Available online at: www.cbioresearch.com/journal.html ISSN No. 0973-8207

Immune enhancement of *Oreochromis mossambicus* (Peters) in relation to different doses of *Lactobacillus sporogenes* given as a feed additive

S.Venkatalakshmi[a] and J. Ebanasar[b]

a. PG and Research Department of Zoology, Government College for Women (Autonomous), Kumbakonam, India.
b. PG Department of Zoology & Biochemistry, Government Arts College (Autonomous), Kumbakonam, India.

*Correspondence e-mail: *venkatalakshmi.gpm@gmail.com*

Abstract

The significance of a suitable diet in preserving the health of living organism is widely recognized. In this present study, the microbial probiotic *Lactobacillus sporogenes* was assessed for its immunostimulatory properties when given as feed additive. The probiotic was given in three different doses – $2.5X10^5$, $5X10^5$ and 10^6 CFU as a feed supplement in the form of spores. All the three doses enhanced the specific antibody response to heat killed. *Aeromonas hydrophila,* activated neutrophils, total and differential white Blood Cell Count significantly. 100% survival was observed in 10^6 CFU fed groups and the other two lower doses gave 80% survival against *Aeromonas hydrophila* infection. The gut colonization was also tested in the treated groups. A dose dependent survival of *Lactobacillus sporogenes* was recorded in the tank water and gut of *Oreochromis mossambicus*. From the result of study *Lactobacillus sporogenes* can be prescribed as an efficient microbial feed supplement.

Keywords

probiotic, *Lactobacillus sporogenes, Aeromonas hydrophila,* feed supplement, immunostimulants

Introduction

Nutrition has a great influence on the health and immune response of fish (Blazor and Wolke, 1984). The significance of a suitable diet in preserving the health of living organisms is widely recognized (Lygren *et al.,* 1998). In recent years the increasing consumer concern about the residues of antibiotic resistant strains have led to the use of biological or probiotic feed additives in the animal feeds.(Uma *et al.,* 1999) probiotics have been defined as a live microbial feed supplement, which beneficially affect the host animal by improving its intestinal microbial balance (Fuller, 1989). Recently the definition has been improved as microbial dietary adjuvants that beneficially affect the host physiology by modulating the mucosal and systemic immunity (Rodriguez *et al.,* 2004).

In all these studies, though the probiotics caused significant stimulation of immune system, only a transient colonization (Nikoskalainen *et al.,* 2003 and Joborm, 1998 Gatesoupe,1999) or *in vitro* adhesion in the gut (Villamil *et al.,*2002) was shown to the best of our knowledge, so the present study was aimed at selecting a lactobacillus strain that can permanently colonize the get and also stimulates the immune system.

Materials and methods

Animal maintenance

Oreochromis mossambicus a common fresh water cichlid fish was used for the study. Fish procured from local fish farms were stocked in large fiber tanks. The experiments were carried out in plastic tubs (vol. 70 lt). Fish of both sexes weighing 20-25gm were used in the study. Water was changed frequently to avoid stress due to ammonia accumulation. The animals were fed

ad libitum with a balanced fish diet prepared in our laboratory. Temperature of the water was not controlled because data collected in the preliminary studies indicated only minor daily fluctuations (28 ± 1 °C).

Preparation of feed and Experimental protocol

Lactobacillus sporogenes was supplemented in the form of spores. The viability of the spores was determined by plate count method as colony forming units(CFU) on MRS agar (Titan Biotech, Mumbai) The formulated feed prepared in our laboratory served as a base in which the different number of spores were added and were palletized individually. The number of Lactobacilli in the feed were estimated again by plate count method on MRS agar by homogenizing 1g of feed in 9 ml of sterile PBS pH 7.2 and spreading appropriate dilution from 10^{-1} to 10^{-12} on MRS plates and the plates were incubated at 37 °C for two days (Nikoskalainen *et al*., 2003). Feeds with 3 different CFU were selected for the study - $2.5X10^4$ (T1), $5X10^4$ (T2) and 10^5 (T3) CFU. A control feed with PBS alone was used. Feeds were stored at 4^0C until use. Fish were fed with the supplemented feed for seven days. Then the fish were switched over to normal unsupplimented diet.

Immunization

After seven days of exposure to LAB treatment, fish were immunized with heat killed *Aeromonas hydrophila* (10^8 cells/fish).

Serum collection

The fish were bled serially using one ml tuberculine syringe with 26 gauge needle from the common cardinal vein at regular intervals of seven days after immunization (Michael and Priscilla ,1994) for studying the antibody response. The blood drawn was collected in micro centrifuge tubes (Torson). The serum was separated and decomplemented at 47 °C for 30 min and then stored at -20 °C until use.

The immunization and serial bleeding were done between 14 hrs and 16 hrs throughout the investigation to avoid the possible influence of circadian rhythmic variation in the immune response (Hurshesky, 1984; Michael and Priscilla, 1994).

Intestinal colonization of LAB

The microbial analysis was done before the trial, at the end of week 1, 2, 3, 4 and 8. The fish were starved 24 hrs before sampling. The fish were sacrificed with a blow to the head, opened aseptically and the whole intestine was removed and weighed. The intestine was crushed and ground well in phosphate buffer saline. The microbial analysis was performed by spreading appropriate dilution in PBS form 10^{-1} to 10^{-12} on a selective media for lactic acid bacteria (MRS, Titan Biotech). The plates were incubated at 37 °C for 2 days. The presence of *L. sporogenes* in tank water was also determined on the same days (Nikoskaleinen *et al.,* 2003).

Specific antibody response

The anti-*Aeromonas* antibody titre was determined by bacterial agglutination assay in 96 well 'U' bottom micro titre plates (Torson) (Roberson, 1990).

NBT assay

The NBT assay followed was that of Anderson (1992) except that distilled water was used instead of saline to prepare the NBT solution (Stasiak and Baumann, 1996). The NBT assay was done on 2^{nd}, 4^{th}, 6^{th}, 8^{th}, 10^{th} and 12^{th} days post immunization.

Total and differential white blood cell counts

Total WBC was counted in a Neubauer counting chamber using Natt-Herrings solution as the diluting fluid and differential count was done using Leishman stained blood smears.

Host resistance test

Fish were treated with LAB for seven days prior to vaccination with heat killed *A. hydrophila* whole cells (10^8 cells/fish). A challenge dose of 2 x 10^6 cells/fish of virulent *A. hydrophila* always resulted in less than 50% survival in control fish group and was used as the standard challenging dose in this experiment. An untreated, vaccinated control group and an untreated, unvaccinated, saline injected control group were maintained. Then all the groups of fish (n=30/group) were experimentally infected with the challenging dose of virulent *A. hydrophila*, four weeks after vaccination. Mortalities were recorded after 24 hrs and degree of protection was assessed by calculating the relative percent survival (Logambal *et al.,* 2000).

$$RPS = 1\text{-}n \frac{\%\ \text{experimental mortality}}{\%\ \text{control mortality}} \times 100$$

Statistical analysis

Two way ANOVA was performed when more than one variables are compared. One way ANOVA was done

when a single variable was analyzed. Student's t-test was used for comparing two particular means. MS-EXCEL was used for computing the data and the statistical analyses.

RESULTS

Specific antibody response

Significant stimulation of antibody response by LAB was observed ($p<0.001$) on the peak day (day 14). T1 did not show significant stimulation while T2 & T3 enhanced significantly ($p<0.001$) (Fig 1)

No. of activated neutrophils

The dose dependent enhancement was observed (Fig 2) on the number of activated neutrophils on day 4 by T3 (40 ± 2.1) and minimum in T1 on day 6 (18 ±1.03). The peak day was in advance shifted to 4th day in T2 and T3. The effect of the probiotic was highly significant on the number of activated neutrophils ($p < 0.001$).

Total while blood cell count

When compared to the control, the LAB supplemented diets have enhanced the peripheral leukocyte count significantly ($p<0.005$). T3 enhanced maximally, but T2 advanced the peak day to day 4 post immunization (Fig 3).

Lymphocyte count

Fig 4 revealed that all doses enhanced the lymphocyte count significantly ($p<0.05$) . The lymphocyte count was maximum on the 12th day.

Granulocytes count

There was no significant modulation in the granulocyte count ($p>0.05$) (Fig 5)

Monocyte count

When compared to the control the number of monocytes was decreased significantly by all the doses tested (fig 6).

Host resistance test

Relative percent survival was significantly increased by all the doses examined ($p<0.001$) maximum protection was offered by T3 (Fig 7) T1&T2 were equal in their protection efficiency.

Intestinal colonization

A dose dependent colonization was observed in all the treated groups. And a significant number of CFU were recorded till 60 days after discontinuation of the LAB treatment ($p<0.001$) (Fig 8).

Survival of LAB in tank water

A Significant dose dependent survival of LAB was observed in tank water till 60 days post treatment ($p<0.001$) (Fig9).

DISCUSSION

In fish as in other aquatic organisms, the whole micro organisms administered have mainly been bacterial species, which in the form of feed additives, have been shown to improve the intestinal microbial balance and increase the health states of fish, seemingly by colonizing the gut and acting as antagonists to pathogens and so increasing resistance to pathogens. (Gatesoupe 1990, Fuller, 1989 and Tannock 1997).

Good gut colonization potential is a prerequisite for a microbial candidate to be considered as a probiotic (Gatesoupe, 1999) This is the fist report to the best of our knowledge that the lactobacillus administered through feed was able to be isolated even 60 days after the fish were switched over to unsupplemented diet. Earlier reports were there for a dose dependent colonization but when changed to unsupplimented feed, the number of bacteria dropped dramatically after one week in the intestine, skin and tank water (Nikoskalainen *et al.*, 2003). A full washout of the probiotic bacteria was observed by Joborn , 1998. Similarly, viability of *Carnobacterium sp* in the gastrointestinal tract of salmonid fingerlings and fry was reported only for 4 days and 10 days respectively (Robertson *et al.,* 2000). But in out study, the probiotic *Lactobacillus sporogenes* was isolated in significant numbers even on 60th day after discontinuation of probiotic treatment. Similar results were recorded when *lactobacillus sporogenes* was administered as water additive (unpublished data)

Schiffrin *et al.,* 1997 observed that strains that are able to adhere and survive in the gut mucosa are more efficient at stimulating phagocytic cells. So according to our results, *Lactobacillus sporogenes* is a better probiotic than any other species studied so far.

The amount of viable lactobacilli in tank water samples were related to the dose of LAB in the feed. This result was in agreement with earlier studies (Nikoskalainen *et al.,* 2003). But the probiotic count

decreased when fish were changed to unsupplimented diet.

In our studies significant number of cells persists till the end of the study .The reason might be that, the species we studied is a spore forming strain. So they were able to survive better than the other lactobacillus species. In addition instead of the vegetative cells, we used the spores. The spores are 2-8 times more resistant to heat and other adverse conditions than the vegetative cells.

Once the fish were changed to normal diet, and though the water was frequently changed, the number of bacteria in tank water persists and was in correlation with the number of colonized cells in the intestine. So the source of LAB in tank water after the fish were changed to normal unsupplimented diet should have been from feces. Since Majeed & Prakash 1998 reported that the spores of *Lactobacillus sporogenes* are executed slowly via the feces for approximately seven days after discontinuation of administration.

Lactobacillus has been shown to induce antibody production in humans (Malin *et al.,* 1996, Ogawa *et al.,*, 2001). Recently handful of studies are there for immunostimulatory effects of probiotics in fish. (Cuesta 2002, Nikoskalainen *et al.,* 2003, Villamil *et al.,*2003, Rodriguez *et al.,* 2004). However though it was proved that administration of bacteria improves the survival of some fish and shell fish after challenge with pathogens, it is not completely accepted that, this is the result of direct stimulation of the immune system (Gatesoupe, 1999)

However recent molecular studies support the direct immuno modulatory role of Lactobacillus. The purified chromosomal DNA from 12 strains of Lactobacillus acidophilus induced proliferation of splenic B lymphocytes. The cloned and amplified DNA from *L.gassori* JCM 131j induced the B lymphocyte mitogenic activities (Kitazawa *et al.,* 2001) *L.rhamnosus* was recorded to have the activity of interferon promotion and interleukin IL- 4 & IL –5, monokines (IL-12,IL-18) (Cross *et al.,*., 2002) similarly the dietary intake of *Lactobacillus rhamnosus* HN001 enhances the production of both Th1 and Th2 cytokines in antigen primed mice(Cross *et al.,* 2002). The enhanced antibody production could be correlated with its ability to stimulate the number of activated macrophages and there by enhancing antigen presentation (Logambal *et al*., 2000) and the effect on the immunopoietic cells themselves.

The fish immune system is well developed and comparable to the mammalian immune system, as it consists of both T cell and B cell mediated immunity and constituting the cellular and humoral components (Iwana and Nakanishi, 1996). Hence the total WBC and differential counts were used as simple tools for assaying the immune status of fish (Logambal and Michael, 2001; Misra *et al.,* 2006).The increase in WBC count can be explained by the direct stimulatory effect of Lactobacillus on B cells and T cells and other interleukins, and monokines (Cross *et al.,* 2002, Kitazawa *et al.,* 2001).

There is no much significant modulation in differential count. This might be because it is a less sensitive tool (Anderson, 1996) but significant enhancement was observed on B lymphocyte numbers.

The decrease in number of monocytes in blood can be justified by the fact that the monocytes are differentiated into tissue macrophages and they migrate towards the site of infection or inflammation phagocytosis and antigen processing & presentation. Hence the decrease in monocytes can be expected to be indirectly proportional to the stimulation of the specific and non specific defense mechanism.

In fish the major granulocytes are neutrophils. Granulocytes in blood can be greatly increased with in 24 hrs of stressing fish (Secombes, 1996). In our study we carried over the assay only after 48 hrs. So this incorrect sampling time might be the reason for the insignificant modulation of the count by the probiotic.

Since Svobodova (1991) reported that ichthohaematology would be useful in the assessment of suitability of feeds and feed mixture, evaluation of fish conditions, determination of toxic effect of substances as well as diagnosis of disease, the present study showed the efficiency of the probiotic supplemented feed.

In recent years there has been great interest in the use of probiotic bacteria in aquaculture to improve disease resistance, water quality and/or growth of farmed fish(Verschuere *et al.,* 2000) most of the works with probiotics in fish has been focused on protection of fish against infectious diseases(Nikoskalainen *et al.,* 2003). Several probiotics have been reported for

FIG 1.EFFECT OF LACTOBACILLUS SPOROGENES ON PRIMARY ANTIBODY RESPONSE IN OREOCHROMIS MOSSAMBICUS

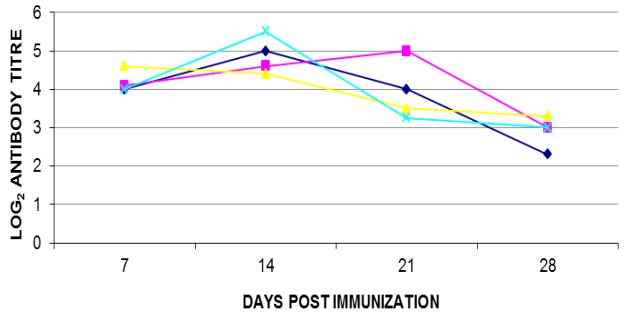


FIG 2. EFFECT OF LACTOBACILLUS SPOROGENES ON ACTIVATED NEUTROPHILS IN PHERIPHERAL BLOOD OF OREOCHROMIS MOSSAMBICUS

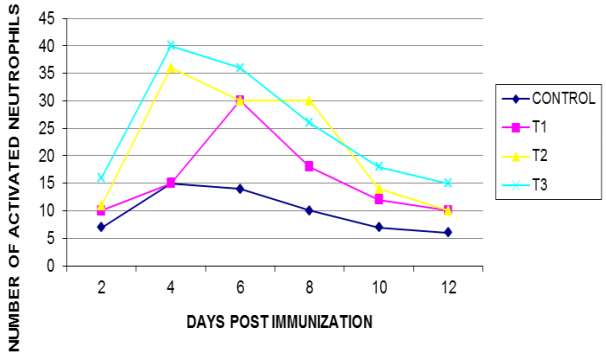


FIG 3. EFFECT OF LACTOBACILLUS SPOROGENES ON WBC COUNT IN PHERIPHERAL BLOOD OF OREOCHROMIS MOSSAMBICUS

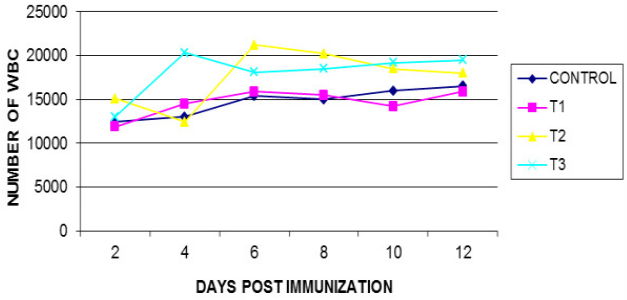


FIG 4. EFFECT OF LACTOBACILLUS SPOROGENES ON LYMPHOCYTES COUNT IN PHERIPHERAL BLOOD OF OREOCHROMIS MOSSAMBICUS

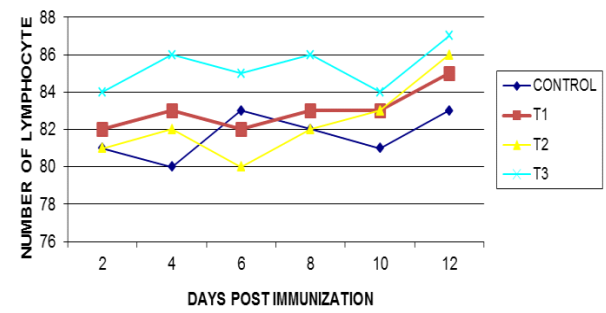


FIG 5. EFFECT OF LACTOBACILLUS SPOROGENES ON GRANULOCYTES COUNT IN PHERIPHERAL BLOOD OF OREOCHROMIS MOSSAMBICUS

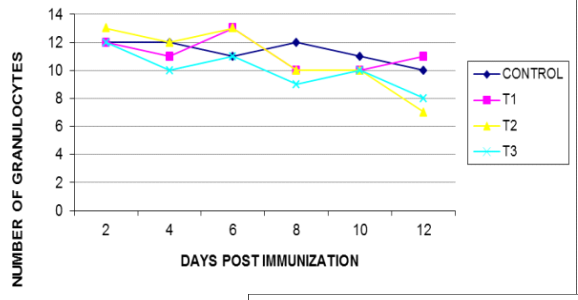


FIG 6. EFFECT OF LACTOBACILLUS SPOROGENES ON MONOCYTES COUNT IN PHERIPHERAL BLOOD OF OREOCHROMIS MOSSAMBICUS

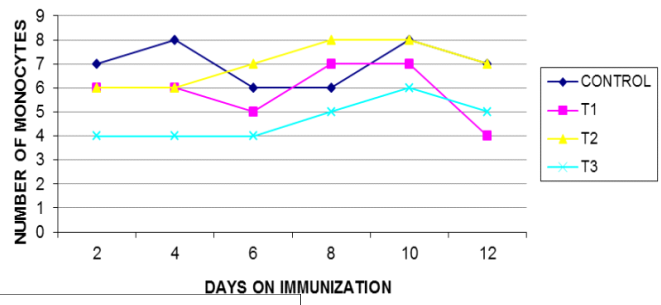
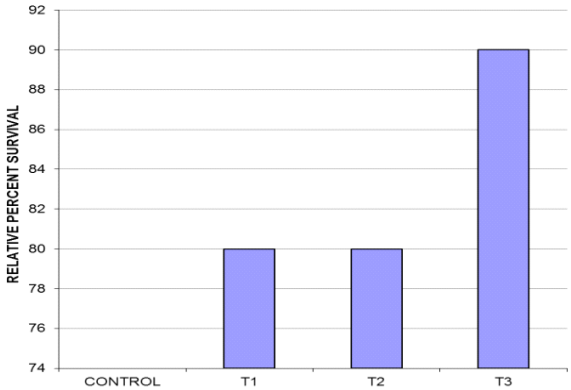


FIG 7. EFFECT OF LACTOBACILLUS SPOROGENES ON HOST RESISTANCE IN OREOCHROMIS MOSSAMBICUS AGAINST THE PATHOGEN AEROMONAS HYDROPHYLLA



increasing disease resistance in mammals and fish (Gatesoupe 1999, Uma *et al.*,., 1999, Niloskalainen *et al.,* 2003, Robertson *et al.,* 2000, Rodriguez *et al.,* 2004)

In agreement with these studies *Lactobacillus sporogenes* increased the disease resistance against *Aeromonas hydrophila* infection. The mechanisms by which probiotics exert their effect by modifying gut pH (Galindo, 2003) antagonizing pathogens through production of anti microbial and anti bacterial compounds(Villamil *et al.,* 2003), competing for pathogen binding and receptor sites (Villamil *et al.,*2002) as well as for available nutrients and growth factors(Gatesoupe 1999), stimulating immunomodulatory cells(Nikoskalainen *et al.,* 2003,Villamil *et al.,* 2002, Rodriguez *et al.,* 2004, Panigrahi 2004) and producing lactase (Veschuere *et al.,* 2000, Hoolihan, 2001).

Hence *Lactobacillus sporogenes* has been proved to be an efficient probiotic for aquaculture as it enhances antibody response. Galindo, 2003 showed that Lactobacillus populations were scarcely detected in farmed fish, while is a natural inhabitant in wild fish. He proposed a mathematical kinetic model describing the numeric abundance of Lactobacillus in the faces of tilapia in response to the dose and frequency of *L. plantarum* supplementation. It showed that higher the retention time in intestine higher the reduction in number of harmful bacteria (Gildberg *et al.,* 1997)and in prolonging their health effects (Fuller 1989, Ouwehand *et al.,* 1999) most of the probiotics are completely excreted in the following days after ingestion(Robertson *et al.,* 2000). So Nikoskalainen *et al.,* 2003 recommended that the probiotic containing feed must be given continuously to retain the probiotic bacteria in the gut, skin, and tank water, but based on our results, *L. sporogenes* supplemented diet can be given once in 2 months. Hence it is more cost effective. At the same time the higher colonizing efficiency of *L. sporogenes* qualifies itss as the better probiotic for the aquaculture industry than any other prescribed so far.

REFERENCES

Anderson, D.P. 1992. In vitro immunization of fish spleen sections and NBT, phagocytic, PFC and antibody assays for monitoring the immune response. In: Techniques in Fish Immunology, Stolen JS, Fletcher TC, Anderson DP, Kaattari SL and Rowley AT (Eds) SOS Publications, Fair Haven, USA 79-87.

Anderson, P. 1996. Environmental factors in fish health In: Iwama G., Nakanishi T(ed) The fish immune system, organism, pathogen and environment, Academic press, London, 289-310.

Blazer, V.S. and Wolke, R.E. 1984. The effect of tocopheral on the immune response and non specific resistance factors of rainbow trout (*Salmo gairdneri* Richardson) Aquaculture 31 (1): 1-9.

Cross Martin, L., Mortensen Rikke, R., Kudsk Jane and Gill Harsharnjit, S. 2002. Dietary intake of Lactobacillus rhamnosus HN001 enhances production of both Th1 and Th2 cytokines in antigen-primed mice, *Medical Microbiology and Immunology,* 191(1): 49 – 53.

Cuesta, A., Ortuno, J., Rodriguez, A., Esteban, M., and Meseguer, J. 2002. Changes in innate immune defence of seabream (*Sparus aurata* L) induced by retinol acetate. *Fish and Shell fish Immunology*; 13: 279-91.

Fuller, R. 1989. Probiotics in man and animals: a review. *J. Appl. Bacteriol.* 68: 365-378.

Galindo, A.B. 2003. *Lactobacillus plantarum* 44A as a live feed supplement for fresh water fish. Ph.D thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Gatesoupe, F. J. 1990. The continuous feeding of turbot larvae, *Scophthalmus maximus* and control of the bacterial environment of rotifers. Aquaculture 89:139–148.

Gatesoupe, F.J. 1999. The use of probiotics in aquaculture. *Aquaculture* 180: 147-165.

Gildberg, A., Mikkelsen, H., Sandaker, E. and Ringo, E. 1997. Probiotic effect of lactic acid bacteria in the feed on growth and survival of fry and atlantic cod (*Gadus morhua*). *Hydrobiologia* 352: 279-85.

Hoolihan, L.K. 2001. Prophylatic and therapeutic uses of probiotics. *J Am. Diet. Assoc.* 101 (2): 229-241.

Hurshesky, W.J.M. 1984. Chemotherapy timing an important variable in toxicity and response In: Pretty M.C and Yorbo (eds) Toxicity of chemotherapy. Gruine and Stration Inc.,New York, 447-449.

Iwana, G., and Nakanishi, T. 1996. The fish immune system- Organism, pathogen and environment, Academic press, Inc. USA, 1-360.

Joborn, A .1998. The role of gastro intestinal microbiota in the prevention of bacterial infections in fish. Ph.D thesis, Goteborg University, Goteborg, Sweden.

Kitazawa, H., Veha, S., Itoh, S., Watanabe, H., Konnok, Kawai, Y., Saito, T., Itoh, T. and Yamaguchi T. 2001. AT oligonucleotides including â-lymphocyte activation exist in probiotic

Lactobacillus gasseri. Int. J Food Mic. 65 (6): 149-162.

Logambal, S.M. and Michael, R.D. 2001. Azadirachtin-An immuno-stimulant for *Oreochromis mossambicus* (Peters). *J. Aqua. Trop.*, 16: 339–347.

Logambal, S.M., Venkatalakshmi, S. and Michael, R.D. 2000. Immuno- stimulatory effects of leaf extracts of *Ocimum sanctum* Linn. in *Oreochromis mossambicus* (Peters). Hydrobiologia 430: 113-120.

Lygren, B., Sveier, H., Hjelthes, B. and Waagbo, R. 1998. Investigation of Lactoferin and vitamin C as dietary immunostimulants in atlantic salmon (Salmo salar) In: Kane A.S and S.C.Poynton (Eds) proceedings of the international symposium on Aquatic Animal Health, Baltimore, USA, p 226.

Majeed, M. and Prakash, L. (1998). Lactospore: The effective probiotic Piscataway. NJ, Nature Science Publishes, Inc.

Malin, M., Suomalainen, H., Saxelin, M. and Isolauri E. 1996. Production of IgA immune response in patients with Crohn's disease by oral bacteriotherapy with lactobacillus GG. Ann. Nutr. Metb. 40: 137-145.

Michael, R.D. and Priscilla, A.S. 1994. Diel variation in antibody response to bovine serum albumin in Oreochromis mossambicus (Peters) *Ind. J. Exp. Biol.,* 32: 474-477.

Misra, C.K., Dasa, B.K., Mukerjee, S.C. and Meherc, P.K. 2006. The immunomodulatory effects of tuftsin on the non specific immune system of Indian Major carp *Labeo rohita.Fish and Shell fish Immunol*. 20 (5): 728-738.

Nikoskelainen, S., Ouwehand, A.C., Bylund, G., Salminen, S. and Lilius, E.M. 2003. Immune enhancement in rainbow trout (*Oncorhynchus mykiss*) by potential probiotic bacteria (*Lactobacillus rhamnosus*). *Fish and Shellfish Immunol*. 15: 443-452.

Ogawa, M., Shimizu, K., Nomoto, K., Takahashi, M., Watanuki, M., Tanaka, R., Tanaka, T., Hamabata, T., Yamasaki, S. and Takeda, Y. 2001. Protective effect of *Lactobacillus case* strain Shirota on Shiga toxin producing *Escherichia coli* 0157:H7 infection in infant rabbits. *Infection and Immunity* 69 (2): 1101-1108.

Ouwehand, A.C., Niemi, P. and Salminen, S.J. 1999. The normal faecal microflora does not affect the adhesion of probiotic bacteria in vitro. *FEMS Microbiol Lett*.177: 35-8.

Panigrahi, A., Kiron, V., Kobayashi, T., Puangkaew, J., Satoh, S. and Sugita, H. 2004. Immune responses in rainbow trout *Oncorhynchus mykiss* induced by a potential probiotic bacteria *Lactobacillus rhamnosus* JCM 1136. *Vet. Mmunol. & Immunopathol.,* 102 (4): 379-388.

Roberson, S. Bob. 1990. Bacterial agglutination, In: Techniques in fish immunology J.S.Stolen, T.C.Fletcher, D.P.Anderson, B.S.Roberson and W.B.Van muiswinkel (eds) SOS Publications, 43 De Normandie Ave, Fair Haven, NJ 07704-03303 USA pp 81-86.

Robertson, P.A.W., Dowd, C.O., Burrells, C., Williams, P. and Austin, B. 2000. Use of *Carnobacterium sp*. as a probiotic for Atlantic salmon (*Salmo salar* L) and rainbow trout (*Onchorhynchus mykiss*, Walbaum). *Aquaculture,* 185 (3-4): 253-263.

Rodriguez, A., Cuesta, A., Esteban, M.A. and Meseguer, J. 2004. The effect of dietary administration of the fungus *Mucor cincinelloides* on non specific immune responses of gilt head sea bream. *Fish and Shellfish Immunol*., 16: 241-249.

Schiffrin, E.J., Brassart, D., Servin, A., Rochat, F. and Donnet-Hughes, A. 1997. Imunomodulation of blood leucocytes in human by lactic acid bacteria: criteria for strain for strain selection. *Am. J. Clin. Nutr*., 66: 515S-520S.

Stasiack, A. Steward and Baumann C. Paul. 1996. Neutrophil activity as a potential indicator for contaminant analysis. *Fish and Shellfish Immunol*., 6: 537-539.

Svobodova, D., Ravds, J. and Palackova, L. 1991. Unified method of haematological examination of fish. Research Institute of Fish culture and hydrobiology. Vodamy, Czechoslovakia.

Tannock, G.W. 1997. Modification of the normal microbiota by diet, stress, antimicrobial agents, and probiotics In: Mackie R.I, With B.A., Isaacson R.E (eds) Gastrointesitnal microbes and host interactions. International Thomson Publishing, New York, pp 434-465.

Uma, A., Jawahar, A.T., Prince, J.M.J. and Sundararaj, V. 1999. Effect of probiotic feed supplement on performance and disease resistance of Indian white shrimp *Penaeus indicus* H. Milne Edwards. *J. Aqua. Trop.,* 14 (2): 159-164.

Verschuere, L., Rombaut, G., Sorgeloos, P. and Verstraete, W. 2000. Probiotic bacteria as biological control agents in aquaculture. *Microbiol. Mol. Biol. Rev*., 64: 655-671.

Villamil, L. Taffalla, C., Figueras, A. and Novoa, B. 2002. Evaluation of immunomodulatory effects of lactic acid bacteria in turbot (*Scophthalmus maximus*) Clinical and diagnostic laboratory Immunology. 9 (6): 1318-1323.

Villamil, L., Figueran, A. and Novoa, B. 2003. Immunomodulatory effects of nisin in turbot (*Scophthalnus maximus* L) *Fish and Shellfish Immunol*. 14 (2): 157-169.