



## **Assessment of Probiotic in Aquaculture: Functional Changes and Impact on Fish Gut**

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### **Authors' contributions**

*This work was carried out in collaboration among all authors. Author MAAM designed the study, performed the statistical analysis, wrote the protocol and prepared the first draft of the manuscript. Authors SN, SSR and MJS managed the literature searches. Authors PD and KVA managed the gut histology of Rohu. All authors read and approved the final manuscript.*

### **Article Information**

DOI: 10.9734/MRJI/2019/v29i130156

#### Editor(s):

(1) Dr. Sabina Fijan, University of Maribor, Slovenia Fakulteta Za Zdravstvene Vede, Faculty of Health Sciences, Žitna ulica, Slovenia.

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(4) Hideharu Shintani, Chuo University, Japan.

Complete Peer review History: <http://www.sdiarticle4.com/review-history/51672>

**Review Article**

**Received 28 July 2019**

**Accepted 30 September 2019**

**Published 09 October 2019**

### **ABSTRACT**

One of the most studied or researched sector is probiotics. The probiotic sector has generated hundreds of publications, products and also created awareness of public health benefits. Probiotics means "live microorganisms (usually bacteria) that are similar to beneficial microorganisms found in the human gut that are taken as dietary supplements or found in foods." Such work will include,

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competitive exclusion, generating inhibitory components, a striving for the same nutrients and interference with quorum sensing mechanism and improved immunity. Probiotic one of the most reliable approach to combat fish disease for sustainable aquaculture. This microbial intervention approach can boost fish yield by improving feed utilization, nevertheless provide protection from pathogens by different modes of action. The use probiotic containing food has the beneficial properties has been known for centuries in human health however research in probiotics for aquaculture was a journey from last two decades. Here we are discussing the role of probiotics in aquatic animal and aquaculture environment, particularly focusing on their functional changes such as growth promoter, biocontrol and bioremediation agents, interrupt the action of pathogens by producing inhibitory substances. Finally gut morphology of rohu, *Labeo rohita* were compared between probiotic and basal diet fed fishes.

**Keywords:** Probiotics; gut morphology; aquaculture; mode of action; *Labeo rohita*.

## 1. INTRODUCTION

Aquaculture is one of the fastest growing food production sectors in the world including India. Global fish production peaked at about 171 million tonnes in 2018, with aquaculture representing 47 percent of the total fisheries production [1]. Excluding aquatic plants, aquaculture output in 1970 accounted for 3.9 percent of total fisheries production, by 2001 that proportion had increased to 29 percent and by 2006 and 2014 to 36 and 44 percent respectively. It is expected that global aquaculture production will reach to 50 percent of the total fisheries production between the years 2025-2030 [2]. Intensification has come up as a boon to meet the increasing food demand [3]. However, the growth of aquaculture industry where extensive, semi-intensive system transferred into intensive and super-intensive form resulting sudden and onset mortalities, are mainly caused by virulent microorganisms [4,5]. The best way to prevent diseases and commercial losses of fish would be to improve their resistance to infections in addition to improving husbandry with good health management.

Intensive aquaculture system lead to the risk of microbial diseases and there is a urgent interest to find alternatives to antibiotic prophylaxis for fish health management as unwise use of antibiotics in aquaculture industry has grown to the resistant in antibiotic. Besides, antibiotic in the environment may transfer R plasmid to human intestinal microbiota [6]. Nevertheless, antibiotic applications do not prevent virulent microorganisms in the aquaculture environment. Moreover, the application of antibiotics may drastically alter the gut microbiota of the aquatic

animal, resulting break down of first line defence mechanism. An alternative method to antibiotic use would be the use of probiotics that overthrow the pathogens from the host body by its different mode of action [7,8]. With this brief background, probiotics can be a substitute to resist infectious pathogens and pave the way for empirical fish production. Probiotics are well known and routinely used additives in the main livestock species now a day's practicing in aquaculture industry. Therefore a brief review is taken to address the probiotics assessment in aquaculture with an evidence in gut structure of Indian Major Carp, *Labeo rohita* fed with *Lactobacillus rhamnosus*, is a short Gram positive probiont.

## 2. ETYMOLOGY OF 'PROBIOTIC' AND ITS DEFINITIONS

Probiotic came from Greek words, Pro (favour) and Bios (life) simply 'for life'. It refers to organisms and substances that favour life. Dr. Elie Metchnikoff, a Russian scientist was first conceptualize and describe in 1905. It was Lilly and Stillwell [9], who modified the original word 'probiotika' as 'probiotic'. Gatesoupe [10] suggested a modification in the definition of probiotics as used in aquaculture. He defined probiotics 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. He further classified the microbial preparation used in aquaculture into three types-biocontrol agents, probiotics and bioremediation agents. Probiotics have several functional ways in conferring their health benefits in fish. The inclusion of probiotic bacterial through feed is a better method to ensure the better gastrointestinal (GI) tract of fish.

## 2.1 Selection of Potential Candidates for Use as Probiotics

Probiotics have been classified as biological preparations under the proposed Aquaculture Drug Regulation of India. An 11-point criterion has been set out to regulate the use of these preparations. The use of probiotics in aquaculture is a clear case where commercial use started before there was research backing to support its use. Time and again, companies have borrowed the probiotic preparations used in animal husbandry, and are directly applied in aquaculture systems. So far, adverse impacts on animal health and environment have not been reported [11].

## 2.2 Probiotic Selection Criteria

Various researcher had enunciated of different thinking on necessary criteria to choose microbes as probiotics or probionts [12] or applications in aquaculture. Merrifield et al. [13] described the same features and elaborate the characteristics to below points (where E indicates an essential criterion and F indicates a favorable criterion). The probiont:

- Must not be pathogenic with host as well as other flora and fauna (E)
- Must be free of plasmid-encoded antibiotic resistance genes (E)
- Must be resistant to enzymes and acid environment of host (E)
- Stable in the intestinal environment (F)
- Should be recognized as safe for use as a feed additive (F)
- Should act as growth promoter (F)
- Should exhibit antagonistic properties towards one or more key pathogens (F)
- Should produce relevant extracellular digestive enzymes (F)
- Should be local to the host or culturing environment (F)
- Should remain viable while storage and industrial process (F)
- Anti-inflammatory, antimutagenic, immunostimulatory (F)

## 2.3 Types of Probiotics and its Application Methods

Gatesoupe [10] classified the microbial preparations used in aquaculture into three types- a) biocontrol agents, biocontrol agents are those methods of treatment using the antagonism among microbes to kill or reduce the

number of pathogens in the aquaculture environment. Those bacterial treatments which improve the water quality and thus called b) bioremediation agents. Recently, several commercial products have sought to exploit the idea that bacteria, which improve water quality, may be useful to animal health. Among farmers in India, these products are known as water or soil-probiotics and most of them contain nitrifying bacteria and/or *Bacillus* spp. [11]. The widespread usage of probiotic bacteria in India has used either with feed known as gut probiotics or in water to prevent the proliferation of pathogenic bacteria. Commercial probiotics are coming with two forms- powder or liquid forms. Powder forms can be added in feed and fed to the fishes whereas liquid form probiotics generally speeded in the aquaculture farms. Research suggested that liquid probiotics work faster than the dry probiotics [14].

## 2.4 Source of Probiotics and Commercial Preparations

In a review by Lazado et al. [15] stated selection of inappropriate probiotic can lead to imbalance the nutrients uptake of the host. An ideal probiotic should adhere to the gastrointestinal tract of the host. But very often these probiotic become useless because of their stability in host because of non-host origin. Till now many probiotics such as lactic acid bacteria, *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Leuconostoc*, *Carnobacterium*, *Bacillus*, *Vibrio*, *Aeromonas*, *Saccharomyces boulardii*, *Alteromonas* had evaluated in aquaculture [11]. Searching of new probiotic candidate is a continuous endeavor. Because of host specificity, a particular probiotic may useful for one host however may not for others. Today, a search for host associated probiotic has an increasing trend in scientific community. Host mucosal surfaces of gastrointestinal tract is the place of several useful probiotic bacteria can be isolate for further commercialization.

## 3. MODES OF ACTION/APPLICATIONS OF PROBIOTICS IN AQUACULTURE

### 3.1 Growth Promoter

In the beginning interest was focused on the application of probiotics as growth promoters and to improve the health of animals [4]. Essa et al. [16] reported better growth, feed utilization and improved enzyme activities of amylase, protease and lipase in Nile tilapia when diets were

supplemented with a probiotic, *L. plantarum*. Supplementation of a probiotic, *Lactobacillus plantarum* and with an organic acid, citric acid, alone and in combination lead to significant weight gain, growth, survival, feed conversion ratio (FCR), specific growth rate (SGR) in *Labeo rohita* [17]. Similar results have been reported in the fish *Labeo rohita* fed with probiotic *L. plantarum*, at  $10^8$ ,  $10^{10}$  cfu g<sup>-1</sup> with significant increase in daily weight growth, FCR, SGR in *Epinephelus coioides* [18], *Tilapia nilotica* (*Oreochromis niloticus*) [19].

### 3.2 Competition for Space

Inclusion of *L. plantarum* in feed produces certain bacteriocins which inhibit the growth of Gram positive and Gram negative bacteria including *Staphylococcus aureus*, *Escherichia coli*, *Listeria innocua* and *Pseudomonas aeruginosa* [20]. A significant reduction in the adhesion of *Aeromonas hydrophila* and *Aeromonas salmonicida* was evident when administered with *L. plantarum* [21,22]. It is suggested that lactic acid bacteria along with other bacteria that belong to the autochthonous (indigenous) microbiota of fish might be an important part of the defence mechanisms against colonization of fish pathogens in the gastro-intestinal tract. Moreover, probiotic bacteria plays an important role in the prevention of colonization of parasites, bacteria and fungi.

### 3.3 Production of Inhibitory Substances

Bactericidal or bacteriostatic substances such as bacteriocins, lysozymes, proteases hydrogen peroxide, siderophores are produce by probiotic bacteria [23,24]. In addition, some probiotic bacteria produce organic acid and volatile fatty acids (e.g. lactic, acetic, butyric and propionic acids), that can result into the reduction of pH in the gastrointestinal lumen, thus preventing growth of opportunistic pathogenic microorganisms [23]. Compound like indole (s, 3-benzopyrrole) is very effective against pathogens.

### 3.4 Antibacterial Properties of Probiotics

Many authors had revealed that microbial intervention as probiotics in aquaculture have been shown antibacterial activity against known pathogens. The antibacterial compounds produced by *Lactobacillus* have a broad spectrum of activity against bacteria that are closely related to the producer. It has been shown that *L. plantarum* produces inhibitors

against *Vibrio* sp. when the culture was grown in the presence of *Bacillus thuringiensis*. *Bacillus* species are known to produce different kinds of antibiotics, such as Bacitracin, Polymixin, Trycodin, Gramicidin and Circulin. Many authors have reported the antagonistic effect of *Bacillus* species against gram negative microorganisms.

### 3.5 Antiviral Properties

Ibrahem [25] reviewed on the antiviral effects of some probiotic bacteria. Laboratory tests indicated that the inactivation of viruses can occur by chemical and biological substances, such as extracts from marine algae and the bacterial extracellular products. It has been reported that strains of *Pseudomonas* sp., *Vibrio* sp. *Aeromonas* sp. and groups of *coryneforms* isolated from salmonid hatcheries, showed antiviral activity against infectious hematopoietic necrosis virus (IHNV) with more than 50% plaque reduction [26]. Similar findings also reported by Li et al. [27] where they revealed that pacific white leg shrimp fed with a *Bacillus megaterium* strain increased the resistance to white spot syndrome virus (WSSV). Harikrishnan et al. [28] reported that the administration of supplemented diet with *Lactobacil* individually or mixed with *Sporolac* act as immunostimulants that enhance the innate immune response and disease resistance in lymphocystis disease virus (LCDV) infected olive flounder, *Paralichthys olivaceus*.

There was a report on the passive immunization of the tiger prawn, *Penaeus monodon*, using rabbit antisera to *Vibrio harveyi* Lee et al. [29]. In this method, outer membrane of bacterium, *V. harveyi* strain 820514, was separated and afterwards purified and immunized into rabbit. The antibody conferred the protective immunity against WSSV in black tiger shrimp [30].

### 3.6 Antifungal Activity

Much research is not available on antimycotic effect of probiotics. Lategan et al. [31] isolated *Aeromonas media* (strain A199) from eel (*Anguilla australis*) culture water and were observed to have a strong inhibitory activity against *Saprolegnia* sp. Lipińska et al. [32] reported antifungal activity of *Lactobacillus pentosus* in the presence specific media.

### 3.7 Competition for Chemicals or Energy

An astonishing findings noted that nutritional components were utilize by probiotic bacteria

otherwise taken by virulent microbes. Competition for nutrients can play an important role in the composition of the microbiota of the digestive system or ambient environment of the cultured organisms. Many bacteria including the known probiotic group lactic acid bacteria (LAB) consume the nutrients that are essential for the growth of a number of pathogens hence make them unavailable to the pathogen [33].

### 3.8 Maintaining the Water Parameter

In an extensive review by Sahu et al. [14] reported that probiotics could help in improving the water quality in aquaculture ponds. This is due to the ability of the probiotic bacteria to participate in the turnover of organic nutrients in the ponds. Application of Gram positive bacteria, such as *Bacillus* spp. is beneficial in improving the quality of the water system. *Bacillus* spp. have a more efficient ability in converting organic matter into carbon dioxide. Several bacteria e.g. *Nitrosomonas*, convert ammonia to nitrite and other bacteria e.g. *Nitrobacter*, further mineralize nitrite to nitrate [14].

### 3.9 Effect on Algal Plankton

Verschuere et al. [34] mentioned that many bacterial strains could have a significant algicidal effect on many species of microalgae. A strain of *Flavobacterium* sp. isolated from natural seawater during the decline period of an algal bloom had a strong algicidal effect on the red tide plankton, *Gymnodinium mikimotoi* [35]. Therefore these results indicate that bacterial effects should be taken into account to obtain stable mass culture of food microalgae.

### 3.10 Increase Nutrient Digestibility

Hundreds of published paper revealed that probiotic had a positive interaction in gastrointestinal process of aquatic animals [36]. In fish, it has been noted that Bacteroides and *Clostridium* sp. have contributed to the host's nutrition, especially by supplying fatty acids and vitamins [37]. Considerable research findings revealed that probiotic helps in digestion of the host by releasing digestive enzymes as well as other growth promoting elements such as essential amino acids, fatty acids and vitamins [38,39]. Haroun et al. [40] conducted to examine probiotic treatment in the fingerling diet of Nile tilapia *Oreochromis niloticus* (L.). Five types of experimental diet was used viz., 0.5%, 1%, 1.5%, 2% and 2.5% to evaluate the nutrient utilization of Nile tilapia. Results indicated weight gain,

specific growth rate, protein efficiency ratio, protein productive value and energy retention were significantly ( $P < 0.01$ ) higher in the treatment receiving probiotic (Biogen<sup>®</sup>) than the control diet. Therefore probiotic microorganisms increase nutrient digestion when applied together with feed components.

### 3.11 Interference of Quorum Sensing

In a review by Miller and Bassler [41] stated that Gram positive and Gram negative bacteria use quorum sensing (QS) communication circuits to regulate a diverse array of physiological activities. These processes include symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation, and biofilm formation [41]. The disruption and destruction of QS is considered a high potential anti-infective strategy in aquaculture [42]. Some probiotic bacteria such as *Lactobacillus*, *Bifidobacterium* and *Bacillus cereus* strains degrade the signal molecules of pathogenic bacteria by enzymatic secretion or production of auto inducer antagonists [43]. In biofilm mode when bacteria are more resistant to heat [5], antibiotics, surfactants, antibodies and phagocytic cells can do exchanging their necessary communication by quorum sensing signaling. The protein expression is also different in biofilm mode and planktonic mode of bacteria. So, blocked this communication (QS) is very vital when we are going to kill the pathogenic bacteria. Probiotic bacterial when applied in feed can disrupt the quorum sensing signaling rendering destruction of the pathogen.

### 3.12 Enhancement of Immune Response

The non-specific immune system can be stimulated by probiotics. Sakai et al. [43] reported that oral administration of *Clostridium butyricum* bacteria to rainbow trout enhanced the resistance of fish to vibriosis, by increasing the phagocytic activity of leucocytes. *Bacillus* sp. (strain S11) has provided disease protection by activating both cellular and humoral immune defenses in tiger shrimp [44]. Probiotics trigger the immune system of hosts, which include the stimulation of cytokines on the activity of immune cells, increasing the phagocytic activity, levels of antibodies, acid phosphatase, lysozymes, complement, cytokines, tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), gamma interferon (IFN- $\gamma$ ), and antimicrobial peptides [45]. The *Lactobacillus plantarum* influenced both the cellular and humoral immune defences in the shrimp. *L. plantarum* was known to enhance

the phenoloxidase (PO) activity, prophenoloxidase (ProPO) activity, respiratory bursts, superoxide dismutase (SOD) activity and clearance efficiency of *Vibrio alginolyticus*, peroxinectin mRNA transcription, and better survival rate after challenge with *V. alginolyticus* [25].

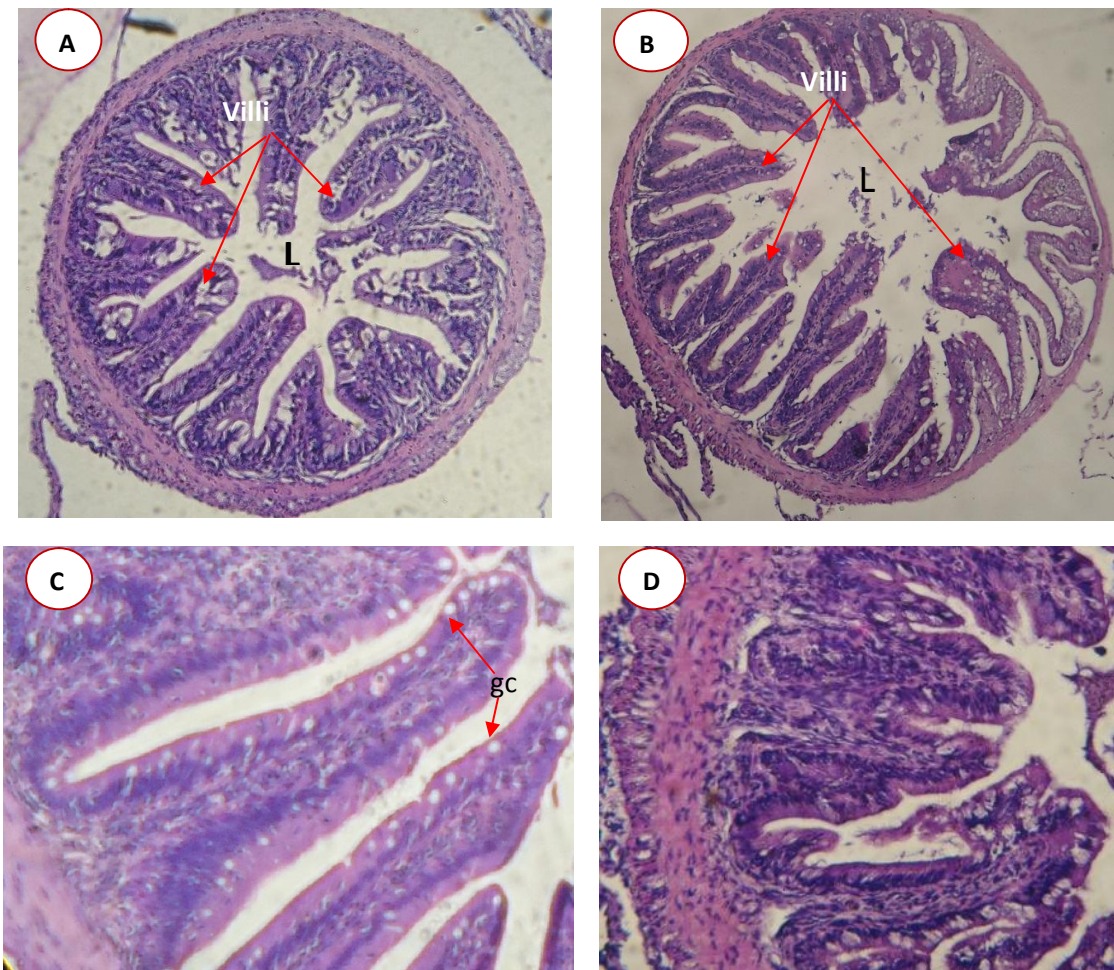
### 3.13 Stress Salvation

Disease outbreak is a major constraint in intensive aquaculture systems resulting in mortality and reduced yield. Intensive aquaculture offers an increased opportunity for spreading of infectious diseases at all stages of production. Infectious diseases due to viruses, bacteria, fungi and parasites are taking heavy toll

with significant economic loss [46]. Stress can be ranked as number one which is responsible for the mass mortality of aquatic organisms. According to Brett [47] stress is defined as a condition produced by a biotic or abiotic factor(s) which extends the adaptive responses of the individual beyond the normal range, such that its chances of survival are significantly reduced. By application of probiotic bacteria it can be upregulated the immune system and therefore reducing the detrimental effects of different stressors [48].

### 3.14 Impact on Reproduction

There has been scanty of probiotic research article on reproductive performance and gamete



**Fig. 1. (A:10 X; C: 40 X) Light microscopic view of villi in the mid gut from rohu (*Labeo rohita*) fed *L. rhamnosus*, note that villi are long and lumen space is very less (A) with adequate number of conspicuous goblet cells (C). (B:10 X; D: 40 X) gut microscopy of control fish having short and stout villi and wide lumen area (B) and presence of fewer goblet cells (D)**

quality of fish. Aydın and Çek-Yalnız [49] reviewed probiotic impact on fish reproductive performance and gamete quality in fish and evaluates further applications of probiotics in reproduction of fish. Abasali and Mohamad [50] reported inclusion of commercial probiotic, enhanced the reproductive performance of Swordtail, *Xiphophorus hellerifor*. Supplementation of *L. rhamnosus* can upregulated the fecundity of zebrafish [51]. Probiotic supplementation on reproductive performance of fish was reported out by Ghosh et al. [52] using a probiotic strain of *B. subtilis* isolated from intestine of *Cirrhinus mrigala*, incorporated at different doses to four species of ornamental fishes, in a one-year experiment. The results showed that using *B. subtilis* concentrations of  $10^6$ – $10^8$  cells  $g^{-1}$  of food, produced increases in the reproductive indices, i.e., gonadosomatic index, fecundity, viability, and production of fry from the females of all four species [52].

### 3.15 Probiotics on Gut Health

A study was taken to evaluate the gut intestine of Rohu, *Labeo rohita* for 60 days. Treatment groups were fed the *Lactobacillus rhamnosus* containing pelleted diet @  $10^9$  cfu/g. Production and nutrient uptake were significantly elevated in fish receiving probiotics than in those fed the basal diet. The inclusion of probiotic feed exerted some effect on the intestinal morphology and gut immunity. Histological evidence of probiotic effect of gut are shown in Fig. 1. Nile tilapia (*O. niloticus*) fed with dietary vitamin C showed intact architecture of intestine with increased villi heights, number of goblet cells [53] which is an indication of strong immune system. More number of goblet cells produce more amount of mucus which can trap the pathogenic microorganism can lead to destruction of pathogens. Intestinal tract are in direct contact with the external environment and are thus continuously exposed to the large numbers of microorganisms. To cope with the substantial microbial exposure, epithelial surfaces produce a diverse arsenal of antimicrobial proteins that directly kill or inhibit the growth of microorganisms [54].

## 4. CONCLUSION AND RECOMMENDATION

The use of food containing live microorganisms such as curd and yogurt having beneficial properties has been known for centuries for

human health aspects. Now probiotic has become widely acceptable and well recognized item in veterinary health management. In aquaculture probiotic research topic has got much attention from the last two decades. Presently, several probiotic products are marketed in Indian market and farmers often applied in fish and shrimp farms. Therefore probiotics become an alternative to chemotherapeutics especially to antibiotics which is well known for its negative impact on living biota. However, much work is still needed in aquaculture sector. Because satisfactory results could not be achieved by many research findings. It might be due to the alteration of environmental factors. As the application of probiotic industry is likely to rise the production cost, cost-benefit analysis must be prioritized and emphasized. Finally it is recommended that i) More funding support be given to research on probiotics ii) Intensified research needed to investigate the fate of probiotic organisms in the environment and in the animal iii) Manufacturers should abide by the rules of Aquaculture Authority and iv) Commercial probiotic manufacturers and marketers should get their products tested by a competent research laboratory before they market their product.

## ACKNOWLEDGEMENTS

First two authors are grateful to the ICAR for providing Netaji-Subhas International Fellowship. We also thank Dr. KS Ramesh, Head of the Aquatic Health Management Laboratory, College of Fisheries, Mangalore, for the support.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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