viz. injection, immersion, spray and oral (bio and micro encapsulated). Various researchers have had variable success with these methods.

Khalifa and Post (1976), was one of the first to demonstrate protective immunity in rainbow trout fry and the fishes were immunized which had been fed with 0-3g vaccine for period of 23 days against \textit{A. liquefaciens}. This resuls suggest that after a period of 3 months, the vaccinated fish had a mortality of 25% when compared with 75% of increased mortality rate in the controls.

Thorburn and Jansson (1988), investigated the effects of booster vaccination and fish size on protective immunity in rainbow trout (\textit{Salmo gairdneri}) against \textit{V. anguillarum}. The fishes 4.1g and 6.3g were bath immunized either once or twice and bath challenged one month after the second vaccination. Result indicated that booster dose did not increase the survival rate and maturation of (6.3g) fishes but increase the survival rate when compared the fish vaccinated at 4.1g. With regard to the above studies conducted with booster studies, our study results also showed concurrence when booster doses were employed.

Karunasagar \textit{et al.}, (1991), recorded very high titres of antibodies in the fingerlings of Indian major carps to \textit{A. hydrophila} vaccine after three boosters and at the end of the study the results revealed that there was no significant difference observed between the immunized batches.
Durbin et al., (1999), evaluated the rainbow trout *Oncorhynchus mykiss* were protected (relative percent survival >80%) after use of a formalin-killed whole-cell furunculosis vaccine, grown in iron-depleted conditions, administered by intraperitoneal injection followed by an oral boost. Result indicate that humoral antibodies were produced against the OMP (maximum titer = 1:2,560 at day 105) and iron-regulated outer membrane proteins (IR-OMP; maximum titer = 1:12,800 on day 105) of *A. salmonicida* after vaccination through intraperitoniial route accompanied by oral boost.

Vinitnantharat *et al.*, (1999) and Hastein *et al.*, (2005) both have suggested that during the past few decades, more suitable vaccines were developed and more refined straggles were developed in order to control pathogen specific disease in regard to aquaculture.

Esteve *et al.*, (2004), prepared a vaccine against *V. vulnificus* which is found to protect eels against vibriosis during the course of the study these eels were vaccinated through triple prolonged immersion. Protection lasted for at more than 6 months, but later, there was a decrease in the level of protection and eels were found to be suffering stress-related vibriosis. So, therefore the author has designed an oral vaccine that can be used for reimmunization at any developmental stage of eel. The protection and the immune response in serum, mucus and bile were evaluated in reimmunized and control fish for a period of 60 days. Reimmunization strategy significantly increased protection level and antibody titres. This was performed after bath infection was challenged with the pathogen.

Plant *et al.*, (2011), discusses the disease prevention is essential to the continued in the development of aquaculture around the world. Vaccination is the most effective method of combating disease and currently there are a number of
vaccines commercially available for use in fish. The majority of aquatic vaccines are delivered by injection, which is by far the most effective method when compared to oral or immersion deliveries. However it is labour intensive, costly and not feasible for large numbers of fish fewer than 20 g.

Ali et al., (2014), investigated that the immune response of Indian major carp, *Labeo rohita* (Hamilton, 1822) in regard to age and size. Different size groups (5-8 cm, 9-12 cm and 13-16 cm) of same aged fishes were immunized against *A. hydrophila* vaccine by injection and immersion methods. Results revealed that immersion and injection immunization elicit same level of antibody titers in small sized fishes, but a distinct different level of antibody titers was observed in large sized fishes. Therefore, it was concluded that the Immune response of Indian major carp *L. rohita* mainly depend on size rather than age.

2.4.1 WHOLE CELL VACCINE (WC)

Bacterial vaccines used in the aquaculture till now were half killed or inactivated vaccines produced from the broth culture of specific strain subjected to formalin inactivation (Newman, 1993; Toranzo., et al., 1997). A killed vaccine was biological safety to both aquatic animals and environment. Several investigators have attempted to study the immunization of WC vaccine in different types of fish that was reviewed and presented below.

Salati et al., (1987), reviewed techniques and procedures for vaccinating eels against *Edwardsiella tarda* and found that two basic types of vaccines used were whole cell bacterins and bacterial extracts. Result showed that vaccination triggers antibody production and phagocytosis in eels upon injection with Formalin-killed bacterial and Lipopolysaccharide vaccines of *Edwardsiella tarda*.
Piganelli (1999), developed the whole cell vaccine against *R. salmoninarum* and the vaccine was used by using this bacteria where it was formalin fixed at 37°C, this fixation of the bacteria decreased the bacterial hydrophobicity. The Coho Salomon which is known as the *Oncorhynchus kisutch* were vaccinated both intrpertionially and also through intramuscular injection. There were two experiment groups in this study, the first group was injected with Freund’s Incomplete Adjuvant (FIA) and in the second group was vaccinated with extracellular protein (ECP) which is the concentrated supernatant of FIA and the fishes were bath immersed in the vaccine. At the end of the study the results show that ECP vaccination was found to be more effective in immunizing the fishes against the pathogen *Renibacterium salmoninarum*.

Kozinska and Antychowicz (2001), have evaluated the influence of monovalent *A. hydrophila* and *A. sobria* vaccines on the induction of protective immunity against heterogenous strains of Aeromonas in carp. Separate groups of carp were immunized with 1S-95 (*A. hydrophila*) or 4R-96 (*A. sobria*) antigens by intraperitionial injection or by immersion method. The immunization efficacy was evaluated by using challenge tests with various heterologus strains of *Aeromonas*. Fish immunized by immersion demonstrated a particularly high level of immunity.

Anbarasu and Chandran (2001), demonstrates on immunostimulatory effect of vitamin C on the humoral and cell mediated immunity of the catfish, (*Mystus gulio*) using different bacterins of *A. hydrophila*. The vitamin supplemented lipopolysaccharide vaccinated group exhibited greater immune responses than its formalin killed and heat killed bacterin vaccinated counter parts. Challenge study suggest that the relative percent survival was found to be the same for both formalin killed and LPS immunized vitamin treated groups while lower for the heat killed immunized vitamin treated group.
Kwon et al., (2006), stated that *E. tarda* formalin-killed bacterial vaccine and bacterial ghosts was used to vaccinate tilapia fish by intraperitoneal injection method against Edwardsiellois. The results revealed that high protective ability, serum agglutination titers and bactericidal activity of both vaccines was compared with control. The higher effects were observed in fish vaccinated with the bacterial ghosts.

Mai et al., (2008), studied the three types of formalized whole culture *A. hydrophila* vaccine (FWC) were prepared, FWC vaccine, FWC vaccine mixed with Freund's complete adjuvant (FCA) and FWC vaccine mixed with Freund's incomplete adjuvant (FIA), tested for sterility and administered to female Nile tilapia (*O. niloticus*) using two methods of delivery. Micro-agglutination and the double immune diffusion tests were performed on serum, mucus and eggs to evaluate maternal immunity. The RLP was calculated after challenge infection.

Lucienne et al., (2010), evaluated an inactivated *S. agalactiae* vaccine in tilapia for the control of streptococcal disease outbreaks. One group of tilapia (treatment 1) received one vaccine dose and the other group of tilapia (treatment 2) received two doses, with an interval of 21 days. Immunized and control tilapia were intraperitonially challenged at 30 days post vaccination. The fish were monitored daily for disease signs and for mortality for 16 days post challenge. A statistically significant difference (P=0.0045) was found between the mortality of treatments 1 and 2. The value of relative per cent of survival of 83.6% and 96.4%, respectively, indicate that this vaccine was efficient in Nile tilapia.

Prasad and Areechon (2010), evaluated the humoral response in red tilapia against formalin-killed *A. hydrophila* and *Streptococcus* sp. vaccine administered by intraperitoneal injection and immersion method. The result
indicated that *A. hydrophila* vaccine induced significantly (P<0.05) high antibody titers and protective response than the *Streptococcus sp.* vaccinated group.

Dash *et al.*, (2011), studied the dose dependence specific and non-specific immune response of Indian major carp *Labeo rohita* to intraperitoneal injection of formalin killed *Aeromonas hydrophila* whole cell vaccine. Three different doses (10\(^5\) cfu/ml, 10\(^7\) cfu/ml 10\(^10\) cfu/ml) were administered intraperitoneally for 1 month. Results revealed that the highest antibody titer and RPS was recoded up to 80% at highest dose of 10\(^10\) cfu/ml. Therefore, highest dose of formalin killed cells was found to be the most effective dose for vaccination which increased the immunity in Indian major carp (*L. rohita*) to a larger extent.

Anany *et al.*, (2014), studied the efficacy of using *A. hydrophila* and *P. fluorescence* formalized killed vaccines in African Catfish (*Clarias garpeinus*). Monovalent and Bivalent vaccine was given by injection, immersion and oral method at 7, 14, 21 and 28\(^{th}\) day. Challenge infection was done after 30 days of vaccination. The humoral immune response was detected by RPS, Mortality rate and ELISA. Results indicated that maximum RPS and antiboby titer (ELISA) values in vaccinated groups when compared to the control groups.

Sen *et al.*, (2014), studied the efficacy of three antigenic preparations (FAH, FAH+A, ECP) from the fish pathogen *A. hydrophila* was evaluated as a vaccine candidate in rohu. At the end of 10, 20, 30 days post vaccination, fishes were challenged with *A. hydrophila* and RPS was recorded. Result showed that, the immununological parameters like, specific leukocyte proliferation, nitric acid production, super oxide anion were increased in FAH+A (Formalin inactivated *A. hydrophila* group + Freunds complete adjuvant) and a higher level of survival in all the vaccinated groups.
Li et al., (2016), investigated the various vaccine preparations including formalin, phenol, chloroform and heat-killed whole cell bacterins and subcellular lipopolysaccharides (LPS), as well as different administration routes, Silver Sea Bream (Sparus sarba) against Vibrio alginolyticus. Fish immunized with the subcellular LPS exhibited the best protection (RPS-100), while fish immunized with whole cell bacterins displayed varying degrees of protection (RPS ranged from 80 to 28), formalin-killed, phenol-killed, heat-killed, chloroform-killed bacterins respectively. Regarding various administration routes, fish immunized with two injections exhibited the best protection, and the RPS values were 100 or 85 upon higher or lower doses of pathogenic V. alginolyticus challenges. Both oral vaccination and a combination of injection/immersion trial were also effective, which achieved relatively high protection (RPS 45 to 64.3). Marked elevations of serum agglutinating antibody titer were detected in all immunized fish.

Based on all the above studies, it was conferred that WC vaccines prepared from both bacterial pathogens showed significant difference in protection when administered with or without adjuvant. Booster doses elicited more immune response but in all the experimental groups, when compared with OMP vaccines, the WC vaccines were found to be less effective in eliciting the immunoprotective level against the pathogens.

2.4.2 OUTER MEMBRANE PROTEIN (OMP)

The outer membrane protein (OMP) of Gram-negative bacteria has an important role in the interaction between bacteria with hosts in terms of adherence, uptake of nutrients from the host, and subverting host defense mechanisms (Harikrishnan et al., 2011). OMP has been considered to be the Noval vaccine due to its ability to play a role as molecular adhesion molecule and also their exposed
epitopes on the surface. Some investigators have attempted to study the immunization of OMP vaccines in different groups of fish, the literature on these studies were presented below.

Rahman and Kawai (2000), analysed the expression of OMPs, of *A. hydrophila* *in vivo* and *in vitro* to seek a correlation between the antibody responses in goldfish with high protective levels. It was observed that the *in vivo* OMP of *A. hydrophila* have protective immunogenicity against infection by this bacterium, but, no apparent differences were found between the fish groups immunized with *in vivo* or *in vitro* OMPs and concluded that OMPs of *A. hydrophila* have protective immunogenicity and it may be useful to develop vaccines by selecting such OMP antigens.

Rahman *et al.*, (2002), studied the outer membrane fraction (OMF) of *Flavobacterium psychrophilum* induces protective immunity in rainbow trout and ayu. This OMF induced significantly higher antibody titers and protection against cold water disease in rainbow trout (*O. mykiss*) and Ayu (*P. altivelis*) compared to inactivated whole cell *Flavobacterium psychrophilum* bacterium. The above results were similar to the findings in our present study.

Khushiramani *et al.*, (2007), immunized Indian major carp, *L. rohita* using a purified 37 kDa temperature sensitive OMP (OMP TS) of *A. hydrophila*. The protein induced antibodies with mean titers of 1:4000 on day 14 and 1:12,000 on day 28 indicating that the protein is highly immunogenic in fish and that the gene is a potential candidate for vaccine development.
Mao et al., (2007), have produced the recombinant OMP from *Vibrio harveyi* and *Vibrio parahaemolyticus* were found to protect yellow croaker *P. crocea* from infection with virulent strains of both bacteria.

Wang et al., (2011), identified and evaluated an outer membrane protein OmpU from a pathogenic *V. harveyi* isolate as vaccine candidate in turbot (*S. maximus*). The ompU gene encoded a 35 kDa protein, which was purified by Ni-NTA His-Bind Resin column. Turbot were injected intramuscularly with the purified OmpU protein and the recombinant PEGFP-N1/ompU plasmid, respectively. The fish vaccinated with the purified OmpU protein were completely protected with a RPS of 100% against pathogenic *V. harveyi* infection. Efficient protection was also found in the pEGFP-N1/ompU (DNAvaccine) vaccinated group, with a RPS of 51.4%. Significant specific antibody responses were detected in the vaccinated turbot by indirect enzyme-linked immunosorbent assay. Various OMP bands were observed in our studies which needs further investigation in future.

Sun et al., (2011), studied that the effect of Lipopolysaccharide (LPS) and Outer membrane protein (OMP) vaccines on protection of Grass Carp (*Ctenopharyngodon idella*) against *A. hydrophila*. The fishes were immunized with OMPs and LPs by injection method. Immune response was assessed after 21st and 28th day of vaccination and challenge study was carried out after 5 weeks post vaccination. The results reveal that increased in respiratory burst, phagocytic activities in head kidney leucocytes and serum lysozyme activity. Relative percent survival was increased to 83.3% in LPs and 72.2% in OMPs when compared with control.

Thanga viji et al., (2012), have evaluated that an Outer Membrane Protein (OMP) of *Aeromonas hydrophila* as a vaccine to provide protection against the
pathogen in goldfish (*Carassius auratus*) by using the extract of *A. racemosus* tuber powder as an adjuvant in the vaccine preparation. Survival and immunological response of the vaccinated fishes (30 and 60 dpv), were evaluated after challenge with virulent *A. hydrophila*. The vaccine treated experimental groups significantly improved (p > 0.05) the survival at 50% compared to the controls and had improved immunological responses including phagocytosis, albumin-globulin ratio, serum lysozyme activity and serum bactericidal activity.

Divya *et al.*, (2015), studied the comparative effects of bacterial outer membrane protein encoding gene clone (BOMPG) and bacterial outer membrane protein (BOMP) vaccination to *C. auratus* against *A. hydrophila* and vaccinated by intra peritoneal injection method in every 15 days interval. At the end of the experiment, the immunized and control fishes were challenged with virulent strain of *A. hydrophila* and assessed the biochemical, haematological, and immunological parameters. *C. auratus* succumbed to death 100% at five days when no vaccination was given whereas the vaccinated groups survived significantly (F=34.64; P ≤ 0.001) of 70 and 80% respectively in BOMPG and BOMP after 10 days of challenge. The biochemical parameters, haematological and immunological parameters were also improve significantly (P ≤ 0.001) in the BOMP vaccinated fishes due to the immune enhancement by the vaccines. Among the two different BOMP deliveries, the BOMP highly influenced to improve the immune system against *A. hydrophila* challenge than BOMPG.

### 2.4.3 MONOVALENT AND POLYVALENT VACCINES

During past few decades, much attention has been given to design and develop suitable monovalent and polyvalent vaccines based on the antigenic nature of the used strains, dose and route of administration for controlling diseases in
aquaculture (Busch 1997; Evelyn 1997). The success of polyvalent vaccines was regulated by the concentration of individual antigens, cross reactivity and competition among different antigens (Swain et al., 2003).

Thune and Plumb (1982), found that both sac fry and swim-up fry vaccinated by immersion in sonicated polyvalent bacterin were protected against challenge with homologous bacteria.

Vera et al., (2002), compared the Protection against atypical furunculosis in spotted wolfish vaccinated with monovalent or multivalent vaccines containing different strains of atypical A. salmonicida. Vaccinated fishes were challenged with three different A. salmonicida strains. Significant protection was obtained against both homologous challenges. The best protection, regardless of the strain used for challenge was obtained with the multivalent vaccine composed of three different bacterial strains. Also, some of the monovalent vaccines resulted in significant protection.

Swain et al., (2007), demonstrated the immune response to mixed whole cell antigens of A. hydrophila, E. tarda and P. fluorescens, the common Gram negative bacterial pathogens associated with diseases of Indian major carps. The rohu yearlings were either immunized with antigens from single bacterial strain, A. hydrophila, E. tarda and P. fluorescens or a combination of all three by injection method. RPS and serum agglutination responses were analysed after immunization in vaccinated and control groups. Results revealed that there was increase in the RPS and antibody level in the vaccinated groups. Similarly, no significant difference (p > 0.05) in the antibody level was found between groups immunized with single and mixed bacterial antigens.
Silva et al., (2009), studied the efficacy of a polyvalent bacterin vaccine against *Aeromonas hydrophila*, *P. aeruginosa* and *E. durans* administered by different routes in Nile tilapia by analyzing hematological and immunological parameters 7 and 21 days after vaccination. Vaccinated fish groups presented higher agglutination titer, hematocrit, number of erythrocytes and leukocytes than the nonvaccinated group. The different vaccine administration routes stimulated hematological and immunological responses in Nile tilapia 21 days post-vaccination, but intraperitoneal vaccination presented higher total number of leukocytes, lymphocytes and serum agglutination titer. The results of silva et al., were identical to our results where in the polyvalent vaccines administration to the experimental groups produced higher agglutination titre and high leukocyte count than the non vaccinated groups.

Osman et al., (2009), prepared different vaccine formulations for vaccination of Tilapia species were tried by adding formalin to the bacterial culture (bacterin) and used by immersion and oral routes. Fish were vaccinated by using monovalent, bivalent and polyvalent vaccines (*A. hydrophila, A. sorbia, A. caviae* and *P. fluorescence*) and the efficacy of these vaccines were tested by using the challenge test with the detection of RPS and by using indirect ELISA for estimation of the immune response of fish during and after vaccination. The results of fish vaccination showed easier administration and of higher efficacy (RPS) and it were effective against more than one type of bacteria.

Bailone et al., (2010), evaluated the effects of formalin inactivated polyvalent vaccination (*A. hydrophila* (ATCC 7966), *P. aeruginosa* (ATCC 27853) and *E. durans* ATCC (1949)) by injection route in Nile tilapia against *A. hydrophila*. RPS, hematological and serum agglutination responses were analysed ten days after immunization in vaccinated and control group. Results revealed that there was
increase in the RPS, Leucocyte counts and agglutination titer values in the vaccinated groups when compared to the control group.

Sun et al., (2011), obtained the most protection of the *E. orientalis* against the disease by the bivalent vaccine of *E. tarada* and *V. anguillarum* along with Freund's incomplete adjuvant.

Yun et al., (2011), demonstrated the multivalent killed whole cell vaccines with protective effect against two or more of the pathogens (*E. tarda* TX$_1$, *V. anguillarum* C$_{312}$, *Staphylococcus iniae* SF$_1$ and *V. harveyi* T$_{4D}$) in Japanese flounder (*P. olivaceus*). The results suggest a humoral immunity-based mechanism of protection induced by inactivated WC vaccines, and that there exists a mutual and specific immunostimulatory effect between *Edwardsiella tarda* TX$_1$ and *V. anguillarum* C$_{312}$, which enables the divalent to induce effective protective immunity against *E. tarda* TX$_1$, *V. anguillarum* C$_{312}$.

Craige et al., (2012), tested the ability of a killed bivalent *S. iniae* and *V. vulnificus* vaccine delivered through intraperitonal injection to protect sex reversed hybrid tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) against challenged each bacterium, independently. In two independent trials, vaccination of tilapia with the bivalent vaccine conferred protective immunity against *V. vulnificus* and *S. iniae*. RPS values ranged from 79 to 89% for *V. vulnificus* and 69 to 100% for *S. iniae* following challenge of bivalent vaccinated fish. Use of this bivalent formulation may be a cost-effective strategy to reduce losses in tilapia coinfected with these two important bacterial pathogens.

Zheng et al., (2012), investigated the immune enhancing effects of different adjuvants used in a pentavalent vaccine for turbots. The pentavalent
vaccine consisted of inactive bacterial cells from five common pathogenic strains (\textit{V. anguillarum}, \textit{V. scophtalmi}, \textit{E. tarda}, \textit{V. harveyi} and \textit{V. alginolyticus}) and the adjuvants were Astragalus Polysaccharides (APS), propolis and FCA. Turbots were immunized with the pentavalent vaccine alone or with one of the adjuvants. Fishes were also challenged with the pathogens after immunization and the RPS was assessed. The results showed that APS, propolis and FCA had significant immune-enhancing effects on turbots as shown by the higher titers of antibodies against the pathogens and enhanced RPS after challenge with pathogens.

2.4.4 IMMUNO ADJUVANT (\textit{ASPARAGUS RACEMOSUS})

In most vaccines, adjuvants are a vital ingredient for efficacy. Various adjuvants have been used in fishery and they induce better and more long lasting protection than non-adjuvant vaccine. In general natural products were found to be less toxic, bio degradable, harmless for the fish and human health and safer than chemical products. Recently, the herbal immunoadjuvant, \textit{Asparagus racemosus} has been shown to improve vaccine delivery against aquatic pathogens (Kumaran 2010).

Edelman (1980), stated that, ideal adjuvants should be stable with long shelf life, bio-degradable, cheap to produce, not induce immune responses against themselves and promote an appropriate immune response (\textit{i.e.}, cellular or humoral immunity depending on the requirements for protection).

Anbarasu \textit{et al.}, (1998), have suggested that formalin inactivated vaccines were found to be superior to heat killed preparation, especially when the bacterins were injected along with adjuvant.
Cohen and Bioterrorism (2001), have suggested that saponins based adjuvants (*Asparagus racemosus*) have the ability to modulate the cell mediated immune system as well as enhance the antibody production.

Gautam *et al.*, (2004), evaluated the immunoadjuvant potential of *A. gracemosus* aqueous root extract in experimental animals immunized with diphtheria, tetanus, pertussis (DTP) vaccine. Immunized animals (treated and untreated) were challenged with *B. pertussis* 18323 strain and the animals were observed for 14 days. Immunostimulation was evaluated using serological and hematological parameters. Results indicate that the treated animals did show significant increase in antibody titers as compared to untreated animals after challenge (*P* = 0.002).

Citarasu *et al.*, (2006), reported that the increase in survival and resistance to White Spot Syndrome Virus (WSSV) infection in black tiger shrimp, *P. monodon* feeding immunostimulant herbal supplemented diets. The results have indicated that, when the shrimps fed with 800 mg/kg mixed diet had more survival (74%) and reduction in viral load which was found to be significant (*p*<0.0001).

Gopalakannan and Arul (2006), investigated that immunomodulatory effect of dietary intake of chitin, chitosan and levamisole on the immune system of *C. carpio* and control of *Aeromonas hydrophila* infection in ponds. Results revealed that there was an increase in the WBC count, RPS after feeding the common carp with immunostimulants like Chitin.

Rao *et al.*, (2006), demonstrated the dietary supplementation of *A. aspera* seed stimulated immunity and enhanced resistance to *A. hydrophila* infection of *L. rohita*, rohu fingerlings. Results have revealed that *A. aspera* seed had enhanced
the survival rate and increased fish growth rate, serum protein, lysozyme, serum protein, A/G ratio and phagocytosis.

Sahu et al., (2007), evaluated the efficacy of dietary doses of Magnifera indica (mango) kernel on the immune response and disease resistance of L. rohita fingerlings against the bacterial pathogen A. hydrophila. Fish was challenged with A. hydrophila, furthermore 60 days post feeding and the rate of mortality was recorded for a period of 10 days that is during the post-infection period. The results demonstrate that fish fed with mango kernel showed enhanced superoxide anion production, lysozyme, serum bactericidal, serum protein, and albumin (P < 0.05) compared with control group. The survivability was higher in experimental diets. These results indicate that mango kernel stimulates the immunity and makes L. rohita more resistant against A. hydrophila infection.

Pachanawan et al., (2008), tested the dietary supplementation of P. guajava against A. hydrophila in Tilapia (O. Niloticus). Result have revealed that increased survival rate after challenging the fish with A. hydrophila in tilapia fed diets containing either dry leaf powder or ethanol extract of P. guajava leaf.

Thangaviji et al., (2013), studied that, different vaccines (WC, ECP, OMP and BF) prepared from A. hydrophila to provide protection against the pathogen in C. auratus (Goldfish) by using A. racemosus tuber powder extract as an adjuvant. Relative Percent Survival and immunological responses of the vaccinated fishes (25 and 50dpv) were observed after challenge with virulent strain of A. hydrophila. The results showed that, the vaccinated groups significantly improve the survival rate (p >0.05) compared to the control groups and also enhances the Biochemical, haematological and immunological parameters. Based on the results, the immunoadjuvant A. racemosus helped to improve the efficiency of vaccines.
Arunvasu et al., (2013), evaluated the efficacy of different dietary doses of Z. officinale powder for the immune response and the disease resistance of the Indian major carp (C. catla) infected by A. hydrophila. Haematological, biochemical and immunological studies were performed on fish and were analysed different days of feeding trial. The SGR, RPS, total erythrocyte, leukocyte count, haemoglobin content and total serum protein were significantly (P<0.05) enhanced in Z. officinale supplemented groups. The results concluded that the Zingiber officinale powder was used in this study which acted as immunostimulant, in order to enhance the non-specific immunity and disease resistance of C. catla to A. hydrophila infection.

All the above literature findings based on using immunoadjuvants along with mono and polyvalent vaccines produced concurrent results in our studies by eliciting immune response, verified by increased RPS rate and decreased mortality value. A significant increase was found in agglutination titre and blood leukocyte counts in immunoadjuvanated groups than non-immunoadjuvanated groups.

2.4.5 QUALITATIVE PROTEIN ANALYSIS BY SDS-PAGE METHOD

SDS-PAGE Method offers a rapid and relatively accurate way to determine the protein molecular weights in bacterial vaccines.

Kawai et al., (2004), reported the immunogenic response of 37 kDa OMP and induction of protective immunity against E. tarda infection in Japanese flounder. The protein profile was analysed by SDS – PAGE method. In this study, common carp fishes vaccinated with purified recombinant OMP A protein elicited a significant immune response and the vaccinated fish when challenged with pathogenic Edwardsiella tarda revealed a higher survival rate (60%) when compared to un-immunized fish.
Maji et al., (2006), characterized the outer membrane proteins (OMP) of *A. hydrophila* to identify suitable immunoreactive components. A total of 10 fractions were generated from crude OMP preparation. Primarily a 57 kDa polypeptide and a 23 kDa polypeptide, showed maximum sero-reactivity, even higher than the crude OMP. They concluded that the 57 kDa and 23 kDa polypeptides of the OMP of *A. hydrophila*, possessed high immunoreactivity, and should be given due attention while preparing immunodiagnostic and immunoprophylatic tools against *Aeromonas* infections in goldfish.

Maiti et al., (2011), studied the recombinant OMP A of *E. tarda* was used as a vaccine candidate in common carp. The 12% SDS-PAGE was used to analyse the OMP protein profiles and obtaind the molecular weight of 36 and 44Kda. These OMPs were highly immunogenic in fish.

Divya et al., (2015), studied the comparative effects of bacterial outer membrane protein encoding gene clone (BOMPG) and bacterial outer membrane protein (BOMP) vaccination to *C. auratus* against *A. hydrophila*. The bacterial outer membrane protein (BOMP) profile was analysed by the method of laemelli using 10% SDS-PAGE.

SDS-PAGE protein profile showed difference in both *E. tarda* and *P. flourescence*. The molecular weights of all protein bands were verified by band analysis method using G image software. The specific protein band that elicited immune response needs further investigaton which could be taken up as broad study in future.
2.4.6 QUANTITATIVE PROTEIN ANALYSIS BY LOWRY’S METHOD

Lowry’s method is commonly used to determine the total protein concentration of the sample. This technique is used for estimate the proteins present in the fish vaccines.

Thangaviji et al., (2013), studied the immunization of OMP provides protection against Aeromonas hydrophila in goldfish. In this study, prepared OMP was quantified following the protocol of Lowry’s method and used for immunization. The vaccine treated experimental groups significantly improved (P <0.05) the survival at 50% when compared to the control.

Saurabh et al., (2016), studied the Aeromonas hydrophila Omp W PLGA Nanoparticle oral vaccine induced immunity in rohu fish. In this study, the protein concentration was measured as described by Lowry’s method and used for vaccine preparation.

2.4.7 METHOD OF VACCINATION - IMMERSSION METHOD

According to Newman 1993, immersion immunization methods are associated with variable efficacy, yet they offer the benefits of low labour input, minimal handling stress, easy administration of large number of small fish and stimulation of the immune system via the natural route of the pathogen entry. Prolonged immersion, however, may suggest a greater level of protection. Prolonged immersion has been considered in rainbow trout (Moore et al., 1998; Ototake et al., 1999).

Johnsons et al., (1982), vaccinated the fry of several Salmonoid species by direct immersion with either Yersinia ruckeri or Vibrio anguillarum and
monitored the level of protective immunity induced by the survival of the fish after bath challenge with virulent organisms. The duration of the protective immunity varied with the bacterin concentration and size and species of fish.

Rodgers (1990), studied the immersion vaccination used rainbow trout Salmo gairdneri and compared the efficacy of a vaccine with other vaccine containing liposome particles against a natural challenge of fish furunculosis. Results have shown that fry could be protected with a 3 component vaccine consisting of whole cells, 'toxoided' extracellular products and lipopolysaccharide that can significantly enhance the efficiency that also contained liposomes.

Oleson (1991), has detected the antibody response in rainbow trout following immersion vaccination with Y. ruckeri bacterins by ELISA and passive immunization. This test was proved to be more sensitive than the agglutination test and passive immunization studies which reveal that humoral factors have played a significant role in protective immunity after immersion vaccination with Y. ruckeri bacterins.

Swain et al., (2002), have studied the bath immunization of spawns, fry and fingerlings of Indian major carps using a particulate antigen and determined the age, dose and duration of antigen exposure. Result showed a significant resistance against virulent E. tarda bacteria and significant antibody titre was recorded in fry and fingerlings that were exposed to $10^9$ CFU/ml bacterin concentration for a time period of 45 and 60 minutes respectively.

Arijo et al., (2015), investigated the protection of cultured Sole, Solea senegalensis Sub sp. Piscicida was vaccinated by divalent vaccine against V. harveyi and Photobacterium damselae. Formalized whole cells and extra cellular
products of virulent strains of both microorganisms and administered by immersion route. Result revealed that the high protection afforded by the divalent vaccine in sole lasted for 4 months after which the RPS values against both pathogens decreased significantly.

Sajjad *et al.*, (2013), stated that formalin-killed, heat-killed and lipopolysaccharide vaccines against *A. hydrophila* and a bivalent formalin-killed vaccine against *A. hydrophila*, *A. veroni* and *A. sobria* were tested in rainbow trout (*O. mykiss*). The evaluation of trout fish immune response after vaccination with *Aeromonads* bacterins by immersion and bath challenge route was undertaken using an indirect ELISA. To test the strength of protection, the challenge process was examined using 10 cells of the live bacteria/ml of *A. hydrophila*. The results showed that the RPS in the trout fish groups vaccinated by heat-killed type of vaccine were significantly higher (P<0.05) than that the other types of vaccines (84%). In addition, the fish vaccinated with the bivalent vaccine of *A. hydrophila*, *A. veroni* and formalin-killed vaccine showed a high percentage of RPS (67%), while it was measured as 34% for the LPS vaccine.

One of the major advantages of immersion method in our present study was cost effective, less handling stress, less man power, highest RPS rate, higher agglutination titre and leukocyte counts which made us inquisitive in taking up this study which produced good results.

### 2.4.8 IMMUNOLOGICAL STUDIES - BLOOD LEUKOCYTE COUNT & SERUM ANTIBODY TITRE (AGGLUTINATION TEST)

Immunisation primes the immune system of the host against pathogens encountered during infections (Thomson and Adams, 2004). Haematological
parameters of fish blood are useful tools that aids in diagnosis of disease. It can also be used to study immunopotentiators. Moreover, Leucocytes are one of affecting factors in immunity of fish and leucocyte numbers or the proportion of different cell types has been used as indicators of health of aquatic animals (Duncan et al., 1996). The humoral response of carps measured as antibody titres (Sundick and Rose 1980). Several reports are available on the ability of very young fish to produce humoral immune response.

Harikrishnan et al., (2003), studied that the hematological and biochemical parameters of common carp (Cyprinus carpio) infected by pathogenic strain of A. hydrophila. The infected fishes were dip treated with an aqueous Azadirachta indica leaf extract for 30 days until the lesions healed completely. The hematological and biochemical parameters of the infected and control fishes were monitored on the 10th, 20th and 30th day. The result showed that the leukocyte number was significantly increased in control group which are not treated than treated groups.

Plumb and Areechon (1990), Chen and Light (1994), Yildirim et al., (2003), revealed that fish are the most primitive vertebrate group to present acquired immune system, and the ability to produce antibody after antigenic stimuli. The agglutination is a clumping reaction between specific antibodies and a particulate antigen, such as erythrocytes or bacterial cells suspension, and it is usually applied in the study of adaptive immune responses to evaluate the antibody production.

Mercy (2006), observed the influence of leaf extract of Phyllanthus emblica (Linn) on immunological, hematological and biochemical responses in freshwater fish, C. mrigala. The fish was artificially infected with bacterial pathogen, Pseudomonas fluorescens. The result showed that the significant increase
in hemagglutination antibody titers was observed in *Cirrhinus mrigala* when compared with control group.

Silva *et al.*, (2009), studied the efficacy of a polyvalent vaccine against *A. hydrophila, P. aeruginosa* and *Enterococcus durans* administrated by different routes in Nile tilapia was assessed by analyzing hematological and immunological parameters 7 and 21 days after vaccination. The different vaccine administration routes stimulated haematological, and immunological responses in Nile tilapia 21 days post vaccination, but intraperitoneal vaccination presented higher total number of leukocytes, lymphocytes and serum agglutination titer.

Yin *et al.*, (2009), investigated that the effect of Chinese herbs (*A. radix* and *Ganoderma lucidum*) on immune response of carp. *A. hydrophila* vaccinated or unvaccinated fishes were fed with Chinese herbs for a period of 5 weeks and these fishes were challenged with *A. hydrophila*. Result revealed that feeding vaccinated or unvaccinated carp with combination of *A. radix* and *Ganoderma lucidum* stimulated immunological parameters and increases the RPS rate (90%) when compared to control group.

Sharma *et al.*, (2010), observed the stimulatory effect of dietary doses of *Withania somnifera* (Ashwagandha) root on immunity and disease resistance against *Aeromonas hydrophila* infection in Indian major carp, *L. rohita* fingerlings. The dietary doses were given in different dosages that include 0 g kg$^{-1}$ (control), 1 g kg$^{-1}$, 2 g kg$^{-1}$ (T2) and 3 g kg$^{-1}$ (T3) for a time period of 42 days. The result of the study show that fishes that were fed with *Withania somnifera* showed an increase in Phagocytic activity, total Immunoglobulin level and lysozyme activity was found to be significant when compared to control group. Furthermore, RPS was found to higher and was found to be significant when compared to control group. The fishes
which were fed with 2 g kg\(^{-1}\) concentration of \(W. \text{ somnifera}\) are known to have a higher protection rate in fishes against \(A. \text{ hydrophila}\).

Sugahara and Eguchi, (2012), investigated the assessment of agglutinating antibody titer is an easy approach to measure circulating antibodies in serum samples collected from fish previously immunized with particulate antigen preparations.

Sivagurunathan \textit{et al.}, (2012), has evaluated the Immunomodulatory effect of dietary \textit{Nelumbo Nucifera} (Lotus) in growth and haematology of \textit{C. mrigala} Challenged with \textit{Pseudomonas aeruginosa}. Immunostimulant potential of \textit{N. nucifera} (Lotus) in formulated diets with different concentrations of ethanolic extract of \textit{N. nucifera} (D1=0%, D2=1% and D3=2%) were fed to \textit{C. mrigala} for 40 days, the Specific Growth Rate (SGR) and Feed Conversion Ratio (FCR) were calculated significant increase was observed in SGR and FCR. Then the experimental fishes were challenged with \textit{P. aeruginosa} and the results revealed that, the haematological parameters like Total Erythrocyte count (TEC), Haemoglobin, Total leucocyte count (TLC), Differential leucocyte count (DLC), serum total protein, serum albumin and globulin levels were increased significantly in D3 diet fed fishes.

Rakesh \textit{et al.}, (2013), studied that the efficiency of water extracts of \textit{Ocimum sanctum} Linn (Tulsi) on the immune response and disease resistance of \textit{Labeo rohita} (Hamilton) fingerlings against the \textit{A. hydrophila} infection. Result revealed that significant increase in WBC counts and RSP (\(p < 0.05\)) in treatment groups when compared with control group. These results indicate that \textit{O. sanctum} leaf extract stimulates the immunity and makes \textit{L. rohita} more resistant to bacterial infection.
Sujatha (2013), carried out a study on *C. catla* fish which was vaccinated by heat killed *A. hydrophila* vaccine with or without Montanide Adjuvant (ISA 763 AVG) by dip immersion method. The effects of heat killed antigen vaccine and vaccine with adjuvant on the leucocyte count of fish *C. catla* was studied. The results shown that differential counts of leucocytes were found to higher in the fishes treated with vaccine and adjuvant than fishes treated with heat killed antigen, vaccine only group and control group. Microscopic observation of the diluted *A. hydrophila* isolate and the serum collected from the vaccinated fishes showed the clumps of antigen-antibody molecules confirming bacterial agglutination.

Rahimi et al., (2015), evaluated the Effects of different levels of Vitamin C and E administration on Leucocyte counts in Rainbow trout (*O. mykiss*) fingerlings during 60 days of feeding trails. The results revealed that administration of Vitamin C and E can be led to significant increase in leucocytes that may influence immune response and resistant against disease. Our studies also revealed that monovalent and polyvalent vaccines presented higher agglutination titre and leukocyte counts in OMP+A when compared with other experimental groups.