EFFECT OF DIETARY SUPPLEMENTATION OF *Bacillus subtilis* AND *Terribacillus saccharophillus* ON THE INNATE IMMUNE RESPONSES OF A TROPICAL FRESHWATER FISH, *Labeo rohita*

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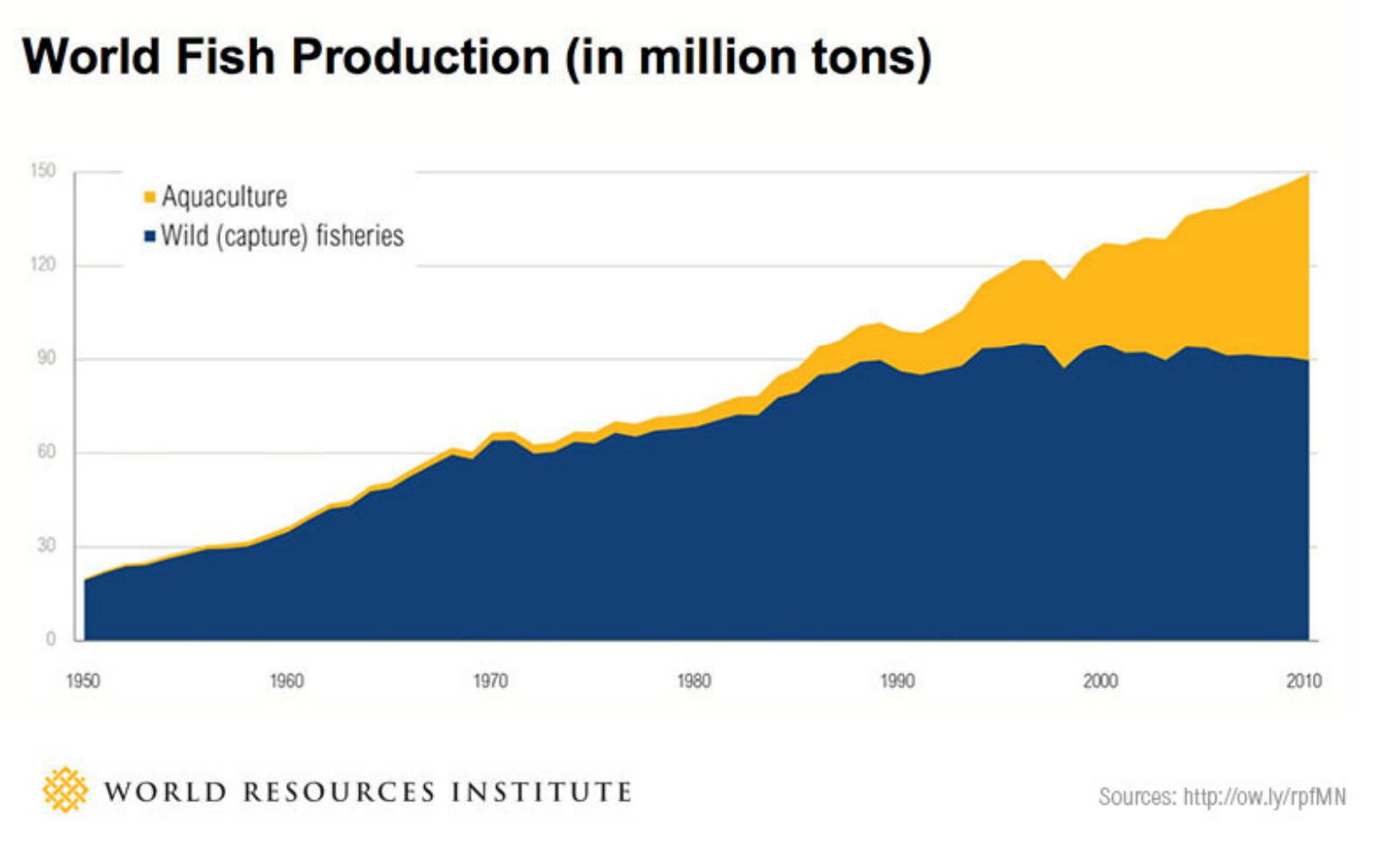
Fish production



Aquaculture

- **The most promising and fastest- growing industries.**
- **World fish production: in 2012 US$144.4billions)**
- **India stands second largest producer of fish (90.48 lakh tonnes-2012-13) in the world after China.**
- **Andhra Pradesh province In India, ranks second in fish production in the country.**

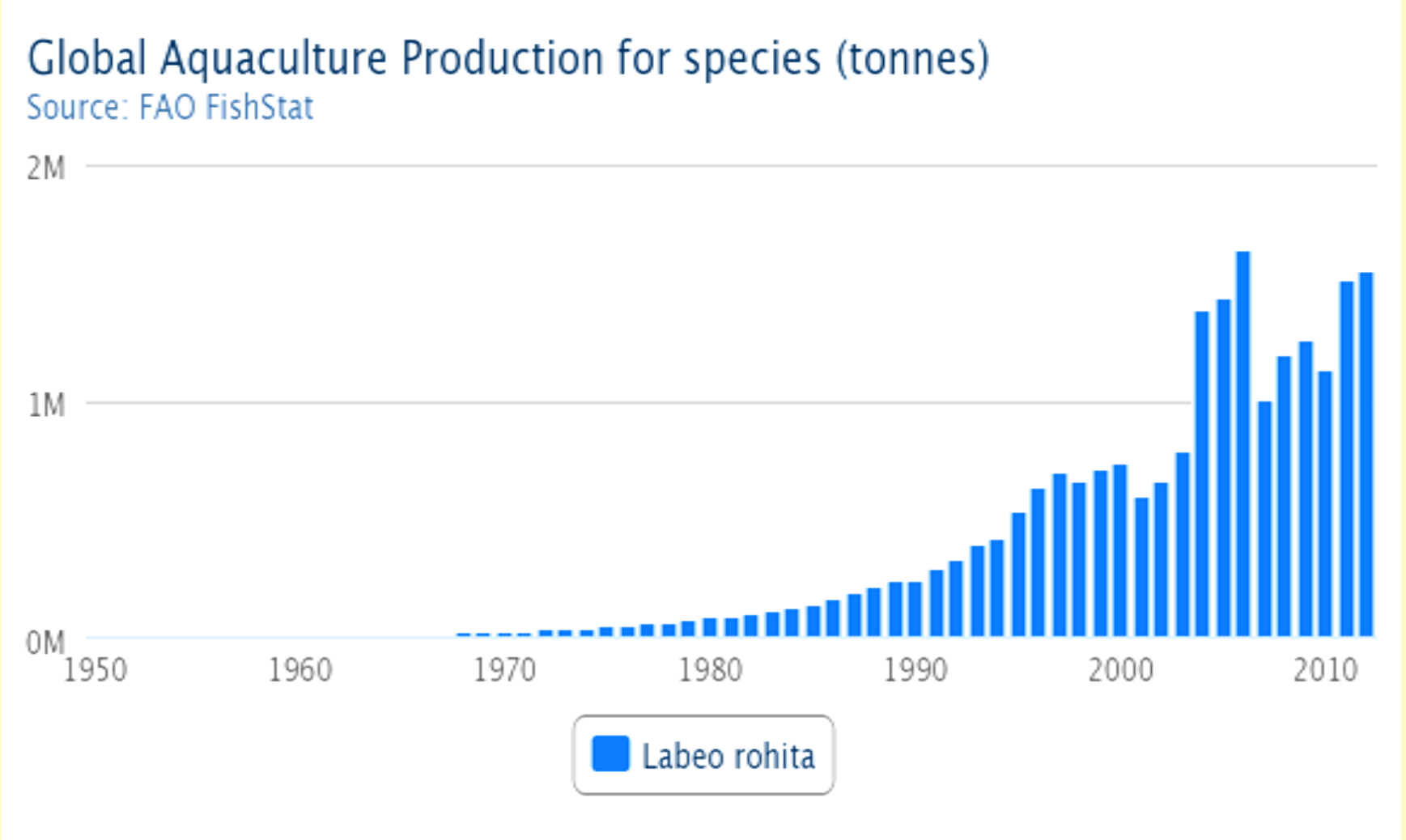
Indian Major Carps (IMCs)

Labeo rohita

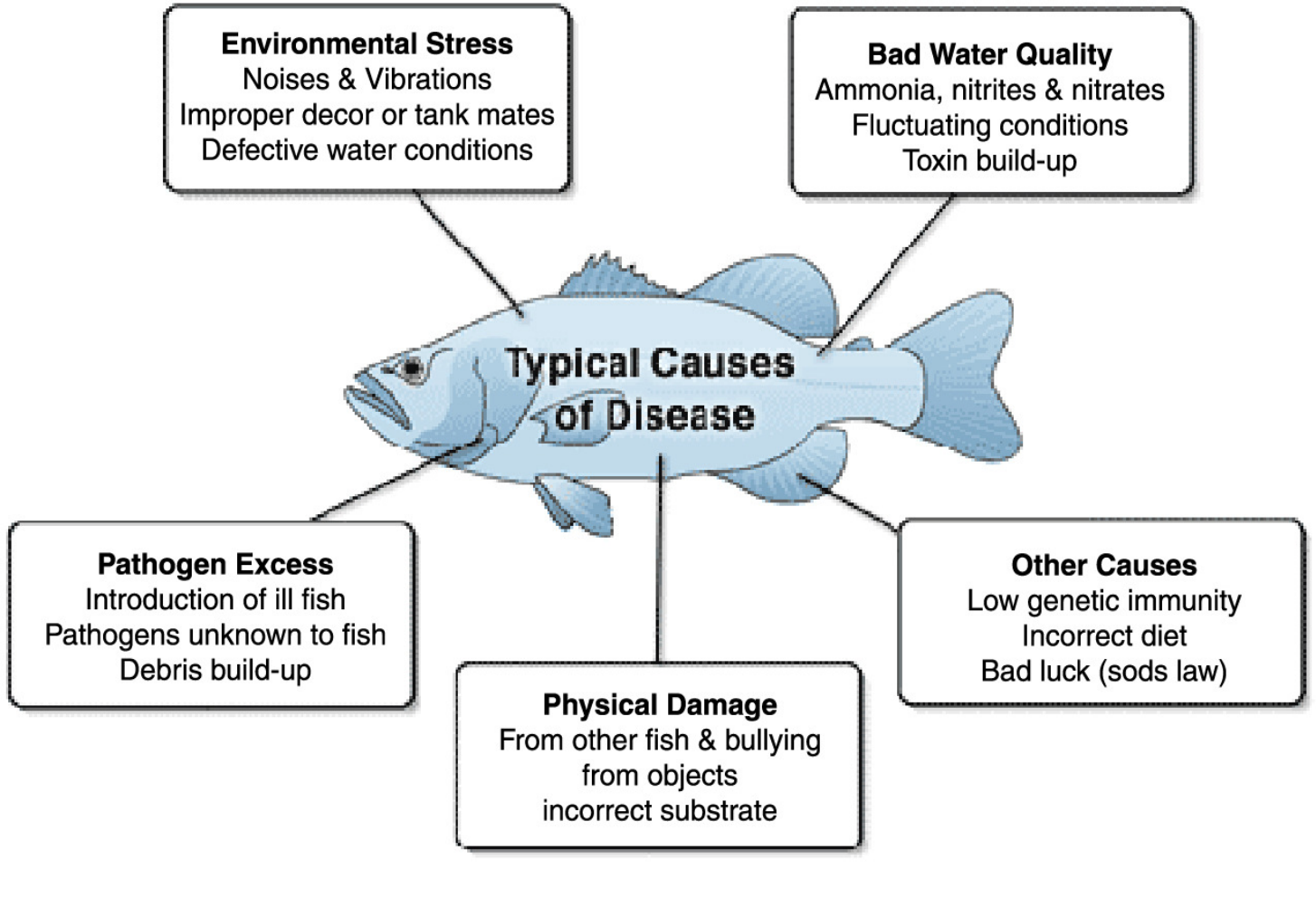
Catla catla

Cirrhinus mrigala

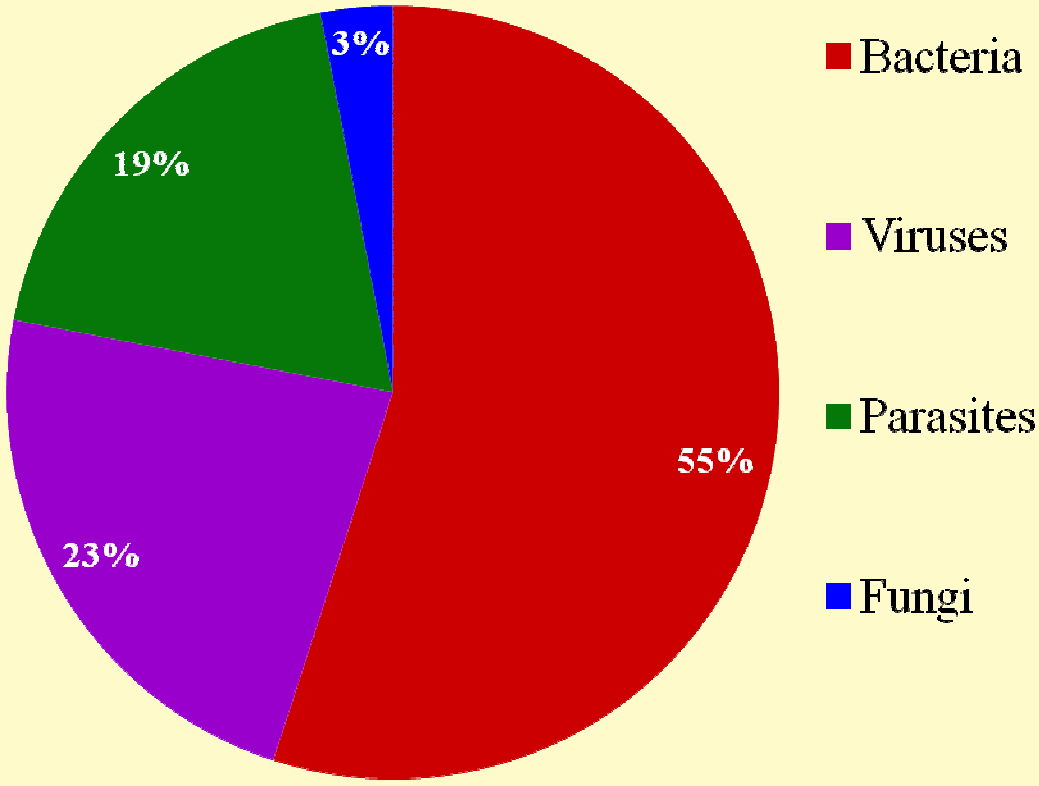
Fish Production



Typical causes of Diseases



Fish diseases -Profile

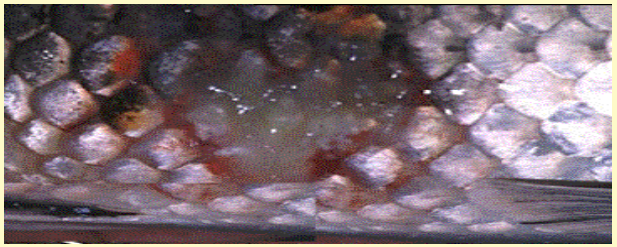


Gill disease →

Fin and Tail rot disease →

Ulcers →

Saprolegniasis (Fungal disease) →



Ichthyophthiriasis (protozoa disease) →

Therapeutic / Treatment measures – Associated Problems

MEASURES	LIMITATIONS
WATER BATH TREATMENTS - Short term - long term (Ex: Formalin, Salt, Copper Sulfate)	Not effective to treat internal infections
ANTIBIOTICS (Navare *et al., 2008)* Erythromycin, Streptomycin Tetracycline, Vancomycin Oxytetracycline **VACCINATION** Recombinant DNA (Biering *et. al.*, 2005), Commercial DNA vaccines (2005) and Reverse engineered & multiepitope vaccines (2007)	- Pathogens develop resistance - Kills beneficial bacterial flora (Gonzalez *et al.*, 2000; Gomez-Gil *et al.*, 2000). Not Cost effective; Difficult to develop

Fish-Immune System

- Thymus
- Anterior or Head Kidney
- Spleen

Non-specific Immunity- Physical Barriers; Non-specific cellular toxicity: AMP; Phagocytosis; Complement pathway: TNF; Lysozymes, Interferons, Interleukins, cytokines, Natural Antibodies etc.,

The search for effective disease control/prevention strategies in the last few decades has led to the practice of some modern approaches:

- **Use of non-specific immunostimulants (e.g. β-1,3 glucan)**
- **Dietary supplements (e.g. vitamin C),**
- **Probiotics**
- **development of genetically disease-resistant stock,**
- **and restriction on the movement of infected stock other than the traditional use of vaccines and antibiotics (Irianto and Austin, 2002; Austin and Austin,2007).**

Among these one of the methods gaining recognition for controlling pathogens within the aquaculture industry is the use of beneficial or probiotic bacteria

➢**Probiotics** :

✓ Microbial adjuncts which are defined as “live micro-organisms that confer a health benefit on the host” .

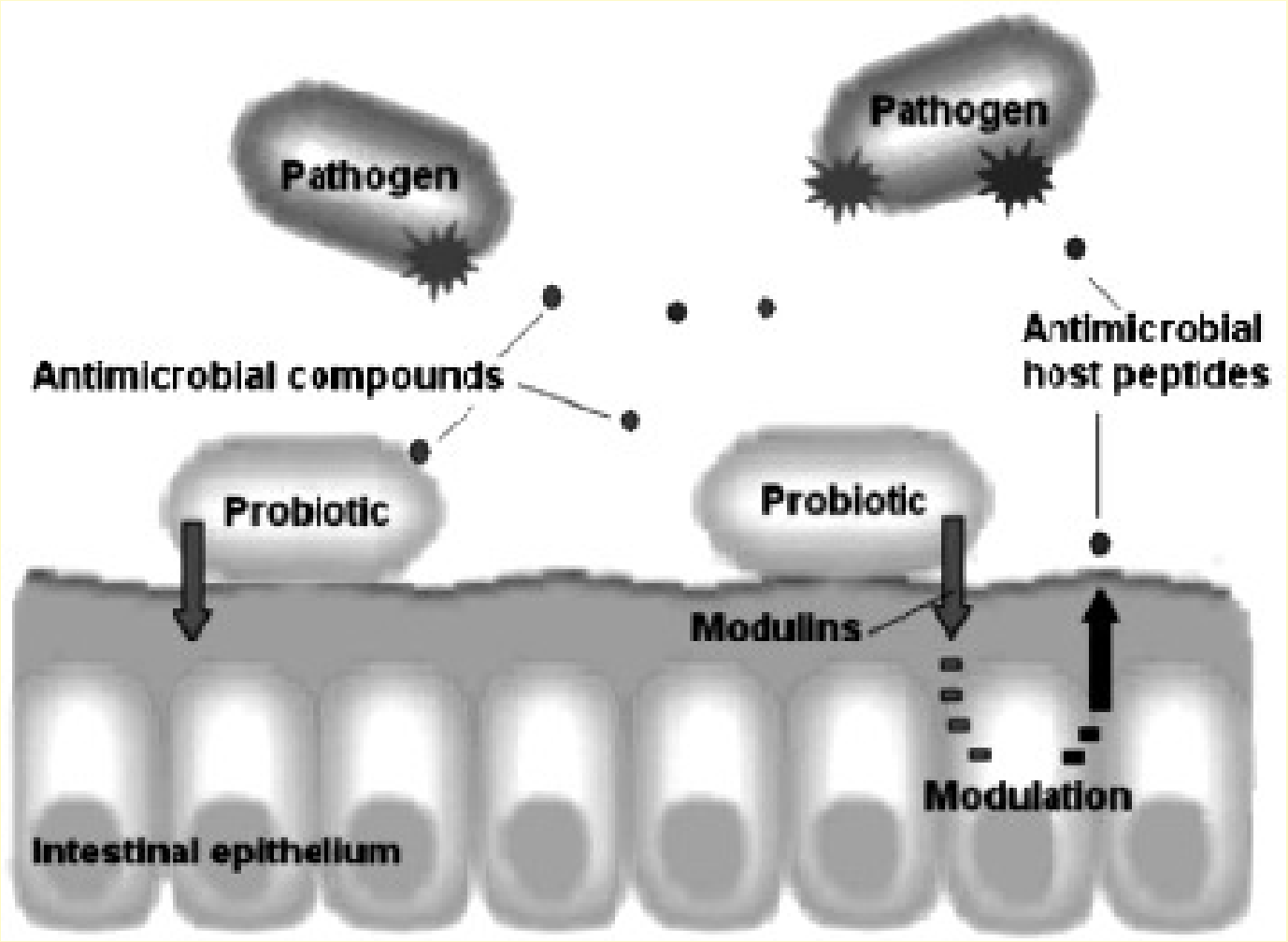
✓ Intestinal microflora were noticed to result in Health improvement and promotion of growth (Moriarty,1999 & Roberson et al.,2000).

✓ These were found to prevent proliferation of pathogens (Moriarty 1998; Gomez; Gil *et. al*., 2000; Verschuere *et. al*., 2000; Decamp and Moriaty, 2005)

✓ Strengthen immune system (Kailaspathy and Rybka, 1997).

Hence, of late, biocontrolling theory of using probiotics in aquaculture to treat infections is more focused.

Proposed mode of action of probiotics in the intestinal tract of a host (Balcazar *et al.*, 2006)



Probiotics	Test sps	Obervation	Reference
Bacillus sps	Zebrafish	Antagonistic/inhibitory activity	Sumathi et al., 2012.
Lactobacillus sps	Grouper & Nile tilapia	Enhanced the growth, innate immune responses, and disease resistance	Son *et al.*, 2009, Ngamkala *et al.*, 2010.
Lactobacillus rhamnosus	Rainbow trout	Stimulated respiratory burst	Nikoskelainen et al., 2003
Bacillus sp.	White shrimp	Stimulation of the immune system by increasing phagocytosis and antibacterial activity	Balcazar 2003
Bacillus subtilis	Gilthead seabream	Increases phagocytic activity	Salinas et al., 2005
Bacillus coagulans	Tilapia	Increase immune responses such as MPO and respiratory burst activities	Sakai et al., 1999

- **However, more attention has to be paid on the selection of a suitable probiotic in order to achieve the desired benefit in the host species.**

- **Use of bacteria with probiotic nature isolated from the host claimed as an elegent logic.**

- **Studies on exploration of probiotic nature of gut bacteria as well as their long term administration are very limited.**

Tests/ Isolates	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Gram's Staining/ Shape	**+/** rod	**+ /** rod	**+ /** rod	**- /** rod	**- /** rod	**- /** rod	**- /** rod	**+ /** rod	**+ /** rod
Motility	**+**	-	**+**	**+**	**+**	**+**	**+**	**+**	**+**
Catalase	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**
Indole	-	-	-	**+**	**-**	-	-	-	-
Methyl Red	**+**	**+**	**+**	-	**-**	-	**-**	**+**	**+**
Voges - Proskauer	**+**	**+**	**+**	-	-	-	-	**+**	**+**
Citrate test	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**

Isolates	Closest relative (obtained from BLAST search)	Similarity (%)
Culture 1	*Bacillus pumilus strain B-2*	100
Culture 2	*Terribacillus saccharophilus strain JP44SK46*	100
Culture 3	*Bacillus safensis strain IHB B 14387*	100
Culture 4	*Aeromonas hydrophila strain ZHYYZ-4*	99
Culture 5	*Pseudomonas aeruginosa strain NY3*	99
Culture 6	*Pseudomonas stutzeri strain SLG510A3-8*	99
Culture 7	*Achromobacter xylosoxidans strain IHB B 6801*	97
Culture 8	*Bacillus subtilis strain 10A*	94
Culture 9	*Bacillus cereus strain RCTy3*	83

Earlier Observations

Bacteria isolated from the gut of *L.rohita* showed antimicrobial activity against ***Vibrio harveyi,* *Pseudomonas fluorescens, Aeromonas hydrophila* and *Escherichia coli*** .

***B. subtilis* and *T. saccharophillus* showed higher inhibition of above pathogens compared to the rest.**

Hence these two bacteria were chosen for isolation and characterization of peptides (Nicin-like Bacteriocins) responsible for antimicrobial action.

- **HPLC purified peptides were assessed through antimicrobial assay.**

- **Through SDS and MALDITOF analysisthe molecular mass of antimicrobial peptides *was determined.***

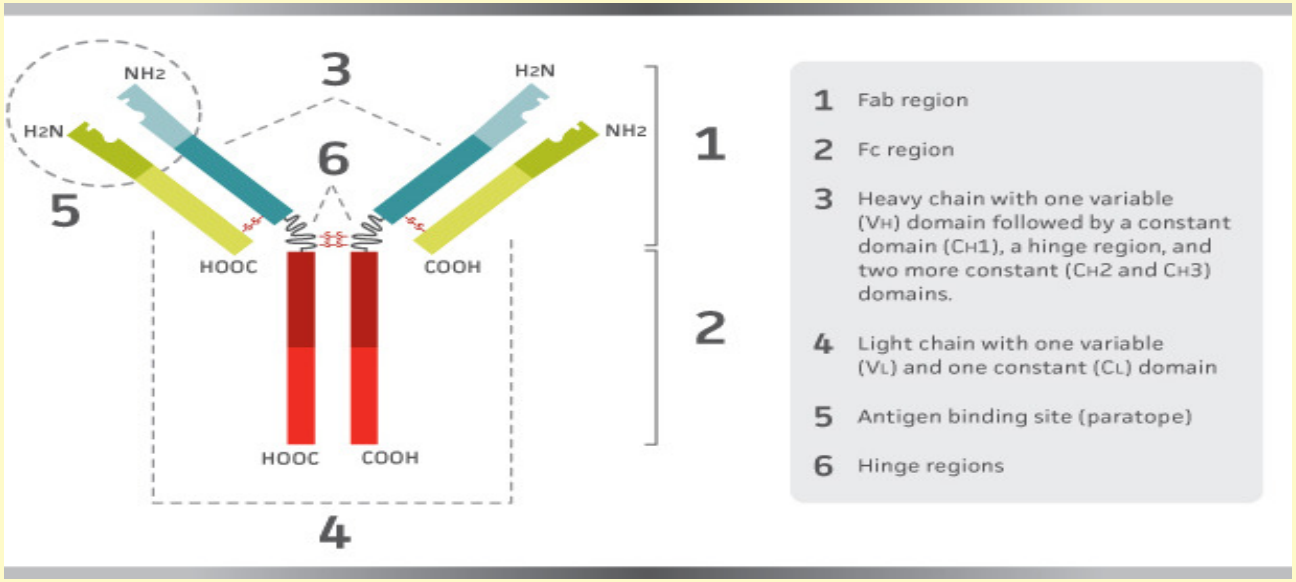
- **Antimicrobial peptide functional groups were determined through FTIR and assessed stability of antimicrobial peptides by using varient temperatures, pH and surfactants.**

OBJECTIVES

- To assess the impact of *B. subtilis* and *T. saccharophillus* diet on serum levels of IgM in *L. rohita.*
- To observe the effect of probiotics on the levels of phagocytic, Myeloperoxidase and Respiratotory burst activities.
- To assess the impact of probiotics on the production of serum lectins, haemagglutination and haemolytic activity.

IMMUNOGLOBULINS

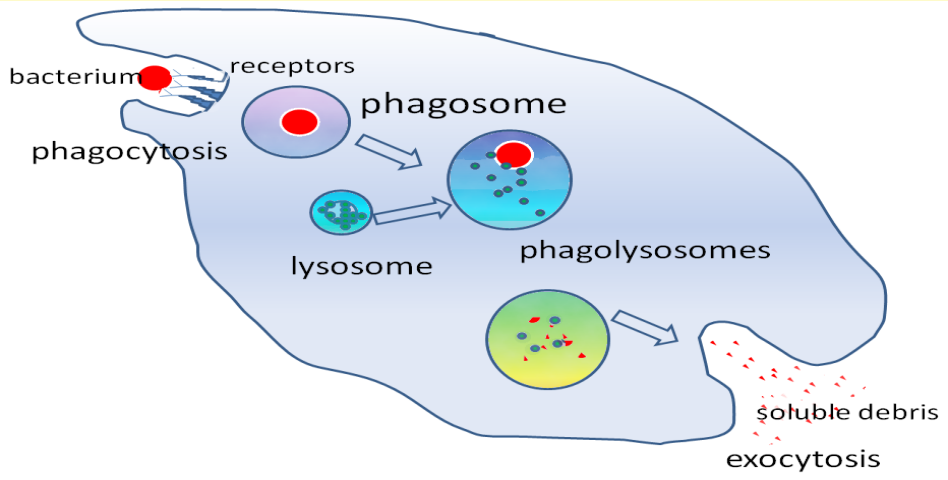
- Teleostean IgM - tetramer containing eight combining sites (Acton *et al*., 1971).
- Secreted mainly by plasmablasts and plasma – like cells of head kidney and elicit memory responses (Ye *et al*., 2011)- major components of humoral immune system.
- Known to participate in pathogen recognition and activation of the innate immune system (Shoemaker *et al*., 2005).



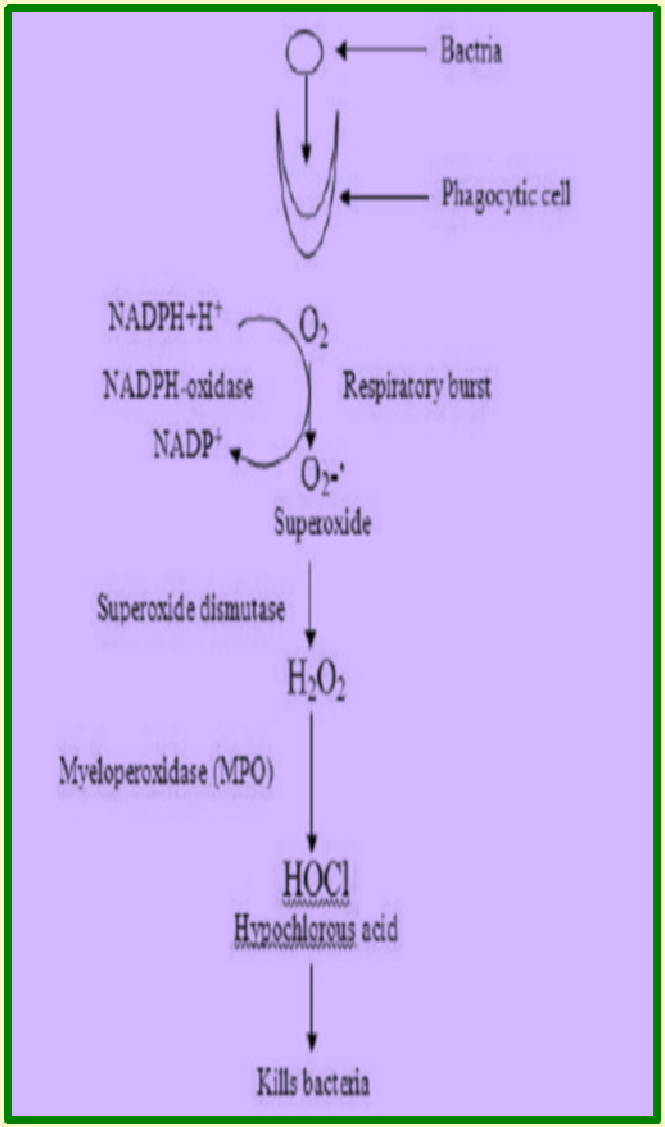
- IgD, IgG & IgT are also found to play an important role in teleostean fishes

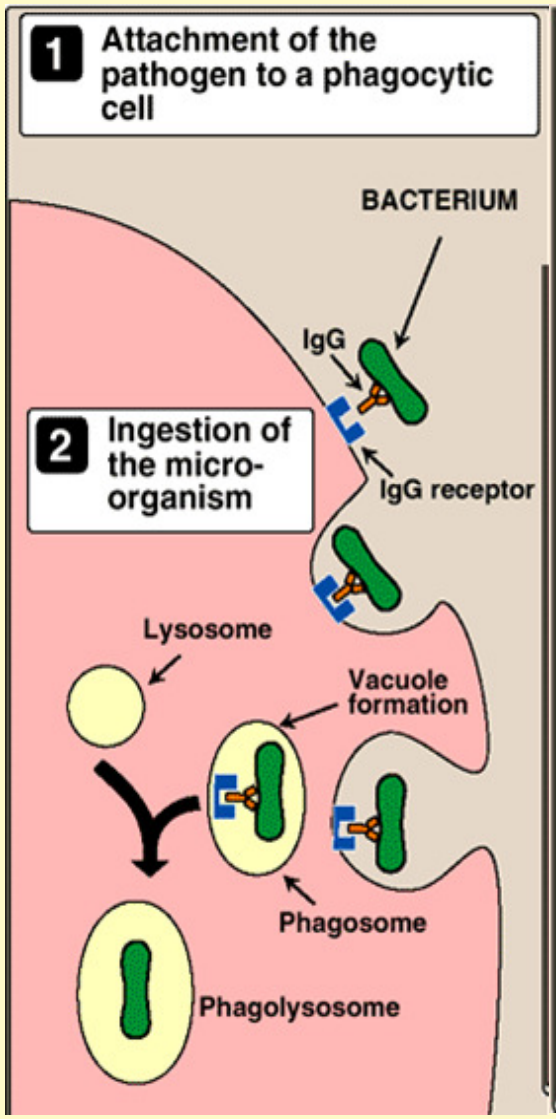
PHAGOCYTOSIS

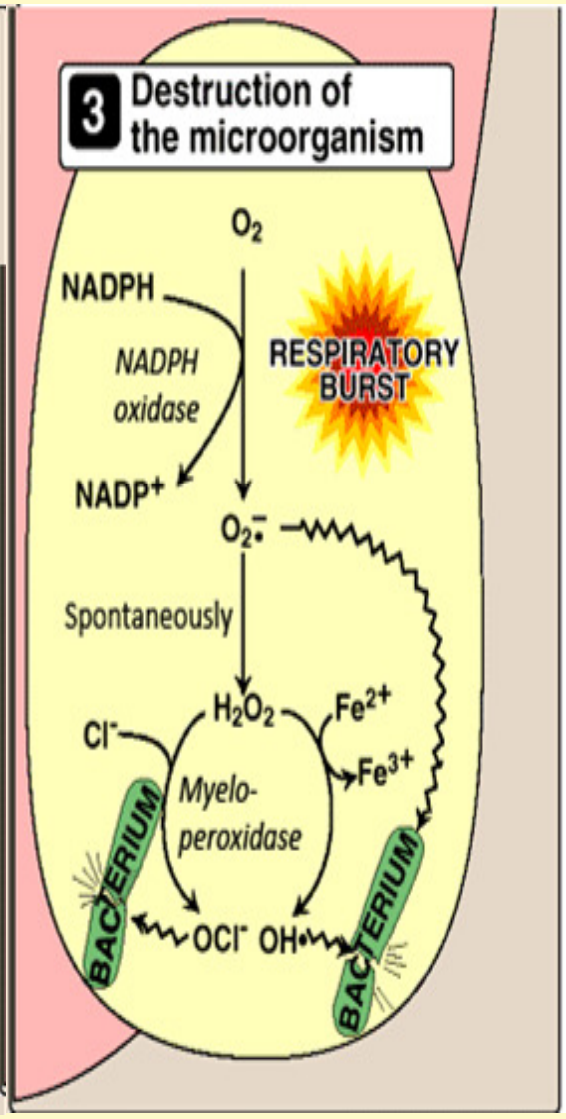
- The most important processes in all poikilothermic animals (Blazer, 1991; Lange and Magnadottir, 2003; Magnadottir, 2006).
- Granulocytes, monocytes and macrophages, neutrophils are specialized for the pursuit, capture, ingestion and intracellular destruction of invading microbes by phagocytosis.
- The main cells involved in phagocytosis in fish are neutrophils and macrophages (Secombes and Fletcher, 1992). These cells remove bacteria through a phenomenon called “respiratory burst”.



PHAGOCYTOSIS







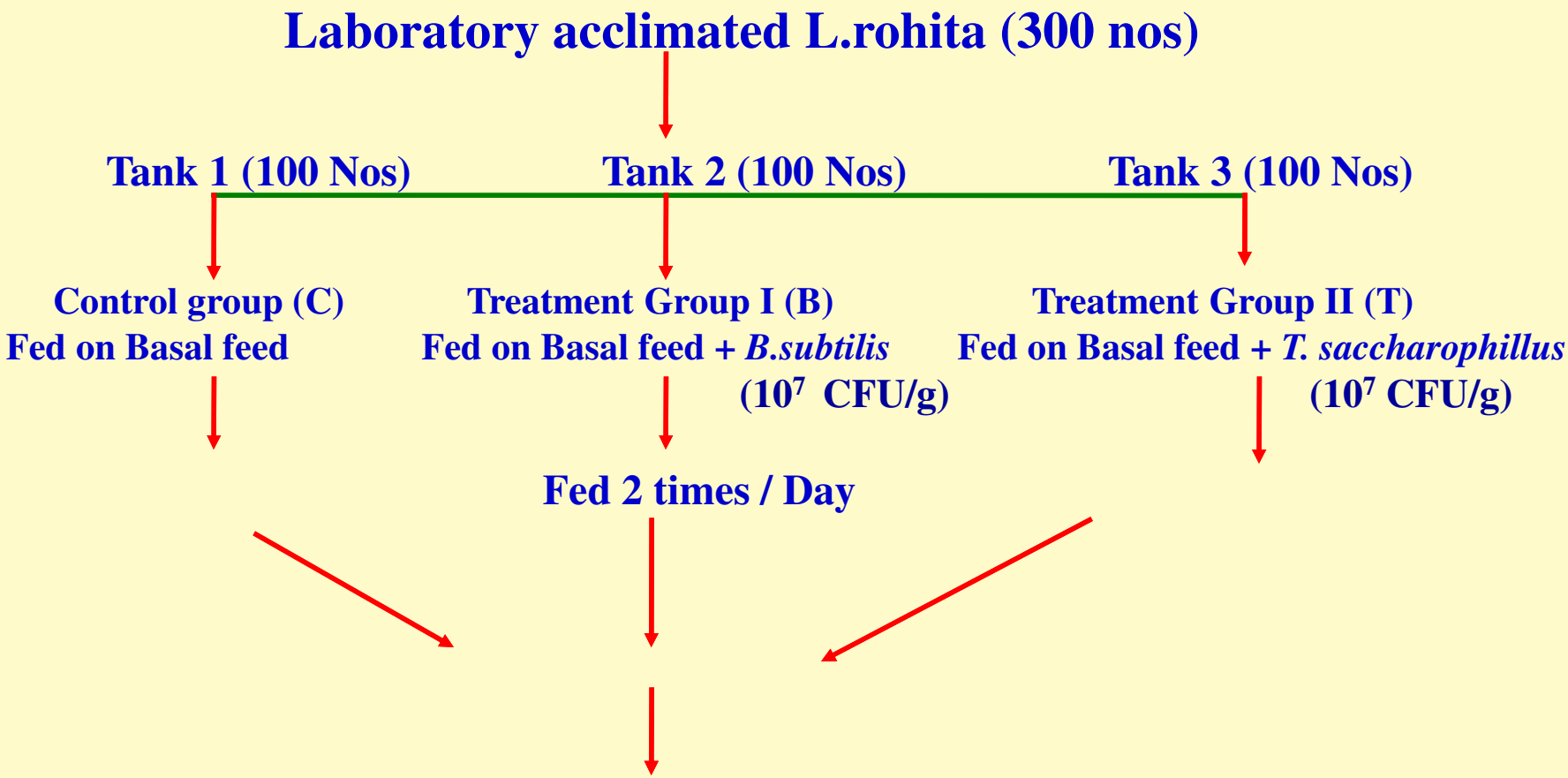
LECTINS

- Lectins are carbohydrate binding proteins found in viruses, prokaryotes and eukaryotes.

- These proteins are known to agglutinate foreign cells, precipitate membrane polysaccharides, glycoproteins or glycolipids mediating different biological processes such as cell – cell interaction, induction of apoptosis, antibacterial and antiviral actvitity etc.

- Lectins are believed to mediate pathogen recognition in fish immune system with important role in innate immune response.

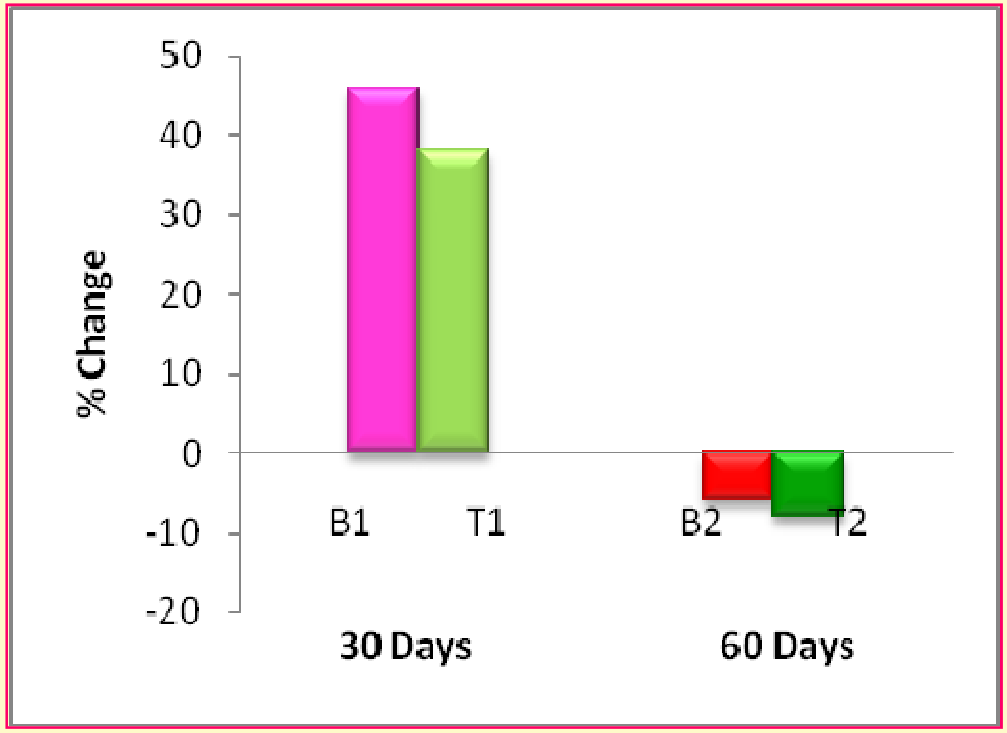
Experimental Design



Assessement of Immune response parameters on Day 0 (control group)
Day 30 (B_1 / T_1 group)
and Day 60 (B_2 / T_2 group)

RESULTS

1. Percent change in Ig M levels of *L. rohita* fed on *B. subtilis* and *T.saccharophillus* formulated diets for 30 (B_1 / T_1) and 60 (B_2 /



- **Probiotics act as immunomodulators and enhance IgM levels in fish (Carroll & Prodeus 1998; Amar *et al*., 2000; Nikoskelainen *et al*., 2003).**

- **Serum IgM levels increased by 26 and 47% in grouper fish upon administration of *B. pumilus* and *B. clausii* supplemented diets respectively for 30d but decreased by 20 and 15% respectively with continued administration till 60d (Sun *et al*., 2010)**

- **120% increase in serum IgM levels in fingerlings of *L. rohita* fed on a mixture of *B. subtilis, L. lactis* and *S. cerevisiae* supplemented diets for 15d (Mohapatra *et al*., 2014).**

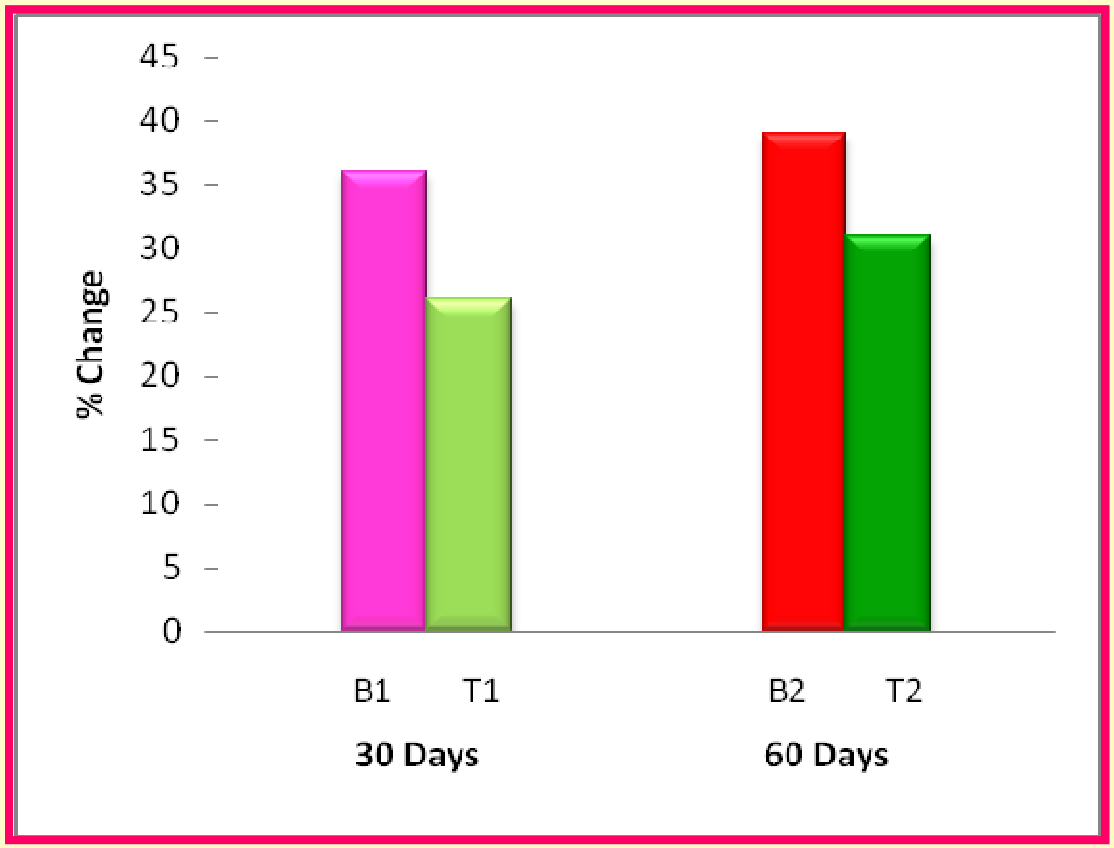
- **Live yeast, *Debaryomyces hansenii* administered through diet for 5 weeks increased IgM levels by 15% in the leopard grouper, *Mycteroperca rosacea* (Reyes – Becerril *et al*., 2011)**

- **Dietary administration for 105d of *L. acidophilus* increased IgM levels by 4% in *Catla catla* (Krishnaveni *et al*., 2013); dietary supplementation for 126d of the probiotic bacteria, *Pediococcus acidilacticion* enhanced IgM levels by 24% in the ornamental fish Oscar (Safari *et al*., 2013)**

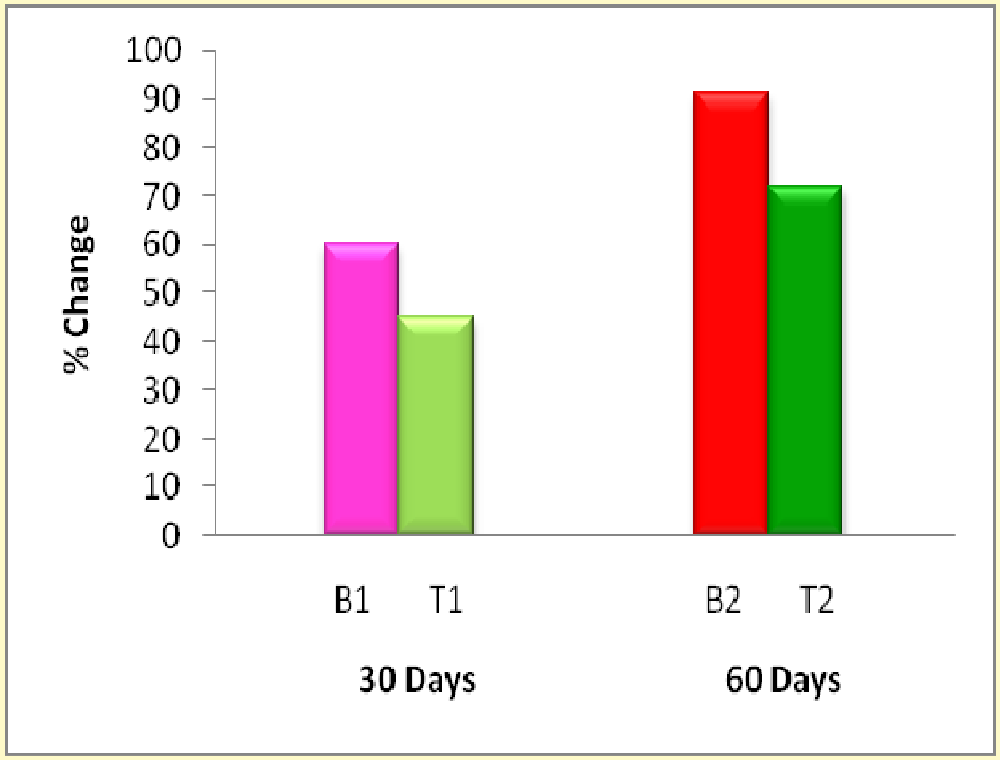
- ***L. rohita*** **showed 24% increase and 9% decrease upon administration of** ***P. aeruginosa*** **supplemented diets for 30 and 60d respectively (Giri** ***et al.,*** **2012).**

- **Rainbow trout,** ***O. mykiss,*** **showed significant increase in plasma immunoglobulin upon 20d administration of** ***L. rhamnosus*** **supplemented diet which decreased significantly thereafter with continued probiotic treatment (Panigrahi et al., 2005).**

2a . Percent change in Phagocytic cell number of *L. rohita* fed on *B. subtilis* and *T. saccharophillus* formulated diets for 30 (B_1 / T_1) and 60 (B_2 / T_2) days compared to control group fed on basal diet.

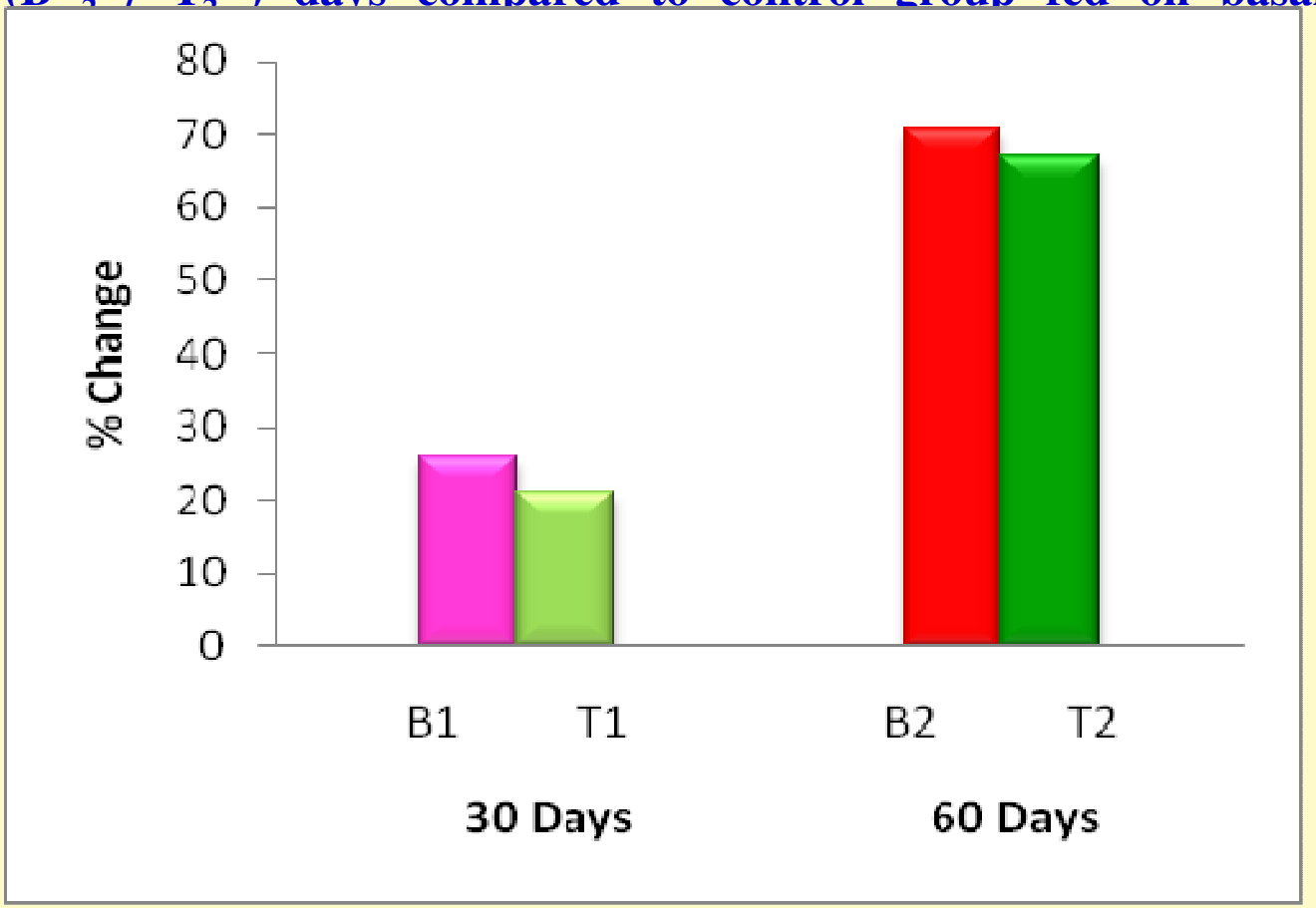


2 b Percent change in Respiratory Burst activity of *L. rohita* fed on *B. subtilis* and *T.saccharophillus* formulated diets for 30 (B_1 / T_1) and 60 (B_2 / T_2) days compared to control group fed on basal diet



***B. subtilis* : 60 / 90% : *T. saccharophillus* : 45 / 72%**

2c. Percent change in Myeloperoxidase activity of *L. rohita* fed on *B. subtilis* and *T. saccharophillus* formulated diets for 30 (B_1 / T_1) and 60 (B_2 / T_2) days compared to control group fed on basal diet.



- **Increased Phagocytic activity 39 & 27% in grouper fish fed on *B. pumilus* and *B. clausii* for 15days respectively (Sun *et al.,* 2010);**

- **by 62% in *O. niloticus* fed on *Micrococcus sps.* 15d (Osman *et al.,* 2010);**

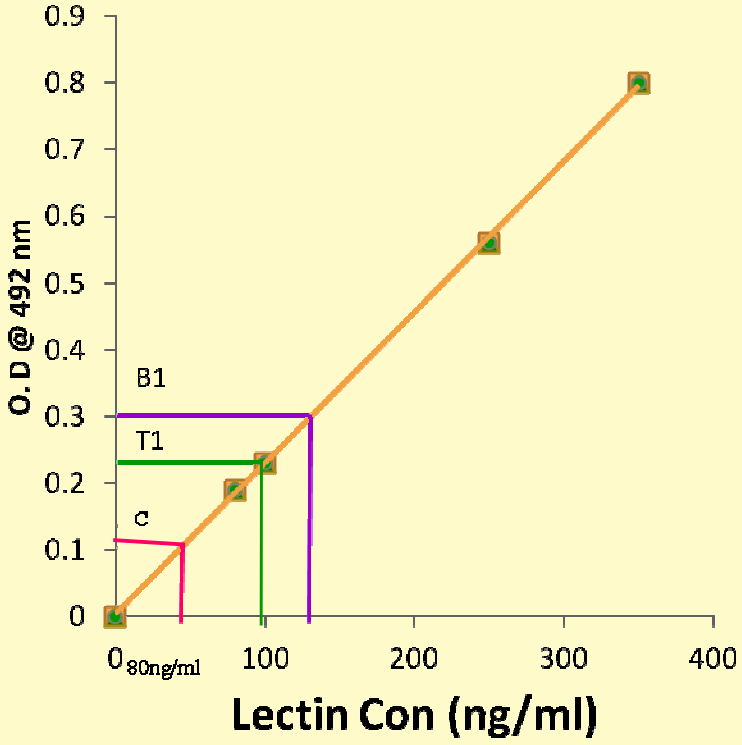
- **by 34% in Nile tilapia fed on *L. plantarum* supplemented diets for 15d (Dotta *et al*., 2011).**

- **25% increase in the fingerlings of catfish (Krishnaveni *et al*., 2013) upon supplementation with 3% probiotic mixture of *L. acidophilus* and *S. cerevisiae* for 105d.**

- **130% increase in phagocytic activity, 50% increase in respiratory burst along with 62% increase in myeloperoxidase activity were observed in rainbow trout fed on *B. subtilis* supplemented diet for 14d (Newaj – Fyzul *et l*., 2007).**

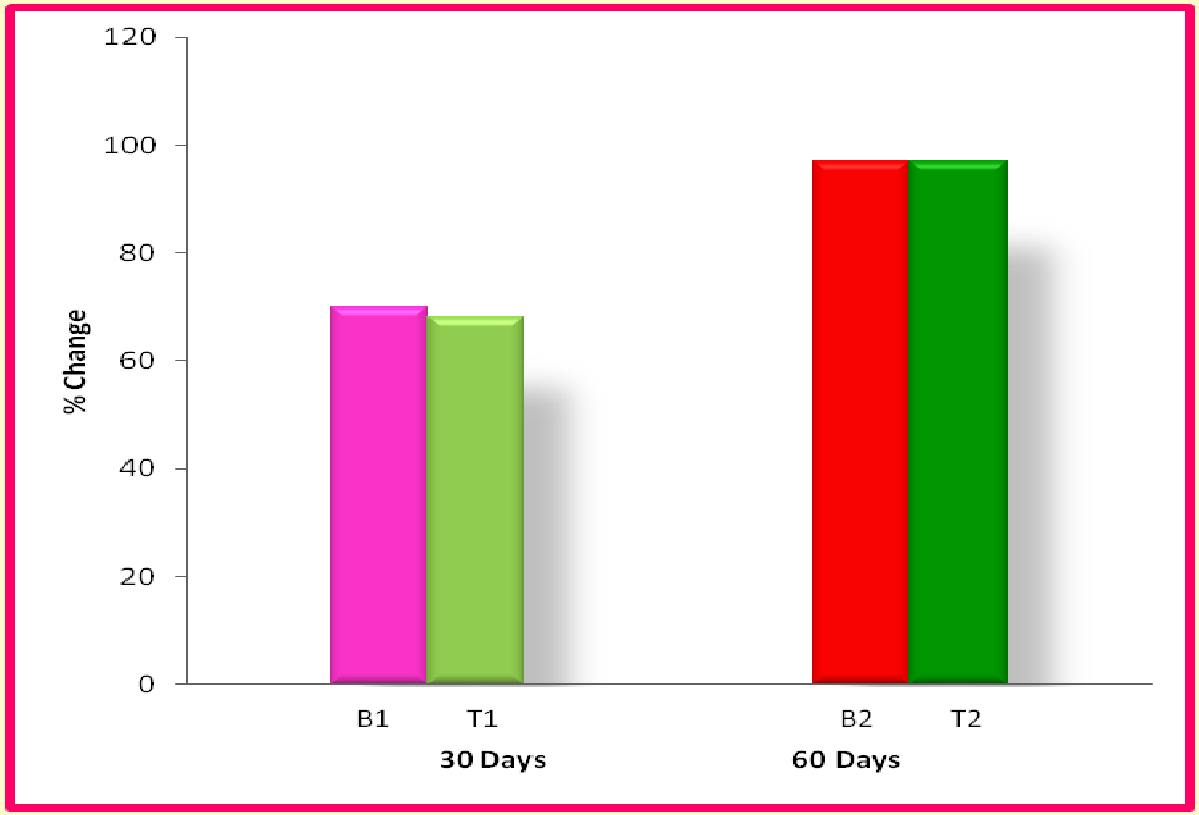
- **75% increase in respiratory burst and 50% increase in myeloperoxidase activity were observed in tilapia fed for 40d on *L. lactis* supplemented diet (Zhou *et al.,* 2010)**

3. Serum lectin levels of *L. rohita* fed on *B. subtilis* and *T. saccharophillus* formulated diets for 30 (B_1 / T_1) and 60 (B_2 / T_2) days compared to control group fed on basal diet.
(Values are Mean ± SD of 5 individual observations)

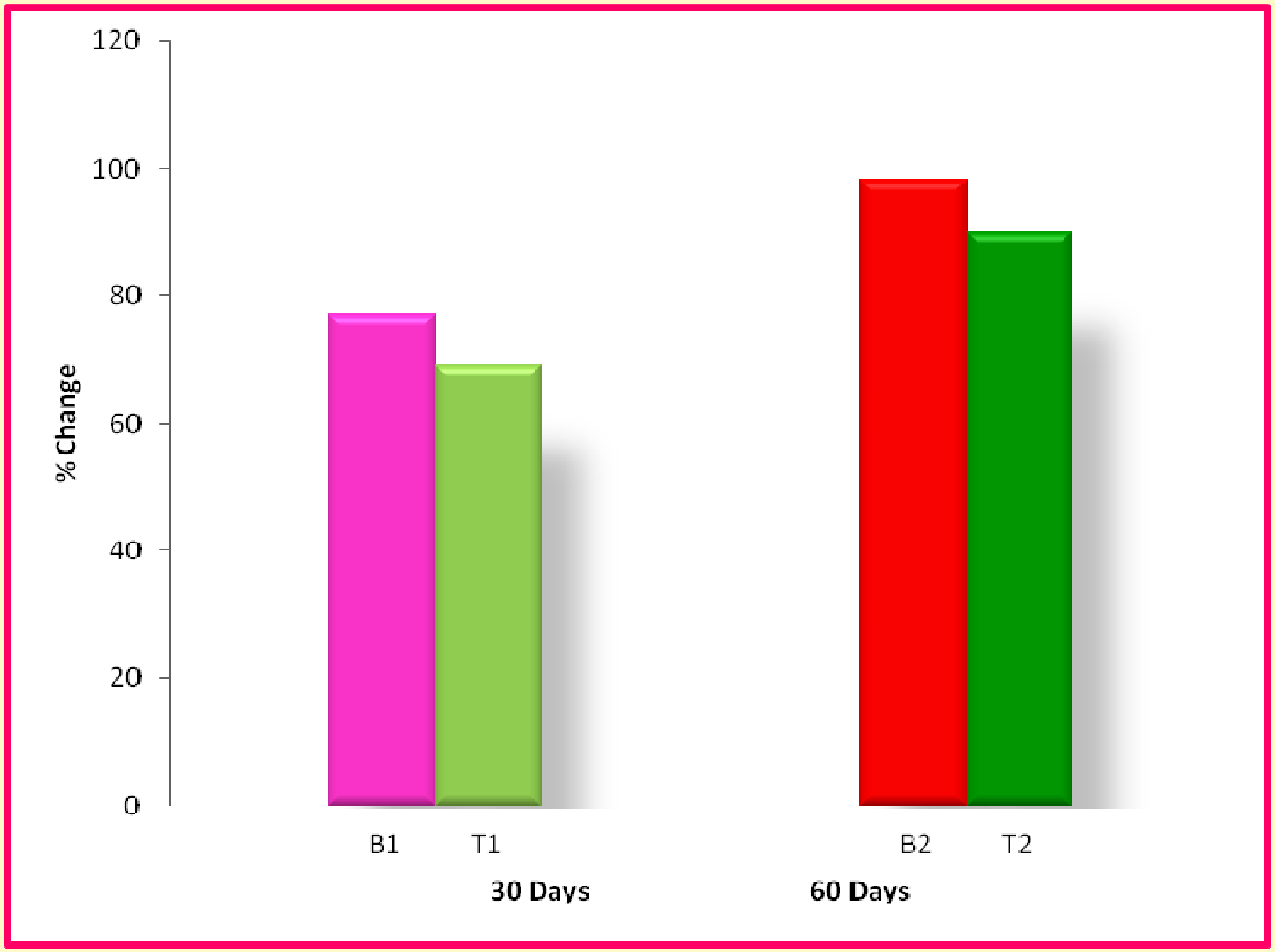


Test samples	Concentration of Lectins (ng/ml)
C	80
B_1	120
T_1	100
B_2	180
T_2	160

3b. Percent change in Haemagglutination activity (HA) of *L. rohita* fed on *B. subtilis* and *T. saccharophillus* formulated diets for 30 (B_1 / T_1) and 60 (B_2 / T_2) days compared to control group fed on basal diet



3c Percent change in Haemolytic activity of *L. rohita* fed on *B. subtilis* and *T. saccharophillus* formulated diets for 30 (B_1 / T_1) and 60 (B_2 / T_2) days compared to control group fed on basal diet



- **ELISA studies on hemolymph of freshwater prawns, *Macrobrachium rosenbergii* using antibodies raised against purified lectin showed a positive correlation between haemagglutination activity and concentration of lectin (Agundis *et al*., 2000).**

- **Sangvanich *et al.* (2007) observed strongest haemagglutination activity against rabbit erythrocytes on using a phytochemical. Curcumin, a known potent immunostimulatory agent in mice, horse, and other higher vertebrates including humans (Behera *et al*., 2010).**

- **Zhang *et al.* (2012) clearly demonstrated the sharp rise in spleen mannose – binding lectin expression in channel catfish challenged with a Gram -Negative bacterium. While action of maanose – binding lectins in stimulating complement system was explained by Presanis *et al.* (2003).**

CONCLUSIONS

- **Administration of either *B. subtilis* or *T. saccharophillus* can significantly increase phagocytic activity, respiratory burst and respiratory burst activities, enhancing ability of the fish to act against invading pathogenic organisms.**

- **Further both *B. subtilis* and *T. saccharophillus* were found to contribute to increased production of IgM & serum lectins indicating the improvement in non-specific immunity.**

- **Promotion in haemagglutination & haemolytic activities demonstrated the ability of pathogen clearance.**

- **This study clearly showed that, the genus *Terribacillus* which has not been explored so far is non-pathogenic and antagonistic to *V. harveyi, P. fluorescens, A. hydrophila* and *E. coli* and may also be used as a diet supplement in aquaculture industry similar to *Bacillus* for suppressing the growth of pathogenic bacteria and for promotion of non- specific innate immunity.**

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- **Centre for DNA finger printing Technology, Hyderabad**


AGAINST HUMAN PATHOGENS


Thank You