Probiotic Induced Immunomodulation: Investigation into the Cellular and Molecular Mechanism Involved

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Abstract

Commensalisms or symbiosis or the concept of probiosis is a prospective outcome of the dynamic co evolution of host-bacterial relationships. The use of antibiotics is discouraged in aquaculture as it has led to the appearance of drug-resistant bacteria, immunosuppression in animals besides harmful effects on the environment and concerns on food safety.

Strain specific immunomodulation by probiotic bacteria was determined experimentally. The feeding of different strains of probionts Lactbacillus rhamnosus, Bacillus subtilis, Eterococcous faecium was found to influence immune response both at local and systemic level. The non-specific cellular immune response increased with probiotic feeding as reflected in the increase of phagocytosis and super oxide anion of head kidney leucocytes in rainbow trout and humoral response as lysozyme activity. The upregulation of cytokines like, Interleukin (IL 1), Tumor necrosis factor (TNF), Transforming growth factor (TGF) isolated from head kidney leucocytes and other tissues of the probiotic fed groups were observed compared to that of the control fish. Cytokines induced by these probionts may thus be important regulators of gut-associated immune system. Probiotic as a factor for immunomodulation including the immune gene modulation which in turn is correlated to the health status of this lower vertebrates, could probably be explored for developing alternate health management strategy in aquaculture.

Keywords: Probiotic, cellular, humoral, immunomodulation, immune mechanism.

Introduction

The interest in probiotics for aquaculture follows their use in human medicine and agriculture in which the microorganisms are generally administered as live supplements in feed.[6] Probionts like LAB Vibrionacea, Pseudomonads and Bacillus are found to give protection to turbot, salmon, cod and prawns and oysters. Probiotics for aquatic organisms have been defined as microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive, with the aim of improving health.[7] Taking in to account the mode of action, Verschuere et al[17] defined probiotic as a live microbial adjunct which has a beneficial effect on the host by (i) modifying the host-associated or ambient microbial community, (ii) ensuring improved use of the feed or enhancing its nutritional value, (iii) enhancing the host's response towards disease or (iv) improving the quality of its ambient environment.

A wide range of microalgae (Tetraselmis), yeasts (Debaryomyces, Phaffia and Saccharomyces) and Gram-positive (Bacillus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Micrococcus, Streptococcus and Weissella) and Gram-negative bacteria (Aeromonas, Alteromonas, Photorhodobacterium, Pseudomonas and Vibrio) has been evaluated for their probiotic properties. The targeted groups are mostly the importrant candidate species which includes Pacific oysters, Penaeid shrimp, Atlantic cod, common snook flounder, tilapia, turbot and salmonids. The adoption of best management practices is a practical way to approach environmental management of aquaculture. Microbial intervention like this brings alternate solution to the health related problems in aquaculture especially WSSV and other viral outbreaks and vibriosis associated with shrimp industry. Pseudomonas fluorescens (AH2) was shown to be strongly inhibitory against Vibrio anguillarum where the mortality rate in rainbow trout was significantly reduced by the addition of this probiotic bacterium.

However, the mode of action of the probiotics is rarely investigated, but possibilities include competitive exclusion, i.e. the probiotics actively inhibit the colonization of potential pathogens in the digestive tract by antibiosis or

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by competition for nutrients and/or space, alteration of microbial metabolism, and/or by the stimulation of host immunity.[8, 13, 14] It is required to understand how microbes including probiotics can drive host immunity in positive directions. The immune system of fish is classified as humoral immune response and cell mediated immune response which can customarily be divided further into the innate (non-specific) and the acquired (specific) immune system; however there exists a combinational system also. Probiotics induced cellular and humoral immune response include some nutritional advantage. The immune gene especially the cytokine gene expression pattern from the immune organs showed differential expression pattern with strains of probiotic bacteria. The innate immune response is the only defense weapon of invertebrates and a fundamental defense mechanism of fish plays an instructive role in the acquired immune response and homeostasis. Probiotic bacteria can induce immunomodulation involving one or several components of an immune response e.g. humoral, cellular or nonspecific immunity. The precise mechanisms by which these immunomodulatory components operate remain to be investigated. This study attempt to investigate the immune response involved at humoral, cellular and molecular level upon probiotic feeding in fish.

Material and Methods

Collection and Culture of the Probionts: The bacterium Lactobacillus rhamnosus, Bacillus subtilis and Enterococous faecium were obtained from Japan Collection of Microorganisms (JCM), Institute of Physical and Chemical Research (Riken), Japan in freeze-dried form. The culture was revived in Man, Rogosa and Share MRS[3] and nutrient broth by cultivating for 48 h at 30 °C, subsequently preserved in glycerin at – 80 °C and kept as stab for further use. After a day by inoculating a pure strain, it was harvested by centrifuging at 16,500 × g for 10 min and washing three times with sterile peptone water (NaCl 0.85% and polypeptone, 0.1%).

Forms of bacteria: Three different kinds of bacteria were used in this study being incorporated in the diet as freeze-dried form. The viability was checked by plating on MRS agar three times. The freeze-dried form was prepared by keeping the bacterial suspension for 60 h at -20 °C in a REL 206 freeze-drier (Kyowa Vacuum Tech., Tokyo, Japan). The freeze-dried powder and suspension form was enumerated for bacterial number per g and ml of the product respectively. The freeze-dried form was vacuum packed before preserving at -20 °C until further use.

Diet formulation and probiotic supplementation: The diets were formulated incorporating both probiotic and prebiotic (10 % fructooligosachharides, FOS) components. The experimental diets were formulated with 50 % defatted fishmeal (DFM) as the protein source and linseed oil as the lipid source. To reduce the microflora associated with the DFM, it was autoclaved and dried before incorporation in the diet. For preparation of the diets, the ingredients were mixed mechanically (ACM-50 LAT, Aikohsha Mfg., Tokyo, Japan) and sterilized water was added prior to pelletizing (AEZ12M, Shimadzu, Kyoto, Japan). The pellets were dried in a REL 206 freeze-drier and stored at -20 °C until further use.

The formulated rainbow trout diet was used as the basal diet for the supplementation of probiont L. rhamnosus, B subtilis and E. faecium. The freeze-dried form was included (by weight to get the identical density) along with the basal ingredients during the diet preparation stage. Care was taken to maintain sterile conditions through all procedures. The probionts incorporated feed Bacillus subtilis and Enterococcous faecium are fed to the three treatments namely, Tr1, Tr2 and Tr3 along with the Co group fed with the control basal diet.

Experimental design: The experiment is designed and conducted following the guidelines for animal care existing at the Tokyo University of Marine Science and Technology. In brief the rainbow trout were reared in flow through system in 60 L tanks in triplicate. The experimental animal rainbow trout (av. weight 100 ± 12 g) was previously grown on commercial feed. The fish were offered the basal control diet for a 2-week conditioning period. The experimental diet was given two times daily to satiation for 3 weeks. The average temperature of the rearing water was 12 °C.

Sample preparation: Sampling was scheduled initially, 3 weeks after probiotic feeding. At each time point the 3 fish were taken randomly each day from one of the triplicate tanks of each treatment. A total of 9 fish were collected per treatment at the end of each sampling term. Blood was drawn from the caudal vein of individual fish after anaesthetization. The whole blood collected with the non-heparinised syringes was allowed to clot for an hour in micro tubes at room temperature followed by 5 h at 4 °C and later centrifuged at 1500 × g for 5 min at 4 °C for the serum samples and preserved at – 80 °C prior to analysis.

To collect leucocytes from head kidney, the organ was aseptically removed from the fish after partial decapitation to expose the trunk kidney area. The leucocytes were separated and enriched according to the techniques of Chung and Secombes[5]. The macrophage-rich cell suspension was adjusted to 10^7 cell ml^{-1} in L-15 medium (Leibovitz; Sigma-Aldrich, Japan)-penicillin-streptomycin solution (P/S; Sigma) for assaying phagocytosis and superoxide production respectively.

Phagocytosis assay: Phagocytic activity of leucocytes was determined as described earlier.[12] Briefly, a 300-µl volume of the leucocyte suspension in L-15 medium (Sigma-

Aldrich) containing $2x10^6$ cells was seeded into a chamber slide (Lab-Tek Nalge Nunc International, IL, USA) and after incubation and removal of the non-adherent cells, further incubated for 1 h after adding opsonized fluorescent latex beads (2 l; Sigma) to each chamber to maintain the cells to beads ratio of 1:10. After washing and fixation, the slides were stained with Diff Quick solution (Kokusai Shiyaku Hyougo, Japan). The phagocytic activity (PA) was expressed as the percentage of phagocytic cells quantified from 300 adherent cells under microscope. The phagocytic index (PI) was expressed as the average number of particle beads ingested by each phagocytic cell.

Superoxide anion production: The superoxide anion production by the head kidney leucocytes was determined based on the reduction of nitroblue tetrazolium (NBT) as described by Puangkaew et al[15].

Cytokine gene expression: The expression of genes interleukin-1β 1 and β 2, tumor necrosis factor 1 and 2 and transforming growth factor-β were examined in the spleen isolated from the fish at the end of the feeding period.

RNA extraction: The leucocytes were collected from the head kidney and sonicated in the presence of 1mL trizol (Invitrogen, Carlsbad, USA), in order to disrupt the cells and release the RNA. The resulting suspensions were passed repeatedly through a 21-in needle to break the genomic DNA. It was then placed on ice and 0.2 mL chloroform was added.

After vigorous shaking and incubation at room temperature for 5 min, the samples were centrifuged in micro-tubes at 13,500 rpm at 4 °C for 15 min. The lower phase and white protein inter-phase were discarded, while the clear upper phase containing the RNA was aspirated and placed in a fresh tube. An equal volume of cold isopropanol was added and the solution was allowed to stand at room temperature for 5–10 min before being centrifuged. The supernatant was discarded and the pellet washed in 1mL cold 75% diethylpyrocarbonate ethanol (DEPC; Sigma, St. Louis, USA) followed by centrifugation as earlier for 5 min. After the final wash, the ethanol was removed and the pellet was air-dried for 10–15 min and redissolved in DEPC-treated water. The extracted RNA was diluted 35 times and 70 µl was used to determine the total RNA concentration and purity of the samples by a GeneQuant spectrophotometer (Pharmacia Biotech, Freiburg, Germany). The RNA was stored at 80 °C.

RT- PCR: Total RNAs were quantified by RT-PCR using 5µg of total RNA per reaction as described by the manufacturer (Invitrogen life Technology, CA, US). Primers specific to β-actin and various cytokines are listed in table 1a. The reaction conditions (Table 1b) were first-strand cDNA synthesis at 65 °C for 5 min, reverse-transcriptase inactivation at 70 °C for 15 min and reaction conditions of PCR with specific primers RT-PCR products were run on a 2% agarose gel containing ethidium bromide (100 ng ml^{-1}) and subjected to UV visualisation and densitometric analysis with a ATTO Gel Doc system (Tokyo, Japan). Densitometric scanning of agarose gel images using UVP gel imaging system and UVP gel works LD advance software was performed to quantify the relative levels of RNA for each gene. Cytokine product: β actin produced was subsequently calculated for each gene of interest and the expression pattern was assessed.

Statistical analysis: Statistical analyses were conducted by using the SPSS 11.0 microcomputer software package (SPSS, Chicago, IL, USA). Analysis of variance was performed by a one-way ANOVA, followed by Duncan's test. Level of significance was set at $p< 0.05$.

Results and Discussion

Probiotic bacteria are found to elicit the innate immune response in fish. Our previous studies indicated an enhancement of humoral parameters with regard to oral delivery of probiotic bacteria like *L. rhamnossus*. The hematocrit value increased significantly compared to that of the control. Similiarly with regard to the serum lysozyme activity, there were changes and the serum alternate complement activity increased significantly after probiotic feeding. Feeding *Lactobacillus rhamnossus* at a density of

Table 1a
Primers selected for the expression study

Name of Cytokine gene	Accession no.	Primer sequence
β-Actin	AF157514	F:ATG GAA GAT GAA ATC GCC
		R:TGC CAG ATC TTC TCC ATG
TNF 1	AJ277604	F: CAA GAG TTT GAA CCT TGT TCA A
		R: GCT GCT GCC GCA CAT AGA C
TGF β	X99303	F: AGT TGC CTT GTG ATT GTG GG
		R: CAA TCA TAT TGG GCA CCT GC

1 X 10^8 CFU/ g of feed could elicit the complement activity. Similarly, probiotic treatment of other probionts including Tr1, Tr2, Tr3 also showed same trend. A consistently increasing trend was observed in the series of experiments with regard to the complement activity.

These lower vertebrates display cellular immune response with leucocytes and lymphocytes activation. It was established that the phagocytic activity of the head kidney leucocytes is elevated in the probiotic fed groups. All our previous experiments established that the phagocytic activity of these leucocytes increased significantly after feeding probiotic *L. rhamnossus* incorporated feed. In case of the other probionts like *B. subtilis* and *E. faecium* also, there is increase in this phagocytic property of these immune cells. Macrophages phagocytose infected cells present with T and B cells and produce cytokines and chemokines that modulate immune response.[10] The ability of head kidney leucocytes to phagocytize latex beads was used as a measure of innate immunity. Phagocytic activity of the head kidney leucocytes elevated significantly upon LAB feeding at 8 week of probiotic feeding. Ingestion of *Lactobacillus acidophilus* strain La1 or *Bifidobacteriaum bifidum* strain Bb 12 for 3 weeks in fermented milk increased phagocytic activity of peripheral blood leucocytes in human subjects. Similiarly, the probiotic fed groups showed higher head kidney leucocyte superoxide anion production upon stimulation compared to that of the control groups. Nikoskelainen et al[11] demonstrated that the administration of probiotic *L. rhamnossus* ATCC 53101 in feed stimulated the respiratory burst activity, which is a measure of phagocytosis after 2 weeks of feeding.

The viability of the probiotic bacteria is always under scrutiny as per its role in immunomodulation and how far the cellular envelop and components help increase the immune response even in a nonviable probionts. Our studies revealed that viability of the probionts is definitely a factor in inducing immunomodulation.[13] In our study the feed was prepared with fructooligosacharide, a prebiotic component which gives additional advantage. FOS are naturally occurring non-digestible carbohydrates that in combination with the probionts promote long-term health via gastrointestinal immune system. Viable LAB is better than non-viable bacteria in inducing non-specific immune responses like phagocytosis and serum alternate complement activity in rainbow trout.[12]

The feeding of different strains of probionts in Tr1, Tr2 and Tr3 are found to influence immune response both at local and systemic level. The non-specific cellular immune response increased with probiotic feeding as reflected in the increase of phagocytosis and super oxide anion of head kidney leucocytes and lysozyme activity after 3 weeks of probiotic feeding.

Molecular immune response: Cytokines are intercellular signaling molecules which play a central role in the modulation of immunological and physiological events in animal under both homeostatic and abnormal condition. The leucocytes which are the important vehicle for the cellular immune response, elicit many genes especially the cytokines. present in the head kidney The TNF and TGF β genes were found to be upregulated in the probiotic exposed fish. There is many fold increase in the TGF β expression in the Lr and Bs whereas it is 12 fold in case of Ef. With regards to the Interferon, highest fold increase was observed in case of Tr3 group followed by the Tr1 and Tr2 groups respectively.

Lactic acid bacteria (LAB) is reported to influence the immune response of the host animal by promoting secretary Ig, enhancing phagocytosis, augmentation of natural killer cell activity, lysosomal enzyme secretion, modulation of cytokine gene expression, adjuvant effect, regression of tumor, carcinogen production, reduction in serum cholesterol concentration etc. Probiotic feeding is a function of cytokine gene expression which in turn can be correlated to the health status of fish. It could probably be one milestone to elucidate the immune mechanism involved and formulate strategy for cytokine based therapeutics in aquaculture through the harmless probiotic bacteria.

Recently, probiotics is being used in all aquaculture system. Mostly the shrimp farming have evolved as zero/ less water exchange probiotic culture system. According to Austin et al[1], the bacteria *Vibrio alginolyticus* was found to reduce diseases in Atlantic salmon caused by infection of common pathogenic strains (*Aeromonas salmonicida*, *Vibrio anguillarum*). Aquafarmers add different bacterial mixtures in water as "water additives" or "soil additives" and sometimes as "feed additives" having a beneficial effect on aquaculture production. Queiroz and Boyd[16] reported that a commercially prepared bacterial mixture of *Bacillus* spp. mixed into the rearing water increased survival and production of channel catfish (*Ictalurus punctatus*). It has been suggested previously that bacterial cell wall and cytoplasmic components modulate the action of immune cells via their receptors and cytokines[14]. The probiotics impact as nutritional immunomodulator varies depending on individual strain, its state, its level of consumption and ambient environmental conditions.

Conclusion

Microbial interventions in aquaculture can also improve water and soil quality by reducing levels of phosphorus and nitrogen in aquaculture waste and can be evaluated. Acceptance of microbial intervention concept by regulatory bodies will be possible only if the mechanisms of their action are well explained. The expressions of cytokines isolated from immune tissues and also from the leucocytes of the probiotic fed groups were more compared to that of

Table 1b

Target	Cycling Protocol				
	Product size	Denature	Anneal	Elongation	No. of Cycles
β-actin	262 bp	95°C- 5 min 95°C- 1 min	- 54 °C- 30 sec	 72 °C- 1:30 sec 72 °C- 5 min	1 24 1
IL-1 β 1	399 bp	95°C- 5 min 95°C- 1 min	 58 °C- 30 sec	 72 °C- 1:30 sec 72 °C- 5 min	1 35 1 1
TNF α 2	212 bp	95°C- 5 min 95°C- 1 min	59 °C- 30 sec	72 °C- 1:30 sec 72 °C- 5 min	1 30 1
TGF β	492 bp	95°C- 5 min 95°C- 1 min	59 °C- 30 sec	72 °C- 1:30 sec 72 °C- 5 min	1 30 1

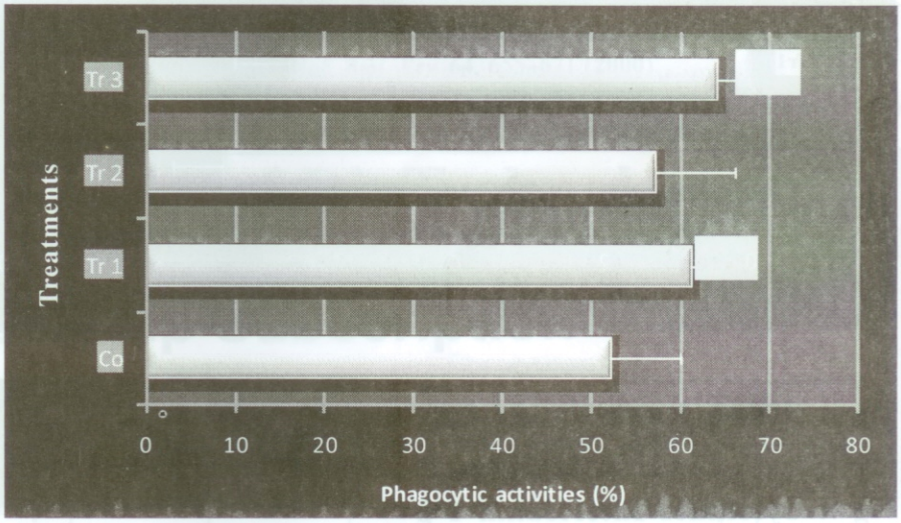

Fig 1a: Phagocytic activity and superoxide anion production (c) of the head kidney leucocytes from Tr1, Tr2and Tr3 and the control diet fed groups after 3 weeks of probiotic feeding. Data shown as mean with standard deviation as error bars; significant difference (P< 0.05) between groups is indicated by asterisk mark.

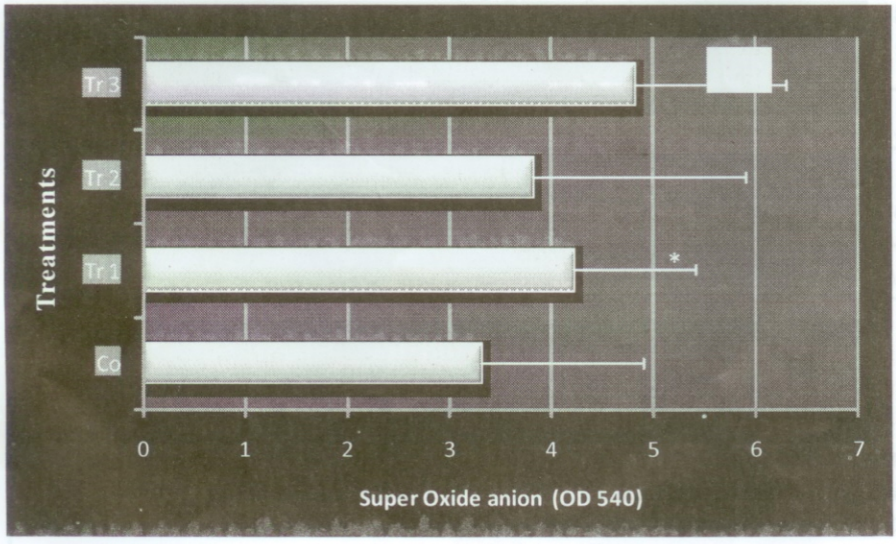

Fig 2: Superoxide anion production of the head kidney leucocytes from Tr1, Tr2and Tr3 and the control diet fed groups after 3 weeks of probiotic feeding. Data shown as mean with standard deviation as error bars; significant difference (P< 0.05) between groups is indicated by asterisk mark.

the control fish. Also the cellular and humoral immune parameters are showing considerable elevation. Cytokine induced by these probionts may thus be important regulators of gut-associated immune system. The probiotics impact as nutritional immunomodulator varies depending on individual strain, its state and its level of consumption.

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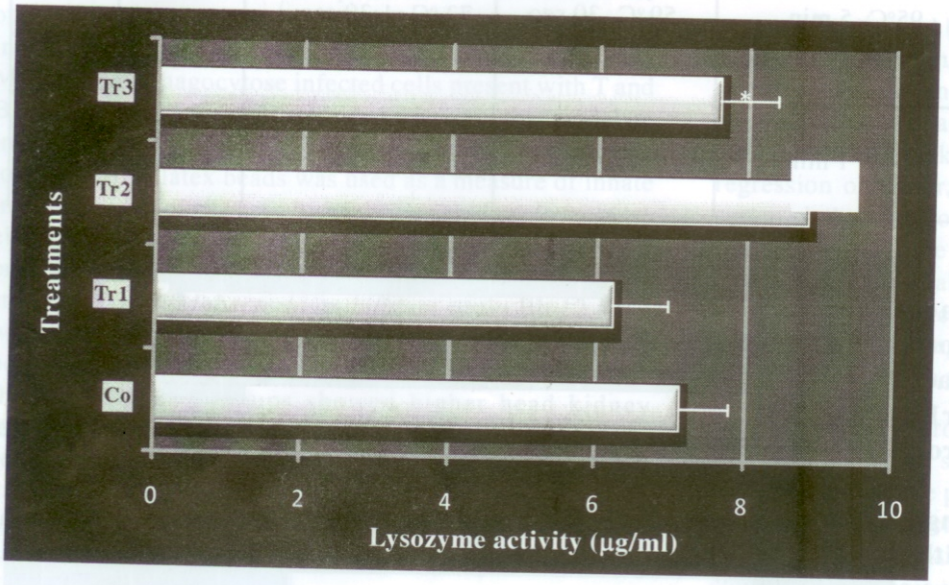

Fig 3: Lysozyme activity from Tr1, Tr2and Tr3 and the control diet fed groups after 3 weeks of probiotic feeding. Data shown as mean with standard deviation as error bars; significant difference (P< 0.05) between groups is indicated by asterisk marks.

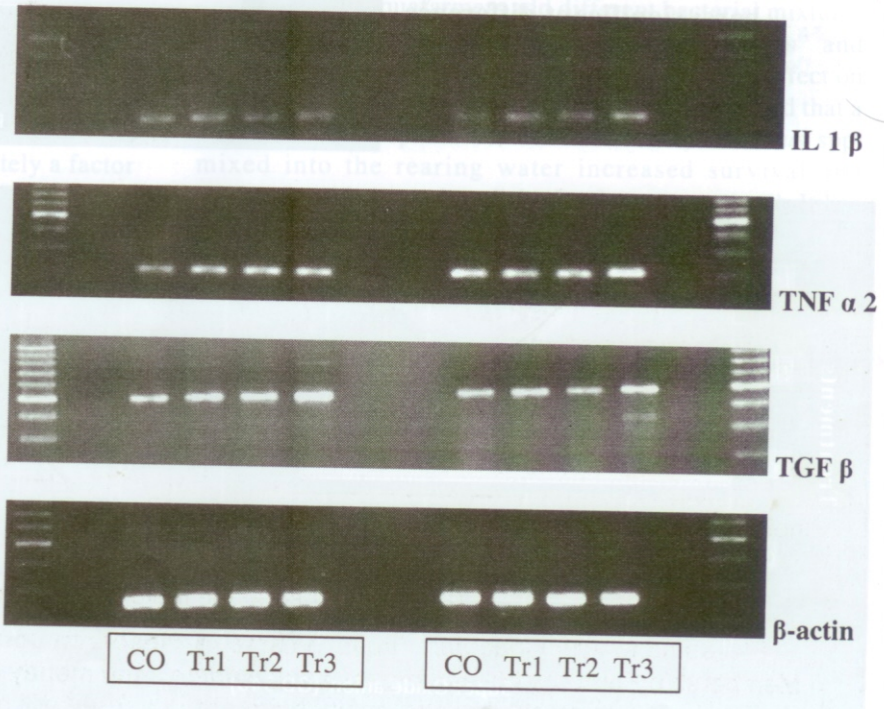

Fig 4: Cytokines Interleukin 1 β (IL 1 β), Tumor Necrosis Factor (TNF α) and Transforming growth factor (TGF β) gene expression in the kidney of the control and probiotic fed rainbow trout; densitometric quantification of cytokine gene expression relative to β-actin transcript.

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